Cellules endothéliales circulantes et progéniteurs endothéliaux circulants : biomarqueurs de l'angiogénèse tumorale et des traitements anti-angiogéniques et anti-vasculaires

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CELLULES ENDOTHÉLIALES CIRCULANTES ET PROGÉNITEURS ENDOTHÉLIAUX CIRCULANTS : BIOMARQUEURS DE L'ANGIOGÉNÈSE TUMORALE ET DES TRAITEMENTS ANTI-ANGIOGÉNIQUES ET ANTI-VASCULAIRES

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SOMMAIRE

« On ne peut vaincre la nature qu'en lui obéissant »

Francis Bacon (extrait de *Novum Organum*)

Préambule

De part son rôle bien établi dans la croissance, la progression tumorale et la dissémination métastatique, l'angiogénèse est une des cibles majeures des nouveaux traitements anti-cancéreux (1-3). Deux classes thérapeutiques ont émergés : les agents anti-angiogéniques agissant sur la néo-angiogénèse tumorale et les agents anti-vasculaires (ou *vascular disrupting agents* (VDA)) visant à détruire les vaisseaux tumoraux en place. Malgré ces avancées thérapeutiques, le mécanisme d'action précis des stratégies ciblant les vaisseaux sanguins tumoraux, les raisons de leur efficacité et de leur synergie en association à la chimiothérapie cytotoxique, ainsi que les mécanismes de résistance à ces drogues sont encore mal compris (4-7). L'identification de marqueurs biologiques capables de refléter l'angiogénèse tumorale ou les effets sur la vascularisation tumorale des traitements ciblés est l'objet de nombreux travaux (8-11). Dans ce contexte, la mise en évidence de cellules endothéliales présentes dans la circulation sanguine, potentiels « biomarqueurs » facilement accessibles, chez des patients atteints de cancer suscite beaucoup d'intérêt.

Deux populations cellulaires, les cellules endothéliales matures circulantes (*circulating endothelial cells*, (CEC)) et les progéniteurs endothéliaux circulants (*circulating endothelial progenitors* (CEP)) sont aujourd'hui activement évaluées. Les CEC sont des cellules endothéliales matures détachées de la paroi des vaisseaux sanguins et leur présence en nombre élevé est considérée comme un indicateur direct de la lésion vasculaire (12, 13). Très rares chez des sujets sains, des taux relativement élevés de CEC ont été décrits chez des patients atteints de différents cancers (14). Issues de la moelle osseuse, les CEP sont mobilisés dans un mécanisme compensatoire de réparation des parois vasculaires lésées et participent à la néoangiogénèse au niveau des sites d'ischémie (15-17). Ces deux populations cellulaires sont des acteurs clés des processus de lésion et dysfonctionnement vasculaire (12, 18, 19). Compte tenu de leur importance dans des pathologies vasculaires, ces cellules ont d'emblée été soupçonnées de jouer un rôle dans la néoangiogénèse tumorale et pressenties comme des candidats intéressants pour

être des biomarqueurs de réponse aux stratégies ciblant la vascularisation tumorale (14). Des travaux menés chez la souris ont montré que les CEP contribuent à degré variable à la vasculogénèse tumorale (20-23). Plus récemment, des données émergentes soutiennent aussi l'hypothèse du rôle des CEP dans la résistance aux traitements anti-angiogéniques, dans la promotion et la progression de métastases, et possiblement dans la résistance aux traitements cytotoxiques standards par un effet de rebond vasculaire (24-27). Cependant, chez l'homme, l'importance des CEP dans ces processus reste à éclaircir.

Afin de mieux comprendre l'implication des CEC et CEP dans l'angiogénèse tumorale et dans les mécanismes de résistance aux stratégies thérapeutiques ciblant les vaisseaux tumoraux, nous avons étudié le rôle de ces cellules dans différents contextes. Dans un premier temps, nous avons exploré l'intérêt de ces cellules en tant que biomarqueur de l'angiogénèse dans des tumeurs pédiatriques (28), ainsi que leur rôle en tant que biomarqueurs de traitement par des agents anti-angiogéniques chez des sujets adultes atteints de cancer (29, 30). Ces travaux ont permis de mettre en lumière l'intérêt des CEP et ont été à la source d'un travail plus « mécanistique » chez l'animal où nous avons étudié les rôles respectifs de ces cellules dans un modèle particulier de thérapies ciblées qui est celui des VDA (31). Compte tenu du mécanisme d'action de ces drogues qui consiste à détruire des vaisseaux tumoraux établis, nous avons émis l'hypothèse que les CEC et CEP, en tant que marqueurs de lésion et de réparation vasculaire, pourraient être respectivement des biomarqueurs d'activité et de résistance au VDA. Plus particulièrement, nous avons étudié dans différents modèles murins le rôle des CEC et CEP dans le mécanisme d'action des VDA ainsi que le rôle fonctionnel des CEP dans la résistance à ces molécules et avons exploré des stratégies d'association de VDA aux agents anti-angiogéniques destinées à augmenter l'activité anti-tumorale des VDA via l'inhibition des CEP (31).

Ces hypothèses se fondaient sur l'expérience acquise au préalable dans le laboratoire chez l'homme et chez l'animal, ainsi que sur différents travaux que j'ai pu mener ou auxquels j'ai pu participer dans le cadre mon Master 2 et des trois années de thèse passés au sein de l'équipe de Biologie des Cellules Circulantes du Laboratoire de Recherche Translationnelle, Unité INSERM U981, de l'Institut Gustave Roussy. Cette thèse a été aussi l'opportunité d'un travail de réflexion sur les stratégies anti-angiogéniques dans le domaine de la cancérologie pédiatrique. Le fruit de ce travail a fait l'objet de 8 publications (28-35), 3 présentations orales (dont une en session plénière à la *Société Internationale d'Oncologie Pédiatrique* (SIOP) 2009), et 2 communications affichées à l'AACR (*American Association for Cancer Research*). Ces travaux

seront présentés succinctement avec le travail principal publié récemment dans *Cancer Discovery* (31).

Avant d'aborder la partie consacrée aux Résultats, ce manuscrit comporte une partie introductive permettant d'avoir une vue d'ensemble sur les dernières avancées concernant le rôle des CEC et CEP dans l'angiogénèse tumorale dans un premier temps, et leur intérêt en tant que biomarqueurs des traitements anti-angiogéniques et des agents anti-vasculaires dans un second temps. Certains paragraphes pourraient être considérés comme étant à la marge du sujet de thèse. Cependant, ils permettent selon moi d'aborder brièvement des aspects importants autour des controverses sur le rôle des CEC et CEP dans le cancer, liés en grande partie aux difficultés techniques de leur détection, et qui peuvent ainsi avoir un intérêt dans la compréhension globale de la problématique et des choix méthodologiques abordés durant ce travail.

1. INTRODUCTION

1.1 Problématique scientifique actuelle

L'émergence des thérapies moléculaires ciblant l'angiogénèse tumorale a modifié pendant la dernière décennie de façon majeure le pronostic de certains cancers. L'approbation par la FDA (*Food & Drug Administration*) en 2003 du premier agent anti-angiogénique (36), le bévacizumab (Avastin®, Genetech, Inc), un anticorps monoclonal recombinant humanisé anti-VEGF-A (*Vascular Endothelial Growth Factor*) (37) en combinaison avec la chimiothérapie standard dans le cancer colorectal métastatique (38, 39) est emblématique de la pertinence de cette approche. D'autres exemples entrés dans la pratique oncologique quotidienne comme le bévacizumab dans le cancer du poumon non à petites cellules (40) et le cancer du sein métastatique (41), ou encore les inhibiteurs de l'activité tyrosine kinase des récepteurs VEGFR (*Vascular Endothelial Growth Factor Receptor*) dans les cancers du rein métastatiques (42, 43), les hépatocarcinomes avancés (44), et les tumeurs stromales gastro-intestinales résistantes à l'imatinib (45) illustrent également le succès de ces approches (2, 5). Résultant d'une meilleure connaissance des mécanismes fondamentaux des processus d'oncogenèse et de l'identification de molécules clés inhérentes à la mise en place de la vascularisation tumorale, les thérapies ciblant l'angiogénèse correspondent à un changement essentiel dans le développement des agents antitumoraux dans la mesure où l'agent est spécifiquement évalué pour sa capacité d'action sur sa cible (2, 46). Cependant, comme les traitements conventionnels, ces nouvelles stratégies anti-angiogéniques sont encore caractérisées par un fort taux d'échec et une efficacité modeste et présentent d'importants désavantages. Les gains limités en termes de survie sans progression et de survie globale qui se chiffrent le plus souvent en mois et sont souvent associés à des mécanismes de résistance, la toxicité des traitements (47) et leur coût (48), ont mis en évidence une double nécessité qui est d'une part de sélectionner les patients et d'autre part de mieux comprendre les mécanismes d'action de ces molécules (5, 8-10, 49).

Il est rapidement apparu essentiel d'identifier des marqueurs biologiques (biomarqueurs) qui permettraient de sélectionner les patients réellement à même de bénéficier des thérapies ciblant l'angiogénèse (7-9, 49). A ce jour, les critères de prescription des thérapies anti-angiogéniques sont imprécis. Le bévacizumab peut être proposé aux patients sur simple critère diagnostique de cancer colorectal (39, 50). La sélection des patients sur la base de biomarqueurs prédictifs de réponse au traitement pourrait améliorer substantiellement la prise en charge thérapeutique

d'une population spécifique de patients tout en évitant aux autres patients un traitement toxique et inutile dont ils ne vont retirer aucun bénéfice. Pour cette seconde catégorie de patients, ces sur-traitements retardent les traitements efficaces tout en ayant un coût important pour la société. Le bévacizumab coûte plus de 100 000 US dollars pour 1 an de traitement (51). Au Royaume-Uni, le NICE (*National Institute for Health and Clinical Excellence*) ne prend plus en charge de nombreux agents anti-angiogéniques approuvés aux Etats-Unis en raison du rapport coût-bénéfice limité pour les patients (48). Ces données à la fois biologiques, éthiques et économiques constituent un rationnel fort en faveur de l'introduction des biomarqueurs dans les essais y compris aux stades les plus précoces du développement des drogues. L'identification de biomarqueurs permettant de mieux sélectionner les patients pourrait de plus accélérer le processus d'approbation des drogues en permettant le développement d'une drogue dans une population spécifique. Selon la NIH (*National Institute of Health*), un biomarqueur est défini comme « une caractéristique mesurée de façon objective et évaluée comme un indicateur d'un processus biologique normal ou pathologique ou une réponse pharmacologique à une intervention thérapeutique » (52). Plusieurs types de biomarqueurs (prédictifs, pronostiques, pharmacodynamiques….) susceptibles de fournir des informations différentes ont été définis (10, 53). Ils doivent être à la fois validés au niveau analytique et qualifiés au plan clinique avant d'être intégrés définitivement dans les essais cliniques. Dans ce contexte, l'optimisation et la standardisation des tests de mesure de ces biomarqueurs, indispensables à la comparaison des résultats des essais sont un enjeu important et une étape préalable à leur qualification clinique dans des études prospectives et rétrospectives.

Bien que l'identification de nouveaux biomarqueurs soit devenue une priorité et est l'un des domaines d'investigation les plus actifs en oncologie, la validation de biomarqueurs des thérapies anti-angiogéniques s'est avérée être très difficile. Premièrement, le succès de l'identification d'un biomarqueur prédictif de réponse d'un agent suppose d'avoir élucidé son mécanisme d'action. Or, à ce jour, ces stratégies utilisées seules ou en association avec des traitements conventionnels présentent des mécanismes d'action différents en fonction de la classe thérapeutique à laquelle ils appartiennent et qui dans certains cas sont mal connus, conduisant à définir de nouveaux objectifs d'évaluation de leurs effets (7, 10, 54). Par exemple, les critères conventionnels d'évaluation de réponse ou de non-réponse selon les critères radiographiques standards (comme le RECIST *(Response Evaluation Criteria in Solid Tumors*)) peuvent être pris à défaut, des stabilisations radiologiques ayant été associées à des bénéfices cliniques et en terme de survie chez des patients ayant un cancer colorectal métastatique traité

par l'association du bévacizumab avec la chimiothérapie (55). Deuxièmement, le développement et la validation d'un biomarqueur doivent prendre en compte les caractéristiques d'un « bon » biomarqueur, qui incluent le rapport coût-bénéfice et -efficacité, un taux bas à l'état basal chez des individus sains, un seuil de sensibilité du test approprié, l'accessibilité par des moyens non-invasifs comme un prélèvement sanguin ou urinaire, ainsi que la robustesse clinique et la reproductibilité dans de multiples centres cliniques et les laboratoires (10, 49, 56). Bien que des méthodes non-invasives soient le but ultime pour identifier des biomarqueurs pertinents, les informations biologiques issues de la tumeur au moment du diagnostic et qui s'appuient de ce fait sur une méthode invasive (biopsie ou chirurgie) sont actuellement capitales pour le choix de la stratégie thérapeutique initiale. L'évaluation des caractéristiques moléculaires de la tumeur obtenue par analyse d'une biopsie peut être particulièrement intéressante pour déterminer la nécessité d'une thérapie ciblée contre cette anomalie. Ce paradigme est illustré par l'administration d'un inhibiteur de la protéine ALK (*Anaplastic Lymphoma Kinase*), le crizotinib, chez les patients ayant un cancer du poumon présentant une mutation ou un remaniement du gène *ALK* (57). Cependant, une limitation reconnue de cette stratégie est l'erreur d'interprétation inhérente à la représentativité de l'échantillon tumoral. Compte tenu de l'hétérogénéité tumorale, les analyses moléculaires réalisées sur un seul prélèvement de la tumeur du patient peuvent ne pas refléter l'ensemble de la pathologie maligne. De surcroît, différentes zones de réponse ou de non-réponse/rechute peuvent coexister au sein de la tumeur traitée. Une difficulté supplémentaire vient de la nature dynamique inhérente au cancer et de son hétérogénéité spatio-temporelle, où les caractéristiques de la tumeur primitive peuvent être différentes de celle des métastases, et dont l'ensemble peut varier en fonction de la progression tumorale et des traitements. Ces différents aspects peuvent retentir sur l'identification de biomarqueurs. Dans le cas des agents ciblant la vascularisation tumorale, la corrélation de données anatomo-pathologiques et moléculaires analysées au sein de la tumeur avec des marqueurs dans le sang du patient pourrait peut-être guider la sélection de biomarqueurs représentatifs de l'état de l'angiogénèse tumorale locale, voire de l'angiogénèse tumorale générale en cas de maladie disséminée (49). Même s'il ne s'agit pas dans le cas des agents anti-angiogéniques d'adapter un traitement à une anomalie moléculaire donnée, la question est bien de personnaliser le traitement en identifiant pour chaque patient la molécule susceptible d'avoir le plus d'efficacité. Le caractère non-invasif du biomarqueur prend alors toute son importance car il permettrait des mesures répétées à différents stades de la prise en charge thérapeutique avec un véritable « monitoring » du patient qui pourrait s'avérer crucial pour l'adaptation des stratégies anti-angiogéniques. Si divers types de marqueurs (cellules circulantes, facteurs

protéiques circulants, marqueurs tissulaires ou imagerie) ont été activement explorés, aucun n'est encore validé aujourd'hui au niveau clinique.

Les travaux menés dans le Laboratoire de Recherche Translationnelle, Unité INSERM U981, de l'Institut Gustave Roussy où j'ai effectué ma thèse de sciences ont été en grande partie consacrés à la mise au point de stratégies de mesure de deux populations cellulaires circulantes dans le sang, les CEC et les CEP, et à explorer leur intérêt clinique dans divers types tumoraux à l'occasion d'études avec des agents anti-angiogéniques. Compte tenu de leur importance dans des pathologies vasculaires, ces cellules ont été pressenties comme des candidats intéressants pour être des biomarqueurs de réponse aux stratégies ciblant la vascularisation tumorale (12, 13, 19, 22). Au sein du laboratoire, un effort considérable a été fourni pour valider des stratégies de mesure de ces deux populations cellulaires avec des méthodes de détection répondant aux critères requis pour la détection des évènements rares. Dans ce travail, nous apportons des arguments en faveur du rôle potentiel de ces cellules en tant que biomarqueurs de l'angiogénèse et des traitements ciblant ce processus (28-30). Plus particulièrement, nous montrons que les CEP pourraient avoir le potentiel de prédire l'existence d'une maladie métastatique, de prédire le bénéfice clinique des traitements anti-angiogéniques inhibiteurs de l'activité tyrosine kinase, et d'identifier des mécanismes de résistance aux traitements anti-vasculaires (31).

1.2 L'Angiogénèse tumorale

L'angiogenèse est défini comme l'ensemble des processus cellulaires et moléculaires aboutissant à la formation de néovaisseaux à partir d'un réseau vasculaire préexistent. Dérégulé, ce processus physiologique devient un évènement essentiel à la croissance et à la dissémination tumorale (58). L'observation que l'angiogénèse se produisait autour de tumeurs a été faite il y a plus de 100 ans (59). Pourtant, la publication princeps en 1971 d'un jeune chirurgien, Judah Folkman, qui émettait l'hypothèse selon laquelle « *les tumeurs ont besoin de générer leur propre réseau vasculaire afin d'obtenir l'oxygène et les nutriments indispensables à la croissance* », suscita critiques et scepticisme de la part de la communauté scientifique (60). L'hypothèse de Folkman ne sera confirmée qu'en 1992 lorsque ce même chercheur montra que l'inhibition de l'angiogénèse pouvait être une stratégie antitumorale efficace (61). Plus de 40 ans après, le bien-fondé de sa théorie qu'il défendait avec obstination, est maintenant clairement

établi et a été à l'origine d'une intense recherche autour des mécanismes et molécules régulatrices de l'angiogénèse (2, 46, 58).

La régulation de l'angiogénèse est un processus complexe, résultant de l'action de facteurs pro- et anti-angiogéniques, activant des voies de signalisation en partie connues et conduisant à l'activation ou à l'inhibition de la croissance des cellules endothéliales des vaisseaux. Les cellules endothéliales qui constituent la paroi des vaisseaux sont maintenues dans un état de quiescence par la présence concomitante de facteurs pro- et anti-angiogéniques. Cet équilibre peut basculer en faveur de l'angiogénèse sous l'effet d'un excès de facteurs pro-angiogéniques ou un défaut de facteurs anti-angiogéniques par un mécanisme de commutation angiogénique ou « *switch angiogénique* » (62). Par ce mécanisme, une tumeur peut induire l'angiogénèse afin de disposer d'apports supplémentaires en oxygène et nutriments, indispensables à son développement. La taille d'une tumeur non vascularisée est inférieure à $2mm^3$ et ses besoins en nutriments et en oxygène sont alors assurés par diffusion simple. Au fur et à mesure de la croissance tumorale, un état hypoxique intratumoral est généré qui induit l'activation de facteurs de transcription inductibles par l'hypoxie (*Hypoxia-inducible factors*, HIF), en particulier HIF-1α, la transcription de gènes cibles et la sécrétion de ces facteurs de croissance pro-angiogéniques (46, 58, 63). L'hypoxie induit l'expression par les cellules tumorales et le microenvironnement tumoral de facteurs pro-angiogéniques, principalement le VEGF-A (46), qui enclenche le « switch angiogénique ». Via ce « switch », la tumeur acquiert la capacité d'induire le bourgeonnement et la croissance de nouveaux vaisseaux à partir des vaisseaux préexistants (46, 62). D'autres signaux capables d'initier ce « switch » incluent le stress métabolique ou mécanique, ou des réponses immunitaires ou inflammatoires, qui vont, via l'activation de l'angiogénèse, permettre l'apport en oxygène et en nutriments nécessaires à la croissance de la tumeur (58). Les facteurs pro- et anti-angiogéniques peuvent émaner des cellules tumorales elles-mêmes mais aussi des cellules endothéliales et stromales, du sang et de la matrice extracellulaire (64). Leur contribution relative peut changer selon le type et la localisation tumorale, ainsi qu'en fonction de la progression tumorale, la régression ou la rechute. Ces facteurs sont des cytokines ou des facteurs de croissance qui agissent directement au niveau des cellules endothéliales ou indirectement via des cellules « accessoires » (monocytes, macrophages, péricytes et cellules stromales) (65-68).

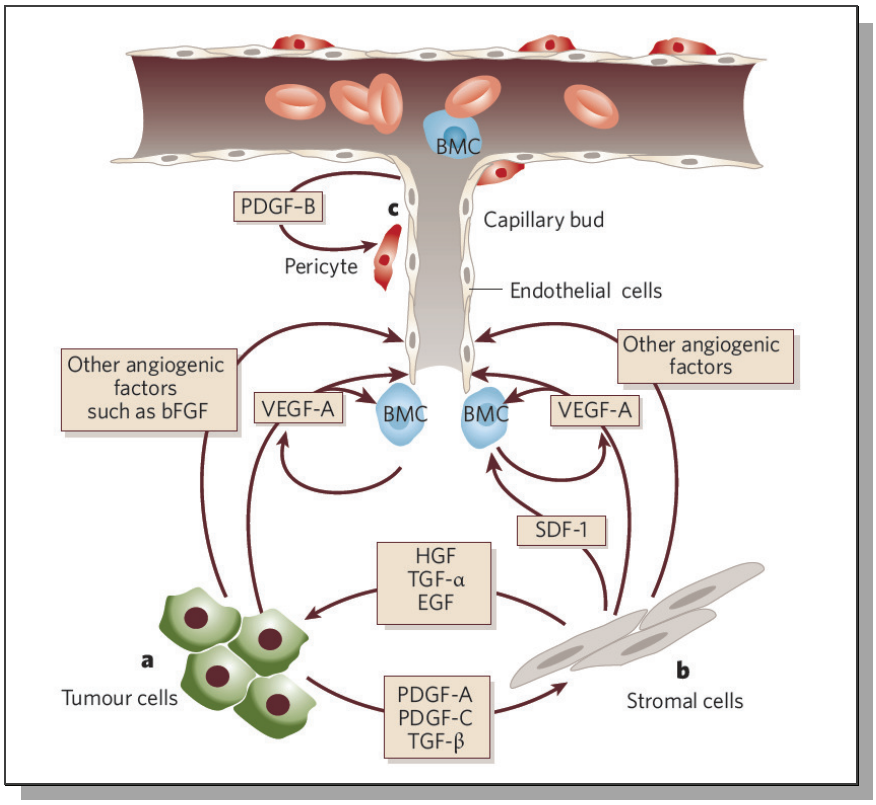

Figure 1. Mécanismes moléculaires et cellulaires de l'angiogénèse (extrait de Ferrara & Kerbel Nature 2005 (1)). Les cellules tumorales et les cellules du stroma produisent du VEGF-A ainsi que d'autres facteurs de croissance pro-angiogéniques qui vont stimuler la prolifération et la migration des cellules endothéliales.

Facteur clé de l'angiogénèse, la famille de VEGF joue un rôle essentiel dans la migration et la prolifération des cellules endothéliales (69). Au sein de cette famille de protéines comprenant les VEGF-A à –E, mais aussi les facteurs placentaires PlGF (*Placental Growth Factor*) 1 et 2, le VEGF-A ($VEGF_{165}$) est l'acteur principal. Les effets biologiques «du» VEGF nécessitent sa fixation sur des récepteurs à activité tyrosine kinase (VEGFR -1 à -3) ou sans activité kinase (neuropilines NRP1 et 2). Le récepteurs VEGFR-1 (dont les ligands incluent le VEGF-A, -B, et le PlGF) et VEGFR-2 (dont les ligands incluent le VEGF-A, -C, et –D) sont impliqués dans les effets mitogènes, dans l'angiogenèse et dans la perméabilité vasculaire du VEGF, tandis que VEGFR-3 est plus spécifiquement impliqué dans la lymphangiogenèse (46, 69). Les récepteurs VEGFR-1 et -2 sont exprimés de façon prédominante sur l'ensemble des cellules endothéliales sauf celles des vaisseaux cérébraux. Le VEGF est produit essentiellement par les cellules tumorales et le stroma et agit de façon prédominante via le récepteur VEGFR-2 pour stimuler la prolifération et la migration endothéliale. Le VEGFR-1 est aussi exprimé sur des cellules souches hématopoïétiques, les cellules musculaires lisses vasculaires, les monocytes et bien que sa contribution à l'angiogénèse ne soit pas parfaitement éclaircie, il a été montré que ce récepteur coopère directement avec le VEGFR-2 par une hétérodimerization (46).

D'autres facteurs pro-angiogéniques impliqués lors des différentes étapes de l'angiogénèse peuvent être regroupés suivant leur principal rôle physiologique, dont quelques-uns sont cités ci-dessous:

- Les facteurs de croissance : PDGF (*Platelet-Derived Growth Factor*), FGF (*Fibroblast Growth Factor*), HGF (*Hepatocyte Growth Factor*), TGF-α et TGF-β (*Tumor Growth Factor*), EGF (*Epidermal Growth Factor*), IGF-1 (*Insulin Growth Factor*);
- Les médiateurs de l'inflammation : TNF-α (*Tumor Necrosis Factor*), IL-8, IL-3 (*Interleukin*);
- Les facteurs hématopoïétiques : Erythropoïétine, G-CSF (*Granulocyte Colony Stimulating Factor*) et GM-CSF (*Granulocyte Macrophage Colony Stimulating Factor*).

De nombreux inhibiteurs de l'angiogénèse ont également été identifiés comme l'angiostatine, l'endostatine, la TSP-1 (*Thrombospondin-1*), l'IFN-α, -β et –γ (*Interféron*), l'IL-12, l'Ang-2 (*Angiopoïétine-2*), les TIMP-1 et -2 (*Tissue Inhibitor of Metalloproteinase*) (69).

Les vaisseaux sanguins tumoraux diffèrent des vaisseaux sanguins normaux : ils sont immatures et de diamètre irrégulier, sont fragilisés et désorganisés, manquant de la stabilité conférée par des cellules perivasculaires (58) et peuvent même dans certains cas manquer de cellules endothéliales (70). La perméabilité des vaisseaux est anormale et excessive, favorisant le phénomène d'extravasation plasmatique et de ce fait, la formation de métastases à distance. Plusieurs mécanismes sont décrits et semblent être impliqués de façon non-exclusive dans l'angiogénèse tumorale (58) (Figure 2). Les vaisseaux sanguins tumoraux peuvent se développer par co-option, bourgeonnement (« *sprouting* ») des vaisseaux sanguins existants et recrutement de cellules endothéliales (71). Il a aussi été montré que les vaisseaux tumoraux pouvaient être composés de cellules tumorales ou d'une mosaïque de cellules tumorales et de cellules endothéliales. Dans des modèles murins de tumeurs de côlon spontanées ou issues de xénogreffes, 15% des vaisseaux sont en mosaïques composées de cellules tumorales et endothéliales (72). La transdifférenciation de cellules tumorales en cellules endothéliales, appelée « vasculo-mimétisme » a été décrite par Maniotis (73). De plus, il y aurait une coopération entre les cellules d'origine tumorale et les vaisseaux sanguins (74).

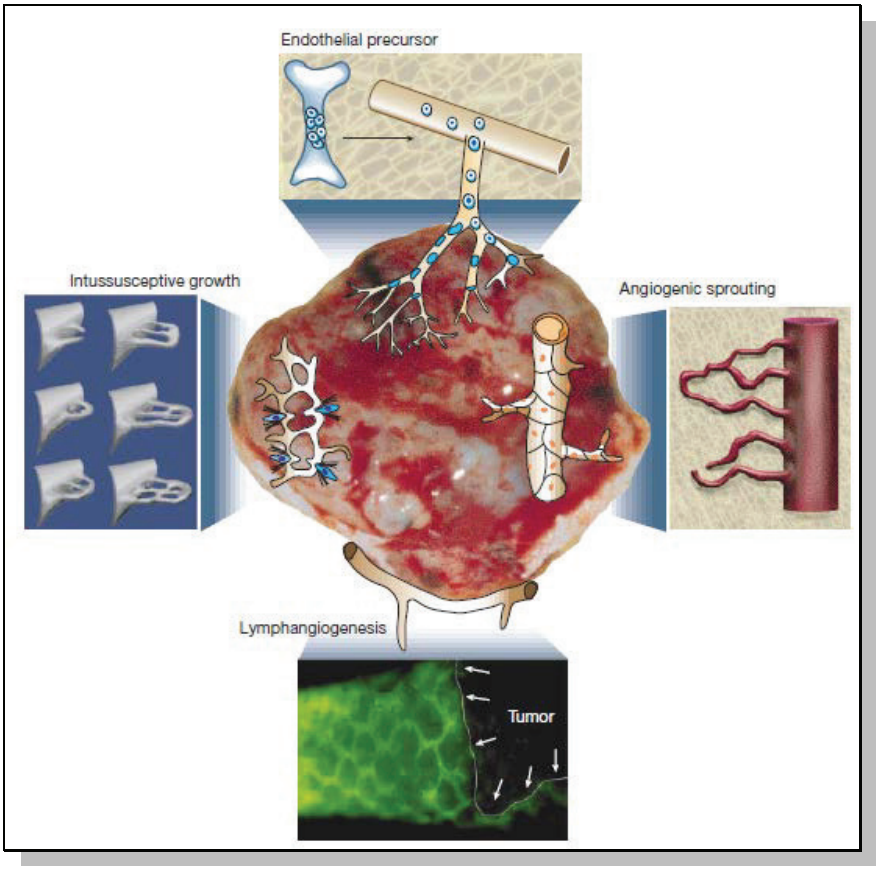

Figure 2. Mécanismes impliquées dans l'angiogénèse tumorale (extrait de Carmeliet & Jain Nature 2000 (58) : « sprouting » ou bourgeonnement de cellules endothéliales pour former de nouveaux réseaux vasculaires; remaniement par insertion de tissu interstitiel dans la lumière de vaisseaux préexistants appelé « intussusception » ; contribution fonctionnelle de progéniteurs endothéliaux mobilisés depuis la moelle osseuse ; les vaisseaux lymphatiques participent au drainage des liquide interstitiel et permettent l'échappent des cellules tumorales pour disséminer à distance.

L'angiogénèse a longtemps été interprétée comme un processus exclusivement local, considéré comme le principal paradigme pour la néo-vascularisation tumorale. Cependant depuis une dizaine d'années, de nouveaux éléments ont amené la révision de ce modèle. De multiples populations cellulaires recrutées à distance par ces facteurs pro-angiogéniques circulent dans le sang et sont capables de migrer au site tumoral où elles assurent des fonctions spécifiques dans la néoangiogénèse (20-22). Parmi celles-ci, les CEP et les CEC ont été l'objet d'un intérêt particulier parce qu'ils étaient susceptibles de refléter directement un processus actif de néoangiogénèse voire de jouer un rôle physique dans la formation des vaisseaux tumoraux (14, 75, 76).

1.3 Les traitements anti-angiogéniques

L'émergence de la notion de thérapie anti-angiogénique dans les années 1970 s'avère être une approche séduisante puisqu'il s'agit de s'attaquer aux vaisseaux tumoraux indispensables à la croissance et la progression tumorale et composés de cellules endothéliales ayant proliféré et migré en réponse à des stimuli induits par la tumeur. Ces stratégies ont été développées pour inhiber la néovascularization et/ou détruire des vaisseaux tumoraux existants directement par l'inhibition des cellules endothéliales ou indirectement par l'inhibition des molécules pro-angiogéniques produites par la tumeur (1). Les stratégies anti-angiogéniques impliquent aussi un accès direct des agents thérapeutiques sur leur cible mais aussi un moindre risque de résistance, les cellules endothéliales étant supposées stables sur le plan génétique (2).

Le rôle essentiel du VEGF a très tôt été mis en exergue comme cible moléculaire de choix mais il faudra attendre 2003 pour que le concept de thérapie moléculaire ciblant l'angiogénèse soit confirmé avec la validation clinique du bévacizumab dans le cancer colorectal métastatique en association avec une chimiothérapie (38). Depuis, un grand nombre d'essais cliniques de phase III avec le bévacizumab en combinaison avec une chimiothérapie ont démontré un bénéfice en terme de survie globale ou survie sans progression dans les cancers métastatiques colorectaux, du sein, et du poumon (5, 38, 40, 41, 77). Si la preuve de l'intérêt du ciblage moléculaire de l'angiogénèse dépendante de la voie VEGF/VEGFR est venue de l'approche par anticorps monoclonal se liant au ligand VEGF (bévacizumab) (36), il convient de noter que la majorité des nouvelles thérapeutiques moléculaires actuellement développées ou en cours de développement dans ce domaine repose maintenant sur des inhibiteurs multi-cibles dirigés contre l'activité tyrosine kinase des récepteurs VEGFR et des autres récepteurs de facteurs de croissance pro-angiogéniques. Parmi les agents ciblant le VEGFR, deux molécules, le sunitinib et le sorafenib, méritent d'être soulignées dans le contexte de ce travail. Le sunitinib (Sutent®) est un inhibiteur multikinase de VEGFR 1-3, de c-Kit (le récepteur au *stem cell factor*) et de PDGFRα/β. L'étude de phase III ayant permis l'autorisation du sunitinib en 1ère ligne de traitement des cancers du rein métastatiques a comparé la molécule au schéma de référence avec l'interféronα et a montré un avantage significatif pour le sunitinib sur l'interféronα à la fois en termes de réponse objective (31% *versus* 6%, p<0,001) et de survie sans progression (11 mois *versus* 5 mois, p<0,001) (42). D'autre part, le sorafenib (Nexavar®) est un inhibiteur multikinase de VEGFR 2-3, PDGFR, Raf kinase et de c-Kit. Cette molécule se situe aujourd'hui en deuxième ligne dans le cancer du rein métastatique après échec d'une immunothérapie suite

aux résultats d'une étude de phase III contre placébo montrant une différence significative en termes de survie médiane sans progression, mais sans amélioration de la survie globale (43). Dans un travail présenté plus loin, nous avons évalué les taux de CEC et CEP chez des patients ayant d'un cancer du rein métastatique et traités par un de ces deux agents anti-angiogéniques (voir Résultats : Article 2).

Malgré une efficacité thérapeutique avérée qui se traduit par une stabilisation ou plus rarement une fonte tumorale dans de nombreux types tumoraux, l'utilisation en clinique des agents anti-angiogéniques soulève de nombreuses questions (5, 6, 8-10, 78). Compte tenu de la multiplicité des voies moléculaires impliquées dans la néoangiogénèse et vasculogénèse tumorale, de leur complexité et de leur redondance, les mécanismes d'action des agents anti-angiogéniques actuellement approuvés restent mal compris. Plusieurs mécanismes d'actions non exclusifs dont l'importance est probablement variable selon les types de tumeurs sont supposés et comprennent : (i) l'inhibition de la formation de nouveaux vaisseaux intra-tumoraux; (ii) une régression de la vascularisation tumorale en place; (iii) une modification de la perméabilité vasculaire et une vasoconstriction avec un effet de « normalisation » du système vasculaire (79) permettant potentiellement une meilleure délivrance des cytotoxiques ; (iv) le blocage du recrutement au site tumoral des populations cellulaires issues de la moelle osseuse et en particulier des progéniteurs endothéliaux d'origine médullaire; et (v) possiblement enfin une action directe sur les cellules tumorales (8, 9). L'hypothèse selon laquelle les agents anti-angiogéniques entraîneraient une « normalisation » des vaisseaux tumoraux augmentant ainsi leur capacité à délivrer la drogue cytotoxique a longtemps prévalu pour expliquer leur efficacité thérapeutique en association à la chimiothérapie (8, 79). Cette hypothèse ne permettait pas cependant d'expliquer pourquoi certains agents, comme le bévacizumab, en combinaison avec une chimiothérapie entrainent un bénéfice clinique dans certains types de tumeurs et pas d'autres, ni pourquoi d'autres drogues (notamment les inhibiteurs de l'activité tyrosine kinase) présentent une efficacité moins aboutie dans cette situation, leur activité principale étant observée en monothérapie (5, 8, 80).

De plus, dans la très grande majorité des cas et en dépit de traitements anti-angiogéniques puissants et d'une réponse initiale au traitement, les tumeurs repoussent et la maladie progresse. Dans d'autres cas plus rares, aucun bénéfice n'est observé. Selon Bergers et Hanahan, deux principaux modes de résistance aux agents ciblant la voie du VEGF (et possiblement d'autres inhibiteurs de l'angiogénèse) pourraient rendre compte de ces observations cliniques (4). Le

premier cas est une situation de non réponse par résistance intrinsèque préexistante au traitement. Dans le second cas, la progression tumorale qui succède à une période initiale mais transitoire de contrôle de la maladie reflète un mécanisme adaptatif de résistance par lequel la tumeur va s'adapter à la présence de l'anti-angiogénique et développer des moyens alternatifs et compensatoires pour se développer en contournant l'inhibition de la cible thérapeutique. Quatre mécanismes principaux de résistance adaptative ont été proposés : (i) l'activation de voies de signalisation pro-angiogéniques alternatives, (ii) le recrutement de cellules pro-angiogéniques d'origine médullaire et en particulier les CEP, (iii) l'augmentation de la couverture vasculaire en péricytes susceptibles d'atténuer la dépendance au VEGF, et (iv) l'activation de processus d'invasion et de métastases pour accéder au système vasculaire normal avoisinant (4).

L'identification de biomarqueurs pourrait ainsi aider à répondre aux nombreuses interrogations soulevées par l'utilisation des agents anti-angiogéniques en clinique. Comment améliorer les effets bénéfiques de ces drogues ? Pourquoi sont-ils limités à certaines pathologies ou certains patients ? Comment et quand associer ces drogues pour améliorer leur efficacité ? Est-il possible de stratifier et sélectionner les patients en fonction de biomarqueurs qui permettront de prédire ou orienter vers le traitement qui aura le plus de chance de succès ? La réussite des stratégies anti-angiogéniques repose très certainement sur l'identification et la validation de biomarqueurs qui permettront de déterminer la dose optimale biologique, d'identifier la séquence appropriée et les schémas thérapeutiques d'association optimaux, de prédire la réponse à ce type de traitement, d'anticiper l'absence de réponse ou l'échappement, voire d'orienter vers une autre molécule anti-angiogénique de 2ème ligne (7). L'efficacité de ces molécules ne sera aussi véritablement améliorée que si les mécanismes de résistance sont décryptés et que des drogues qui les ciblent sont intégrées dans l'arsenal thérapeutique avec une chronologie efficace. De ce fait, des biomarqueurs de résistance ou de non réponse offrant une explication mécanistique sont tout aussi importants que des biomarqueurs de réponses aux traitements anti-angiogéniques (10, 11, 49, 54, 78).

Les travaux réalisés dans le Laboratoire de Recherche Translationnelle, Unité INSERM U981, et dans le cadre de cette thèse à la fois chez l'homme (voir Resultats : Articles 2 et 3) et chez la souris (voir Resultats : Article 4) apportent des arguments en faveur du rôle potentiel des CEC et CEP en tant que biomarqueurs prédictifs de réponse aux stratégies anti-angiogéniques et de possiblement de résistance.

1.4 Les agents anti-vasculaires

A la différence des agents anti-angiogéniques qui empêchent la formation de néovaisseaux, les agents anti-vasculaires ciblent les vaisseaux tumoraux préexistants dans l'objectif d'entraîner leur déstabilisation et leur occlusion (81). Le terme d'agents anti-vasculaires tend actuellement à être remplacé dans la littérature par une dénomination plus précise de leur mécanisme d'action : *vascular disrupting agents* (VDA) (81, 82).

La spécificité d'action des VDA qui épargnent les vaisseaux des tissues normaux, est expliquée par une activité préférentielle sur les cellules endothéliales en prolifération au sein des lésions tumorales et la vulnérabilité particulière des vaisseaux tumoraux, immatures et caractérisés par une architecture, une perméabilité et une fragilité anormales (81-83). Le collapsus vasculaire résulterait de l'effet de changements morphologiques et fonctionnels au niveau du cytosquelette des cellules endothéliales et de l'augmentation accrue de la perméabilité vasculaire des vaisseaux tumoraux. C'est l'augmentation de la perméabilité vasculaire qui serait un des principaux mécanismes entrainant une diminution importante du flux sanguin et qui déterminerait la susceptibilité individuelle des différents types de tumeurs aux VDA (83-85). L'administration d'un VDA est accompagnée d'autres phénomènes, dont l'activation plaquettaire, la coagulation, l'extravasation et l'augmentation des molécules d'adhésion sur les cellules endothéliales pour l'attraction de polynucléaires neutrophiles, qui peuvent contribuer aux effets cytotoxiques de ces drogues (81).

Le groupe le plus important de composés développés dans le domaine des agents VDA est représenté par la famille des combrétastatines, un dérivé naturel du *Combretum caffum* (arbre sud-africain), qui cible la tubuline en se liant sur le même site que la colchicine (81). La combrétastatine A-4 était le composant le plus actif mais c'est une forme plus soluble, la combrétastatine A-4 phosphate (CA-4-P) qui a été développé et étudié plus activement car moins toxique (83). Le mécanisme d'action des agents ciblant la tubuline est de prévenir la polymérisation de la ß-tubuline et donc d'inhiber la formation des microtubules, éléments essentiel et dynamique du cytosquelette. Pour une dose modérée de CA-4-P, les modifications rapides de la morphologie et des propriétés d'adhésion des cellules endothéliales induites par la drogue sont responsables d'une réduction significative du flux sanguin détectable dans les modèles animaux dans les 5 minutes qui suivent son administration, avec un collapsus complet du flux sanguin intra-tumoral après 20 minutes (83). De nombreux agents thérapeutiques utilisés en chimiothérapie conventionnelle présentent une activité anti-tubuline: les vinca-alcaloïdes, les

taxanes et la colchicine (81). Ces composés présentent sur des modèles expérimentaux une action déstabilisante des cellules endothéliales et ont suscité un grand intérêt pour leurs effets potentiels anti-angiogéniques et anti-vasculaires en clinique (86).

En entraînent un collapsus brutal des vaisseaux tumoraux déjà établis, les agents VDA entraînent une privation des cellules tumorales en oxygène et en nutriments et induisent une ischémie et une nécrose importantes, particulièrement marquées dans les zones centrales de la tumeur (81, 82, 84, 87). Cependant, ces drogues présentent la caractéristique d'épargner en périphérie de la tumeur des cellules dont la nutrition est assurée par les vaisseaux normaux avoisinants situés au contact de la lésion et supposés moins sensibles aux VDA (81, 83, 87, 88). L'observation expérimentale de la repousse rapide des tumeurs à partir de cette couronne périphérique a été immédiatement corrélée à une efficacité très limitée de ces drogues administrées seules (81, 84, 87, 89). Ce constat a conduit à poser d'emblée la question de la place stratégique de ces drogues et de leur association à d'autres thérapies (88).

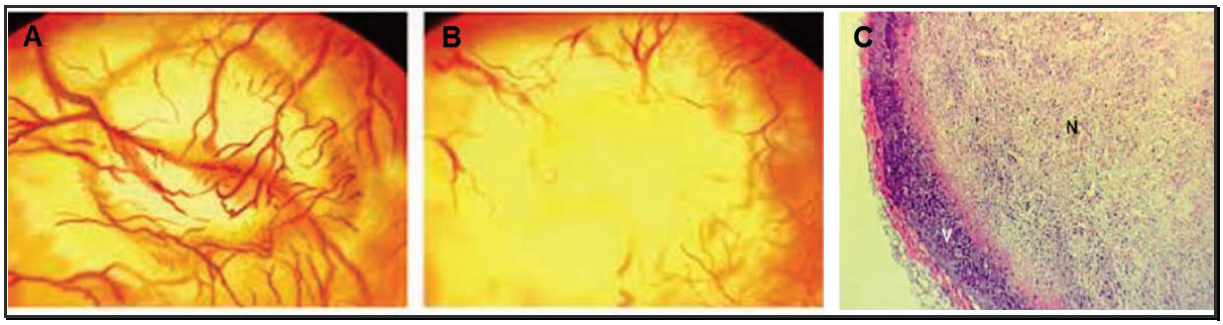

Figure 3. Visualisation des effets des agents anti-vasculaires dans un modèle murin (extrait de Tozer *et al*. Nat Rev Canc 2005 (81). Image d'une tumeur en chambre « optique » avant traitement par VDA (A) et 1h après (B) avec collapsus vasculaire central intra-tumoral. (C) Coupe histologique en H&E d'une tumeur après traitement par VDA avec couronne périphérique de cellules tumorales viables.

Différentes stratégies visant à inhiber la repousse tumorale à partir de cette couronne périphérique ont été explorées à la fois en préclinique (90-92) et en clinique (93-95). L'association d'un VDA avec un agent anti-angiogénique trouvait son rationnel dans le fait que cette repousse tumorale est dépendante d'un processus angiogénique (81, 88) qui pouvait être inhibé par le bévacizumab (96) ou le sunitinib (97) dans certains travaux précliniques. Une hypothèse mécanistique à ces données a été fournie par les travaux de Shaked *et al*. montrant que chez des animaux porteurs de tumeurs, un traitement par un VDA induisait une mobilisation accrue et immédiate (en 4 heures) de CEP qui étaient recrutés au niveau de la couronne

périphérique tumorale et semblaient capables d'y promouvoir la croissance tumorale (76). Le blocage des CEP avec un anticorps dirigé contre le VEGFR-2 augmentait l'activité anti-tumorale du VDA mettant ainsi en lumière le rôle des CEP dans les mécanismes de résistance à ces types de drogues. Ce dogme mécanistique de mobilisation immédiate de CEP induite par la drogue VDA a longtemps prévalu. A la suite du travail publié par Shaked *et al*., les travaux de notre équipe ont montré pour la première fois chez l'homme, la mobilisation de CEP chez des patients traités par un VDA, l'ombrabuline, dans un essai de phase I (98). Au cours de mon travail de thèse, j'ai exploré dans différents modèles murins le rôle des CEC et CEP dans le mécanisme d'action des VDA ainsi que le rôle fonctionnel des CEP dans la résistance à ces drogues. Grâce à ce travail, notre équipe a démontré pour la première fois l'existence d'une mobilisation tardive de CEP induite par le VDA et spécifique de la présence de tumeur dans les modèles murins ainsi que chez l'homme. Comme développé plus loin, nous avons montré que cette mobilisation tardive de CEP étaient, contrairement aux travaux de Shaked *et al*., probablement la véritable source des phénomènes de résistance et de réparation vasculaire induites par la drogue et donc la cible préférentielle des stratégies d'association aux anti-angiogéniques destinées à potentialiser l'activité anti-tumorale des VDA (31).

1.5 Les cellules endothéliales circulantes et les progéniteurs endothéliaux circulants

1.5.1 Les cellules endothéliales circulantes (CEC)

1.5.1.1 Définition des CEC

Des cellules avec une morphologie endothéliale ont été observées dans la circulation sanguine il y a plus de 40 ans (99). La nature endothéliale de ces cellules a été confirmée 20 ans après par des travaux d'immunohistochimie (IHC) (100, 101), et leur énumération a démontré que des taux élevés de cellules endothéliales circulantes (CEC) étaient observés dans de multiples situations cliniques où existent des lésions vasculaires (12, 13, 102).

Les CEC sont définies comme des cellules matures qui se détachent des vaisseaux en réponse à divers stimuli physiopathologiques et sont emportés dans la circulation (12, 13, 18). Grâce au développement d'anticorps monoclonaux, ces cellules ont été caractérisées et identifiées comme un marqueur secondaire à un traumatisme mécanique de l'endothélium (101). Elles présentent

une grande taille (10-50µm), expriment le facteur de von Willebrand (vWF) et plusieurs marqueurs membranaires endothéliaux, en particulier le CD146 (S-Endo1) (101), mais aussi le CD31 (PECAM-1), CD144 (VE-cadhérine), CD202-b (Tie-2) ainsi que le CD34, CD13, CD105 (endogline), CD117 (c-kit) et les récepteurs au VEGF, notamment le récepteur 2 (VEGFR-2, KDR ou Flk-1) (13, 14, 18, 103). Elles sont négatives pour les marqueurs hématopoïétiques CD45 et CD14 et les marqueurs des progéniteurs CD133 (13, 103).

1.5.1.2 Détection des CEC

Les CEC représentent entre 0,01% et 0,001% des cellules mononuclées et appartiennent à la catégorie des évènements très rares du sang périphérique ce qui rend leur quantification précise très difficile (104, 105).

Les techniques de détection des CEC, initialement basées sur l'examen de la morphologique et le marquage par immunofluorescence du vWF ont considérablement évolué grâce au développement d'anticorps monoclonaux (101, 106). L'identification du marqueur S-Endo1 (CD146) par Dignat-George *et al*. a conduit au développement d'une méthode d'immunocapture à base de billes magnétiques recouvertes de ce marqueur (101). Cette stratégie d'immuno-séparation magnétique (IMS) est considérée comme la technique de référence: elle est utilisée depuis plus de 15 ans par les équipes travaillant sur les pathologies vasculaires et a récemment été standardisée (103). Etant donné que le CD146 est aussi exprimé par une large sous-population de cellules non-endothéliales, en particulier des lymphocytes T, cette méthode doit être associée à une seconde étape de marquage en fluorescence avec un marqueur spécifique de l'endothélium et un comptage en microscopie pour identifier les éléments endothéliaux parmi les cellules isolées. Les valeurs de CEC mesurées en IMS sont inférieures à 10 cellules/ml chez le sujet sain (12, 18, 101, 105). Cependant, elle est techniquement lourde et donc peu adaptée à l'évaluation en routine pour des études cliniques.

Des méthodes en cytométrie de flux (CMF) présentent l'avantage d'être plus souples et plus faciles à mettre en œuvre pour des études multicentriques, et adaptées aux prélèvements réalisés en routine. Compte tenu de l'absence de marqueur spécifique pour la détection de CEC, la CMF présente l'avantage de pouvoir combiner plusieurs marqueurs pour distinguer cette sous-population. Au vue de la rareté des cellules endothéliales circulantes (CEC et CEP), la CMF nécessite l'utilisation de procédures très rigoureuses de marquage et d'analyse adaptées à la

quantification d'évènements très rares, procédures qui sont éventuellement précédées d'une étape d'enrichissement (104, 107). Néanmoins, à la différence de l'IMS qui a montré une bonne reproductibilité inter-laboratoire pour l'énumération des CEC (12, 103, 105), il n'y a pas encore de consensus sur la combinaison de marqueurs et sur les stratégies de marquage et d'analyse des CEC en CMF (14, 107-109).

Utilisant une méthode de marquage sur sang total suivie d'une analyse en CMF, une équipe italienne a décrit pour la première fois la présence de taux très élevés de CEC dans le cancer, rapportant des valeurs moyennes de 39 100 CEC/ml chez des patientes porteuses de cancer du sein métastatiques et de 7900 CEC/ml chez des individus sains (110). Cette publication a été le point de départ d'une série de publications rapportant avec cette méthode de CMF la présence de taux très élevés de CEC chez des patients atteints de cancers métastatiques (14, 111, 112). Les différences très importantes des taux de CEC obtenus par les deux techniques, en particulier chez des sujets sains avec des valeurs environ mille fois plus élevée par CMF que par IMS ont été à l'origine d'une importante controverse opposant les tenants de la méthode en CMF décrite en 2001 (110) et les spécialistes des pathologies vasculaires utilisant l'IMS (103, 105, 113). Ce n'est qu'en 2007 que les travaux de Strijbos *et al*. ont formellement établi que les cellules détectées par la méthode de CMF décrite en 2001 n'étaient pas des cellules endothéliales mais en fait des plaquettes géantes (114). Cette controverse a révélée deux choses : (1) l'ensemble des publications décrivant des CEC détectées par la stratégie en CMF décrite en 2001 chez des patients ayant un cancer était à considérer avec un regard très critique et rapportait probablement des données fausses ; (2) la nécessité d'une méthodologie standardisée pour la détection de CEC.

1.5.1.3 Les CEC, marqueurs de lésion vasculaire

Les CEC sont considérées aujourd'hui comme un marqueur de l'intégrité vasculaire et le nombre de CEC détecté dans le sang périphérique peut être considéré comme un indicateur *ex vivo* de l'importance de la lésion vasculaire (12, 13, 18, 19, 113) .

Chez l'homme, une élévation des taux de CEC attestent dans un large spectre de maladies de l'existence d'une altération ou d'un dysfonctionnement de l'endothélium : maladies cardiovasculaires, ischémiques, infectieuses ou encore auto-immunes (vascularites, transplantation (rein) et greffe (moelle allogénique)) (12, 13, 18, 19, 102, 115, 116). Des taux

élevés de CEC sont associés à une élévation des marqueurs plasmatiques reflétant l'activation endothéliale comme le vWF, le t-PA, la thrombomoduline soluble ainsi que des molécules d'adhésion solubles (ICAM-1, VCAM-1) (117). De façon générale, qu'il s'agisse d'une réponse aigüe, inflammatoire ou d'un processus chronique, le taux de CEC reflète directement l'importance de la lésion endothéliale et de l'activité de la maladie. Outre l'aspect quantitatif, les CEC sont extrêmement hétérogènes sur un plan cytologique, probablement en raison de la variété des lits vasculaires d'origine, mais aussi de la variété des stimuli pathologiques. Leur libération dans le circulation sanguine peut résulter de plusieurs mécanismes qui ne sont pas exclusifs, tels des agressions mécaniques mais aussi de multiples stimuli pathologiques (cytokines pro-inflammatoires, facteur de croissance pro-angiogéniques, stress oxydatif), la diminution des propriétés d'adhésion faisant intervenir des molécules de la famille des intégrines, la diminution de l'attachement aux protéines de la matrice (fibronectine, laminine) ainsi que l'activation du programme d'apoptose cellulaire (13, 113).

1.5.2 Les progéniteurs endothéliaux circulants (CEP)

1.5.2.1 Physiopathologie des CEP

En 1997, les travaux de Asahara *et al*. ont montré pour la première fois que des progéniteurs endothéliaux originaires de la moelle osseuse pouvaient circuler, s'incorporer à des vaisseaux pour complémenter l'angiogénèse assurée par l'endothélium existant et ainsi contribuer à la néo-vascularisation sur des sites d'ischémie par un mécanisme de vasculogénèse, paradigme qui était auparavant limité à l'embryogénèse (16, 17). La fraction des progéniteurs endothéliaux détectée dans la circulation, les progéniteurs endothéliaux circulants (CEP), est dotée d'un potentiel prolifératif très élevé et leur intérêt thérapeutique dans la vascularisation d'organe et la régénération tissulaire est activement étudié (75). Mobilisés sous l'effet de facteurs comme le VEGF (23, 118) et le SDF-1 (119, 120), les CEP sont recrutés par un phénomène de « homing » vers les sites de lésion vasculaire ou d'ischémie, où leur incorporation aux néovaisseaux est corrélée à une amélioration de la fonction et de la viabilité tissulaire (15, 121). Depuis ces premiers travaux, les CEP ont été largement explorés pour leur rôle dans la réparation des parois vasculaires, la néoangiogénèse au niveau des sites d'ischémie, ainsi que dans la croissance et la néoangiogénèse tumorale et le développement de métastases (21, 23, 75, 122-124).

1.5.2.2 Détection des CEP

La fraction circulante des CEP est significativement inférieure à celle des CEC et représente environ 0,0001% des cellules mononuclées du sang périphérique (19, 125, 126). Les CEP peuvent être identifiés grâce à des caractéristiques phénotypiques et fonctionnelles (17, 127-129). Ces cellules co-expriment des marqueurs vasculaires (CD146, CD144, CD202b, VEGFR2) ainsi que des marqueurs communs aux cellules hématopoïétiques et endothéliales (CD34, CD117, CD133) (19, 128-131). Le niveau d'expression de CD45 (négatif ou intermédiaire) sur ces cellules est controversé et varie selon les groupes, le débat étant de déterminer si les CEP sont identifiés dans la fraction de progéniteurs d'origine médullaire $CD45^{dim}$ ou la fraction très rare de progéniteurs $CD45^{-}$ (14, 17, 128, 132-134). La découverte de CD133, un antigène qui identifie des cellules souches primitives, a permis dans un premier temps de distinguer les CEP des CEC puisque ces derniers semblaient perdre l'expression de CD133 au cours de leur maturation (104, 135). Cependant, l'expression de CD133 sur des CEP évalués dans des tests de culture a été par la suite remise en question (136, 137). *In vitro*, les progéniteurs endothéliaux d'origine médullaire ont la capacité de proliférer et de se différencier en cellules adhérentes appartenant à la lignée endothéliale. Ils constituent cependant un groupe hétérogène de cellules (129, 137, 138). En utilisant des cultures *in vitro* et des essais fonctionnels *in vivo*, Yoder *et al*. ont redéfini deux populations distinctes de progéniteurs endothéliaux d'origine médullaire : (1) une sous-population d'origine myéloïde caractérisée par un délai d'apparition en culture rapide, un potentiel prolifératif limité et l'expression de marqueurs à la fois monocytaires et endothéliaux. Ces cellules, plus fréquentes, sont les « *colony forming unit endothelial cells »* (CFU-EC) qui facilitent l'angiogénèse mais ne forment pas de colonies de cellules endothéliales ni de vaisseaux in vivo; (2) une autre population nommée «*endothelial colony forming cells*» (ECFC), dont la fréquence est très faible dans la circulation, correspond aux véritables progéniteurs endothéliaux dotés d'un fort potentiel de prolifération, d'une capacité de différentiation endothéliale stricte et d'un potentiel vasculogénique in vivo (129).

L'hétérogénéité phénotypique et fonctionnelle des CEP, le manque de consensus sur une définition stricte des CEP, l'expression commune de certains marqueurs hématopoïétiques utilisés pour détecter ces cellules ainsi que l'absence de corrélation entre les tests de culture et la CMF, communément utilisée pour détecter ces cellules, ont été sources d'immunophénotypes divergents pour détecter et énumérer de « potentiels » CEP et ont générés des résultats

contradictoires sur le rôle de ces cellules dans les modèles précliniques et chez l'homme (107, 112, 128, 132, 133, 139, 140). La confusion sur l'identité et la fonction des CEP est illustrée par le débat autour du niveau d'expression de CD45 sur ces cellules (28, 29, 98, 134). Gehling *et al*. avaient montré que des cellules progénitrices $CD133^+$ purifiées avaient la capacité de se différencier en cellules endothéliales (141). Après des travaux rapportant que des cellules $CD133^+$ et $VEGFR2^+$ présentes dans la circulation avaient des propriétés fonctionnelles de CEP (135, 141), les CEP ont été définis (et communément acceptés) comme des cellules $CD34^+CD133^+VEGFR2^+$ chez l'homme (14, 135, 142). Cependant, Case *et al*. ont récemment rapporté que cette population ne formaient pas de colonies dans les essais clonogéniques endothéliales ni de vaisseaux *in vivo* mais formaient des colonies dans des conditions hématopoïétiques et exprimaient le marqueur spécifique hématopoïetique CD45 (137). Malgré l'absence de consensus sur la combinaison de marqueurs la plus pertinente et sur les stratégies de marquage et d'analyse en CMF des CEP, qui s'avère être indispensable dans ce domaine (46), l'exploration et l'énumération des CEP en CMF restent très actives dans la recherche sur la néovascularisation tissulaire et la régénération endothéliale dans les modèles expérimentaux et en clinique (133, 134, 143, 144).

1.6 Les cellules endothéliales circulantes et les progéniteurs endothéliaux circulants : biomarqueurs de l'angiogénèse tumorale ?

1.6.1 Les CEC et l'angiogénèse tumorale

De part leur importance dans de multiples pathologies caractérisées par des anomalies vasculaires, les CEC ont rapidement suscité un grand intérêt et ont été activement évaluées en tant que biomarqueurs potentiels de l'angiogénèse tumorale (7, 14). Dans des modèles animaux, des taux élevés de CEC ont été observés par rapport à des animaux sains et étaient corrélés au volume tumoral, suggérant ainsi un rôle dans la néoangiogénèse tumorale (14). Des taux relativement élevés de CEC ont aussi été décrits chez des patients atteints de différents types de cancers comme le lymphome, le mélanome, les gliomes, le cancer du sein, de l'estomac, du colon, du rein, et de la prostate (14, 110, 111, 145-147). Cependant, l'ensemble des travaux étant basés sur la technique de CMF décrite en 2001 et ne pouvant être pris en compte, il y a aujourd'hui peu de données fiables décrivant des CEC chez des patients porteurs de tumeurs (108).

Une stratégie de détection des CEC en CMF a été mise au point dans le laboratoire de Françoise Farace pour mesurer les CEC chez l'adulte et chez l'enfant (28, 148), et est présentée en détail plus loin (voir Méthodes). Dans une première étude, ce test a été utilisé pour mesurer les CEC dans une cohorte de patients adultes porteurs de cancer métastatique ainsi que dans une petite cohorte d'individus sains (148). La valeur médiane des CEC mesurées chez 20 sujets sains était de 6,5 CEC/ml (range, 0-15) et dans l'ordre de grandeur des valeurs obtenues par IMS, globalement inférieures à 10 CEC/ml. Par contre, cette valeur était de 15 CEC/ ml (range, 0-179) chez les 125 patients atteints de cancers métastatiques et significativement différente de celle des sujets sains ($p<0,001$) (148). Dans chaque type de cancer, certains patients présentaient des taux bas de CEC, similaires à ceux des sujets sains.

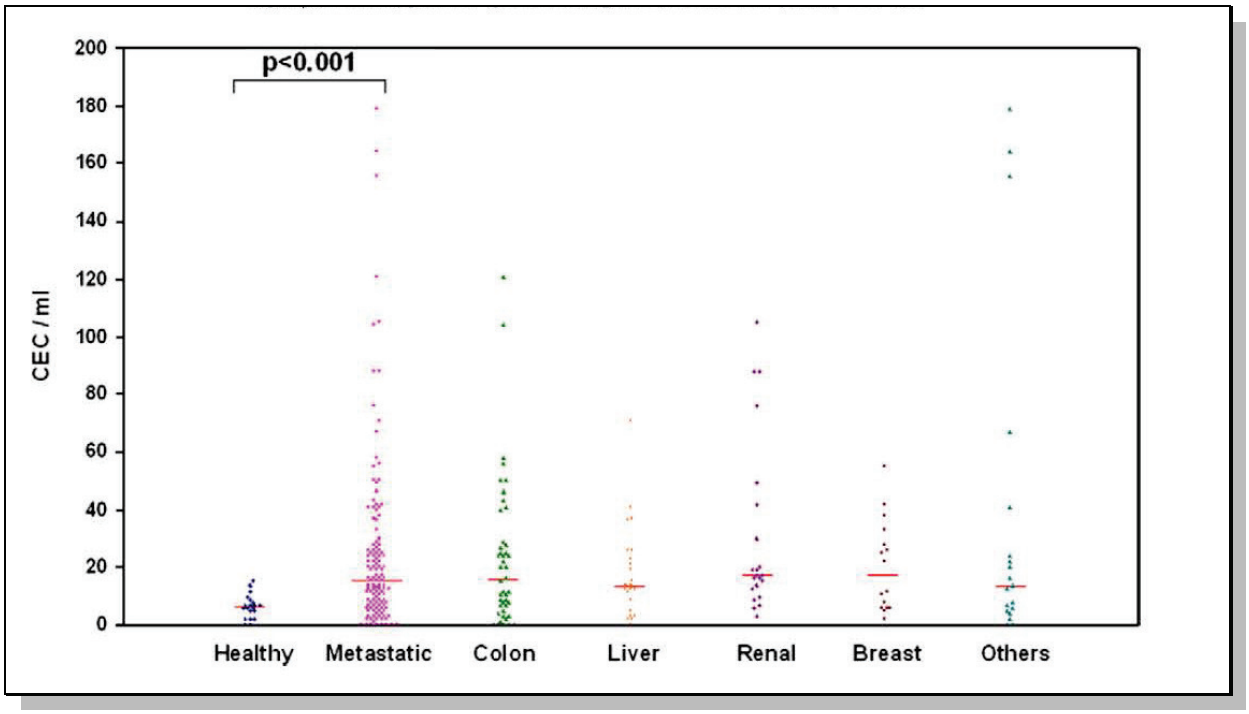

Figure 4. Taux de CEC mesurés en CMF 4 couleurs chez 125 patients atteints de cancers métastatiques et 20 sujets sains (extrait de Jacques *et al*. J Immunol Methods 2008 (148)).

Dans un autre travail, l'équipe de Françoise Farace a comparé dans 154 prélèvements de patients ayant un cancer métastatique les taux de CEC obtenus avec leur stratégie en CMF et les taux de CEC mesurés par le système CellSearch™ (Veridex), une approche semi-automatisée qui repose sur le même principe d'enrichissement par des microbilles magnétiques couvertes de l'anticorps CD146 et le marquage en fluorescence que l'IMS, et qui s'est récemment développée dans les laboratoires spécialisés en oncologie (149). Bien que les deux tests ne détectent pas le même

phénotype ($CD31^+CD146^+CD45^-7AAD^-$ *versus* $CD105^+CD146^+CD45^-7AAD^-$), une bonne corrélation entre les deux méthodes a été observée ($r=0,6$; $p<0,0001$), des taux anormalement élevés de CEC étant généralement également repérés par les deux méthodes avec des valeurs proches de celles rapportées par l'IMS. Dans l'étude réalisée chez 45 patients porteurs de tumeurs solides pédiatriques et 20 enfants sains (voir Résultats : Article 1), nous avons observé que les taux de CEC des patients et des sujets sains étaient proches des valeurs obtenues chez les adultes sains, mais dans cette cohorte plus restreinte, nous n'avons pas observé de différence significative entre les taux de CEC mesurées chez les patients et les enfants sains (28). Depuis, d'autres travaux réalisés dans le laboratoire ont mesuré de façon prospective les CEC chez des patients atteints de cancer, notamment dans le cadre d'études multicentriques, et ont permis de confirmer la stratégie de détection des CEC développée en CMF ainsi que l'existence de taux élevés de CEC avant traitement chez des patients atteints de cancers métastatiques ou avancés (29, 30, 150).

Malgré l'intérêt suscité par ces cellules, la signification clinique des CEC dans le cancer reste encore mal comprise. Même si nos travaux et d'autres de la littérature (145, 146, 149) attestent de l'existence de taux de CEC supérieurs à la normale chez des patients atteints de cancer métastatique ou évolutif, il n'a pas encore été formellement démontré que ces cellules étaient reliées à l'agressivité de la tumeur ou à l'évolutivité de la maladie. L'origine des CEC dans le cancer ainsi que les mécanismes qui aboutissent à leur relargage dans la circulation ne sont pas complètement élucidés. Nous pouvons avancer deux hypothèses non exclusives pour ces observations. D'une part, l'activation prolongée de l'endothélium est à l'origine de perturbations vasculaires chez des patients atteints de cancer. Le «switch angiogénique» associé à la croissance tumorale et la production robuste de facteurs de croissance pro-angiogéniques, tels le VEGF, bFGF, HGF par les cellules tumorales et autres cellules de l'hôte, pourraient être responsables du chimiotactisme des CEC. L'existence d'une régulation autocrine par le VEGF au niveau des cellules endothéliales (151) pourrait expliquer en partie l'accroissement du remodelage vasculaire. Dans ce contexte, les CEC observés à des taux élevés dans différentes pathologies tumorales pourraient provenir des vaisseaux tumoraux et être la conséquence d'un important «turnover» et remodelage de l'endothélium vasculaire tumoral. Alternativement, en tant que marqueur de l'intégrité vasculaire, les CEC pourraient provenir de vaisseaux normaux activés par ces mêmes facteurs pro-angiogéniques et être le reflet d'un processus généralisé et systémique d'activation endothéliale chez les patients ayant un cancer. Dans ce cas, les CEC pourraient être une manifestation non spécifique de la pathologie tumorale à un stade

relativement avancé sans être directement de véritables acteurs du processus de néoangiogénèse tumorale.

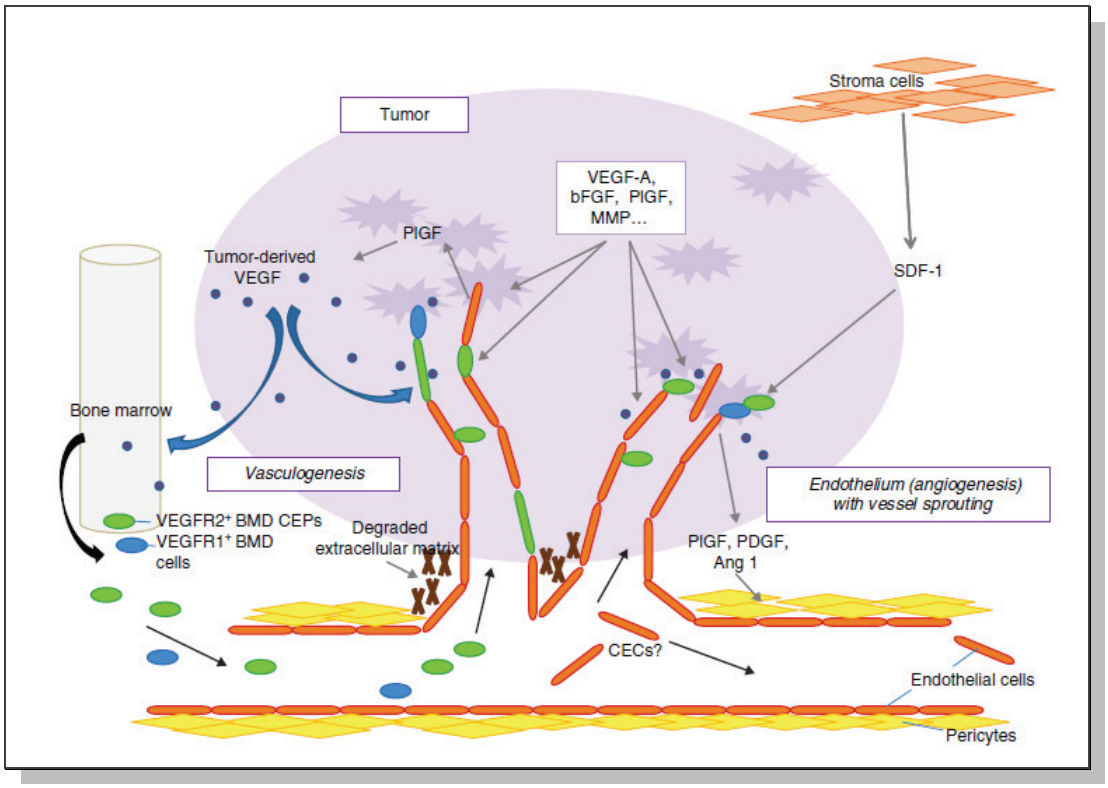

Figure 5. Différents mécanismes de néoangiogénèse tumorale impliquant les CEC et CEP. Les deux principaux modes de vascularisation tumorale des tumeurs solides, le bourgeonnement des vaisseaux avec la migration de cellules endothéliales avoisinantes et la vasculogénèse avec le recrutement de sous-population originaires de la moelle osseuse incluant les CEP VEGFR2^{+} et les hémangioblastes VEGFR1^{+} (extrait de Taylor *et al*. Expert Opin Investig Drugs 2010 (35)).

1.6.2 Le rôle des CEP dans l'angiogénèse tumorale

La première démonstration du rôle des CEP dans la vasculogénèse tumorale a été réalisée en 2001 par l'équipe de Lyden et Rafii (23). Utilisant des souris mutantes pour les facteurs de transcription *Id1* et *Id3*, Lyden *et al*. montrèrent que le recrutement et l'incorporation aux vaisseaux tumoraux de CEP exprimant le VEGFR2 étaient conditionnés par l'expression de ces gènes et indispensables à la néo-vascularisation tumorale (23). Depuis ces premières observations, les CEP ont été largement explorés dans la croissance et néoangiogénèse tumorale ainsi que dans le développement de métastases (22, 123, 124).

Dans le système murin, le rôle de la vasculogénèse dans la néo-vascularisation tumorale a fait l'objet d'une importante controverse centrée sur la contribution des CEP et les mécanismes par lesquels ces progéniteurs intervenaient, par incorporation directe et fonctionnelle dans le vaisseau ou par des effets paracrines au niveau de l'environnement périvasculaire. Une contribution variable des CEP à l'endothélium tumoral a été rapportée dans divers modèles de tumeurs non traitées pouvant aller de moins de 5% jusqu'à 10% voire 50%, la majorité des études montrant des contributions dans les valeurs basses (152-154). Pour tenter de résoudre en partie cette controverse, plusieurs travaux dans des modèles de souris (mutantes pour les facteurs de transcription *Id1* et *Id3)* ont montré que l'intégration fonctionnelle des CEP aux vaisseaux tumoraux était variable selon le grade de la tumeur (plus marquée dans les tumeurs peu différenciées) et le stade de vascularisation (122, 153). De surcroît, l'influence des facteurs pro-angiogéniques sur le devenir des CEP mobilisés était variable selon le site d'implantation de la tumeur. En utilisant un modèle murin de gliome, Aghi *et al*. ont montré que le SDF-1α produit par les cellules tumorales jouait un rôle important dans la mobilisation des CEP et déterminait leur différentiation en péricytes dans une tumeur sous-cutané ou en cellules endothéliales incorporées aux néovaisseaux tumoraux dans une tumeur orthotopique (119). Dans l'ensemble de ces modèles, la nature des tumeurs et en particulier leur capacité à sécréter des cytokines et des facteurs pro-angiogéniques et à créer un microenvironnement capable de promouvoir la vasculogénèse semblent être un facteur déterminant (119, 155).

En 2008, utilisant divers modèles de souris génétiquement modifiées développant des métastases pulmonaires, Gao *et al*. ont montré que les CEP, par l'expression de *Id1*, avaient un rôle capital dans le « switch angiogénique » et la progression des micro-métastases en macro-métastases (124). En complétant les travaux antérieurs de l'équipe de Rafii et Lyden, cette étude soulignait de plus l'importance des interactions entre CEP et progéniteurs hématopoïétiques dans la formation du microenvironnement tumoral et le rôle de ces derniers dans l'initiation de la niche pré-métastatique (124, 156). En parallèle, plusieurs études (157) et en particulier les travaux de Purhonen *et al*. (158) et de De Palma *et al*. (66) rapportaient un rôle très mineur voire inexistant des CEP, réfutaient totalement leur capacité à s'incorporer dans la lumière des vaisseaux tumoraux et mettaient en avant le potentiel pro-angiogénique de précurseurs macrophagiques et/ou des péricytes d'origine médullaire. Plusieurs éléments peuvent rendre compte de ces divergences de résultats comme la variété des modèles tumoraux utilisés et des profils pro-angiogéniques produits, ou les difficultés techniques à localiser précisément ces cellules dans la lumière des vaisseaux ou en position périvasculaire. De plus, la majorité de ces

travaux s'appuient sur des souris génétiquement modifiées pour des gènes spécifiques de l'angiogénèse (*Id1* et *Id3*) ou de l'endothélium (comme Tie2 ou VE cadhérine) pour cibler ou identifier les CEP (66). Cependant, en ciblant potentiellement aussi les progéniteurs hématopoïétiques (qui expriment *Id*) ou les cellules endothéliales vasculaires (qui expriment Tie2 ou VE cadhérine), il est difficile de savoir si l'inhibition de l'angiogénèse observée dans ces études est due exclusivement à la diminution de l'incorporation fonctionnelle des CEP ou si cela résulte de l'absence associée des autres progéniteurs hématopoïétiques ayant un rôle paracrine. Même si l'ensemble de ces travaux illustrent le rôle important des progéniteurs hématopoïétiques dans la néoangiogénèse tumorale, aucun ne permet de confirmer ou écarter formellement le rôle des CEP dans la modulation de ce processus. La principale origine de cette controverse réside donc probablement dans la difficulté à définir phénotypiquement et fonctionnellement les CEP chez la souris et de les distinguer des autres sous-populations de progéniteurs hématopoïétiques avec lesquels elles partagent de nombreux marqueurs en commun et dont les caractéristiques phénotypiques et le rôle restent aussi à éclaircir avec précision (128, 140). Le rôle et la contribution des CEP à la néoangiogénèse tumorale restent à ce jour un sujet très controversé (128, 129, 140, 158-160).

Chez l'homme, l'importance de la vasculogénèse dans la néovascularisation tumorale n'est pas élucidée et les moyens d'aborder cette question restent limités. Plusieurs travaux suggèrent que le rôle des CEP pourrait être plus modeste chez l'homme que chez la souris. Dans un travail réalisé chez des patients ayant développé des cancers secondaires après avoir reçu une greffe de moelle d'individus de sexe opposé, il a été montré que les cellules d'origine médullaire détectées par FISH ne représentaient que 5% des cellules de l'endothélium tumoral (161). Une autre étude montre que chez des patients atteints de cancers du poumon, les CEP $CD133^+$ sont détectés avec une très faible fréquence (0,2%) sur des coupes histologiques de tumeurs (162). L'ensemble de ces résultats sont en accord avec ceux publiés par Lin *et al*. montrant que, chez l'adulte sain, les CEC originaires des parois vasculaires constituent la grande majorité (95%) des cellules angiogéniques circulantes (163). Les CEP sont aujourd'hui activement évalués en clinique en tant que marqueurs de l'intégrité vasculaire, plus particulièrement dans le contexte des maladies cardiovasculaires (19, 164).

Chez les patients atteints de cancer, la plupart des travaux ont consisté à mesurer les CEP par marquage avec une combinaison de marqueurs de surface pour une analyse en CMF. Cependant, comme pour les CEC, il n'y a pas de consensus sur la combinaison de marqueurs la plus

pertinente et sur les stratégies de marquage et d'analyse en CMF, et la majorité des études ont employé la stratégie développée par l'équipe italienne selon les mêmes principes que celle établie pour la détection des CEC (14, 165, 166). Les discordances entre les phénotypes et les taux rapportés de « CEP » potentiels identifiés dans toutes ces études ont abouti à une grande variabilité des données, source de désaccord entre les études et les laboratoires (14, 107, 112, 167-169). Peu de laboratoires se sont réellement donné les moyens de développer une approche en CMF adaptée à la détection d'évènements aussi rares et en particulier d'utiliser des méthodes d'enrichissement pour la mesure des CEP (107, 109, 134, 143, 144).

Comme présentée plus loin, une stratégie de détection des CEP a été mise au point dans le laboratoire selon les mêmes principes que celle établie pour la mesure des CEC, et a servi à l'évaluation de ces cellules chez des patients adultes et des patients pédiatriques porteurs de tumeurs (28-30, 98). Le travail réalisé chez les patients présentant une tumeur pédiatrique, et dont les principaux résultats sont présentés dans la partie Résultats : Article 1, a été le premier à explorer les taux de CEC et de CEP dans le domaine de l'oncologie pédiatrique. Les taux élevés de CEP objectivés chez des patients atteints de différents types de tumeurs grâce aux études réalisées dans le laboratoire ont permis d'avancer l'hypothèse que les CEP pourraient refléter un processus angiogénique agressif et une évolution défavorable et pourraient ainsi avoir un rôle potentiel en tant que biomarqueurs de l'angiogénèse.

Récemment, de nombreux travaux ont montré que d'autres lignées cellulaires pouvaient, comme les CEP, être mobilisées depuis la moelle osseuse par les facteurs pro-angiogéniques produits par le microenvironnement tumoral ou la tumeur en réponse à l'hypoxie comme le SDF-1α (20, 21). Il est ainsi rapporté que des progéniteurs hématopoïétiques d'origine médullaire (*hematopoietic stem cells* (HSC)) peuvent aussi être mobilisés pour contribuer directement ou indirectement à la néoangiogénèse d'origine ischémique ou tumorale (170-176). Parmi celles-ci, les progéniteurs hématopoïétiques $CD34^+$ circulants pourraient moduler l'angiogénèse et des taux élevés ont été corrélés au risque de rechute tumorale (126, 170). Les progéniteurs des péricytes sont eux aussi susceptibles de s'incorporer physiquement aux parois vasculaires (66). Des cellules appartenant à la lignée myéloïde comme les macrophages (*tumor-associated macrophages* (TAM)), les monocytes exprimant le récepteur Tie2 (TEM), et les cellules myéloïdes $GR1^+CD11b^+$ à activité immunosuppressive peuvent aussi être recrutées dans le microenvironnement tumoral périvasculaire et avoir, par des mécanismes paracrines, de puissants effets facilitateurs de la formation de néovaisseaux et de promotion de la

néoangiogénèse (66, 177, 178). Certaines de ces populations cellulaires, même si elles sont présentes en très petit nombre comme les CEP ou les TEM, peuvent avoir des effets extrêmement puissants, « catalytiques » de la progression tumorale. Toutes ces populations sont susceptibles d'intervenir à certaines étapes du processus de néoangiogénèse, d'avoir des fonctions spécifiques dans certains types tumoraux, et d'être les acteurs de mécanismes redondants de néoangiogénèse susceptibles d'assurer à la tumeur un système vasculaire viable et versatile.

1.7 Les cellules endothéliales circulantes et les progéniteurs endothéliaux circulants : biomarqueurs des traitements anti-angiogéniques et anti-vasculaires ?

1.7.1 CEC et CEP : biomarqueurs des traitements anti-angiogéniques

Les CEC et CEP, par leurs rôles et leurs capacités supposés à refléter un effet global résultant du traitement anti-angiogénique, semblent émerger comme des biomarqueurs potentiels très prometteurs. La grande majorité des travaux publiés sur la valeur prédictive des CEC et/ou CEP chez des patients traités par des agents anti-angiogéniques ayant utilisé la méthode de CMF publiée par Bertolini (110) ou une variante (112, 179) et ne pouvant être pris en compte, il existe peu de données fiables évaluant le rôle des CEC et CEP en tant que biomarqueurs des traitement anti-angiogéniques.

Une étude menée dans le laboratoire chez des patients atteints de cancer du côlon et traités par chimiothérapie et bévacizumab, montre que les taux de CEC sont prédictifs de l'efficacité anti-tumorale du traitement combiné (150). Dans cette étude, les patients avec un taux élevé de CEC prétraitement ou après un cycle de chimiothérapie avaient une survie sans progression défavorable à 6 mois ($p<0,01$) (150). Ces résultats confirmaient et étendaient les données publiées peu de mois auparavant par l'équipe de Ronzoni *et al*. dans une cohorte de 40 patients analysés en CMF (180). En utilisant la technique de CellSearch™ pour mesurer des CEC chez 31 patients atteints de cancer colorectal et traités par bévacizumab, Matsusaka *et al*. ont observé que la survie sans progression et la survie globale étaient réduite de manière significative ($p=0,003$ et $p=0,027$, respectivement) chez les patients avec un taux prétraitement de CEC élevé et que le taux de CEC prétraitement était un facteur prédictif de réponse au bévacizumab (181).

Dans un autre travail réalisé dans le laboratoire évaluant les taux de CEC et de facteurs plasmatiques avant et à jours 3, 15 et 60 d'un traitement par bévacizumab en monothérapie chez 43 patients atteints d'hépatocarcinome cellulaire avancé (182), nous avons aussi observé que les taux de CEC à J15 étaient corrélés à la réponse au traitement par bévacizumab (p=0.04), bien qu'ils n'aient pas été associés à la survie sans progression ou la survie globale. Quelques études utilisant la méthode CellSearch™ rapportent des résultats intéressants sur le potentiel des CEC en tant que biomarqueur mais qui restent à confirmer (181, 183).

Deux études menées dans de petites séries de patients atteints de cancer du rein métastatique rapportent qu'une augmentation du taux de CEC à J14 d'un traitement par inhibiteur de tyrosine kinase, le sunitinib, est associée à la survie sans progression (132, 184). Le travail que nous avons mené chez 55 patients atteints de cancer du rein métastatique et traités par inhibiteurs de tyrosine kinase (dont 46 par sunitinib) n'a pas confirmé ce résultat. Cependant, nous avons observé qu'un taux élevé de CEP était le seul marqueur avant traitement corrélé à la survie sans progression et la survie globale, suggérant que les taux de CEP dans cette pathologie pourraient être un biomarqueur prédictif permettant de discriminer des patients avec une évolution défavorable lors d'un traitement par inhibiteurs de tyrosine kinase (29). Ce travail sera développé plus loin dans la partie Résultats : Article 2. Nous avons aussi évalué les taux de CEC et de CEP chez 71 patients inclus dans quatre études de phase I testant de nouveaux agents anti-angiogéniques ou anti-vasculaires (voir Résultats : Article 3). Alors qu'aucune corrélation n'a été observée entre les taux de CEC et la survie globale, celle-ci était significativement plus courte chez les patients présentant un taux prétraitement élevé de CEP (9 mois *versus* 17 mois, p=0,03) (30). Ces résultats corroborent ceux observés dans d'autres travaux réalisés dans le cancer du poumon non à petites cellules (CBNPC) et dans l'hépatocarcinome traités par inhibiteur de tyrosine kinase, où des taux bas de progéniteurs hématopoïétiques exprimant le CD133 étaient associés à une survie sans progression augmentée (185, 186).

L'ensemble de ces données suggèrent que la mesure des taux de CEC avant et après un cycle de traitement pourraient, dans certains types de cancers comme le cancer colorectal ou l'hépatocarcinome, aider à la stratification des patients qui vont bénéficier d'un traitement anti-angiogénique mais illustrent aussi la complexité des mécanismes d'action de ces agents et des traitements combinés. De même, des taux de CEP prétraitement pourraient refléter une réponse systémique de l'hôte, particulièrement agressive sur le plan de l'angiogénèse, et être de ce fait un facteur prédictif de non réponse voire de résistance aux inhibiteurs de tyrosine kinase.

En parallèle à l'évaluation des CEC et CEP, de multiples biomarqueurs candidats ont été explorés pour prédire la réponse aux traitements anti-angiogénique dans des modèles précliniques ou la survie chez des patients traités ayant un cancer (5, 10, 54, 187). L'identification de cytokines prédictives de réponse à chaque famille de molécule anti-angiogénique reflétant si possible son effet direct sur le processus de néoangiogénèse et sur les cellules endothéliales, a été largement étudiée. Cependant, compte tenu de la complexité des voies moléculaires impliquées dans la néoangiogénèse et la vasculogénèse tumorale déjà abordées dans ce travail, l'identification de biomarqueurs protéiques s'est avèrée être très ambitieux. Le VEGF circulant a naturellement été le biomarqueur circulant le plus activement étudié. De nombreux résultats contradictoires ont été publiés sur la valeur du taux prétraitement de VEGF dans des essais de phase II et aucune corrélation avec l'efficacité du bevacizumab n'a été confirmée dans quatre essais de phase III (188). De plus, l'augmentation des taux de VEGF circulants à des temps précoces de l'administration de ces agents semblent être une caractéristique commune des drogues ciblant cette voie. Chez les patients traités par bevacizumab, les résultats varient en fonction de la méthode de dosage puisque l'on observe une augmentation des taux de VEGF complexé à la drogue et une chute des taux de VEGF libre (54). Chez les patients traités par des inhibiteurs de tyrosine kinase, l'augmentation des taux de VEGF, PlGF et de facteurs pro-angiogéniques ainsi que la diminution du taux de VEGFR2 est maintenant considérée comme une signature de ce type de drogue (10, 189). Une étude particulièrement intéressante chez des animaux sains traités par le sunitinib a montré que la production massive de facteurs de croissance pro-angiogéniques induite par ce traitement, dans la circulation et dans les tissus, était indépendante de la présence d'une tumeur (190). Elle apparaît donc comme une réponse systémique de l'hôte sans valeur prédictive, susceptible d'avoir un intérêt pharmacodynamique et d'être éventuellement en rapport avec des mécanismes de résistance par la mobilisation de CEP et d'un « rebond » vasculaire tumoral. Dans le cas des inhibiteurs de tyrosine kinase, la dynamique des CEC et plus particulièrement CEP pourrait donc être le miroir de celles des facteurs de croissance pro-angiogéniques.

1.7.2 CEC et CEP : biomarqueurs des traitements anti-vasculaires

Peu d'études ont exploré le rôle potentiel des CEC et des CEP en tant que biomarqueurs des agents anti-vasculaires. Dans les modèles murins, cette hypothèse a été abordée exclusivement sur le versant des CEP par Shaked *et al*. et dont les travaux ont déjà été présentés pages 21-22

(27, 76). De façon similaire, Daenen *et al*. ont évalué les taux de CEP, et non de CEC, après administration d'un VDA en explorant les possibilités de bloquer la mobilisation de cette population avec une chimiothérapie métronomique (91). Chez l'homme, Beerepoot *et al*. ont observé une élévation en quelques heures des taux de CEC chez des patients inclus dans une étude de phase I et recevant un VDA, le ZD1626 (191). Dans le laboratoire, Françoise Farace a rapporté pour la première fois la mobilisation de CEP induite par l'injection de la drogue chez des patients inclus dans une étude de phase I combinant un VDA, l'ombrabuline, avec la cisplatine (98). Plus récemment, une étude de phase I évaluant l'association de la combrétastatine A4-phosphate en combinaison avec le bévacizumab chez 15 patients a observé une augmentation des taux de progéniteurs $CD34^+$ et $CD133^+$ après administration de la drogue (93). Nous avons étudié dans différents modèles murins le rôle des CEC et CEP dans le mécanisme d'action des VDA ; ce travail est présenté dans les Résultats : Article 4 (31).

2. CONTEXTE ET BUT DU TRAVAIL

La stratégie de détection des CEC et CEP en CMF établie dans le laboratoire de Recherche Translationnelle, Unité INSERM U981, a été développée pour répondre aux standards requis pour la détection des évènements rares. Les caractéristiques de cette méthodologie sont développées dans la partie Matériel et Méthodes. Pour les CEC, des expériences de quintuple marquage avec le CD144, marqueur typiquement endothélial ont confirmé l'origine endothéliale des cellules identifiées comme des CEC et la reproductibilité et la linéarité de la méthode ont été démontrées (148). Les valeurs de CEC retrouvées avec cette méthode chez des volontaires sains sont comparables à celles retrouvées avec la méthode de référence d'immunocapture IMS (103, 105). Une stratégie similaire a été développée pour la détection des CEP chez l'homme (98). La mise au point de ces outils fiables était une étape indispensable avant d'aborder les questions autour du rôle de ces cellules dans le cancer et ses traitements. Depuis, de nombreuses études exploratoires ont été menées dans le laboratoire visant à étudier l'intérêt clinique potentiel des CEC et CEP dans divers types de cancers et différentes situations cliniques.

Le premier volet de mon travail de thèse a consisté à évaluer le rôle des CEC et CEP en tant que biomarqueurs de l'angiogénèse et des traitements anti-angiogéniques chez l'homme. A la différence des tumeurs adultes, d'origine épithéliale souvent, peu vascularisée et à temps de prolifération lente, les tumeurs solides pédiatriques sont en majorité indifférenciées, d'origines embryonnaires, richement vascularisées et caractérisées par un taux élevé de prolifération. Compte tenu du rôle important de l'angiogénèse dans les tumeurs solides pédiatriques, j'ai choisi ce modèle pour étudier le rôle CEC et CEP en tant que biomarqueurs de l'angiogénèse tumorale. Ce travail a permis de mettre en évidence pour la première fois l'existence d'une corrélation entre un taux élevé de CEP dans le sang périphérique des patients et l'existence d'une maladie métastatique (voir Résultats : Article 1 (28)). J'ai aussi participé à deux travaux analysant le rôle des CEC et CEP en tant que biomarqueurs des traitements anti-angiogéniques dans les tumeurs solides adultes. Nous avons montré chez des patients ayant un cancer du rein métastatique (mRCC) recevant un traitement anti-angiogénique que le taux de CEP initial était corrélé à la réponse tumorale au traitement et qu'un taux de CEP initial élevé était prédictif d'une absence de réponse clinique et d'un mauvais pronostic (voir Résultats : Article 2 (29)). Nous avons aussi analysé la valeur pronostique des CEC et CEP chez des patients atteints de cancers avancés inclus dans des études de phase I (voir Résultats : Article 3 (30)).

Ces premiers résultats ont mis en exergue le rôle potentiellement important des CEP en tant que biomarqueurs de l'angiogénèse et des traitements anti-angiogéniques. Par conséquent, le deuxième volet de mon travail de thèse a consisté à étudier dans des modèles murins les mécanismes impliqués dans la mobilisation des CEP qui pouvaient être recrutés dans un processus de réparation vasculaire et être des acteurs clés de la revascularisation, de la repousse et de ce fait de la résistance tumorale. Les CEP sont mobilisés en réponse aux cytokines pro-angiogéniques produites par la tumeur en situation d'ischémie ou en cas de lésion/réparation des vaisseaux. D'un point de vue mécanistique, il nous est paru que la destruction vasculaire et l'ischémie tumorale occasionnées par un VDA constituaient typiquement une situation de lésion vasculaire suivie de réparation et de régénération vasculaire susceptible de mobiliser les CEP. A la suite de l'étude de Françoise Farace rapportant la mobilisation de CEP chez des patients traités par un VDA (98) et de l'observation de taux élevés de CEC chez des patients traités par une autre VDA (191), nous avons émis l'hypothèse que le VDA pouvait être un modèle particulier pour étudier les rôles respectifs de ces cellules dans l'angiogénèse tumorale, où les taux de CEC pourraient un biomarqueur pharmacodynamique capables de refléter directement l'importance de la lésion vasculaire induite par la drogue et donc refléter l'activité biologique du VDA, alors que les CEP, recrutés dans un mécanisme de réparation vasculaire, pourraient être acteurs clés de la revascularisation et donc être des biomarqueurs de résistance au VDA. De plus, les CEP pourraient constituer des cibles thérapeutiques dans des stratégies d'association des VDA avec d'autres drogues et le suivi de leurs taux dans le sang périphérique pourrait identifier des schémas thérapeutiques efficaces.

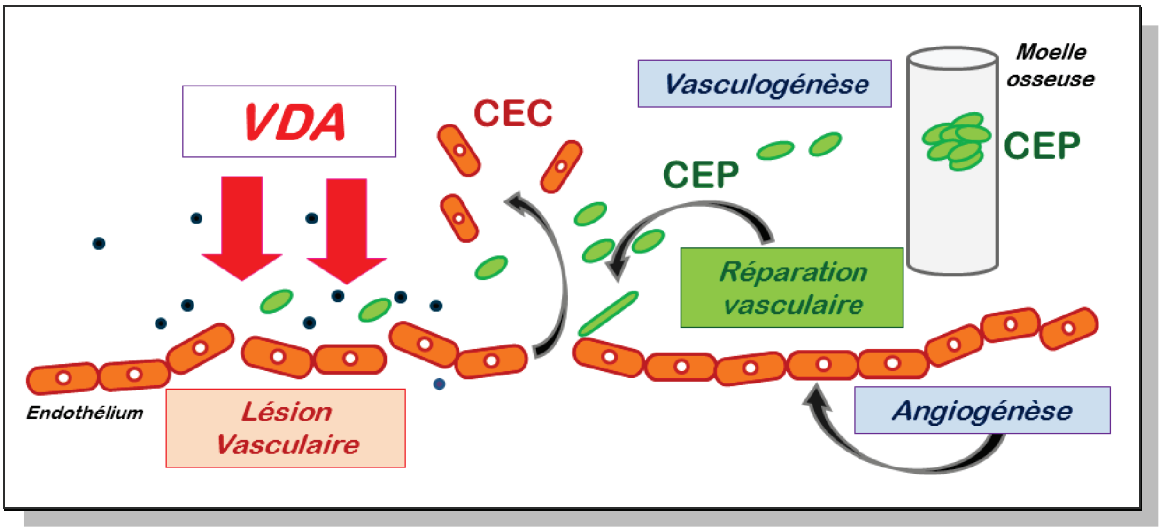

Figure 6. Hypothèse sur les rôles des CEC et CEP en tant que biomarqueurs des VDA.

Cette exploration plus « mécanistique » dans des modèles murins a consisté en l'étude du rôle des CEC et CEP dans les mécanismes d'action des VDA sur trois versants :

1) En caractérisant avec précision les CEC et CEP en tant que biomarqueur pharmacodynamique en relation avec les changements morphologiques et fonctionnels induits par la drogue dans la tumeur ;
2) En étudiant le rôle fonctionnel des CEP dans la résistance à ces drogues ;
3) En explorant les facteurs de croissance pro-angiogéniques régulant la mobilisation de ces cellules.
4) En identifiant des stratégies d'association VDA et agent anti-angiogénique qui en ciblant les CEP mobilisées par la drogue, avaient une efficacité anti-tumorale optimale.

3. MATÉRIEL ET MÉTHODES

3.1 Détection des CEC et CEP en cytométrie de flux 4 couleurs chez l'homme

Le développement de la détection des CEC et des CEP en cytométrie de flux 4 couleurs chez l'homme a été réalisé dans le laboratoire de Recherche Translationnelle par deux techniciennes, Nathalie Jacques et Nadège Vimond (98, 148). La stratégie de détection des CEC et CEP en CMF établie par Françoise Farace a été développée pour répondre aux standards requis pour la détection des évènements rares et présente les caractéristiques suivantes : (1) le marquage d'un volume de sang important, (2) l'acquisition d'un nombre élevé d'évènements (environ $5x10^6$) pour permettre une analyse statistique, (3) l'utilisation de billes fluorescentes de taille 10 µm afin d'exclure les évènements de petite taille, (4) l'utilisation d'un marqueur de viabilité, et (5) des contrôles FMO (« *fluorescence minus one* ») permettant de mesurer avec précision le bruit de fond dans les fenêtres sélectionnées selon les recommandations publiées (143, 192). Pour les CEC, la reproductibilité et la linéarité de cette méthode a été montré, de même que l'origine endothéliale des cellules identifiées comme des CEC a été confirmée dans des expériences de quintuple marquage avec le CD144, marqueur typiquement endothélial (148). Etant donné que les techniques de mesure de CEP en culture cellulaire nécessitaient des volumes sanguins importants (>50 ml) et inadaptés à l'évaluation des patients en routine dans des essais cliniques, une méthode de détection en CMF 4 couleurs semblable à celle établie pour les CEC a été développée. Cependant, compte tenu de la rareté des CEP, ces cellules étaient mesurées à partir d'un volume sanguin de 10 ml après une étape d'enrichissement en cellules progénitrices qui sera détaillée plus loin.

3.1.1 Prélèvements sanguins pour l'étude des CEC et CEP chez l'homme

Tous les prélèvements sanguins des sujets étudiés ont été réalisés à l'Institut Gustave Roussy, sauf ceux de la population pédiatrique de volontaires sains qui ont été réalisés dans le service d'endocrinologie pédiatrique de l'hôpital Bicêtre (voir Résultats : Article 1 (28)). Un consentement éclairé et signé était recueilli pour chaque patient avant le prélèvement sanguin qui a été réalisé dans la majorité des cas en même temps que d'autres prélèvements systématiques nécessaires à la prise en charge du patient. Pour l'étude des CEC, un prélèvement de sang veineux était recueilli dans un tube CellSave™ Preservative (Immunicon, PA, USA)

après élimination des 2 ml de sang initiaux suivant la ponction veineuse. Le volume sanguin minimum requis pour l'étude des CEC est de 1,6 ml de sang veineux. Pour l'étude des CEP, un prélèvement initial de 10 ml de sang veineux était prélevé sur un tube hépariné. Dans le souci de déterminer le volume sanguin minimal nécessaire à l'étude des CEP dans une population pédiatrique, nous avons diminué ce volume de sang à 5 ml pour les enfants de moins de 15 kg.

3.1.2 Méthode de détection des CEC et CEP chez l'homme en cytométrie de flux 4 couleurs

3.1.2.1 Procédure de marquage de sang total pour la détection des CEC

La stratégie établie identifie les CEC dans le sang total comme des évènements viables ayant pour immunophénotype $CD31^{+}CD146^{+}CD45^{-}7AAD^{-}$ (148). Les anticorps utilisés sont le CD31-FITC (clone WM59, BD Pharmingen, NJ, USA), le CD146-PE (clone P1H12, BD Pharmingen), et le CD45-APC (clone T29/33, DakoCytomation, Denmark) alors que les contrôles isotypiques de fluorochromes conjugués (IgG1-FITC, IgG1-PE, et IgG-APC de souris) ont été achetés chez DakoCytomation. Le marqueur de viabilité utilisé est le 7-aminoactinomycine-D (7AAD). Les anticorps ont été titrés sur 1 ml de sang total afin de déterminer la dose optimale et afin d'être utilisés en excès. Pour le CD146, la titration était réalisée sur des cellules HUVEC. Le 7AAD (2 µl par 0 ,5 ml de sang total), les réactifs BD FACS Lysing et BD CellFix ont été achetés chez BD Biosciences.

Le sang total était distribué dans 4 tubes polypropylène de 5 ml (BD Falcon Biosciences, MA, USA) : 0,1 ml dans le tube 1, et 0,5 ml dans les tubes 2, 3 et 4 respectivement. Les anticorps, le 7AAD, et les contrôles isotypiques de fluorochromes conjugués ont été ajoutés comme suit :

- Tube 1 : IgG1-FITC, IgG1-PE, IgG1-APC de souris et le 7AAD
- Tube 2 (FMO) : CD31-FITC, IgG1-PE, CD 45-APC et 7AAD
- Tube 3 et 4 (test) : CD31-FITC, CD146-PE, CD45-APC et 7AAD

Trois autres tubes de 0,1 ml de sang total contenant chacun des anticorps seuls et des contrôles isotypiques étaient réalisés afin de vérifier le voltage PMT et la compensation de fluorescence. Les échantillons ont incubé pendant 20 min dans l'obscurité à 4°C avant l'adjonction du réactif FACS Lysing (1ml dans le tube 1 et 4 ml dans les tubes 2, 3 et 4). Les échantillons ont été vortexés soigneusement puis incubés 15 min à 4°C dans l'obscurité. Un lavage au PBS a été

réalisé. Les tubes 3 et 4 ont été poolés et les trois tubes restants lavés encore une fois avant d'être fixés avec 250 µl de CellFix pour être analysés dans les 48 h.

3.1.2.2 Procédure de marquage de sang total pour la détection des CEP

Les CEP sont identifiés comme la fraction viable de cellules progénitrices circulantes d'origine médullaire ($CD45^{dim}CD34^{+}7AAD^{-}$) exprimant le VEGFR2 (98). Les anticorps utilisés sont le CD45-FITC (clone T29/33, DakoCytomation, Denmark), le CD34-APC (clone BIRMA-K3, DakoCytomation), et le KDR (VEGFR2)-PE (clone 89106, R&D Systems, MN, USA). Des contrôles isotypiques conjugués au fluorochrome (IgG1-FITC, IgG1-PE et IgG1-APC de souris) étaient achetés auprès de DakoCytomation. Les anticorps ont été titrés afin de déterminer la dose optimale et afin d'être utilisés en excès. Les réactifs 7AAD (2µl par tube), FcR blocking reagent (Miltenyi Biotec, Germany) et BD CellFix ont été achetés chez BD Biosciences.

Les CEP ont été identifiés dans des fractions de cellules mononuclées enrichies en progéniteurs par la méthode RosetteSep® (StemCell Technologies) à partir de 10 ml (ou 5 ml chez les enfants de <15 kg) de sang total. Grâce à un mélange pré-établi d'anticorps RosetteSep®, des rosettes sont formées en liant les cellules non désirées et les hématies qui précipitent sur gradient Ficoll® au cours d'une centrifugation à 2500 rpm pendant 20 minutes. Cette étape d'enrichissement en cellules progénitrices (hématopoïétiques et endothéliales) permet d'éliminer les cellules mononuclées différenciées. Après lavage, la fraction enrichie a été distribuée dans trois tubes polypropylène de 5 ml (BD Falcon Biosciences, MA, USA) : 0,1 10^6 cellules mononuclées dans le tube 1 (contrôle isotypique) et le restant des cellules récupérées réparti en quantité égale dans les tubes 2 et 3, à savoir le contrôle FMO et le test CEP respectivement. La quantité de cellules distribuée dans les tubes 2 et 3 dépendait de la récupération cellulaire après enrichissement. Le réactif FcR blocking reagent destiné à saturer les récepteurs Fc pour éviter une fixation non spécifique des anticorps a été ajouté aux trois tubes (2µl/tube) puis les anticorps, le 7AAD et contrôles isotypiques conjugués aux fluorochromes ont été distribués de la manière suivante :

- Tube 1 : IgG1-FITC, IgG1-PE, IgG1-APC de souris et 7AAD
- Tube 2 (FMO) : CD45-FITC, IgG1-PE, CD34-APC et 7AAD
- Tube 3 (test) : CD45-FITC, KDR (VEGFR2)-PE, CD45-APC et 7AAD.

Les échantillons ont incubés 20 minutes à 4°C avant d'être lavés une fois au PBS. Enfin, ils ont été fixés avec 250µl de CellFix et analysés dans les 48h.

3.1.2.3 Acquisition et analyse en cytométrie de flux

L'acquisition des cellules marquées a été réalisée sur un FACSCalibur (BD Biosciences). Les tubes 1, les contrôles isotopiques et les tubes contenant les anticorps seuls ont été utilisés pour l'ajustement du voltage PMT et pour les compensations de fluorescence. Occasionnellement, ces derniers étaient aussi ajustés avec des Compbeads (BD Biosciences) en déterminant la médiane des populations positives et négatives. Des microbilles Flow-Check (Beckman-Coulter) étaient ajoutées au tube contenant les contrôles isotypiques et ce tube était acquis en premier afin d'ajuster précisément le voltage FSC et identifier les évènements cellulaires ayant une taille supérieure à 10 µm. Toutes les cellules contenu dans le contrôle FMO (tube 2) et le tube test CEC ou CEP ont été acquis. Cela correspondait à environ $2,5x10^6$ et $5x10^6$ évènements pour les tubes FMO et test CEC ou CEP respectivement. La vitesse d'acquisition ne dépassait pas 4000 évènements/seconde. Une fenêtre de sauvegarde a été utilisée afin d'exclure une partie des granulocytes et débris. Des lavages ont été réalisés entre chaque acquisition. Les données ont ensuite avec le logiciel CellQuest 3.2.

Les résultats de CEC mesurées sont exprimés en nombre de CEC/ml de sang. Les taux de CEP mesurés sont exprimés en pourcentage de cellules progénitrices $CD45^{dim}CD34^{+}7AAD^{-}$ totales exprimant le VEGFR2 (KDR). Des expériences préliminaires réalisées dans le laboratoire ont montré que parmi les cellules identifiées comme des CEP, une sous-population exprimait aussi le marqueur CD133. Nous avons donc estimé que le marquage pour le CD133 n'apportait pas d'information supplémentaire et n'a donc pas été retenu pour une utilisation en routine.

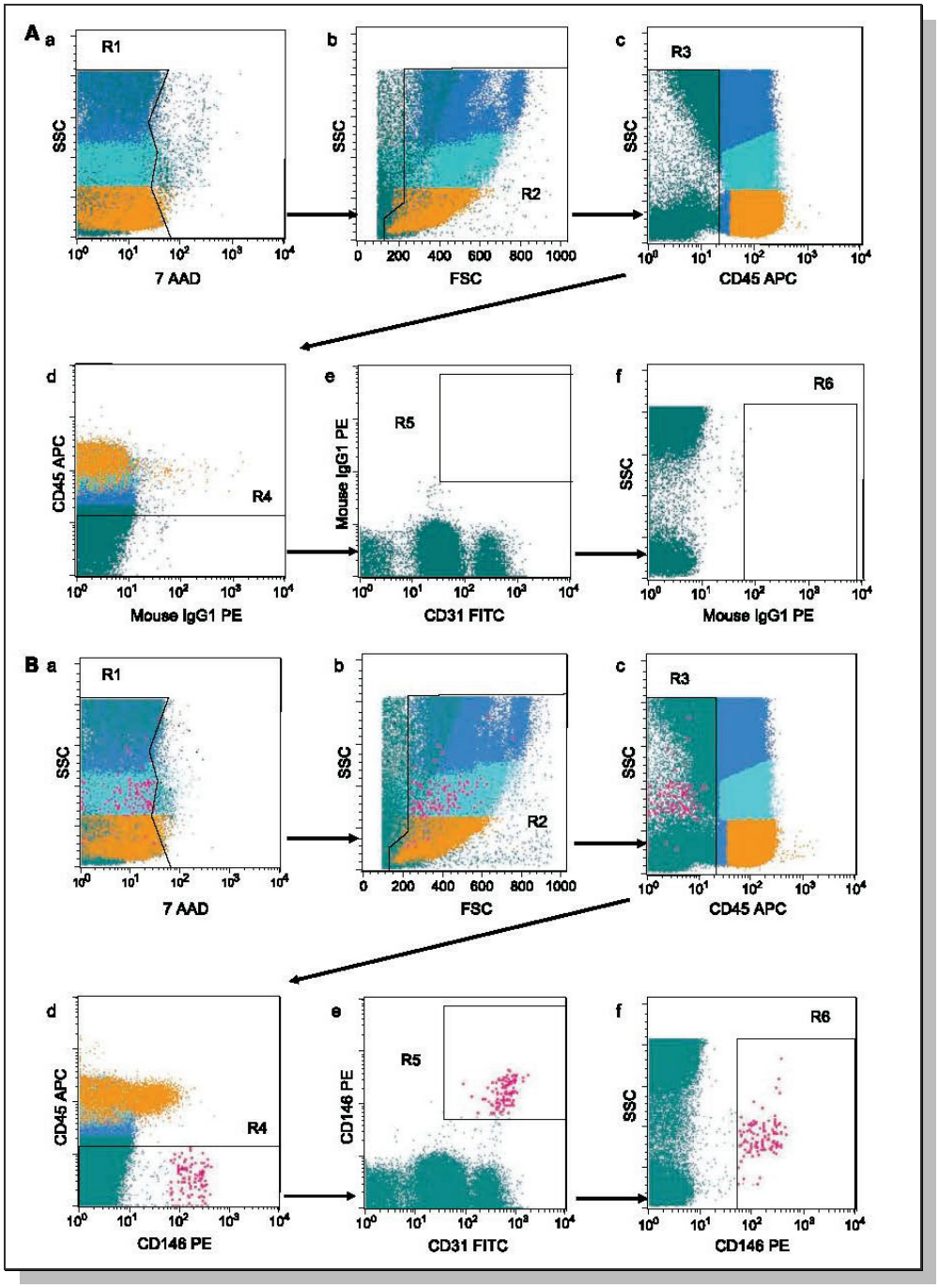

Figure 7. Stratégie de détection en CMF 4 couleurs des CEC chez l'homme. (A) Analyse du contrôle FMO PE (CD45-APC/CD31-FITC/mouse IgG1-PE/7AAD). (B) Analyse du test (CD45-APC/CD31-FITC/CD146-PE/7AAD). Les panels montrent 100% des évènements ($2{,}5x10^6$ évènements pour le contrôle et $5x10^6$ évènements pour le test). Les panels de (a) à (f) sont identiques pour le contrôle FMO-PE et le tube test. (a) sélection des évènements viables $7AAD^-$ (R1). (b) la fenêtre R2 permet d'éliminer les débris et la majorité des granulocytes sans exclure des cellules de grande taille. (c) et (d) les fenêtres R3 et R4 sont placées pour exclure les évènements $CD45^+$. (e) R5 : cellules $CD31^+CD146^+7AAD^-$ dans la fenêtre $CD45^-$. (f) la fenêtre R6 résulte du croisement des fenêtres de sélection successives pour compter les CEC. Les CEC sont présentées en gras et identifiées comme des cellules $CD31^+CD146^+$ $CD45^-7AAD^-$.

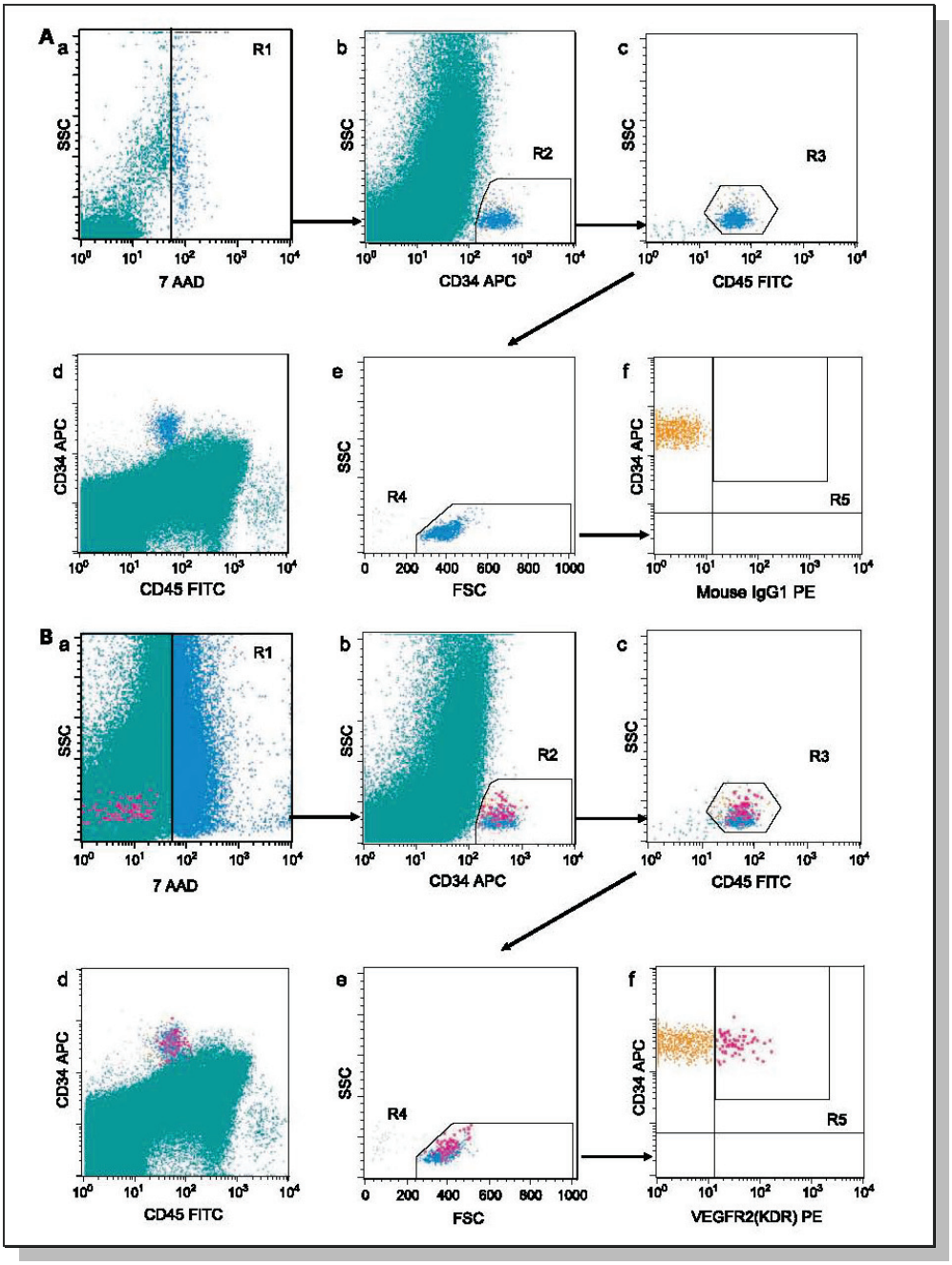

Figure 8. Stratégie de détection des CEP en CMF 4 couleurs chez l'homme. (A) Analyse du contrôle FMO-PE (CD45-FITC/mouse IgG1-PE/CD34-APC/7AAD) pour le placement des fenêtres de sélection. (a) Seulement 1% des évènements est présenté pour la sélection des évènements viables $7AAD^-$ (R1). Les autres panels montrent 100% des évènements. (B) Analyse du test (CD45-FITC/VEGFR2(KDR)-PE/CD34-APC/7AAD) pour lequel 100% des évènements sont présentés. (b) la fenêtre R2 sélectionne les évènements $CD34^+$. (c) la fenêtre R3 est placée pour sélectionner les évènements $CD45^{dim}$ dans R2. (d) évènements viables $CD45^{dim}CD34^+7AAD^-$. (e) la fenêtre R4 est utilisée pour éliminer des débris de R3. (f) la fenêtre R5 résulte du croisement des fenêtres de sélection successives pour compter les cellules $CD45^{dim}CD34^+7AAD^-VEGFR2^+$. Les fenêtres de sélection de (a) à (f) sont identiques pour le contrôle FMO-PE et le tube test. Les CEP sont présentées en gras et identifiées comme des cellules $CD45^{dim}CD34^+7AAD^-VEGFR2^+$.

3.2 Méthodes de détection des CEC et CEP en cytométrie de flux 4, 5 et 6 couleurs chez la souris

La mise au point de la détection et l'énumération des CEC et CEP en CMF 4, 5 et 6 couleurs chez la souris a été réalisée par une technicienne du laboratoire Fanny Billiot et par moi-même. La stratégie de détection établie par Françoise Farace était strictement similaire à celle mise au point dans le laboratoire pour la détection des CEC et CEP chez l'homme en CMF. A la différence des autres équipes travaillant dans ce domaine, il a été choisi de ne pas détecter les CEC et CEP chez la souris dans le même tube de sang ce qui a conduit à la mise au point de deux combinaisons d'anticorps : l'une pour les CEC, et l'autre pour les CEP. Cette approche plus lourde permettait de mieux caractériser ces cellules d'un point de vue phénotypique mais nécessitait plus de sang, et donc nécessitait de doubler les expériences pour avoir 2 cohortes d'animaux à chaque temps expérimental : l'une pour les CEC et l'autre pour les CEP. Le volume de sang total nécessaire à la mesure des CEC ou des CEP était de 250µl, et était prélevé par voie rétro-orbitaire ou par ponction intracardiaque.

3.2.1 Procédure de marquage de sang total pour la détection des CEC et CEP

Les CEC sont identifiées dans le sang total de souris comme des évènements viables ayant pour immunophénotype $CD31^{+}CD45^{-}$Flk-1$(VEGFR2)^{+}MECA\text{-}32^{+}7AAD^{-}$. Les anticorps monoclonaux rat anti-souris utilisés étaient : CD31-FITC, CD45-PerCP, Flk-1(VEGFR2)-PE, MECA-32 biotine et streptavidine-APC (tous de chez BD Pharmingen). Le marqueur nucléaire de viabilité utilisé était le DAPI (4', 6'-diamidino-2-phenylindole). Les anticorps ont été titrés afin de déterminer la dose optimale et afin d'être utilisés en excès. Les anticorps MECA-32-biotine et Flk-1-PE ont été titrés sur des prélèvements sanguins présentant des taux élevés de CEC issus d'animaux traités par colchicine. Les CEP sont identifiés dans le sang total après un marquage avec les anticorps monoclonaux de rat anti-souris suivant : CD45-PerCP, CD117-APC, Sca-1-FITC et Flk-1 (VEGFR2)-PE (tous de chez BD). Le marqueur de viabilité utilisé ici était le DAPI. Des contrôles isotypiques conjugués au fluorochrome (IgG1-FITC, IgG1-APC, IgG1-PerCP, et IgG1-PE de rat achetés chez BD Pharmingen) ont étaient établis. De même, des contrôles FMO pour chaque fluorochrome et pour la streptavidine (CD45-PerCP / CD31-FITC / Flk-1-PE / Streptavidin-APC) ont été réalisés dans du sang total pour les stratégies d'identification des CEC et CEP (5 tubes FMO pour le test CEC et 4 tubes FMO pour le test

CEP) afin de mesurer le bruit de fond et ajuster les fenêtres d'analyse avec précision selon les recommandations (143, 192).

Les échantillons de sang étaient distribués dans des tubes polypropylène de 5 ml (BD Falcon Biosciences, MA, USA) et incubés pendant 20 minutes à 4°C avec le Fc Block (BD Biosciences) afin d'éviter la fixation non-spécifique des anticorps. L'adjonction des anticorps nécessaires aux tubes correspondant aux contrôles FMO et au tube test CEC ou CEP étaient réalisés exactement selon la même stratégie établie chez l'homme. Après une incubation des anticorps pendant 20 minutes à 4°C, la solution BDPharmLyse (BDBiosciences) a été rajoutée à chaque tube pour lyser les globules rouges. Les échantillons ont été lavés au PBS puis acquis immédiatement sans adjonction de CellFix.

3.2.2 Acquisition et analyse en cytométrie de flux

L'acquisition des cellules marquées a été réalisée sur un FACSCalibur (BD Biosciences) ou sur un FACSCanto II (BD Biosciences). Les contrôles isotopiques et les tubes contenant les anticorps seuls ont été utilisés pour l'ajustement du voltage PMT et pour les compensations de fluorescence. Ces derniers étaient aussi ajustés avant chaque acquisition avec des Compbeads (BD Biosciences) en déterminant la médiane des populations positives et négatives. Des microbilles Flow-Check (Beckman-Coulter) étaient utilisées pour ajuster précisément le voltage FSC. Toutes les cellules contenu dans les contrôles FMO (4 par test) et le tube test CEC ou CEP ont été acquis afin d'obtenir au moins 150,000 événements mononuclées pour chaque tube. La vitesse d'acquisition ne dépassait pas 4000 évènements/seconde. Les cellules non viables et débris apoptotiques ont été exclus par des fenêtres d'analyse en FSC et une fenêtre de sélection des évènements négatifs pour le DAPI (1 µl/tube rajouté immédiatement avant l'acquisition). Des lavages ont été réalisés entre chaque acquisition. Les données ont été analysées avec les logiciels CellQuest 3.2 ou BD FACS Diva (BD Biosciences), avec pour ce dernier une représentation bi-exponentielle des data.

Les CEC ont été identifiées comme des évènements viables $CD45^{-}CD31^{+}$ $Flk\text{-}1^{+}MECA\text{-}32^{+}DAPI^{-}$. Les CEP ont été identifiées comme des évènements viables $CD45^{-/dim}CD117^{+}Sca\text{-}1^{+}Flk\text{-}1^{+}DAPI^{-}$. Les taux de CEC et de CEP absolus ont été calculés à partir de numérations formules sanguines déterminées sur un compteur MS9-3 (Melet Schlœsing, Osny, France) pour chaque échantillon de sang murin prélevé et selon la formule suivante : taux absolu de CEC ou

CEP / µl de sang = CEC ou CEP comptées au FACS x (lymphocytes + monocytes à la NFS/lymphocytes + monocytes comptés au FACS).

Des expériences complémentaires en CMF 5 et 6 couleurs ont été réalisées pour mieux caractériser les cellules identifiées comme des CEP avec l'aide d'un ingénieur, Phillipe Rameau, Service Commun de Cytométrie et d'Imagerie, IRCIV, de l'Institut Gustave Roussy. Pour ces expériences, les anticorps monoclonaux de rat anti-souris suivant ont été utilisés : Gr-1-PE Cy7 (eBiosciences), CD11b-FITC et CD34-FITC (BD Pharmingen), CD4-PE (BD Pharmingen), CD2-, CD3-, CD4-, CD11b-, CD14-, CD19-, et CD20-PerCP Cy5.5 (tous BD Pharmingen), F4/80-PerCP Cy5.5 (eBiosciences), CD144 (VE cadhérine)-biotine (Biolegend) et streptavidine-FITC (BD Pharmingen).

3.3 Modèles animaux

La séquence précise des évènements morphologiques et fonctionnels induits par une VDA, la combrétastatine A4-phosphate (CA4P) a été étudiée dans différents modèles de xénogreffes chez la souris. Ce travail a été réalisé avec l'aide de Fanny Billiot, technicienne du laboratoire de Recherche Translationnelle, et avec Olivia Bawa et le Pr Paule Opolon de l'unité UMR8121 ainsi que le Service Commun d'Expérimentation Animale (SCEA) de l'Institut Gustave Roussy.

3.3.1 Lignées tumorales et établissements de tumeurs sous-cutanées

Les lignées de cellules tumorales humaines utilisées étaient des lignées de prostate (PC-3, IGR Cap-Luc, et MDA PCA), de cancer de poumon (A549, H69), de neuroblastome (IGR-N91), et de carcinome de rein à cellules claires (786-0). Des xénogreffes ont été établis chez des souris immunodéficientes athymiques swiss nudes nu/nu (élevage Institut Gustave Roussy) femelles ou males (pour les lignées de prostate) âgés de 6 à 8 semaines par une seule injection de 2,5 10^6 cellules en sous cutanée au niveau du flanc droit ou gauche. L'étude des différentes xénogreffes a permis de déterminer que la lignée humaine de prostate PC3 donnait des tumeurs très vascularisées et est de ce fait très sensible aux VDA. Le modèle murin de xénogreffe de PC3 a été le modèle privilégié pour caractériser les changements morphologiques et fonctionnels induits *in situ* par ce traitement.

La lignée syngénique de cancer pulmonaire Lewis Lung Carcinoma (LLC) a été utilisée pour l'établissement de tumeurs sous-cutanées chez des souris immunocompétentes. Pour ces greffes, $5\ 10^6$ cellules LLC ont été injectées en sous cutanée au niveau du flanc de souris C57Bl/6 (Charles River, France) ou de souris C57Bl/6 chimériques

3.3.2 Etablissement d'un modèle de souris chimérique au niveau médullaire

Afin de caractériser la fonctionnalité des CEP recrutées au niveau de la tumeur, nous avons ensuite établi un modèle de souris chimérique exprimant la « *green fluorescent protein* » (GFP) au niveau médullaire. Pour cela, des souris C57Bl/6 âgées de 6-8 semaines ont été irradiées létalement (950 rad) puis transplantées par injection au niveau de la veine caudale avec 10^7 cellules médullaires GFP^+ prélevées depuis les tibias et les fémurs de souris transgéniques C57Bl/6-Tg(ACTB-EGFP)1Obs/j. Quatre semaines après la transplantation, un prélèvement rétro-orbitaire a été réalisé pour détecter le nombre de cellules GFP^+ en CMF et a montré que les souris présentaient un chimérisme sanguin de l'ordre de 75-80%. Après confirmation de la stabilité du chimérisme, les animaux ont été greffés avec la lignée syngénique LLC puis utilisés dans des expériences identiques avec injection de la CA4P associée ou non à un agent anti-angiogénique.

3.3.3 Analyse des tumeurs

3.3.3.1 Analyses histologiques

Après euthanasie des animaux, les tumeurs sous-cutanées ont été prélevées à différents temps expérimentaux pour des analyses histologiques et immunohistochimiques qui ont été réalisées avec l'aide d'Olivia Bawa et le Pr Paule Opolon, de l'unité UMR8121. Nous avons analysé la nécrose tumorale, la microdensité vasculaire et l'hypoxie tumorale sur de images de sections tumorales entières acquises sur un microscope Zeiss Axiophot (25X) et enregistrées avec un scanner Nikon SuperCoolscan 8000 ED équipé d'un porteur de lames FH-8G1 (Nikon, Champigny-sur-Marne, France). Les quantifications de nécrose, de microdensité vasculaire et de cellules tumorales hypoxiques (positive pour l'expression de CA-IX) ont été déterminées grâce à une procédure automatisée utilisant le logiciel d'analyse d'image PixCYT. Avant l'analyse, les régions d'intérêts ont été sélectionnées afin de n'inclure que le tissu tumoral et exclure du tissu non-tumoral ou des débris/artéfacts. La nécrose a été analysée après une coloration des sections

tumorales (4-6 µm) à l'hématoxyline et l'éosine (H&E) et quantifiée par un pathologiste expérimenté (P.O.) selon des critères morphologiques et exprimée comme un % de la surface de la section tumorale. Pour l'analyse de la microdensité vasculaire et de l'expression de CA-IX, les régions nécrotiques ont été exclues afin de ne considérer que la partie viable de la tumeur. La microdensité vasculaire a été mesurée comme la surface des vaisseaux marqués avec un anticorps rat anti-souris contre le CD34 (marqueur vasculaire) (1:20, Hycult Biotechnology) révélé par un anticorps secondaire de lapin anti-rat (1:400, Southern Biotech) et a été exprimée comme une fraction de surface (%) de la tumeur viable. L'hypoxie tumorale a été déterminée par le taux de CA-IX (visualisé avec un anticorps de rat dirigé contre le CA-IX (MN antigen, Slovak Academy of Sciences, Bratislava, Slovak Republic)) et exprimée comme un % de la surface tumorale viable.

3.3.3.2 Analyses en immunofluorescence

L'étude de la fonctionnalité des CEP par la détection de cellules GFP^+ intégrées dans des vaisseaux sanguins des tumeurs LLC et présentant des marqueurs membranaires endothéliaux a nécessité la mise au point de marquage en fluorescence sur coupe épaisse de tumeur afin de mettre en évidence avec précision la co-localisation des signaux GFP et du marquage par un anticorps anti-CD31 spécifique de l'endothélium. Ces mises au point ont été réalisées avec l'aide de Virginie Marty et du Dr Philippe Vielh (Module Histocytopathologie du Laboratoire de Recherche Translationnelle, IGR) et de Sophie Salomé-Desnoulez et du Dr Corinne Laplace-Builhé du Service Commun de Cytométrie et d'Imagerie, IRCIV (IGR).

Les tumeurs LLC ont été prélevées à partir des souris C57Bl/6 chimériques avant traitement, à 3 jours et à 6 jours après traitement par combrétastatine puis ont été congelées. Après une réhydratation avec du PBS pendant 30 min, des sections tumorales de LLC ont été incubées avec différents anticorps et visualisées avec un microscope confocal Leica TCS SPE (Leica, Germany) et un objectif sec 20X. Les images fluorescentes ont été collectées au travers de filtres spécifiques : DAPI (405 nm excitation), AlexaFluor 546 (532 nm excitation) pour le marquage CD31, Alexa fluor 633 (635 nm excitation) pour le marquage CD41, Alexa fluor 647 (635 nm excitation) pour le marquage CD11b et F4/80, et GFP (488 nm excitation) pour les cellules médullaires GFP^+. Pour l'évaluation du nombre d'évènements GFP^+ dans la tumeur et dans les vaisseaux tumoraux, des Z-stacks de 10 sections optiques consécutives (2-2.5 µm d'épaisseur) ont été capturés (20X) grâce au logiciel confocal Leica avec des paramètres d'acquisition

identiques. La quantification des cellules GFP^+ dans chaque Z-stack a été réalisé avec le logiciel ImageJ (http://rsbweb.nih.gov/nih-image/). Dans un second temps, des cryosections épaisses (40-50 µm) de LLC ont été évalué pour la présence de cellules exprimant le marqueur myéloïde CD11b, le marqueur macrophagique F4/80, et le marqueur plaquettaire CD41 parmi les cellules GFP^+ d'origine médullaire infiltrant la tumeur et intégrant les vaisseaux tumoraux.

Des Z-stacks entiers de sections optiques consécutives (0.8-1µm par pas de section) de la totalité des cryosections épaisses (50µm) de LLC ont été capturés avec le logiciel Leica confocal, avec des programmes d'acquisition identique et une densité de pixel de 1024x1024. Le grossissement utilisé était de X20 et X63. Des projections en intensité maximale des images confocaux de Z-stacks ont été réalisées grâce au logiciel ImageJ. Des reconstructions 3D de l'ensemble des Z-stacks obtenus pour chaque cryosection ont été réalisées grâce au logiciel Imaris (Bitplane, Zurich, Suisse). Des animations des sections confocaux en 2D ont été réalisées en utilisant le logiciel Imaris.

3.4 Mesure des facteurs plasmatiques de l'angiogénèse

Chez l'homme, les taux plasmatiques de VEGF et de sVEGFR2 (soluble VEGFR2) ont été déterminés grâce à des kits ELISA (R&D Systems, Mineapolis, MN) et selon les recommandations indiquées. Tous les échantillons de plasma des patients ont été analysés en duplicats. Les valeurs de densité optique étaient considérées comme significatives si celles-ci étaient au moins deux fois supérieures à la valeur du bruit de fond.

Chez la souris, les taux sériques de VEGF, MMP9 (*matrix metallopeptidase 9*), SDF-1 (*stromal-derived factor-1*) et de G-CSF ont été déterminés avec des kits ELISA (R&D Systems) selon les recommandations indiquées. Etant donné que les recommandations sont d'utiliser le sérum pour les mesures de SDF-1 et de G-CSF, toutes les mesures de facteurs pro-angiogéniques ont été réalisées sur le sérum et non le plasma chez la souris. Les prélèvements sanguins ont été réalisés sur tube sec et centrifugés, puis le sérum a été aliquoté, congelé puis conservé à -70°C. Les taux de SDF-1 ont aussi été déterminés dans des échantillons de moelle osseuse. Pour cela, les fémurs et tibias ont été prélevés immédiatement après sacrifice de la souris aux différents temps de mesure, et la moelle a été purgée avec une aiguille (21G) montée d'une seringue dans 100 µl de milieu RPMI (Gibco, Paisley, UK) supplémenté avec 10% de sérum de veau fœtal (Gibco).

La suspension de moelle osseuse a été centrifugée puis le surnageant a été congelé et conservé à -80°C. Les valeurs de densité optiques étaient considérées comme significatives si celles-ci étaient au moins deux fois supérieures à la valeur du bruit de fond.

3.5 Drogues

Pour les modèles murins de xénogreffes, la combretastatin-A4 3-O phosphate (CA-4-P) a été fourni par Toroma Organics (Saarbruecken, Germany) et a été conservé à 4°C. La formulation a été reconstitué pour obtenir une concentration de 30 mg/mL dans du sérum physiologique (chlorure de sodium 0.9%) et a été administré comme une dose unique de 50 mg/kg ou 100 mg/kg en injection au niveau de la veine caudale. Les groupes contrôles ont reçu du sérum physiologique selon les mêmes modalités. Le SU11248/sunitinib malate a été fourni par Pfizer et gardé à température ambiante. La formulation a été resuspendue dans de la carboxy-methylcellulose de sodium (0.5% poids/vol), du NaCl (1.8% poids/vol), du Tween 80 (0.4% poids/vol), et de l'isopropanole (0.9% poids/vol) pour obtenir une concentration de 12 mg/mL. Le sunitinib a été donné par voie orale à la dose de 40 mg/kg (100µL) une fois par jour avec des schémas d'administrations différentes. Les groupes contrôles ont reçu de la methylcellulose selon les mêmes modalités. Le DC101, un anticorps monoclonal de rat dirigé spécifiquement contre le VEGFR-2/flk-1 de souris, a été administré par voie intra-péritonéale (i.p.) à une concentration de 800µg/souris. Les traitements administrés étaient donné comme le CA-4-P, le sunitinib, ou le DC101, seul ou en association avec le sunitinib ou le DC101 et ce, 24 heures avant l'injection de CA-4-P ou avant le deuxième pic de CEP.

3.6 Analyses statistiques

Les valeurs de CEC et de CEP sont dans l'ensemble exprimées comme des valeurs médianes (±écarts-types). Les stratégies d'expression des résultats et les tests statistiques utilisés sont présentés en détails dans chaque article dans la section consacrée à la méthodologie.

4. RÉSULTATS

Article 1 : ***Clinical Cancer Research. 2009 July 15;15(14) :4561-71***

High levels of circulating VEGFR2^{+} bone –marrow derived progenitor cells correlate with metastatic disease in patients with pediatric solid malignancies.

Taylor M, Rössler J, Geoerger B, Laplanche A, Hartmann O, Vassal G, Farace F.

Nous avons mesuré les taux de CEC et CEP chez 45 enfants et adolescents atteints de tumeurs solides pédiatriques localisées (n=23) ou métastatiques (n=22) avant tout traitement, ainsi que chez 20 sujets sains d'une population pédiatrique afin de déterminer si ces cellules pouvaient être de potentiels biomarqueurs de l'angiogénèse tumorale dans ce type de tumeur (28). Les taux de CEC et CEP chez les patients ont été analysés ainsi que les taux de protéines plasmatiques pro-angiogéniques, le VEGF et le sVEGFR2.

Contrairement aux tumeurs adultes, les tumeurs pédiatriques évaluées dans cette cohorte n'étaient pas associées à des taux élevés de CEC. La valeur médiane des CEC mesurée chez les 45 enfants présentant une tumeur solide était de 7 CEC/ ml (range, 0-152/ml) et n'était pas significativement différente de celle des sujets sains et de 7,5 CEC/ ml (range, 0-56/ml) ($p<0,71$) (Figure 9). Nous n'avons pas observé de différence entre les taux de CEC et le stade localisée ou métastatique de la maladie : 7 CEC/ ml (range, 0-52/ml) *versus* 7,5 CEC/ml (range, 0-152/ml), respectivement ($p<0,26$). Les taux de CEC n'étaient pas corrélés au sexe ni à l'âge des patients (coefficient de corrélation, 0,015; $p<0,91$).

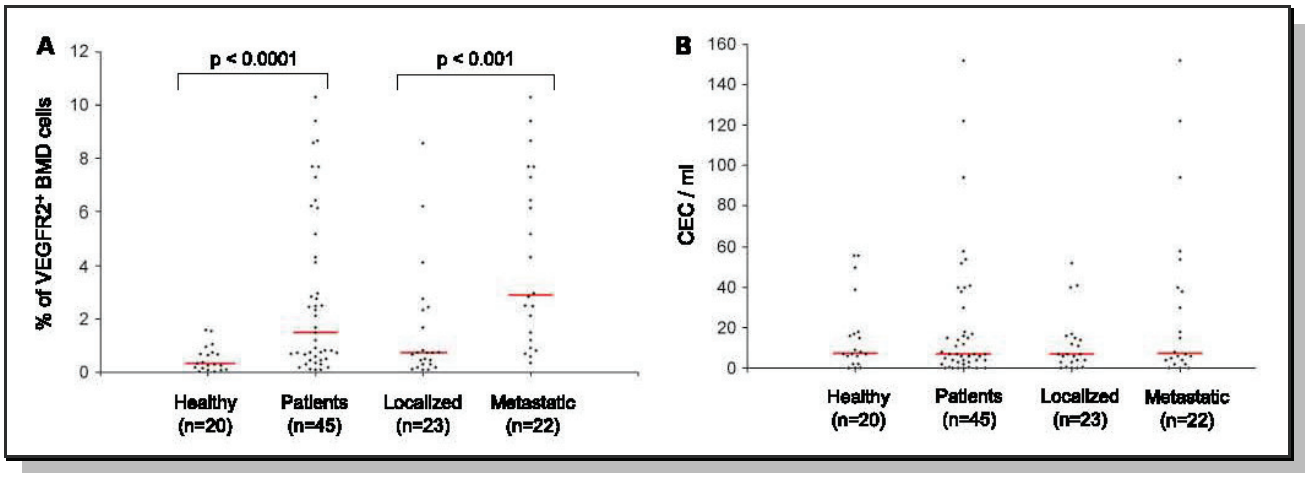

Figure 9. Taux de CEC et de CEP (identifiées par le phénotype CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}) mesurés chez 45 patients ayant une tumeur solide pédiatrique localisée (n=23) ou métastatique (n=22) ainsi que chez 20 sujets sains.

Nous avons analysé le taux d'expression membranaire de VEGFR2 parmi les fractions $CD45^{-}CD34^{+}7AAD^{-}$ et $CD45^{dim}CD34^{+}7AAD^{-}$ de progéniteurs circulants chez les patients et les sujets sains. La fraction $CD45^{-}CD34^{+}VEGFR2^{+}7AAD^{-}$ était indétectable (<0,003% des progéniteurs $CD34^{+}$ circulants) dans ces deux groupes. En revanche, la valeur médiane de la fraction $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ représentait 0,3% (range, 0-1.6%) et 1,5% (range, 0-32,5%) des progéniteurs $CD34^{+}$ circulants parmi les sujets sains et les patients avec une tumeur solide pédiatrique, respectivement. De plus, nous avons observé que cette population de CEP, de phénotype $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$, était significativement plus exprimée chez les patients par rapport aux sujets sains ($p<0.0001$) (Figure 9). Les valeurs de CEP étaient aussi significativement plus élevées dans le groupe de patients avec une maladie métastatique que dans le groupe de patient avec une maladie localisée : valeurs médianes 2.9% (range, 0,6-10,3%) *versus* 0.7% (range, 0-8,6%) des cellules $CD34^{+}$ totales, respectivement ($p<0.001$) (Figure 9). La différence entre les valeurs de CEP observées chez les patients ayant une maladie métastatique et ceux avec une maladie localisée restait significative lorsque ces valeurs étaient exprimées en valeur absolue : 50 cellules/ml (range, 4-89/ml) *versus* 8 cellules (range, 0-135/ml), respectivement ($p<0,007$). Aucune corrélation entre les taux de progéniteurs $CD34^{+}$ totales et le stade de la maladie n'a été observée. Enfin, les taux plasmatiques médians de VEGF et sVEGFR2 mesurés chez les 45 patients étaient de 52 ng/ml (range, 0-988) et de 9817 ng/ml (range, 6601-21113), respectivement. Ces taux n'étaient pas corrélés avec le stade de la maladie ou les taux de CEC ou de CEP.

Seules les valeurs de CEP corrélaient fortement avec le statut de la maladie alors qu'aucune corrélation n'était observée avec les taux de CEC, de VEGF ou de VEGFR2. Etant donné que l'identification de l'incorporation de CEP est difficile à mettre en place d'un point de vue technique chez l'homme, nous n'avons pas pu affirmer l'existence d'une incorporation des CEP dans le néo-endothélium des métastases. Néanmoins, la corrélation entre les taux de CEP avec la sévérité de la maladie, et ce malgré la diversité des types de tumeurs explorées, suggérait que la vasculogénèse pouvait être en effet un mécanisme important de la vascularisation des tumeurs pédiatriques, plus particulièrement dans la maladie métastatique.

Ce premier travail a été à la source d'une réflexion générale sur l'intérêt des stratégies anti-angiogéniques dans les tumeurs pédiatriques, en particulier dans le neuroblastome. Les quatre articles publiés sur ce thème sont présentés en Annexe (33-35).

High Levels of Circulating VEGFR2$^+$ Bone Marrow–Derived Progenitor Cells Correlate with Metastatic Disease in Patients with Pediatric Solid Malignancies

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Abstract

Purpose: Pediatric solid malignancies display important angiogenic potential, and blocking tumor angiogenesis represents a new therapeutic approach for these patients. Recent studies have evidenced rare circulating cells with endothelial features contributing to tumor neovascularization and have shown the pivotal role of bone marrow–derived (BMD) progenitor cells in metastatic disease progression. We measured these cells in patients with pediatric solid malignancies as a prerequisite to clinical trials with antiangiogenic therapy.

Patients and Methods: Peripheral blood was drawn from 45 patients with localized (n = 23) or metastatic (n = 22) disease, and 20 healthy subjects. Subsets of circulating vascular endothelial growth factor receptor (VEGFR)2$^+$-BMD progenitor cells, defined as CD45$^-$CD34$^+$VEGFR2(KDR)$^+$7AAD$^-$ and CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ events, were measured in progenitor-enriched fractions by flow cytometry. Mature circulating endothelial cells (CEC) were measured in whole blood as CD31$^+$CD146$^+$CD45$^-$7AAD$^-$ viable events. Data were correlated with VEGF and sVEGFR2 plasma levels.

Results: The CD45$^-$CD34$^+$VEGFR2(KDR)$^+$7AAD$^-$ subset represented <0.003% of circulating BMD progenitor cells (≤0.05 cells/mL). However, the median level (range) of the CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ subset was higher in patients compared with healthy subjects, 1.5% (0%-10.3%) versus 0.3% (0%-1.6%) of circulating BMD progenitors (P < 0.0001), and differed significantly between patients with localized and metastatic disease, 0.7% (0%-8.6%) versus 2.9% (0.6%-10.3%) of circulating BMD progenitors (P < 0.001). Median CEC value was 7 cells/mL (0-152 cells/mL) and similar in all groups. Unlike VEGFR2$^+$-BMD progenitors, neither CECs, VEGF, or sVEGFR2 plasma levels correlated with disease status.

Conclusion: High levels of circulating VEGFR2$^+$-BMD progenitor cells correlated with metastatic disease. Our study provides novel insights for angiogenesis mechanisms in pediatric solid malignancies for which antiangiogenic targeting of VEGFR2$^+$-BMD progenitors could be of interest.

Pediatric solid malignancies have an overall better survival and outcome compared with adult cancers (1). However, prognosis remains grim for patients with metastatic or relapsed solid malignancies in spite of aggressive therapy associating surgery, radiation, and intensive chemotherapy often including high-dose regimens with autologous hematopoietic stem cell support. Novel therapeutic approaches are urgently needed to improve prognosis in this pediatric population (2). Angiogenesis, the process of new–blood vessel formation from preexisting vasculature, plays a key role in the growth, development, and metastatic dissemination of solid malignancies (3). Antiangiogenic therapy is emerging as one of the most significant advances in clinical oncology and, although most clinical studies have focused on adult malignancies (4), antiangiogenesis represents an appealing therapeutic strategy in pediatric oncology as well. Unlike adult solid malignancies, which derive mainly from epithelial tissue and have low proliferation rates and vascularity, pediatric solid malignancies display a strong angiogenic profile because they are nearly exclusively undifferentiated tumors characterized by high proliferation rates and increased vascularity. Preclinical data and early phase I clinical trials support the potential role of antiangiogenic agents in treating pediatric solid malignancies (5, 6). Subsequently, understanding the angiogenic processes involved could help guide the optimal use of antiangiogenic strategies in the clinical setting.

Tumor vascularization is dependent on the sprouting of nearby blood vessels, with migration and differentiation of

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Translational Relevance

Pediatric solid malignancies display important angiogenic potential, and blocking tumor angiogenesis represents a new therapeutic approach for these patients. This is the first report evaluating circulating endothelial cells, bone marrow–derived (BMD) endothelial progenitor cells, and angiogenic plasma proteins in the peripheral blood of patients with pediatric solid malignancies. We observed that strikingly high levels of BMD endothelial progenitors correlated with metastatic disease. These results support and extend recent preclinical findings indicating that these cells may play a pivotal role in metastatic disease progression. Our data suggest that monitoring and targeting of BMD endothelial progenitor cells could be of interest to guide the optimal use of antiangiogenic treatments in patients with pediatric solid malignancies. Finally, our findings raise important questions about clinical situations that mobilize hematopoietic, and possibly endothelial progenitors, because these cells may adversely promote tumor vasculogenesis after anticancer treatment associated with stem cell growth factors.

existing mature endothelial cells (angiogenesis), and on the recruitment of mobilized bone marrow–derived (BMD) endothelial progenitor cells (vasculogenesis; refs. 7, 8). Rare circulating BMD endothelial progenitors (commonly referred to as CEP) contribute to tumor neovessels in cancer-bearing animals (7, 9–13) as well as in humans (14, 15). Moreover, inhibition of circulating endothelial progenitor (CEP) recruitment prevents tumor growth in animal models (9, 11, 12), thus establishing their significance in tumor progression. Besides true CEP structurally incorporating growing vessels, several subsets of BMD progenitor cells are also involved in tumor vasculogenesis (9, 11, 16–19). Hematopoietic (VEGFR1$^+$) and endothelial (VEGFR2$^+$) BMD progenitors collaborate in disease progression, the first to initiate the premetastatic niche and the second to promote the vascularization of metastatic lesions (20–22). For these reasons, circulating BMD endothelial progenitors and mature endothelial cells have been intensively studied in cancer patients. However, whereas these rare cells have been documented in adult patients and are currently being investigated as potential biomarkers of antiangiogenic therapies (8, 23), there exists no data in a pediatric population.

The aim of the present study was to provide the first evaluation of levels of circulating VEGFR2$^+$-BMD progenitor cell subsets and circulating endothelial cells (CEC), as well as plasma levels of VEGF and sVEGFR2, in patients with various pediatric solid malignancies as a prerequisite to future clinical trials with antiangiogenic agents. Results were compared with data from healthy volunteers of similar ages. Whereas CECs, VEGF, and sVEGFR2 levels were not correlated with disease status, a subset of VEGFR2$^+$-BMD progenitors, immunophenotypically defined as CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ viable events, was found in significantly higher levels in patients with metastatic disease.

Patients and Methods

Patients and blood sample collection. Informed parental consent and, when appropriate, child consent were obtained for all patients and healthy volunteers. The present study was approved by our Institutional Review Board and local ethics committee. Patients ages 1 to 25 y with localized or metastatic pediatric solid malignancies were included either at initial diagnosis or at relapse. Patients undergoing anticancer therapy, or with a known history of vascular disease or deep venous thrombosis, were excluded. All patients were sampled before anticancer treatment to avoid influencing results with potential therapy-induced vascular lesions. For this, patients at initial diagnosis were sampled before treatment and at least 2 wk after minor surgery (central venous catheter or tumor biopsy). Also, patients at relapse were included only if the minimum delay between the end of prior anticancer treatments and blood sampling was 2 mo. Patients with lymphoma were distinguished according to Ann Arbor staging as having localized (stage I or II) or metastatic disease (stage III or IV). Standard clinicopathologic information was recorded for all patients.

Healthy volunteer subjects of similar ages were recruited from a local general pediatric department and were free of inflammatory, infectious, autoimmune, or vascular disease. Blood sampling for the study was done during routine blood workup.

After discarding the first 2 mL after venupunction, peripheral blood samples were drawn: 2 mL of whole blood was collected in Cellsave preservative tubes (Immunicon) for CEC analysis, and 10 mL whole blood was collected in standard heparin tubes for circulating VEGFR2$^+$-BMD progenitor cell analysis. Cellsave tubes contain EDTA and a cell preservative agent that stabilizes fragile cells, such as CECs, and have been previously validated in our laboratory for CEC measurement by flow cytometry (24).

Measurement of circulating VEGFR2$^+$-BMD progenitors. Circulating subsets of VEGFR2$^+$-BMD progenitor cells were measured in 10 mL of progenitor-enriched whole blood by four-color flow cytometry as previously reported (14). Briefly, Ficoll-gradient mononuclear cells were enriched with the use of the RosetteSep antibody cocktail

Table 1. Characteristics of patients with pediatric solid malignancies (N = 45)

Patients	Localized	Metastatic
n	23	22
Male	15	17
Median age, y	9.1	12.9
Range, y	1-22.5	2.7-25
Initial diagnosis	22	12
Relapsed disease	1	10
Tumor type		
CNS	8	2
Sarcoma	8	9
Lymphoma	2	3
Other*	5	8
Metastatic site		
Lung		8
Bone		5
Bone marrow		7
Other		8

*Other: neuroblastoma, nephroblastoma, hepatoblastoma, and primitive neuroectodermal tumor.

Table 2. Results of CEC, VEGFR2$^+$-BMD progenitor, VEGF, and sVEGFR2 levels for 45 patients with localized or metastatic pediatric solid malignancies

Patient	Age/Sex	Localized disease (n = 23)	Initial diagnosis/ relapsed disease (n = 34/11)	CEC/mL	% VEGFR2$^+$-BMD*,†,‡,§ (absolute value/mL)	CD34$^+$/mL‖	VEGF (ng/mL)	sVEGFR2 (ng/mL)
CNS tumor								
P1	1.3/M	Glioneuronal tumor	I	16	0.5 (15)	3,230	841	10,313
P2	2.7/M	Brain stem pilocytic astrocytoma	I	0	0.7 (24)	3,330	988	7,977
P3	2.7/M	Low grade glioma (ventricles)	I	41	0.3 (4)	1,405	532	9,170
P4	9.4/F	Brain stem glioma	I	6	1.7 (ND)	ND	0	9,492
P5	11.5/M	Atypical teratoid rhabdoid tumor	I	17	4.1 (ND)	ND	60	9,781
P6	6.3/M	Medulloblastoma	I	14	0.1 (3)	2,366	72	11,942
P7	9.5/F	Medulloblastoma	I	0	0.7 (135)	19,957	27	12,708
P8	18/F	Medulloblastoma	I	7	2.3 (ND)	ND	28	7,784
Sarcoma								
P9	4.7/M	Rhabdomyosarcoma	I	52	0.3 (6)	1,975	0	9,045
P10	13/M	Rhabdomyosarcoma	I	6	0.7 (16)	2,550	52	7,401
P11	9/F	Undifferentiated sarcoma (liver)	I	0	0.7 (4)	514	152	11,304
P12	9.3/F	Synovialosarcoma	R	12	6.2 (78)	1,360	44	13,164
P13	17/F	Primitive neuroectodermal tumor	I	3	2.7 (16)	610	0	6,988
P14	14.3/M	Ewing's tumor	I	15	0 (0)	ND	0	11,566
P15	16.5/M	Ewing's tumor	I	0	0.4 (ND)	ND	28	10,392
P16	22.5/M	Ewing's tumor	I	3	0.1 (4)	3,910	21	10,541
Lymphoma¶								
P17	5.3/F	Hodgkin's disease (I)	I	7	2.4 (30)	1,495	15	10,352
P18	16.8/M	Burkitt lymphoma (I)	I	0	0.8 (8)	1,030	32	8,431
Other								
P19	1/F	Hepatoblastoma	I	7	0.4 (5)	1,028	726	10,958
P20	1.4/M	Hepatoblastoma	I	40	0.2 (2)	1,345	30	10,284
P21	1/M	Nephroblastoma (bilateral)	I	4	0.2 (3)	1,773	241	9,467
P22	5.8/M	Nephroblastoma	I	4	0.7 (19)	2,608	124	9,526
P23	5.7/M	Neuroblastoma (NMYC+)	I	11	8.5 (76)	1,020	110	7,602

Patient	Age/Sex	Metastatic disease (n = 22)	Initial diagnosis/ relapsed disease (n = 34/11)	Metastatic site	CEC/mL	% VEGFR2$^+$-BMD*,†,‡,§ (absolute value/mL)	CD34$^+$/mL‖	VEGF (ng/mL)	sVEGFR2 (ng/mL)
Sarcoma									
P24	11/M	Osteosarcoma	I	Bone	122	1.5 (55)	3,676	103	12,598
P25	14.9/M	Osteosarcoma	I	Lung, bone	38	7.3 (23)	380	79	11,216
P26	15/M	Osteosarcoma	I	Lung	152	6.4 (71)	1,190	95	9,817
P27	15/F	Ewing's tumor	I	Lung	30	6.1 (18)	370	0	7,309
P28	10/M	Alveolar Rhabdomyosarcoma	R	BM	15	9.4 (16)	295	59	11,209
P29	17/M	Osteosarcoma	R	Lung	40	2.8 (46)	1,780	9	9,931
P30	18.5/M	Osteosarcoma	R	Lung	8	10.3 (33)	410	4	6,813
P31	19.2/M	Osteosarcoma	R	Lung	4	0.7 (4)	645	28	7,759
P32	19.9/M	Osteosarcoma	R	Lung	7	4.3 (ND)	ND	15	7,976
Lymphoma¶									
P33	3.5/M	Anaplasic lymphoma (III)	I	Pleural	58	2.1 (ND)	ND	37	9,439
P34	11.5/F	Anaplasic lymphoma (III)	I		2	2.9 (60)	2,575	0	21,113
P35	14.3/M	Hodgkin's disease (IV Bb)	I	Lung, bone	5	0.8 (ND)	ND	240	10,064

(*Continued on the following page*)

Table 2. Results of CEC, VEGFR2$^+$-BMD progenitor, VEGF, and sVEGFR2 levels for 45 patients with localized or metastatic pediatric solid malignancies (Cont'd)

Patient	Age/Sex	Metastatic disease (n = 22)	Initial diagnosis/ relapsed disease (n = 34/11)	Metastatic site	CEC/mL	% VEGFR2$^+$-BMD*,†,‡,§ (absolute value/mL)	CD34$^+$/mL‖	VEGF (ng/mL)	sVEGFR2 (ng/mL)
CNS tumor									
P36	4/F	Glioblastoma	I	Leptomeningeal	0	1.2 (ND)	ND	72	12,295
P37	20.7/M	Medulloblastoma	R	Brain, BM	2	2.5 (63)	2,595	0	11,761
Other									
P38	2.7/M	Neuroblastoma (NMYC-)	I	BM, bone	6	0.9 (ND)	ND	69	10,133
P39	3/F	Neuroblastoma (NMYC-)	I	BM, liver	4	0.4 (15)	4,068	545	6,601
P40	3/M	Neuroblastoma (NMYC-)	I	Lymph nodes	6	2.5 (ND)	ND	521	11,067
P41	5/M	Neuroblastoma (NMYC-)	I	BM, bone	18	0.6 (88)	14,812	988	11,070
P42	7/F	Neuroblastoma (NMYC-)	R	BM	94	8.6 (50)	582	134	8,470
P43	25/M	Neuroblastoma (NMYC-)	R	BM, liver	54	5.2 (60)	1,161	76	8,695
P44	10/M	Hepatoblastoma	R	Endovascular	0	7.9 (89)	1,285	30	7,695
P45	21/M	Nephroblastoma (2nd relapse)	R	Pancreas	0	7.7 (ND)	ND	30	7,939

Abbreviations: BM, bone marrow; I, initial diagnosis; R, relapsed disease; ND, not determined.
*VEGFR2$^+$-BMD progenitor cells were defined as CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ events.
†P values: all 45 patients, localized versus metastatic disease, $P < 0.001$.
‡All 45 patients, initial versus relapsed disease, $P < 0.0001$.
§Patients at initial diagnosis (n = 34), localized versus metastatic disease, $P < 0.02$.
‖CD34+ cell counts determined as CD45$^{dim/-}$CD34+ events.
¶Patients with lymphoma were distinguished according to Ann Arbor staging as having localized (stage I or II) or metastatic disease (stage III or IV).

(StemCell Technologies Inc.) and then distributed into control and test tubes before treatment with FcR blocking reagent (Miltenyi Biotec). Staining was done with the following monoclonal antibodies: CD45-FITC (clone T29/33; DakoCytomation), CD34-APC (clone BIRMA-K3; DakoCytomation), KDR-PE (clone 89106; R&D Systems), and 7AAD (BD Biosciences). The viability dye 7AAD (BD Biosciences) was used to eliminate background noise occasioned by dead cells. Antibody batches were titrated rigorously to determine the optimal dosage and to be used in excess, except KDR-PE, which was used according to manufacturer's recommendations. Control tubes included isotypic and fluorescence-minus-one controls for each of the fluorochromes. A control tube, including a mouse IgG1-PE reagent (CD45-FITC/mouse IgG1-PE/CD34-APC/7AAD), was done to measure background noise accurately and to adjust the gates precisely. The IgG1-PE reagent was purchased from the same manufacturer and used in the exact same quantity as the KDR-PE. Cells were acquired on a FACSCalibur system (BD Biosciences), and data were analyzed with the use of CellQuest 3.2 software. Additionally, absolute circulating CD34$^+$ cell counts (CD45$^{dim/-}$CD34$^+$ events) were determined for each blood sample according to the BD Procount progenitor cell enumeration kit (BD Biosciences). Results were expressed as the percentage of VEGFR2$^+$ cells among circulating BMD progenitor (CD45^{dim}CD34$^+$7AAD$^-$ and CD45$^-$CD34$^+$7AAD$^-$) cells, and as the absolute values of CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ and CD45$^-$CD34$^+$VEGFR2$^+$7AAD$^-$ progenitor cells per milliliter, respectively.

Measurement of CECs. CECs were measured in 1 mL whole blood by four-color flow cytometry according to a method we previously reported (24). Briefly, immunofluorescent staining was done with the following monoclonal antibodies: CD31-FITC (clone WM59; BD Pharmingen), CD146-PE (clone P1H12; BD Pharmingen), and CD45-APC (clone T29/33; DakoCytomation). Antibody batches were titrated rigorously. An IgG-PE control was done in 0.5 mL of whole blood (CD45-APC/CD31-FITC/mouseIgG1-PE/7AAD) to measure background noise and to adjust the gates precisely. Flow-Check microbeads (Beckman Coulter) were added to a tube containing isotypic controls to adjust forward scatter (FSC) voltage and to identify cell events with a size >10 μm. Cells were analyzed on a FACSCalibur system (BD Biosciences). To ensure statistical analysis, all of the cells contained in the IgG-PE control tube and in the CEC test tube were acquired, representing ~2.5 × 10^6 and 5 × 10^6 events, respectively. Data were analyzed with the use of CellQuest 3.2 software.

Determination of plasma VEGF and sVEGFR2 levels. Plasma levels of VEGF and sVEGFR2 were determined with the use of commercially available ELISA kits (R&D Systems) and according to manufacturer's guidelines. All plasma samples were assayed in duplicates. The absorbance values obtained were plotted against standard curves generated on the ELISA plate with a correlation coefficient >0.99. Absorbance values were considered significant if found to be at least twice as high as background noise.

Statistical analysis. Results are expressed as medians and range. Groups were compared with the use of a two-sided nonparametric Mann-Whitney test. Data were analyzed with SPSS software (version 15.0). A P value ≤0.05 was considered as statistically significant. Correlations were measured with the use of the Pearson correlation coefficient.

Results

Patient characteristics. Levels of VEGFR2$^+$-BMD progenitor cells, CECs, plasma VEGF, and sVEGFR2 were measured in 45 patients, of whom 31 were male. Thirty-four patients were at initial diagnosis, whereas 11 patients had relapsed disease, of which 10 of 11 were at metastatic sites. At the time of evaluation, 23 patients had localized disease and 22 patients had metastatic disease. Median (range) age was 10 (1-25 y) years among patients and did not differ significantly between localized or metastatic populations (9.1 and 12.9 y, respectively; $P < 0.07$). Patients more than the age of 18 y (8 of 45) were in majority those with relapsed disease (6 of 8). Median

time between blood sampling and the end of prior anticancer treatments for relapsing patients was 5.4 (3.7-19 mo) months. The clinical and biological characteristics of the 45 patients evaluated are presented in Tables 1 and 2.

Levels of CECs and VEGFR2$^+$-BMD progenitor cells were measured in 20 healthy subjects, of whom 8 were male. Median age of healthy subjects was 8.7 (1.7-18.2 y) years and did not differ significantly from that of patients with pediatric malignancies ($P < 0.81$).

Levels of circulating VEGFR2$^+$-BMD progenitors. Subsets of VEGFR2$^+$-BMD progenitor cells were measured in progenitor-enriched whole blood and were identified as the rare fraction of circulating BMD progenitor cells (CD45^{dim}CD34$^+$7AAD$^-$ and CD45$^-$CD34$^+$7AAD$^-$) expressing the VEGFR2(KDR) receptor. VEGFR2 was not significantly expressed in the CD45$^-$CD34$^+$7AAD$^-$ subset, because the CD45$^-$CD34$^+$VEGFR2$^+$7AAD$^-$ subset represented <0.003% of circulating BMD progenitor cells (≤0.05 cell/mL) in both patient and healthy control populations. However, VEGFR2 was expressed in the CD45^{dim}CD34$^+$7AAD$^-$ subset with a median level of 0.3% (0%-1.6%) of circulating BMD progenitors in healthy subjects and a median level of 1.5% (0%-10.3%) of circulating BMD progenitors in all 45 patients (Fig. 1A). The median level of CD45^{dim}CD34$^+$-VEGFR2$^+$7AAD$^-$ cells was significantly higher in patients with pediatric solid malignancies compared with healthy subjects ($P < 0.0001$). Median absolute values of CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ cells, available for 50 subjects, also differed significantly between 34 patients, 18.5 cells/mL (0-135.0 cells/mL), and 16 healthy subjects, 5.0 cells/mL (0-25.0 cells/mL; $P < 0.001$).

Among patients, the median level of CD45^{dim}CD34$^+$-VEGFR2$^+$7AAD$^-$ cells was 0.7% (0%-8.6%) of circulating BMD progenitors for those with localized disease but was 2.9% (0.6%-10.3%) of circulating BMD progenitors and was significantly higher for patients with metastatic disease ($P < 0.001$; Fig. 1A). Patient characteristics and respective CD45^{dim}CD34$^+$-VEGFR2$^+$7AAD$^-$ values are presented in Table 2. Median absolute values of CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ cells also differed significantly between patients with localized ($n = 19$) and metastatic ($n = 15$) disease: 8.0 (0-135.0 cells/mL) and 50.0 cells/mL (4.0-89.0 cells/mL), respectively ($P < 0.007$). No variability in total CD34$^+$ counts was observed between localized or metastatic populations ($P < 0.27$; Table 2).

Moreover, the 11 patients with relapsed disease had significantly higher levels of CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ cells compared with the 34 patients at initial diagnosis: 6.2% (0.7%-10.3%) and 0.8% (0%-8.6%) of circulating BMD

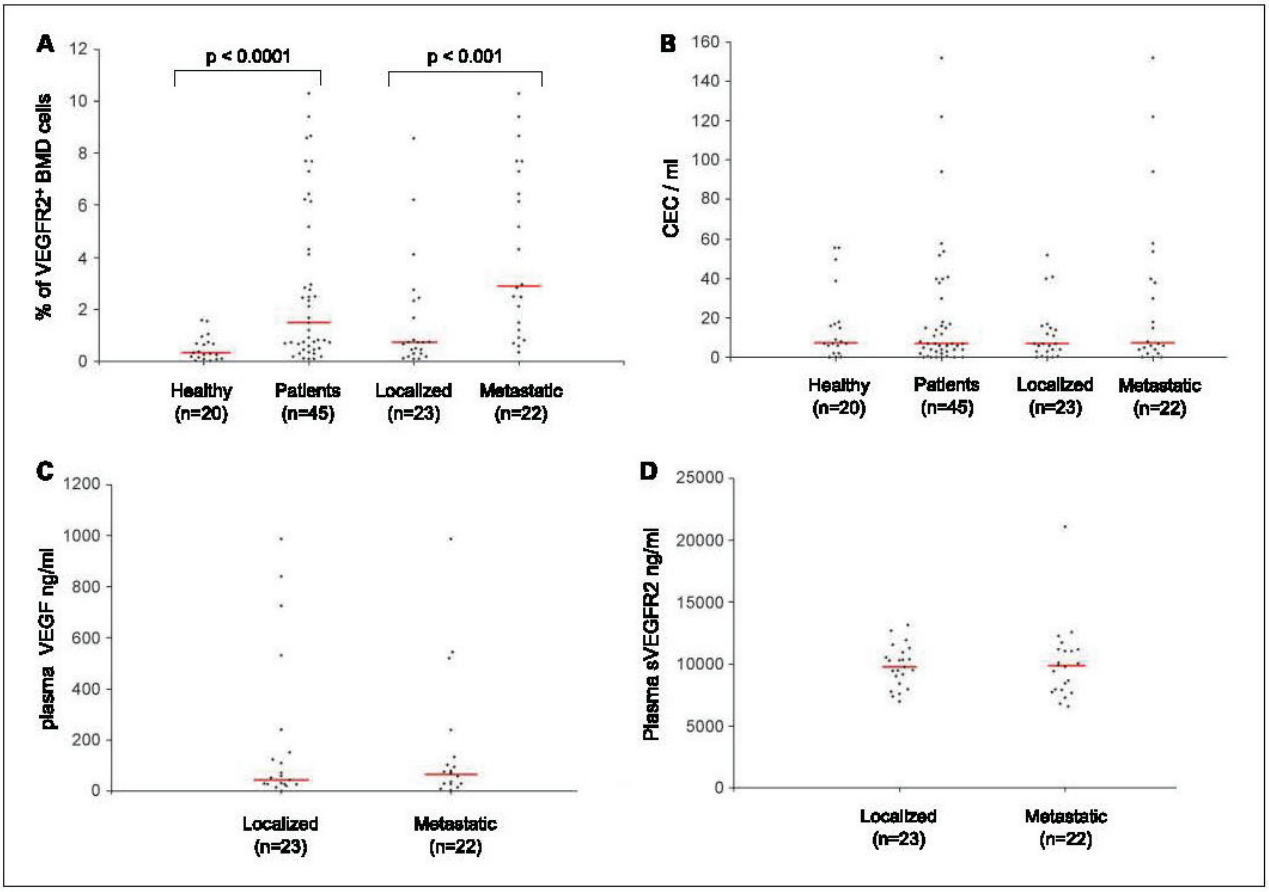

Fig. 1. Measurement of levels of CECs, VEGFR2$^+$-BMD progenitor cells, plasma VEGF, and sVEGFR2 in 45 patients with pediatric solid malignancies and 20 healthy subjects. Comparison of median levels of VEGFR2$^+$-BMD progenitor cells (*A*), CECs (*B*), plasma VEGF (*C*), and plasma sVEGFR2 (*D*) in healthy subjects and patients with localized versus metastatic disease. Horizontal line, median values.

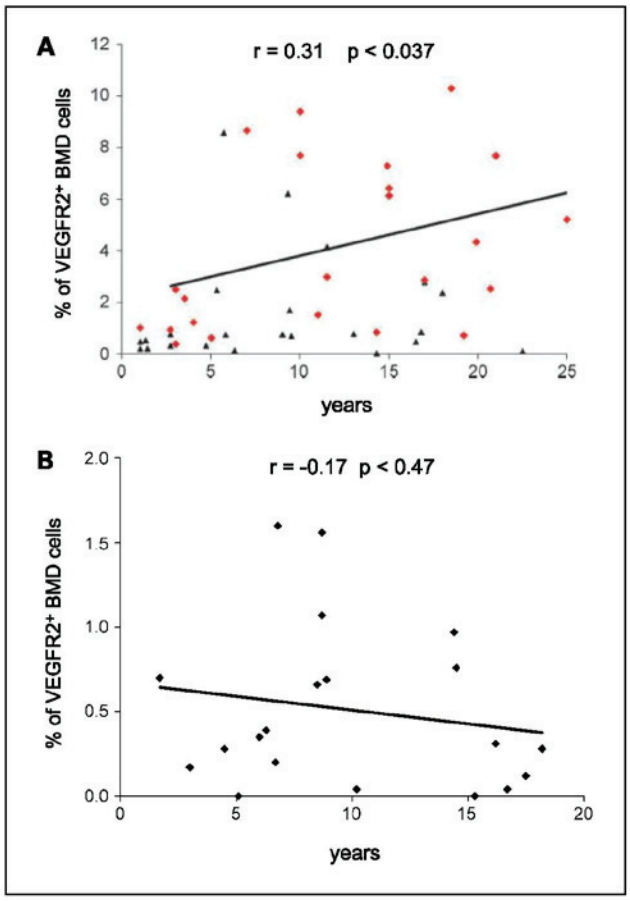

Fig. 2. Distribution of VEGFR2$^+$-BMD progenitor cells according to age in 45 patients with localized (*black triangle*) and metastatic (*red diamond*) pediatric solid malignancies (*A*) and in 20 healthy control subjects (*B*).

progenitors, respectively ($P < 0.0001$; Table 2). Because relapsing patients were older, we studied if the value of CD45^{dim}CD34$^+$-VEGFR2$^+$7AAD$^-$ cells was linked with age (Fig. 2). The correlation coefficient between levels of CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ cells and age was 0.31 ($P < 0.037$) for the 45 patients with pediatric solid malignancies (Fig. 2A). However, no correlation was observed among healthy subjects ($P < 0.47$), suggesting a lack of association between age and VEGFR2$^+$-BMD progenitor cell levels (Fig. 2B). No differences were observed between VEGFR2$^+$-BMD progenitor cell levels and gender ($P < 0.66$; data not shown). Also, we excluded relapsing patients and evaluated levels of CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ cells among 34 patients at initial diagnosis. In this subgroup, the median level of CD45^{dim}CD34$^+$-VEGFR2$^+$7AAD$^-$ cells was higher among 12 patients with metastatic disease compared with 22 patients with localized disease: 1.8% (0.3%-7.3%) and 0.7% (0%-8.6%) of circulating BMD progenitors, respectively ($P < 0.015$). Finally, as the biology of dissemination in lymphoma may be distinct from that of solid tumors, statistical analysis was done after excluding 5 patients with lymphoma and showed similar differences in levels of CD45^{dim}CD34$^+$-VEGFR2$^+$7AAD$^-$ cells between localized ($n = 21$) and metastatic ($n = 19$) populations: 0.6% (0%-8.6%) and 3.6% (0.3%-10.3%) of circulating BMD progenitors, respectively ($P < 0.001$).

It is noteworthy that the two patients with localized disease and displaying the highest levels of CD45^{dim}CD34$^+$VEGFR2$^+$-7AAD$^-$ cells had particular clinical features: patient P12 had locally relapsed synovialosarcoma, and patient P23 had stage III neuroblastoma with *MYCN* gene amplification. Results for a representative patient are shown in Fig. 3 according to the gating strategy used to identify circulating VEGFR2$^+$-BMD subsets by four-color flow cytometry.

Levels of CECs. CECs were identified as CD31$^+$CD146$^+$ CD45$^-$7AAD$^-$ viable events in whole blood by four-color flow cytometry (24). CEC values for the 45 patients evaluated are presented in Table 2. Median CEC levels did not differ between patients with pediatric solid malignancies and healthy subjects: 7.0 (0-152.0 cells/mL) and 7.5 cells/mL (0-56.0 cells/mL; $P < 0.71$; Fig. 1B). Also, although significant interpatient variability was observed, median circulating endothelial values were not different between patients with localized or metastatic disease: 7.0 (0-52.0 cells/mL) and 7.5 cells/mL (0-152.0 cells/mL), respectively ($P < 0.26$; Fig. 1B). CEC levels were not correlated with age (coefficient correlation, 0.015; $P < 0.91$) or with gender (data not shown). Also, no significant correlation was observed between CEC levels and initial or relapsing disease status, primary or metastatic tumor sites, or time elapsed between sampling and minor surgery (data not shown). To illustrate the gating strategy established to identify CECs by four-color flow cytometry, the results for P26, who had osteosarcoma of the leg with skin perforation and probable concurrent vascular lesions, are presented in Fig. 4.

Plasma levels of VEGF and sVEGFR2. Median levels of plasma VEGF and sVEGFR2 for the 45 patients were 52 ng/mL (0-988 ng/mL) and 9,817 ng/mL (6,601-21,113 ng/mL), respectively. Levels of plasma VEGF and sVEGFR2 did not differ between localized and metastatic disease populations (Fig. 1C and D). Also, no significant correlation was observed between plasma VEGF or sVEGFR2 levels and CECs, VEGFR2$^+$-BMD progenitor levels, age, or primary or metastatic tumor sites. Table 2 displays these data.

Discussion

We describe the first quantitative analysis of subsets of circulating VEGFR2$^+$-BMD progenitor cells and CECs in the peripheral blood of children and young adults with various pediatric solid malignancies. Due to the absence of data in healthy children, our results were compared with VEGFR2$^+$-BMD progenitor cell and CEC levels measured in a control population of 20 healthy subjects of similar ages. We found that levels of circulating VEGFR2$^+$-BMD progenitor cells, characterized as CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ events, were significantly higher in patients with pediatric solid malignancies compared with healthy subjects. Moreover, among patients, strikingly elevated levels of VEGFR2$^+$-BMD progenitor cells correlated with metastatic disease. Preclinical models have provided solid evidence that circulating endothelial progenitors play an important role in tumor progression (25). However, the variable degrees of incorporation of endothelial progenitors shown in different tumor models have led to controversy about the extent of their actual involvement in tumor vascularization (10, 13, 19, 26, 27). Identification of CEP is highly complex and has been hampered by the extreme rarity of these cells as well as the overlapping antigenic similarities between hematopoietic progenitors and

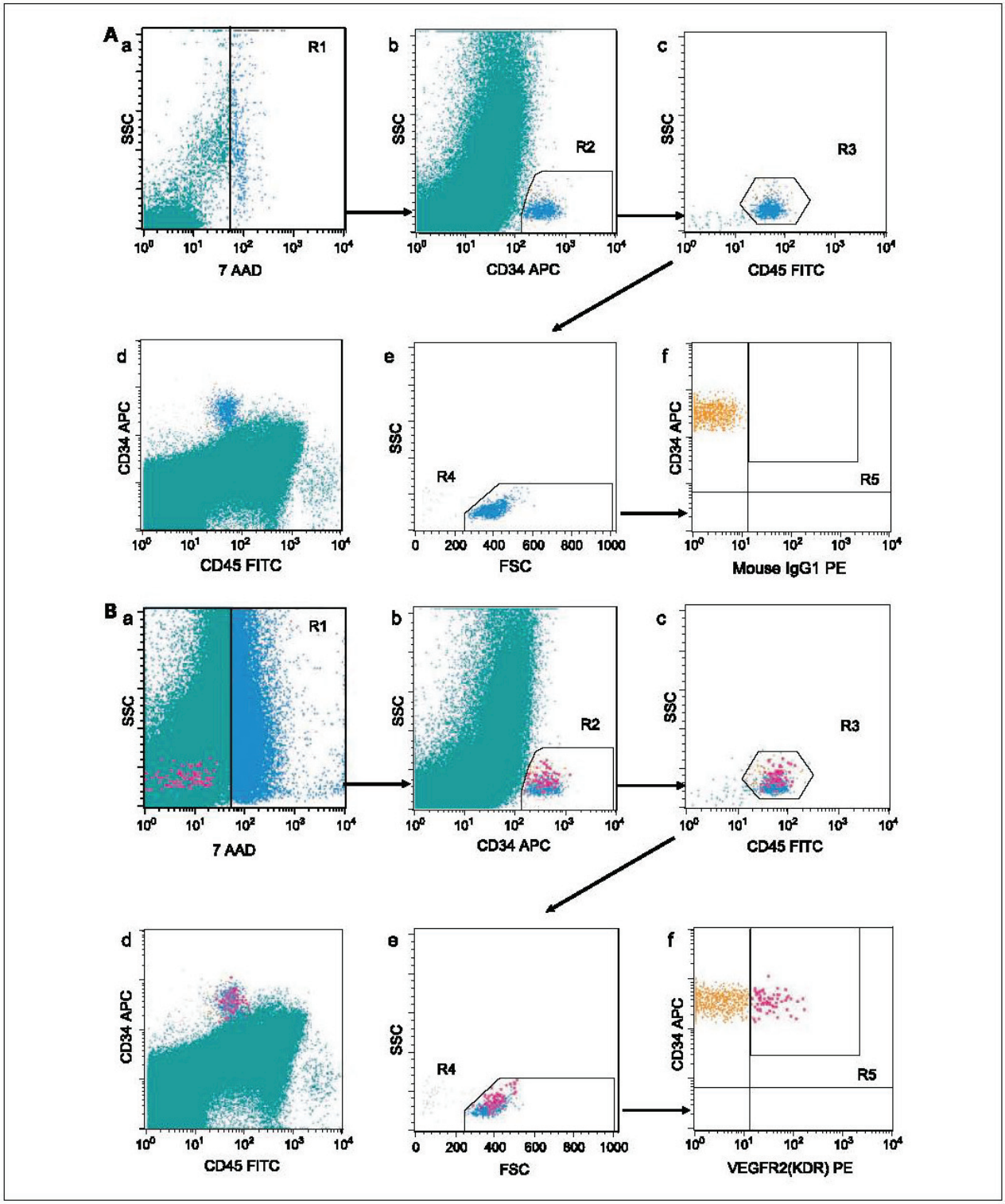

Fig. 3. control for flow cytometric detection of circulating $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor cells. *A*, analysis of the control (CD45-FITC/mouse IgG1-PE/CD34-APC/7AAD) tube. The control tube is used to place accurately the successive gates. Only 1% of the events are shown (*a*) to select the gating of viable $7AAD^{-}$ events (R1). Other panels, 100% of events. *B*, analysis of the test (CD45-FITC/KDR-PE/CD34-APC/7AAD) tube, for which 100% of events are shown (*all panels*). *b*, the R2 gate used to select $CD34^{+}$ events; *c*, the R3 gate used to select $CD45^{dim}$ events from the R2 gate; *d*, $CD45^{dim}CD34^{+}7AAD^{-}$ viable events; *e*, the R4 gate used to eliminate debris from the R3 gate; *f*, the R5 gate resulting from the crossing of all successive gates and used to count $VEGFR2^{+}$-BMD progenitor cells. *a* to *f* are identical for both the control and test tube. $VEGFR2^{+}$-BMD progenitor cells (*bold*) are characterized as $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ viable events.

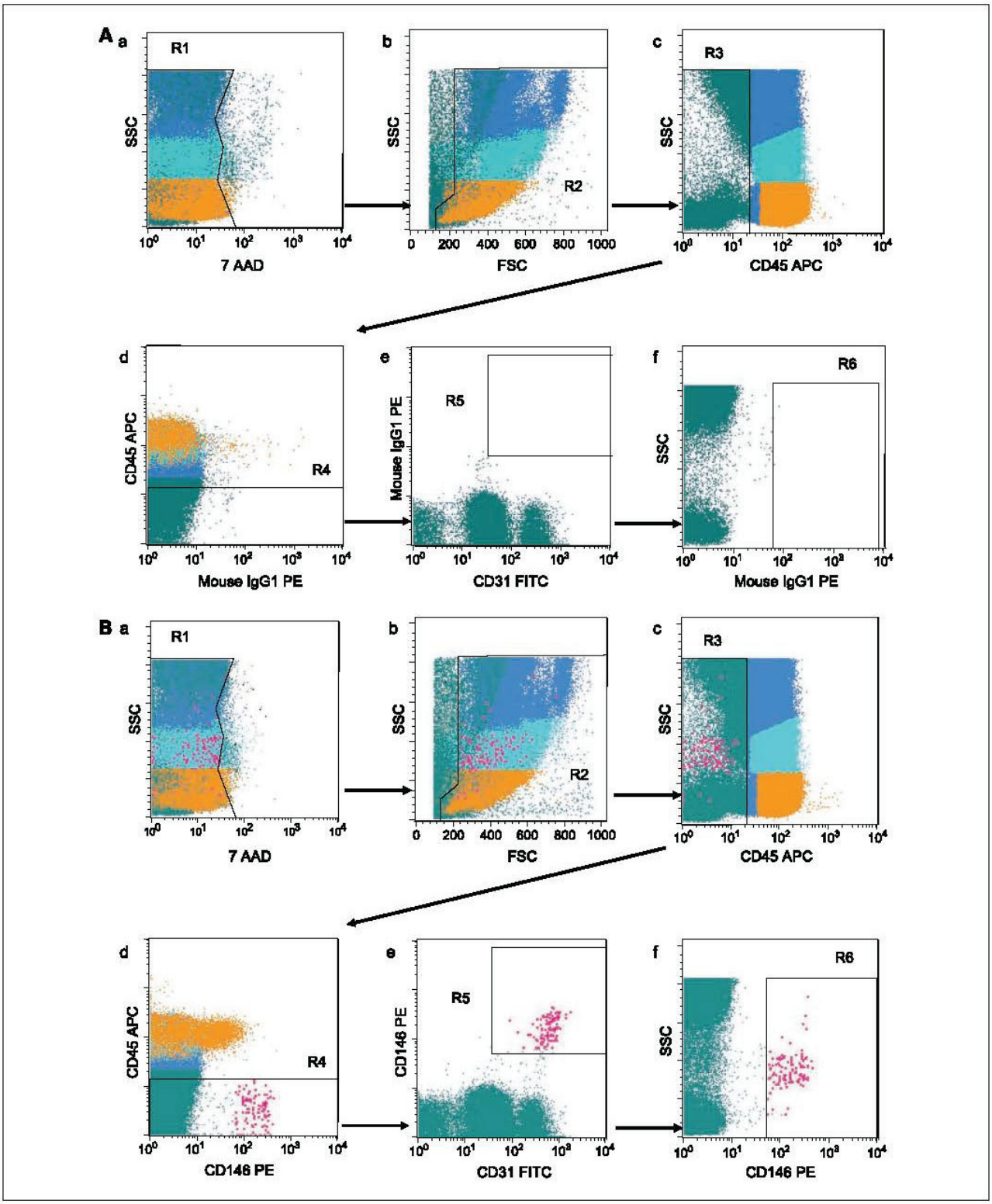

Fig. 4. Gating strategy for flow cytometric detection of CECs. *A*, analysis of the control (CD45-APC/CD31-FITC/mouse IgG1-PE/7AAD) tube. *B*, analysis of the test (CD45-APC/CD31-FITC/CD146-PE/7AAD) tube. Histograms, all stored events (2.5×10^6 events for the control and 5×10^6 events for the CEC test). *a* to *f* are identical between the control and test tube. *a*, gating of viable $7AAD^-$ events (R1); *b*, the large gate R2 used to exclude most debris and granulocytes without excluding very large cells; *c* and *d*, the R3 and R4 gates used to exclude $CD45^+$ events; *e*, $CD31^+CD146^+7AAD^-$ CECs in the $CD45^-$ gates; *f*, the R6 gate resulting from crossing all of the successive gates and used to count CECs. CECs (*bold*) are characterized as $CD31^+CD146^+CD45^-7AAD^-$ viable events.

true CEP (28, 29) that are involved in tumor vasculogenesis (11, 16, 17, 19, 26). In cancer patients, the importance of the involvement of these cells remains controversial, in part because of the lack of consensus on their phenotypic definition (30, 31). Circulating BMD endothelial progenitors are typically identified and enumerated by flow cytometry as cells coexpressing CD34, CD133, and VEGFR2 markers (8, 32, 33). Several studies have analyzed CEP levels in 50 to 100 μL of peripheral blood with the use of flow cytometry in cancer patients (34–36) but have reported discordant results. Specialists in the field agree on the extreme rarity of these cells, which represent 0.0001% to 0.003% of circulating mononuclear cells (30, 31, 33). This rarity should incite careful interpretation of CEP measurements done in small volumes of blood (100 μL). In accordance with recommendations for the identification of extremely rare events, we developed a rigorous four-color flow cytometry assay to identify subsets of circulating VEGFR2$^+$-BMD progenitors in 10 mL of progenitor-enriched whole blood. The methodologic characteristics presented here that are used to detect this rare population include: (*a*) sampling of an important volume of blood, (*b*) a pre-enrichment step, (*c*) use of a viability marker, and (*d*) a multiple gating strategy (14).

Because the expression level of CD45 (either "dim" staining or negative) on circulating BMD progenitors with endothelial features is still matter of debate, we analyzed VEGFR2$^+$ expression in both the CD45^{dim}CD34$^+$7AAD$^-$ and the CD45$^-$CD34$^+$7AAD$^-$ subsets. VEGFR2$^+$ expression was not significantly present in the CD45$^-$CD34$^+$7AAD$^-$ fraction. However, we observed that the rare CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ subset was the only population identified by flow cytometry to significantly express the VEGFR2 receptor. This finding is consistent with the fact that hematopoietic and endothelial progenitors derive from a common precursor, the hemangioblast (28), and thus share the CD45 hematopoietic stem cell marker. In preliminary experiments, we showed that the vast majority of these cells were also positive for the CD133 marker (data not shown). Importantly, although there is no consensus, the CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ immunophenotype coincides with current definitions of BMD progenitors showing typical endothelial features described by others (8, 32, 37).

The lack of consensus on the appropriate method for CEC measurement has yielded conflicting data and hampered the understanding of their role in cancer patients (38). CECs have been detected with the use of flow cytometry (39, 40) but have been observed in very high numbers in cancer patients (1-39,000 cells/mL) and in healthy individuals (1-7,900 cells/mL) compared with the values reported with the immunomagnetic separation method of reference (<10 cells/mL; ref. 41). These findings have raised considerable questioning of the reliability of the flow cytometry method for CEC measurement (38, 42, 43). Recently, in 2007, Strijbos et al. argued that cells identified as CECs by flow cytometry in these studies were, in fact, large platelets (42). In a pediatric phase I trial with the VEGF neutralizing antibody bevacizumab (5), "mature CEC levels" were monitored in six children undergoing antiangiogenic therapy for refractory solid tumors and were found to range from 0 to 12,000 cells/mL with the use of flow cytometry. Previously, we have shown that median CEC levels were 6.5 cells/mL (0-15.0 cells/mL) in healthy adults and 15.0 cells/mL (0-179.0 cells/mL) in patients with metastatic carcinoma ($P < 0.001$; ref. 24). In the present study, the median CEC values observed in healthy subjects [7.0 cells/mL (0-56.0 cells/mL)] and in patients with localized [7.0 cells/mL (0-52.0 cells/mL)] or metastatic [7.5 cells/mL (0-152.0 cells/mL)] pediatric solid malignancies were within a similar range as the values reported in healthy adults with the use of the immunomagnetic separation method (<10 cells/mL; refs. 38, 41).

VEGF and sVEGFR2 have been evaluated as markers of endothelial cell mobilization (7, 11, 18, 44) and have been shown previously to correlate with tumor burden in cancer-bearing animals (45, 46) as well as in humans (47). In this study, we observed no correlation between angiogenic factor levels and CECs, circulating VEGFR2$^+$-BMD progenitor cells, or clinical status. Our study population did not allow for comparison of tumor burden between patients and could explain the absence of association between angiogenic factors and disease status.

We observed significant interpatient variability in both CEC and VEGFR2$^+$-BMD progenitor cell levels, which may be inherent to the heterogeneity of our population. However, we recently published a study on CEC levels in 125 adult patients with various metastatic solid malignancies, in which CEC levels also displayed significant interpatient variability despite the similarity of tumor types and stage (24). These observations highlight the biological complexity of variables influencing CEC and VEGFR2$^+$-BMD progenitor levels in cancer patients as well as the difficulties in understanding the significance of these rare cells in tumor angiogenesis.

Interestingly, we found that patients with metastatic or relapsing disease had high levels of circulating VEGFR2$^+$-BMD progenitor cells. Recent studies have shown that circulating VEGFR2$^+$-BMD endothelial progenitors play a major and catalytic role in tumor progression, which could be maximal in metastatic (20) and relapsing disease (25) by promoting the progression of avascular micrometastases to vascularized macrometastases (20). In the present study, differences in levels of VEGFR2$^+$-BMD endothelial progenitors were independent of age, gender, therapeutic intervention, or therapy-induced vascular lesions, suggesting that the results observed might be associated with the clinical behavior of pediatric solid malignancies. Mobilization of VEGFR2$^+$-BMD progenitors could result from two possible mechanisms. First, bone marrow involvement or proximity of bone metastases could locally stimulate the release of VEGFR2$^+$-BMD progenitors into the circulation via inflammation or tumor-derived cytokines. Second, VEGFR2$^+$-BMD progenitors could be actively recruited by proangiogenic characteristics that underline metastatic disease. Because metastatic lesions are almost never resected, we could not do immunohistochemistry to analyze if VEGFR2$^+$-BMD progenitors incorporated metastatic neoendothelium. Our data are not sufficient to affirm that VEGFR2$^+$-BMD progenitor cell elevations are independently associated with metastatic progression because the initial design of the study and population size did not allow for multivariate assessment of the clinical and biological factors that may also influence CECs and VEGFR2$^+$-BMD progenitor cell levels in patients. However, the significantly higher levels of VEGFR2$^+$-BMD progenitors found in numbers paralleling clinical severity, despite the diversity of tumor types investigated, raise great interest about the general relevance of these cells in metastatic disease progression. Further investigations in larger and more homogenous study populations will be necessary to analyze other covariates that may be responsible for the association with VEGFR2$^+$-BMD progenitor cell levels.

Finally, our data may raise concern about clinical situations that mobilize hematopoietic, and possibly endothelial, progenitors because these cells might induce unintended adverse effects. Fast tumor regrowth has been observed with increased levels of circulating BMD progenitors induced by stem cell growth factor (48, 49) as well as by vascular trauma resulting from radiation (16) or from vascular disrupting agents in tumor-bearing animals (25). Recently, we showed for the first time that vascular disrupting agent–induced VEGFR2$^+$-BMD progenitor mobilization is also present in human patients (14). Consequently, "vasculogenic rebounds" induced by anticancer therapies and possibly sustained by stem cell growth factor could adversely promote BMD progenitor mobilization and tumor angiogenesis, and, therefore, could increase the risk of relapse, perhaps at metastatic sites (50).

Pediatric solid malignancies present strong angiogenic potential and hopes to improve prognosis in these patients rely on antiangiogenic strategies. This is the first study to show evidence of increased levels of circulating VEGFR2$^+$-BMD progenitor cells in patients with metastatic pediatric solid malignancies. This finding may offer new insights to angiogenesis mechanisms in pediatric malignancies, in which monitoring and targeting of circulating VEGFR2$^+$-BMD progenitor cell levels could be of interest to guide the optimal use of antiangiogenic treatments in patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Levels of circulating $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cells correlate with outcome in metastatic renal cell carcinoma patients treated with tyrosine kinase inhibitors.

Farace F, Gross-Goupil M, Tournay E, **Taylor M**, Vimond N, Jacques N, Billiot F, Mauguen A, Hill C, Escudier B.

Nous avons mesuré de façon prospective les CEC et CEP chez 55 patients atteints de cancers du rein métastatique (mRCC) traités à l'Institut Gustave Roussy par des inhibiteurs de tyrosine kinase (sunitinib, n=46 patients, et sorafenib, n=9 patients) afin de déterminer si ces cellules pouvaient être de potentiels biomarqueurs des traitements anti-angiogéniques ciblant la voie du VEGF (29). Les CEC et CEP étaient mesurés avant traitement (J1) et à J14 du traitement en parallèle à des protéines plasmatiques pro-angiogéniques comme le VEGF, le sVEGFR2, le SDF-1 et endothéliales comme le sVCAM-1. Nous avons étudié s'il existait des corrélations entre les taux de CEC, CEP, les protéines plasmatiques à J1 ainsi que leurs modifications entre J1 et J14 et la réponse clinique au traitement, la survie sans progression (SSP) et la survie globale (SG).

Aucun patient n'a présenté une réponse complète au traitement anti-angiogénique; une réponse partielle était observée dans 19% des cas (n=10 patients), une stabilisation de la maladie dans 53% des cas (n=28 patients) et une progression de la maladie dans 28% des cas (n=15 patients). Les valeurs médianes de SSP et de SG étaient de 6 mois et 21 mois, respectivement. Les taux de CEC avaient une valeur médiane prétraitement de 13/ml (range, 0-119/ml) et n'étaient pas modifiés par le traitement. La valeur médiane de CEP prétraitement était de 0,5% des progéniteurs $CD34^{+}$ totales (range, 0-24,3%) et bien que nous ayons observé une tendance à l'augmentation des taux de CEP à J14 de traitement, celle-ci n'était pas significative (p=0.08).

Aucune corrélation n'a été observée entre les taux prétraitement de CEC et des protéines plasmatiques (VEGF, sVEGFR2, SDF-1, sVCAM-1) et la réponse au traitement. De même, aucune corrélation n'a été observée avec la SSP et la SG. Seuls les taux de CEP prétraitement étaient associés de façon significative à la SSP (p=0,01) et à la SG (p=0,006) (Figure 10).

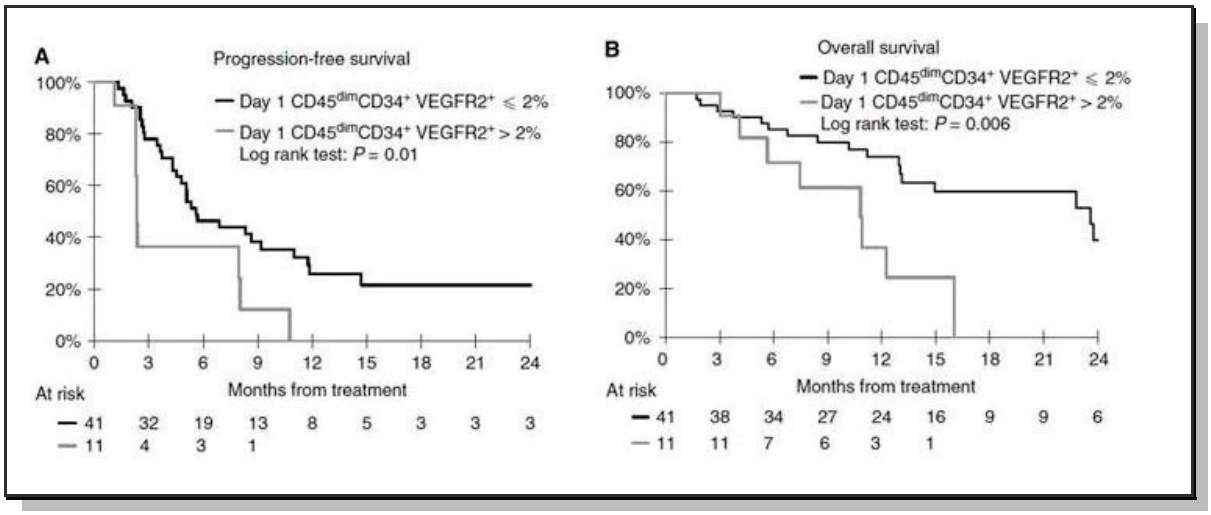

Figure 10. Courbes Kaplan-Meier de survie sans progression (A) et de survie globale (B) en fonction des taux de CEP (définis par le phénotype $CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ mesurés avant traitement chez des patients atteints de cancer du rein métastatique traités par agents anti-angiogéniques.

Nous n'avons pas observé de corrélation entre les variations des taux de CEC entre J1-J14, les taux de sVEGFR2 et de sVCAM-1 et la réponse au traitement. En revanche, les modifications des taux de CEP entre J1 et J14 étaient associées à la SSP (p=0,03). Les patients avec un taux de CEP stable ou augmenté entre J1 et J14 avaient un risque de progression significativement plus bas que ceux ayant un taux de CEP qui diminuait pendant cette intervalle (HR=0,3 et 0,5 respectivement, p=0,03). Les variations de taux de SDF-1 étaient corrélées à la SSP et la SG (p=0,002 et p=0,007, respectivement) (Figure 11). Aussi, les changements de VEGF entre J1 et J14 étaient associés à la SG (p=0.02) (Figure 12). Comme attendu avec ce type de traitement, les niveaux de sVEGR2 ont fortement chuté sous traitement mais ils n'étaient pas corrélés à la réponse au traitement, ni à la SSP ou la SG.

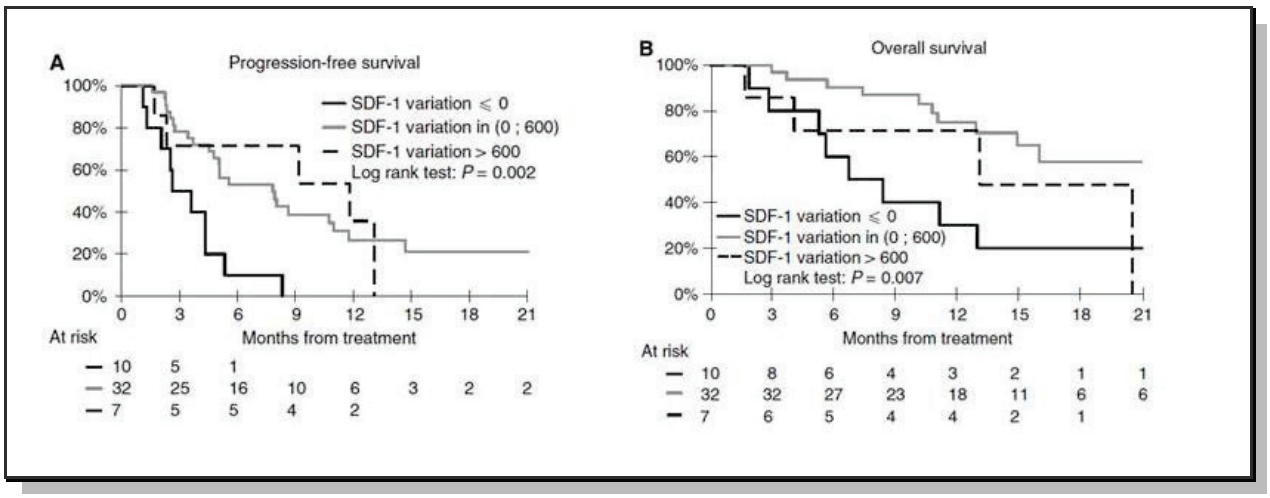

Figure 11. Courbes Kaplan-Meier de survie sans progression (A) et de survie globale (B) en fonction des modifications des taux plasmatiques de SDF-1 mesurés entre J1 et J14 chez des patients atteints de cancer du rein métastatique traités par agents anti-angiogéniques.

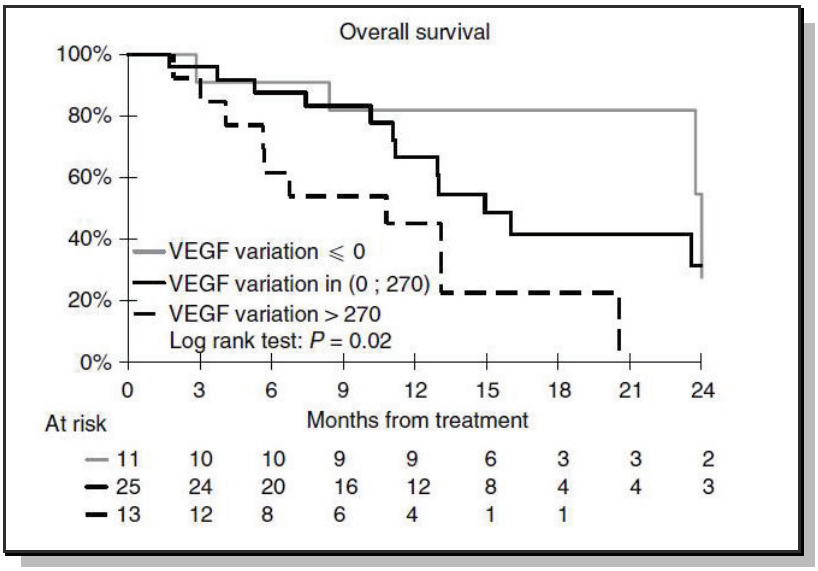

Figure 12. Courbes Kaplan-Meier de survie globale en fonction des modifications des taux plasmatiques de VEGF mesurés entre J1 et J14 chez des patients atteints de cancer du rein métastatique traités par agents anti-angiogéniques.

Nous avons donc observé qu'un taux élevé de CEP est le seul marqueur avant traitement corrélé à la SSP et la SG, ce qui suggère que les taux de CEP pourraient être des biomarqueurs prédictifs permettant de discriminer des patients avec une évolution défavorable lors d'un traitement par inhibiteurs de tyrosine kinase. Cependant, dans cette étude, l'absence de bras contrôle ne permettait pas de déterminer la valeur prédictive des CEP. Les patients présentant un taux stable ou augmenté de CEP pendant le traitement avaient un risque significativement plus bas de progression mais l'association entre les variations des taux de CEP et la SG n'a pas atteint la significativité statistique (p=0.07). Les modifications induites par le traitement des niveaux des deux protéines clés, le VEGF et le SDF-1 qui contrôlent la mobilisation des CEP, étaient corrélées à la survie des patients. Nous pouvons donc émettre l'hypothèse que ces modifications de CEP, VEGF, et SDF-1 pourraient être induites par l'hypoxie générée par la régression vasculaire durant le traitement anti-angiogénique et être donc la manifestation d'un mécanisme de résistance à ce type de traitement.

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Levels of circulating CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cells correlate with outcome in metastatic renal cell carcinoma patients treated with tyrosine kinase inhibitors

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BACKGROUND: Predicting the efficacy of antiangiogenic therapy would be of clinical value in patients (pts) with metastatic renal cell carcinoma (mRCC). We tested the hypothesis that circulating endothelial cell (CEC), bone marrow-derived CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cell or plasma angiogenic factor levels are associated with clinical outcome in mRCC pts undergoing treatment with tyrosine kinase inhibitors (TKI).

METHODS: Fifty-five mRCC pts were prospectively monitored at baseline (day 1) and day 14 during treatment (46 pts received sunitinib and 9 pts received sorafenib). Circulating endothelial cells (CD45^{-}CD31^{+}CD146^{+}7-amino-actinomycin (7AAD)$^{-}$ cells) were measured in 1 ml whole blood using four-color flow cytometry (FCM). Circulating CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} progenitor cells were measured in progenitor-enriched fractions by four-color FCM. Plasma VEGF, sVEGFR2, SDF-1α and sVCAM-1 levels were determined by ELISA. Correlations between baseline CEC, CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} progenitor cells, plasma factors, as well as day 1–day 14 changes in CEC, CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} progenitor, plasma factor levels, and response to TKI, progression-free survival (PFS) and overall survival (OS) were examined.

RESULTS: No significant correlation between markers and response to TKI was observed. No association between baseline CEC, plasma VEGF, sVEGFR-2, SDF-1α, sVCAM-1 levels with PFS and OS was observed. However, baseline CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-} progenitor cell levels were associated with PFS ($P=0.01$) and OS ($P=0.006$). Changes in this population and in SDF-1α levels between day 1 and day 14 were associated with PFS ($P=0.03$, $P=0.002$). Changes in VEGF and SDF-1α levels were associated with OS ($P=0.02$, $P=0.007$).

CONCLUSION: Monitoring CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cells, plasma VEGF and SDF-1α levels could be of clinical interest in TKI-treated mRCC pts to predict outcome.

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Keywords: circulating endothelial progenitor cells; circulating endothelial cells; biomarker; angiogenesis; metastatic renal cell carcinoma; tyrosine kinase inhibitors

Molecular Diagnostics

Metastatic renal cell carcinoma (mRCC) is generally resistant to chemotherapy and hormonal therapy and marginally sensitive to immunotherapy. Insights on the genetics and biology underlying RCC especially the role of the von Hippel-Lindau tumour-suppressor gene (*VHL*), have provided the rationale to target this pathway in VHL-deficient RCC and supported antiangiogenic strategies in this disease (Rini and Small, 2005). Sunitinib is a multitargeted tyrosine kinase inhibitor (TKI) of vascular endothelial growth factor A receptors (VEGFRs), platelet-derived growth factor receptors (PDGFRs), stem cell factor receptor (KIT), glial cell-line-derived neurotrophic factor receptor (rearranged during transfection), FMS-like tyrosine kinase-3 (FLT3) and the receptor for macrophage colony-stimulating factor (CSF-1R) (Mendel *et al*, 2003). Sorafenib is a potent inhibitor of Raf-1, a member of the RAF/MEK/ERK signalling pathway, and of TKI receptors including VEGFRs, PDGFRs, FLT3 and KIT (Wilhelm *et al*, 2004). Both antiangiogenic drugs have shown clinical activity in metastatic clear-cell RCC and have been approved worldwide for the treatment of metastatic RCC (Escudier *et al*, 2007; Motzer *et al*, 2007).

Tumour vascularisation is dependent on the sprouting of blood vessels with migration of endothelial cells and the recruitment of mobilised bone marrow-derived (BMD) cells (Asahara *et al*, 1999). Circulating endothelial cells (CECs) are mature cells detached from vessel walls and high levels are observed in clinical diseases

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hallmarked by vascular insult including cancer (Blann *et al*, 2005; Goon *et al*, 2006). Bone marrow-derived cells include several categories of hematopoietic and vascular progenitors recruited to sites of tumour neovascularisation in both cancer-bearing animals (Lyden *et al*, 2001) and humans (Peters *et al*, 2005). One subset, circulating endothelial progenitor cells (referred to as CEP), was reported to incorporate into tumour neovessels (Lyden *et al*, 2001; Spring *et al*, 2005; Nolan *et al*, 2007) and pre-clinical studies have identified a critical role for these cells in promoting the angiogenic switch and metastatic progression (Gao *et al*, 2008). Although the precise role of CEP as well as their phenotypic and functional definition is still debated (Purhonen *et al*, 2008; Yoder and Ingram, 2009), numerous studies have highlighted the importance of interactions between hematopoietic ($VEGFR1^{+}$) and endothelial ($VEGFR2^{+}$) BMD progenitor subsets in disease progression (Lyden *et al*, 2001; Purhonen *et al*, 2008; Yoder and Ingram, 2009), and vascularisation of metastatic lesions (Kaplan *et al*, 2005).

In clinical practice, the use of these vascular-targeted therapies is challenged by the absence of validated biomarkers allowing to predict response to treatment or toxic effects, select optimal dosage, or elucidate potential mechanisms of action and resistance. Among candidate biomarkers of antiangiogenic drugs, circulating BMD progenitors (including CEP) and CEC measurements have raised considerable interest (Jubb *et al*, 2006). However, because of technical difficulties for their measurement, reliable data on their actual prognostic or predictive value in cancer patients (pts) undergoing antiangiogenic treatment are lacking (Strijbos *et al*, 2008). Also, despite the fact that circulating levels of angiogenic growth factors generally indicate poor prognosis, their significance in terms of predicting antiangiogenic drug efficacy and clinical benefit is unclear.

In this study, we investigated whether the levels of CEC, of $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cell subset as well as of several proangiogenic/endothelial plasmatic factors (VEGF, sVEGFR-2, SDF-1α, sVCAM-1), measured before and after 2 weeks of therapy, are associated with clinical outcome in mRCC pts undergoing TKI therapy.

PATIENTS AND METHODS

Patients and blood sample collection

All pts had mRCC and were treated at the Institut Gustave Roussy, France, from October 2006 to January 2009. Patients received either sunitinib or sorafenib as antiangiogenic treatment. Before treatment, all pts had a detailed history, physical examination and baseline laboratory parameters. Pretreatment baseline tumour status was evaluated with CT scans of the brain, chest, abdomen and pelvis. Data collected included standard demographics and disease characteristics, first date of treatment, best response to treatment and date of progression, date of death or last follow-up. Tumour evaluation was performed after 12 weeks of sunitinib or after 2–3 months of sorafenib. Responses were documented according to Response Evaluation Criteria in Solid Tumours (RECIST). Patients were followed by their physician (BE) every 4–6 weeks. Informed consent was obtained for all pts. This prospective study was approved by our institutional review board.

Peripheral blood sampling was performed at baseline day 1 (before treatment initiation) and at day 14 of antiangiogenic therapy: 2 ml of whole blood was collected in Cellsave Preservative tubes (Immunicon, Huntingdon Valley, PA, USA) for CEC analysis, and 10 ml whole blood was collected in standard heparin tubes for $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor cell and plasmatic protein analysis.

Measurement of $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cells

$CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor cells were measured in 10 ml of progenitor-enriched whole blood according to a four-color FCM assay previously reported (Farace *et al*, 2007; Taylor *et al*, 2009). Ficoll-gradient mononuclear cells were enriched in progenitor cells using the RosetteSep antibody cocktail (StemCell Technologies Inc., Vancouver, Canada). Progenitor-enriched mononuclear cells were distributed into control and test tubes and treated with FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Staining was performed with monoclonal antibodies CD45-FITC (clone T29/33, DakoCytomation, Glostrup, Denmark), CD34-APC (clone BIRMA-K3, Dako Cytomation), KDR-PE (clone 89106, R&D Systems, Minneapolis, MN, USA) and 7AAD (BD Biosciences, San Jose, CA, USA). Control tubes included a control PE tube (CD45-FITC/mouse IgG1-PE/CD34-APC/7AAD) performed to measure accurately background noise and to adjust the gates precisely. Cells were acquired on a FACSCalibur (BD Biosciences). Data were analysed using CELLQuest 3.2 software (BD Biosciences). Results were expressed as the percentage of $VEGFR2^{+}$ cells among circulating $CD34^{+}$ progenitor ($CD45^{dim}CD34^{+}7AAD^{-}$ and $CD45^{-}CD34^{+}7AAD^{-}$) cells. The multigating strategy used to identify circulating $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor cells by four-color FCM is shown in Supplementary Figure S1.

Measurement of CECs

Circulating endothelial cells were measured in 1 ml whole blood by four-color FCM according to a method we previously established (Jacques *et al*, 2008). Immunofluorescent staining was performed with monoclonal antibodies CD31 FITC (clone WM59, BD Pharmingen, San Diego, CA, USA), CD146 PE (clone P1H12, BD Pharmingen), CD45 APC (clone T29/33, DakoCytomation). An IgG-PE control was performed in 0.5 ml of whole blood (CD45-APC/CD31-FITC/mouse IgG1-PE/7AAD) to measure background noise and to adjust the gates precisely. All of the cells contained in the IgG-PE control tube and in the CEC test tube were acquired, representing approximately 2.5×10^6 events and 5×10^6 events, respectively. Data were analysed using CELLQuest 3.2 software. Using this method, we found that median CEC levels were 6.5 ml^{-1} (0–15 ml^{-1}) in healthy adults ($n = 20$) and 16.0 ml^{-1} (0–179 ml^{-1}) in pts with metastatic carcinoma ($n = 125$) ($P < 0.001$) (Jacques *et al*, 2008).

Plasmatic factors

Plasma levels of VEGF, sVEGFR-2, sVCAM-1 and SDF-1α were determined using commercial ELISA kits (R&D Systems). Plasma samples were assayed in duplicates. Optical density values were considered significant if found to be at least twice as high as background noise.

Statistical analysis

Correlation between markers and clinical response to treatment (progressive *vs* non-progressive) were tested using the Wilcoxon–Mann–Whitney test. The Wilcoxon signed-rank test was used to test differences between marker levels at baseline and day 14. Overall survival (OS) was calculated from the start of treatment to the date of death or the last follow-up (censored data). Progression-free survival (PFS) was calculated from the start of treatment to the date of disease progression, death or the last follow-up (censored data).

Overall survival and PFS rates were estimated using the Kaplan–Meier method for survival curves. The relationships between survival and the different markers were tested using the log-rank test. The hazard ratios yielded by the Cox model were provided.

Values at baseline and day 14 were dichotomised according to the third quartile cut-off. As levels of $CD45^{dim}CD34^{+}VEGFR2^{+}$ cells in normal individuals and certain pts are very low (Taylor *et al*, 2009) and close to the detection limit of the method used, a

cut-off at a low or even at the median value might not have allowed to discriminate pts with the highest risk *vs* pts with a lowest risk because of an overlap between these two groups. We therefore decided to select a threshold at two-thirds of the values and to compare the third of the pts with the highest values with the two-thirds remaining with lower values.

Variations between baseline and day 14 were classified as increased, decreased or stable. All tests were two-sided and a *P*-value <0.05 was considered statistically significant. The statistical analysis was performed using SAS software (Release 9.1; SAS Institute, Cary, NC, USA).

RESULTS

Patient characteristics and baseline levels of CEC, CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cells and plasma proangiogenic factors

A total of 55 pts with mRCC were included in this study: 46 (84%) pts received sunitinib and 9 pts (16%) received sorafenib. Tumour histology (43 pts had clear cell renal carcinoma *vs* 12 with non-clear cell), clinical characteristics at baseline and response to treatment are presented in Table 1. A majority of pts received TKIs as first-line therapy (38 out of 55). No patient reached a complete response after treatment. The partial response rate to treatment was 19% (10 pts). Stable disease was achieved in 28 pts (53%) and progression was observed in 15 pts (28%). Two pts were not evaluable for response because of early cessation because of toxicity. Kaplan–Meier curves for PFS and OS for the 55 pts are presented in Supplementary Figure S2. Median PFS and median OS were 6 and 21 months, respectively.

Table 1 Description of patient characteristics, treatment and outcome ($n=55$)

	No.	%
Male	43	78
Age (years)		
Median (range)	58	(34–86)
ECOG performance status		
0	23	42
1	28	51
2	4	7
Histology		
Clear cell	43	78
Non-clear cell	12	22
Metastatic sites		
1	13	24
2	26	47
>3	16	29
Treatment		
Sunitinib	46	84
Nexavar	9	16
Best response		
PR	10	19
SD	28	53
PD	15	28
MSKCC category		
Low	17	31
Intermediate	36	65
Poor	2	4

Abbreviations: ECOG = Easter Cooperative Oncology Group; MSKCC = Memorial Sloan-Kettering Cancer Center; PD = progressive disease; PR = partial response; SD = stable disease.

Levels of CEC, CD45^{dim}CD34^{+}VEGFR2^{+}7AAD progenitor cell, plasma VEGF, sVEGFR-2, SDF-1α and sVCAM-1 were monitored at baseline and at day 14 (Table 2). Circulating endothelial cells were identified as CD31^{+}CD146^{+}CD45 7AAD viable events in whole blood by four-color FCM (Jacques *et al*, 2008). In the present cohort of mRCC pts, the median CEC level at baseline was 13 ml^{-1} (range 0–119 ml^{-1}) (Table 2). We analysed VEGFR-2 (KDR) expression in both CD45^{dim}CD34^{+}7AAD and CD45 CD34^{+}7AAD progenitor cell subsets. At baseline, the CD45 CD34^{+}VEGFR2^{+}7AAD subset represented $<0.005\%$ of circulating CD34^{+} progenitor cells. However, the median level of the CD45^{dim}CD34^{+}VEGFR2^{+}7AAD subset was of 0.5% of circulating CD34^{+} progenitor cells (range 0–24.3%). Median levels of plasma VEGF, sVEGFR-2, SDF-1α and sVCAM-1 at baseline were 151 pg ml^{-1} (range 0–1706 pg ml^{-1}), 9523 pg ml^{-1} (range 5410–17 680 pg ml^{-1}), 2726 pg ml^{-1} (range 1210–3948 pg ml^{-1}) and 673 ng ml^{-1} (range 279–1610 ng ml^{-1}), respectively (Table 2).

Changes in levels of CEC, CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cell and plasma proangiogenic factors under treatment

Absolute counts of CEC did not significantly change between day 1 and day 14 ($P=0.12$) (Table 2). Also, CD45^{dim}CD34^{+}VEGFR2^{+}7AAD progenitor cell levels were not significantly modified between day 1 and day 14 (0.5 *vs* 1.7%, $P=0.08$). As expected, TKI treatment induced an increase in plasma VEGF levels (median values: 151 *vs* 273 pg ml^{-1}, $P<0.0001$), which was associated with a concomitant decrease in plasma sVEGFR2 levels (9523 *vs* 6229 pg ml^{-1}, $P<0.0001$). Both SDF-1α and sVCAM-1 plasma levels significantly increased at day 14 (2726 *vs* 2931 pg ml^{-1}, $P<0.0001$, 673 *vs* 720 ng ml^{-1}, $P=0.04$).

Association between levels of CEC, CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cells and plasma proangiogenic factors and clinical outcome

For all markers, values at baseline and absolute variations between day 14 and baseline values were compared between non-progressive (stable and partial responses, $n=38$) and progressive ($n=15$) pts. No significant association between CEC, CD45^{dim}CD34^{+}VEGFR2^{+}7AAD progenitor cell, plasma VEGF, sVEGFR-2, SDF-1α, sVCAM-1 values and clinical response to TKI treatment was observed (Table 3).

No significant correlation was observed between baseline CEC, plasma VEGF, sVEGFR-2, SDF-1α, sVCAM-1 levels and PFS or OS (Table 3). However, CD45^{dim}CD34^{+}VEGFR2^{+}7AAD progenitor cell levels at day 1 were associated with PFS and OS. Patients with a CD45^{dim}CD34^{+}VEGFR2^{+}7AAD progenitor cell level at baseline $>2\%$ had a higher risk of progression (HR = 2.5, $P=0.01$) (Figure 1A) and had poorer prognosis compared with those pts whose CD45^{dim}CD34^{+}VEGFR2^{+}7AAD progenitor cell levels at baseline were $\leqslant 2\%$ (HR = 3.3, $P=0.006$) (Figures 1B).

No significant association between day 1 and day 14 changes in CEC, sVEGFR-2, sVCAM-1 levels was observed. Patients whose CD45^{dim}CD34^{+}VEGFR2^{+}7AAD progenitor cell levels were stable ($<-2\%$ or $\leqslant +2\%$) and pts whose CD45^{dim}CD34^{+}VEGFR2^{+}7AAD progenitor cell levels increased ($\geqslant 2\%$) between baseline and day 14 had a lower risk of progression compared with pts whose CD45^{dim}CD34^{+}VEGFR2^{+}7AAD progenitor cell levels decreased over the same period ($<2\%$) (HR = 0.3 and 0.5, respectively, $P=0.03$) (Table 3). The variation in VEGF levels between day 1 and day 14 levels was correlated with OS: pts whose VEGF values increased more than 270 pg ml^{-1} between day 1 and day 14 had a poorer OS (HR = 4.9, $P=0.02$) (Figure 2). The

Table 2 Median levels of CEC, CD45^{dim}CD34^{+} VEGFR2^{+} cells and plasmatic factors at baseline and day 14

	Median levels at		
Markers	**Day 1**	**Day 14**	**Changes day 1–day 14 P^a**
CEC (ml^{-1})	13 ($n=55$) (0–119)[b]	17 ($n=51$) (0–157)	0.12
CD45^{dim}CD34^{+} VEGFR2^{+} cells (%)	0.5 ($n=52$) (0–24.3)	1.7 ($n=48$) (0–28.4)	0.08
VEGF (pg ml^{-1})	151 ($n=54$) (0–1706)	273 ($n=49$) (1–3765)	<0.0001
sVEGFR-2 (pg ml^{-1})	9523 ($n=54$) (5410–17 680)	6229 ($n=49$) (2609–10 393)	<0.0001
SDF-1α (pg ml^{-1})	2726 ($n=54$) (1210–3948)	2931 ($n=49$) (1400–4433)	<0.0001
sVCAM-1 (ng ml^{-1})	673 ($n=53$) (279–1610)	720 ($n=48$) (325–1796)	0.04

Abbreviations: CEC = circulating endothelial cells; SDF-1α = stroma-derived factor -1α; sVCAM-1 = soluble vascular cell adhesion molecule-1; VEGF = vascular endothelial growth factor; VEGFR2 = vascular endothelial growth factor receptor 2. [a]Wilcoxon signed-rank test. [b]Range.

Table 3 Levels of significance of associations between markers and clinical outcome (response, PFS, OS)

	Clinical outcome		
Markers	**Response P^a**	**PFS P^a**	**OS P^a**
Day 1			
CEC (ml^{-1})	0.47	0.69	0.87
CD45^{dim}CD34^{+} VEGFR2^{+} cells (%)	0.12	**0.01 (0.005)**	**0.006 (0.001)**
VEGF (pg ml^{-1})	0.12	0.62	0.30
sVEGFR-2 (pg ml^{-1})	0.16	0.45	0.48
SDF-1α (pg ml^{-1})	0.15	0.08	0.47
sVCAM-1 (ng ml^{-1})	0.26	0.35	0.10
Changes day 1–day 14			
CEC (ml^{-1})	0.27	0.53	0.75
CD45^{dim}CD34^{+} VEGFR2^{+} cells (%)	0.83	**0.03 (0.05)**	0.07
VEGF (pg ml^{-1})	0.75	0.48	**0.02 (0.02)**
sVEGFR-2 (pg ml^{-1})	0.38	0.87	0.92
SDF-1α (pg ml^{-1})	0.18	**0.002 (0.01)**	**0.007 (0.009)**
sVCAM-1 (ng ml^{-1})	0.65	0.09	0.78

Abbreviations: CEC = circulating endothelial cells; OS = overall survival; PFS = progression-free survival; SDF-1α = stroma-derived factor -1α, sVCAM-1 = soluble vascular cell adhesion molecule-1; VEGF = vascular endothelial growth factor; VEGFR2 = vascular endothelial growth factor receptor 2. [a]Log rank test. Values in brackets present levels of significance of associations in the 43 patients with metastatic clear cell renal carcinoma.

variation in SDF-1α levels between day 1 and day 14 was correlated with both PFS and OS (Table 3). Patients whose SDF-1α values increased between 0 and 600 pg ml^{-1} and pts whose SDF-1α values increased more 600 pg ml^{-1} between day 1 and day 14 had a lower risk of progression (HR = 0.3 and 0.2, respectively, $P=0.002$) and a lower risk of death (HR = 0.3 and 0.6, respectively, $P=0.007$) compared with pts with decreased SDF-1α values (Figures 3A and B).

The analysis of associations between levels of CEC, CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cells and plasma proangiogenic factors and clinical outcome was repeated in the 43 pts with metastatic clear cell carcinoma. As shown in Table 3, baseline CD45^{dim}CD34^{+}VEGFR2^{+}7AAD progenitor cell levels were associated with PFS ($P=0.005$) and OS ($P=0.001$) in this specific histologic subtype. Similarly, changes in CD45^{dim}CD34^{+}VEGFR2^{+}7AAD progenitor cell levels and in SDF-1α levels between day 1 and day 14 remained associated with PFS ($P=0.05$, $P=0.01$). Changes in VEGF and SDF-1α levels were also associated with OS ($P=0.02$, $P=0.009$) in pts with metastatic clear cell carcinoma. Given the preponderance of sunitinib-treated pts, the analysis was finally repeated in pts with this specific subtype receiving this single treatment modality (i.e., sunitinib). All of the above associations remained significant in this small cohort of 34 pts (data not shown).

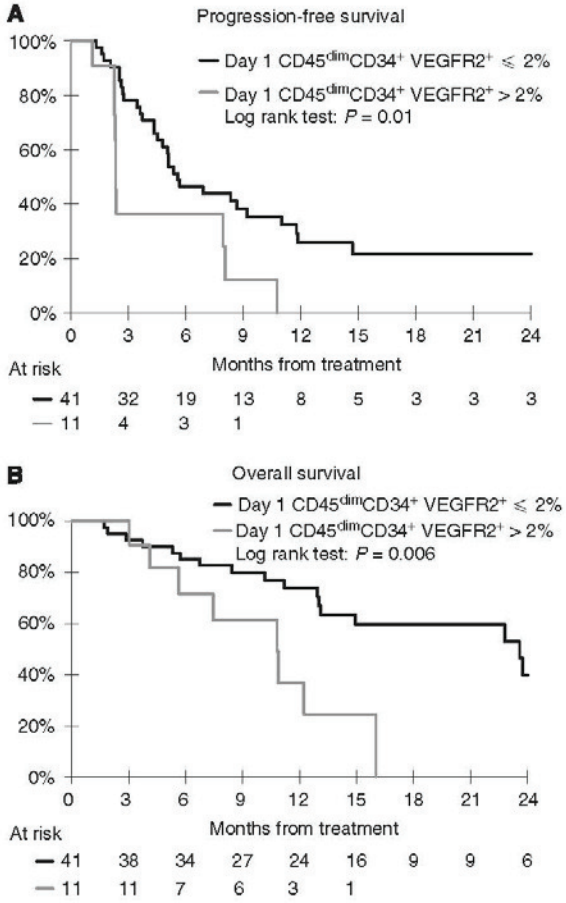

Figure 1 Progression-free survival and OS according to day 1 CD45^{dim}CD34^{+} VEGFR2^{+} progenitor cell levels. (**A**) Progression-free survival according to day 1 CD45^{dim}CD34^{+} VEGFR2^{+} progenitor cell levels. (**B**) Overall survival according to day 1 CD45^{dim}CD34^{+} VEGFR2^{+} progenitor cell levels.

DISCUSSION

Drugs targeting the VEGF pathway have made a major impact in the treatment of many types of cancer. Currently, multitargeted TKI, such as sunitinib or sorafenib, are considered as the standard of care for therapy in pts with mRCC. However, these agents clearly demonstrate therapeutic heterogeneity in terms of both efficacy and toxicity (Escudier *et al*, 2007; Motzer *et al*, 2007).

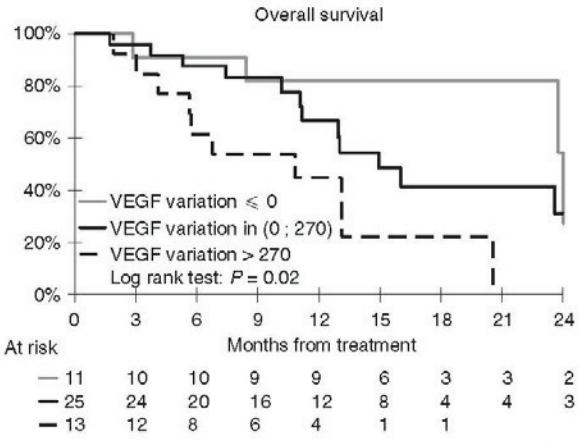

Figure 2 Overall survival according to changes in day 1 day 14 VEGF levels.

Thus, any biomarker that can predict clinical benefit would be of great value. To date, none of the expected biological markers, such as *VHL* status or VEGF plasma levels, has predicted response to targeted therapies in mRCC. In the present exploratory study, we reported the potential interest of a BMD progenitor cell subset, identified by the CD45^{dim}CD34^{+}VEGFR2^{+} phenotype in a cohort of 55 mRCC pts treated with multitargeted TKI. Interestingly, we observed a correlation between pretreatment CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cell levels phenotype and both PFS and OS. Early (i.e., within the two first week of treatment) changes in this progenitor cell subset and in plasma VEGF and SDF-1α levels were also associated with PFS or OS.

Increased numbers of CEC are considered as a useful marker of vascular integrity in pts with vascular disorders (Blann *et al*, 2005) although their role in tumour neoangiogenesis is less clear. The rarity of CEC and the controversy concerning the reliability of their measurement using flow cytometry have yielded conflicting as well as limited data in cancer pts (Strijbos *et al*, 2008). In this study, no association between CEC levels and outcome was observed. Whether CEC recruitment is an intrinsic characteristic of some tumour types or a marker of drug class effect or associations, or whether our method lacks sensitivity to discriminate different patient outcomes, remains an open question.

Circulating endothelial progenitor can home to sites of neovascularisation and differentiate into endothelial cells, a process called postnatal vasculogenesis that was widely proposed as a mechanism for vascular repair, and tumour metastasis, neoangiogenesis and growth promotion (Lyden *et al*, 2001; Spring *et al*, 2005; Nolan *et al*, 2007; Gao *et al*, 2008). Initial (Lyden *et al*, 2001) and subsequent studies have indeed identified CEP incorporation into the endothelial layer of tumour neovessels (Spring *et al*, 2005; Nolan *et al*, 2007), and their critical role in promoting progression of micro- to macro-metastases (Gao *et al*, 2008). Other historical data have refuted such a contribution (Purhonen *et al*, 2008; Yoder and Ingram, 2009). Despite this debate, all of these studies agree on the existence of a robust recruitment of both endothelial and hematopoietic BMD progenitor cells into the neoangiogenic perivasculature, thus supporting the important role these cells may play in the microenvironmental molecular and cellular events necessary for tumour invasion and metastasis. In humans, the phenotypical and functional characterisation of CEP has been hampered by the extreme rarity of these cells, the lack of consensus on surface marker phenotype, the important phenotypical overlap with hematopoietic progenitors as well as the absence of standard *in vitro* or *in vivo* assays for

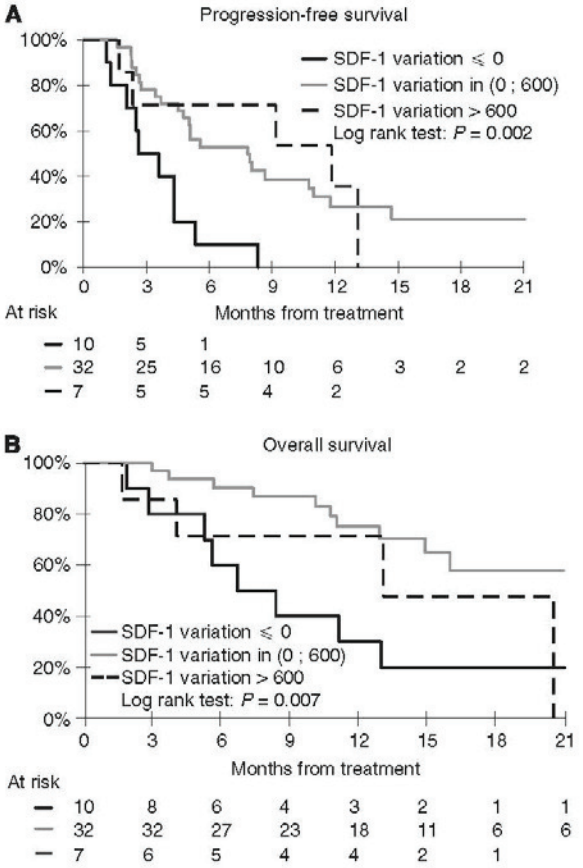

Figure 3 Progression-free survival and OS according to changes in day 1 day 14 SDF-1α levels. (**A**) Progression-free survival according to changes in day 1 day 14 SDF-1α levels. (**B**) Overall survival according to changes in day 1 day 14 SDF-1α.

functional characterisation (Yoder and Ingram, 2009). In light of this, we preferred to refer to the specific sub-population investigated herein as CD45^{dim}CD34^{+}VEGFR2^{+} cells (rather than to CEP). We used a rigorous four-color FCM assay to detect the circulating CD45^{dim}CD34^{+}VEGFR2^{+}7AAD cell subset in 10 ml of progenitor-enriched whole blood, which characteristics included (i) sampling of an important volume of blood, (ii) a pre-enrichment step, (iii) use of a viability marker (7AAD) and (iv) a multiple gating strategy (Farace *et al*, 2007; Taylor *et al*, 2009). In contrast to a recent report in pts with non-small cell lung cancer, we did not detect any sub-population with a CD45 CD34^{+} VEGFR2^{+}CD133 phenotype harbouring size and structural characteristics of viable cells in our FCM analyses (Vroling *et al*, 2010). We observed that a high level of CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cells (>2%) was the single baseline marker associated with poor PFS ($P=0.01$) and OS ($P=0.006$) thus suggesting that baseline CD45^{dim}CD34^{+}VEGFR2^{+} progenitor cell levels could allow to discriminate mRCC with poor outcome. Furthermore, we observed that pts with stable or increased levels of CD45^{dim} CD34^{+}VEGFR2^{+} progenitor cells between baseline and day 14 had a lower risk of tumour progression ($P=0.03$). However, the association between day 1 and day 14 changes in CD45^{dim} CD34^{+}VEGFR2^{+} progenitor cell levels and OS did not reach statistical significance ($P=0.07$). The present exploratory study was conducted in a small cohort of 55 pts with mRCC and the

absence of a control group (i.e., non-TKI-treated pts) did not allow to determine whether $CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ progenitor cell levels were prognostic or predictive. Furthermore, several cell sub-populations and plasma markers were evaluated, thus introducing potential biases of multiple testing. When the analysis was repeated in the 43 pts with metastatic clear cell carcinoma and in the 34 pts with this single histologic subtype receiving a single modality of treatment (i.e., sunitinib), these associations between levels of $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cells and clinical outcome remains significant. Biomarkers for RCC would be best developed in specific histologic subtypes, that is: clear cell RCC or non-clear cell RCC, especially because the biology of these tumour types is different and may differently influence the tumour microenvironment and have different biomarkers relevant to their predictive and prognostic value. Future studies in a cohort of mRCC pts with a single tumour histology (i.e., clear cell carcinoma) receiving a single antiangiogenic treatment (i.e., sunitinib) are needed to confirm our results and to determine the eventual prognostic or predictive value of $CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ progenitor cell levels in this tumour subtype.

Early and more recent studies, including the pivotal phase III trial of sorafenib in advanced mRCC (Jacobsen *et al*, 2000; Escudier *et al*, 2009), have indicated that baseline VEGF levels correlate with disease activity and prognosis. However, other studies have produced inconclusive results. It currently remains uncertain whether VEGF status alone is an adequate predictive marker for efficacy of VEGF-targeted therapy in mRCC. Herein, baseline VEGF and other factor levels did not correlate with PFS or OS. Unlike a previous study reporting larger changes in VEGF levels in pts demonstrating objective tumour responses during sunitinib treatment (Deprimo *et al*, 2007), we observed that the magnitude of increase in VEGF levels under treatment was associated with OS ($P=0.02$). Surprisingly, a decrease in SDF-1α levels was also found to be strongly associated with both PFS ($P=0.002$) and OS ($P=0.007$). Plasma factor changes, that is, increased levels in circulating VEGF and concomitant decreased levels in sVEGFR2 have been reported as class effects of TKI in pts. Recently, the observation of increased levels of multiple proangiogenic factors in sunitinib-treated tumour-free mice has implied a systemic multi-organ endocrine response to VEGF and PDGF inhibition in normal tissues (Ebos *et al*, 2007). Elucidating underlying mechanisms with respect to the multiple targets directly or collaterally affected by such agents may prove difficult. VEGF and SDF-1α are critical regulators governing the mobilisation and recruitment of a heterogeneous population of BMD proangiogenic vascular and hematopoietic progenitor cells to tumours. Initial studies have shown that VEGF-targeted therapy may inhibit the mobilisation of CEP (Willett *et al*, 2004) and induce the increase in SDF-1α blood levels during tumour escape (Batchelor *et al*, 2007). Our results do not support these findings although the dynamics of these processes remain to be determined under continued therapy. Overall, our results suggest that inadvertent changes induced by TKI treatment, that is, upregulation of VEGF, decrease in SDF-1α, and subsequent changes in BMD $CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ progenitor cell levels are associated with poor outcome in mRCC pts treated with TKI.

None of the six angiogenesis/endothelial markers evaluated correlated with tumour response as documented by RECIST. As angiogenesis/endothelial markers are mainly host-derived, their changes during antiangiogenic treatment are expected to reflect the angiogenic–antiangiogenic balance resulting from a complex interplay between the biological actors of angiogenesis and the therapeutic agent. These markers may not be as informative for tumour shrinkage as could be tumour-derived factors. Clinical results of VEGF-targeted therapy have shown little evidence of tumour shrinkage and have rather suggested a cytostatic effect, therefore tumour response criteria by RECIST may not be a good indicator of clinical benefit of antiangiogenic agents. An important issue in identifying biomarkers is the endpoint of the actual response in pts. Progression-free survival or OS may be more accurate clinical read-outs for host angiogenesis marker evaluation.

Escape from antiangiogenic therapy has been demonstrated in both pre-clinical and clinical settings (Bergers and Hanahan, 2008). Emerging data have implicated evasive resistance mechanisms where host adaptive responses circumvent the specific angiogenic blockade. Low oxygen tension, HIF-1α accumulation and subsequent SDF-1α and VEGF effectors were reported to promote angiogenesis and tumour growth via the recruitment of various proangiogenic BMD sub-populations (Bergers and Hanahan, 2008). Evidence supporting the link between therapy-induced hypoxia and BMD proangiogenic cell populations stemmed from a study demonstrating that vascular disrupting agents (VDA) induced vasculogenic 'rebounds' that homed to the vasculature of treated tumours thereby promoting tumour neovascularisation and subsequent re-growth (Shaked *et al*, 2006). In a previous clinical study, we observed the presence of $CD45^{dim}$ $CD34^{+}$ $VEGFR2^{+}$ progenitor cell mobilisation in a small series of cancer pts included in phase I trial combining a VDA (AVE8062, Sanofi-Aventis, Antony, France) with chemotherapy (Farace *et al*, 2007). Further studies are needed to determine whether sub-populations of BMD progenitors may be biomarkers of response or resistance to antiangiogenic therapies.

In conclusion, our study shows for the first time an association between baseline levels of a BMD $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cell subset and outcome in mRCC pts treated with TKI. Also, we present novel data on therapy-induced changes of $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cell, VEGF and SDF-1α levels that were also associated with PFS or OS. Large prospective studies in a homogeneous cohort of mRCC pts are warranted to confirm our results as it will allow necessary multivariate analysis to assess the eventual prognostic or predictive value of these markers in mRCC pts treated by TKI.

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Prognostic value of circulating VEGFR2+ bone marrow-derived progenitor cells in patients with advanced cancer.

Massard C, Borget I, Le Deley MC, **Taylor M**, Gomez-Roca C, Soria JC, Farace F.

Nous avons émis l'hypothèse que les CEC et CEP pourraient être chez des patients atteints de cancers avancés résistants aux traitements conventionnels, une caractéristique biologique commune capable de refléter l'agressivité de la tumeur ou la résistance aux traitements, et de ce fait être un facteur pronostique potentiellement utile à la décision clinique dans le contexte des essais thérapeutiques précoces (30). Pour tester cette hypothèse, nous avons étudié la corrélation entre les taux prétraitement de CEC et CEP, les facteurs cliniques et biologiques usuels ainsi que le score pronostique RMH (« *Royal Marsden Hospital* »)(193), et la SG chez 71 patients inclus dans quatre études de phase I testant de nouveaux agents anti-angiogéniques et anti-vasculaires.

Pour les 71 patients évalués, la valeur médiane de CEC était de 12 CEC/ml (range, 0-154/ml). Nous avons analysé le taux d'expression membranaire de VEGFR2 parmi les fractions $CD45^{-}CD34^{+}7AAD^{-}$ et $CD45^{dim}CD34^{+}7AAD^{-}$ de progéniteurs circulants chez 58 patients. Alors que la fraction $CD45^{-}CD34^{+}VEGFR2^{+}7AAD^{-}$ était indétectable (<0,005% des progéniteurs $CD34^{+}$ circulants), la valeur médiane de la fraction $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ (identifiée comme des CEP) représentait 1,3% (range, 0-32,5%) des progéniteurs $CD34^{+}$ totaux circulants. En analyse uni-variée, il n'y avait pas de corrélation entre les taux de CEC et de progéniteurs $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ et le nombre de sites métastatiques ou le score RMH. Les valeurs médianes de survie sans progression (SSP) et de survie globale (SG) étaient de 2,7 mois (IC 95%, 2-3,9 mois) et de 10 mois (IC 95%, 7,6-13,3 mois), respectivement. Nous n'avons pas observé de corrélation entre les taux de CEC ou de CEP et la SSP dans notre cohorte. De même, aucune corrélation n'a été observée entre les taux de CEC et la SG.

En revanche, la SG était significativement plus courte chez les patients présentant un taux élevé de CEP (>1% des progéniteurs $CD34^{+}$ circulants), et était de 9 mois *versus* 17 mois (p=0,03). Elle était aussi significativement plus courte chez les patients avec un score RMH supérieur à 0 (9 mois *versus* 24,2 mois, p=0,02). En analyse multi-variée, la valeur pronostique du taux de CEP prétraitement restait significative (p=0.02) et non celle du RMH (Figure 13).

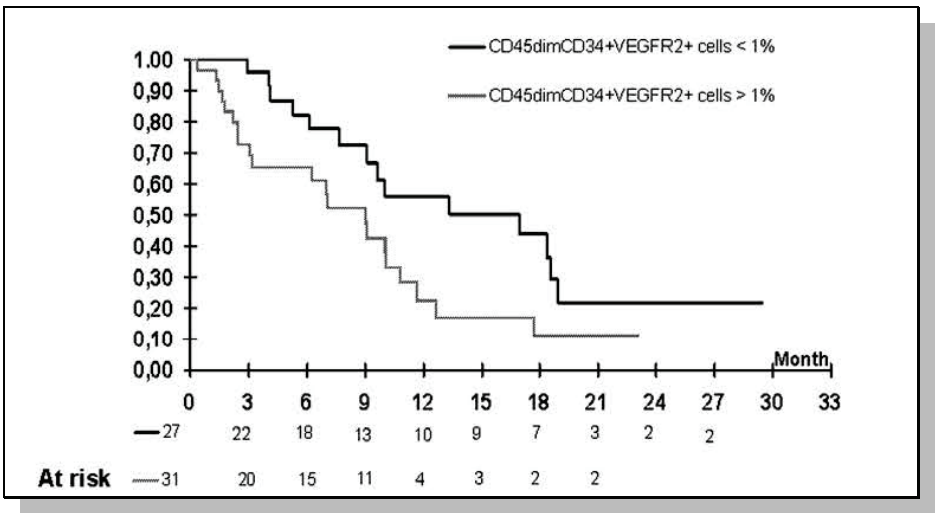

Figure 13. Courbe Kaplan-Meier de survie globale en fonction des taux de CEP (définis par le phénotype $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$) mesurés avant traitement chez 58 patients atteints de cancers avancés.

Nous avons développé un score composite combinant le score RMH avec les taux de CEP mesurés chez les patients, et calculé par l'ajout d'un point lorsque le taux de CEP était > à 1%. Le score composite était égal au score RMH si le taux de CEP était < à 1%. La SG était significativement diminuée chez les patients présentant un score combiné « taux de CEP/RMH » ≥ à 2 par rapport à ceux présentant un score combiné égal à 0 ou 1 : survie médiane de 9 mois *versus* 18,4 mois, respectivement (HR=2.6 (IC 95%, 1,2-5,8, p=0,02)) (Figure 14).

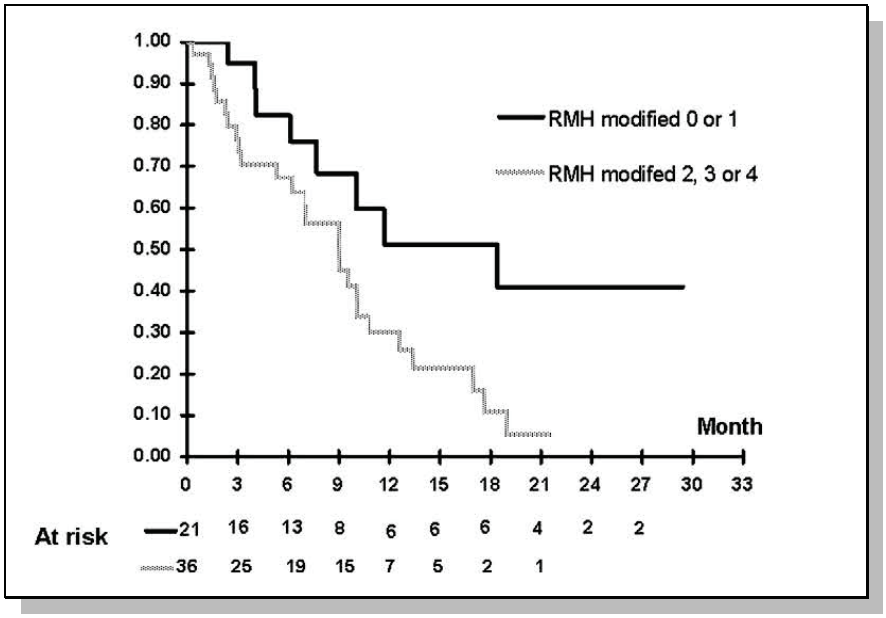

Figure 14. Courbe Kaplan-Meier de survie globale en fonction du score composite associant le taux de CEP et le score RMH chez 58 patients atteints de cancers avancés.

Ces résultats suggéraient donc que de forts taux de CEP, mobilisés en réponse à des processus hypoxiques, pourraient constituer un marqueur général d'agressivité tumorale chez des patients ayant un cancer avancé. De ce fait, les CEP pourraient constituer un marqueur pronostique dont l'utilité ne se cantonnerait pas aux agents anti-angiogéniques ou anti-vasculaires. Dans cette étude, l'association entre le taux de CEP et la survie globale indépendamment du type de traitement (anti-angiogénique ou anti-vasculaire) suggère que ces cellules pourraient constituer un marqueur pronostique plutôt que prédictif. Dans le contexte des essais thérapeutiques précoces, les CEP pourraient apporter une information complémentaire aux paramètres cliniques et biologiques et une aide précieuse à la sélection des patients susceptibles de retirer (ou non) un bénéfice clinique de ces traitements.

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Prognostic value of circulating VEGFR2$^+$ bone marrow-derived progenitor cells in patients with advanced cancer

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KEYWORDS
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Circulating endothelial cells
Angiogenesis biomarker
Prognosis
Advanced cancers

Abstract We hypothesised that host-related markers, possibly reflecting tumour aggressiveness, such as circulating endothelial cells (CEC) and circulating VEGFR2$^+$ bone marrow-derived (BMD) progenitor cells, could have prognostic value in patients with advanced cancer enrolled in early anticancer drug development trials.

Baseline CECs (CD45 CD31$^+$CD146$^+$7AAD cells) and circulating VEGFR2$^+$-BMD progenitor cells (defined as CD45^{dim}CD34$^+$VEGFR2$^+$7AAD cells) were measured by flow-cytometry in 71 and 58 patients included in phase 1 trials testing novel anti-vascular or anti-angiogenic agents. Correlations between levels of CECs, circulating VEGFR2$^+$-BMD progenitor cells, clinical and biological prognostic factors (i.e. the Royal Marsden Hospital (RMH) score), and overall survival (OS) were studied.

The median value of CECs was 12 CEC/ml (range 0–154/ml). The median level of VEGFR2$^+$-BMD progenitor cells was 1.3% (range 0–32.5%) of circulating BMD-CD34$^+$ progenitors. While OS was not correlated with CEC levels, it was significantly worse in patients with high VEGFR2$^+$-BMD progenitor levels (>1%) (median OS 9.0 *versus* 17.0 months), and with a RMH prognostic score >0 (median OS 9.0 *versus* 24.2 months). The prognostic value of VEGFR2$^+$-BMD progenitor levels remained significant (hazard ratio (HR) = 2.3, 95% confidence interval (CI), 1.1–4.6, $p = 0.02$) after multivariate analysis. A composite VEGFR2$^+$-BMD progenitor level/RHM score $\geqslant 2$ was significantly associated with an increased risk of death compared to scores of 0 or 1 (median OS 9.0 *versus* 18.4 months, HR = 2.6 (95% CI, 1.2–5.8, $p = 0.02$)).

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High circulating VEGFR2$^+$-BMD progenitor levels are associated with poor prognostics and when combined to classical clinical and biological parameters could provide a new tool for patient selection in early anticancer drug trials.

1. Introduction

Recent studies have evidenced that rare circulating cells with endothelial features contribute to tumour neovascularisation and possibly metastatic dissemination, therapeutic inhibition of which has yielded considerable interest, and has been validated in the clinic. Angiogenesis is mediated not only by the 'sprouting' of pre-existing differentiated vascular endothelial cells, but also by a systemic host process involving the mobilisation and homing of various bone marrow derived (BMD) progenitors, which include several categories of haematopoietic and vascular progenitors recruited to sites of tumour neovascularisation.[1] One subset called circulating endothelial progenitor cells (referred to as CEP), for which the precise role and phenotypic definition is still debated[2,3] was reported to incorporate tumour neovessels[4,5] and to promote the angiogenic switch, key to metastatic progression.[6] For these reasons, circulating endothelial cells (CEC)- mature cells shed from existing vessels for which high levels are observed in clinical diseases hallmarked by vascular insult including cancer[7–9] – and BMD progenitor cell subsets are being investigated actively as prognostic and/or predictive biomarkers in patients with cancer as these cell populations could reflect the underlying biological processes regulating tumour growth, potential metastatic dissemination and consequently, the 'angiogenic aggressiveness' of a given tumour.

We hypothesised that patients with advanced solid tumours who are resistant or who have exhausted conventional therapies might share general biological processes reflecting tumour aggressiveness or resistance to therapy. In spite of tumour heterogeneity, patients with advanced solid tumours eligible for phase 1 trials may have such underlying host characteristics contributing to a particularly 'aggressive' tumour phenotype. Despite the number and importance of phase 1 trials for the development of new therapies, medical oncologists are still lacking objective prognostic parameters that could help to foresee which patients will have worse outcome and help guide optimal treatment strategies.[10–12] A few retrospective studies have identified clinical or biological prognostic factors to help identify patients eligible in phase 1 trials.[13–18] However, biological parameters reflecting a systemic host-response to an aggressive tumour such as angiogenesis markers may help the oncologist decide on the type of potentially innovative anticancer therapy to be tested in patients. Moreover such a general parameter could help in the decision to exclude patients with the shortest life expectancy unnecessarily exposed to a considerable risk of toxicity since the new agent could substantially affect their quality of life and outcome.

In light of our previous results showing the potential clinical interest of monitoring VEGFR2$^+$-BMD progenitor cell mobilisation[19–21] and CEC[22] and in order to investigate new biomarkers of advanced solid tumours, we prospectively investigated levels of BMD progenitor cell subset and CEC levels in cancer patients enrolled in phase 1 trials testing new anti-angiogenic or anti-vascular agents at our institution. While CEC levels were not correlated with overall survival (OS), we observed that high baseline levels of circulating VEGFR2$^+$-BMD progenitor cells were associated with worse prognosis in patients with advanced cancer.

2. Patients and methods

2.1. Study design and patients eligibility

This study considered all consecutive patients treated in five phase 1 trials at the Service des Innovations Thérapeutiques Précoces (SITEP) Institut Gustave Roussy, France, between October 2005 and April 2009. All patients had given their informed consent to take part in phase 1 trials approved by our institutional Review Boards, who also granted approval for the present analysis. Standard clinico-pathological parameters were recorded at study entry, including tumour type, age, sex, Eastern Cooperative Oncology Group (ECOG) performance status (PS), full blood count, biochemistry (including serum albumin and lactate dehydrogenase (LDH)), number and sites of metastasis and number of prior cancer treatments. The Royal Marsden Hospital (RMH) score which is based on the serum albumin level (lower than 35 g/L), LDH level (greater than the upper limit of normal (ULN)), and the number of metastatic sites was calculated for each patient.[18] Blood sampling for CEC and progenitor cell enumeration was performed before treatment. After discarding the first 2 mL following venupunction, 2 mL of whole blood was collected in Cellsave™ preservative tubes (Immunicon Inc., Huntingdon Valley, PA) for CEC analysis[8] and 10 mL whole blood was collected in standard heparin tubes for CD45^{dim}CD34$^+$VEGFR2$^+$ progenitors as previously described.[19–21]

2.2. Measurement of BMD-VEGFR2$^+$ progenitor cells

BMD-VEGFR2$^+$ progenitor cells were measured in 10 mL of progenitor-enriched whole blood according

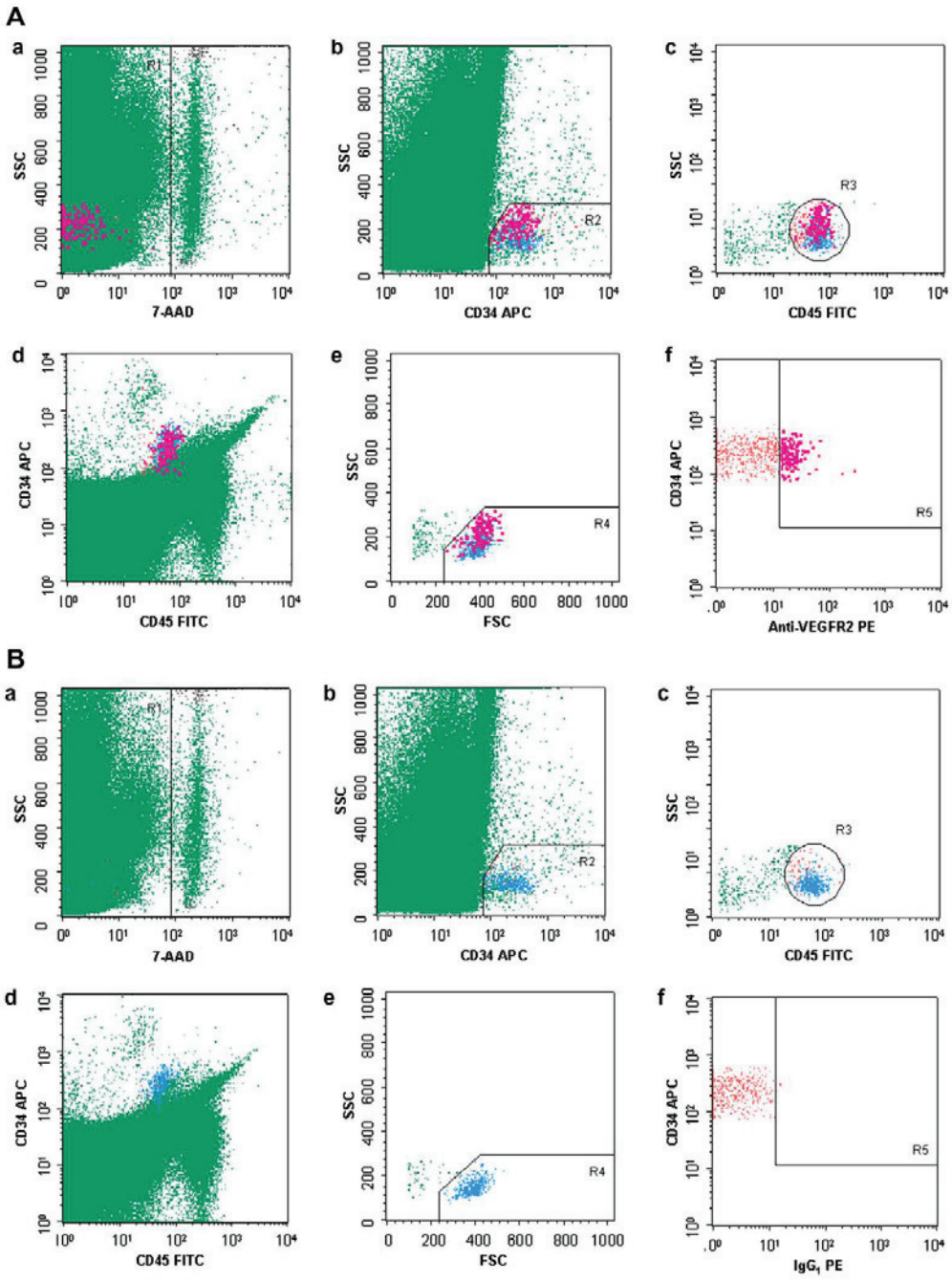

Fig. 1. Flow cytometric detection of circulating $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor cells in a representative patient. *Fig. 1A:* Analysis of the test (CD45-FITC/KDR-PE/CD34-APC/7AAD) tube. $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor cells are highlighted in bold. (a) selection of viable ($7AAD^{-}$) events (R1 gate); (b and c) gates (R2 and R3) used to select $CD34^{+}$ and $CD45^{dim}$ cells; (d) cells according to CD34 and CD45 expression; (e) gate (R4) used to exclude debris; (f) gate used to detect $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ cells resulting from crossing R1, R2, R3 and R4 gates. *Fig. 1B:* Analysis of the control PE (CD45-FITC/mouse IgG1-PE/CD34-APC/7AAD) tube. (a) selection of viable ($7AAD^{-}$) events (R1 gate), 1% of events are shown; (b and c) gates (R2 and R3) used to select $CD34^{+}$ and $CD45^{dim}$ cells; (d) cells according to CD34 and CD45 expression; (e) gate (R4) used to exclude debris (f) gate used to detect $CD45^{dim}CD34^{+}Ig^{+}7AAD^{-}$ cells resulting from crossing R1, R2, R3 and R4 gates.

to a four-colour flow cytometry (FCM) assay we previously reported.[19–21] Briefly, ficoll-gradient mononuclear cells were enriched in progenitor cells using the RosetteSep® antibody cocktail (StemCell Technologies Inc., Vancouver, Canada), then distributed into control and test tubes and treated with Fc receptors (FcR) blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Staining was performed with the following monoclonal antibodies: CD45-FITC (clone T29/33, Dako, Glostrup, Denmark), CD34-APC (clone BIR-MA-K3, Dako), KDR-PE (clone 89106, R&D Systems, Minneapolis, MN) and 7AAD (BD Biosciences, San Jose, CA). Antibody batches were titrated rigorously to determine optimal dosage and in order to not be used in excess, except KDR-PE which was used according to the manufacturer's recommendations. Control tubes included a control PE tube (CD45-FITC/mouse IgG1-PE/CD34-APC/7AAD) were performed to measure accurately background noise and to adjust the gates precisely. The IgG1-PE reagent was purchased from the same manufacturer and used in the exact same quantity as the KDR-PE. Cells were acquired on a FACSCalibur (BD Biosciences) and data were analysed using CELLQuest 3.2 software (BD Biosciences) (Fig. 1). Results were expressed as the percentage of $VEGFR2^+$ cells among circulating $CD34^+$progenitor cells ($CD45^{dim}CD34^+7AAD^-$ and $CD45^-CD34^+7AAD^-$) cells.

2.3. Measurement of CECs

CECs were measured in 1 mL whole blood by four-colour FCM according to a method we established previously.[8] Briefly, immunofluorescent staining was performed with the following monoclonal antibodies: CD31 FITC (clone WM59, BD Pharmingen, NJ, United States of America (USA)), CD146 PE (clone P1H12, BD Pharmingen), CD45 APC (clone T29/33, Dako). An IgG-PE control was performed in 0.5 mL of whole blood (CD45-APC/CD31-FITC/mouse IgG1-PE/7AAD) to measure background noise and to adjust the gates precisely. To ensure statistical analysis, all of the cells contained in the IgG-PE control tube and in the CEC test tube were acquired, representing approximately 2.5×10^6 events and 5×10^6 events, respectively. Data were analysed using CELLQuest 3.2 software (BD Biosciences). Using this method, we found that median CEC levels were 6.5/mL (0–15/mL) in healthy adults and 16.0/mL (0–179/mL) in patients with metastatic carcinoma ($p < 0.001$).[8]

2.4. Statistical analysis

OS was calculated from the initiation of phase 1 treatment to the date of death or the last follow-up (censored data). Progression-free survival (PFS) was calculated from the start of treatment to the date of disease progression, death, or the last follow-up (censored data). OS and PFS rates were estimated using the Kaplan–Meier method for survival curves. The relationships between survivals and the different markers were tested using the log-rank test. The hazard ratios yielded by the Cox model are presented. All tests were two-sided and a p value <0.05 was considered statistically significant. The statistical analysis was performed using SAS software version 9.1.

3. Results

3.1. Patients characteristics

Pre-treatment CEC and circulating $VEGFR2^+$-BMD progenitor cell levels were measured respectively in 71

Table 1
Patient characteristics ($n = 71$).

Characteristics		N	%
Age			
Median (yrs)	50		
Range (yrs)	21 78		
Sex			
Female		29	41
Male		42	59
ECOG (PS)			
0		28	39
1		42	60
2		1	1
LDH level			
<250 UI/L		45	63
⩾250 UI/L		26	37
Albumin level (1 missing)			
<35 g/L		30	43
⩾35 g/L		40	57
Number of metastatic site			
0 or 1		20	28
⩾2		51	72
RMH score (1 missing)			
0		15	21
1		31	44
2		23	32
3		1	1
Antiangiogenic or antivascular therapy			
Vascular disrupting agents (VDA)		38	54
Dual HER1-2 and VEGFR2 TKI		22	31
VEGFR TKI		11	15
Tumour types			
NSCLC		12	17
Renal cancer		7	10
Colon cancer		7	10
Others		45	6

ECOG PS, Eastern Cooperative Oncology Group performance status; RMH score, Royal Marsden Hospital score; HER, human epidermal receptor; VEGFR, vascular endothelial growth factor; NSCLC, non small cell lung cancer; TKI, tyrosine kinase inhibitors.

and 58 patients, for whom clinical characteristics are shown in Table 1. Sixty-eight patients had metastatic disease and three patients had a locally advanced tumour. All these patients received previously standard chemotherapy regimens, and patients had a median of three prior systemic therapies (range, 1–7).The most common primary tumours were lung cancer (17%), renal cancer (10%), and colon cancer (10%). The most common sites of metastasis were lymph nodes ($n = 30$, 42%), lung ($n = 48$, 68%), liver ($n = 27$, 38%) and bone ($n = 14$, 20%). Twenty-five (35%) patients had more than two metastatic sites (median 2, range (0–6)). Baseline biochemistry showed decreased albumin levels (albumin < 35 g/L) in 30 (42%) patients, and LDH levels greater than the ULN (>250 UI/L) in 26 (37%) patients. Fifteen (21%) patients had a RMH score of 0, 31 (44%) patients had a RMH of 1 and 24 (33%) had a RMH $\geqslant 2$ (one missing data).

3.2. Trial characteristics

During the 4-year study period, all patients included were treated within one of five anti-angiogenic or anti-vascular phase 1 trials (Table 1). The novel drugs evaluated included vascular endothelial growth factor (VEGFR) small molecule/tyrosine kinase inhibitors in 15% of cases, dual human epidermal receptor 1-2 (HER1-2) and VEGFR2 small molecule/tyrosine kinase inhibitors in 31% of cases and anti-vascular (also referred to as vascular disrupting agent (VDA)) agents in 54% of cases.

3.3. Baseline levels of CEC and circulating VEGFR2$^+$-BMD progenitor cells

For all 71 patients, the median baseline absolute count of CECs was 12/ml (range 0–154/ml). The membrane expression of VEGFR2 (KDR) was examined at baseline in both $CD45^{dim}CD34^+7AAD^-$ and $CD45^-CD34^+7AAD^-$ progenitor cell subsets of 58 patients. The $CD45^-CD34^+VEGFR2^+7AAD^-$ subset was undetectable (<0.005% of circulating $CD34^+$ progenitor cells). However, the median value of the $CD45^{dim}CD34^+VEGFR2^+7AAD^-$ subset represented 1.3% (range 0–32.5%) of circulating $CD34^+$ progenitors (Fig. 1). Univariate analysis indicated that there was no clear association between CEC and $CD45^{dim}CD34^+VEGFR2^+$ progenitor cell levels and the number of metastatic sites or RMH score (data not shown).

3.4. Association of CEC and circulating VEGFR2$^+$-BMD progenitor cell levels with outcome

During the median follow-up period of 21.5 months (range 0–39 months), 45 patients died and 66 patients had disease progression. The median PFS was 2.7 months (95% confidence interval (CI), 2–3.9 months) and the median OS was 10 months for the entire cohort (95% CI, 7.6–13.3 months). Univariate analysis revealed no association between the levels of CEC and of circulating VEGFR2-BMD progenitor cells, and PFS (data not shown). A worse outcome was associated with an elevated level of $CD45^{dim}CD34^+VEGFR2^+$ progenitor cells (hazard ration (HR) 2.0, 95% CI = [1.04–4.1], $p = 0.03$) and a RMH score higher than 0 (HR 2.9, 95% CI = [1.1–7.4], $p = 0.02$) (Table 2). The risk of death was significantly increased in patients who had a prognostic RMH score greater than 0 as compared to patients who had a score equal to 0 (median OS 9.0 months *versus* 24.2 months, respectively) (Fig. 2) and in patients who had increased $CD45^{dim}CD34^+VEGFR2^+$ progenitor cell levels (>1%) as compared to those who had lower values (median OS 17.0 months *versus* 9.0 months, respectively) (Fig. 3). In the multivariate analysis, only the level of $CD45^{dim}CD34^+VEGFR2^+$ progenitor cells was associated significantly with risk of death (HR = 2.3, 95% CI, 1.1–4.6, $p = 0.02$) (Table 2). We did not observe a relationship between CEC levels and outcome (Table 2). We developed a composite score combining the RMH score with $CD45^{dim}CD34^+VEGFR2^+$

Table 2
Univariate and multivariate analysis of prognosis factors on overall survival.

Characteristics		N	Median OS		Univariate analysis			Multivariate analysis		
			Months	95% C1	HR	95% C1	P*	HR	95% C1	P*
RMH score (Group)	0	15	24.2	(11.7 38.6)	1			1		
	1-2-3	55	9.0	(7.0 10.1)	2.9	(1.1 7.4)	0.02	3.9	(0.92 16.3)	0.06
CEC (/mL)	⩽20/mL	51	10.0	(6.9 13.3)	1					
	>20/mL	19	18.9	(7.1 38.0)	0.6	(0.3 1.4)	0.26			
$CD45^{dim}CD34^+VEGFR2^+$ progenitors (%)	⩽1%	27	17	(9.1 18.9)	1		0.03	1		
	>1%	31	9.0	(3.2 10.8)	2.0	(1.04 4.1)		2.3	(1.1 4.6)	0.02
Combined score	0-1	21	18.4	(8.4 38.0)	1					
	2-3-4	36	9.0	(5.3 10.0)	2.6	(1.2 5.8)	0.02			

OS, overall survival; RMH score, Royal Marsden Hospital score; CEC, circulating endothelial cells; HR, hazard ratio; CI, confidence interval.
* Logrank test.

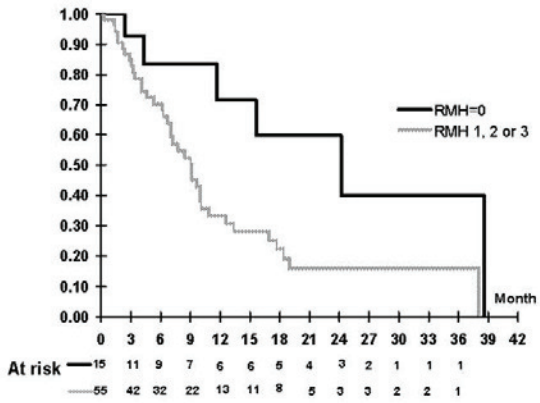

Fig. 2. Kaplan Meier curves of overall survival stratified according to the Royal Marsden Hospital (RMH) score.

progenitor cell levels. As a majority of patients (31/58) had $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cell levels greater than 1% of circulating $CD34^{+}$ progenitors, this combined $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cell level/RHM score was calculated by adding one point to the RMH score when the $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cell level was greater than 1%, and not adding a point otherwise (combined RHM score = RHM score if $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cell levels <1%). The risk of death was significantly increased in patients with a combined $CD45^{dim}CD34^{+}VEGFR2^{+}$ progenitor cell levels/RMH score at least equal to 2 as compared to patients with score equal to 0 or 1 (median OS 9.0 months *versus* 18.4 months respectively, HR = 2.6 (95% CI, 1.2–5.8, $p = 0.02$)) (Table 2) (Fig. 4).

4. Discussion

In the present exploratory, we investigated a novel biological paradigm, – whether cells from the tumour microenvironment such as $VEGFR2^{+}$-BMD progenitor cells and CECs-, could inform on the prognosis given the role of these cells in neoangiogenesis and tumour metastasis. Interestingly, a significant association between $VEGFR2^{+}$-BMD progenitor cell levels and

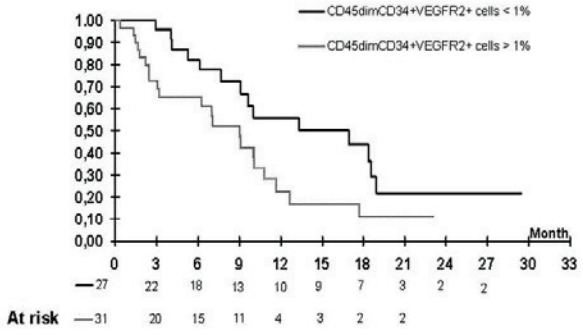

Fig. 3. Kaplan Meier curves of overall survival stratified according to pre-treatment levels of $CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ progenitor cells.

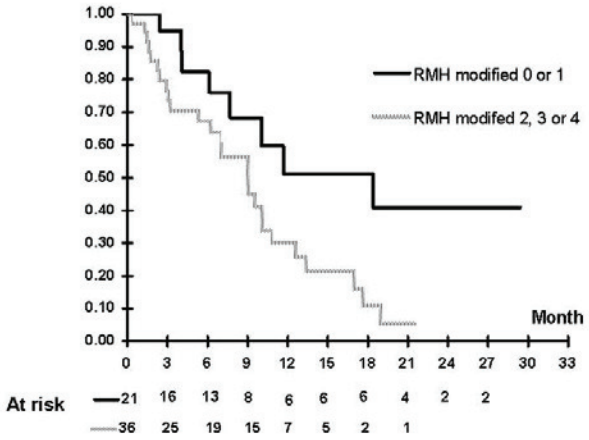

Fig. 4. Kaplan Meier curves of overall survival stratified according to the composite score combining the the Royal Marsden Hospital (RMH) score and pre-treatment $CD45^{dim}CD34^{+}$ $VEGFR2^{+}$ progenitor cell levels.

the risk of death was observed in a cohort of patients included in phase 1 trials suggesting that this marker might indeed reflect a general aggressive tumour-behaviour, and may help to better identify candidates for phase 1 trials.

During tumour growth, local hypoxic conditions trigger the mobilisation and recruitment to sites of neovascularisation of a heterogeneous population of BMD cells, which include haematopoietic and endothethial progenitors and immune modulatory cells.[1,4,5] Among BMD progenitor cells, CEP were initially reported to directly incorporate the endothelial layer of tumour neovessels.[5] Subsequent studies have proposed a pivotal role for these cells in promoting progression of micro- to macro-metatases in murine models.[6] Although conflicting results have questioned the exact role of CEP[2,3] all of the studies agreed on the critical and complementary role of BMD cell populations in supporting tumour growth and promoting metastasis development and dissemination. In humans, the identification of CEPs is complex because of the extreme rarity of these cells, the important phenotypical overlap with haematopoietic progenitors and the absence of standard assay for functional characterisation.[2] In light of this, the specific subpopulation investigated herein was referred to BMD-VEGFR2$^+$ cells rather than CEP. In previous studies, we explored VEGFR2$^+$-BMD progenitor cell subsets in the blood of patients with metastatic cancer by using a rigorous four-colour FCM assay that, in accordance with recommendations for the identification of extremely rare cells, included sampling of an important volume of blood (10 mL), a pre-enrichment step in progenitor cells, use of a viability marker (7AAD) and a multiple gating strategy.[19–21] In a small series of phase 1 cancer patients, we previously observed that CD45^{dim}CD34$^+$VEGFR2$^+$ progenitor cells were mobilised in high levels in response to acute tumour insult and severe hypoxia induced by a treatment combining an anti-vascular agent (VDA) with chemotherapy.[19] Recently, we reported an association between high pre-treatment CD45^{dim}CD34$^+$VEGFR2$^+$ progenitor cells levels and poor PFS and OS in a cohort of 55 metastatic renal cell carcinoma (mRCC) patients treated with tyrosine kinase inhibitors (TKI) thus supporting the potential utility of this progenitor cell subset to predict the outcome in TKI treated mRCC patients.[21] In our series of patients receiving different anti-angiogenic or anti-vascular agents in phase 1 trials, high baseline CD45^{dim}CD34$^+$VEGFR2$^+$ progenitor cell levels were associated with a higher risk of death regardless of tumour type and previous treatment. High levels of circulating CD45^{dim}CD34$^+$VEGFR2$^+$ progenitor cells might reflect a general aggressive tumour-behaviour and associated tumour hypoxia-driven processes that orchestrate their recruitment to sites of neoangiogenesis and possibly their catalytic role in metastatic tumour growth.[20] This hypothesis of 'tumour aggressiveness' would support its prognostic value in patients and would render VEGFR2$^+$-BMD progenitor cell levels a universal marker of poor prognosis, not only restricted to anti-vascular or anti-angiogenic treated patients. The prognostic value of VEGFR2$^+$-BMD progenitor cell levels in our population of patients enrolled in phase 1 trials may be extrapolated to other phase 1 patients, and this hypothesis must be validated in a large prospective cohort of patients with advanced cancer.

Alternatively, pre-treatment VEGFR2$^+$-BMD progenitor cell levels might be targeted by anti-vascular agents or anti-angiogenic agents undergoing phase 1 evaluation. In this respect, VEGFR2$^+$-BMD progenitor cell level measurement might help to select patients eligible for such targeted trials and might inform on the differential impact of such treatments, thereby offering a predictive value. Angiogenesis TKI might exert their antitumour effect in part through the suppression of these cells. In contrast, anti-vascular agents leading to massive disruption of the established tumour vasculature would cause increases in BMD progenitors mobilised for vascular repair, as observed in experimental models[24] as well as in human patients.[21] Because anti-vascular agents and anti-angiogenic agents are expected to have different impacts on VEGFR2$^+$-BMD progenitor cell levels and because we observed high levels correlated with OS regardless of treatment, the hypothesis of a prognostic rather than a predictive value of these cells in our phase 1 patient population, seems to be privileged here.

Retrospective studies have attempted to identify clinical prognostic factors to help select patients for phase 1 trials according to their life expectancy. Such prognostic factors include the performance status, the number of metastatic sites and lymphopenia.[13–17] The Royal Marsden Hospital (RMH) Phase 1 Unit developed and prospectively validated a simple clinical prognostic score based on three parameters and demonstrated that low serum albumin (<35 g/L), high serum LDH (>ULN) and >2 metastatic sites were independent prognostic factors in multivariate analysis. The number of poor prognostic variables in patients was expressed as a RMH score ranging from 0 to 3.[18] More recently, the same group developed a new score which included baseline circulating tumour cell (CTC) counts and allowed to identify a group of patients with a worse prognosis and a median OS of 13 weeks (95% CI 12–15 weeks).[23] In our present study, we observed a significant association between CD45^{dim}CD34$^+$VEGFR2$^+$ progenitor cell levels and the risk of death in our cohort which remained significant in the multivariate analysis which included the RHM score. Moreover, we created a single composite score that was strongly correlated with outcome and could easily be used by clinicians. Our results suggested that measurements of VEGFR2$^+$-BMD

progenitor cell levels when combined to classical clinical and biological parameters could provide a new tool which may have the advantage of being easier to use by oncologists than the RMH score and % VEGFR2-BMD progenitor levels considered independently for selecting candidates for in phase 1 trials. The association of the level of $VEGFR2^{+}$-BMD progenitor cells to the RMH score, or the composite score alone, may offer better insight to clinicians in order to stratify patients according to their OS for participation or not in phase 1 studies. In particular, patients with a better OS might be candidates for personalised therapeutic alternatives in early clinical trials.

The incorporation of predictive/prognostic biomarkers in early clinical anticancer drug evaluation is expected to improve the development of new drugs.[25–27] Circulating BMD progenitors (including CEP) and CEC measurements have raised considerable interest as candidate biomarkers to predict the efficacy of anti-angiogenic treatments in phase II studies.[28,29] Studies have described high pre-treatment levels of $CD45^{dim}CD34^{+}CD133^{+}$ progenitor cells (a phenotype of haematopoietic progenitor cells) correlated with a worse outcome in cancer patients treated by TKIs[30,31] while conflicting data on CEC and cells referred to as CEP were reported.[29,32,33] In the present study, no association between CEC levels and outcome was observed. Whether our method lacks sensitivity or our cohort is too small to discriminate different patient outcomes or whether the mechanisms leading to high levels of CEC are not relevant in the present patient population remains an open question. Furthermore, although not statistically significant, patients with high CEC levels had an OS longer than patients with low CEC levels. This finding could appear contra-intuitive as high CEC levels (without evidence of vascular disease or damage) might reflect a high rate of remodelling and turnover of the tumour endothelium and might therefore be considered as a biological marker of tumor aggressiveness. On the other hand, one can also hypothesise that tumours from these patients (with high CEC levels) might be more sensitive to agents targeting the tumour vasculature such as VDA or antiangiogenic drugs. According to this latter hypothesis, patients with high CEC levels might benefit more from the treatment. Finally, current research has evidenced that BMD progenitor cells may play a role in tumour angiogenesis-driven escape mechanisms such as resistance to standard cytotoxic chemotherapy or resistance to anti-vascular agents through hypoxia-driven processes.[34] Whether subpopulations of BMD progenitors may also act as biomarkers of resistance to these treatments, offers an attractive perspective for further research.

The present exploratory study investigated the hypothesis that host-related markers from the tumour environment, and in particular those related to tumour-hypoxia driven mechanisms possibly reflecting tumour aggressiveness in patients resistant to standard cytotoxic chemotherapies, would be correlated to outcome in patients bearing aggressive tumours included in phase 1 trials. We observed that high baseline $VEGFR2^{+}$-BMD progenitor cell levels were associated with worse prognosis and could additionally provide complementary information to classical clinical and biological parameters for medical oncologists concerned about OS in patient selection. Further prospective studies are needed to confirm these results in a larger population of phase 1 patients. Our data suggest a new biological prognostic tool of clinical utility for patients with aggressive advanced tumours which could be targeted by new anti-angiogenic therapies.

Conflict of interest statement

None declared.

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Reversing resistance to vascular-disrupting agents by blocking late mobilization of circulating endothelial progenitor cells.

Taylor M, Billiot F, Marty V, Rouffiac V, Cohen P, Tournay E, Opolon P, Louache F, Vassal G, Laplace-Builhé C, Vielh P, Soria JC, Farace F.

Dans un travail antérieur réalisé dans le laboratoire (98), la cinétique des CEP observés chez des patients traités en phase I par un VDA, l'ombrabuline, est apparue d'emblée très différente de celle rapportée chez la souris par Shaked *et al.* (76) (présentée page 21-22). En effet, ces auteurs montraient chez la souris une mobilisation immédiate de CEP, 2 à 4 heures après l'injection du VDA alors que l'équipe de Françoise Farace avaient observé chez les patients une mobilisation de CEP plus tardive, 4 à 7 jours après le traitement par ombrabuline (98). De plus, cette mobilisation immédiate décrite par Shaked *et al*. (76) nous semblait peu cohérente avec un véritable processus de différentiation et recrutement de CEP en réponse à la lésion vasculaire induite par le VDA. Nous avons donc décidé d'étudier dans des modèles murins la chronologie des évènements cellulaires et moléculaires induits par la drogue dans la tumeur, et en parallèle celle de CEC et CEP dans le sang (31).

Nous avons tout d'abord sélectionné un modèle de xénogreffe tumorale (lignée prostatique humaine PC3) qui présentait une microdensité vasculaire élevée, de ce fait probablement une grande sensibilité au traitement par VDA, et pratiquement aucune nécrose spontanée. Dès 4h après l'injection du VDA, (100 mg/kg de combrétastatine), les tumeurs PC3 présentaient une importante nécrose intra-tumorale (médiane 73% *versus* 4%, $p<0,001$) qui culminait à 24h, se maintenait jusqu'à 120h et était associée dès 4h à une réduction importante de la microdensité vasculaire (0,7% *versus* 3,7%, $p<0,03$). L'expression dans la tumeur de CAIX, protéine de l'hypoxie en aval de HIF-1α, était maximale à 24h et concomitante au plus faible niveau de microdensité vasculaire (Figure 15).

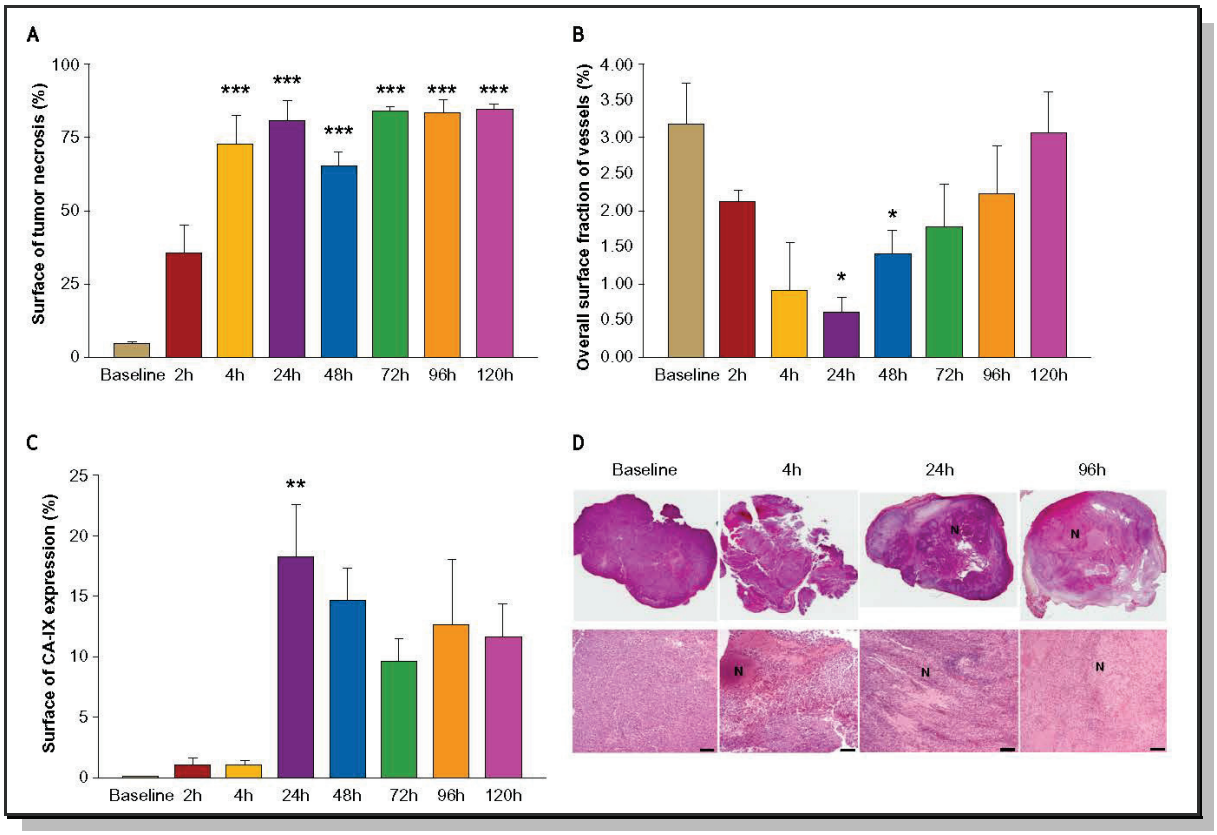

Figure 15. Taux de nécrose (A), de microdensité vasculaire (B) et d'expression de CA-IX (marqueur d'hypoxie) (C) mesurés dans des xénogreffes de PC3 à différents temps (0-120 h) après injection i.v. de 100 mg/kg de combrétastatine (CA-4-P). Images histologiques en H&E représentatives (D) de sections tumorales montrant l'étendue de la nécrose intra tumorale au cours du temps.

Lorsque nous avons étudié avec précision la cinétique des CEC et CEP, nous avons observé un pic bref de CEC à 4h présent exclusivement chez les animaux porteurs de tumeurs et qui était cohérent avec les changements histologiques observés. L'injection de VDA induisait aussi un pic de CEP à 2h et 4h, présent à la fois dans les animaux porteurs de tumeurs et les animaux sains comme rapporté par Shaked *et al*. (76) et qui était antérieur à celui des CEC. La chronologie des évènements dans la tumeur et dans le sang suggérait donc que ce pic précoce de CEP observable dès 2h, et donc avant l'apparition des CEC et la survenue des stigmates de la lésion vasculaire dans la tumeur mesurés par la nécrose et la microdensité vasculaire, ne pouvait pas refléter un processus de réparation vasculaire spécifique mais vraisemblablement une réponse systémique et immédiate de l'hôte à l'injection de la combrétastatine. Nous avons donc émis l'hypothèse qu'un pic de CEP devait être mobilisé plus tardivement dans un processus spécifique de réparation vasculaire. Des prélèvements successifs ont effectivement permis de vérifier cette hypothèse en mettant en évidence un pic tardif (à 96h) de CEP deux fois plus important que le pic initial qui était spécifique des animaux porteurs de tumeurs (Figure 16).

Nous avons ensuite confirmé l'existence de ces deux pics de CEP dans un autre modèle tumoral (H69) et montré que leur intensité dépendait de la dose de VDA injectée.

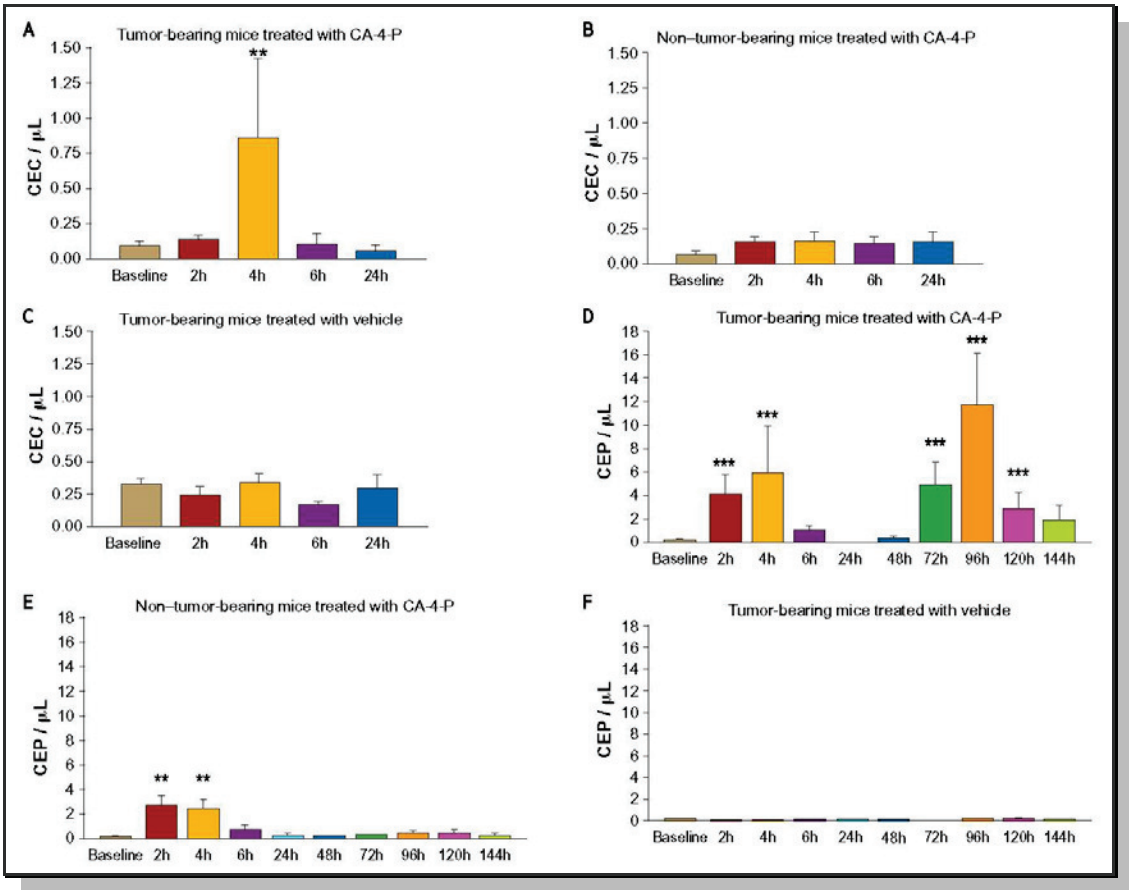

Figure 16. Cinétique des taux de CEC et CEP chez des souris nude porteuses de xénogreffes PC3 prélevées à différents temps après traitement par la combrétastatine (CA-4-P, 100mg/kg). (A-C) Taux de CEC mesurés chez des souris (n=6-8 par temps dans chaque groupe) à des temps allant de 0 à 24h après traitement par le CA-4-P (A) chez des souris porteuses de tumeur, (B) chez des souris non porteuses de tumeur, et (C) après traitement par le tampon de reconstitution de la drogue chez des souris porteuses de tumeur. (D-F) Dans des expériences séparées, les taux de CEP ont été mesurés dans des souris (n=8-10 par temps dans chaque groupe) à des temps allant de 0 à 144h après traitement avec le CA-4-P chez (D) des souris porteuses de tumeur, (E) des souris non porteuses de tumeur, et (F) dans un groupe contrôle de souris porteuses de tumeur recevant le tampon de reconstitution de la drogue. Les résultats sont exprimés en moyenne±SD. La signification des tests est représentée par * (0,05>p>0,01), ** (0,01 ≥p>0,001) ou *** (p≤0,001).

Afin d'étudier les facteurs de croissance pouvant être impliqués dans la mobilisation des CEP après traitement par VDA, nous avons mesuré les taux sériques de G-CSF, MMP9 et SDF-1α ainsi que les taux de SDF-1 médullaire à différents temps après traitement avec 100 mg/kg de CA-4-P chez des souris porteuses de tumeur et des souris non porteuses de tumeur. Nous avons observé que les taux de G-CSF et de MMP9 sériques ainsi que les taux médullaires de SDF-1α présentaient une cinétique similaire à celle des CEP avec deux pics distincts survenant à 2-4h et à 72-96h après traitement et exclusivement chez les souris porteuses de tumeur (Figure 17).

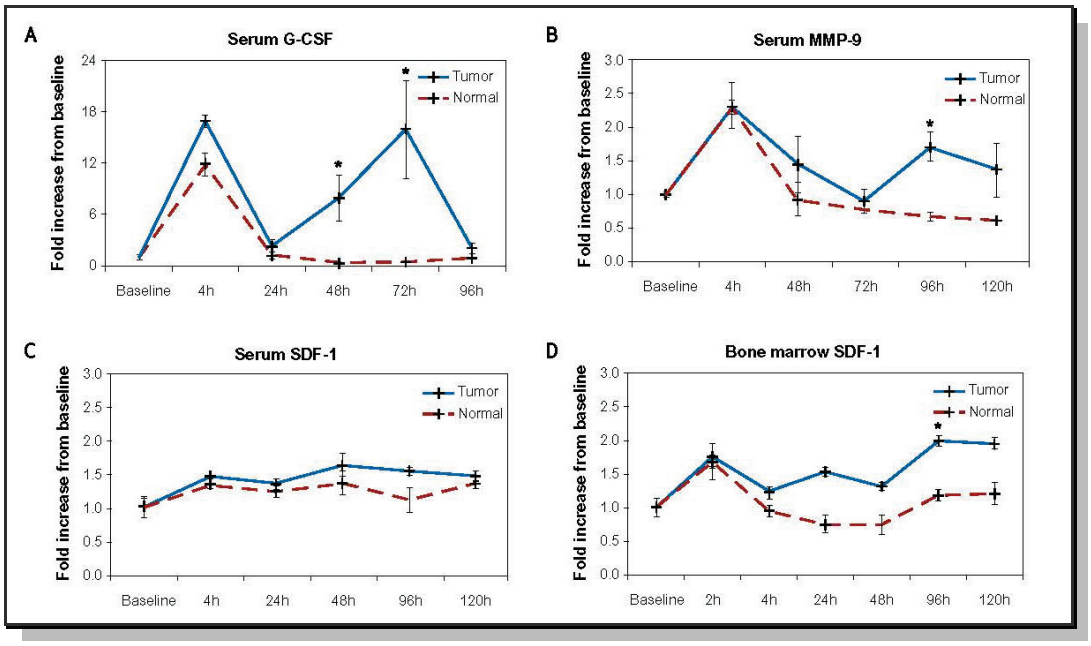

Figure 17. Taux sériques de G-CSF (A), de MMP9 (B) et de SDF-1α (C) et taux médullaires de SDF-1α (D) mesurés à différents temps après traitement avec 100 mg/kg de CA-4-P chez des souris porteuses de tumeur et des souris non porteuses de tumeur (n=4-6 souris par temps dans chaque groupe). Les résultats sont exprimés en moyenne±SD et par rapport aux valeurs avant traitement (baseline). La signification des tests est représentée par * (0,05>p>0,01), ** (0,01 ≥p>0,001) ou *** (p≤0,001).

Compte tenu des discordances dans la littérature autour du phénotype désignant de « potentiels » CEP déjà abordé dans ce travail, nous avons entrepris de multiples marquages en CMF 5 et 6 couleurs pour mieux caractériser la population identifiée comme des CEP. Ces expériences ont permis de montrer premièrement que les cellules $CD45^{-/dim}CD117^{+}Sca1^{+}Flk\text{-}1^{+}DAPI^{-}$ identifiées comme des CEP exprimaient le CD34 (spécifique du phénotype des progéniteurs) et la VE-cadhérine (spécifique du phénotype endothéliale). Deuxièmement, nous avons comparé notre stratégie d'identification des CEP basée sur la sélection d'une population exprimant le $CD45^{-/dim}$ avec une stratégie différente qui consiste à détecter des populations n'exprimant pas le marqueur de Lineage (Lin). Cette stratégie « Lin^{-} » permettait de sursoir aux débats autour de l'identification des CEP basée sur le marqueur CD45. En utilisant un « dump-channel » pour les populations $Lin(^{+})$ à la place du marqueur CD45, nous avons montré que le nombre de CEP identifiées par notre stratégie était semblable au nombre d'évènements $Lin^{-}CD117^{+}Sca1^{+}Flk\text{-}1^{+}DAPI^{-}$ détectés. Enfin, nous avons démontré que la fraction viable de cellules $CD45^{-/dim}CD117^{+}DAPI^{-}$ n'exprimait pas des marqueurs myéloïdes ou monocytaires, à savoir le Gr-1, le CD11b ou le CD14.

Nous avons ensuite mis au point des expériences d'association de la combrétastatine avec le sunitinib, un agent anti-angiogénique pouvant cibler le VEGFR-2 porté par les CEP, dans le

même modèle de xénogreffe PC3. Alors que le sunitinib n'avait aucun effet sur le premier pic (immédiat et possiblement non-spécifique) de CEP, nous avons observé que le sunitinib pouvait bloquer de façon très significative le second pic de CEP, et que ceci était associé à une augmentation très importante de l'activité anti-tumorale du VDA et à un contrôle prolongé de la croissance tumorale. Par ces expériences, nous avons pu ainsi déterminer que le schéma thérapeutique optimal d'association d'un agent anti-angiogénique avec le VDA consistait à administrer le sunitinib après le VDA, probablement grâce à un effet inhibiteur ciblé sur le second pic de CEP (Figure 18).

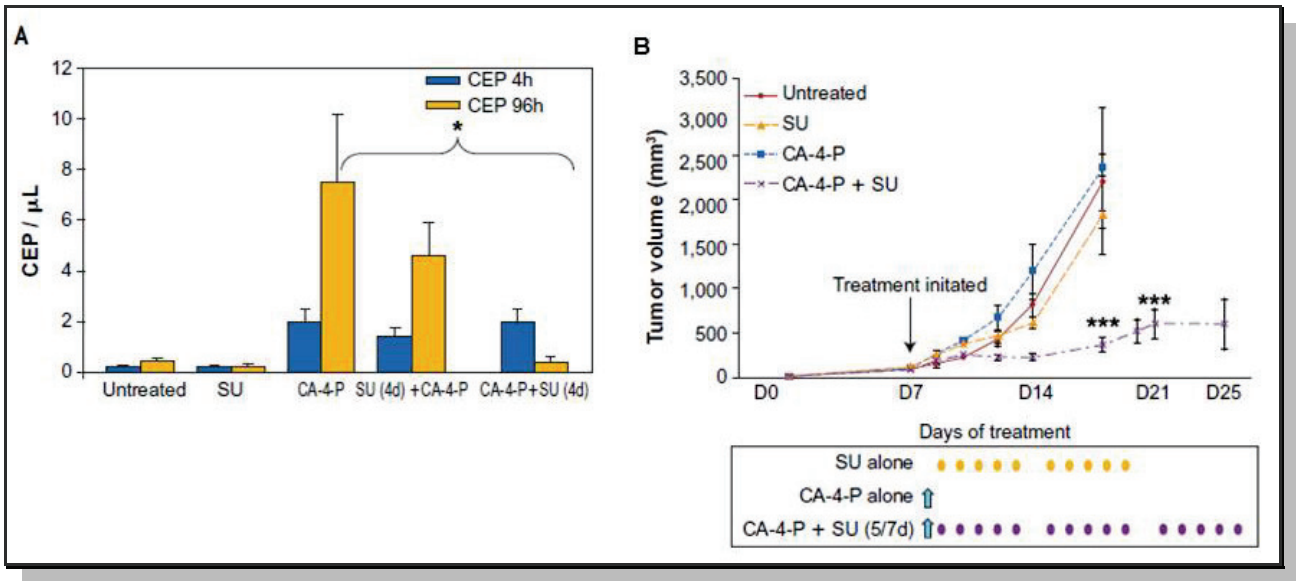

Figure 18. Le blocage du second pic de CEP par le sunitinib est associé à un contrôle prolongé de la croissance tumorale. Des souris nude (n=6-8) porteuses de xénogreffes PC3 ont été traités avec le sunitinib (40 mg/kg) seul pendant 4 jours (SU), le CA-4-P (100 mg/kg) seul ou combiné au sunitinib pendant 4 jours avant (SU(4d)+CA-4-P) ou après l'injection de CA-4-P (CA-4-P+SU(4d)). (A) Mesure des CEP à 4h et 96h après l'injection de CA-4-P (n=5 souris par groupe) et après le début du sunitinib dans le groupe SU. (B) La progression tumorale a été évaluée chez des souris (n=5 par groupe) non traitées, traitées par le sunitinib (40 mg/kg/j), le CA-4-P (100 mg/kg) seul ou combiné au sunitinib (40 mg/kg/j) administré après le CA-4-P. Les résultats sont exprimés en moyenne±SD. La signification des tests est représentée par * (0,05>p>0,01), ** (0,01 ≥p>0,001) ou *** (p≤0,001).

Dans le but de caractériser le rôle fonctionnel des CEP, nous avons mis au point un modèle de souris chimérique exprimant la GFP au niveau médullaire et réalisé de nouvelles expériences de blocage avec 2 agents anti-angiogéniques, le sunitinib et l'anticorps rat anti-souris dirigé contre le VEGFR-2, le DC101, en association avec la combrétastatine. Lorsque les agents anti-angiogéniques étaient administrés 24h avant chacun des pics de CEP induits par le VDA, nous avons observé que les deux agents étaient capables de bloquer de manière spécifique le second pic de CEP et non le premier. L'analyse des tumeurs traitées par combrétastatine seule a montré

que le nombre de cellules pro-angiogéniques GFP$^+$ totales était doublé 3 jours après le premier pic alors qu'il était multiplié par 8 avec une infiltration massive de cellules GFP$^+$ 3 jours après le second pic de CEP détecté dans le sang. A la différence des données publiées par Shaked *et al*. (76), l'analyse de la co-localisation des signaux GFP$^+$ et CD31$^+$ (marqueur des vaisseaux) a montré que le nombre de cellules GFP$^+$ par vaisseau n'était pas très différent entre les tumeurs non traitées et les tumeurs prélevées 3 jours après le premier pic de CEP. En revanche, le nombre de cellules GFP$^+$ par vaisseau était significativement supérieur à jour 6 (soit 3 jours après le second pic) avec une incorporation luminale des cellules GFP$^+$ dans les vaisseaux tumoraux (Figure 19).

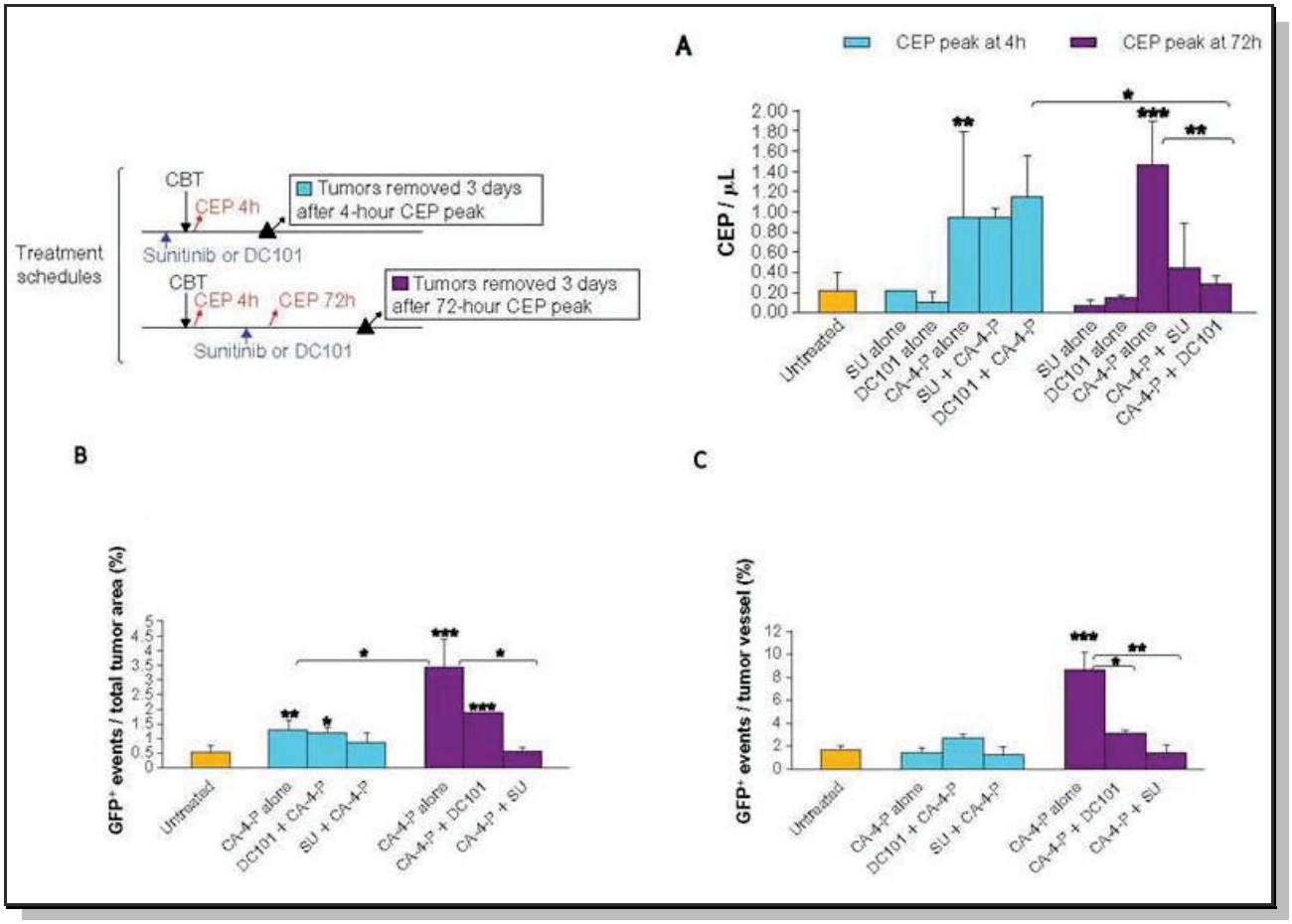

Figure 19. Des souris C57Bl/6 transplantées avec des cellules médullaires GFP$^+$ et porteuses de tumeurs LLC ont été traitées avec le CA-4-P, DC101, sunitinib (SU) ou l'association des drogues comme indiqué ci-dessus (n=5-13 souris à chaque temps par groupe). Les CEP ont été mesurées à 4h ou 72h selon les traitements indiquées (A). Trois jours après les pics de CEP à 4h ou 72h, les tumeurs ont été réséquées et des coupes préparées pour déterminer le nombre de cellules GFP$^+$ dans les tumeurs (B) et par vaisseau tumoral (C). Les résultats sont exprimés en moyenne±SD. La signification des tests est représentée par * (0,05>p>0,01), ** (0,01 ≥p>0,001) ou *** (p≤0,001).

Afin d'apporter un niveau de preuve supplémentaire sur l'incorporation des cellules GFP$^+$ dans les vaisseaux des tumeurs traitées par VDA, nous avons réalisé des analyses en immunofluorescence sur des cryosections épaisses de tumeurs permettant de visualiser avec précision le recrutement intraluminal et donc fonctionnel des cellules GFP$^+$ aux parois des vaisseaux lésés (Figure 20). Des expériences de co-marquage ont permis de démontrer que ces cellules GFP$^+$ d'origine médullaire infiltrant les tumeurs 6 jours après le traitement par combrétastatine n'exprimaient pas des marqueurs de macrophages (F4/80), ni de cellules myéloïdes (CD11b) et n'étaient pas des plaquettes (marquées en CD41).

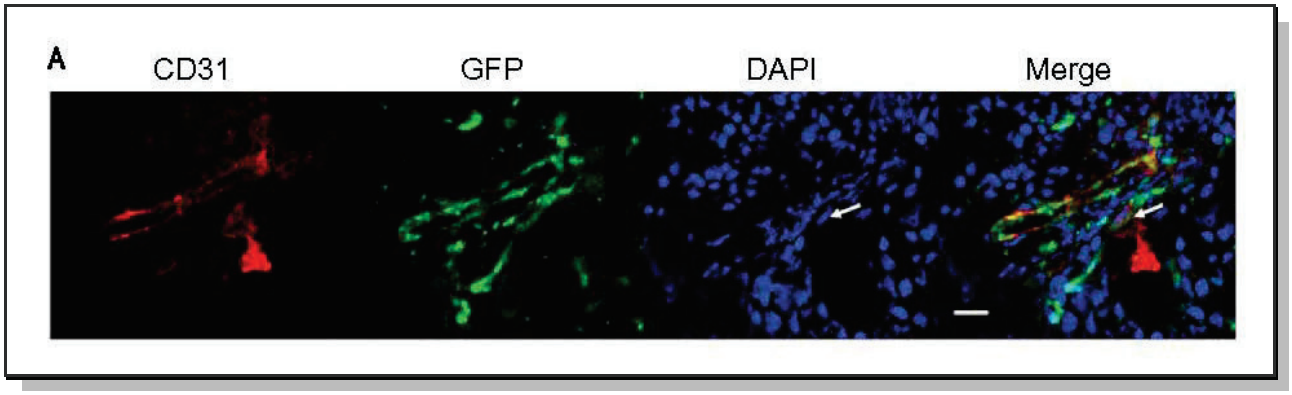

Figure 20. Image en fluorescence de cellules GFP$^+$ d'origine médullaires co-localisées dans des vaisseaux tumoraux d'une tumeur LLC 6 jours après traitement par le CA-4-P. L'image représente une projection d'intensité maximale de z-stacks entiers d'images confocales obtenues à partir d'une section tumorale épaisse de 40-50µm. La flèche blanche indique le noyau d'une cellule GFP$^+$ d'origine médullaire exprimant le marqueur endothélial CD31 et incorporée à la lumière d'un vaisseau. La ligne d'échelle est de 20 µm.

Les expériences d'association du VDA avec un agent anti-angiogénique ciblé contre le VEGFR-2 porté par les CEP ont montré que seul le blocage du second pic permettait de réduire l'infiltration tumorale en cellules GFP$^+$ et leur recrutement dans les vaisseaux tumoraux. Ces résultats montraient pour la première fois l'existence d'une vague tardive de CEP, exclusivement présente chez des animaux porteurs de tumeurs, qui pourraient être de véritables effecteurs du processus de réparation vasculaire et de reprise tumorale. Nos résultats indiquaient aussi que ces deux vagues de CEP pourraient être contrôlées par des mécanismes moléculaires différents : le premier pic pourrait correspondre à un efflux passif de CEP déjà présents dans le compartiment médullaire qui pourrait être la conséquence d'un effet direct du VDA sur les vaisseaux médullaires, le second pic pouvant résulter de la différenciation et maturation de CEP spécifiquement activés par une cascade de chémokine VEGF-dépendante, elle-même déclenchée

par l'hypoxie intense occasionnée par la lésion vasculaire. Bien que le niveau de CEP présents dans les tumeurs non traités soit faible, nos résultats montrent aussi qu'en réponse à l'ischémie aigüe occasionnée par la lésion vasculaire dans le modèle très particulier des VDA, des CEP ont véritablement la capacité de s'incorporer dans la lumière des vaisseaux et de contribuer physiquement à leur formation. Enfin, ces données précliniques de pic précoces de CEC et de vague tardive de CEP après administration de VDA ont été confirmées chez l'homme dans un groupe de patients ayant un cancer et traités par un VDA dans le cadre d'une étude de phase I (Figure 21).

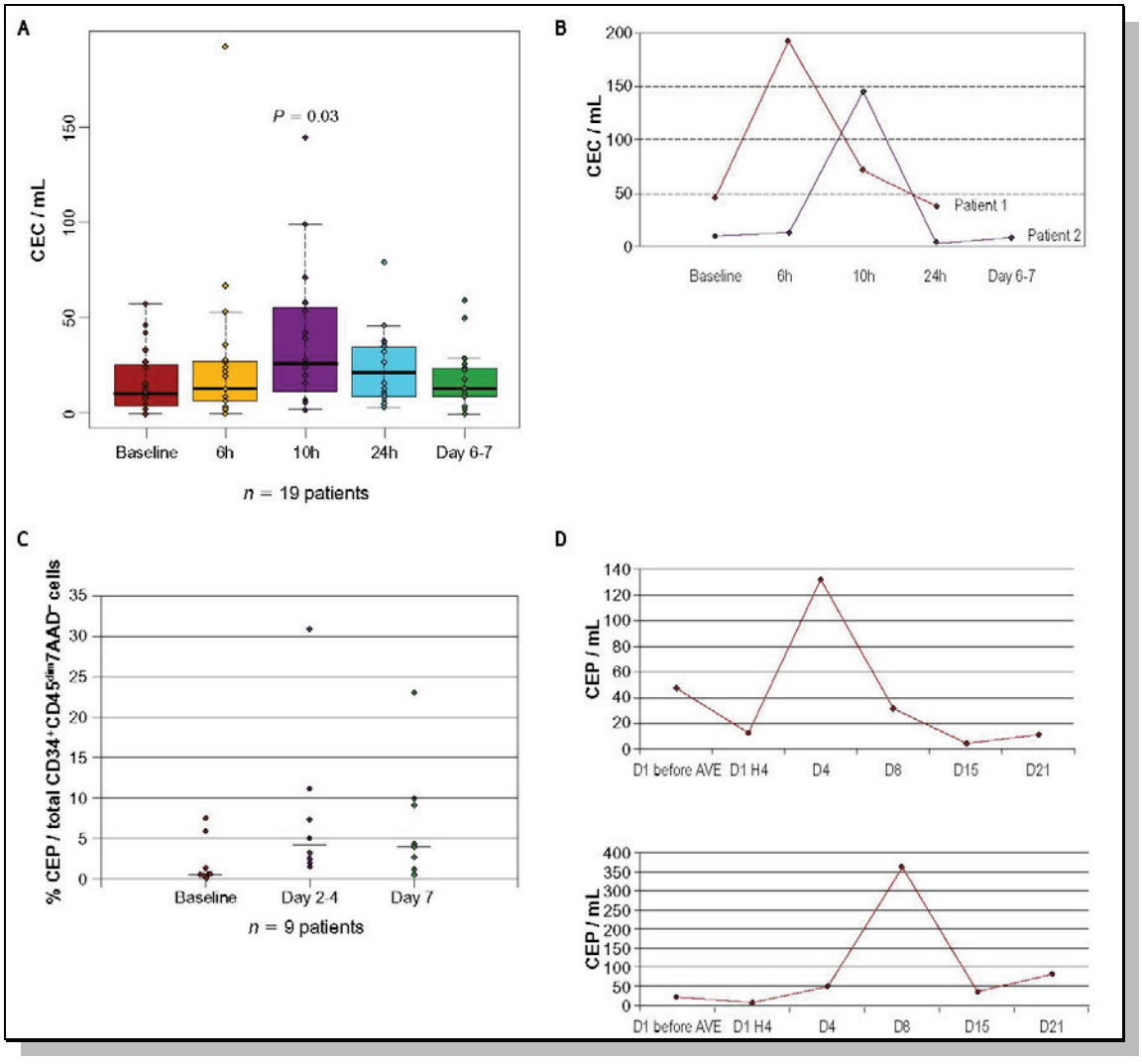

Figure 21. Cinétique des CEC (A, B) et des CEP (C, D) chez des patients ayant un cancer avancé et traités par un VDA, l'ombrabuline, dans le cadre d'essais de phase I. Les valeurs médianes sont représentées par des barres horizontales. Les taux CEC mesurés chez 19 patients présentaient un pic significatif à 10h après l'injection du VDA. La cinétique des CEP mesurés chez 9 patients montrait une mobilisation tardive, entre 4 et 7 jours après administration du VDA.

N.B. Les « Supplemental data » de cet article sont présentés en Annexe.

IN THE SPOTLIGHT

Circulating Endothelial Progenitors and Tumor Resistance to Vascular-Targeting Therapies

Michele De Palma[1,2] *and Silvia Nucera*[2,3]

Summary: Acute mobilization of circulating endothelial progenitors has been implicated in tumor resistance to vascular-disrupting agents. In the current issue of *Cancer Discovery*, Taylor and colleagues provide novel insight into the kinetics of endothelial progenitor mobilization by vascular-disrupting agents in both mouse tumor models and cancer patients. *Cancer Discov; 2(5); 395–7. ©2012 AACR.*

Commentary on Taylor et al., p. 434 (12).

INTRODUCTION

Endothelial cells (EC) are highly specialized cells that form a monolayered tissue, called endothelium, which lines the inner surface of the blood vessels. ECs also circulate (albeit at low numbers) in the mouse and human blood (1). Although most of these circulating ECs represent mature ECs that are possibly shed from the endothelium as a consequence of microvascular damage or remodeling, a small population of immature ECs also has been detected in the blood that originates from the bone marrow (1). The latter have been termed endothelial progenitor cells or circulating endothelial progenitors (CEP) and are distinguished from mature circulating ECs on the basis of their high proliferative potential and expression of defined cell-surface markers (2–4).

CEP counts are low in the blood of both naïve and tumor-bearing mice, with figures generally below or around 1 cell per microliter of blood in most mouse strains (5). The exceedingly low frequency of CEPs, together with the difficulty of distinguishing them from circulating hematopoietic progenitor cells, has generated significant uncertainty and controversy regarding their phenotype, enumeration, and functional properties (3). Nevertheless, EC colonies growing as monolayers can be established by culturing blood-derived mononuclear cells under conditions that favor EC proliferation, indicating that the blood is in fact a reservoir of rare CEPs with high clonogenic potential (2–4).

In early studies, researchers suggested that bone marrow–derived CEPs represent an essential source of vascular ECs in rapidly growing mouse tumors (2). Subsequent studies in which authors scrutinized the contribution of CEPs to tumor blood vessels have had conflicting results, and a consensus on the extent and functional significance of this phenomenon is still lacking (6–8). In more recent studies, investigators also have suggested that certain cytotoxic drugs, such as vascular-disrupting agents (VDA) and taxanes, increase the number of CEPs in the peripheral blood of mice and enhance their incorporation in the tumor vasculature (9, 10). VDAs are microtubule-destabilizing drugs that cause the rapid collapse of intratumoral blood vessels, leading to tumor necrosis and shrinkage. However, tumors rapidly regrow after an initial response phase (11). Interestingly, the acute recruitment of bone marrow–derived CEPs to VDA-treated tumors has been causally linked to tumor revascularization and relapse in mouse tumor models (9, 10).

Shaked and colleagues (9) have previously shown that a single VDA dose induces a transient CEP spike in the mouse circulation as soon as 4 hours after drug administration. Neutralization of this CEP spike with the use of a blocking antibody against vascular endothelial growth factor receptor-2 (VEGFR-2) inhibited the incorporation of CEPs in the tumor blood vessels and delayed tumor revascularization and regrowth (9). These findings suggest that the therapeutic efficacy of VDAs may be improved by blocking drug-induced CEP rebounds into the tumor vasculature. In this issue of *Cancer Discovery*, Taylor and colleagues (12) report carefully performed studies that provide novel insight into the involvement of CEPs in tumor resistance to the VDA combretastatin-A4-phosphate (CA-4-P).

STUDY RESULTS

Taylor and colleagues (12) have extended previous findings by Shaked and colleagues (9, 10) by demonstrating that a single dose of CA-4-P induces 2—rather than 1—distinct CEP spikes in treated mice. Although the first CEP spike occurs a few hours after the administration of the VDA in both naïve and tumor-bearing mice, as previously reported (9), the second CEP spike occurs 3 to 4 days after the administration of VDA and is specifically observed in tumor-bearing mice (12). Of importance, the authors also detected bimodal CEP kinetics in cancer patients treated with a clinically validated VDA (ombrabulin). At variance with earlier findings (9, 10), anti-VEGFR-2 antibodies or sunitinib (an inhibitor of VEGFR

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and platelet-derived growth factor-receptor kinase activity) did not block the early CEP spike but effectively blunted the late CEP spike. Repeated administrations of sunitinib inhibited tumor regrowth for 2 to 3 weeks after a single dose of CA-4-P, whereas CA-4-P or sunitinib alone had no detectable antitumor activity in the same experimental tumor models. Together, these data suggest that the late CEP spike supports tumor regrowth after VDA therapy.

The late CEP spike may be induced by soluble factors that are upregulated in the tumor after VDA-induced ischemic damage. The authors observed significant serum elevation of granulocyte colony-stimulating factor (G-CSF) and matrix metalloproteinase-9 (MMP-9), which paralleled the late CEP spike in the VDA-treated mice. These data suggest that tumor-derived G-CSF and/or MMP-9 had promoted the second wave of CEP mobilization to the peripheral circulation. Although not experimentally validated by the authors, this hypothesis would be consistent with previous studies in which investigators showed that MMP-9 activity is required for CEPs to egress from the bone marrow (2).

To investigate whether VDA-induced CEP mobilization also leads to their enhanced incorporation in the tumor blood vessels, the authors used a bone marrow transplantation strategy that included green fluorescent protein (GFP)-tagged bone marrow cells. They found that vascular incorporation of GFP-tagged ECs is extremely infrequent in untreated tumors, in agreement with previous reports (9, 13–15). However, the occurrence of vessel-incorporated bone marrow–derived ECs increased in CA-4-P–treated mice a few days after the late CEP spike, possibly suggesting a direct relationship between increased CEP numbers and their enhanced rate of incorporation. As shown in previous studies (9, 10), anti-VEGFR-2 or sunitinib decreased the incorporation of bone marrow–derived ECs in the blood vessels of VDA-treated tumors. Because combined VDA/antiangiogenic treatment delays tumor regrowth, these findings suggest that CA-4-P–induced CEP mobilization and subsequent incorporation in the tumor blood vessels are important determinants of tumor revascularization and regrowth after VDA-based therapy.

OUTSTANDING QUESTIONS

The study of Taylor and colleagues (12) attests to the significance of "CEP rebounds" for tumor resistance to VDA therapy in mouse models of cancer. However, it also raises important questions. One key issue is represented by the ill-defined nature of the CEPs described in this study (12). Indeed, it is unclear from the data presented whether the second wave of cells mobilized in response to the VDA (late CEP spike) truly represent *bona fide* cell progenitors, that is, immature cells endowed with clonogenic activity. Does treatment with VDA increase the frequency of colony-forming unit ECs (3) in the blood of tumor-bearing mice? If yes, do colony-forming unit ECs specifically increase concomitant to the late CEP spike? Furthermore, the anatomic site at which CEPs proliferate in response to VDA treatment has not been defined. Do CEPs proliferate in the bone marrow or in the tumors? If CEPs proliferate after tumor colonization, does this occur before or after they become incorporated in the tumor endothelium? Finally, do CEPs undergo a differentiation program and lose expression of "progenitor" markers (e.g., c-kit or Sca-1) once they integrate in the tumor endothelium? At present, these questions remain largely unanswered. Perhaps most importantly, future studies should also formally demonstrate that CEPs give rise to the bone marrow–derived ECs that incorporate in the tumor blood vessels. Because CEP-tracing experiments (16) were not performed, the current study (12) provides only indirect evidence that the late CEP spike gives rise to the pool of bone marrow–derived ECs.

Another unresolved issue, which may merit further investigation, is the modality by which CEPs promote tumor revascularization after VDA therapy. The authors report occasional luminal incorporation of bone marrow–derived (GFP-tagged) ECs in the blood vessels of the VDA-treated tumors, with less than 10% of the tumor blood vessels displaying at least one cell apparently incorporated in the endothelial layer. These data argue against the possibility that the "building-block" function of CEPs (i.e., their ability to structurally contribute to the tumor endothelium) could account for their blood vessel–forming activity in the VDA-treated tumors. As properly pointed out by the authors, antiangiogenic treatment by VEGFR-2 blockade or sunitinib not only suppressed the late CEP spike and bone marrow–derived EC incorporation in the tumor blood vessels, but it also dramatically reduced tumor infiltration by bone marrow–derived hematopoietic cells, which represent the vast majority of the cells recruited from the bone marrow after treatment with the VDA. These bone marrow–derived cells include macrophages and other myeloid-lineage cells, which are known to provide proangiogenic and/or reparative signals/factors to the tumor vasculature after radiation- or VDA-induced tumor damage, as shown in recent studies (14, 17, 18).

The finding that antiangiogenic drugs can reverse VDA-induced tumor infiltration by macrophages and other bone marrow–derived hematopoietic cells is of special interest. Of note, sunitinib or VEGFR-2 blockade did not affect hematopoietic cell infiltration in tumors that had not received the VDA. These observations may suggest that VDAs can sensitize tumors to the activity of the antiangiogenic drugs, whose crucial targets—besides CEPs—are the angiogenic tumor blood vessels and their associated perivascular cells (e.g., pericytes). VDA-induced tumor sensitization to antiangiogenic drugs may thus directly improve angiogenesis inhibition and also limit the extravasation of (proangiogenic/reparative) bone marrow–derived hematopoietic cells in the tumor. In such a scenario, CEP blockade might not critically account for the antiangiogenic and antitumoral effects of the combined treatments.

In conclusion, the study by Taylor and colleagues (12) reports exciting and thought-provoking findings that may have important implications for the design of more effective vascular-targeting treatments based on optimized drug scheduling. Moreover, it corroborates the notion that CEPs represent useful biomarkers of drug activity and/or resistance in mouse tumor models (4, 5, 9, 10). Nevertheless, further studies are needed to more rigorously establish the functional importance of CEPs and to better explore the biologic mechanisms whereby CEPs contribute to tumor resistance to VDA-based therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. De Palma
Writing, review, and/or revision of the manuscript: M. De Palma, S. Nucera

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RESEARCH ARTICLE

Reversing Resistance to Vascular-Disrupting Agents by Blocking Late Mobilization of Circulating Endothelial Progenitor Cells

Melissa Taylor[1,2], Fanny Billiot[1], Virginie Marty[1], Valérie Rouffiac[3], Patrick Cohen[9], Elodie Tournay[4], Paule Opolon[5], Fawzia Louache[6], Gilles Vassal[7], Corinne Laplace-Builhé[3†], Philippe Vielh[1], Jean-Charles Soria[2,8], and Françoise Farace[1,2]

ABSTRACT

The prevailing concept is that immediate mobilization of bone marrow–derived circulating endothelial progenitor cells (CEP) is a key mechanism mediating tumor resistance to vascular-disrupting agents (VDA). Here, we show that administration of VDA to tumor-bearing mice induces 2 distinct peaks in CEPs: an early, unspecific CEP efflux followed by a late yet more dramatic tumor-specific CEP burst that infiltrates tumors and is recruited to vessels. Combination with antiangiogenic drugs could not disrupt the early peak but completely abrogated the late VDA-induced CEP burst, blunted bone marrow–derived cell recruitment to tumors, and resulted in striking antitumor efficacy, indicating that the late CEP burst might be crucial to tumor recovery after VDA therapy. CEP and circulating endothelial cell kinetics in VDA-treated patients with cancer were remarkably consistent with our preclinical data. These findings expand the current understanding of vasculogenic "rebounds" that may be targeted to improve VDA-based strategies.

SIGNIFICANCE: Our findings suggest that resistance to VDA therapy may be strongly mediated by late, rather than early, tumor-specific recruitment of CEPs, the suppression of which resulted in increased VDA-mediated antitumor efficacy. VDA-based therapy might thus be significantly enhanced by combination strategies targeting late CEP mobilization. *Cancer Discov; 2(5); 434–49. ©2012 AACR.*

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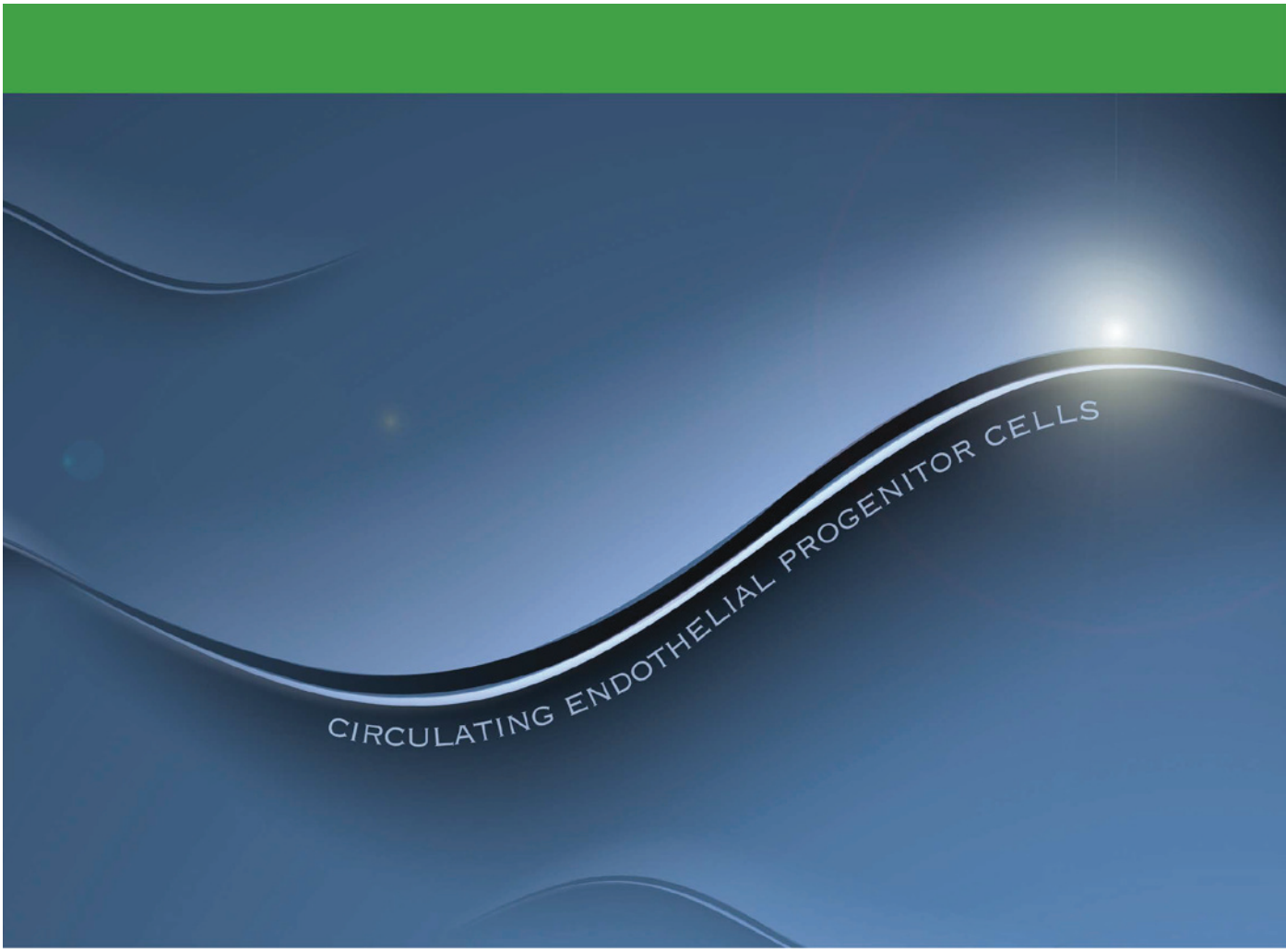

INTRODUCTION

Rapid tumor revascularization and repopulation compromise the expected clinical efficacy of vascular-disrupting agent (VDA)–based therapy (1–4). VDAs selectively target the already-established tumor vasculature and cause acute vascular shutdown (2, 5, 6), effectively resulting in severe tumor hypoxia, ischemia, and cell death (1, 7). Although VDAs are characterized by extensive necrosis of the tumor core, a viable peripheral rim is typically spared from which tumor cell repopulation and regrowth rapidly resumes (2, 5, 7). Tumor recovery at the periphery, representing treatment resistance, appears to be a consistent finding and may explain the minimal tumor shrinkage and lack of clinical benefit observed with VDAs when given as single agents (1, 2, 5).

For these reasons, combination strategies with cytotoxic agents or radiotherapy aiming to interfere with the tumor recovery that ensues after VDA therapy have been actively evaluated both preclinically (1, 8–10) and clinically (4, 11–13). The future success of VDAs will most likely rely on advances in deciphering the mechanisms underlying VDA-induced tumor resistance and determining optimal agents and schedules that will improve the antitumor activity of these drugs.

VDA-induced tumor repopulation is angiogenesis dependent, suggesting that combining VDAs with drugs targeting angiogenesis may be effective (2, 7). Initial studies in xenograft tumor models showed that the anti-VEGF antibody, bevacizumab (14), or a potent inhibitor of VEGF receptor-2 (VEGFR-2)–associated tyrosine kinase effectively enhanced the antitumor activity of VDAs (15). A mechanistic rationale for these findings was provided in a pioneering study by Shaked and colleagues (16) in which they showed that the treatment of tumor-bearing mice with VDAs led to an acute and immediate (within 4 hours) mobilization of circulating endothelial progenitor cells (CEP), which were reported to home to the viable peripheral rim and promote tumor regrowth. Blockade of this VDA-induced CEP mobilization with a monoclonal antibody to mouse VEGFR-2 (DC101) enhanced the antitumor efficacy of the VDA, thus highlighting the potential role of CEPs in tumor recovery and treatment escape after VDA therapy (16). On the basis of these findings, it seemed reasonable to postulate that combined complementary antiangiogenic strategies would readily translate into substantial tumor control and clinical benefit for patients (11, 17). However, in preclinical studies authors also have underscored the importance of schedule

and sequence to optimize the putative additive effects of combination approaches (18).

Accumulating evidence suggests that bone marrow-derived CEPs are pivotal mediators of tumor progression. Initial studies (19, 20) in preclinical models of acute vascular injury first showed how CEPs are mobilized and home to ischemic sites, incorporate into neovessels, and complement angiogenesis afforded by preexisting endothelium (21, 22). Since then, CEPs have attracted considerable interest and have been actively investigated for their role in angiogenesis-mediated tumor growth (21, 23, 24). CEP mobilization is regulated in part by proangiogenic cytokines released into the circulation by the tumor cell, or after ischemic lesions or vessel wall injury for vascular repair (23).

Although in some preclinical studies authors have shown the incorporation of CEPs in tumor neovessels (24–27), the contribution and general relevance of CEPs to tumor angiogenesis and progression have been controversial and the subject of intense scrutiny as the result of several studies in which investigators reported contradictory data (28–32). Alternatively, in other preclinical studies, authors have suggested that CEPs are not only key contributors to tumor neoangiogenesis and tumor growth (24–26) but are also critical regulators of the angiogenic switch promoting metastatic progression (33). Another subpopulation of endothelial cells that has been considerably studied in the context of vascular trauma and cancer is mature circulating endothelial cells [CEC (34, 35)]. CECs are shed from blood vessel walls into the circulation as the result of vascular injury. Although their role in tumor angiogenesis is less clear, the number of CECs in peripheral blood directly reflects the extent of endothelial insult, and CEC counts are currently considered as a useful biomarker of vascular damage in patients with vascular disorders (36).

We hypothesized that CECs and CEPs might have the potential of being unique tools, possibly biomarkers, reflecting the pathophysiologic effects of VDAs. Previously, we observed the presence of CECs (13) and, more importantly, of VDA-induced CEP mobilization in a small series of cancer patients included in a phase I trial combining a VDA, ombrabulin (AVE8062; Sanofi-Aventis), with cisplatin and in whom CEP levels peaked 3 to 7 days after drug injection (37). CECs have also been documented in cancer patients in a phase I study evaluating another VDA, ZD6126 (38). These preliminary clinical findings supported the mechanistic hypothesis that CEC levels might directly reflect the antivascular and antitumor activity of the VDA, whereas CEP levels might express tumor resistance via vasculogenic rebounds and could therefore be crucial to identify possible targets as well as optimal schedules for combined strategies.

Herein, we show for the first time that VDA therapy induces not only an instantaneous CEP peak as previously described (16) but also a delayed yet more dramatic tumor-specific burst of CEPs that were recruited to VDA-disrupted tumor vessels. Blockade of this late CEP burst significantly increased the antitumor efficacy of the VDA. Taken together, these findings provide novel insight into host-reactive responses and vasculogenic "rebounds" mediating resistance to VDA therapy which might be more efficaciously targeted by optimally scheduled antiangiogenic strategies.

RESULTS

Treatment with CA-4-P Induces Early and Massive Necrosis with Hypoxia-Related Microvessel Destruction Followed by Revascularization

Preliminary experiments with tumors of different histologic types (see Supplementary Methods) showed that PC3 tumors displayed an important level of microvessel density with little to no spontaneous necrosis and were very sensitive to VDAs compared with other xenograft tumor types (data not shown). To investigate the precise cascade of events triggered by VDAs in tumors, PC3 xenograft tumors established in male nude mice were evaluated for necrosis, microvessel density, and upregulation of carbonic hydrase IX (CA-IX) expression (an indicator of hypoxia) after treatment with a potent VDA, combretastatin-A4-phosphate [CA-4-P (39)]. Once PC3 tumor volumes reached an average of 300 to 500 mm^3, mice ($n \geq 5$) were randomized into groups and culled for tumor harvesting at different time points ranging from 2 to 120 hours after a single i.v. injection of 100 mg/kg CA-4-P.

Four hours after CA-4-P injection, PC3 tumors displayed significant and marked intratumoral necrosis compared with baseline values (73 ± 10% vs. 4.6 ± 1%, $P < 0.0003$; Fig. 1A). By 24 hours, necrosis was extensive (81% ± 7%, $P < 0.0001$ compared with baseline; Fig. 1A and D) and was associated with a significant reduction in the surface fraction of $CD34^+$ vessels (microvessel density; 0.7% ± 0.3% vs. 3.7% ± 1% at baseline, $P < 0.03$; Fig. 1B and E), consistent with the effects of VDA described in different tumor models (2, 7, 39). Tumor expression of CA-IX peaked significantly 24 hours after CA-4-P ($P < 0.005$ compared with baseline values; Fig. 1C) and was concomitant to the lowest level of microvessel density. Microvessel density increased again by 48 hours and mirrored the downregulation of CA-IX expression, suggesting the existence of revascularization processes after immediate and extensive hypoxia-related tumor and vascular damage that ensued after CA-4-P treatment.

VDA-Induced Tumor Vascular Disruption Correlates with Peaks in CEC and CEP Levels

Next, we investigated whether VDA-induced tumor alterations and vascular injury were concomitantly reflected by changes in CEC and CEP levels, with special focus on the mobilization of CEPs that mediate vascular repair. We established flow cytometry assays to enumerate CECs and CEPs in mouse peripheral whole blood in accordance with the strategy we previously established in human samples [37, 40, 41 (Supplementary Data and Supplementary Figs. S1–S5)]. CECs were identified as

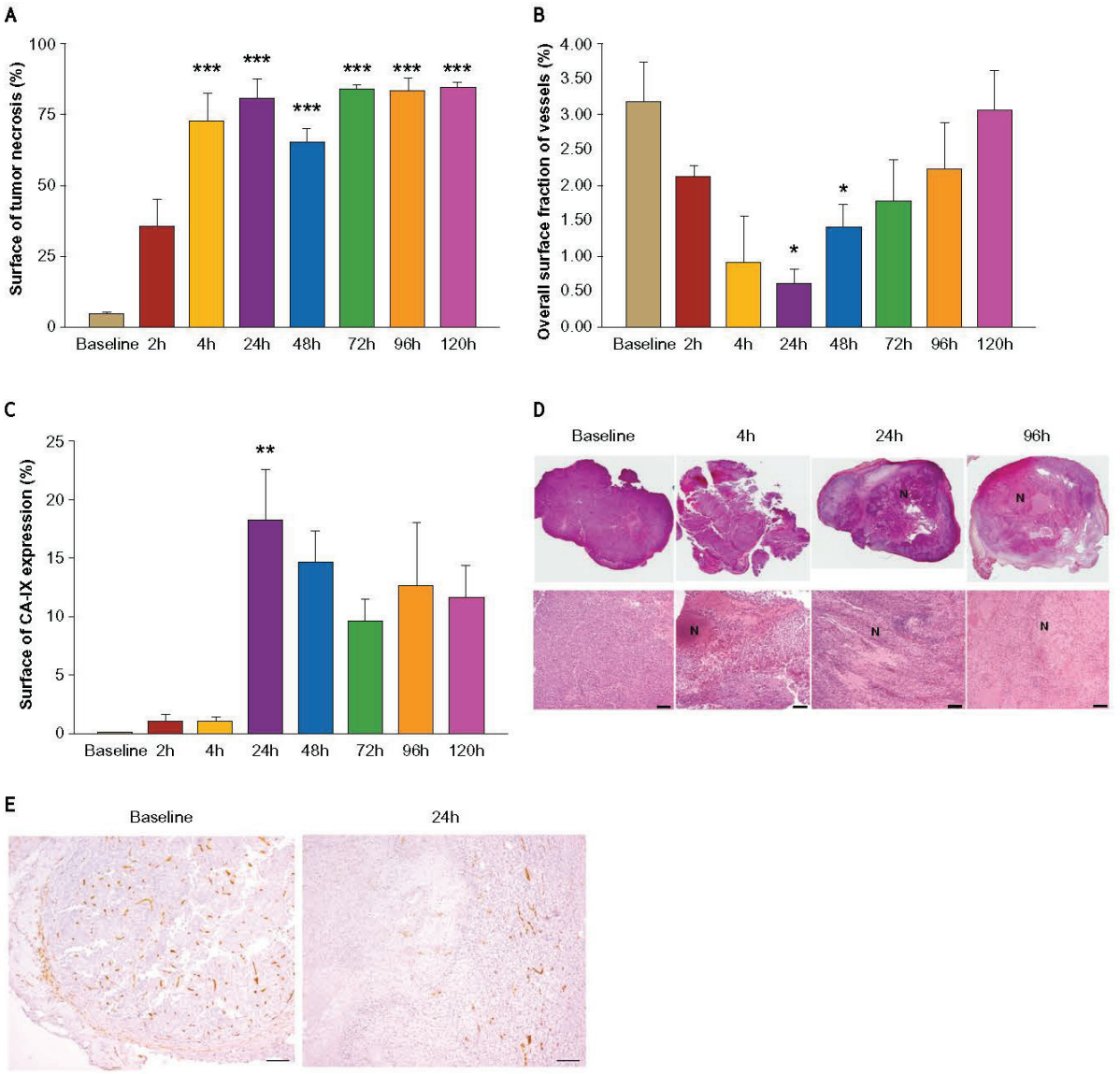

Figure 1. CA-4-P-treated PC3 xenografts show rapid extensive necrosis and hypoxia-related vascular damage. Six- to 8-week-old nude mice bearing PC3-xenografts of 300 to 500 mm^3 established subcutaneously 15 days previously were culled at indicated times ranging from baseline to 120 hours ($n = 5$ mice per group) after a single i.v. injection of 100 mg/kg CA-4-P, at which times tumors were harvested for analysis. **A-C,** summary graphs for the percentage of (**A**) intratumoral necrosis, (**B**) surface fraction of $CD34^+$ vessels (microvessel density), and (**C**) area containing expression of CA-IX after CA-4-P treatment are presented. Data are expressed as mean ± SD. Significance was set as $0.05 > P > 0.01$ (*), $0.01 \geq P > 0.001$ (**) or $P \leq 0.001$ (***) compared with baseline values. **D,** representative hematoxylin and eosin images of tumor sections show increased necrosis (N) over time (baseline to 96 hours) after CA-4-P treatment. Scale bars at 100 μm for lower images. **E,** representative images of immunohistochemical staining of the endothelial cell marker CD34 showing reduced and peripheral microvessel density 24 hours after CA-4-P (right) compared with baseline (left). Scale bars, 100 μm. Original magnification, ×25.

viable $CD45^-Flk\text{-}1^+CD31^+MECA\text{-}32^+$ cells. CEPs were identified as viable $CD45^{-/dim}CD117^+Sca\text{-}1^+Flk\text{-}1^+$ cells. Complementary flow cytometry experiments further characterized CEPs as expressing $CD34^+$, as recently described by Yang and colleagues (42), and importantly, vascular endothelial (VE)-cadherin, which directly supports the endothelial phenotype (Supplementary Fig. S2).

A rapid and significant peak in CEC levels was observed in PC3 tumor-bearing mice 4 hours after a single injection of 100 mg/kg CA-4-P ($P < 0.008$ compared with baseline values; Fig. 2A). In contrast, no changes in CEC levels were detected in control groups of non-tumor-bearing mice receiving CA-4-P (Fig. 2B) or tumor-bearing mice receiving vehicle (Fig. 2C). These data were consistent with our

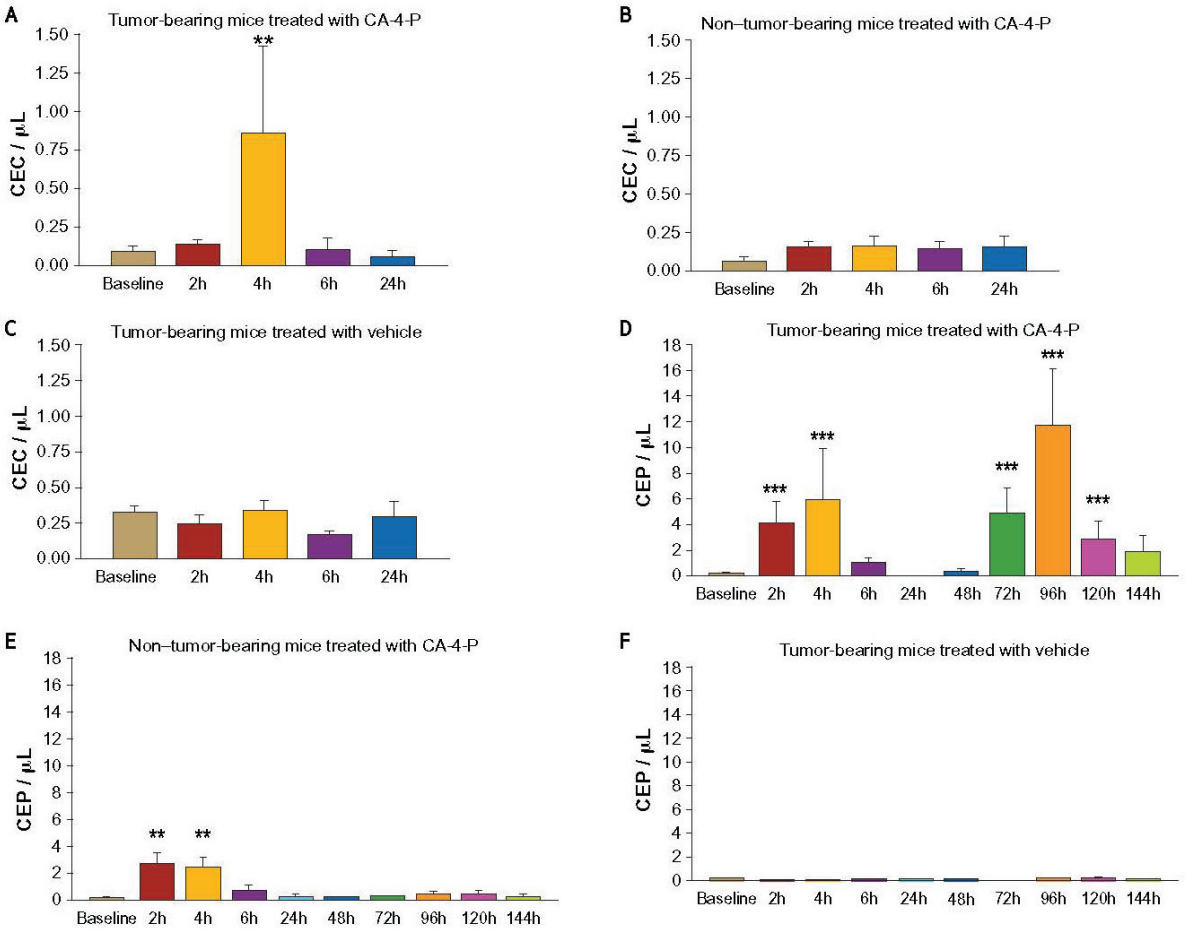

Figure 2. CA-4-P–induced tumor vascular damage is associated with peaks in CEC and CEP levels and a characteristic delayed tumor-specific burst in CEPs. CEC and CEP levels were evaluated using 4-color flow cytometry in mice bearing PC3-xenografts of 300 to 500 mm^3 at sequential time points after treatment with 100 mg/kg CA-4-P. **A–C,** CEC levels were evaluated in mice (n = 6–8 per time point in each group) at times ranging from baseline to 24 hours after CA-4-P (**A**) in tumor-bearing mice and (**B**) in non–tumor-bearing mice, or (**C**) in tumor-bearing mice receiving vehicle. Data are expressed as mean ± SD. Significance was set as $0.05 > P > 0.01$ (*), $0.01 \geq P > 0.001$ (**) or $P \leq 0.001$ (***) compared with baseline values. **D–F,** in a separate experiment, CEP levels were measured in mice (n = 8–10 per time point in each group) at sequential time points ranging from baseline to 144 hours in (**D**) tumor-bearing and in (**E**) non–tumor-bearing mice after CA-4-P treatment and in (**F**) a control group of tumor-bearing mice receiving vehicle. Data are expressed as mean ± SD. Significance was set as $0.05 > P > 0.01$ (*), $0.01 \geq P > 0.001$ (**) or $P \leq 0.001$ (***) compared with baseline values unless expressed otherwise.

histologic findings and suggested that the CEC peak observed in tumor-bearing mice was the direct consequence of the tumor vascular injury induced by CA-4-P.

Similarly, a single injection of 100 mg/kg CA-4-P caused an acute increase in CEP levels in tumor-bearing mice at 2 and 4 hours after treatment ($P < 0.0001$ and $P < 0.0004$, respectively, compared with baseline values; Fig. 2D), which then returned to basal levels by 24 hours. CEP levels also rapidly increased in non–tumor-bearing mice at 2 and 4 hours after CA-4-P ($P < 0.002$ compared with baseline values; Fig 2E), as previously described (16). Interestingly, the CEP levels observed at 2 and 4 hours after CA-4-P did not significantly differ between tumor-bearing and non–tumor-bearing mice ($P = 0.66$ and $P = 0.86$, respectively; Fig. 2D and E). Also, in tumor-bearing mice, the increase in CEPs occurred before the peak in CEC levels. These results suggested that the early peak in CEP levels—observed immediately after CA-4-P in both tumor-bearing and non–tumor-bearing mice but before the peak in CEC levels—did not actually reflect a VDA-induced tumor vascular-repair process but was most likely a general host response to the injection of CA-4-P. Alternatively, the significant peaks in CECs observed exclusively in tumor-bearing animals 4 hours after CA-4-P treatment were highly suggestive of tumor-specific VDA-induced vascular lesions. We therefore hypothesized that the mobilization of CEPs recruited for tumor-specific vascular repair might occur at later time points after CA-4-P administration.

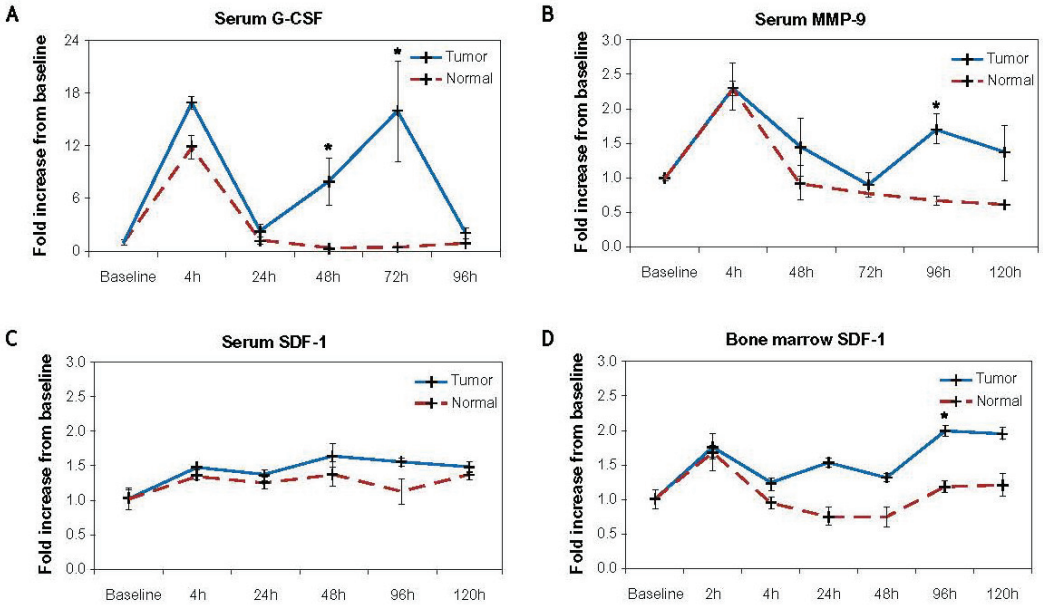

Figure 3. Circulating G-CSF, MMP-9, SDF-1 serum levels, and bone marrow SDF-1 content differ in CA-4-P–treated tumor-bearing and non–tumor-bearing mice. **A–D,** blood samples collected from PC3–tumor-bearing and non–tumor-bearing mice at baseline, 4 hours, 24 hours, 48 hours, 72 hours, and 96 hours after administration of 100 mg/kg CA-4-P were evaluated with the use of ELISA for serum levels of (**A**) G-CSF, (**B**) MMP-9, and (**C**) SDF-1, as well as levels for (**D**) bone marrow SDF-1 content ($n = 4$–6 mice per time point). Results obtained were plotted as a fold increase from the baseline value. Data are expressed as mean ± SD. Significance was set as $0.05 > P > 0.01$ (*), $0.01 > P \geq 0.001$ (**) or $P \leq 0.001$ (***) compared with baseline values.

To test this hypothesis, previous experiments were repeated in separate cohorts of tumor-bearing and non–tumor-bearing mice to extend CEP measurements from 4 to 144 hours after CA-4-P administration. Tumor-bearing mice displayed a dramatic burst in CEP levels 96 hours after CA-4-P treatment ($P <$ 0.0001 compared with baseline values) that was 2-fold greater than the previous CEP peak at 4 hours (Fig. 2D). In contrast, control groups showed no changes in CEP levels after 24 hours (Fig. 2E and F) suggesting that the CA-4-P–induced burst in CEPs at 96 hours was specific to tumor-bearing animals.

Comparable experiments in a different xenograft-tumor model (H69) yielded similar results with a rapid increase in CEC levels (within 6 hours) and 2 distinct CEP peaks at 4 and 72 hours after treatment of H69-tumor-bearing mice with 100 mg/kg CA-4-P (Supplementary Fig. S6). Administration of different doses of CA-4-P (50 mg/kg or 100 mg/kg) to H69 tumor-bearing animals showed that CEC and CEP levels varied in a drug-dose dependent manner (Supplementary Fig. S6). These results showed that VDA-induced tumor vascular lesions were reflected by changes in CEC and CEP levels that were both tumor-specific and drug-dose dependent.

CEP Kinetics Correlate with Serum Matrix Metallopeptidase-9, Granulocyte Colony-Stimulating Factor, and Bone Marrow Stromal-Derived Factor-1 Levels

To investigate which growth factors may be responsible for the mobilization of CEPs after VDA treatment, we measured circulating levels of murine VEGF, granulocyte colony-stimulating factor (G-CSF), matrix metallopeptidase-9 (MMP-9), and stromal-derived factor-1 (SDF-1) in serum samples obtained from tumor-bearing and non–tumor-bearing mice at various time points after the administration of CA-4-P (Fig. 3). The results in Figure 3A and B show that the kinetics of G-CSF and MMP-9 levels paralleled those observed for CEPs with an initial unspecific rise at 4 hours in both non–tumor-bearing and tumor-bearing CA-4-P–treated mice followed by a second increase in G-CSF and MMP-9 levels at 72 to 96 hours exclusively in tumor-bearing mice. VEGF levels at baseline were similar among tumor-bearing and non–tumor-bearing mice and were not correlated to changes in CEP levels in tumor-bearing animals (data not shown) as reported in a previous study describing CEP mobilization after VDA-like chemotherapeutic agents (43).

Systemic induction of SDF-1 accounts for the rapid mobilization of CEPs induced by VDAs and by paclitaxel in non–tumor-bearing mice (43). Here, we found that serum SDF-1 levels did not change after treatment with CA-4-P and did not differ between tumor-bearing and non–tumor-bearing mice (Fig. 3C). SDF-1 levels measured directly within bone marrow samples showed, however, a rapid increase at 24 hours after the administration of CA-4-P in tumor-bearing and non–tumor-bearing animals as well as a significant increase at 96 hours exclusively in tumor-bearing mice ($P < 0.03$ compared with baseline values; Fig. 3D), suggesting that the second burst of CEPs was associated with changes in bone marrow and not serum SDF-1 content.

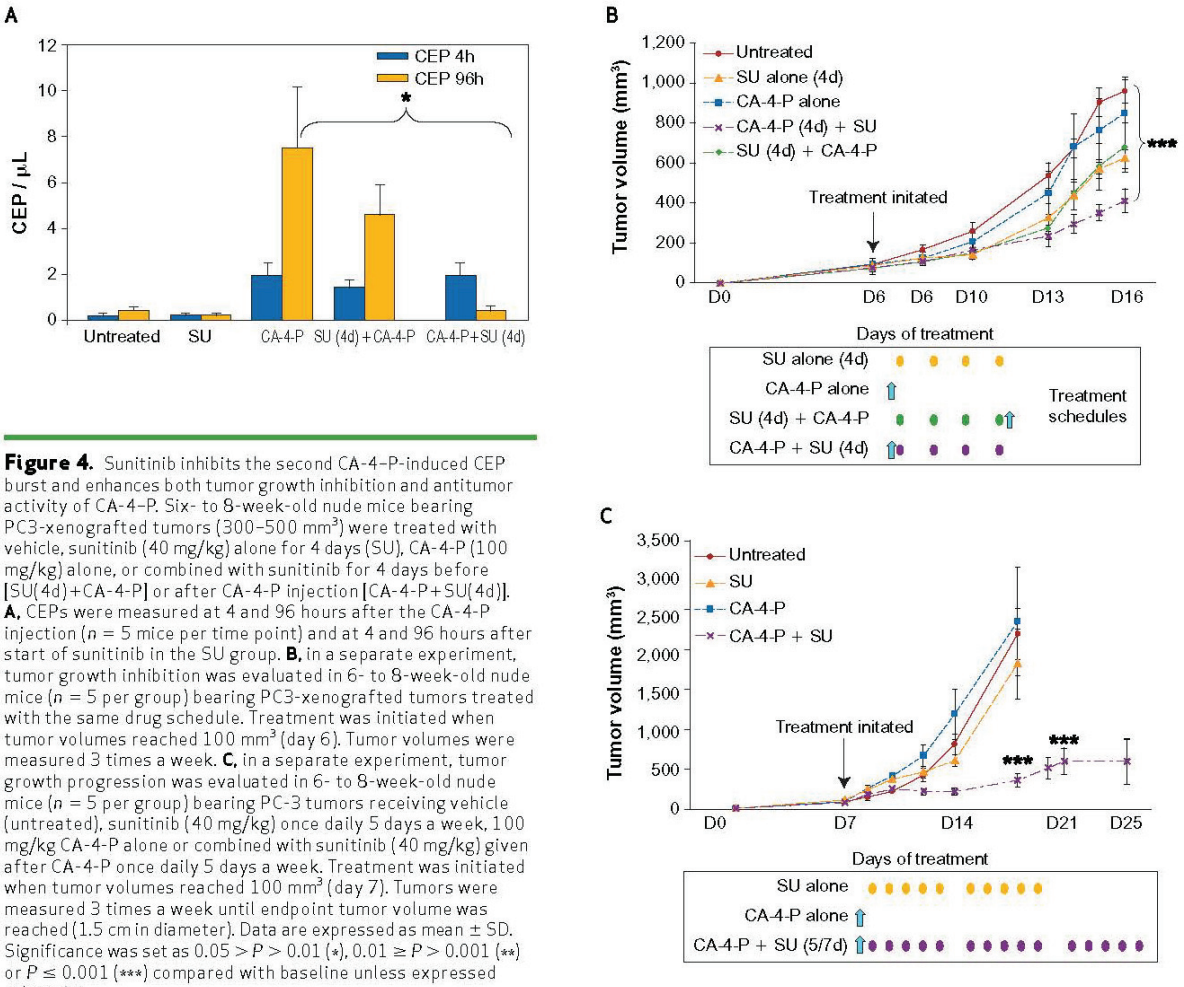

Figure 4. Sunitinib inhibits the second CA-4-P-induced CEP burst and enhances both tumor growth inhibition and antitumor activity of CA-4-P. Six- to 8-week-old nude mice bearing PC3-xenografted tumors (300–500 mm^3) were treated with vehicle, sunitinib (40 mg/kg) alone for 4 days (SU), CA-4-P (100 mg/kg) alone, or combined with sunitinib for 4 days before [SU(4d)+CA-4-P] or after CA-4-P injection [CA-4-P+SU(4d)]. **A,** CEPs were measured at 4 and 96 hours after the CA-4-P injection ($n = 5$ mice per time point) and at 4 and 96 hours after start of sunitinib in the SU group. **B,** in a separate experiment, tumor growth inhibition was evaluated in 6- to 8-week-old nude mice ($n = 5$ per group) bearing PC3-xenografted tumors treated with the same drug schedule. Treatment was initiated when tumor volumes reached 100 mm^3 (day 6). Tumor volumes were measured 3 times a week. **C,** in a separate experiment, tumor growth progression was evaluated in 6- to 8-week-old nude mice ($n = 5$ per group) bearing PC-3 tumors receiving vehicle (untreated), sunitinib (40 mg/kg) once daily 5 days a week, 100 mg/kg CA-4-P alone or combined with sunitinib (40 mg/kg) given after CA-4-P once daily 5 days a week. Treatment was initiated when tumor volumes reached 100 mm^3 (day 7). Tumors were measured 3 times a week until endpoint tumor volume was reached (1.5 cm in diameter). Data are expressed as mean ± SD. Significance was set as $0.05 > P > 0.01$ (*), $0.01 \geq P > 0.001$ (**) or $P \leq 0.001$ (***) compared with baseline unless expressed otherwise.

Antiangiogenic Treatment Blocks the Delayed CEP Burst and Is Associated with Enhanced Antitumor Activity

We next evaluated the capacity of an antiangiogenic agent to inhibit the 2 CEP bursts induced by CA-4-P treatment. Because preclinical studies have investigated therapies that combine VDAs with an antiangiogenic drug targeting VEGFR-2 (15), and previous reports have shown that antiangiogenic drugs can suppress the mobilization of CEPs (16, 43), we postulated that sunitinib, a potent receptor tyrosine-kinase inhibitor including VEGFR and PDGFR, might target the VEGFR-2 receptor expressed on CEPs.

PC3-tumor–bearing mice were treated with vehicle alone or with CA-4-P (100 mg/kg) alone or in combination with sunitinib. We reasoned that the antiangiogenic agent had to be given sufficiently in advance to be able to suppress the VDA-induced CEP peaks. To selectively target either of the CEP peaks and do so with an equivalent treatment period, sunitinib was given to tumor-bearing mice orally once daily for 4 days either before or after the CA-4-P injection. As shown in Figure 4A, treatment with sunitinib before CA-4-P did not block the early CEP peak. Alternatively, sunitinib given after CA-4-P completely abrogated the second CEP peak at 96 hours ($P < 0.008$ compared with CA-4-P alone). In mice receiving sunitinib before the injection of CA-4-P, a moderate inhibition of CEP levels at 96 hours was observed and was probably attributable to a sustained effect of sunitinib despite its discontinuation. CEP levels were unchanged in mice treated with sunitinib alone. These results suggested that only the second tumor-specific CA-4-P-induced CEP burst could be blocked by the combination of an antiangiogenic agent.

To assess whether inhibition of VDA-induced CEP peaks with an antiangiogenic agent was associated with therapeutic antitumor activity, we evaluated tumor growth in mice receiving combined therapy. PC3-xenograft tumors were allowed to reach approximately 100 mm^3, at which point treatment with vehicle, sunitinib, CA-4-P alone, or combined with sunitinib was initiated according to the same drug combination and schedule described previously. The results in Figure 4B show that the most significant tumor growth inhibition was observed in tumor-bearing mice treated with CA-4-P followed by 4 days of sunitinib as compared with those receiving vehicle

($P < 0.0001$), CA-4-P alone ($P < 0.002$) or sunitinib before CA-4-P ($P < 0.02$). Tumors treated with sunitinib alone displayed moderate growth delay at day 16 compared with untreated tumors ($P < 0.02$), which suggested a mild local antiangiogenic effect of sunitinib despite its known lack of antitumor activity in the PC3-xenograft model (44). Overall, the best tumor growth suppression was observed with a sequence that combined sunitinib after CA-4-P administration and that was concurrent with marked abrogation of the second burst of CEPs.

Next, the long-term antitumor efficacy of this combination strategy was evaluated. When tumor volumes reached 100 mm^3, PC3-xenograft tumors received a single dose of CA-4-P followed by sunitinib given once a day, 5 days a week, until tumor progression. As shown in Figure 4C, this combination sequence yielded substantial antitumor effect manifested by prolonged control of tumor progression at day 18 compared with tumor-bearing mice treated with vehicle ($P < 0.0001$), CA-4-P, or sunitinib alone ($P < 0.001$). Tumor control was maintained at day 25 compared with controls receiving vehicle for which the tumor end point was reached by day 18 ($P < 0.005$; Fig. 4C). Sunitinib alone showed only a delay in tumor growth by a few days. CA-4-P alone resulted in potent tumor regrowth with volumes that were comparable with untreated tumors, most likely attributable to VDA-induced tumor repopulation (Fig. 4B and C). It is of note that the combination treatment with sunitinib given before CA-4-P injection was not evaluated here because this strategy did not prove relevant in previous experiments.

Taken together, these results indicated that in our PC3-xenograft model, selective blocking of the late CA-4-P–induced CEP peak was significantly associated with marked tumor growth suppression and the optimal therapeutic combination scheme, allowing for this enhanced antitumor efficacy relied on administrating sunitinib after CA-4-P.

The Late VDA-Induced CEP Peak Correlates with Bone Marrow–Derived Cell Tumor Infiltration and Vessel Incorporation That Can Be Blocked by Antiangiogenic Drugs

To further characterize host-derived vascular repair mechanisms caused by VDAs and determine the functional role of bone marrow–derived CEPs, we focused subsequent experiments on a syngeneic C57Bl/6 mouse model transplanted with GFP^+-tagged bone marrow (GFP^+ bone marrow). First, Lewis lung carcinoma (LLC) tumors established in immunocompetent C57Bl/6 mice were allowed to reach 300 to 500 mm^3, at which point CA-4-P treatment was initiated. As observed in nude mice bearing PC3 xenografts, CEP levels peaked in LLC-tumor–bearing C57Bl/6 mice at 2 distinct times after CA-4-P, with an early CEP peak at 4 hours ($P < 0.002$ compared with baseline) followed by a delayed but significantly greater (by 7-fold) tumor-specific CEP burst at 72 hours ($P < 0.0001$ compared with untreated controls; Fig. 5A). The lower CEP values and slightly earlier occurrence of the second CEP burst in C57Bl/6 mice compared with nude mice were likely the result of inherent differences in immunogenic background. Initial studies by Shaked and colleagues (16) showed that DC101 administered 24 hours before VDA treatment could block the early-occurring CEP surge and resulted in increased antitumor efficacy.

We evaluated the capacity of a single dose of DC101 or sunitinib, administered 24 hours before, to suppress the successive CEP mobilizations in CA-4-P–treated LLC-tumor–bearing mice. As shown in Figure 5A, neither DC101 nor sunitinib blocked the early CEP peak. A single dose of DC101 or sunitinib given 24 hours before the second CEP peak, however, completely blunted this late CEP mobilization ($P < 0.006$ compared with CA-4-P alone; Fig. 5A). These data extended our results observed with combined treatment in the PC3-xenograft model and, taken together, implied that (i) only the second delayed CA-4-P–induced tumor-specific CEP burst could in fact be blocked by antiangiogenic agents in combined strategies, and (ii) the mechanisms underlying the mobilization of the two distinct CEP bursts were different.

These experiments were repeated to explore the capacity of CA-4-P–mobilized CEPs to home to tumors and incorporate to the tumor vasculature of LLC tumors established in GFP^+ bone marrow–transplanted C57Bl/6 mice (Fig. 5B–D). After CA-4-P treatment, tumors were removed 3 days after each CEP peak (at day 3 or day 6). Immunohistochemical analyses of tumors from untreated control mice showed moderate incorporation of GFP^+ bone marrow–derived cells (Fig. 5B and D). Mice treated with sunitinib or DC101 alone also showed mild incorporation of GFP^+ bone marrow–derived cells in tumors, similar to that observed in untreated tumors (data not shown). CA-4-P–treated tumors presented a 2-fold increase in total GFP^+ bone marrow–derived cell numbers at day 3 ($P < 0.009$ compared with untreated controls), which increased further by 8-fold with significant tumor infiltration at day 6 ($P < 0.0003$ compared with untreated controls; Fig. 5B and D). These results suggested a correlation between the CEP peaks detected in peripheral blood and the level of GFP^+ bone marrow–derived cells observed in tumors and supported the finding that GFP^+ bone marrow–derived cells infiltrating VDA-treated tumors could, at least in part, derive from the $VE\text{-}cadherin^+$-CEP bursts.

In contrast to previously reported data (16), the number of GFP^+ bone marrow–derived cells recruited to $CD31^+$-tumor vessels did not differ between untreated tumors and CA-4-P–treated tumors removed at day 3 (Fig. 5C). Tumors treated with sunitinib or DC101 alone showed levels of GFP^+ bone marrow–derived cells incorporated into tumor vessels that were comparable with untreated tumors (data not shown). In contrast, a substantial increase in GFP^+ bone marrow–derived cells per tumor vessel was observed at day 6 after CA-4-P ($P < 0.0004$ compared with untreated controls; Fig. 5C). Because the early CEP peak could not be suppressed, it was not possible to determine whether the greater number of GFP^+ bone marrow–derived cells detected at day 6 in CA-4-P–treated tumors resulted from the accumulation of both CEP bursts, from the intratumoral proliferation of GFP^+ bone marrow–derived cells already present at day 3, or were exclusively derived from the second CEP burst. The finding that GFP^+ bone marrow–derived cell numbers significantly increased in tumor vessels 3 days after the delayed CEP burst suggested, however, that these cells were recruited to injured tumor vessels and were most likely, in part, direct descendents of the second CEP burst.

To investigate this hypothesis, we asked whether the administration of antiangiogenic agents could block the recruitment of GFP^+ bone marrow–derived cells to the tumor and tumor vasculature after CA-4-P to a similar extent as was observed with the CEP peaks. Treatment with DC101

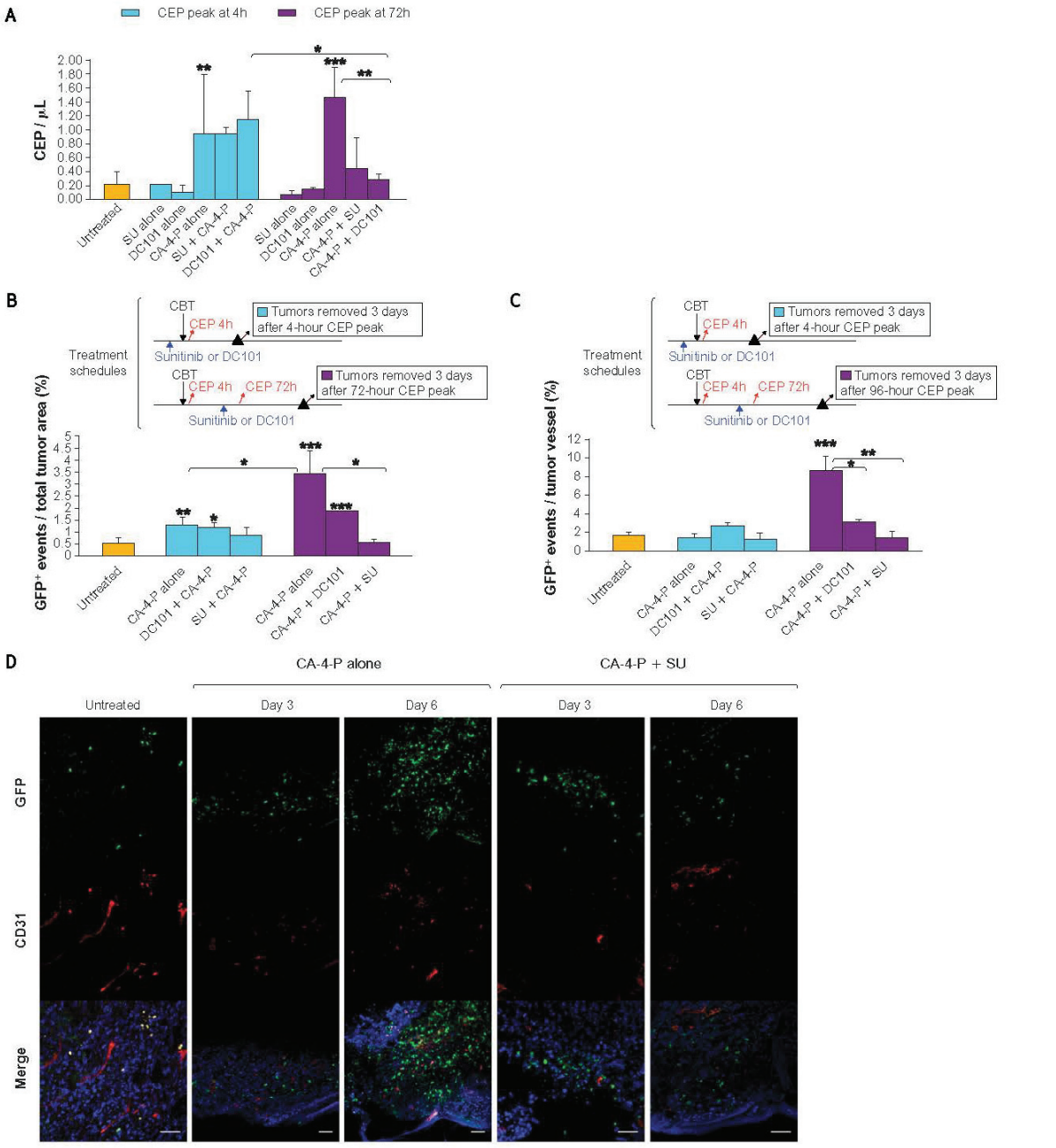

Figure 5. Homing and incorporation of GFP$^+$ bone marrow–derived cells in tumors and tumor vessels parallel levels of CEPs in blood of tumor-bearing mice after CA-4-P and can be blocked by antiangiogenic treatment. **A,** 8- to 10-week-old C57Bl/6 mice bearing 300 mm^3 LLC tumors were monitored for CEP levels at 4 and 72 hours after treatment with CA-4-P (100 mg/kg i.v.), sunitinib (SU; 40 mg/kg), DC101 (800 μg i.p.), or a combination of the drugs. Combination treatment consisted in CA-4-P administered with sunitinib ($n = 5$ mice per time point) or with DC101 ($n = 13$ mice per time point) given 24 hours before the 4-hour or the 72-hour CEP peak. Control groups received vehicle ($n = 5$ mice), CA-4-P alone ($n = 8$ mice per time point), or sunitinib or DC101 alone given 24 hours before blood sampling ($n = 5$ mice per time point). CEPs were measured in all groups at 4 and 72 hours after CA-4-P or at 4 and 72 hours after start of sunitinib or DC101 alone. Summary graphs of CEP levels are presented. Data are expressed as mean ± SD. Significance was set as $0.05 > P > 0.01$ (*), $0.01 > P > 0.001$ (**) or $P < 0.001$ (***) compared with baseline unless expressed otherwise. **B–D,** LLC cells (0.5×10^6) were injected subcutaneously to GFP$^+$ bone marrow–transplanted C57Bl/6 mice. When tumors reached 300 to 500 mm^3, mice were treated with CA-4-P, DC101, sunitinib, or combinations of the drugs at the same doses and schedule presented in **A.** Three days after the 4- or 72-hour CEP peak, tumors were removed (at day 3 and day 6, respectively), and cryosections were prepared for assessment of GFP$^+$-cell colonization (green), CD31$^+$ endothelial cells (red) representing blood vessels, and colocalization of CD31$^+$ and GFP$^+$ cells. DAPI-staining (blue) for nuclei. Summary graphs of the quantifications of (**B**) GFP$^+$ bone marrow–derived events in LLC tumors and (**C**) of GFP$^+$ bone marrow–derived events per tumor vessel are presented ($n = 5$ mice per group). Data are expressed as mean ± SD. Significance was set as $0.05 > P > 0.01$ (*), $0.01 \geq P > 0.001$ (**) or $P \leq 0.001$ (***) compared to baseline unless expressed otherwise. **D,** representative immunofluorescent images of LLC tumors after treatment with CA-4-P or CA-4-P combined with suntinib (SU) as described in **B** and **C** are presented; original magnification, ×20. Scale bar, 50 μm.

or sunitinib 24 hours before the early CEP peak had no effect on the total number of GFP^+ bone marrow–derived cells per tumor or tumor vessel detected at day 3 (Fig. 5B and C) whereas DC101 or sunitinib administered 24 hours before the second CEP burst markedly reduced the amount of GFP^+ bone marrow–derived cells infiltrating tumors and incorporating tumor vessels at day 6 (Fig. 5B–D). Previous treatment with DC101 or sunitinib had no effect on the number of other circulating GFP^+ hematopoietic cell populations in CA-4-P-treated mice (Supplementary Fig. S7). These data further suggested that the delayed CA-4-P-induced VE-cadherin$^+$-CEP burst was an important source of proangiogenic bone marrow–derived cells that were mobilized in response to tumor vascular injury and were recruited to tumor neovessels to promote tumor regrowth and recovery via angiogenesis mechanisms.

To further characterize GFP^+ bone marrow–derived cells recruited to tumor vessels, we sought to identify the luminal incorporation of these cells into CA-4-P-treated tumor vessels. Thick cryosections of LLC tumors established in GFP^+ bone marrow–transplanted C57Bl/6 mice were harvested at day 6 after CA-4-P and were analyzed by the use of high-resolution confocal/Z-stack microscopy. Analyses of multiple Z-stacks showed the presence of GFP^+ bone marrow–derived cells that were incorporated into the vessels of CA-4-P-treated tumors (Fig. 6) and colocalized with the $CD31^+$ endothelial marker (Fig. 6A). GFP^+ platelets may also express CD31 and can be closely associated to $CD31^+$ blood vessels or vessel debris, especially after VDA treatment (Supplementary Fig. S8 and Supplementary Movie SM2). In some instances, the nuclei of GFP^+ bone marrow–derived cells coexpressing $CD31^+$ could not be clearly identified within the endothelial layer (Fig. 6A). Nuclei were observed in the immediate perivascular environment of these cells. This limitation could be attributable to technical aspects (nucleus situated above or below the cryosection). The presence of such GFP^+CD31^+ signals could, however, also result from aggregated platelets which, in these cases, could prevent the quantification of CEP incorporation in blood vessels. GFP^+ bone marrow–derived cells luminally incorporated into vessels did not coexpress the macrophage marker F4/80 or myeloid marker CD11b (Fig. 6B–D and Supplementary Movie SM1). $GFP^{+or}F4/80^+$ and $GFP^{+or}CD11b^+$ cells consistently occupied a perivascular location.

The amount of GFP^+ bone marrow–derived cells detected in CA-4-P-treated tumors was not correlated with the extent of necrosis and was therefore not caused by unspecific GFP^+ monocyte and macrophage infiltrates (Supplementary Table 1). $CD41^+$ or $CD31^+$ platelets were observed within the lumen of damaged $CD31^+$ blood vessels in close proximity to GFP^+ platelets (Supplementary Fig. S8 and Movie SM2). GFP^+ bone marrow–derived cells incorporated into blood vessels and identified as CEPs presented a nucleus and did not colocalize with the CD41 platelet-specific marker (Fig. 6E and F, Supplementary Movies SM3 and SM4). On the basis of the proximity of $CD31^+$ and $CD41^+$ platelet signals, it is impossible to exclude, however, that GFP^+CD31^+ signals could, in some instances, originate from platelets and lead to artifactual assessment of CEPs.

Cancer Patients Treated with VDAs Display Acute Peaks in CEC Levels and Delayed CEP Bursts

To evaluate whether our preclinical findings were extended to the clinical setting, we measured CEC and CEP levels in patients with advanced solid tumors enrolled at our institute in phase I clinical studies evaluating a novel VDA, ombrabulin (Fig. 7). Sequential measurements of CEC levels in patients treated with ombrabulin alone ($n = 19$) showed an early and significant increase in CEC levels occurring between 6 and 10 hours after drug injection ($P < 0.03$), which returned to baseline values at 24 hours (Fig. 7A and B) and were very similar to the CEC kinetics observed in mice after CA-4-P. We have previously reported preliminary data on the changes in CEP levels in 5 patients receiving treatment with ombrabulin combined with CDDP (37).

CEP monitoring in this ongoing study has now been extended to 9 patients, in whom CEP levels dramatically increased by 4-fold at days 2 to 4 and remained increased by 3-fold at day 7 (Fig. 7C). Finally, close monitoring of CEP kinetics was performed in 2 patients included in an ongoing dose-escalating study evaluating ombrabulin combined with bevacizumab and in whom CEP levels increased significantly in a delayed fashion between day 4 and day 8 after VDA, similar to our preclinical observations (Fig. 7D). Of note, CEP levels in these 2 patients did not peak at 4 hours after ombrabulin (low doses of 8.5 and 11.5 mg/m^2) administration, in contrast to previously published data; this was not attributable to the effect of combined bevacizumab therapy, which was administered 24 hours after ombrabulin. Also, we did not observe an inhibition of the delayed CEP burst in these 2 patients receiving combined antiangiogenic therapy, but this may be the result of low initial doses of bevacizumab at 5 mg/kg or the fact that CEP inhibition could be effective only after several cycles of this combined therapy. These clinical findings were, nonetheless, remarkably consistent with our preclinical data and strongly support our mechanistic hypothesis favoring the existence of a late (rather than early) tumor-dependent CEP mobilization recruited specifically after VDA-induced tumor vascular injury.

DISCUSSION

Results from clinical trials evaluating VDAs in combination strategies have highlighted the urgent need to identify VDA-induced resistance mechanisms that lead to treatment escape (4, 17, 37). Our findings expand the current understanding of the mechanisms that underscore tumor vascular injury and revascularization, as well as subsequent host-reactive resistance responses triggered by VDA therapy. By studying the cascade of histologic and functional events that ensue VDA treatment in a highly VDA-sensitive PC3-xenograft tumor model, we show that VDA-induced vascular shutdown correlated with an acute and tumor-specific peak in CEC levels, thereby rendering these cells a potential pharmacodynamic biomarker of VDA activity.

Importantly, we describe for the first time 2 timely yet very distinct VDA-induced CEP bursts. The initial CEP peak, present as early as 2 to 4 hours after VDA administration and previously described (16), was apparently an unspecific, general, and transient chemokine-mediated response to the VDA—a finding emphasized by the fact that this initial peak was similarly detected in both tumor and non-tumor-bearing animals and occurred before the increase in CEC levels resulting from tumor vascular injury in tumor-bearing animals. Remarkably, a second delayed yet

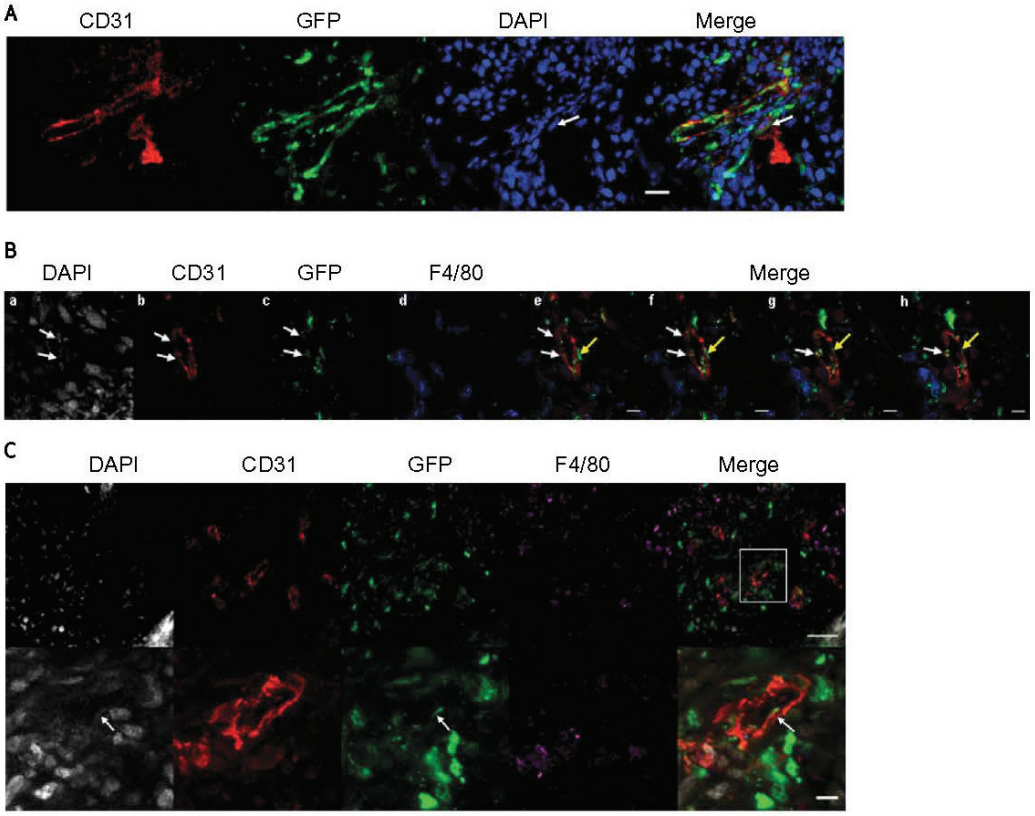

Figure 6. Incorporation of GFP$^+$ bone marrow–derived cells in LLC tumor blood vessels after CA-4-P treatment. Confocal immunofluorescence analysis was performed in LLC tumors grown subcutaneously in GFP$^+$ bone marrow–transplanted C57Bl/6 mice and harvested 6 days after treatment with CA-4-P (100 mg/kg). Maximum intensity projections of full z-stacks of confocal images obtained from 40- to 50-μm thick tumor samples are shown individually for indicated antibodies or nuclear staining with DAPI (4′,6′-diamidino-2-phenylindole) and after merging (merge). **A,** representative image of GFP (green) expression in LLC cryosections stained with anti-CD31 (red) and DAPI (blue). CD31$^+$ endothelial cells of a blood vessel carried GFP$^+$ signals for which nuclei were not located in the endothelial layer. The white arrow indicates the nucleus of a GFP$^+$ bone marrow–derived CD31$^+$ endothelial cell incorporated into the endothelial layer of the blood vessel. Scale bar, 20 μm. **B,** LLC cryosections stained with anti-CD31 (red), anti-F4/80 (blue), DAPI (white), and analyzed for GFP (green) expression. Representative confocal images from a full z-stack are shown (see Supplementary Movie SM1). **E** is a merging of **A**–**D**. **F**–**H** represent successive optical sections after **E**. Yellow arrow shows a GFP$^+$ bone marrow–derived cell incorporating the endothelial lining of a damaged blood vessel. White arrows show GFP$^+$CD31$^+$-expressing cells within the vascular lumen and corresponding nuclei. GFP$^{+/-}$F4/80$^+$ macrophages were located perivascularly. Scale bar, 10 μm. **C,** maximum intensity projection of a full z-stack of confocal images from an LLC cryosection stained with anti-CD31 (red), anti-F4/80 (magenta), DAPI (white), and analyzed for GFP (green) expression. GFP$^{+/-}$F4/80$^+$ macrophages were distributed throughout the tumor mass. Note the perivascular proximity of GFP$^+$ bone marrow–derived cells. White arrow indicates an incorporated bone marrow–derived cell (GFP$^+$CD31$^+$) with a nucleus that does not express F4/80; scale bar, 50 μm. The bottom panels show a greater magnification of images shown in the inset above; merged images are on the right. Scale bar, 10 μm.

more important burst in CEP levels was unexpectedly observed 72 to 96 hours after drug administration exclusively in tumor-bearing animals and subsequently verified in different mouse strains and tumor models, suggesting that this delayed but tumor-specific CEP mobilization was the actual VDA-induced tumor-dependent response for vascular repair and regrowth.

Functional analyses in the syngeneic C57Bl/6 mouse model transplanted with GFP$^+$-tagged bone marrow showed a significant correlation between the late CEP burst detected in peripheral blood and tumor recruitment and infiltration of GFP$^+$ bone marrow–derived cells, with a fraction of these cells incorporating into the lumen of tumor neovessels. Inhibition of the second VDA-induced CEP burst via the use of combined treatment with antiangiogenic agents (sunitinib or DC101) was associated with markedly reduced GFP$^+$ bone marrow–derived cell tumor colonization and recruitment to vessels and with significantly enhanced antitumor activity and efficacy of CA-4-P treatment, thereby suggesting a distinct but relevant role of the late VE-cadherin$^+$-CEP burst in tumor recovery after VDA treatment.

Despite the consensus on the important role of bone marrow–derived cells in solid tumors, controversy has shifted to the critical role played by CEPs and their contribution to tumor neovessels as illustrated by studies evidencing variable extent to which CEPs participate in the generation of tumor endothelium (24, 25, 28, 30). It is of note that many of these studies supporting such a role of CEPs have focused on tumor models analyzed in untreated steady states or in spontaneous conditions (27, 29, 45), which may be very different from the biologic processes triggered by anticancer therapies. Although CEP levels were indeed typically low in untreated PC3, H69, and LLC tumors, we found that these levels could increase dramatically

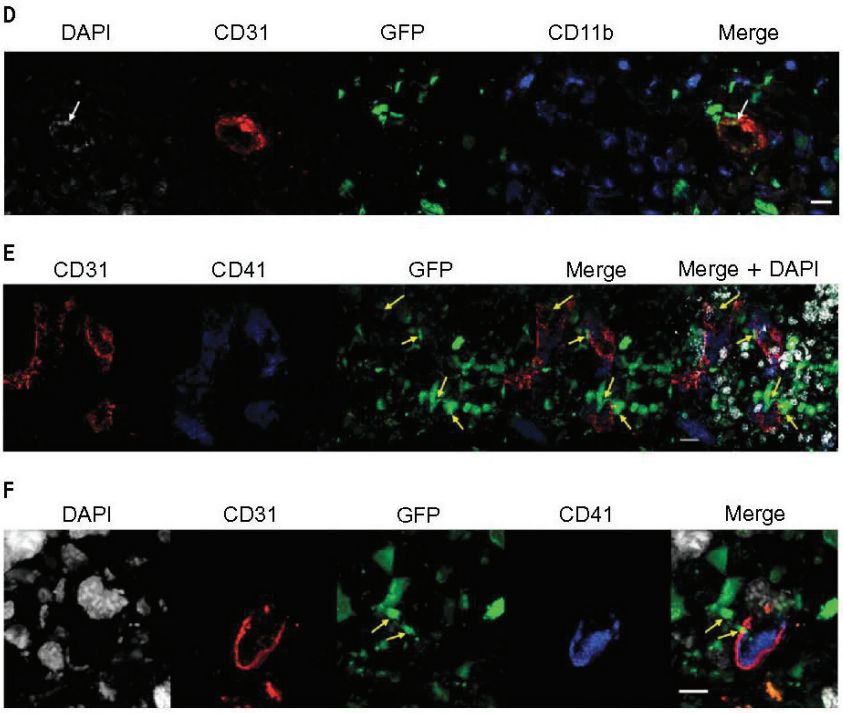

Figure 6 *continued*. **D,** maximum intensity projection of a full z-stack of confocal images from an LLC cryosection stained with anti-CD31 (red), anti-CD11b (blue), DAPI (white), and analyzed for GFP (green) expression. White arrow indicates an incorporated GFP$^+$CD31$^+$ bone marrow–derived cell, and corresponding nucleus, which does not express CD11b. CD11b$^+$ myeloid cells were located in the perivascular surrounding. Note that several CD11b$^+$ myeloid cells did not express GFP and none incorporated the CD31$^+$ blood vessel. Scale bar, 10 μm. **E-F,** LLC cryosections stained with anti-CD31 (red), anti-CD41 (blue), DAPI (white), and analyzed for GFP (green) expression. Maximum intensity projections of full z-stacks of confocal images are shown. CD41$^+$ platelets were observed within the vessel lumen and were closely associated to damaged CD31$^+$ blood vessels but did not co-stain for CD31. **E,** the GFP$^+$ bone marrow–derived cells (yellow arrows) replacing the damaged endothelial wall presented a nucleus and did not express CD41 platelet-specific marker. Note that a minority of CD41$^+$ platelet aggregates expressed GFP. Scale bar, 50 μm. **F,** yellow arrows show a GFP$^+$ bone marrow–derived cell that does not express CD41 and is incorporated into a damaged CD31$^+$ blood vessel. Scale bar, 10 μm. See Supplementary Movies SM3 and SM4.

in peripheral blood and tumor in response to acute stress and vascular injury, such as that caused by VDA treatment.

Confocal microscopy experiments showed that GFP$^+$ bone marrow–derived cells were incorporated into tumor vessels, colocalized with the CD31-endothelial marker, and did not express CD11b-myeloid, F4/80-macrophage, or CD41-platelet markers. Platelets from the bone marrow of GFP$^+$-bone marrow–transplanted mouse models can aggregate to damaged blood vessels, especially in the context of VDA. The presence of GFP$^+$ platelets is an important limitation that can interfere with the assessment of CEP incorporation in tumors and tumor blood vessels, as described herein and elsewhere (16, 43). Our findings supported, however, that bone marrow–derived cells including CEPs are recruited to tumor neovessels and may therefore have a catalytic role in tumor recovery following VDA therapy.

This is, to our knowledge, the first study investigating delayed biologic and cellular effects (vasculogenic rebounds) after VDA therapy. Vascular repair processes after VDA treatment have been previously analyzed in preclinical models but within a limited time-frame almost always restricted to the first 24 hours after drug administration (16, 46, 47). We confirmed the existence of an early (within 2–4 hours) VDA-induced CEP peak in both non–tumor-bearing and tumor-bearing animals. In our hands, however, disruption of this immediate VDA-induced CEP peak using DC101 or another antiangiogenic agent (sunitinib) was not observed. The variations in CEP levels reported herein were synchronized with changes in levels of G-CSF, MMP-9, and SDF-1, 3 key proteins known to orchestrate CEP mobilization (21, 31, 43, 48, 49). Interestingly, although antiangiogenic strategies had no effect on the initial CEP peak, their administration after CA-4-P completely abrogated the late CEP burst and showed the most potent antitumor activity with concomitant disruption of GFP$^+$ bone marrow–derived cell tumor infiltration.

One hypothesis for the differential effects observed when aiming to block these VDA-induced CEP peaks with antiangiogenic agents may be that these 2 distinct CEP bursts rely on different molecular pathways for mobilization. VDA therapy may have a direct and an indirect effect on CEP and

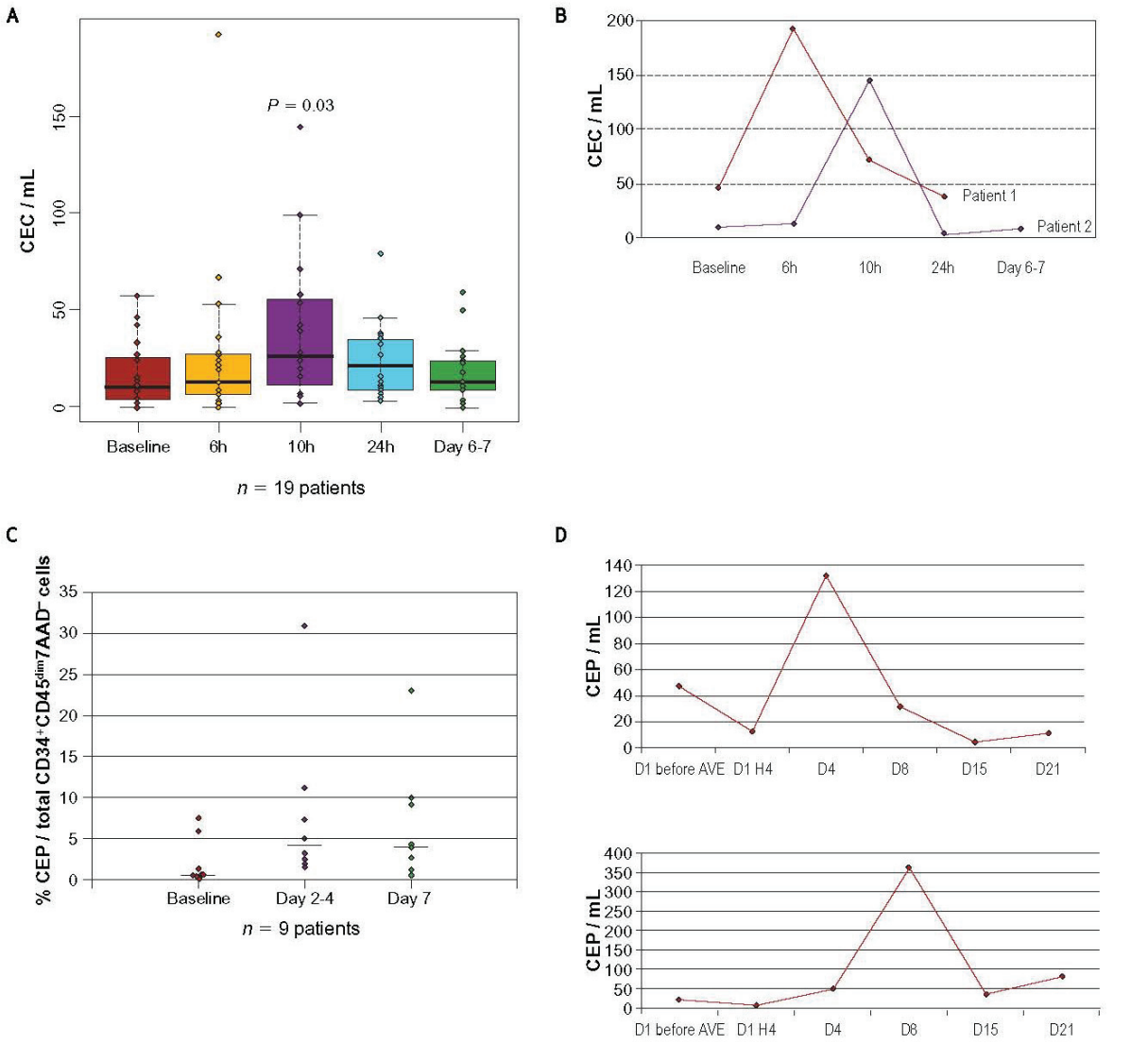

Figure 7. Kinetics of CEC and CEP levels in VDA-treated cancer patients. **A,** cancer patients treated with ombrabulin alone ($n = 19$) were monitored for CEC levels following drug administration. CECs were identified as $CD31^+CD146^+CD45^-7AAD^-$ viable events. Median values are represented by horizontal lines. **B,** CEC kinetics after ombrabulin are presented for 2 patients. **C,** in a separate study, CEP levels were monitored in cancer patients ($n = 9$) at baseline, day 2 to 4 and day 7 after treatment with ombrabulin combined with a single dose of 75 mg/m^2 CDDP. CEP levels were measured as the fraction of total $CD34^+CD45^{dim}7AAD^-$ viable events expressing VEGFR2. Median values are represented by horizontal lines. **D,** in a different study combining ombrabulin with bevacizumab (5 mg/kg) administered 24 hours after the VDA, CEP levels were closely monitored in 2 cancer patients and showed delayed bursts of CEP at 4 or 8 days after ombrabulin.

bone marrow–derived cells: first, the VDA may trigger an instantaneous and violent, but unspecific vascular effect on bone marrow vessels resulting in a passive "efflux" of CEPs already present within the bone marrow compartment; second, the intense hypoxia resulting from the tumor vascular injury might drive a VEGFR-dependent chemokine cascade powered to stimulate CEP proliferation, differentiation, and maturation for mobilization and subsequent recruitment for tumor-specific regrowth. This proposed mechanism would explain why the early CEP peak (or efflux) could not be blocked.

Taken together, our data suggest the existence of powerful regenerative mechanisms involving a complex chemokine cascade via G-CSF (46), MMP-9 (48, 49) and bone marrow SDF-1 after VDA therapy, instigated to restore tumor endothelium integrity and culminating into a relevant burst of mobilized CEPs 4 days after VDA-induced vascular injury. Importantly, combined antiangiogenic strategies scheduled after VDA and associated with complete blunting of the late VE-cadherin$^+$ CEP mobilization resulted in enhanced antitumor activity of the VDA-based strategy.

Several studies support the emerging concept that bone marrow–derived cells recruited to sites of tumor neoangiogenesis are composed of various proangiogenic subsets that all help to promote tumor growth and neovascularization, even when

present in small numbers (50). These different bone marrow–derived cell subsets include not only CEPs but also myeloid progenitors, especially $Gr1^+CD11b^+$ (51), and tumor-associated macrophages (52), including a particular subset expressing $Tie2^+$ (TEM), which contribute indirectly to neovascularization by incorporating perivascularly or by delivering cytokines essential to angiogenesis (29). Recently, Welford and colleagues (47) examined the role played by TEMs in the early stages after CA-4-P treatment. The authors reported that TEMs were detected within 4 to 24 hours after drug injection and were critical for perivascular support during tumor recovery.

Our results do not exclude the potential contribution of other bone marrow–derived cells to tumor revascularization and recovery after CA-4-P treatment. In fact, a complementary role between various bone marrow–derived subsets that indirectly respond to VEGFR-2–mediated processes is most probable. We observed that antiangiogenic agents, DC101 and sunitinib, blocked the late burst of CA-4-P-induced CEPs in peripheral blood and were associated with a significant reduction of GFP^+ bone marrow–derived cell infiltrates in tumors and tumor vessels. The precise mechanism by which DC101 treatment—which is supposed to specifically target the $VEGFR2^+$-CEP population—broadly inhibited the recruitment of GFP^+ bone marrow–derived cells (other than CEPs) to tumors remains unclear, although this phenomenon has been previously observed after VDA treatment (16) and chemotherapy (45).

Treatment with DC101 and sunitinib after CA-4-P did not affect the levels of other circulating GFP^+ hematopoietic cell populations. Therefore, these antiangiogenic drugs may exert a broader effect on bone marrow–derived cells and may block their intratumoral recruitment, possibly through the inhibition of local VEGFR-2–angiogenic pathways. The improved tumor control observed with combined antiangiogenic agents most likely involves additional mechanisms including the inhibition of endothelial cell proliferation from pre-existing blood vessels, which might also be more sensitive to antiangiogenic drugs after the administration of VDA. Our data showed, nevertheless, that combined antiangiogenic agents scheduled after VDA therapy and associated with blockade of the delayed CEP burst could critically impact the systemic recruitment of bone marrow–derived cells (including CEPs) to tumors to an extent that was sufficient to achieve significant anticancer efficacy with VDA therapy.

Our preclinical findings were extended by data obtained from cancer patients enrolled in dose-escalating clinical trials with a VDA, ombrabulin, where CEC peaks were observed in patients 4 hours after drug injection. Importantly, we also observed delayed and dramatic bursts in CEP levels in patients monitored over time after VDA administration. The delayed CEP bursts we report in cancer patients here and elsewhere (37) is strongly supported by other clinical studies documenting the kinetics of CEP mobilization in response to vascular injury (53, 54). Research evaluating CECs or CEPs has focused on their role during antiangiogenic treatment and despite the need to identify biomarkers of drug activity and resistance mechanisms, studies validating these cells as surrogate biomarkers in cancer patients are few (13, 23, 37, 55). Although VDAs are a particular model of vascular injury and repair, we observed in our study that the acute shedding of CECs directly reflected the vascular lesion and could be a pharmacodynamic biomarker of VDA activity, as shown by the exclusive peak in VDA-treated tumor-bearing animals and by CEC kinetics in cancer patients. Importantly, the late CEP burst could be considered as a remarkable host response and biomarker of resistance to VDA treatments.

The results presented here provide complementary insights to the intricate host responses and vasculogenic rebounds instigated after VDA treatment and call for a reconsideration of the current paradigm offered by initial studies supporting the role of very early (within hours) CEP responses after VDA therapy. Our data strongly suggest that the delayed VDA-induced tumor-specific burst in $VE\text{-}cadherin^+$-CEP levels is a crucial mediator that needs to be targeted for enhanced anticancer efficacy with VDAs. Because the future of VDA-based treatments in clinical oncology may rely exclusively on combination strategies, optimal scheduling is critical. Our findings provide a rationale for improving the sequence of administration of combined antiangiogenic agents in order to target the second CEP burst, which may prove to significantly enhance the clinical efficacy of VDA treatment.

METHODS

Blood Samples Obtained from Cancer Patients

Blood samples were collected from patients with advanced solid tumors who had given consent to participate in phase I studies evaluating escalating doses of a novel VDA, ombrabulin (Sanofi-Aventis), at the Service des Innovations Thérapeutiques Precoces (SITEP), Institut Gustave Roussy, France. Patients were enrolled in 1 of 3 trials evaluating ombrabulin alone ($n = 19$ patients) or combined with CDDP ($n = 9$ patients) or with bevacizumab ($n = 2$ patients). These studies were approved by our institutional ethical review board, who also granted approval for CEC and CEP analyses. Written informed consent was obtained from all patients. Blood samples were collected from patients at baseline and at indicated times after ombrabulin treatment. CECs and CEPs were quantified using 4-color flow cytometry according to previously published methods (37, 40, 41).

Tumor and Animal Models

PC3-xenografts established in male Foxn1-immunodeficient athymic Swiss nude nu/nu mice (Gustave Roussy Institute breeding) and LLC tumors established in immunocompetent C57Bl/6 mice or C57Bl/6 mice previously transplanted with GFP^+-tagged bone marrow cells were done according to procedures described in the Supplementary Methods.

Drug Administration and Scheduling

Treatment was given as CA-4-P (100 mg/kg via tail injection), SU11248/sunitinib malate (40 mg/kg orally), or DC101 (800 μg/mouse administered intraperitoneally) administered alone or in combination according to schedules described in the Supplementary Methods.

Evaluation of CEC and CEP Levels by Flow Cytometry

Viable CEC and CEP levels were measured each in 250 μL of whole blood using flow cytometry. In summary, immunofluorescent staining for CEC measurement was performed with the following rat antimouse monoclonal antibodies: MECA-32 biotin, CD31-FITC, CD45-PerCP, Flk-1-PE, and streptavidin-APC (all from BD Pharmingen). For CEP measurement, the following rat antimouse monoclonal antibodies were used: CD45-PerCP, CD117-APC, Sca-1-FITC, and Flk-1-PE (all from BD Pharmingen; refs. 16, 23, 43). The detailed staining procedures, acquisition, and analyses of CECs and CEPs as well as complementary characterization of CEPs are described in the Supplementary Methods and Supplementary Figures S1 to S4.

Determination of Proangiogenic Factor Levels in Serum and Bone Marrow Samples

Murine serum levels of VEGF, MMP-9, SDF-1, G-CSF, and levels of bone marrow SDF-1 were determined using commercially available mouse ELISA kits (R&D Systems) as described in the Supplementary Methods.

Tissue Processing

Tumors were fixed and paraffin-embedded for analysis of tumor necrosis, microvessel density, and hypoxia or immediately frozen (LLC tumors) for GFP visualization using confocal microscopy as described in the Supplementary Methods.

Image Acquisition and Analysis

The measurement of necrosis, microvessel density (CD34-stained endothelial cells), and CAIX-stained hypoxic tumor cell surfaces from digitalized whole histological tumor sections was done as described in the Supplementary Methods. A protocol to measure GFP$^+$bone marrow–derived cell numbers in LLC tumors and tumor blood vessels using a Leica SPE confocal microscope was established as detailed in the Supplementary Methods. To summarize, full Z-stacks of consecutive optical sections (0.8–1 μm step size) of thick (40–50 μm thickness) tumor cryosections were captured at a magnification of ×20 or ×63 using the Leica confocal software. Maximum intensity projections of full Z-stacks of confocal images were done using ImageJ software and are presented.

Statistical Analysis

Data are expressed as mean ± SD. The Wilcoxon–Mann–Whitney test was used to compare the distribution of marker levels between tumor and control groups or to compare levels at baseline and at a specific time. All tests were 2-sided and P values below 0.05 were considered to denote statistical significance. Data were analyzed by the use of SAS software (V 9.1, SAS institute). Significance was set as $0.05 > P > 0.01$ (*), $0.01 \geq P > 0.001$ (**) or $P \leq 0.001$ (***).

Disclosure of Potential Conflicts of Interest

J-C. Soria is a consultant for Sanofi-Aventis. No potential conflicts of interest were dislosed by the other auhors.

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Conception and design: M. Taylor, J-C. Soria, F. Farace
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5. DISCUSSION ET CONCLUSION

Nous pouvons tirer plusieurs conclusions des travaux présentés ici. Tout d'abord, ces études ont montré que notre approche en CMF pour la détection des CEC présentait un niveau de sensibilité suffisant pour mesurer ces cellules chez des patients atteints de différents types de cancers (101, 105). Nous avons constaté que l'utilité clinique des CEC pourrait fortement dépendre du type de cancer étudié. Dans les tumeurs pédiatriques, les taux de CEC mesurés chez les patients ne différaient pas de manière significative avec ceux mesurés dans la population pédiatrique de sujets sains. Compte tenu de la taille limitée de la cohorte étudiée, nous n'avons pas pu déterminer s'il existait une corrélation entre le taux de CEC et l'existence d'une maladie métastatique et si le taux de CEC pouvait refléter un remodelage et un « turnover » de l'endothélium tumoral et être la caractéristique d'une tumeur pourvue d'une forte activité angiogénique (28). Dans le cancer du rein métastatique et dans les tumeurs très avancées, les CEC semblaient avoir aussi un moindre intérêt prédictif ou pronostique que les CEP (29). En revanche, d'autres travaux réalisés dans le laboratoire dans le cancer du rein métastatique ont permis dans de plus grandes cohortes de patients adultes de corréler le taux de CEC avec une évolution défavorable et sans bénéfice d'un traitement standard associant la chimiothérapie au bevacizumab (150), laissant supposer que les taux élevés de CEC étaient la manifestation d'une tumeur à forte activité pro-angiogénique, ayant activé des voies alternes de néoangiogénèse, et que le blocage de l'angiogénèse en ciblant uniquement la voie du VEGF pourrait ne pas avoir une efficacité suffisante chez les patients présentant un taux prétraitement de CEC élevé. Ainsi, bien que les CEC soient en nombre plus élevé que chez les sujets sains dans la plupart des tumeurs solides métastatiques, elles ne pourraient être un véritable marqueur d'agressivité biologique ou de néoangiogénèse tumorale que dans certains cancers comme le cancer du côlon en relation avec des caractéristiques intrinsèques, mécanismes de vascularisation et profil de cytokines angiogéniques particuliers à cette tumeur.

Inversement, nos résultats montrent que les CEP pourraient être des biomarqueurs de l'angiogénèse tumorale et plus particulièrement de maladie métastatique dans les tumeurs pédiatriques. Depuis notre publication (28), seulement deux autres études se sont penchées sur l'évaluation des cellules circulantes dans les tumeurs pédiatriques. La première a évalué les taux de CEC et CEP chez 18 patients jeunes ayant un ostéosarcome et 7 sujets sains (194), mais la stratégie de mesure des CEC et CEP en CMF utilisée et semblable à celle publiée par l'équipe

italienne et controversée (110, 114), nous incite à ne pas prendre en compte leurs résultats. La deuxième étude de Pradhan *et al*. a évalué différentes populations circulantes de cellules endothéliales et de progéniteurs hématopoïétiques et endothéliaux pro-angiogéniques $CD45^{dim}$ $CD34^{+}$ $CD31^{+}$ chez 19 patients ayant une tumeur solide pédiatrique avant traitement et chez 24 sujets sains (195). Même si la stratégie de détection et l'immunophénotype des cellules décrites comme étant des progéniteurs pro-angiogéniques dans ce travail diffèrent de notre population identifiée comme des CEP, il est intéressant de noter que les auteurs ont observé des taux élevés de progéniteurs hématopoïétiques pro-angiogéniques $CD45^{dim}CD34^{+}CD31^{+}CD133^{+}$ chez les patients par rapport à ceux mesurés chez les volontaires sains (195). L'analyse des taux de ces populations n'a pas été faite en fonction du stade de la maladie. Cependant, Pradhan *et al*. rapportent une augmentation des taux de progéniteurs hématopoïétiques pro-angiogéniques et de progéniteurs endothéliaux (identifiés comme des ECFC dans ce travail) après un cycle de chimiothérapie. Corroborant nos propres résultats, les auteurs concluent que les progéniteurs hématopoïétiques et endothéliaux pro-angiogéniques pourraient être des biomarqueurs pertinents dans les tumeurs pédiatriques (195). Le caractère très vascularisé des tumeurs pédiatriques est bien connu et est corrélé au mauvais pronostic dans certains types de tumeurs comme le neuroblastome (35). Nos données suggèrent que la vasculogénèse pourrait être un mécanisme d'angiogénèse tumorale particulièrement important dans ces pathologies et que le ciblage des CEP avec des traitements anti-angiogéniques pourraient être une stratégie anti-tumorale prometteuse dans les tumeurs pédiatriques (35).

Nos résultats montrent, de plus, que les CEP pourraient avoir une valeur pronostique et/ou prédictive de l'efficacité d'un traitement anti-angiogénique dans plusieurs types de cancers métastatiques. Dans le cancer du rein métastatique, de fortes mobilisations de CEP avant traitement anti-angiogénique étaient associées à une SSP et SG défavorables, suggérant que la mesure de ces cellules pourrait être utilisée pour identifier des sous-groupes de patients susceptibles de ne pas bénéficier de ce traitement (29). Dans cette étude, nous pouvons remarquer que le taux de CEP était, parmi les différents biomarqueurs de l'angiogénèse testés, l'unique paramètre corrélé avant traitement à une évolution clinique défavorable. Dans notre cohorte de patients traités en phase I par différents agents anti-angiogéniques ou anti-vasculaires, des niveaux élevés de CEP avant traitement étaient associés à un risque plus élevé de décès quelque soit le type de tumeur et les traitements antérieurs (30). En phase avec les travaux chez la souris montrant le rôle « catalytique » des CEP dans le développement de métastases (24), nos données suggèrent que chez certains patients atteints de cancers

pédiatriques, de cancer du rein ou de cancers avancés, des niveaux élevés de CEP mobilisés par de processus orchestrés par l'hypoxie tumorale, pourraient être un marqueur de l'agressivité tumorale et avoir une valeur pronostique. Nos résultats chez les patients de phase I traités par des drogues exerçant des mécanismes d'action différentes (agents anti-vasculaires versus agents anti-angiogéniques) soutiennent l'hypothèse que les CEP pourraient avoir une valeur pronostique (plutôt que prédictive) et constituer un marqueur universel de mauvais pronostic qui pourrait ne pas être restreint aux patients traités par des agents anti-angiogéniques ou anti-vasculaires. De ce point de vue, les CEP gagneraient vraisemblablement à être étudiés chez les patients présentant une maladie localisée, en tant que reflet du potentiel métastatique de la tumeur. Cependant, nos résultats dans le cancer du rein ne nous permettent pas d'exclure l'hypothèse que des taux élevés de CEP pourraient être la caractéristique d'une population de patients « mauvais répondeurs » dont la tumeur possède des mécanismes alternatifs et compensatoires de vascularisation tumorale difficilement contrôlables par les traitements anti-angiogéniques et, dans ce sens, les CEP pourraient avoir une valeur prédictive de l'efficacité de ces traitements. L'augmentation de l'hypoxie induite par la régression vasculaire causée par les agents anti-angiogéniques est, par des mécanismes probablement similaires, capable d'activer des voies pro-angiogéniques alternatives et de mobiliser des CEP qui pourraient aussi être des marqueurs de résistance, hypothèse que nous n'avons pas encore abordée en clinique (78).

Nos travaux chez l'homme et chez la souris montrent que les CEC ont probablement un véritable intérêt en tant que biomarqueur pharmacodynamique de l'activité des VDA (31). La nature éphémère des pics de CEC et la variabilité des réponses d'un patient à l'autre nécessite cependant la réalisation d'une cinétique qui reste lourde à la fois pour le patient et d'un point de vue technique, et coûteuse. De plus, la variabilité des réponses d'un patient à l'autre reste difficile à interpréter ; l'absence de CEC (ou des taux très faibles) pourrait être considérée comme une non « sensibilité » du patient mais il n'est pas exclu qu'un pic de CEC très éphémère n'ait pas été détecté. Il est clair que ces données biologiques gagneront à être analysées en parallèle à des données radiologiques fonctionnelles. Ceci pourrait nous aider à déterminer s'il existe vraiment des patients et des types de tumeurs chez lesquels la drogue n'est pas active, données qui pourraient permettre de mieux sélectionner les patients traités par ces agents.

Les travaux réalisés chez la souris apportent des éléments originaux sur le rôle complexe des réponses de l'hôte et des rebonds « vasculogéniques » de CEP dans le mécanisme de la

résistance aux VDA (31). En effet, nos résultats mettent en évidence pour la première fois l'existence d'une vague tardive de CEP, dépendante de la présence d'une tumeur, capable de s'intégrer fonctionnellement aux néovaisseaux tumoraux et dont le blocage par des agents anti-angiogéniques est associé à une augmentation substantielle de l'efficacité thérapeutique du VDA. L'existence de ces deux vagues de CEP, répondant probablement à des mécanismes d'action distincts de mobilisation, a aussi pu être mise en évidence dans des prélèvements longitudinaux de patients. Ces données amènent à reconsidérer le paradigme actuel qui repose sur les travaux initiaux de Shaked *et al*. et qui supporte le rôle de la mobilisation précoce de CEP dans la revascularisation tumorale après VDA (76). En dépit d'une puissante activité anti-tumorale, les VDA ont une efficacité clinique limitée lorsqu'ils sont administrés en monothérapie (81, 82, 84, 87, 88). L'avenir de ces drogues en oncologie repose donc sur des stratégies d'association avec des molécules qui permettront de contrôler les puissants mécanismes de résistance responsables de la revascularisation et la repousse tumorale (88, 96, 97). Dans ce contexte, nos résultats apportent un rationnel mécanistique pour associer un agent anti-angiogénique au VDA et optimiser la séquence d'administration des drogues afin de cibler la seconde vague de CEP. Nos données n'excluent pas la contribution d'autres populations d'origine médullaires comme les TEMs à la repousse tumorale après VDA comme cela a été récemment montré par l'équipe de M. De Palma (196), bien que ces travaux n'aient analysé que des mobilisations cellulaires à des temps très précoces et jamais au-delà de 24h (66, 196). Nos observations montrent cependant que dans le modèle très particulier des VDA, en réponse au stress aigu occasionné par la lésion vasculaire, des CEP mobilisés plus tardivement (à 96h chez la souris et à J2-4 à J8 chez l'homme) dans un puissant processus de réparation orchestré par l'hypoxie ont la capacité d'infiltrer les tumeurs, de s'incorporer massivement dans la lumière des vaisseaux et d'avoir ainsi probablement un rôle catalytique de la repousse tumorale (31).

Le rôle particulièrement important voire possiblement catalytique des CEP observé dans l'ensemble de nos travaux soulèvement de nombreuses interrogations sur d'autres situations de stratégies anti-tumorales en clinique. Premièrement, certaines chimiothérapies cytotoxiques, comme le cyclophosphamide administré aux doses maximales tolérées, les taxanes et les vinca-alcaloïdes, semblent être dotées d'une puissante activité anti-tumorale semblable à celle des VDA (86, 197) et être de ce fait capables d'induire une mobilisation de CEP (26, 197). Mêmes si ces travaux n'ont explorés que des temps précoces après l'administration des drogues, nos résultats précliniques et chez l'homme soutiennent l'hypothèse d'une possible mobilisation de CEP et d'un rebond vasculaire tardif après certaines chimiothérapies qui pourraient contribuer à

la revascularisation et la repousse tumorale durant l'intervalle libre qui sépare les cures de chimiothérapie. Nos données ainsi que celles de la littérature soutiennent le fait que la mobilisation tardive de CEP est orchestrée en partie par le G-CSF (27). L'utilisation de facteurs de croissance hématopoïétique chez des patients ayant un cancer et recevant une chimiothérapie à haute-dose ou à dose proche de la dose maximale tolérée mérite alors réflexion puisque le soutien hématologique par G-CSF recombinant pourrait non seulement potentialiser la mobilisation des CEP déjà induite par la chimiothérapie, mais pourrait aussi augmenter le recrutement des populations myéloïdes suppresseurs $CD11b^{+}Gr1^{+}$ qui peuvent promouvoir l'angiogénèse (27, 65, 177, 198). Ainsi, dans certaines situations, ces traitements pourraient générer des effets paradoxalement contreproductifs, peut-être plus néfastes encore chez des patients présentant un taux initial de CEP élevé et donc un profil angiogénique d'emblée agressif. Enfin, qu'en est-il alors dans le cas de greffes de cellules souches hématopoïétiques (CSH) chez des patients conditionnés par une chimiothérapie à haute dose, recevant une greffe de progéniteurs hématopoïétiques $CD34^{+}$ pouvant comporter entre autre des CEP, et traités par des injections quotidiennes de G-CSF pendant plusieurs semaines jusqu'à reconstitution hématopoïétique ? Serait-il possible d'imaginer là aussi un possible effet contre-productif ou aggravant ? Même si l'identité et la définition phénotypique des CEP restent controversées, la majorité des études s'accordent sur l'observation que des progéniteurs hématopoïétiques d'origine médullaires pro-angiogéniques et exprimant le marqueur CD34 peuvent promouvoir l'angiogénèse tumorale dans les modèles précliniques (22, 23, 143, 156, 170). Pouvons-nous, au vue de ces données, améliorer le conditionnement d'une greffe de CSH et le risque de rechute de la maladie en associant une stratégie anti-angiogénique qui pourraient inhiber les CEP ?

La chimiothérapie métronomique apparait comme une stratégie anti-angiogénique intéressante puisque l'administration régulière de dose moins toxique de chimiothérapie sans intervalle libre pourrait non seulement prévenir la mobilisation des CEP et le « rebond vasculaire » mais pourrait agir aussi sur les cellules endothéliales différenciées au sein du réseau vasculaire tumoral (199-202). Dans un modèle préclinique associant un VDA, le OXi4503, avec de faibles doses de cyclophosphamide, Daenen *et al*. rapportent le blocage du pic (précoce) de CEP induit par le VDA et une augmentation de l'efficacité anti-tumorale du VDA lorsque celui-ci est administré en combinaison avec la chimiothérapie métronomique (91). Des stratégies par chimiothérapie métronomique sont activement évaluées dans des modèles précliniques et en clinique (201-203), et plus particulièrement dans les tumeurs pédiatriques (204-208). Un travail récent rapporte l'efficacité anti-tumorale d'une chimiothérapie métronomique par topotécan

associé à un agent anti-angiogénique dans des modèles murins de tumeurs cérébrales et que cette efficacité était corrélé à une diminution des taux de CEC et de CEP (209). Les stratégies anti-angiogéniques de chimiothérapies métronomiques chez l'adulte et chez l'enfant gagneront probablement à être analysées en parallèle des taux de CEP chez les patients.

L'utilisation à bon escient des traitements anti-angiogéniques soulèvent de nombreuses questions, en particulier au vue de leur efficacité modeste en terme de survie et les observations décevantes de résistance et de progression tumorale sous traitement, phénomènes qui semblent être dûs à la fois à des réponses émanant de la tumeur mais aussi de l'hôte (25, 78). Le bénéfice limité de ces traitements lorsqu'ils sont administrés en situation néo-adjuvante dans une maladie à stade précoce ou localisé laissent d'autant plus perplexe (210). Des données précliniques récentes suggèrent de plus que les traitements anti-angiogéniques pourraient être paradoxalement responsables d'une maladie plus invasive et infiltrante, en initiant de nombreux mécanismes compensatoires au niveau du microenvironnement tumoral et de l'hôte qui contribueraient non seulement à annihiler l'efficacité du traitement mais aussi à favoriser la dissémination métastatique (211, 212). Paez-ribes *et al*. ont observé dans un modèle murin une augmentation du nombre de métastases après un traitement inhibiteur de VEGF; ces résultats n'étaient observés qu'après une efficacité locale sur la tumeur primitive qui avaient permis d'obtenir une augmentation de la survie globale des animaux (212). Des données cliniques tendent à corroborer ces observations précliniques en témoignant de rechutes rapides ou de repousses tumorales focales ou disséminées accélérées à l'arrêt du traitement anti-angiogénique (213-215). Ainsi, malgré un effet bénéfique initial et global en terme de survie, les traitements ciblant l'angiogénèse tumorale pourraient induire des réponses systémique au niveau de l'hôte qui faciliteraient l'induction d'une maladie invasive et disséminé (25, 78).

Parmi ces réponses systémiques de l'hôte, l'induction de multiples cytokines, chemokines et facteurs de croissance pouvant favoriser la croissance tumorale pourraient être en partie responsables de la progression tumorale à court et à long terme. Si l'augmentation des taux plasmatiques circulants comme le VEGF et le PlGF, ainsi que la diminution du sVEGFR-2 sont aujourd'hui reconnues comme un effet de classe universel des inhibiteurs de tyrosine kinase observés chez les patients (189, 216) mais aussi des sujets sains (217) traités, des données précliniques et cliniques rapportent que les traitements anti-angiogéniques peuvent aussi induire des modifications de cytokines « collatérales » comme le G-CSF, le SDF-1α et l'ostéopontine (190), connus pour leurs effets sur la promotion de métastases. Des taux élevés de G-CSF et de

SDF-1α pourraient rapidement mobiliser des populations de progéniteurs pro-angiogéniques (119, 218), dont les CEP (27, 31, 76) qui pourrait accélérer les processus de réparation vasculaire et de repousse tumorale, et ainsi compromettre le bénéfice initial de ces traitements voire promouvoir l'invasion et la dissémination tumorale. En agissant de manière additive ou en synergie avec des facteurs produit localement par la tumeur, ces modifications systémiques de l'hôte pourraient compromettre le bénéfice initial des traitements anti-angiogéniques, voire accélérer la rechute ou la progression métastatique. Il apparaît dans ce contexte, que la mesure des modifications des cytokines en tant que biomarqueurs des traitements anti-angiogéniques est moins pertinente que le « monitoring » des cellules potentiellement réellement effectrices de la revascularisation tumorale et la rechute locale ou disséminée. L'identification de biomarqueurs permettant de mieux cibler, administrer et suivre ces traitements, et peut-être à fortiori de mieux détecter les réponses de l'hôte en cas de progression ou de rechute, est devenue une priorité absolue pour l'avenir des stratégies anti-angiogéniques. Dans ce contexte, le rôle des CEP dans l'angiogénèse tumorale et la résistance aux traitements anti-angiogéniques et anti-vasculaires mis en avant dans nos travaux soutiennent la pertinence de mesurer, suivre et cibler ces cellules dans ces stratégies thérapeutiques.

Nos données sur le rôle des CEC et des CEP en tant que biomarqueurs de l'angiogénèse et des traitements ciblant l'angiogénèse tumorale, pourraient dans ce contexte aider à apporter une meilleure visibilité sur la sélection des patients à même de bénéficier d'un traitement anti-angiogénique et offrir un rationnel mécanistique pour optimiser les schémas thérapeutiques actuels des traitements anti-angiogéniques, anti-vasculaires, voire des chimiothérapies standards et stratégies métronomiques ainsi que comprendre les mécanismes de résistance à ces traitements.

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ANNEXES

Articles présentés en Annexe :

« Supplemental Data » de l'Article 4 : ***Cancer Discovery 2012 May;2(5):434-49.***

Article 5 : ***Current Cancer Drug Targets. 2008 Feb;8(1):76-85. Review.***

Small molecule tyrosine kinase inhibitors: potential role in pediatric malignant solid tumors.

Rössler J, Geoerger B, **Taylor M**, Vassal G.

Article 6 : ***European Journal of Cancer. 2008 Aug;44(12):1645-56. Review.***

Angiogenesis as a target in neuroblastoma.

Rössler J, **Taylor M**, Geoerger B, Farace F, Lagodny J, Peschka-Süss R, Niemeyer CM, Vassal G.

Article 7 : ***Clinical Cancer Research. 2009 Nov 1;15(21):6740; author reply 6740-1.***

Quantification of circulating vascular endothelial growth factor receptor-3-positive lymphatic/vascular endothelial progenitor cells.

Farace F, **Taylor M**, Rössler J, Loriot Y, Besse B.

Article 8 : ***Expert Opinion Investigational Drugs. 2010 Jul;19(7):859-74. Review.***

New anti-angiogenic strategies in pediatric solid malignancies: agents and biomarkers of a near future.

Taylor M, Rössler J, Geoerger B, Vassal G, Farace F.

Supplementary Data for

Reversing resistance to vascular disrupting agents by blocking late mobilization of circulating endothelial progenitor cells

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Supplementary Data

Supplementary Data include Supplementary Methods, Supplementary References, one Supplementary Table, eight Supplementary Figures and four Supplementary Movies.

Supplementary Methods

Tumor and animal models

Human tumor cell lines included: prostate cancer (PC-3, IGR Cap-luc and MDA PCA; all kind gifts from Dr A. Chauchereau), lung carcinoma (A549 and H69; kindly provided by Dr E. Deutsch), neuroblastoma (IGR-N91, a kind gift from Dr J. Rössler), and renal clear-cell carcinoma (786-0; kindly provided by Dr E. Deutsch). Lewis Lung carcinoma (LLC) cells were kindly provided by Dr K. Benhioud. Cells were grown in cell cultures as per manufacturer's directions or as previously described (1-3, 6). No further testing or authentification was done by authors.

Xenografts were established by subcutaneous injection of 2-5x10^6 cells into the left flank of 6-8 week old female or male (for prostate tumor cell lines) Foxn1 immunodeficient athymic swiss nude nu/nu mice (Gustave Roussy Institute breeding). LLC cells (5x10^6) were subcutaneously implanted into immunocompetent C57Bl/6 mice or C57Bl/6 mice (Charles River, France) previously transplanted with GFP^+-tagged bone marrow (BM) cells from donor transgenic mice (C57Bl/6-Tg(ACTB-EGFP)1Osb/J) after lethal irradiation (950 rad).

Mice were randomized into balanced groups of at least 5 mice based on tumor volume such that each time-point to be analyzed was performed in a single group of

animals. Tumor volumes were assessed regularly with vernier calipers using the formula: V (mm^3) = $width^2$ (mm^2) x length (mm)/2. Animals of each group were culled at the indicated time-point for blood sampling and tumor harvesting. Blood samples were obtained by retro-orbital bleeding or cardiac puncture from anaesthetized mice and were collected in heparin tubes for CEC and CEP measurements and in plain dry tubes for serum protein measurements. Institutional guidelines were strictly followed for maintenance of animals and for end-point of tumor studies.

Drug administration and scheduling

Combretastatin-A4 3-O phosphate (CA-4-P) was supplied by Toroma Organics (Saarbruecken, Germany) and stored at 4°C. The formulation was reconstituted to achieve a concentration of 30 mg/mL in sodium chloride 0.9% and was administered as a single dose of 50 mg/kg or 100 mg/kg body weight as indicated via tail vein injection. Control groups received sodium chloride 0.9%. SU11248/sunitinib malate was supplied by Pfizer and stored at room temperature. The formulation was re-suspended in carboxy methylcellulose sodium (0.5% wt/vol), NaCl (1.8% wt/vol), Tween 80 (0.4% wt/vol), and isopropanol (0.9% wt/vol) to achieve a concentration of 12 mg/mL. Sunitinib was administered orally at 40 mg/kg (100µL) once a day, in different schedules. Control groups received methylcellulose at the same volume and schedule. DC101, a rat monoclonal antibody specific for mouse VEGFR-2/flk-1, was administered intra-peritoneal (i.p.) at a concentration of 800µg/mouse. Treatment was given as CA-4-P, sunitinib, or DC101, alone or in combination with administration of sunitinib or DC101 24 hours prior to CA-4-P injection or prior to the second CEP burst.

Evaluation of CEC and CEP levels by flow cytometry

Five-color flow cytometry assays were established to enumerate CECs and CEPs in mouse peripheral whole blood. Methodological characteristics included: -1) sampling of an important volume of blood (≥ 250 µl), 2) accumulation of a large number of events (at least 150,000 mononuclear cells per sample) to ensure statistical analysis, and 3) use of specific fluorescence minus-one (FMO) controls to measure accurately background noise. CECs were measured in whole blood after immunofluorescent staining with the following rat anti-mouse monoclonal antibodies: CD31-FITC, CD45-PerCP, Flk-1 (VEGFR2)-PE, MECA-32 biotin and streptavidin-APC (all from BD Pharmingen) (Supplementary Fig. S1). Antibody batches were titrated rigorously to determine optimal dosage. MECA-32 biotin and Flk-1-PE antibodies were titrated using blood samples presenting elevated levels of CECs obtained from colchicine-treated animals (data not shown). CEPs were measured in whole blood using the following monoclonal antibodies: CD45-PerCP, CD117-APC, Sca-1-FITC and Flk-1-PE (all from BD Pharmingen) (4-6). An isotypic control tube included fluorochrome-conjugated rat anti-mouse isotypic controls. For CEC and CEP identification strategies, fluorescence-minus-one (FMO) control tubes for each fluorochrome were performed in 250µL of whole blood to measure background noise and to adjust the gates precisely according to recommended procedures (7).

Blood samples were pre-incubated with Fc block (BD Biosciences) to avoid nonspecific binding of antibodies and then incubated with intended antibodies. Red cell lysis was done using BDPharmLyse solution (BD Biosciences). Samples were acquired

on a FACSCalibur (BD Biosciences) or a FACSCanto II (BD Biosciences) flow cytometer. Acquired data were analyzed with CELLQuest 3.2 software and BD FACSDiva software version (BD Biosciences). Non-viable cells/apoptotic debris and cell-doublets were excluded by analysis gates in forward and side scatter and by a gate selecting cells negative for nuclear staining with DAPI (4', 6'-diamidino-2-phenylindole). Bi-exponential representations were used for data analysis. To ensure statistical analysis, all of the cells contained in the FMO controls and test tubes were acquired to reach at least 150,000 mononuclear events for each tube. Additionally, white blood cell (WBC) counts were determined for each murine blood sample using a MS9-3 counter (Melet Schloesing, Osny, France). Calculations of absolute CEC and CEP counts were done using the formula: absolute CEC or CEP per µl of blood = CEC or CEP events counted by FACS x (lymphocytes+monocytes in WBC/lymphocytes+monocytes counted by FACS).

Complementary five- and six-color flow cytometry experiments were performed to further characterize cells identified as CEPs. For this, the antibodies included anti-mouse Gr-1-PE Cy7 (eBiosciences), CD11b-FITC and CD34-FITC (BD Pharmingen), CD14-PE (BD Pharmingen), CD45-PE Cy7 (eBiosciences), Sca-1-PE Cy7 (BD Pharmingen), CD2-, CD3-, CD4-, CD11b-, CD14-, CD19-, and CD20-PerCp Cy5.5 (all from BD Pharmingen), F4/80-PerCP Cy5.5 (eBiosciences), CD144 (VE-cadherin)-biotin (Biolegend) and streptavidin-FITC (BD Pharmingen). First, CEPs identified as $CD45^{-/dim}CD117^{+}Sca\text{-}1^{+}Flk\text{-}1^{+}$ cells were shown to express $CD34^{+}$ (n=10 mice) (supporting the progenitor phenotype) and VE-cadherin^{+} (n=10 mice) (supporting the endothelial phenotype) (Supplementary Fig. S2). Secondly, experiments were designed to validate

our strategy identifying CEPs within the $CD45^{-/dim}$ population instead of within lineage-negative (Lin^-) populations. For this, CEPs measured according to our strategy (n=10 mice) were compared to CEPs identified using a strategy with a lineage-"dump" channel (including CD2, CD3, CD4, CD11b, CD14, CD19, CD20 and F4/80) (n=10 mice). As shown in Supplementary Fig. S3, the numbers of CEPs identified as viable $CD45^{-/dim}CD117^+Sca\text{-}1^+Flk\text{-}1^+$ events were similar to the number of CEPs identified as viable $Lin^-CD117^+Sca\text{-}1^+Flk\text{-}1^+$ events. Finally, complementary experiments to validate our strategy showed that the viable $CD45^{-/dim}CD117^+$ population was negative for myeloid and monocytic markers CD11b, Gr-1 and CD14 (n=15 mice) (Supplementary Fig. S4).

Measurement of pro-angiogenic factor levels in serum and bone marrow samples

Murine serum levels of VEGF, matrix metallopeptidase 9 (MMP9), stromal-derived factor-1 (SDF-1) and granulocyte colony-stimulating factor (G-CSF) were determined using commercially available mouse ELISA kits (R&D Systems, Mineapolis, MN). Because manufacturers recommend using serum for both G-CSF and SDF-1 dosages, all protein dosages were performed in serum samples instead of plasma. Blood samples were drawn in dry tubes and centrifuged, after which serum was aliquoted, frozen and stored at -70°C until assayed. SDF-1 levels were also determined in the supernatant of BM samples. For this, femurs and tibias were taken immediately from animals sacrificed at indicated times, and the BM was directly flushed into 100 µl RPMI medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Gibco) using a syringe and 21-gauge needle. The BM cell suspension was centrifuged and the supernatant was immediately frozen and stored at -80°C until assayed. Optical density

values were considered significant if found to be at least twice as high as background noise.

Image acquisition and analysis

Digital images of whole histological tumor sections were acquired using a Zeiss Axiophot microscope (magnification 25X) and recorded using a Nikon SuperCoolscan 8000 ED slide scanner equipped with a FH-8G1 medical slide holder (Nikon, Champigny-sur-Marne, France). Quantifications of necrosis, microvessel density (CD34-stained endothelial cells) or CAIX-stained hypoxic tumor cell surfaces on histological slides were analyzed by an automatic procedure using PixCYT image analysis software (8). The resolution was 4,000 dots per inch with 1 pixel covering an area of 40 μm^2. Prior to computer analysis, regions of interest were drawn to select tumor tissue exclusively and to eliminate both non-tumor tissue and large stained artefact deposits, if any. Necrosis was determined on histological sections by an experienced pathologist (P.V.) according to morphological criteria (reduced cellular density, pale cytoplasm and pyknotic nuclei or disrupted cells with or without red blood cell infiltration). Necrosis was expressed as a % of the total tumor area. For microvessel density and CA-IX expression, necrotic areas were excluded to analyze only the viable part of the tumors. Microvessel density profiles are expressed as the surface fraction (%) of the tumor area while CA-IX levels are expressed as a % of the tumor area.

LLC tumor cryosections of 40-50 µm thickness obtained from GFP^+BM-transplanted syngeneic C57Bl/6 mice were imaged using a Leica TCS SPE confocal microscope (Leica, Germany) with a 20X dry objective lens. Fluorescence images were

collected through fluorescence filters specific for: DAPI (405 nm excitation); alexaFluor546 (532 nm excitation) for CD31 staining; alexafluor633 (635nm excitation) for CD41 staining; alexafluor647 (635 nm excitation) for CD11b and F4/80 staining; and GFP (488 nm excitation) for GFP^+-BM cells. LLC tumors were evaluated at day 3 and day 6 after treatment with VDA (n=4-5 per time point).

For the evaluation of GFP^+-BM cell in tumors and tumor blood vessel, Z-stacks of ten consecutive optical sections (2-2.5 μm thick) were captured at a magnification of 20X using the Leica confocal software, with identical acquisition patterns. The analysis of GFP^+-BM cell numbers in each Z-stack was performed using ImageJ software (http://rsbweb.nih.gov/nih-image/). To do this, we quantified "GFP^+-BM events", defined as GFP^+ pixels for which the intensity of the signal had been thresholded so as to eliminate autofluorescence or non specific signals from the sample. For each tumor, the density of GFP^+-BM events was calculated for the entire tumor section area and for the area around each blood vessel in the Z-stack presenting the most optimal visualisation of blood vessels ($CD31^+$ vessels). First, the tumor area was manually outlined on the chosen Z-stack using the DAPI-stained nuclei image. The total number of pixels for the tumor area and the total number of GFP^+ pixels within this area allowed to calculate the density of GFP^+-BM events per total tumor area, expressed as a percentage. Secondly, each $CD31^+$ blood vessel in the corresponding Z-stack was delineated by a rectangular region of interest, positioned so as to include the longest diameter of the blood vessel. The number of pixels corresponding to each blood vessel area and the number of GFP^+ pixels within this region of interest were counted. The ratio GFP^+ pixels/total pixels for each blood vessel region of interest provide a means of quantifying the density of GFP^+-

BM events detected in or around each blood vessel. Results were expressed as the mean density of GFP^+-BM events per blood vessel for each Z-stack. Finally, all measurements were performed in three consecutive Z-stacks for each tumor and expressed as a mean value; at least 4 tumors per group were included in the analysis.

Secondly, confocal microscopy experiments were performed on thick LLC tumor cryosections (40-50 μm) to evaluate the presence of cells expressing myeloid-cell marker CD11b, macrophage-cell marker F4/80, and platelet-marker CD41 among GFP-tagged BM-derived cells incorporating tumors and tumor blood vessels. Full Z-stacks of consecutive optical sections (0.8-1 μm step size) of entire tumor cryosections were captured using the Leica confocal software, with identical acquisition patterns at a pixel density of 1024x1024. Magnifications of 20X and 63X were used. This methodological approach provided an original means of accumulating information acquired at successive optical sections of the tumor, thereby increasing the probability of observing signal information regarding markers or cells as compared to the analysis of a single optical plane. Maximum intensity projections of full Z-stacks of confocal images were done using ImageJ software. Because the projection of Z-stacks compiles signal information from distinct optical sections, "co-staining" or co-localization of markers was rigorously determined on single optical sections for each tumor and systematically done before performing full Z-stack projections. Three-dimensional reconstructions of Z-stacks were also done using Imaris software (Bitplane, Zurich Switzerland). Animations of consecutive two-dimensional optical sections were performed using Imaris software where each movie frame corresponded to a single Z-stack of 0.8 μm. Representative movie frames were selected for certain figures. It is of note that a technical limitation

sometimes associated with immunofluorescence experiments performed on thick tumor sections (40µm thick) is that antibodies and markers may have more difficulty penetrating the tumor tissue, which can lead to a "weaker" staining. For these reasons, staining and subsequent signals may vary slightly, especially nuclear staining.

Bone marrow transplantation

GFP^+-BM cells (10^7) were isolated from femurs and tibias of mice (C57Bl/6-Tg(ACTB-EGFP)1Osb/J) and infused into the tail veins of lethally irradiated (950 rad) 6-8 week old C57Bl/6 mice. After 4 weeks, blood was collected by retro-orbital bleeding from recipient mice to evaluate BM transplantation efficiency using flow cytometry. Mice with ≥ 95% GFP^+-BM cells (identified as $GFP^+CD45^{dim}CD117^+$ events) were used as recipients of LLC cells ($5x10^6$).

Supplementary References

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Supplementary Table

Supplementary Table 1. GFP^+ bone marrow-derived cells are not non-specific monocytic infiltrates.

LLC tumors were established in GFP^+ bone marrow (BM)-transplanted C57Bl/6 mice. Mice were treated with CA-4-P, DC101, or sunitinib according to the design presented in Figure 5B and C. Three days after the 4 hour or 72 hour CEP peak, tumors were removed (at day 3 and day 6, respectively) and cryosections were prepared for the assessment of GFP^+-cell colonization, CD31-positive endothelial cells (representing blood vessels) and co-localization of $CD31^+$ and GFP^+ cells. Histological sections of LLC tumors were evaluated in parallel for necrosis. The amount of GFP^+BM-derived cells in CA-4-P-treated tumors analyzed at day 3 (n=5 tumors) and day 6 (n=5 tumors) was not correlated with the extent of necrosis, thus excluding that variations in GFP^+BM-derived cells in tumors resulted from unspecific GFP-expressing monocytic and macrophage cell infiltrates due to necrosis.

Supplementary Table 1.

Treatment groups	% of necrosis / total tumor area	GFP^+ BM-derived cell events / total tumor area
Untreated	2%	0.5%
CA-4-P alone		
- *Day 3*	15%	1.4%
- *Day 6*	15%	3.4%
CA-4-P + DC101		
- *Day 3*	45%	1.3%
- *Day 6*	52%	1.9%
CA-4-P + sunitinib		
- *Day 3*	40%	1%
- *Day 6*	65%	0.5%

Supplementary Figures

Supplementary Figure S1

Fig. S1. Strategy for five-color flow cytometric detection of CECs in mouse peripheral whole blood. CEC identification was based on the immunophenotypic co-expression of three markers CD31, Flk-1 (VEGFR2), and MECA-32, and on the absence of the pan-hematopoietic marker CD45. The vascular marker MECA-32 was used to assess the endothelial nature of the cells designated as CECs. **A,** large initial morphological gate to exclude red cell and non-viable/apoptotic debris (only 33% of acquired events are shown). **B** and **C,** cell-doublets and non-viable cells/apoptotic debris are gated-off to select viable (DAPI-negative) events. **D-G**, analysis of a test tube and the successive gates to select CECs characterized as viable $CD45^{-}CD31^{+}MECA\text{-}32^{+}Flk\text{-}1^{+}$ events. Bi-exponential representations are shown. **H-K,** analysis of the FMO controls (events gated in P3 are shown) using a gating strategy identical to that used for the test tube. The gates used to identify CEC in the test tube (**E-G**) are shown for FMO controls for FITC (**H**), streptavidin-APC (**J**) and PE (**I, K**).

Supplementary Figure S2

Fig. S2: Strategy for five- and six-color flow cytometric detection of CEPs expressing CD34 and VE-cadherin in mouse peripheral whole blood. CD45, stem/progenitor cell markers CD117 and Sca-1, as well as endothelial marker Flk-1 (VEGFR2) were used for CEP identification. CEPs identified as $CD45^{-/dim}Sca\text{-}1^{+}CD117^{+}Flk\text{-}1^{+}$ viable events were

further characterized for CD34 and VE-cadherin expression to further support the progenitor and endothelial phenotype.

Fig. S2A. Representative flow cytometry analysis of CEPs expressing CD34 in mouse peripheral whole blood. **A,** large initial morphological gate to exclude red cell and non-viable/apoptotic debris (only 50% of acquired events are shown). **B** and **C,** cell-doublets and non-viable cells/apoptotic debris are gated-off to select viable (DAPI-negative) events. **D-F**, Bi-exponential representations for the analysis of the successive gates used to identify CEPs as $CD45^{-/dim}CD117^{+}Sca\text{-}1^{+}Flk\text{-}1^{+}$ events (highlighted in violet in **F**). **G,** fraction of $CD45^{-/dim}CD117^{+}Sca\text{-}1^{+}Flk\text{-}1^{+}$ events expressing CD34 (highlighted in violet). **H-J**, $CD45^{-/dim}CD117^{+}$ events (gated in P4 and shown in red) were assessed for CD34 expression according to an identical gating strategy. Note that 65% of $CD45^{-/dim}CD117^{+}Sca\text{-}1^{+}Flk\text{-}1^{+}$ events (**F**) expressed $CD34^{+}$ (**I-J**) supporting a progenitor phenotype. **K-M,** analysis of the FMO controls for FITC (**K**) and PE (**L,M**) using a gating strategy identical to that used for the test tube (events gated in P4 are shown).

Fig. S2B. Representative six-color flow cytometry analysis of CEPs expressing VE-cadherin in mouse peripheral whole blood. The initial morphological gates and exclusion of non-viable cells/debris and doublets are the same as presented in Fig. S2A (A-C). **A-D**, Bi-exponential representations for the analysis of the successive gates used to identify CEPs as $CD45^{-/dim}CD117^{+}Sca\text{-}1^{+}Flk\text{-}1^{+}$ events (highlighted in red in **C**) expressing VE-cadherin (**D**). **E-G**, analysis of the FMO controls (events gated in P4 are shown) for PE Cy7 (**E**), PE (**F**) and FITC (**G**) using a gating strategy identical to that used for the test tube.

Supplementary Figure S3

Fig. S3: Representative five-color flow cytometry analysis of CEPs in mouse peripheral whole blood using a dump channel for Lineage markers. The strategy identifying CEPs as $CD45^{-/dim}Sca\text{-}1^{+}CD117^{+}Flk\text{-}1^{+}$ viable events was compared to a strategy that circumvented CD45 expression and detected CEPs within Lineage-negative populations. Lineage–marker expressing cells (including CD2, CD3, CD4, CD11b, CD14, CD19, CD20 and F4/80) were excluded using a "dump" channel (PerCP Cy5.5). The initial morphological gates and exclusion of non-viable cells/debris and doublets were the same as in Fig. S2A (A-C) (not shown). Bi-exponential representations are shown in **A-D**, for the analysis of the successive gates used to identify CEPs as $CD45^{-/dim}CD117^{+}Sca\text{-}1^{+}Flk\text{-}1^{+}$ events (highlighted in blue in **C**) and in **E-H**, for the analysis of $Lin^{-}Sca\text{-}1^{+}CD117^{+}Flk\text{-}1^{+}$ events (highlighted in blue in **G**). The numbers of CEPs detected by both strategies were comparable (**C** and **G**). Analyses of the FMO controls for PE (**D**), and FITC (**H**) (events gated in P4 are shown).

Supplementary Figure S4

Fig. S4: Representative five-color flow cytometry analysis of CEPs in mouse peripheral whole blood stained with myeloid and monocyte markers. Additional staining evidenced that cells designated as CEPs did not express myeloid markers Gr-1 and CD11b or monocyte marker CD14. The initial morphological gates and exclusion of non-viable cells/debris and doublets were the same as in Fig. S2A (A-C) (not shown). Bi-exponential representations are shown. **A-D**, $CD45^{-/dim}CD117^{+}$ events (red) were distinct from $Gr\text{-}1^{+}CD11b^{+}$ events (blue) (**B**), the latter being $CD45^{+}$. **C-D**, analyses of the FMO controls

for PE Cy7 (**C**) and FITC (**D**). **E**, $CD45^{-/dim}CD117^{+}$ events did not express Gr-1. **F-I**, $CD45^{-/dim}CD117^{+}$ events (red) did not express CD14 (**G**), nor CD11b (**H**) and were distinct from $CD14^{+}$ events (blue in **I**).

Supplementary Figure S5

Fig. S5: Assessment of CEC and CEP levels in untreated non-tumor bearing and tumor-bearing mice. **A-B,** six to ten week-old non-tumor-bearing nude mice (n=41) were evaluated for CEC and CEP levels. Orthotopic xenografts were established in six to ten week-old mice by subcutaneous injection of six different tumor cell lines ($5x10^6$) including prostate cancer (PC3 (n=53), IGR CAP (n=48), and MDA PCA 2b (n=20)), lung cancer (A549 (n=49) and H69 (n=14)) and neuroblastoma (N91 (n=15)). Measurements began when tumors achieved a volume of at least 300 mm^3. Blood was obtained by cardiac puncture from anaesthetized mice for CEC and CEP measurements in all groups. Mean levels of CECs (**A**) and CEP (**B**) are represented by a horizontal line. (A) The mean levels of CEC in tumor-bearing mice differed among groups and were significantly higher compared to the CEC levels in non tumor-bearing mice (mean CEC=0.22/µl vs. 0.11/µl respectively, p=0.0004). (B) The levels of CEP differed among groups and were significantly higher in tumor-bearing mice compared to the CEP levels measured in non tumor-bearing mice (mean CEP=0.23/µl vs. 0.11/µl respectively, p=0.008). Significance was set as $0.05>p>0.01$ (*), $0.01\geq p>0.001$ (**) or $p\leq 0.001$ (***).

Supplementary Figure S6

Fig. S6: Drug-dose response of CA-4-P on CEC and CEP levels. (**A-B**) CA-4-P was administered i.v. (50 mg/kg or 100mg/kg) to 6-8 week-old H69-xenografted mice when tumors acheived a volume of at least 300 mm^3. Blood was obtained by cardiac puncture from anaesthetized mice (n = 5 mice per time-point) at sequential time points after treatment ranging from 0-24 hours for CEC (**A**) and from 0-72 hours for CEP (**B**) measurements. Data are expressed as mean ± SD.

Supplementary Figure S7

Fig. S7: GFP^+ hematopoietic cell populations in peripheral blood. GFP^+ bone marrow-transplanted C57Bl/6 mice bearing LLC tumors were treated with CA-4-P alone or combined with DC101 or sunitinib (SU) according to the experimental design presented in Figure 5B and C. Peripheral blood was drawn at 4 and 72 hours after treatment with CA-4-P. Summary graphs of absolute values of GFP^+ lymphocytes, monocytes and neutrophils are presented. Compared to other GFP^+ hematopoietic cell populations, the mean absolute values of GFP^+ CEP values were very small and have been represented by horizontal red lines. DC101 and sunitinib following CA-4-P blocked the late burst of CA-4-P-induced CEPs in peripheral blood but did not alter the levels of other circulating GFP^+ hematopoietic cell populations measured at 4 hours and at 72 hours. This finding also implied that the reduced CEP levels at 72 hours in mice receiving combined treatment was not the indirect result of a general depletion of all GFP^+BM-derived hematopoietic cells due to the antiangiogenic drugs. Data are expressed as mean ± SD.

Supplementary Figure S8

Fig. S8: Confocal immunofluorescence analysis of LLC-tumors grown subcutaneously in GFP^+ bone marrow-transplanted C57Bl/6 mice and harvested 6 days after treatment with CA-4-P (100 mg/kg). Maximum intensity projections of full Z-stacks of confocal images obtained from 40-50µm thick tumor cryosections are shown individually for indicated antibodies or nuclear staining with DAPI and after merging (merge). **A**, LLC cryosections were stained with anti-CD31 (red), anti-CD11b (blue), DAPI (white) and analyzed for GFP (green) expression. Several perivascular $CD11b^+$ myeloid cells did not express GFP and none were found incorporated into the $CD31^+$ blood vessel. Note, small GFP^+ aggregates were observed within the lumen of the damaged $CD31^+$ blood vessel and were closely associated to $CD31^+$ blood vessels and $CD31^+$ aggregates (see Supplementary Movie M2). The small size and absence of nucleus associated with these GFP^+ or $CD31^+$ aggregates suggested that these were GFP^+ or $CD31^+$ platelets adhering to the luminal surface of the damaged vessel. Scale bar, 10 µm. **B**, LLC cryosections stained with anti-CD31 (red), anti-CD41 (blue), DAPI (white) and analyzed for GFP (green) expression. Small GFP^+ platelets were observed within the lumen of highly damaged $CD31^+$ blood vessel and were in close proximity to $CD41^+$ or $CD31^+$ platelets. Co-localization of GFP with CD41 or CD31 for platelet aggregates was not observed on individual optical sections. The "dotted" aspect of the GFP signal of platelets located within the vessel lumen was distinguished from the "dotted" GFP^+ signal of the perivascular GFP^+BM-derived cell pattern as the latter was associated with a distinct nucleus. Scale bar, 10 µm.

Supplementary Movies

Confocal microscopy immunofluorescence analysis was performed in LLC tumors grown subcutaneously in GFP^+ bone marrow-transplanted C57Bl/6 mice and harvested 6 days after treatment with CA-4-P (100 mg/kg). Cryosections were 40-50 μm thick. GFP^+BM-derived cells are shown in green.

Supplementary Movie M1

GFP^+BM-derived cells dynamically replacing the endothelial lining of damaged blood vessels are not macrophages. Immunohistochemistry staining for CD31 (red), F4/80 (blue) and DAPI (white) was performed. The animation shows 17 consecutive Z-stack optical sections (0.8 μm step size) of a cryosection. $F4/80^+$ macrophages were located perivascularly and not all expressed GFP. The movie shows a GFP^+BM-derived cell extending towards the vascular lumen to replace the endothelial lining of the damaged vessel (white arrow). GFP^+CD31^+ cells were also detected within the vascular lumen, co-localizing with CD31 and DAPI staining (blue arrows) on individual optical sections. Representative frames can be seen in Figure 6B.

Supplementary Movie M2

Myeloid cells are located perivascularly. Immunohistochemistry staining for CD31 (red), CD11b (blue) and DAPI (white) was performed. The movie shows a three-dimensional reconstruction of a full (40 μm) Z-stack of confocal images of a damaged tumor blood vessel. Representative images are shown in Supplementary Figure S8. $CD11b^+$ myeloid cells were located in the perivascular surrounding. Note that several $CD11b^+$ myeloid

cells did not express GFP and none incorporated the $CD31^+$ blood vessel. Several small GFP^+ aggregates were observed closely associated and intermingled with the $CD31^+$ damaged blood vessel wall and $CD31^+$ aggregates within the vessel lumen. While none co-stained with CD31, these GFP^+ aggregates were highly suggestive of GFP^+ platelets adhering to the luminal surface of the damaged vessel.

Supplementary Movie M3

GFP^+BM-derived cells converging to and replacing vascular lumen are not platelets. Immunohistochemistry staining for CD31 (red), CD41 (blue) and DAPI (white) was performed. The animation shows a full Z-stack of 36 consecutive optical sections (0.8 µm step size) from a representative cryosection. $CD41^+$ platelets were massively detected within the vessel lumen and were closely associated to $CD31^+$ damaged vessels but did not co-stain with CD31. The movie shows four GFP^+BM-derived cells converging towards damaged vessels (white arrows); the GFP^+BM-derived cell in the bottom center replaces the wall of the disrupted $CD31^+$ blood vessel wall. GFP^+BM-derived cells incorporated to tumor blood vessels had a nucleus and did not express CD41. Representative images are shown in Figure 6E.

Supplementary Movie M4

GFP^+BM-derived cells infiltrate damaged tumor vessels. Immunohistochemistry staining for CD31 (red), CD41 (blue) and DAPI (white) was performed. The animation shows 20 consecutive Z-stack confocal images (0.8 µm step size) of a disrupted $CD31^+$ blood vessel at high magnification. The movie shows a GFP^+BM-derived cell incorporating the

damaged vessel wall that does not express CD41 (white arrows). $CD41^+$ platelets were observed within the vessel lumen and in close association with the $CD31^+$ blood vessel wall. Representative images are shown in Figure 6F.

Figure S1

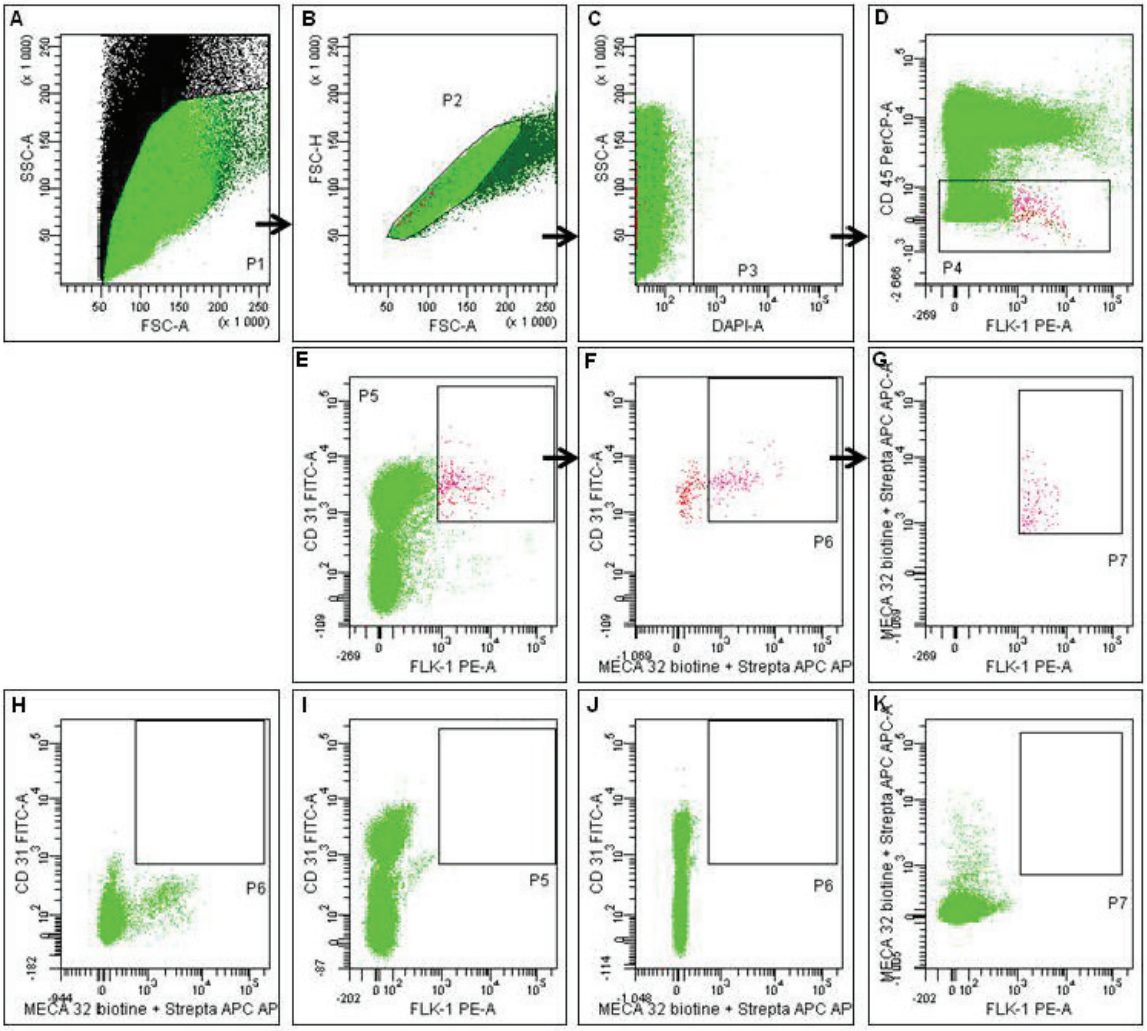
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Figure S2

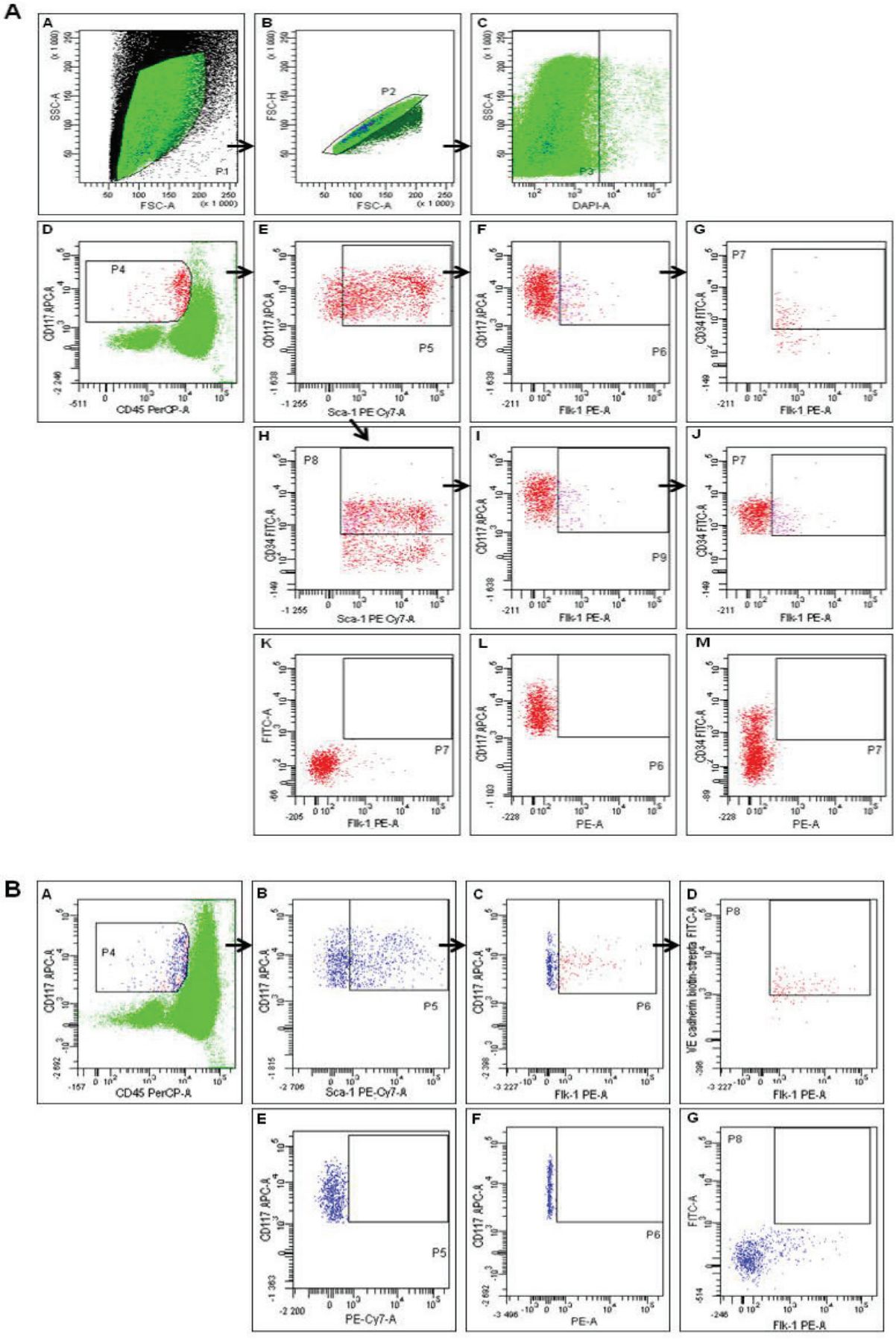
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Figure S3

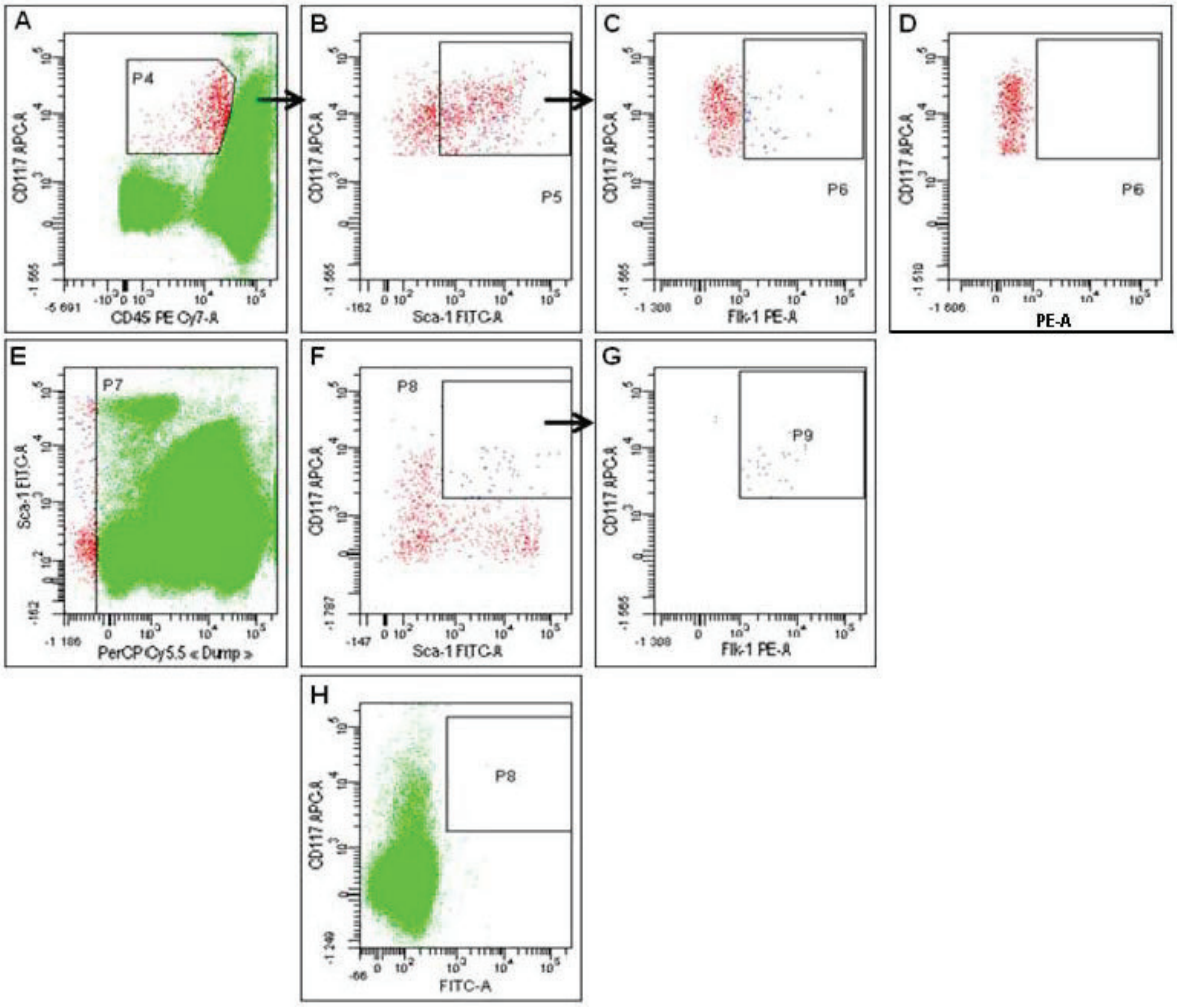
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Figure S4

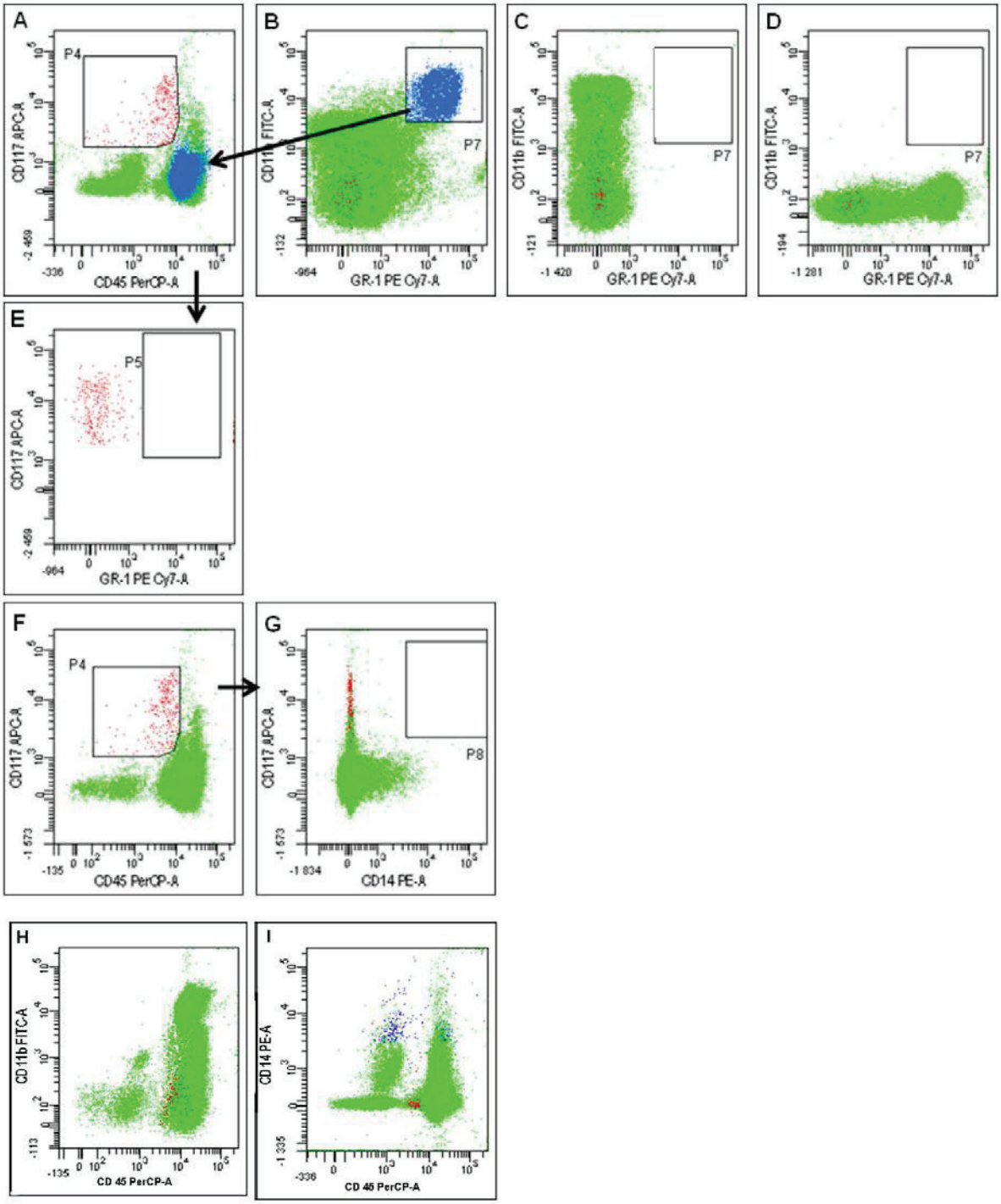
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Figure S5

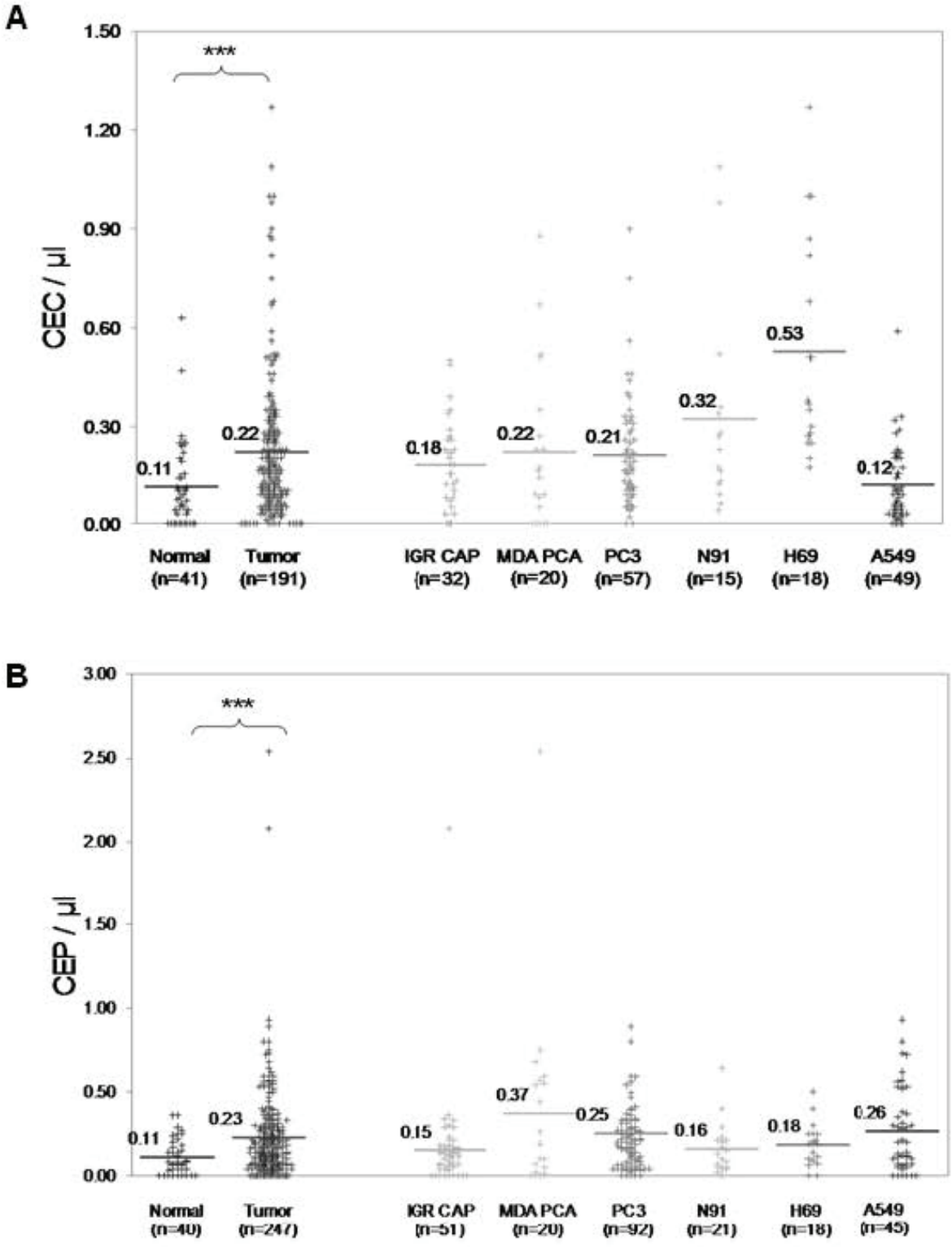
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Figure S6

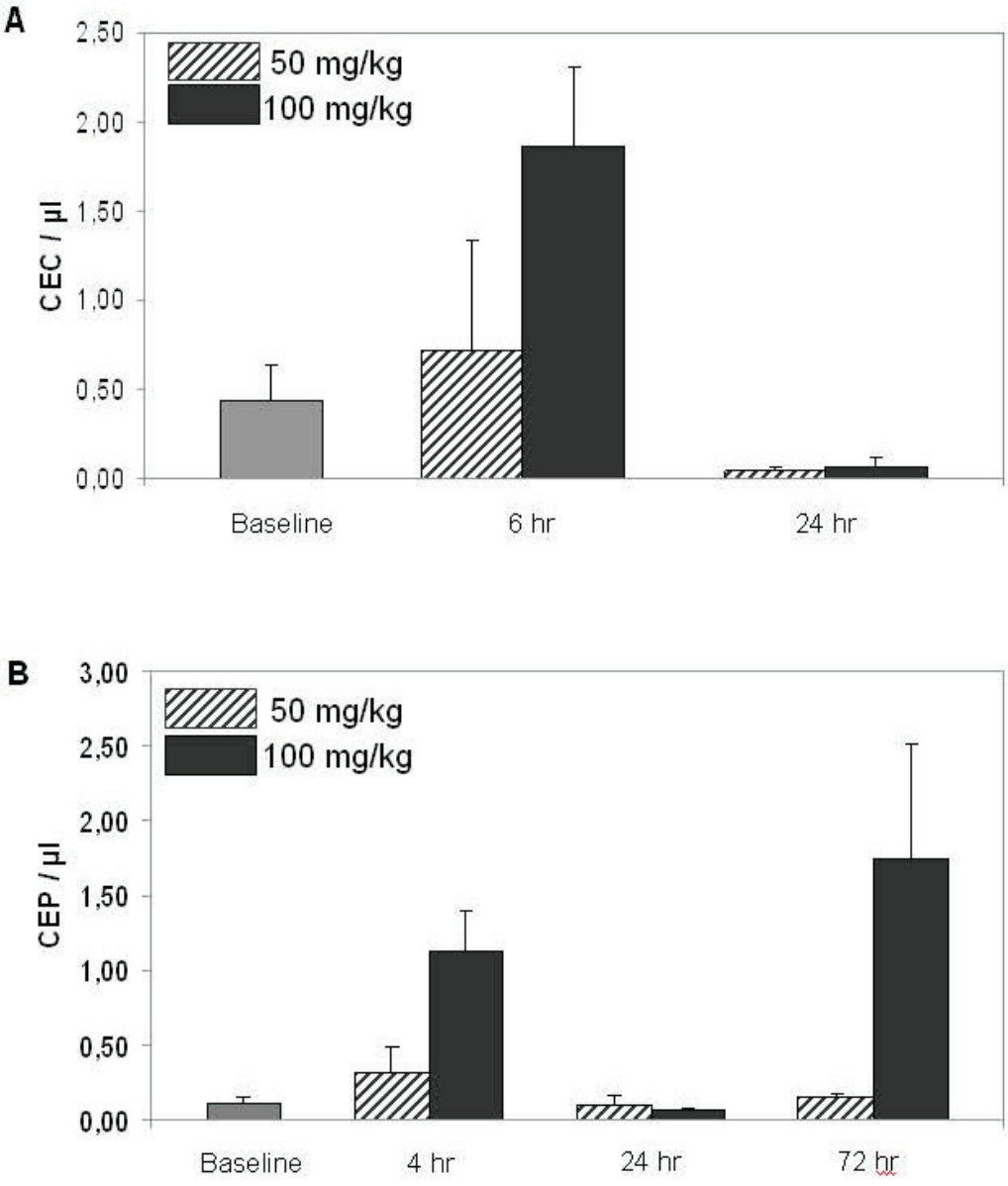
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Figure S7

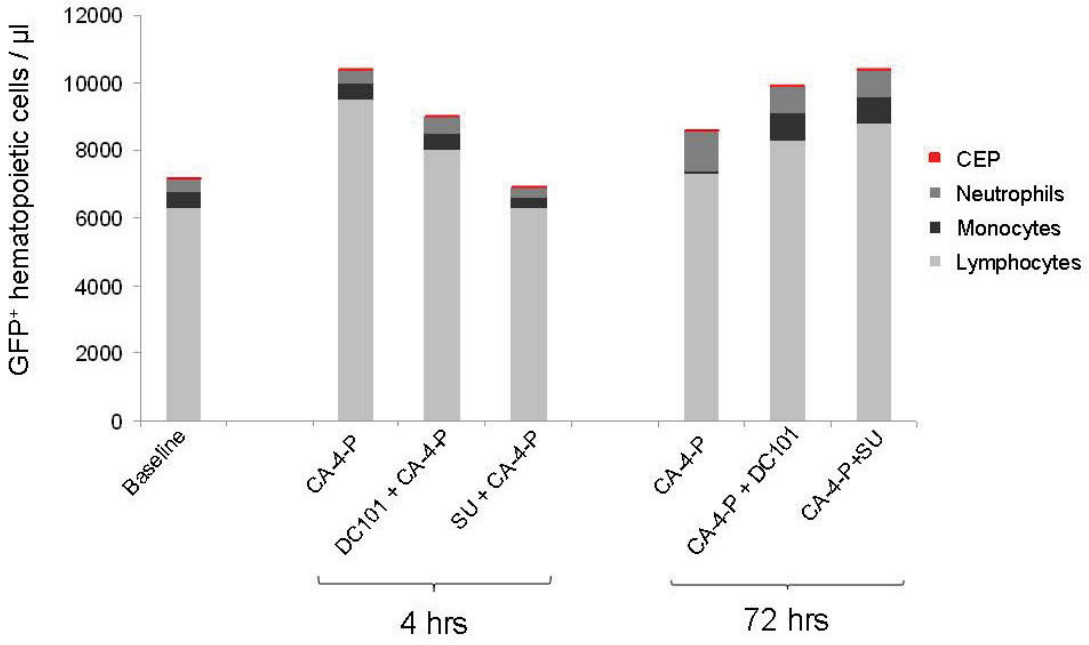
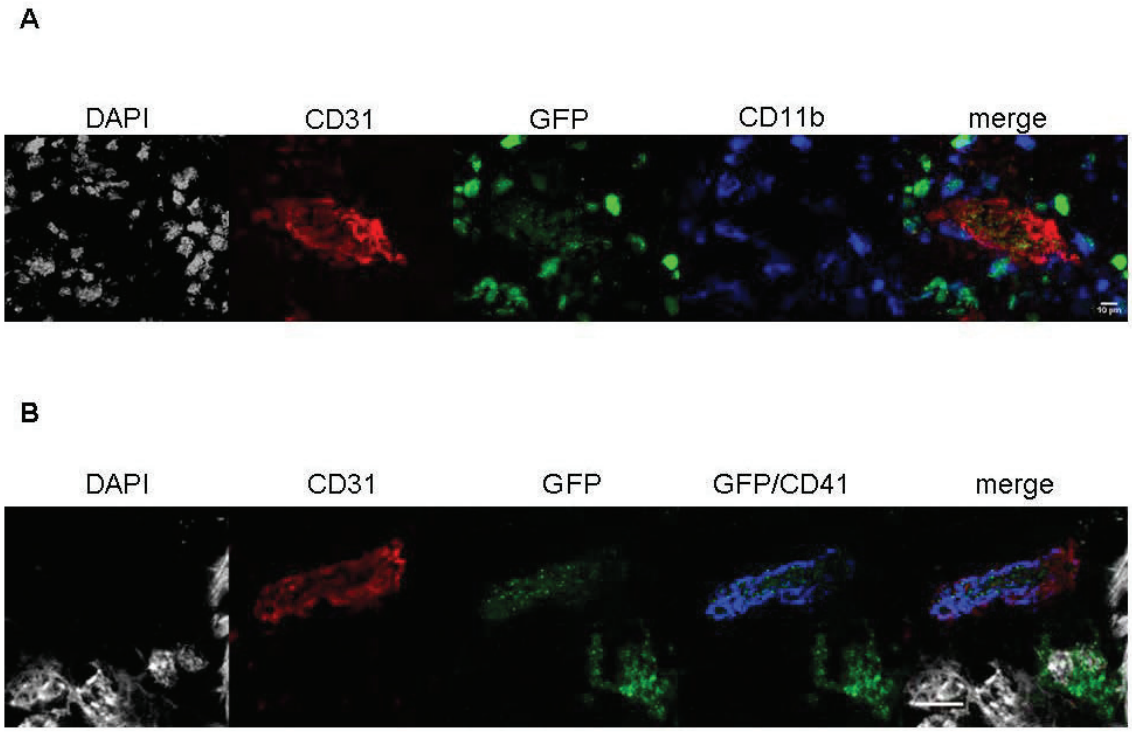


Figure S8



Small Molecule Tyrosine Kinase Inhibitors: Potential Role in Pediatric Malignant Solid Tumors

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Abstract: Tyrosine kinase receptors are expressed on the surface of tumor and/or endothelial cells and represent attractive targets for new anti-cancer treatment strategies. The so-called "small molecule" tyrosine kinase inhibitors have been designed to interact with the intracellular ATP binding site of these receptors, subsequently causing arrest of tumor cell proliferation, as well as induction of apoptosis and tumor migration. Furthermore, these molecules can impact on tumor angiogenesis. Tyrosine kinase inhibitors have been evaluated in several clinical trials for various adult malignant tumor entities and are currently being studied in pediatric solid malignancies. In this review, we will address the data available supporting the potential use of tyrosine kinase inhibitors in solid malignancies of childhood.

Keywords: Tyrosine kinase, tyrosine kinase inhibitors, imatinib, gefitinib, erlotinib, embryonic tumors, sarcomas, pediatric oncology.

INTRODUCTION

Pediatric Malignant Solid Tumors

In Europe, approximately 12,000 childrens are affected by cancer each year [1]. Childhood cancer remains the major cause of death from disease after the age of one year, representing 3,000 childhood deaths from cancer each year.

Pediatric oncology is mainly concerned with tumors of embryonic origin developing from undifferentiated cells of an organ or an organ field. Malignant transformation seems to take place during organ and tissue maturation. While leukemia is the most common childhood malignancy, brain tumors are the most common solid malignancy, representing 21% of all cancers in children, followed by neuroblastoma (8.3%), nephroblastoma (5.9%), bone tumors (4.6%) such as Osteosarcoma, Ewing's, and soft tissue sarcoma (3.7%)[1].

The overall survival rate in pediatric oncology has greatly improved over the past decades, reaching up to 75% because of successful treatment protocols for childhood leukaemia [2]. Enrollment into therapy optimizing protocols is possible for the majority of childhood cancer patients via national (for example GPOH, SFCE, CCLG etc.), and international organizations (SIOP, COG etc.). However, curing children, with metastasized or relapsed disease, remains a major challenge and survival is often achieved at a substantial cost such as organ toxicity, developmental abnormalities and secondary tumors. In view of these outcomes, new therapy options yielding increased efficacy and less toxicity must be evaluated and brought into clinical and translational research.

Tyrosine Kinases

Over the past several years, one of the major goals in cancer research has been to identify the intracellular molecules and related signal transduction pathways responsible for malignant transformation. Among the oncogenes identified, some have protein kinase activity. Aberrant protein kinase activity, caused by mutations or disrupted signaling, has been shown to play an important role in the pathogenesis of human cancer [3]. Therefore, drugs have been developed and specifically designed to inhibit such protein kinases and/or its causal abnormal activity.

More than six hundred protein kinases constitute the human kinome, a large and diverse family of homologous proteins that serve as important regulators of intracellular signal transduction. Protein kinases are enzymes that transfer phosphate from adenosine triphosphate (ATP) to serine, threonine and tyrosine residues on substrate proteins. Phosphorylation of these proteins leads to the activation of various signal-transduction pathways which play a critical role in a large range of fundamental cellular processes such as growth, metabolism, differentiation, adhesion and apoptosis.

Tyrosine kinases (TK) are divided according to their localization into nuclear, cytoplasmatic and membrane-bound kinases. TK may also act as cell surface receptors for growth factors [4]. These TK receptors share common characteristic features: an extracellular ligand-binding, a hydrophobic transmembrane domain and an intracellular domain containing an ATP-binding site responsible for the TK activity [5].

On the cell surface, membrane-bound kinases are present as monomers, but change their conformation and dimerize upon binding the ligand molecule [6]. After dimerization, the enzymatic domain binds ATP and activates the receptor. TK receptor activation leads to phosphorylation of downstream cytoplasmatic signaling proteins, with subsequent signal transduction through the cytoplasm to the nucleus that leads to the triggering of various cellular responses such as cell proliferation.

TYROSINE KINASE INHIBITORS

TK activity can be inhibited either by antibodies that interfere with the extracellular domain of the receptor (suffix –mab, for example cetuximab), or by "small molecules" that interact with the intracellular ATP binding site (suffix –nib, for example imatinib). Inhibition of both of these targets results in the arrest of cell proliferation and migration as well as the induction of apoptosis. TK receptors located on the endothelial cells of tumor vasculature can be targeted by tyrosine kinase inhibitors (TKI) as well. Also, several protein kinases have been shown to be deregulated and overexpressed in human cancers [4]. Consequently, TKI are interesting drugs with potential for direct and indirect inhibition of growth and metastasis of malignant tumors *in vivo*.

Designing molecules that show selective inhibition of specific TK has been one of the major goals in the development of TKI. Though current literature has been enthusiastic about drugs capable of

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[1] Annual report of the German Childhood Cancer Registry, 2004.

1568-0096/08 $55.00+.00

selective targeting, tumor specificity is not always achieved. Most TKI show a certain degree of cross-reactivity, thereby inhibiting more than one TK. Also, other mechanisms such as inhibition of angiogenesis, NK-cell activation, chemotherapy enhancement and radio sensitizing seem to play an important additional role as shown by several pre-clinical and clinical studies. This review will focus on small molecules that target receptors such as c-kit, PDGFRs, EGFRs and VEGFRs as these appear to be the most relevant and well-known candidates for TKI.

Imatinib mesylate (STI-571, Glivec®)

Today, the best known TKI is imatinib mesylate (STI-571, Glivec®). Imatinib was initially designed to inhibit PDGFR. However, it proved to be a specific inhibitor of the gene fusion product BCR-ABL, generated by translocation between chromosomes 9 and 22 (Philadelphia chromosome). By competing with ATP for its specific binding site, imatinib blocks the kinase domain of BCR-ABL and prevents the phosphorylation of downstream substrate proteins. Additionally, imatinib was found to inhibit ABL, ARG, mutated as well as wild type c-Kit, and finally PDGFR-alpha and beta [7-9]. In May 2001, the Food and Drug Administration (FDA) approved imatinib for the treatment of chronic myelogenous leukaemia (CML) and for Philadelphia chromosome positive leukemias expressing the BCR-ABL gene product, including pediatric patients. In February 2002, imatinib was registered for the treatment of gastrointestinal stromal tumors (GIST). The EMEA (European Medical Agency) has recently licensed imatinib for the same indications in Europe. Pre-clinical data suggests that imatinib has cytotoxic and cytostatic effects which effectively inhibits the growth of tumors dependent on abnormal activity of c-Kit, PDGFR-alpha and –beta. Such tumors include dermatofibro-sarcoma protuberans (DFSP) [10], chronic myelomonocytic leukemia [11], chronic myeloproliferative disorders [12, 13] and idiopathic hypereosinophilic syndrome [14, 15]. Furthermore, imatinib has also been reported to inhibit the growth and/or induce apoptosis in other malignancies that potentially express c-Kit/SCF or PDGFR/PDGF *via* autocrine growth loops such as small cell lung cancer [16], pancreatic cancer [17] biliary tract cancer [18] and several pediatric solid tumors (see next paragraph). Interestingly, certain case reports have observed clinical efficacy of imatinib in GISTs lacking typical receptor mutations. Also, dendritic cell (DC)-mediated NK cell activation has been shown to be triggered both *in vitro* and *in vivo* by treatment of DCs with imatinib [19]. Together, these observations suggest that tumors, which are refractory to the anti-proliferative effects of imatinib *in vitro,* may in some cases actually respond *in vivo* to imatinib in an NK cell-dependent manner.

Solid malignancies often exhibit high interstitial fluid pressure (IFP) which acts as a barrier for tumor *trans*-vascular transport and causes the poor uptake of anticancer drugs. While several mechanisms regulate IFP in tumors, activation of PDGFR appears to play an important role in elevating IFP. Also, PDGFR is expressed in various cell types within the tumor microenvironment. Treatment with imatinib has shown to decrease IFP in tumors [20-22], and concomitantly increases the uptake of chemotherapeutic drugs, thereby enhancing their efficacy.

Imatinib can reduce the expression and formation of nuclear foci of Rad51, a critical component of the DNA double-strand breaks repair pathway [23], and consequently sensitize tumors to γ-irradiation. Furthermore, by inhibiting PDGF and its stimulatory effect on smooth muscle cells [24] and pericytes [25], imatinib may have a potential impact on angiogenesis. Further evaluation of anti-angiogenic effects of imatinib is needed. Other TKI targeting PDGFR such as SU101 (Leflunomide, Arava®) or dasatinib (BMS-354825, Sprycel®), an inhibitor of SCR-family kinases, are undergoing pre-clinical and clinical investigation respectively and are summarized in Table **1**.

Gefitinib (ZD 1839, Iressa®) and erlotinib hydrochloride (Tarceva™)

The epidermal growth factor receptor (EGFR) is inhibited by two specific TKIs, gefitinib (ZD 1839, Iressa®) and erlotinib hydrochloride (Tarceva™). These TKI prevent auto-phosphorylation of EGFR/ErbB1 ("HER1") present on tumor cells, and subsequently inhibit the intracellular Ras/Raf/mitogen-activated protein kinase (MAPK) as well as PI3K cascades [26]. As a result, cell division and growth are disturbed. Over expression of EGFR and mutations resulting in constitutive activation of EGFR, have been identified in several tumor entities. In non-small cell lung cancer (NSCLC), breast, glioma, head and neck, gastric genitourinary, and colorectal carcinoma, overexpression of EGFR has been found [27], and most often due to mutations located in exons 19 and 21. The most important EGFR mutation found exclusively in malignant cells is EGFRvIII, (or Δ801EGFR, de2-7EGFR). Some of these mutations sensitize tumor cells to TKI directed against EGFR activity, especially in lung cancer [28-30].

In 2003, gefitinib was given conditional approval by the FDA for the treatment of patients with advanced and chemorefractory NSCLC. However, the negative results of the phase III gefitinib "Iressa Survival Evaluation in Advanced Lung Cancer" (ISEL) trial [31] led the FDA to restrict use of this drug to patients who were already receiving treatment. Although the overall result of the ISEL trial was negative, pre-planned subgroup analyses showed that gefitinib significantly improved the overall survival of non-smokers, women and patients of Asian origin. Also, in the National Cancer Institute of Canada's BR21 trial, erlotinib showed an overall survival benefit compared with placebo. Erlotinib now has full FDA approval for treatment of patients with NSCLC presenting disease progression after treatment with chemotherapy [32]. In December 2006, the EMEA also approved erlotinib for treatment of patients with locally advanced or metastatic NSCLC after failure of at least one prior chemotherapy regimen.

When administered alone, EGFR TKI exert cytotoxic effects on tumor cells. Secondly, anti-angiogenic activity of gefitinib and erlotinib has been found in several *in vivo* tumor models [33]. Thirdly, an additive effect was evaluated pre-clinically by combining gefitinib with radiation or chemotherapy [34-36]. The rationale behind the association of EGFR-TKI with other cytotoxic treatments was to increase cell cycle disruption by additional reduction of S phase and $G_{0/1}$ arrest, as well as the down-regulation of DNA damage repair proteins, respectively [37]. While gefitinib demonstrated an additive effect with radiation or chemotherapy in colon, head and neck and NSCL cancer models, clinical trials combining EGFR TKI with chemotherapy have not demonstrated synergistic or additive effects [38].

Studies on inhibition of EGFR signaling have led to the recent development of BMS-599626, a TKI directed against pan-HER receptors. This drug is a highly potent inhibitor of HER1 and HER2 kinases. By inhibiting HER1/HER2 receptor heterodimerization, BMS-599626 provides an additional mechanism to stop proliferation of tumors in which HER1/HER2 receptor co-expression and heterodimerization play a major role [39]. Other pan-HER inhibitors, such as Tykerb® (lapatinib, GW-572016) and canertinib (CI-1033) are currently under clinical investigation (see Table **1**).

Vatalanib (PTK787/ZK 222584), Sunitinib (SU11248), Sorafenib (BAY43-9006)

The vascular endothelial growth factor (VEGF) family includes six secreted glycoproteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placenta-derived growth factor (PlGF)-1 and PlGF-2 [40]. Its biological effects occur by binding to three different TK receptors: VEGFR-1, VEGFR-2 and VEGFR-3, all of which are present on endothelial cells. Recent studies have reported the expression of these receptors on hematopoietic cell lineages or tumor cells as well [41].

Table 1. Mechanism of Action of TKI and Current Clinical Development for Solid Tumors

TKI	Target TK	Clinical Evidence FDA / EMEA Approval
Imatinib (STI-571, Glivec®)	c-Kit, PDGFR-alpha, PDGFR-beta, c-Abl	First-line treatment of CML BCR-ABL pos.pediatric leukemia GIST Unresectable, recurrent and/or metastatic DFSP that are unresectable
Gefitinib (Iressa®)	EGFR	Only for cancer patients who have already taken the medicine and whose doctor believes it is helping them
Erlotinib (OSI-774, Tarceva™)	EGFR	Locally advanced or metastatic non-small cell lung cancer after failure of at least one prior chemotherapy regimen, Pancreatic cancer in combination with gemcitabine
Sunitinib (SU11248, Sutent®)	PDGFR, VEGFR, Kit, Flt-3	First-line treatment of advanced and/or metastatic renal cell carcinoma GIST after disease progression on or intolerance to imatinib
Sorafenib (BAY 43-9006, Nexavar®)	VEGFR, PDGFR, c-Raf-1, B-Raf	Advanced renal cell carcinoma
Lapatinib (GW-572016, Tykerb®)	EGFR, Her-2 (EGFR-2)	In combination with capecitabine in advanced or metastatic breast cancer overexpressing HER-2 and patients who have received prior therapy including an anthracycline, a taxane and trastuzumab
Vatalanib (PTK787/ZK 222584)	VEGFR, PDGFR, c-kit	*Phase II/III*
Leflunomide (SU101, Arava®)	PDGFR (EGFR, FGFR)	*Phase II/III*
Dasatinib (BMS-354825, Sprycel®)	BCR-ABL, c-KIT, EPHA2, PDGFR-beta	*Phase II/III*
Canertinib (CI-1033)	EGFR, HER-2 (EGFR-2), EGFR-3 (HER-3), EGFR-4 (HER-4)	*Phase I/II*

TKI for VEGFR were originally designed as inhibitors for angiogenesis and target endothelial cell proliferation, migration, tubule formation and block lymphangiogenesis [42].

Vatalanib (PTK787/ZK 222584) is a potent inhibitor of all three VEGFRs. In addition, it may inhibit PDGFR-beta and c-Kit, but at much higher concentrations [43]. Vatalanib inhibits tumor growth and reduces microvasculature as demonstrated by dynamic enhanced magnetic resonance imaging in mouse models [44]. Sunitinib (SU11248) is a new multi-targeted TKI showing both anti-proliferative and anti-angiogenic effects as a result of its inhibiting profile directed against VEGFRs, PDGFR-beta and c-Kit [45]. Several studies using sunitinib in animal models demonstrate successful therapeutic *in vivo* effects [46]. Sunitinib was registered by the FDA and the EMEA for treatment of GIST resistant to imatinib, as well as for the treatment of advanced or metastatic renal cancer after failure of Interferon alpha or Interleukin 2 therapy. The third TKI targeting VEGFR is Sorafinib (BAY 43-9006) and it is currently being evaluated in phase III studies in adult solid tumors. Initially, this bi-aryl urea was developed as a specific inhibitor of the intracellular kinase raf, however, subsequent studies revealed that this compound could also inhibit several other TK involved in tumor progression, including VEGFRs [47]. *In vitro* and *in vivo* experiments have demonstrated anti-tumor efficacy in human xenograft models [48]. Sorafinib has been approved by the FDA in advanced kidney cancer.

Simultaneous inhibition of several TK targets may be necessary to improve the anti-tumor properties of these new compounds and increase their efficacy in patients. Several multi-targeted compounds and other inhibitors of VEGFR are being assessed and undergoing pre-clinical evaluation in adult tumors (see Table **1**). These TKIs include: ZD6474 (Zactima), targeting VEGFR-2, EGFR, FGFR-1 and RET; SU6668, targeting VEGFR-2, PDGFR-beta, FGFR-1, c-kit; AG-013736, targeting VEGFR-1, -2, -3, PDGFR-beta, c-kit; AZD2171, targeting VEGFR-1, -2, -3, PDGFR-beta, c-Kit and finally AEE788, targeting VEGFR-1, -2, EGFR (reviewed in [49]).

PRE-CLINICAL AND CLINICAL STUDIES ON TKI IN PEDIATRIC SOLID TUMORS

Although TK expression in pediatric solid malignancies has been widely evaluated *in vitro*, few studies have analyzed TKI activity *in vivo* in pediatric tumor models. Also, the few studies that do concern pediatric tumors are unfortunately incomplete. For instance, while adult and childhood glioblastoma multiforme (GBM) differ in their tumor biology, certain *in vivo* studies do not specify the tumor origin of the GBM. The pre-clinical studies (*in vitro* and *in vivo*) evaluating TKI directed against c-Kit, PDGFR (imatinib) and EGFR (gefitinib, erlotinib) in different pediatric tumor entities are summarized in Table **2**. Despite different reports on VEGF signaling in solid malignancies of childhood, clinical studies evaluating VEGFR TKIs (vatalanib, sunitinib, sorafinib, etc.) have not been launched until now. Recently, the antibody directed against VEGF, bevacizumab (Avastin©), has demonstrated very promising results in support of anti-angiogenic treatment in pediatric solid tumors[2].

Our intention is to give an overview of current pre-clinical and clinical studies of TKIs in solid pediatric tumors. Data on pediatric solid tumor animal models are presented in Table **2**, while Table **3** summarizes available results from published as well as ongoing phase I and II trials with TKIs.

Imatinib mesylate (STI-571, Glivec®)

Preclinical Studies

Medulloblastoma (MB) is a highly invasive primitive neuroectodermal tumor of the cerebellum. It is the most common malignant tumor of the central nervous system in children. Before an eventual clinical trial with imatinib for MB patients, evaluation of c-Kit expression and mutational status was performed [50]. Expression of c-Kit was identified by reverse transcriptase-polymerase chain reaction in all 10 MB tumor samples and by immunohistochemical

[2] J. L. Glade Bender, P. C. Adamson, S. Baruchel, Y. Shaked, H. X. Chen, J. M. Reid, A. M. Ingle, S. M. Blaney, J. J. Kandel, D. J. Yamashiro, ASCO Annual Meeting Proceedings **2006**.

analysis in 9 of the 10 samples. In addition, tumor samples were screened for mutations in exons 9, 11, and 13 of the c-Kit gene by direct sequencing. No sequence abnormalities were detected in these exons, suggesting that c-Kit activation in MB was not dependent on a mutational status. Interestingly, up-regulated expression of PDGFR and of members of the downstream RAS/MAPK signal transduction pathway were found in MB patients presenting with metastasis [51, 52].

Glioblastoma multiforme (GBM) is a very rare high-grade glioma of childhood. Currently, there is no curative strategy with standard methods such as surgery, radiation and chemotherapy. Preliminary studies indicate that PDGFR signaling contributes to the growth of GBM. In addition, genetic studies have demonstrated the existence of distinct biological subsets. Specifically, in one study evaluating sensitivity to imatinib in 15 human high-grade glioma primary cultures, six out of 15 tumor cultures displayed more than 40% growth inhibition, whereas the seven other cultures showed less than 20% growth inhibition [53]. Sensitivity to imatinib was found to correlate with PDGFR status. Furthermore, expression of the chemokine CXCL12/SDF-1 (stromal cell-derived factor 1) was found to predict sensitivity to imatinib, as identified by supervised analyses of gene expression profiles and real-time PCR. For GBM cell line models presenting reduced sensitivity to PDGFR inhibitors, Kilic *et al.* has described a mechanism involving activation of the ras pathway to explain reduced drug efficacy [54].

Recent studies have shown that imatinib enhances the cytotoxicity of ionizing radiation in both GBM cell lines and a mouse model [55, 56]. This phenomenon could be due to interference with p44/42 MAPK signaling. Combining imatinib with irradiation led to significantly enhanced imatinib-induced apoptosis and caspase 3 cleavage in GBM cells. When administered to tumor-bearing mice in conjunction with daily irradiation, GBM response to imatinib therapy correlated with a greater delay in tumor growth delay and increased survival. Imatinib is known to pass the blood-brain barrier resulting in decreased phospho-PDGFR in GBM. This important pharmacological aspect and pre-clinical data strongly suggest that imatinib could increase GBM response to radiotherapy and thereby improve survival.

Table 2. Pre-Clinical Data on TKI in Pediatric Solid Tumors

TKI	Solid tumor	Experiments performed	Ref.
Imatinib	Neuroblastoma (NB) *in vitro*	Response of NB cell lines irrespective of mRNA status or cell surface expression of c-Kit, PDGFR-alpha and –beta Co-incubation with 9-*cis* RA and γ-irradiation sensitizes NB cell lines to imatinib Pre-treatment with 9-*cis* RA results in resistance of NB cell lines to imatinib	[62]
	in vitro	4 c-Kit-positive NB cell lines sensitive (growth inhibition 30 – 80%) c-Kit-negative NB cell line GI-CA-N was slightly affected, suggesting other imatinib targets operate in regulating NB proliferation Sharp decrease of c-Kit phosphorylation could be demonstrated	[58]
	in vitro/in vivo	Growth inhibition of NB cells *in vitro* and in a NB xenograft model in SCID mice Suppression of PDGFR and c-Kit phosphorylation Inhibition of angiogenesis by down-regulation of VEGF expression	[57]
	in vitro/in vivo	Imatinib tumor concentration in NB tumors independent of administered dose and no correlation with antitumor effect No clear-cut correlation between the levels of expression for imatinib-responsive targets and *in vitro/in vivo* sensitivity	[63]
	Ewing Sarcoma (EWS) *in vitro*	8 EWS cells resistant *in vitro* despite expression of imatinib sensitive tyrosine kinases	[66]
	in vitro	Down-regulation of c-kit phosphorylation and dose response inhibition of EWS cell proliferation (IC_{50}: 12-15 micro M) No induction of apoptosis by 10 μM Increased antitumor effect of chemotherapy by inhibiting the proliferative rate of EWS cells by 15-20% in doxorubicine and by 15-36% in vincristine, and increased apoptotic rate by 15 and 30% when exposed to the same drugs	[67]
	in vitro/in vivo	Interference with growth of all EWS cell lines tested *in vitro* and *in vivo* c-kit expression and phosphorylation in EWS cells Oral administration (every 12 hours for 5-7 days) on primary tumor growth in EWS xenografts in SCID/bg mice	[68]
	Neuroectodermal tumors (EWS, PNET and NB) *in vitro/in vivo*	Decrease in cell proliferation and increase of cell apoptosis in a concentration- and time-dependent manner Inhibition to some extent independent of c-Kit inhibition since cells remained sensitive to SCF stimulation Tumor volume significantly reduced in mice Apoptosis independent on caspase activation Accumulation of reactive oxygen species resulting in cell death	[61]
Gefitinib	Neuroblastoma (NB) *in vitro*	40% to 50% reduction in the number of SY5Y and NLF cells after exposure to 0.5 μM gefitinib Inhibition of auto-phosphorylation of EGFR by EGF in NB cell lines at 0.01 μM Blocked phosphorylation of AKT, but not MAPK, in NLF cells at 0.1 μM	[78]
	Rhabdoid tumor *in vitro/in vivo*	Inhibition of EGFR-phosphorylation and cell growth Induction of apoptosis at high concentrations *in vitro* Cytostatic effect on established xenografts of radiotherapy	[80]
	Juvenile pilocytic astrocytoma (JPA) *in vitro*	Inhibition of proliferation in five JPA short-term primary cell-cultures (IC-50:1.6 – 9.6μM) EGFR protein and mRNA expression undetectable	[81]

The effect of imatinib on *neuroblastoma (NB)* has been reported in several *in vitro* and *in vivo* studies. By inhibiting phosphorylation of c-Kit and PDGFR, imatinib exerts a cytotoxic effect and induces apoptosis via PARP cleavage in NB cell lines [57, 58]. However, for imatinib to have a significant inhibitory effect on NB *in vitro*, much higher concentrations are required compared to those described for CML (IC_{50} ranging from 0.375 to 1.8μM (median 0.6μM)) [59]. Beppu *et al.* treated seven NB cell lines with imatinib for four days and showed that the IC_{50} ranged from 9 to 13μM [57] and it was higher than the plasma concentrations achieved in early clinical trials for CML (4.6μM) with a dose regimen of 400 mg daily [60]. In another report, only partial inhibition of proliferation was observed in four NB cell lines treated with a higher concentration of imatinib (10μM). Furthermore, prolonging treatment with imatinib had no impact on NB proliferation [58]. Another study, however, showed that the proliferation of five NB cell lines was significantly inhibited after exposure to 5 and 10μM imatinib for 72 hours and seven days, respectively [61]. Also, continuous exposure to only 2.5μM imatinib for one week resulted in almost complete inhibition of NB cell clonogenic survival of five NB cell lines[3]. Finally, we conducted a study confirming that the inhibitory effect of imatinib in NB is not dependent on the expression of the targeted TK [62].

Imatinib has also been tested in combination with retinoic acid (RA), a differentiating agent used to treat NB. Vitali *et al.* examined possible synergistic effects between imatinib and all-trans RA (ATRA) in five NB cell lines. The inhibitory effect of 10μM imatinib was not increased when combined with ATRA in concentrations ranging from 0.1 to 5μM, as compared to that of imatinib alone [58]. However, we observed synergistic inhibitory effects of imatinib in combination with 9-*cis* RA in RA sensitive NB cell lines [62]. We also demonstrated a synergistic effect of imatinib in combination with γ-irradiation in the same NB cell lines.

Two studies report the *in vivo* effect of imatinib in NB mouse models [57, 63]. The first study analyzed treatment with imatinib at 50 mg/kg and 100 mg/kg administered orally every 12 hours for 14 days [57]. With this treatment schedule, tumors were significantly smaller than those of control mice treated with vehicle. In the second study, mice were treated with doses of 100, 200, and 400 mg/kg imatinib twice a day for five days for two consecutive weeks [63]. In both studies, the *in vitro* and *in vivo* sensitivities to imatinib were not correlated with the levels of expression of the imatinib-responsive targets. These pre-clinical studies further suggest that the anti-tumor activity of imatinib in NB may involve inhibition of other TK and/or pathways, than those originally targeted.

Osteosarcoma (OS), an aggressive primary bone cancer, is characterized by a high level of expression of PDGF and its receptor [64]. Using three OS cell lines expressing PDGF and PDGF receptors 1 and 2, McGary *et al.* demonstrated both the mitogenic effect PDGF-BB and the capacity of imatinib to inhibit PDGF-mediated proliferation *in vitro* and induce apoptosis. However, imatinib did not show efficacy in an orthotopic *in vivo* model of OS with the v-*KIT-RAS* oncogene transformed KRIB cell line.

In *Ewing Sarcoma (EWS)*, the second most important pediatric bone tumor, amplification of the PDGF or PDGFR genes has rarely been described. However, over expression of PDGF-C and PDGF-A has recently been implicated in EWS [65]. Virtually all EWS and peripheral primitive neuroectodermal tumors (pPNET) have aberrant transcription factors, produced by the fusion of the amino-terminal *EWS* with the DNA binding moiety of an *ETS* transcription factor (FLI-1) in 90% of cases. The resulting hybrid transcription factor, EWS/FLI-1, drives unregulated expression of the *PDGF-C* gene, suggesting that PDGF-C may be a significant mediator of EWS/FLI-1 driven oncogenesis. Consequently, disruption of the PDGF-C signaling could be of therapeutic value. A recent *in vitro* study attempted to demonstrate this hypothesis but showed that 8 EWS cell lines were resistant to imatinib despite TK expression [66].

Conflicting data, however, has been published about EWS response to imatinib. Another study reported dose response inhibition of EWS cell proliferation with down-regulation of c-Kit phosphorylation, observed with imatinib IC_{50} concentrations of 12-15μM [67]. Imatinib did not induce a significant increase in apoptosis when administered alone at 10μM. However, the *in vitro* antitumor effect of 10μM imatinib was increased when combined with doxorubicin and vincristine, drugs commonly used in EWS treatment. Also, Marchent *et al.* observed imatinib-inhibited growth and -mediated apoptosis in EWS cell lines with an IC_{50} of 10-12μM. They also found SCF-mediated c-Kit phosphorylation with an IC_{50} of 0.1-0.5μM [68]. Finally, regression or control of primary EWS tumors was achieved in an orthotopic xenograft model after six days of treatment with 50 and 100 mg/kg of imatinib.

Clinical Studies

Imatinib has been evaluated in clinical trials for children with GIST, a very rare tumor for which clinical behavior is notoriously difficult to predict. A recent review of childhood cases of GISTs suggested a more favorable prognosis and outcome compared to adult cases [69]. As no c-*KIT* or *PDGFR* mutations have been observed to this day in childhood GISTs, it appears that their pathogenesis differs from that of adult GISTs [70]. Interestingly, several case reports testify of the successful use of imatinib in pediatric GIST [71].

Beyond these isolated cases of childhood GIST, two phase II studies have evaluated treatment with imatinib in pediatric solid tumors. The first study was performed by the Children's Oncology Group (COG) and evaluated treatment with imatinib at 440 mg/m^2/day. Only one partial response (PR) in a EWS patient (among 24 EWS) was observed, while all other tumors (10 OS, 10 NB, 1 GIST, 10 DSRCT, and 4 STS) showed no response [72]. The second phase II study was conducted by the European Consortium on Innovative Therapy for Children with Cancer (ITCC), and its results were presented at the ASCO 2006 annual meeting (see Table **3**). One of the inclusion criteria for this trial was tumor expression of at least one imatinib targets, namely c-Kit, PDGFR-A or PDGFR-B. Despite this occurance, no CR or PR was observed. However, five patients (2 fibromatosis, 1 GIST, 1 MB, 1 pseudo-inflammatory tumor) experienced durable stable disease (SD) and were consequently treated for more than twelve months. SD was also achieved in two patients (a brain stem glioma and a renal carcinoma) for ten and seven months respectively. Imatinib toxicity in pediatric patients was similar to that observed in adult patients, i.e. known gastrointestinal disturbances and mild peripheral oedema.

Gefitinib (ZD 1839, Iressa®) and erlotinib (OSI-774, Tarceva™)

Preclinical Studies

The target receptors of gefitinib and erlotinib have been analyzed in both MB and GBM. An immunohistochemical study was performed on 22 MB tumor samples and demonstrated the presence of EGFR-2, (ErbB2, HER-2) and c-EGFR-4 (HER-4) in 10 to 50% of the neoplastic cells of high-grade glial tumors with high immunoreactivity, while EGFR-3 (HER-3) was present in less than 10% of the neoplastically-transformed cells. However, EGFR (HER-1) was not detected suggesting that this receptor does not play a key role in MB [73]. A summary of preclinical data on EGFR inhibitors is described in Table **2**.

In contrast to the extensive studies of EGFR status performed in adult GBM, few reports describe EGFR expression, amplification or prognostic relevance in childhood GBM. One study analyzed EGFR status in a series of 28 pediatric *brain stem gliomas* (BSG) using quantitative PCR and immunohistochemistry [74]. The authors found that EGFR signaling was important for the development of childhood

[3] Palmberg, E., Lindskog, M., Johnsen, J. I., Eriksson, B., Kogner, P., Advances in Neuroblastoma Research Meeting 2004.

BSG and should be further studied as a potential therapeutic target. Also, we have observed both grade-dependent amplification and overexpression of EGFR in childhood BSG, further suggesting complex underlying genetics in this disease (our unpublished results). Another study retrospectively examined EGFR expression and gene amplification in a series of 27 archival pediatric high-grade non-brainstem gliomas by means of immunohistochemistry and competitive PCR [75]. Although elevated immunoreactivity for EGFR was observed in 80% of tumors, only two of the 27 gliomas examined had gene amplification. No difference in outcome was observed between tumors exhibiting extensive EGFR immunoreactivity and those that did not.

EGFR may be over-expressed, but it may be constitutively activated as a result of mutations which consequently sensitize tumor cells to TKI directed against EGFR. It is not known whether EGFR mutations (such as those correlating with responsiveness to EGFR inhibiton in NSCLC) also occur in children. Recently, we have analyzed NB cell lines for mutations in the EGFR gene and we did not observe any of the known mutations in exons 12 and 21 (our unpublished results). EGFR expression has been studied in other pediatric tumors. For instance, cell surface expression of EGFR and HER-2 has been demonstrated *in vitro* as well as *in vivo* in both OS and in EWS [76, 77], but data on the use of gefitinib or erlotinib in these tumors is not available.

Other tumor entities have been evaluated in respect to possible treatment with gefitinib. In a pre-clinical study of gefitinib in NB, authors observed a 40% to 50% reduction in the number of two NB cell lines [78], and it demonstrated an inhibition of EGF mediated auto-phosphorylation of EGFR at low concentrations of 0.01μM. The authors postulated that gefitinib could be a potential inhibitor of NB cell proliferation. Recently, gefitinib was also tested in *atypical teratoid/rhabdoid tumor* (AT/RT), an extremely aggressive malignancy mainly occuring in infants and young children. The most common locations are the kidney and the central nervous system, although AT/RT can also arise in most soft-tissue sites [79]. No curative strategy is currently available for these tumors. One study showed that gefitinib was able to inhibit rhabdoid cell growth *in vitro* by inhibition of EGFR phosphorylation [80]. Another study performed on *juvenile polycytic astrocytoma* (JPA), a brain tumor with good prognosis after gross total resection, demonstrated that the inhibitory effect of gefitinib was independent of EGFR expression [81].

Clinical Studies

Three clinical trials have evaluated treatment with gefitinib in children with refractory solid tumors and CNS malignancies. These trials were reviewed by Freeman *et al.* with special focus on aspects of clinical pharmacology [82]. To this day, gefitinib has been studied in children as a single agent or in combination with radiation and irinotecan (see Table **3**).

The first clinical trial was performed by the COG, in which gefitinib was administered orally at doses of 150-500 mg/m^2 once a day for 28 consecutive days. The maximum tolerated dose (MTD) was of 400 mg/m^2 [83]. Overall, gefitinib was well tolerated with mild side effects similar to adult patients, such as skin rash and diarrhea. Reported results included PR in one EWS after four weeks of treatment, and SD in two nephroblastoma at eight and sixteen weeks and in two BSG at 40 and 60+ weeks, respectively. The authors concluded that gefitinib may enhance multimodal therapy independently of EGFR expression and mutation status.

The second phase I trial was conducted by the Pediatric Brain Tumor Consortium. Gefitinib was administered in combination with radiation therapy to patients with brain stem tumors and incompletely resected supratentorial GBM (reviewed in [82]). In this study, gefitinib was also well tolerated with a MTD of 375 mg/m^2. However, three patients presented intratumoral hemorrhage at the MTD and therefore dosage was decreased to 250 mg/m^2 for the subsequent phase II study. This phase II study aims to determine the therapeutic benefits of gefitinib in children with GBM and is currently ongoing.

The third study was developed by the St. Jude Children's Research Hospital to evaluate the combination of gefitinib and irinotecan (reviewed in [82]). Two days before the initial irinotecan dose, patients received one dose of gefitinib. This administration increased the bioavailability of irinotecan, resulting in greater systemic exposure to SN-38, the active metabolite. Further data is not available at the moment as the study is still ongoing.

Results of a phase I study on erlotinib in pediatric tumor patients were presented recently at the 2006 ASCO meeting (see Table **3**). The recommended phase II pediatric dose determined by this study was found to be 85 mg/m^2/day, whether administered alone or in combination with temozolomide. A COG phase II trial is planned with erlotinib. Also, a study by the ITCC is currently evaluating erlotinib in relapsed brain tumors and pontine glioma in combination with radiation therapy but data is not available yet.

FUTURE DIRECTIONS

Though pre-clinical data appear promising, the potential role of TKIs in childhood solid tumors cannot yet be fully defined. Determining which patients will profit from these new targeted therapies remains a challenge, especially since response to TKIs is not always related to the expression or mutation status of the targeted TK.

Most small molecule TKIs lack substantial benefit when given as monotherapy. Combination therapies such as multiple TKIs (as demonstrated by gefitinib and sunitinib in renal cancer trials) or using small molecule TKIs and antibodies directed against TK (like lapatinib and trastuzumab in breast cancer trials) appear to be more effective. Furthermore, early clinical data suggests that combining TKIs with chemotherapy or radiation increases treatment efficacy, although this synergistic effect was not the initial aim of TKIs development.

New TKIs, such as the pan-HER TKI, are currently under investigation in adult patients with cancer. Childhood tumor entities often present very high angiogenesis. Therefore, targeting tumor angiogenesis with TKI directed against TK on endothelial cells is a particularly exciting concept in treating pediatric tumors. Also, multi-targeted inhibitors, that inhibit both TK present on tumor cells and on endothelial cells, would be particularly interesting to evaluate in pediatric tumor models. New TKI specifically targeting TK expressed on distinct tumor cell are being developed. For example, Trk-A, -B and –C receptors play an important role in NB biology. Expression of Trk-B is associated with higher proliferation and an unfavourable prognosis [84]. CEP-701 is directed against neurotrophin receptors and could be an interesting drug for the treatment of NB. Recently, CEP-701 showed anti-neoplastic activity against NB *in vitro* and *in vivo* pre-clinical studies[4].

Before integrating TKIs into treatment strategies for pediatric malignancies, further evaluation is needed with phase I and II clinical trials. For pediatric phase I trials, a consensus has approved to start with an initial dose equivalent to 80% of the adult MTD, followed by subsequent dose escalation. This design limits the number of children exposed to potentially "un-effective" doses, however, it may be more appropriate to evaluate TKI at a "biological" dose rather than the MTD, as pharmacodynamic endpoints may be particularly crucial in pediatric trials. Also, different TKI appear to have very distinct toxicity profiles. Therefore, close monitoring of these new toxicities will be of utmost importance as it may be very different in the "developing" organism of a young child.

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Table 3. Clinical Data on TKI in Pediatric Solid Tumors

TKI*	Study design	Results	Ref.
Imatinib Phase II study (COG)	2-stage design, pts. less than age 30 years of age with refractory or recurrent solid tumors 440 mg/m^2/day administered daily for 28 day courses. Doses larger than 600 mg divided bi-daily	Cohort: 70 eligible pts enrolled and 59 evaluable (EWS 24, OS 10, NBL 10, GIST 1, DSRCT 10, SS 4). Result: One PR among 24 EWS. Toxicity: Hemorrhagic pleural effusions in 4 pts. With pulmonary PD and in 3 pts. In whom PD was not confirmed. Intratumoral bleeding in 3 pts. Two pts. Discontinued treatment due to unacceptable GI symptoms. Grade 3/4 hematological toxicity in <10% of courses. No other grade ¾ toxicities.	[72]
Dasatinib Phase I study (COG)	single agent in refractory solid tumors (and Ph+ leukemia) at dose levels of 50, 65, 85 and 110 mg/m^2/dose, administered orally twice daily for 28 consecutive days	Cohort: 8 pts with solid tumor (3 pts Ph+ leucemia) Result: a dose of 50 mg/m^2 BID on a continuous 28 day dose schedule	[5]
Imatinib Phase II study (ITCC)	single agent in children and adolescents with refractory or relapsing solid tumors expressing at least one of the TK targeted by imatinib 340 mg/m^2 daily during a total of 168 months (median 1.9 month/patient, range 0.5-19). Ten patients escalated to 440 mg/m^2 due to lack of efficacy	Cohort: 36 pts., median age 13.7 yrs (2.2-22.5 yrs), 12 brain tumors, 6 fibromatosis, 8 mesenchymal/bone tumors, 10 other solid tumors, including 1 GIST and 3 chordoma. 18 expressed c-KIT, 10 PDGFR-alpha, 21 PDGR-beta; 12 expressed more than one receptor. Result: No CR or PR; 5 pts (2 fibromatosis, 1 GIST, 1 MB, 1 pseudo-inflammatory tumor) experiencing durable SD under treatment for more than 12 months, interesting SD during 10 and 7 mths in a BSG and a RCC. Toxicity: during the 1st month, 17 pts. experienced mild toxicity (grade 1 and 2) related to study treatment: GI (n=22), face edema (n=7), asthenia (n=5), tumor induration (n=2), skin toxicity (n=2), thrombocytopenia (n=1).	[6]
Gefitinib phase I (COG)	single agent for recurrent or refractory pediatric solid tumors 150, 300, 400 or 500 mg/m^2 administered orally to cohorts of three to six pts. once daily continuously until disease progression or significant toxicity, evaluation of MTD, DLT and pharmacokinetic behavior	Cohort: 19 pts. evaluable DLT was rush and ALT and AST elevation MTD: 400 mg/m^2 Results: 1 PR in EWS (4 wks), SD in 2 Wilms tumor (8, 16 wks) and in 2 BSG (40, 60+ wks) Toxicity: no additional toxicity other than known from adults (skin rash, dry rash, anemia, diarrhea, nausea, vomiting)	[83]
Gefitinib phase I/II and radiation (Pediatric Brain Tumor Consortium)	Safety and Efficacy in conjunction with irradiation in incompletely resected supratentorial malignant gliomas and BSG Pharmacokinetic studies	MTD: 375 mg/m^2, dose reduction to 250 mg/m^2 for phase II due to 3 intratumoral hemorrhages Phase II ongoing Dexamethasone may enhance gefitinib clearance and reduce systemic exposure	reviewed in [82]
Gefitinib phase I in combination with irinotecan (SJCRH)	Safety daily administration for 21 days in conjunction with i.v. irinotecan daily over a 12 day period in refractory tumors in children Pharmacokinetic studies	Preliminary result: gefitinib increases bioavailability of irinotecan and in consequence an increased systemic exposure of irinotecan.	reviewed in [82]
Gefitinib combined with oral topotecan (TPT) and cyclophosphamide (CPM) (Italy)	oral CPM (50 mg/m^2/day) followed by TPT (0.8 mg/m^2/day) plus G at the fixed dose of 250 mg/day for 14 consecutive days in an outpatient setting. Courses were repeated every 28 days and response was evaluated every two courses.	Cohort: 7 pts, 4 NB relapsed after PBSCT, 3 sarcoma (1 rhabdomyosarcoma, 2 synovial sarcomas) Results: Median PFS was 7 months (range 1-15). Of 4 NB, 2 are progression free at 7+ and 15+ months since inclusion (9 and 22 months after relapse). Toxicity: mainly hematological (neutropenia), grade 4 in 3/7 pts; cutaneous (rash) grade 2 in 1 pt., elevated transaminase grade 3 in 1 pt., 2 episodes of neutropenic fever (1 sepsis)	[7]
Erlotinib phase I followed by combination with temozolomide (TMZ)	Safety as single agent daily in 28 days followed by erlotinib daily and 180 mg/m^2/day TMZ	Cohort: 46 pts with refectory solid tumors MTD: 85 mg/m^2/day erlotinib orally either alone or in combination with TMZ Results: 1 minor response in a STS after one cycle single agent erlotinib, continuing to a PR by cycle 4; 2 NB, 1 neurocytoma had prolonged responses (13-20 mths+) to the combination Toxicity: rash and diarrhea were DLT	[8]
Sorafinib Phase I (ongoing)	Side effects and best dose of in young patients with relapsed or refractory solid tumors or leukemia		www.clinicatrial.gov

*This table is an incomplete list of TKI and studies. There is no single source of listing for pediatric solid tumor TKI trials. Individualized institutions may have other studies available. Further information is provided at the www.clinicatrial.gov homepage.
CD (complete response), PR (partial response), SD (stable disease), MTD (maximal tolerated dose), DLT (dose limitating toxicity), GI (gastroinstestinal)

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In summary, further data on TKI efficacy in pediatric cancer is necessary. Analyzing the results from both adult trials and pre-clinical studies will help to prioritize potential drugs of interest for pediatric trials. Recently, research in this field has been facilitated by the creation of Translational Research Teams. In this context, the National Cancer Institute (USA) and the ITCC (Europe) share common goals: to merge research on biological and pre-clinical evaluation of new drugs and to perform phase I and II clinical studies in pediatric oncology. Furthermore, the new European legislative will help to elaborate clinical studies for pediatric patients on a European level, thereby allowing to enhance the knowledge on new agents such as TKI in pediatric cancer [85].

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ABBREVIATIONS

ASCO = American Society of Clinical Oncology
ATP = Adenosine triphosphate
AT/RT = Atypical teratoid/rhabdoid tumor
BSG = Brain stem glioma
CML = Chronic myeloid tumor
CCLG = Children's cancer and leukemia group
COG = Children's Oncology Group
DC = Dendritic cells
DFSP = Dermatofibrosarcoma protuberans
EGFR = Epidermal growth factor
EMEA = European Medical Agency
EWS = Ewing sarcoma
FDA = Federal Drug Administration
GBM = Glioblastoma mulitforme
GIST = Gastro intestinal stroma tumor
GPOH = Gesellschaft für Pädaitrische Hämatologie und Onkologie
IFP = Interstitial fluid pressure
ISEL = Iressa Survival Evaluation in Advanced Lung Cancer
ITCC = Innovative Therapy for Children with Cancer
MAPK = Mitogen-activated protein kinase
MB = Medulloblastoma
MTD = Maximal tolerated dosis
NB = Neuroblastoma
NSCLC = Non small cell lung cancer
OST = Osteosarcoma
PDGFR = Platelet derived growth factor receptor
PIGF = Placenta derived growth factor
PNET = Primitive neuroectodermal tumor
RA = Retinoic acid
SD = Stable disease
SDF = Stromal derived factor
SIOP = International Society on Pediatric Oncology
STS = Soft tissue sarcoma
TK = Tyrosine kinase
TKI = Tyrosine kinase inhibitor
VEGF = Vascular endothelial growth factor

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Review

Angiogenesis as a target in neuroblastoma

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ABSTRACT

Several research investigations on neuroblastoma (NB) have shown the important dependency of this embryonic tumour on angiogenesis, especially in the advanced and aggressive stages. However, the first pre-clinical data on anti-angiogenic drugs in NB have not been published until recently and clinical trials with anti-angiogenic agents in NB treatment protocols are still missing.

Here, we summarise current knowledge on the important role and mechanisms of angiogenesis in NB, and report available pre-clinical results of anti-angiogenic agents used to treat NB. This review clearly shows that angiogenesis is a target in NB and that clinical trials are urgently needed to bring forward promising anti-angiogenesis treatment strategies into NB therapy.

1. Angiogenesis in neuroblastoma

Angiogenesis represents an attractive new target for tumour therapy and studies on tumour vessel formation have been performed in nearly all types of solid tumours. Whilst the vast majority of this research has focused on adult malignancies, neuroblastoma (NB) has been the focal point of angiogenesis research in paediatric oncology.[1] Although the biological mechanisms that underlie the clinical heterogeneity observed in NB is not completely understood, several recent studies implicate angiogenesis as an essential mechanism regulating NB growth. Therefore, from this point of view, studies on angiogenesis and anti-angiogenic therapies are extremely interesting and necessary in NB. This review summarises recent data on angiogenesis as well as anti-angiogenesis strategies for NB found in the current literature.[2–4]

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1.1. *Angiogenesis*

The first study on tumour vessel formation was reported by Folkman et al. in 1962[5] in which Folkman described the sprouting of new tumour blood vessels from pre-existing ones. Since, the formation of new blood vessels has been shown to be a multi-step process consisting of endothelial cell proliferation, migration and tubule formation. It is now widely accepted that solid tumours require neo-vascularisation for growth beyond a very small size (2–3 mm^3) and for metastatic spread. Within the tumour, this process has been coined 'tumour angiogenesis'. However, as the initial observation was restricted to the formation of blood vessels, a more precise denomination would have been 'tumour blood-angiogenesis'. Dissemination of tumour cells into lymph nodes via the lymphatic vascular system is also a well known problem. Recent research has unravelled another phenomenon named 'tumour lymph-angiogenesis' in which tumour cells not only stimulate the formation of blood vessels, but also the formation of lymphatic vessels as well.[6] Today, understanding the tumour vasculature as a global scheme must take into account both blood and the newly described lymph-angiogenesis processes.

The origin of neo-vessels within the expanding tumour tissue is the result of different mechanisms. Accumulating evidence indicates that in addition to the sprouting of neighbouring pre-existing vessels, tumour angiogenesis is supported by the mobilisation and functional incorporation of endothelial progenitor cells (EPC) that originate from the bone marrow.[7] EPC are highly proliferative cells that can be mobilised to circulate in the peripheral blood and arrest at the site of the developing tumour. Recent reports have described the functional incorporation and contribution of EPC to tumour neovessels, suggesting that the process of 'vasculogenesis' – the progenitor-cell-driven *de novo* generation of new vessels[8] – is not restricted to embryonic development. EPC appear to home to sites of angiogenesis and participate in the generation of new blood vessels in adults as demonstrated by animal models of ischaemia. Other studies have shown that blood vessel formation in tumours may occur primarily through endothelialisation of adjacent and pre-existing blood vasculature, a process called 'co-option'.[9] Exactly how tumours generate new blood vessels is still a matter of debate. Most authors agree that the various mechanisms involved (sprouting, vasculogenesis, or co-option) may not only depend on the nature of the tumour, but may also coexist as a complex and multi-faceted process. To make matters even more confusing, it has been shown that tumour cells may mimic and transform themselves into vascular cells. The term 'vasculogenic mimicry' was recently introduced to reflect this embryonic-like ability of tumour cells to form a blood vessel network.[10] The role and significance of these different angiogenesis mechanisms, recently reviewed by Dome et al.,[11] must be further clarified.

1.2. *Neuroblastoma*

Derived from cells of the primitive neural crest,[12] neuroblastoma (NB) is the most common extra-cranial solid tumour of childhood and the most frequent tumour in children less than one year of age. NB has a wide range of clinical virulence reflective of its underlying biological heterogeneity. Tumour stage, as defined by the International NB Staging System (INSS), and patient age at diagnosis are important clinical prognostic factors that strongly correlate with survival.[13] Genetic abnormalities play a role in determining tumour phenotype and predicting outcome and include amplification of the *MYCN* oncogene, deletion of chromosome 1p, DNA polyploidy, grade of neuronal differentiation and messenger RNA expression level of the neurotrophin receptor Trk-B. Some of these factors have been used to develop a risk-based classification scheme and categorise patients as having low, intermediate or high-risk of tumour relapse in order to help guide treatment protocols.[14] Whilst the majority of children with low-risk NB may be cured by surgery alone, less than 30% of children with high-risk NB become long-term survivors despite aggressive therapy associating surgery, high-dose chemotherapy with autologous stem cell transplantation, radiation and administration of differentiating agents.[15] These children are in desperate need of novel therapeutic strategies. A recent seminar on NB specifically focused on current advances in understanding NB tumour biology as well as potential new approaches to treat this complex paediatric tumour.[16]

2. Mechanisms of angiogenesis in neuroblastoma

Different angiogenesis mechanisms have been described in the formation of NB tumour vasculature. Sprouting of new blood microvessels from pre-existing capillaries under the influence of pro-angiogenic growth factor expression, such as vascular endothelial growth factor-A (VEGF-A), bFGF and angiopoetien (Ang), has been reported.[17–19] A recent study analysing the origin of endothelial cells in NB tumours described the discovery of microvessels harbouring *MYCN* amplification and therefore originating from NB cells. This report provided proof for the existence of 'vasculogenic mimicry' as another mechanism of angiogenesis in NB.[20] Also, we have observed the presence of circulating EPC in patients with NB, demonstrating for the first time that vasculogenesis is also involved in NB tumour vessel formation.[21] Thus, all known mechanisms of angiogenesis described to date are also present in NB. A summary of mechanisms of angiogenesis in NB is given in Table 1.

2.1. *Microvessel density in neuroblastoma*

Microvessel density can be evaluated by counting the number of immunohistochemically identified blood vessels in vascular hot spots of tumour samples. Several other techniques also are available such as Chalkley counting, vascular grading and the use of image analysis systems.[22] Meitar et al. were the first to demonstrate in NB that tumour angiogenesis was associated with a clinical phenotype.[23] They calculated a vascular index, defined as the number of vessels/mm^2 of tissue, and found that a high vascular index correlated strongly with widely disseminated disease, amplification of *MYCN* and unfavourable histology and was associated with poor survival. Multivariate analysis confirmed that tumour vascularity was an important and independent prognostic variable. Another study used a computerised system to determine vas-

Table 1 – Mechanisms of angiogenesis in neuroblastoma

Neuroblastoma	Angiogenesis	Reference
Low risk		
– Favourable histology with presence of Schwann cells	– Highly expressed activin A	Schramm et al.[30]
– Trk-A expression	– Down-regulated angiogenesis factor expression	Eggert et al.[98]
High risk		
– Unfavourable histology with stroma poor in Schwann cells	– Higher vascular index	Meitar et al.[23]
– *MYCN* amplification	– Higher expression levels of VEGF-A, VEGF-B, bFGF, PDGF-A, TGF-alpha	Eggert et al.[18]
	– Down regulation of endothelial cell growth inhibitors	Fotsis et al. [17]
– TrK-B expression	– Increased levels of MMP-2 and MMP-9	Ribatti et al.[27]
	– More highly expressed levels of integrins alpha (v) beta 3 and alpha (v) beta 5	Erdreich-Epstein et al.[28]

cular parameters in hot spots of NB tissue.[24] In contrast to the previous report, the authors did not find that disseminated disease or local relapse was influenced by the angiogenic characteristics of the tumours, which therefore did not demonstrate predictive value for survival. However, it is widely accepted that localised tumours with favourable biological features are often less vascular and have a rich stromal component largely composed of nonmalignant Schwann cells, which have recently been shown to produce potent anti-angiogenic molecules.[25] Further data supporting the importance of the number and the function of microvessels in NB is presented in a study with an orthotopic NB nude mice model in which tumour vessel morphology and vascular perfusion were analysed *in vivo* by ultrasound (Fig. 1).[26] Unlike orthotopic NB tumours, subcutaneous tumours lacked the expression of the highly angiogenic integrin alpha(v)beta3. The authors suggested that the differences observed were due to the impact of the regional microenvironment on tumour biology. Although not fully understood, NB vascularity seems to play a major role in clinical phenotype and be intrinsically related to the underlying tumour biology.

2.2. *Angiogenesis growth factors in neuroblastoma*

Several pro-angiogenic growth factors have been shown to be differentially expressed in NB in a pattern suggesting the promotion of a pro-angiogenic phenotype in high-risk tumours. Significantly higher expression levels of VEGF-A, VEGF-B, bFGF, Ang-2, transforming growth factor alpha (TGF-alpha) and platelet-derived growth factor A (PDGF-A) were found in advanced-stage tumours (stages 3 and 4) compared to low-stage tumours (stages 1, 2, and 4s).[18] In this study, PDGF-A was significantly associated with survival. In a smaller series, VEGF-A was the only factor found to correlate with advanced tumour stage. Matrix metalloproteinases (MMP) and their inhibitors (MMPI) play a key role in maintaining the balance between extra-cellular matrix deposition and degradation especially important for the migration of endothelial cells during angiogenesis.[27] Whilst MMPI has been shown to suppress tumour invasion and angiogenesis, overexpression of MMP-2 and MMP-9 is associated with tumour invasion and metastasis in many types of cancer. In NB, an association between increased levels of MMP-2 and -9 and advanced tumour stage has also been observed. Furthermore, several studies have shown that high levels of MMP-2 expression correlate with poor outcome. Finally, integrins alpha(v)beta3 and al-

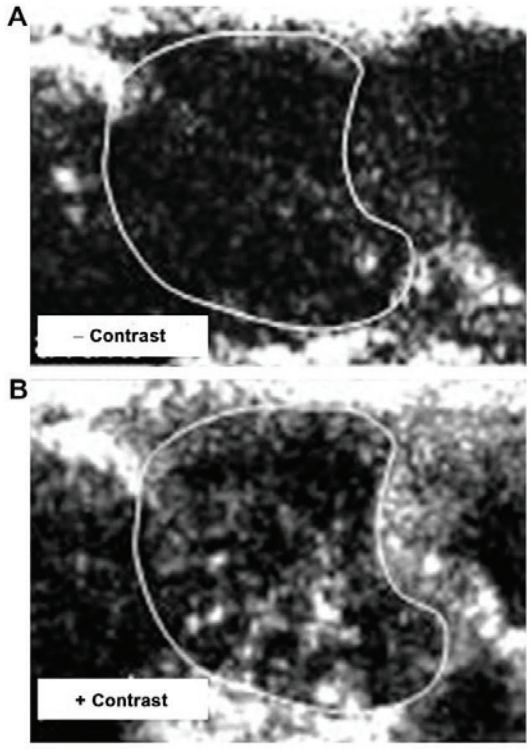

Fig. 1 – Contrast ultrasound to visualise vascularisation of a neuroblastoma (NB) orthotopic tumour. (A) NB cells injected into the right adrenal gland develop into a measurable tumour which can be visualised by ultrasound. (B) After intraocular injection of a contrast agent, vascular perfusion is visible and measurable.

pha(v)beta5 – markers of angiogenic endothelium – were also found to be more highly expressed in blood vessels of high-risk versus low-risk NB tumours.[28]

3. Regulation of angiogenesis in neuroblastoma

Most malignant cells are potently angiogenic as a result of an increased secretion of angiogenic stimulators and a decreased production of inhibitors. However, several other mechanisms appear to play a role in the regulation of angiogenesis in NB, as described below.

3.1. *Oncogene MYCN*

The *MYCN* oncogene is frequently amplified in high-risk NB and is associated with an increased vascular index and poor prognosis.[23] Consequently, it has been hypothesised that *MYCN* might regulate some aspects of NB angiogenesis either by up-regulating angiogenic stimulators or by down-regulating inhibitors. Most investigators have not found a correlation between *MYCN* amplification and increased expression of pro-angiogenic factors such as VEGF, bFGF and MMP. However, recent data suggest that the aggressive biology of *MYCN*-amplified NB is due to suppressed expression of three angiogenesis inhibitors.[17] One of these inhibitors, recently identified as activin A, represses NB growth, endothelial cell proliferation and angiogenesis both *in vitro* and *in vivo*.[29] Activin A is highly expressed in differentiated NB, and increased expression is strongly correlated with favourable outcome.[30] *In vitro* studies demonstrated that *MYCN* inhibited activin A by suppressing its promoter activity.[31] Also, *MYCN* appears to regulate two other inhibitors, interleukin (IL-6) and leukaemia inhibitory factor (LIF),[17,32] which are currently under investigation for their potential as therapeutic targets in NB.

3.2. *Schwann cells*

In contrast to *MYCN*-amplified tumours, low-risk NB is characterised by favourable histology in which a rich stromal component made up of quiescent Schwann cells is frequently observed.[33] Schwann cells produce anti-proliferative and differentiation-inducing factors and a 'cross-talk' between Schwann cells and neuroblasts has been proposed to describe the biology and clinical behaviour of NB tumours.[34] In support of this hypothesis, laboratory studies have shown that Schwann cells also produce several inhibitors of angiogenesis including tissue inhibitor of metalloproteinases (TIMP2), pigment epithelial-derived growth factor (PEDGF) and the secreted protein acidic and rich in cysteine (SPARC).[25,35,36] Therefore, the presence of Schwann cells most likely contributes to the relatively avascular nature of most low-risk NB, further highlighting that differential expression of angiogenesis-related genes contributes to specific phenotype in NB.

3.3. *Hypoxia*

Situations of cellular stress such as hypoxia lead to rapid response mechanisms that ensure adaptive strategies for cellular survival. Genes are rapidly transcribed via the transcription factor hypoxia-inducible factor-1 (HIF) in order to allow for this response[37] and target genes include the pro-angiogenic growth factor VEGF-A. Subsequently, angiogenesis is also regulated by hypoxia.[38] Within the tumour, the initial vasculature is insufficient to cope with the high-index proliferation of tumour cells leading to areas of low oxygen supply. This can be visualised by radiological examinations after minor perfusion of contrast medium into embryonic tumours.[39] We have also demonstrated that hypoxia leads to up-regulated VEGF-A secretion and subsequent stimulation of endothelial cell proliferation in NB cell lines.[19] Furthermore, erythropoietin (EPO) – the prototype of hypoxia-regulated expression via the transcription factor HIF – has a role in erythropoiesis as a stimulator of endothelial proliferation[40] and is also up-regulated in NB cells of neuronal phenotype exclusively.[41,42] However, EPO did not modify NB cell proliferation *in vitro*. More interestingly, in a tissue microarray study, the NB tumour specimens with the highest expression of the EPO receptor had a significantly better overall survival.[43]

4. Lymph-angiogenesis in neuroblastoma

Despite the important role of lymphangiogenesis in tumour growth and metastasis, most studies have focused on evaluating this process in adult cancers, notably in melanoma,[44] and not in children for which very little data are available. Studies concerning embryonic tumours and the relationship between the number of lymph vessels, tumour stage and prognosis are lacking. One study showed significantly higher expression levels of the lymphangiogenesis growth factor VEGF-C in NB of stages 3 and 4 compared to low-stage tumours.[18] In another study using immunohistochemistry, we demonstrated that lymph vessels of different morphology and localization are present in NB tumours.[45] With the help of the antibodies D2_40, specific for lymphatic endothelial cells, and CD34, a surface marker which identifies blood endothelial cells, we could discriminate between both types of tumour vessels in NB tumours (Fig. 2). Lymphatic vessel density was determined by a computer-aided method and ranged from complete absence to as much as 80 lymph vessels per hot spot in available NB specimens. Further studies on lymphangiogenesis are needed as they would help understand its underlying biology and verify its prognostic relevance in NB.

5. Anti-angiogenesis inhibitors in neuroblastoma

Targeting tumour vasculature represents a promising new tool for cancer therapy. The first angiogenic inhibitor, interferon-alpha, was described by Folkman in 1971.[46] Successful treatment of haemangioma, the most frequent vascular tumour in very young infants, was reported with interferon.[47] TNP-470 is the first selective inhibitor of endothelial proliferation and is a synthetic analogue of an antibiotic naturally secreted by *Aspergillus fumigatus* and isolated from an endothelial cell culture contaminated by the fungus.[48] More

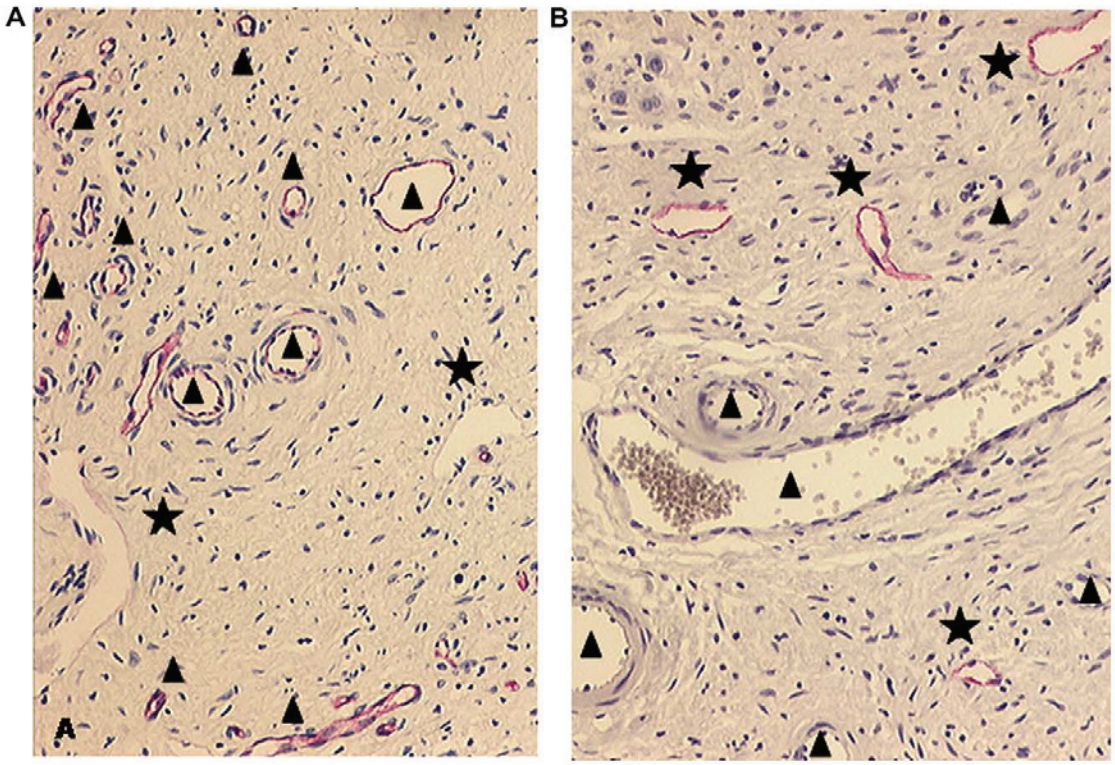

Fig. 2 – Tumor blood and lymphatic vessels identified by immunohistochemistry. (A) The CD34 antibody directed against a cell surface marker of blood endothelial cells shows positively stained tumour blood cells (▲) and negatively stained tumour lymph vessels (★). (B) Immunohistochemistry with the D2_40 antibody marked tumour lymph endothelial cells (★) but negatively stained tumour blood vessels (▲).

than 70 anti-angiogenic molecules are described today. Most of these molecules are being tested in clinical trials, mainly for adult cancer and 18 are currently in phase III studies.[49]

Three classes of angiogenic inhibitors can be distinguished: direct, indirect and mixed inhibitors. Direct angiogenesis inhibitors, such as angiostatin, endostatin or thrombospondin, target microvascular endothelial cells involved in proliferation, migration and formation of new blood vessels. Indirect inhibitors block the production or activity of pro-angiogenic molecules produced by the tumour itself such as VEGF, or the receptors of VEGF or PDGF. The so-called mixed angiogenic inhibitors, such as multipotent tyrosine kinase inhibitors (TKI) or interferon-alpha, affect both tumour endothelial cells and malignant cells.[50]

Recently, a major step in anti-angiogenesis therapy was the approval of bevacizumab (Avastin®) by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) for the treatment of metastatic colorectal cancer with fluoropyrimidine combined regimes.[88] Bevacizumab is a recombinant monoclonal antibody that binds VEGF-A and subsequently blocks the activation of its receptors. Sunitinib (Sutent®, SU11248) was registered by the FDA and the EMEA for the treatment of advanced or metastatic renal cell carcinoma after failure of Interferon alfa or Interleukin 2 therapy, as well as imatinib-resistant gastro-intestinal stromal tumour (GIST). Sunitinib is a multi-targeted TKI showing both anti-proliferative and anti-angiogenic effects as a result of its inhibition of VEGFR, PDGFR-beta and c-Kit.[51] A third anti-angiogenic drug, sorafenib (Nexavar®, BAY 43-9006), has officially been licensed for use in adult oncology after receiving FDA approval for the treatment of advanced renal cell carcinoma. Initially, this bi-aryl urea was developed as a specific inhibitor of the intracellular kinase raf but subsequent studies have shown that this compound inhibits several other TK involved in tumour progression including VEGFR.[52]

Compared to conventional cytotoxic cancer treatment, anti-angiogenic strategies offer several advantages: (1) the damage caused to a single tumour blood vessel impacts a high number of tumour cells dependant on its blood supply; (2) anti-angiogenic therapy appears to normalise tumour vessels, resulting in improved delivery and efficacy of chemotherapy agents; (3) targeting endothelial cells may help control minimum residual disease due to quiescent cells in the $G_{0/1}$ phase which are not accessible to standard chemotherapy; (4) anti-angiogenic drug resistance is rare and contrasts with tumour resistance frequently developed against standard chemotherapy drugs.

Direct angiogenic inhibitors are not currently used in NB therapy protocols. Interestingly, retinoic acid, which is now administered in stage 4 patients after autologous stem cell

transplantation as a maintenance treatment,[53] has not only been chosen for its differentiating effect on immature NB cells, but also for its anti-angiogenic potency as demonstrated in several pre-clinical studies. A summary of pre-clinical studies evaluating anti-angiogenic agents in NB is presented in Table 2.

5.1. *Retinoids*

Retinoids exert their effects by inducing differentiation of NB cells. Furthermore, retinoids and notably fenretinide, a newly developed synthetic retinoid, have demonstrated an anti-angiogenic effect in different experimental models. These effects include prevention of tumour-associated angiogenesis *in vivo*, inhibition of vessel sprouting in chicken chorion allantois membrane (CAM), and tumour cell transformation from an angiogenic to an anti-angiogenic phenotype *in vitro*.[54–56] All these effects are mediated by the inhibition of endothelial migration along with the production of stimulating or inhibiting factors for vessel growth.[54] Furthermore, retinoic acid induced expression of thrombospondin, an important physiological angiogenesis inhibitor, in NB cell lines.[57] Therefore, retrospectively, the use of retinoids in NB is the first anti-angiogenesis strategy to have been used in treating embryonic tumours.

5.2. *TNP-470*

The synthetic derivative of fumagillin TNP-470 (or AGM-1470) inhibits an important enzyme for endothelial cell proliferation and migration known as methionine aminopeptidase-2 (MetAP2).[3] There are several ongoing clinical phase I and II trials with TNP-470 for adult patients with cervical, pancreatic or renal cell carcinoma.[58] When given in combination with other common cytotoxic drugs like cisplatin, paclitaxel or cyclophosphamide, treatment with TNP-470 synergistically potentiates the anti-tumour effects of these molecules.[3] Another MetAP2 inhibitor, A-357300, demonstrated tumour growth suppression in pre-clinical models without the toxicities observed with TNP-470.[59] Several pre-clinical studies report the use of TNP-470 in NB models (Table 2). Altogether, current data suggest that TNP-470 may be useful as adjuvant therapy for high-risk NB patients when administered either between cycles of induction therapy or at the end of cytotoxic chemotherapy, and perhaps more particularly when used in the setting of minimal disease status.

5.3. *Thalidomide*

Thalidomide was first introduced in the 1950s as a nontoxic sedative but withdrawn because of its marked teratogenicity. Recent studies have demonstrated that the aetiology of the limb defects caused by foetal exposure to thalidomide was most likely due to inhibition of blood vessel growth in developing limb buds.[60] Therefore, thalidomide is an anti-angiogenic molecule with potential to treat cancer. In adult oncology, a phase I study showed safety, tolerance and potential efficacy of thalidomide in treatment of recurrent epithelial ovarian cancer.[61] However, in a phase II study for gynaecological sarcoma, thalidomide showed no activity and caused major adverse effects such as constipation, fatigue, drowsiness and worsening of performance status.[62] Consequently, derivatives of thalidomide are currently under development.[63] One study tested thalidomide in a xenotransplant NB mouse model and reported reduced angiogenesis although tumour growth was not altered significantly.[64] The first paediatric clinical trial using thalidomide in combination with radiation in children with brain stem glioma and glioblastoma did not demonstrate efficacy and displayed higher toxicity as shown by increased use of corticosteroids.[65]

5.4. *Matrix metalloproteinase inhibitors*

In normal tissues, a balance between MMP and MMPI exists. However, in tumour tissue, there is an imbalance/switch in favour of the MMP. Consequently, another attempt to control angiogenesis is to use MMPI in order to prevent degradation of the basal membrane and the surrounding connective tissue. Marimastat® (BB-2516) is a synthetic inhibitor for MMP-1, -2, -3, -7 and -9[58] and represents the first orally bioavailable MMPI to be tested in humans.[50,58] Results of phase I, II and III clinical trials using Marimastat® alone or in combination with other chemotherapeutic regimes showed dose-limiting toxicity in musculoskeletal disorders. BMS-275291, another broad-spectrum MMPI, was tested in combination with chemotherapy but showed increased toxicity without improving survival in advanced NSCLC.[66] Therefore, the use of MMPI is considered disadvantageous in this setting. As the expression of MMP-2 and MMP-9 correlates with poor prognosis in ovary, lung, breast and colon cancer, BAY 12-9566 (Tanomastat®) was developed as a selective, non-peptidic and orally available inhibitor of these two MMP. Tanomastat® demonstrated activity in humans without musculoskeletal toxicities in phase I studies,[58] but failed to show efficacy as maintenance therapy in patients with advanced ovarian cancer.[67] On the other hand, RO 28-2653, another MMPI for MMP-2 and MMP-9, showed promising results in a pre-clinical model for pancreatic cancer.[68] In NB, both MMP-2 and MMP-9 seem to play a major role in tumour angiogenesis and studies evaluating MMPI against these two MMP should be performed in pre-clinical models.

5.5. *Endostatin*

Endostatin, a 20 kDa C-terminal fragment of collagen XVIII, inhibits endothelial proliferation *in vitro* and tumour growth *in vivo* when given systemically.[3] Its anti-angiogenic activity is mediated through zinc-binding of endostatin. Results of phase I studies have shown that human endostatin is well tolerated and does not show drug-related toxicity when administered as daily bolus injections. The effects were lower VEGF-A and bFGF urinary levels, reduced tumour blood flow (measured by dynamic magnetic resonance imaging), reduced EPC levels and up-regulation of apoptosis in endothelial cells. However, recent data have questioned the actual efficacy of endostatin in tumour therapy as the reported results have not been reproducible in other laboratories.[50] Differences in terms of storage, handling and purification techniques may be responsible for differential endostatin behaviour.[58] Two studies have tested recombinant endostatin as a possible

Table 2 – Pre-clinical studies on anti-angiogenesis in NB

Drug	Study result	Reference
Retinoids	Pre-treatment of LAN-5 and GI-LI-N cells with 10^{-5} and 3×10^{-5} M RA for 24 h reduced the ability of conditioned medium to stimulate endothelial cell proliferation	Ribatti et al.[56]
	Treatment of SMH-KCNR cells with RA increases both cell associated and soluble forms of thrombospondin within 24 h	Castle et al.[57]
	13-*cis*, 9-*cis* RA and Ro 13-6307 decreased tumour growth in SH-SY5Y xenografts (subcutaneous (s.c.) in nude rats) significantly	Ponthan et al.[99]
TNP-470	Reduction of tumour growth rate and decreased microvascular density in SH-SY5Y xenografts (s.c. in nude rats)	Wassberg et al.[100]
	Induction of metabolic stress, resulting in chromaffin differentiation and apoptosis in SH-SY5Y xenografts (s.c. in nude mice)	Wassberg et al.[101]
	Reduction of primary tumour volume, size of axillary lymph nodes and liver metastases. Improvement of survival in TBJ and C1300 grafts (s.c. murine neuroblastoma (NB) cell lines)	Nagabuchi et al.[102]
	Inhibition of liver metastasis and increased survival of C1300 cells (murine NB cell line) injected in the spleen	Yoshizawa et al.[103]
	Decreased mean rate of tumour growth in NBL-W-N grafts (s.c. in nude mice) with small tumours but not in animals with large tumours. Treatment inversely correlated with tumour burden	Katzenstein et al.[104]
	Inhibition of tumour growth rate of CHP-134 cells inoculation by tail vein injection in mice, increased apoptotic index in treated tumours	Shusterman et al.[105]
Thalidomide	No significant alteration in tumour growth of NGP xenografts (s.c. in athymic mice). Suppression of angiogenesis (as measured by fluorescein angiography, immunohistochemical staining) and induced apoptosis of endothelial cells	Kaicker et al.[64]
Endostatin	Angiogenesis inhibition or immunomodulation alone resulted in only a modest delay in tumour growth of NB cell grafts transfected with endostatin and the immunogene GFP prior to inoculation (in syngenic immunocompetent mice). In combination, prevention of the formation of appreciable tumours	Davidoff et al.[70]
	Tumor growth slowed down only in endostatin-treated SK-N-AS mice (s.c. in mice). No statistically significant difference in endostatin serum levels	Jouanneau et al.[69]
	Continuous administration of recombinant endostatin resulted in more significant tumour regression than intermittent administration in TNB9 xenograft (s.c. in nude mice)	Kuroiwa et al.[106]
Angiostatin	Treatment with AdK3-HSA (a recombinant adenovirus encoding the human angiostatin kringle 1–3 directly fused to human serum albumin HSA) showed no delay in tumour growth of IGR-N835 xenografts (s.c. nude mice)	Jospeh et al.[72]
Bevacizumab (Avastin®)	Alterations in tumour vessel physiology allowing improved delivery and efficacy of chemotherapy in NB-1691 xenografts (orthotopic in SCID mice)	Dickson et al.[79]
	Reduction of SK-N-AS, IMR-32 and SH-SY5Y xenografts (s.c. in mice) without toxicity due to the reduction of angiogenesis. Two–Six-fold increase in serum concentrations of VEGF-A during therapy without faster tumour growth	Segerstrom et al.[78]
DC-101	Inhibition of tumour growth of unfavourable WAC2 xenografts (s.c. in nude mice) potentiated by simultaneous irradiation	Gong et al.[83]
	Significant but transient regression of SK-N-MC, SK-N-AS xenografts, diminished tumour vascularity and inhibited angiogenesis. In combination with low-dose vinblastine: full and sustained regression of large established tumours without an increase in host toxicity or any signs of acquired drug resistance	Klement et al.[82]
SU5416	Reduction in the growth of SH-SY5Y xenografts (s.c. nude mice) without apparent toxicity. Suppression of tumour angiogenesis, despite an increase in plasma VEGF-A levels per ml tumour volume during therapy. Combination with chemotherapy increased efficacy	Beckmann et al.[107]; Svensson et al.[108]
	Inhibition of tumour growth in WAC2 xenografts (s.c. in nude mice) by simultaneous irradiation	Gong et al.[83]

anti-angiogenic drug for NB (Table 2). In one report, only a 'deceleration' in tumour growth was reported in endostatin-treated mice when compared to control mice.[69] However, drug efficacy was increased when endostatin was administered as a continuous infusion or combined with an immunomodulating approach.[70,71]

5.6. *Angiostatin*

Angiostatin is a 38 kDa circulating endogenous protein that mediates its anti-angiogenic activity through binding ATP synthetase on the surface of human endothelial cells. By this, angiostatin causes apoptosis of tumour cells and inhibition of endothelial cell migration and tubule formation.[58] Pre-clinical testing of angiostatin in NB has been evaluated in a gene therapy approach using a recombinant adenovirus encoding the human angiostatin kringle 1–3 directly fused to human serum albumin HSA (AdK3-HSA).[72] Intravenous injection of this vector into the human NB IGR-N835 tumour model showed no delay in tumour growth when compared to tumours treated with the empty virus AdCO1. In early-stage tumours, kinetics of tumour occurrence and tumour growth were similar in

both AdK3-HSA and AdCO1-treated animals, respectively. Because of higher VEGF levels measured in the NB IGR-N835 tumours, the VEGF/VGEFR system has been proposed as a more promising target to inhibit NB angiogenesis.

5.7. *Thrombospondin*

Thrombospondin-1 (TSP-1) is a glycoprotein secreted in extra- and pericellular matrixes capable of inhibiting endothelial cell proliferation and migration. TSP-1 knockout mice display an up-regulation of tumour vascularisation with a subsequent increase in tumour growth.[73] TSP-1 has been shown to be silenced in a subset of undifferentiated, advanced-stage NB tumours and cell lines by promoter methylation.[74] In contrast, expression of this angiogenesis inhibitor was present in most localised NB tumours. ABT-510, a peptide derivative of TSP-1, significantly suppressed the growth of NB xenografts established from two different MYCN-amplified cell lines.[75] In combination with the histone deacetylase inhibitor valproic acid, ABT-510 inhibited even more effectively the growth of small NB xenografts compared to single-agent treatment. In animals with large NB xenografts, total cessation of tumour growth was achieved with this treatment approach. Further evaluation of ABT-510 in NB should be performed in early clinical trials.

5.8. *Monoclonal VEGF-A antibody*

Early studies using a monoclonal antibody against VEGF or using VEGF-TRAP (a composite decoy receptor based on VEGF receptor-1 and -2 fused to an Fc segment of immunoglobulin G1 (IgG1)) in a murine model of human NB were performed by Kim et al.[76,77] By combining the chemotherapy agent topotecan with these anti-VEGF strategies, the authors observed partial suppression of tumour growth and significant inhibition of rebound tumour growth. The first pre-clinical study to evaluate bevacizumab in NB demonstrated a significant reduction in tumour growth *in vivo* without toxicity due to the reduction of NB angiogenesis.[78] Recently, another study showed that bevacizumab-mediated VEGF blockade caused alterations in tumour vessel physiology that, in turn, allowed improved delivery and efficacy of chemotherapy.[79] One can imagine a similar impact in a clinical setting where 'normalisation' of the tumour vasculature and improved tumour perfusion could improve the delivery of systemic chemotherapy. This further emphasises that VEGF blockade in NB is an extremely interesting strategy and should be evaluated as it may help to optimise anti-tumour activity when given with correct drug scheduling.

5.9. *Monoclonal antibodies against VEGF receptors*

Several antibodies against VEGFR-2 (KDR) have been developed. Among them, a high affinity fully human anti-KDR antibody fragment[80] provided proof-of-principle that an anti-VEGFR-2 antibody could strongly inhibit tumour growth.[81] DC-101, a monoclonal rat anti-mouse VEGFR-2 antibody, was shown to inhibit the growth of NB cells without MYCN amplification when administered alone or in combination with low-dose vinblastine.[82] The rationale was to combine the anti-vascular effects of the low-dose chemotherapy which would selectively enhance VEGF survival signals in endothelial cells of neo-vessels that would in turn be simultaneously blocked by the anti-VEGFR-2 antibody. In fact, a full and sustained regression of large established tumours was achieved by this combination. Activity of DC-101 was also shown in a NB cell line bearing MYCN over-expression,[83] in which tumour growth delay was increased by simultaneous irradiation. Finally, another chimeric monoclonal antibody specific for VEGFR-2, IMC-1C11, is currently being evaluated for anti-angiogenesis efficacy.[84]

5.10. *Small molecules as inhibitors of the tyrosine kinase of VEGF receptors*

Sugen 5416 (SU5416, Semoxinal®) is a specific VEGFR-1 (Flt-1) and -2 (KDR) antagonist capable of blocking VEGF-stimulated Flk-1 phosphorylation.[3,58] SU5416 was evaluated in clinical trials for Kaposi's sarcoma, non-small cell lung (NSCLC), ano-rectal, renal cell, adenoid cystic and basal cell carcinomas in which stable disease was observed after six months of treatment.[58] It was reported to be able to induce apoptosis in gastric cancer by inhibiting tumour angiogenesis. SU5416 also exhibits strong inhibitory effects on primitive tumour growth and liver metastasis of gastric cancer in pre-clinical mouse models. Furthermore, SU5416 has shown efficacy as an angiogenesis inhibitor in NB *in vivo* models (Table 2). Efficacy was increased when SU5416 was given in combination with irradiation or chemotherapy. The current status of TKI of VEGFR in oncology has been reviewed.[85] Recently, we have also given an overview of TKI with special focus on their potential in paediatric solid tumours.[86] Although these small molecules are of special interest for their potential use in NB, data concerning their use in NB have not been published to date.

6. Anti-angiogenesis treatment strategies in neuroblastoma

Today, anti-angiogenic treatment is mostly used in combination with standard chemotherapy [87]. In treatment for colorectal cancer, bevacizumab is administered in parallel with standard chemotherapy.[88] However, recent studies have suggested that administration of conventional or high-dose chemotherapy might actually cause vascular rebound with endothelial cell recovery during rest periods, a process that could counteract the anti-cancer effect of chemotherapy.[89] Other schedules have been proposed for anti-angiogenic molecule administration in combination with classical chemotherapy: (i) administration before chemotherapy could increase the efficacy of chemotherapy via normalisation of the tumour vasculature and (ii) administration afterwards could have an effect on CEP that are mobilised during bone marrow regeneration and might contribute to tumour angiogenesis and vascular repair. In the case of NB, addition of an anti-angiogenic drug to efficient combination chemotherapy warrants evaluation in a clinical trial.

Another strategy to target the tumour endothelium is through administration of protracted low doses of chemo-

therapeutic agents in a combination schedule, a concept called metronomic chemotherapy.[90] Several metronomic chemotherapy protocols are available for paediatric solid tumours. In the study by Sterba et al., 22 patients with relapsed solid tumours were treated with the combined oral maintenance biodifferentiating and anti-angiogenic therapy (COMBAT) protocol using celecoxib, 13-cis-retinoic acid and cycles of metronomic temozolomide and low-dose etoposide.[91] Four NB patients were successfully treated with this protocol (2 VGPR, 1 PR and 1 SD). In another study by Stempak et al. using celecoxib in combination with vinblastine or cyclophosphamide, 32 patients with recurrent paediatric solid tumours were treated including three NB patients. Four patients (13%) had durable stable disease (28–78 weeks) although no complete or partial responses were observed.[92] Therefore, metronomic chemotherapy may play a role as a maintenance treatment in NB to consolidate remission and fight minimal residual disease.

A strategy taking into account the formation of lymph vessels and the prevention of tumour cell dissemination via lymph vessels should be taken into account in future anti-angiogenesis strategies. Anti-lymphangiogenesis has been established in animal experiments using antibodies against VEGF-C and VEGF-D[93,94] and as a major result, lymphogenic metastisation could be omitted. Another way to inhibit lymphangiogenesis is via blocking of VEGFR-3 by antibodies or tyrosine kinase inhibitors.[95] However, the efficacy of anti-lymphangiogenesis is controversial as the destruction of lymph vessels could result in a higher interstitial pressure within the tumour leading to increased haematogenous metastisation.[96] Because of limited data, lymphangiogenesis in NB should be further studied in pre-clinical models before evaluating its role in the clinical setting.

Finally, potential toxicity of anti-angiogenic drugs needs to be addressed in the paediatric population. Studies with bevacizumab in adult patients have reported many side-effects including a greater risk of grade 3 hypertension and grade 1 or 2 proteinuria, a slight increase (<2 percentage points) in grade 3 or 4 bleeding and impaired surgical wound healing in patients who undergo surgery during treatment with bevacizumab.[97] Potentially life-threatening events (arterial thrombotic events and gastro-intestinal perforation) have occurred in a small number of patients. In young children and adults free of cardiovascular risk factors, these side-effects may be rare. However, the impact of hitherto unrecognised damage to angiogenesis dependent processes such as skeletal or CNS maturation must be carefully considered when proposing anti-angiogenic therapies in young children.

7. Conclusions and perspectives for anti-angiogenesis in neuroblastoma

Relevant studies on angiogenesis and its regulation in NB is crucial as they highlight the importance of its role in NB tumour biology. The vast majority of pre-clinical study results indicate that specific targeting of a single angiogenic molecule or pathway is most likely to be insufficient and unsuccessful. The most promising anti-angiogenic strategies in NB will most certainly include either drugs that can affect more broadly the different steps of angiogenesis (endothelial cell proliferation, migration and tubule formation), or combinations of agents targeting several mechanisms of tumour angiogenesis (sprouting, co-option, vasculogenic mimicry and vasculogenesis). However, many questions on current concepts as well as on how anti-angiogenic therapies work still have to be addressed in order to optimise their use in the clinical setting.

Only a translational research approach evaluating anti-angiogenesis agents and strategies in regard to standard therapy through pre-clinical models will be able to bring forward the development of anti-angiogenic therapy protocols for NB. Clinical phase I and II trials will answer the question on dosage, toxicity and efficacy of anti-angiogenic drugs in NB.

Conflict of interest statement

None declared.

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Quantification of Circulating Vascular Endothelial Growth Factor Receptor-3–Positive Lymphatic/Vascular Endothelial Progenitor Cells

To the Editor: We read with interest the article by Bogos et al. (1), which showed that circulating lymphatic/vascular endothelial progenitor cells (LVEPC) are significantly increased in small cell lung cancer (SCLC) patients and correlate with clinical behavior. We wish to comment on several unconvincing issues regarding the methodology and patient characteristics presented in the study.

Circulating LVEPC are an extremely rare cell population in peripheral blood that may contribute to lymphangiogenesis and/or angiogenesis and may be phenotypically identified by combining markers CD34, CD133, and vascular endothelial growth factor receptor 3 (VEGFR3). Unfortunately, the quantification of LVEPC reported by Bogos et al. is confusing. Here, authors recovered a mononuclear cell fraction after erythrocyte lysis (instead of the expected whole leucocyte fraction) to measure LVEPC as $CD34^+VEGFR3^+$ events using a two-color fluorescence-activated cell sorting method in which neither the blood volume nor the number of events accumulated for fluorescence-activated cell sorting analysis was specified. Bogos et al. show what they consider to be a typical analysis (Fig. 1A) in which LVEPC represented 95% of total $CD34^+$ cells, but where morphologic characteristics, isotypic controls, and the gating strategy are cruelly lacking. Moreover, Salven et al. (2) initially reported LVEPC levels in healthy adults of 0.2 ± 0.1% of $CD34^+$ cells, representing approximately 5 LVEPC/mL of blood (based on a mean value of 2,500 $CD34^+$ cells/mL). Here, authors report a median value of 1,625 LVEPC/mL in SCLC patients, which is not only inconsistent with those values reported by Salven et al. but also appears highly improbable in light of the fact that LVEPC, like $VEGFR2^+$ circulating endothelial progenitor cells, represent a tiny fraction of circulating $CD34^+$ cells (3, 4).

Moreover, the prognostic value of LVEPC levels in the SCLC patient group analyzed in this study is very questionable. First, multivariate analysis included neither performance status (a major prognostic factor) nor achievement of a complete response (5). Also, no information on the sequence of radiotherapy and chemotherapy was provided although the time to thoracic radiotherapy has been recently highlighted as a potential factor of treatment efficacy. Finally, the overall survival reported may have been affected by the absence of prophylactic cranial irradiation, which is a standard treatment in responding patients.

Finally, Bogos et al. proposed that LVEPC could be surrogate markers to monitor the efficacy of antiangiogenic therapies in SCLC. Although it is likely that circulating endothelial progenitor cells and LVEPC will become biomarkers in cancer, the authors have omitted that there is currently limited data supporting the clinical interest of such agents in SCLC.

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Expert Opinion

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New anti-angiogenic strategies in pediatric solid malignancies: agents and biomarkers of a near future

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Importance of the field: Antiangiogenic strategies are affording considerable interest and have become a major milestone in therapeutics of various adult cancers. However, progress has been slow to expand such therapies to patients with pediatric solid malignancies.

Areas covered in this review: This review discusses the principal pathways for angiogenesis in pediatric solid malignancies and summarizes recent preclinical and clinical data on antiangiogenesis strategies in these tumors.

What the reader will gain: The reader will gain state-of-the-art knowledge in the current advancements of antiangiogenic therapies in pediatric clinical trials in regard to supporting preclinical data, and in the status of potential biomarkers investigated for monitoring angiogenesis inhibitors. Mechanisms of resistance to antiangiogenic therapy will also be discussed. Finally, we describe our experience in the monitoring of circulating endothelial cells and progenitors and their potential role as biomarkers of metastatic disease and resistance to antiangiogenic therapies.

Take home message: Evaluation and development of antiangiogenesis protocols are starting and represent a crucial step in the management of pediatric solid malignancies today. Emphasis should be placed on the development of proper surrogate markers to monitor antiangiogenic activity and on the possible long-term effects of these therapies in a pediatric population.

Keywords: angiogenesis, antiangiogenesis, biomarkers, cancer, children, circulating endothelial cells, circulating endothelial progenitor cells

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1. Introduction

Childhood cancer remains the major cause of death from disease after the age of 1 year, representing approximately 2500 childhood deaths from cancer each year in Europe [1]. The past decades have witnessed a dramatic improvement in the overall prognosis and survival rates of almost all types of pediatric cancer, reaching up to 75% and mostly due to successful advances in treatment protocols for childhood leukemia [2], although not all types of pediatric malignancies have enjoyed this success. The outcome remains grim in case of metastatic, refractory or recurrent solid pediatric tumors, for which cure may be achieved at the cost of substantial organ toxicity, developmental abnormality and risk of secondary tumors and for which only 25% of relapsing children will remain disease-free [3]. In some subtypes of pediatric solid malignancies, the overall survival after relapse or progression can be

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Article highlights.

- Novel therapeutic approaches are needed to improve prognosis in pediatric patients with refractory or metastatic solid malignancies. Emerging preclinical and clinical trials are supporting the potential role of antiangiogenic agents for these patients.
- Tumor angiogenesis is regulated by a number of pro- and antiangiogenic growth factors against which various molecules have been developed for targeted therapies. These are classified as direct, indirect, or mixed angiogenesis inhibitors.
- Current research is striving to validate biomarkers of angiogenesis and antiangiogenesis strategies. Although further work is needed, circulating endothelial cells and endothelial progenitors are very interesting candidates as potential surrogate biomarkers.
- Angiogenic factors and signaling pathways have been explored in many pediatric tumors and support the role for antiangiogenic therapies in these malignancies.
- Antiangiogenic approaches have been investigated at both the preclinical and clinical level. Clinical trials include the evaluation of metronomic chemotherapy, which has resulted in encouraging preliminary data.
- Antiangiogenic strategies in pediatric solid malignancies are starting and have thus far been encouraging, although further clinical evaluations are needed, especially in regard to possible long-term side effects. Emphasis should be placed on identifying possible biomarkers to monitor antiangiogenic drug activity and efficacy.

This box summarizes key points contained in the article.

as low as 5% or less [4]. Understandably, novel therapeutic approaches are urgently needed to improve prognosis in these patients.

One of the most exciting avenues in the current era of cancer research has been antiangiogenic therapy. For over 50 years, cancer therapy has been dominated by the concept that the tumor must be the selected target and that any drug capable of directly killing the tumor cell was, by definition, a candidate for use as chemotherapy in humans. The approval in 2004 of bevacizumab (Avastin), a neutralizing monoclonal antibody directed against VEGF, as the first antiangiogenic drug to treat cancer patients validated the innovative notion introduced by Dr Judah Folkman 30 years earlier that inhibition of tumor angiogenesis might be a valid and effective approach to treat cancer [5]. Over the past decade, targeted therapies have emerged for the most common adult malignancies with regulatory approvals of bevacizumab [5], sunitinib [6], sorafenib [7], and very recently pazopanib [8]. However, little progress has been made to expand such antiangiogenic strategies to children with solid malignancies. Treatment of high-risk pediatric solid malignancies is still based on surgery, radiation, and high-dose cytotoxic chemotherapy with stem cell support, although the strong angiogenic profile of these malignancies would support the rationale for antiangiogenic approaches. Consistent with this prediction, emerging preclinical data and early Phase I/II clinical trials are demonstrating the potential role of antiangiogenic agents in treating pediatric solid malignancies [9,10].

This review will focus on the current understanding of angiogenesis in pediatric solid malignancies and will describe recent preclinical data on antiangiogenic strategies in relation to their clinical development in pediatrics. It will offer an overview of the state of the art in clinical trials evaluating antiangiogenic therapies in pediatrics. It will also address some of the current obstacles to monitoring angiogenesis inhibitors, some emerging hypotheses on mechanisms of resistance to antiangiogenic therapy, as well as some of the questions raised in concern with secondary effects of antiangiogenic therapies in a pediatric population. Finally, the possible role of monitoring circulating endothelial cells, especially in a pediatric population, as potential 'sensors' of angiogenesis or tumor-mediated resistance mechanisms to antiangiogenic strategies will be discussed.

2. Tumor angiogenesis and development of angiogenesis inhibitors

Tumor angiogenesis has emerged as a critical microenvironmental reaction essential for tumor cell survival by guaranteeing the delivery of oxygen, nutrients and growth factors mandatory for tumor growth and metastatic spread. This dynamic process is tightly regulated by a large number of pro-angiogenic and antiangiogenic factors which include growth factors and their receptors, such as VEGF and VEGF receptors (VEGFR1, 2 and 3), adhesion molecules of the integrin, cadherin (VE and N-cadherin) and immunoglobulin families, extracellular matrix proteins, matrix-degrading proteinases, in particular matrix metalloproteinases (e.g., MMP-2, 9), MMP inhibitors (TIMPS), as well as signaling molecules (e.g., Raf, MAPK, PKA, mTOR) and transcription factors (HIFα, NF-κB) [5,11]. Tumors can promote their own neoangiogenesis by upregulating angiogenic cytokines and/or downregulating natural inhibitory proteins within the microenvironment (**Figure 1**). Although the VEGF/VEGFR pathway is the most well known, other angiogenic growth factors are of importance such as fibroblast growth factor (bFGF) which stimulates all major steps in the angiogenesis cascade and is produced by many cells, including macrophages and tumor cells. Placental-derived growth factor (PlGF), a member of the VEGF family that binds to VEGFR1, is released in large amounts by activated endothelial, smooth muscle, inflammatory and tumor cells, and regulates the VEGF-mediated angiogenic process. Platelet-derived growth factor (PDGF) signaling is involved in pericyte, smooth muscle cell and fibroblast recruitment to newly formed vessels for their stabilization and maturation. Finally, angiopoietins-1, -2, and their tyrosine kinase receptors Tie1 and Tie2 are also involved in angiogenesis. Ang-1 acts as an agonist, stimulating the stabilization of blood vessels through the recruitment of mural cells (pericytes and

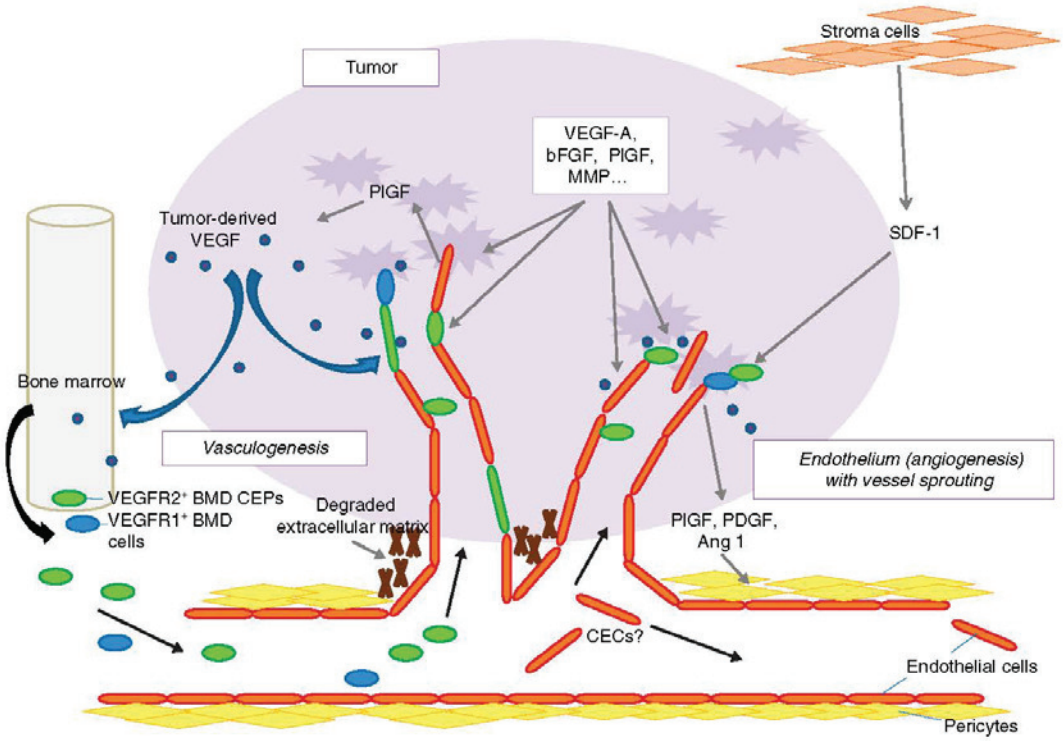

Figure 1. Different mechanisms of tumor angiogenesis. This diagram represents the two main types of vascularization in solid tumors, including sprouting angiogenesis with migration of neighboring mature endothelial cells, and vasculogenesis with the functional incorporation of mobilized VEGFR2$^+$ bone marrow-derived (BMD) circulating endothelial progenitor cells, as well as other bone marrow-derived VEGFR1$^+$ progenitors. Some of the angiogenic growth factors regulating this process are represented.

smooth muscle cells), while Ang-2 is an antagonist with opposite functions (**Figure 1**) [5,11].

The formation of neovessels within the expanding tumor relies on different mechanisms that include the sprouting and co-option of pre-existing vessels with migration of neighboring endothelial cells that organize to create new networks of blood vessels. Pioneering studies evidenced that angiogenesis is also supported by the recruitment and functional incorporation of endothelial progenitor cells that originate from the bone marrow (**Figure 1**) [12,13]. Circulating bone marrow-derived endothelial progenitor cells (CEP) are highly proliferative cells that are mobilized to circulate in the peripheral blood and functionally incorporate into tumor neovessels where they differentiate into endothelial cells, demonstrating that the process of 'vasculogenesis' – the progenitor-cell-driven *de novo* generation of new vessels – is not restricted to embryonic development [12]. Preclinical models have provided solid evidence that CEPs play an important role in tumor progression as well, and contribute to tumor neovessels in cancer-bearing animals [14,15] and in humans [16]. Moreover, inhibition of CEP recruitment prevents tumor growth in experimental models [15,17]. Variable degrees of CEP incorporation in different tumor models have led to controversy about the extent of their involvement in tumor vascularization, although recent studies have begun to recognize the biological significance of CEP contribution [18-20]. Finally, the process of metastasis appears to be angiogenesis-dependent: new entry sites into the circulation are provided by the penetration of neovessels into the primary tumor and by protease-driven disruption of the basement membrane, thereby increasing the tumor's metastatic potential [21]. Moreover, the successful growth of metastases in distant organs relies on a pro-angiogenic microenvironment capable of forming neovessels. Interestingly, CEPs have recently been demonstrated to play a catalytic role in the progression of micrometastases to macrometastases in preclinical tumor models [22].

Inhibition of tumor-induced angiogenesis has therefore attracted great interest as a promising approach to treat cancer. The identification of molecular pathways regulating and mediating angiogenesis, such as the VEGF/VEGFR pathway,

has opened the possibility of selective targeted therapies [5,6]. Over the past decade, hundreds of molecules with antiangiogenic activity in experimental models have been described and about 100 have entered clinical testing in cancer patients [23]. Different strategies are currently under investigation, including antibodies and small molecules directed against angiogenic factors or their receptors, as well as a number of endogenous angiogenesis inhibitors.

Three classes of angiogenesis inhibitors can be distinguished: direct, indirect and mixed inhibitors (**Figure 2**). Direct angiogenesis inhibitors target directly the activation of endothelial cells through mechanisms independent of tumor cell-elaborated angiogenic factors. Indirect inhibitors block the production or activity of pro-angiogenic molecules produced by the tumor itself or their receptors. The so-called 'mixed' angiogenesis inhibitors, such as multipotent receptor tyrosine kinase inhibitors (TKI), affect both tumor endothelial cells and malignant cells. It is of note, however, that although these compounds are categorized as distinct agents, most have overlapping mechanisms of action. Another approach to targeting tumor angiogenesis concerns a different class of drugs known as vascular disrupting agents (VDAs). In contrast to antiangiogenic agents, which prevent the formation of new blood vessels, VDAs target the already existing tumor vasculature, causing acute collapse of established vasculature and extensive tumor necrosis [24]. VDAs will not, however, be discussed in this review.

Compared with conventional cytotoxic cancer treatment, antiangiogenic strategies offer several advantages: i) disruption of a single tumor blood vessel impacts a high number of tumor cells dependent on its blood supply; ii) antiangiogenic therapy appears to 'normalize' tumor vessels, resulting in improved delivery and efficacy of chemotherapy agents; iii) targeting endothelial cells may help to control minimum residual disease due to quiescent cells that are not accessible to standard chemotherapy; and iv) antiangiogenic drugs are not as likely to cause toxicities such as gastrointestinal symptoms and myelosuppression that are characteristic of standard chemotherapy.

The vast majority of antiangiogenic drugs have demonstrated efficacy in patients – but only when used in combination with standard chemotherapy, with the exception of renal cell carcinoma [25] and malignant glioma [26]. One hypothesis to explain this synergy is 'vascular normalization', which proposes that antiangiogenic drugs could have the effect of pruning the number of tumor vessels and of normalizing their function, thereby increasing the delivery and therefore the efficacy of cytotoxic agents [27]. Also, recent studies have evidenced that administration of conventional or high-dose chemotherapy could cause vascular 'rebounds' with endothelial and subsequent tumor cell recovery afforded during drug-free rest periods, a process that could counteract the anticancer effects of chemotherapy [19]. Consequently, combining antiangiogenic molecules with chemotherapy would appear logical; but only if done with timely scheduling so as to target CEPs potentially mobilized during bone-marrow regeneration to restore tumor angiogenesis.

Despite the clinical achievements observed with antiangiogenic therapies targeting the VEGF/VEGFR pathway, these agents are unexpectedly producing only transient clinical responses, as demonstrated by the limited advantage in progression-free survival and no advantage in overall survival observed in clinical trials. After an initial but short-lived clinical response, tumors inevitably begin to regrow and progress, suggesting an emergent resistance to antiangiogenic therapies (reviewed in [28]). This resistance could rely on multiple mechanisms, including therapy-induced pro-angiogenic pathways that circumvent the drug-targeted specific pathway [28,29]. Recently, an emerging body of evidence is indicating that antiangiogenic therapy could have an insidious effect as a driving force promoting tumor dissemination, increased tumor invasiveness and metastasis formation [30,31]. The important clinical implications of these somewhat disturbing reports warrant clinical studies to confirm these findings and perhaps test additional drug combinations aimed at abrogating these paradoxical effects.

3. Biomarkers of antiangiogenic therapies

In spite of the abundance of antiangiogenesis clinical trials, an important dilemma remains: how do we monitor the activity or efficacy of antiangiogenic drugs? Classical biomarkers of response to conventional cytotoxic therapy rely on tumor shrinkage and include measurements of tumor size (WHO or RECIST criteria), disease progression and survival, and may therefore be unsuitable for predicting benefit from antiangiogenic drugs, which are cytostatic and have an indirect effect on tumor growth. Biomarkers of response to antiangiogenic agents should be able to inform on optimal dosage, early or ongoing clinical effect or benefit, and development of resistance in patients, thereby implying noninvasive markers suitable for frequent measurements. Also, in case of tumor escape to treatment, biomarkers should also be able to guide the choice of second-line therapy or help to select efficacious treatment combinations. Various approaches to monitor antiangiogenic effects in patients have been investigated, but no surrogate biomarker has been validated to date for routine use in clinical oncology.

Vascular imaging techniques have been studied for monitoring tumor perfusion, drug efficacy and measurements of objective vascular response to antiangiogenic therapies (reviewed in [32]). Various imaging modalities are being validated in preclinical and clinical studies with good evidence to support the potential of dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), dynamic computed tomography, positron emission tomography, and ultrasound-based techniques. Vascular imaging could be an advantage to assess combined strategies with simultaneous measure of tumor vascularity (marker of antiangiogenic efficacy) and size (marker of cytotoxic efficacy).

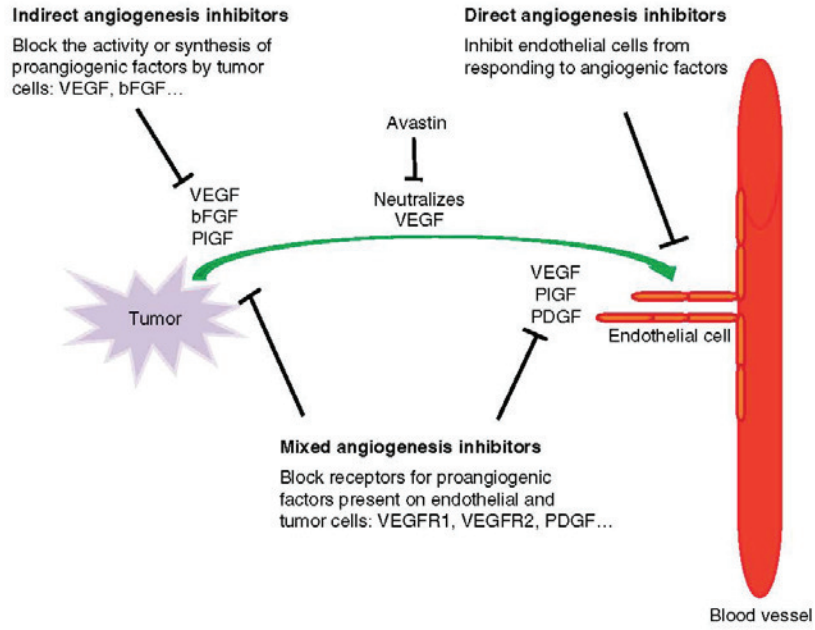

Figure 2. Mechanisms of action of targeted therapies. The three classes of angiogenesis inhibitors are represented, with a schematic illustration of the molecules and targets.

Histological tumor microvessel density is an independent prognostic marker of recurrence, metastasis and survival in various tumors including prostate, gastric, colorectal cancers and melanoma [33]. However, microvessel density requires invasive measures and has not been shown to be a useful predictive marker or indicator of therapeutic efficacy [33]. Circulating levels of angiogenic growth factors such as VEGF and sVEGFR2 have previously been demonstrated to correlate with tumor burden as well as with disease progression in patients with various tumor types [34]. While elevated levels generally indicate poor prognosis, angiogenic factors have poor predictive values of drug activity and efficacy [35,36]. Moreover, rising plasma levels of VEGF and other angiogenic factors have been reported after treatment with antiangiogenic drugs [7], probably via treatment-induced hypoxia and positive feedback mechanisms. Overall, the significance of circulating angiogenic factors in terms of predicting drug efficacy and clinical benefit is unclear, especially since drug-induced increases might be confused with increases due to tumor escape or resistance. Recently, a report by Hanrahan and colleagues investigated 35 plasma biomarkers in patients with NSCLC undergoing combined treatment with vandetanib, a VEGFR2 TKI [37]. This study could be a fruitful approach towards answering which candidate biomarkers will have practical utility and should be explored in patients undergoing antiangiogenic therapy [38].

Because the formation of new blood vessels relies on the recruitment of bone marrow-derived CEPs and possibly on circulating mature endothelial cells (CEC), these cell populations have been explored as potential surrogate biomarkers of tumor-associated angiogenesis and antiangiogenic treatments. In preclinical models, CEC and CEP levels are sensitive to pro- and antiangiogenic influences [39]. CECs are thought to shed from blood vessel walls into the circulation as the result of vascular injury; their numbers in peripheral blood directly reflect the extent of endothelial insult. While CEC counts are currently considered as a useful biomarker of vascular damage in patients with vascular disorders [40] their role in tumor angiogenesis is less clear. CECs have been detected using flow-cytometry (FCM) [41-43], but have been observed in very high numbers in cancer patients (1 – 39,000/ml) and in healthy individuals (1 – 7,900/ml) compared with the values reported with the method of reference, immunomagnetic separation (IMS) (< 10/ml) [44]. These findings, as well as the lack of consensus on the appropriate method for CEC measurement, have yielded conflicting data and raised considerable debate as to the reliability of the FCM method for CEC measurement [41,42,45-47]. Initial data on CECs in patients with pediatric malignancies were reported in a pediatric Phase I trial evaluating bevacizumab [48]. In this trial, 'mature CEC levels' were monitored in six children undergoing antiangiogenic therapy for refractory solid tumors and found

to range from 0 to 12,000/ml using FCM, although the cells identified as 'CECs' here could in fact be large platelets [46]. In a previous study, we identified CECs using a novel four-color FCM assay and showed that CEC levels were within a similar range to the values reported with IMS. Levels of CECs in healthy adults (6.5/ml [0 – 15/ml]) differed significantly from those detected in adult patients with metastatic carcinoma (15.0/ml [0 – 179/ml]), $p < 0.001$) [49]. Moreover, we recently demonstrated for the first time that this novel FCM assay was applicable to a pediatric population, in which CEC levels were easily detected [50]. There is currently no study demonstrating the exact role of CECs in angiogenesis, despite the increased levels of CECs observed in preclinical tumor models and in cancer patients. Most likely, the CECs observed in patients originate from the tumor vessels, where the high degree of vascular instability and anarchic turnover in vessel formation under the influence of pro-angiogenic growth factors leads to an intense egression of CECs from the tumor. This hypothesis would be in line with the fact that higher CEC levels are observed in patients with metastatic disseminated, and consequently a more 'aggressive' disease from a biological perspective.

CEPs have been actively investigated for their role in angiogenesis-mediated tumor growth. In spite of much controversy due to their variable contribution to tumor neovessels, preclinical studies have provided solid evidence that CEPs are not only key contributors to tumor neoangiogenesis [14,15,19], but are also critical regulators of the angiogenic switch promoting metastatic progression [22]. Moreover, preclinical and clinical data have suggested that CEPs may be rapidly recruited to tumor sites in response to VDAs and certain chemotherapy drugs, thus stimulating tumor growth and progression [18,19]. Recombinant G-CSF is often used to reduce myelosuppression in patients receiving chemotherapy, but could adversely promote tumor angiogenesis by increasing the number of CEPs. Consequently, blocking this therapy-induced CEP mobilization may be essential to counteract its negative impact on anticancer efficacy [20,51]. We have reported therapy-induced CEP mobilization in a small series of cancer patients included in a Phase I trial combining the VDA AVE8062 (Sanofi-Aventis) with cisplatin, in whom CEP levels peaked 3 – 7 days after drug injection [52]. Moreover, we demonstrated for the first time that high levels of bone marrow-derived $VEGFR2^+$ cells correlated with metastatic disease in patients with pediatric solid malignancies [50]. Consequently, CEPs could be 'circulating sensors' allowing to monitor the angiogenic status of a patient before or during treatment. Recently, an interesting study by Roodhart and colleagues evaluated the kinetics of CECs, CEPs and modulatory cytokines in adult patients undergoing chemotherapy [53]. At days 7 and 21 after treatment, authors observed a large and important increase in cell levels, independently of the type of chemotherapy. Moreover, the magnitude of the increase in CEC and CEP levels after chemotherapy correlated with response and survival. These preliminary data are promising, and both studies support the biological relevance of these cells in patients' status and prognosis [50,53]. However, the current understanding of the mechanisms that regulate CECs and CEPs is still at an early stage and their respective roles in tumor response or tumor escape are yet to be defined. Finally, CECs and CEPs are being evaluated in adult clinical trials with antiangiogenic therapies for their potential role as biomarkers of response [54,55], although further work is needed in order to validate these cells as surrogate biomarkers of angiogenesis and antiangiogenic strategies.

4. Rationale for targeting angiogenesis in pediatric tumors

Increased microvessel density within primary tumor sites has been shown to correlate with the likelihood of metastasis and to have prognostic value in many cancers, including pediatric solid malignancies such as neuroblastoma [56]. Pediatric solid malignancies are almost exclusively undifferentiated tumors characterized by a high proliferation rate and increased vascularity, contrasting with adult solid malignancies, which derive mainly from epithelial tissue and have low proliferation rates and vascularity. Although it is difficult to predict the clinical effectiveness of angiogenic drugs on the basis of the expression pattern of angiogenic factors, growing preclinical evidence indicates that these factors play an important role in the development and progression of pediatric solid tumors [56,57].

Neuroblastoma (NB) and CNS tumors represent the most frequent solid tumors and have therefore received special attention in regard to research on antiangiogenic strategies. Meitar and co-workers were the first to demonstrate that tumor vascularity was an important and independent prognostic variable in NB, strongly correlated with widely disseminated disease, amplification of *MYCN*, unfavorable histology and poor survival [56]. Recently, we reviewed current data on angiogenesis and antiangiogenesis strategies in NB [10]. The expression of angiogenic factors and signaling pathways has been explored in pediatric CNS tumors (reviewed in [57]) and supports the prediction that these tumors are appropriate candidates for antiangiogenic therapy.

5. Antiangiogenic approaches in pediatric tumor models

A partial list of major angiogenesis inhibitors evaluated in pediatric preclinical models and their presumed mechanism of action is found in **Table 1**. Direct inhibitors of angiogenesis have been investigated in preclinical models of pediatric tumors and include TNP-470, thalidomide, endostatin, angiostatin, and thrombospondin-1 (**Table 1**).

Indirect inhibitors account for a large percentage of the molecules currently used in clinical trials, and all rely to some extent on the tumor's continued secretion of VEGF as the dominant stimulator of angiogenesis. The VEGF family

Table 1. Pre-clinical data on antiangiogenic approaches in pediatric solid tumors.

Agent*	Mechanism of action	Experimental models	Ref.
Direct inhibitors			
TNP-470	Synthetic analogue of fumagillin; inhibitor of an important enzyme for endothelial proliferation and migration, known as methionine aminopeptidase-2 (MetAP2)	Subcutaneous injections of TNP-470 every other day resulting in a reduction of tumor growth rate and microvascular density in NB xenografts TNP-470 administered 3 days/ week for 12 weeks after which 53% of treated mice remained tumor free. Decreased growth rate in treated mice with small NB xenograft tumors but not larger tumors	[94,95]
Thalidomide	Exact mechanism unknown, perhaps modulates integrins	Suppressed angiogenesis, reduction in tumor vasculature, and induced apoptosis of endothelial cells in NB models without reduction in tumor growth Inhibition of tumor growth in malignant glioma model when combined with temzolomide	[96,97]
Endostatin	Proteolytic fragment of collagen XVIII that inhibits endothelial cell proliferation	Significant NB tumor regression when given in continuous infusion	[98]
Angiostatin	Binding of ATP synthase, causing tumor cell apoptosis and inhibition of endothelial migration	No delay in NB xenograft tumor growth in a gene therapy approach using recombinant adenovirus coding the human angiostatin kringle 1 – 3 directly fused to human serum albumin (AdK3-HSA)	[99]
Thrombospondin (TSP-1)	Matrix-bound glycoprtotein, inhibiting endothelial migration and proliferation	ABT-510, a synthetic derivative of TSP-1, significantly suppressed growth of NB xenografts alone and was more efficient in combination with valproic acid	[100]
Indirect inhibitors			
Anti-VEGF antibody		Inhibition of vessel cooption in tumor vasculature and subsequent reduction in tumor growth in xenograft models of NB Inhibition of primary tumor growth and metastasis in Wilm's tumor Inhibition of neoangiogenesis and significant suppression of tumor growth in hepatoblastoma Significant tumor regression of large NB xenografts when given in combination with low-dose vinblastine	[59-61,64]
Mixed inhibitors (TKI) Target TK			
Imatinib (STI-571, Glivec®)	c-Kit, PDGFR-alpha, PDGFR-beta, c-Abl	Enhanced effect of ionizing radiation in models of	[66-69]

*This table is an incomplete list of antiangiogenic agents and studies. There is no single source of listing for pediatric solid tumor anti-angiogenesis trials. Individualized institutions may have other studies available. Further information is provided at the www.cliniclatrial.gov homepage.

Table 1. Pre-clinical data on antiangiogenic approaches in pediatric solid tumors (continued).

Agent*	Mechanism of action	Experimental models	Ref.
		glioblastoma multiforme, with increase in tumor growth delay and survival Reduction of tumor volume in NB mouse models, though sensitivities were not correlated to expression levels of imatinib-responsive targets Regression or control of tumor growth in Ewing's sarcoma model	
Sunitinib (SU11248, Sutent®)	PDGFR, VEGFR, Kit, Flt-3	Potent anti-tumor activity in NB xenograft model both in early stage of tumor and in metastatic disease with synergistic activity when combined with an mTOR	[70]
Sugen (SU5416, Semaxinib®)	VEGFR-1, -2	Antitumor activity in CNS orthotopic xenograft models, implicating that penetration of the blood-barrier was not necessary Efficacy in NB xenograft models alone or in combination with irradiation or chemotherapy	[71,72]

*This table is an incomplete list of antiangiogenic agents and studies. There is no single source of listing for pediatric solid tumor anti-angiogenesis trials. Individualized institutions may have other studies available. Further information is provided at the www.clinicaltrial.gov homepage.

includes seven secreted glycoproteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, PIGF-1 and PIGF-2. Their biological effects occur by binding to three different TK receptors: VEGFR-1, VEGFR-2 and VEGFR-3, all of which are present on endothelial cells [5]. The development of a monoclonal humanized anti-VEGF antibody was first reported to be effective in inhibiting tumor angiogenesis and subsequent growth in preclinical models of human rhabdomyosarcoma and glioblastoma multiforme [58]. Since then, preclinical models of NB, Wilm's tumor, and hepatoblastoma have been demonstrated to respond to inhibitors of the VEGF pathway [59-61], as well as to antiangiogenic scheduling of cytotoxic agents alone or in combination with VEGF blockade [61-64]. Gene therapy approaches to deliver angiogenesis inhibitors have also been explored in pediatric tumors, including NB [65].

Mixed inhibitors of angiogenesis are largely represented by receptor TKI. Available preclinical data evaluating TKI in different pediatric tumor entities are summarized in Table 1 and include studies demonstrating the efficacy of imatinib and sunitinib in experimental pediatric tumor models [66-70]. Synergistic cytotoxicity was observed when sunitinib or SU5416 were combined with irradiation [71], cytotoxic chemotherapy [72], and mammalian target of rapamycin (mTOR) [70]. We recently reported an overview of TKI with special focus on their potential in pediatric solid tumors [9].

Overall, these data emphasize that angiogenic blockade in pediatric tumors is an extremely interesting strategy that deserves further investigation, as it may help to optimize current chemotherapy regimens when given with careful consideration of drug scheduling.

6. Clinical trials in pediatric solid malignancies

In spite of the abundance of promising preclinical data, the number of clinical studies evaluating antiangiogenic therapies in pediatric solid malignancies is limited and several years behind the status of adult malignancies. Some of the clinical studies available today are presented in Table 2.

Thalidomide was evaluated in a Phase II study in combination with radiation in children with brainstem glioma and glioblastoma, but did not demonstrate efficacy and even displayed increased toxicity, as shown by more extended courses of high-dose steroids [73]. In another Phase II trial, thalidomide was tested in combination with cyclophosphamide in 27 patients with refractory solid malignancies with little evidence of efficacy despite modest toxicity [74].

Two Phase II studies evaluated treatment with imatinib in pediatric solid tumors. The first study performed by the Children's Oncology Group (COG) was disappointing, with only one partial response (PR) in a patient with Ewing's tumor and no response in other tumor types [75]. However, the second study of the European Consortium Innovative therapies for Children with Cancer (ITCC) evidenced durable

Table 2. Clinical data on anti-angiogenic strategies in pediatric solid tumors.

Agent*	Study design	Results	Ref.
Thalidomide	Thirteen patients (2 – 14 years old) with glioma or glioblastoma; once daily and continued for 12 months; starting dose 12 mg/kg. None completed the course	Median time to progression (TTP) was 5 months (range 2 – 11 months) and median time to death (TTD) was 9 months (range 5 – 17 months). Addition of thalidomide to radiation did not improve TTP or TTD	[73]
Thalidomide	27 patients, median age 15 years (range 1 – 54 years); thalidomide (6 – 12 mg/kg/day) and CPM (1,200 mg/m2 IV every 28 days)	21 patients assessable: 1 PR, 1 SD, 19 progressive disease. Modest toxicity but little evidence of efficacy	[74]
Imatinib Phase II study (COG)	Patients aged less than 30 years with refractory or recurrent solid tumors; 440 mg/m^2/day administered daily for 28 day courses	59 evaluable patients and only one PR among 24 Ewing's tumor	[75]
Imatinib Phase II study (ITCC)	Single agent in children and adolescents with refractory or relapsing solid tumors; 340 mg/m^2 daily during a total of 168 months (median 1.9 month/patient, range 0.5 – 19) Ten patients escalated to 440 mg/m^2 due to lack of efficacy	36 patients (12 brain tumors, 6 fibromatosis, 8 mesenchymal/ bone tumors, 10 other solid tumors, including 1 GIST and 3 chordoma) No CR or PR; 5 pts experienced durable SD under treatment for more than 12 months	[101]
SU5416 Phase I	Designed to assess MTD and toxicity profiles Dose escalations: stratum I starting at 110 mg/m^2; stratum II started at 48 mg/m^2	33 eligible patients: 23 glial tumors, 4 neural tumors, 4 ependymomas, and 2 choroid plexus carcinomas Prolonged disease stabilization was observed in 4 of 16 stratum I patients with refractory brain tumors	[76]
Bevacizumab	Compassionate use in 15 patients (median age, 14.6 years) with recurrent or progressive solid tumors. 5 – 10 mg/kg IV every 2 – 3 weeks; most patients received chemotherapy in addition	Bevacizumab-related side-effects were mild Radiographic objective responses (partial responses) were observed in two patients suggesting some antitumor activity in heavily pretreated children	[78]
Bevacizumab Phase I	Determination of MTD and DLT, toxicity profiles; 5, 10, 15 mg/kg given IV every 2 weeks in 28-day courses	20 patients aged 1 – 18 year; treatment well tolerated No objective responses were observed, however 5 patients had stabilized disease for more than 3 months	[48]
Bevacizumab	Combination of bevacizumab and irinotecan in 10 patients with multiply recurrent low-grade gliomas	7 patients with objective neuroradiographic response: 1 CR, 3 PR, 3 minor response. Clinical improvements were noted in 7 patients Response was durable and six patients remained on treatment, for up to 22 months	[79]
Cilengitide Phase I	Determination of MTD and DLT, toxicity profiles. Cilengitide over 1 h twice a week for up to 52 weeks at dosages from 120 to 2,400 mg/m^2	31 patients with refractory brain tumors 1 CR in a glioblastoma patient, 2 SD and 1 SD for more than 5 months	[80]

*This table is an incomplete list of antiangiogenic agents and studies. There is no single source of listing for pediatric solid tumor anti-angiogenesis trials. Individualized institutions may have other studies available. Further information is provided at the www.clinicaltrial.gov homepage.
CPM: Cyclophosphamide; CR: Complete response; DLT: Dose limiting toxicity; MTD: Maximal tolerated dose; NB: Neuroblastoma; PR: Partial response; SD: Stable disease.

stable disease (SD) in seven patients treated with imatinib (Table 2).

The results of the first pediatric Phase I study of evaluating SU5416 in patients with CNS tumors were recently reported [76]. While initially designed to assess maximum tolerated doses and toxicity profiles, this study demonstrated that SU5416 displayed potentially interesting activity with prolonged disease stabilization (responses) observed in four of 16 patients with refractory brain tumors. However, the clinical development of SU5416 has been halted in part due to its limited single-agent activity and the significant toxicity related to SU5416-dependent co-administration of cremophor. Other trials of single-agent small-inhibitor therapy in children with CNS tumors, especially high-grade glioma, have demonstrated tolerability but little activity [77], perhaps due to the heterogeneity of tumors evaluated in these trials.

Until 2008, clinical use of bevacizumab in pediatric patients was only reported sporadically. Three studies have, however, evaluated bevacizumab in children with refractory or recurrent solid tumors. Given on a compassionate basis to 15 patients, the first study reported limited side effects and even demonstrated four partial responses in heavily pretreated children and young adults [78]. The second report was a Phase I dose-escalating study performed in 20 patients aged 1 – 18 years [48]. Treatment was well tolerated, with no dose-limiting toxicities among the 18 assessable patients. In five patients, disease could be stabilized for > 3 months, although no objective responses were observed [48]. Packer and colleagues recently reported that children with multiple recurrent low-grade gliomas were responsive to bevacizumab in combination with irinotecan [79]. Among the 10 patients treated, clinical improvements were noted for seven. Also, seven patients had an objective neuroradiographic response (one complete response, three partial responses and three minor responses), which was durable in the majority of cases. An open-label, multicenter, randomized Phase II study has recently opened to evaluate the addition of bevacizumab to chemotherapy in childhood and adolescent patients presenting with metastatic rhabdomyosarcoma and non-rhabdomyosarcoma soft-tissue sarcoma.

Cilengitide is a potent and selective antagonist of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, which allow endothelial cells to attach to the extracellular matrix and are therefore particularly important in angiogenesis. Cilengitide has demonstrated tumor growth inhibition in a dose-dependent manner in various preclinical tumor models. The results of the first trial evaluating cilengitide in pediatric patients with refractory brain tumors reported no dose-limiting toxicities among the 31 assessable patients, although three patients experienced grade 3 or 4 intratumoral hemorrhaging [80]. The responses evidenced were particularly encouraging for this agent in pediatric brain tumors, as one patient with glioblastoma multiforme demonstrated complete response, two patients had stable disease, and one additional patient had stable disease for > 5 months.

Sunitinib was recently evaluated by the Pre-clinical Pediatric Testing Program and demonstrated antitumor activity with tumor growth delay against many of the preclinical models tested, except NB and acute lymphoblastic leukemia [81]. Sunitinib will be evaluated in a Phase I study in children with refractory solid tumors (ccr.nci.nih.gov/trials). Similarly, sorafenib will be investigated in a Phase I study of Raf kinase and RTKI Bay 43-9006 (sorafenib) in children with refractory solid tumors or refractory leukemias (ccr.nci.nih.gov/trials). Also, the COG has a Phase I trial open to evaluate VEGF-Trap in children with refractory solid tumors. Finally, AZD2171, a potent and orally bioavailable selective inhibitor of VEGFR, was screened for activity against preclinical childhood cancer models of the Pediatric Preclinical Testing Program [82]. Antitumor activity was evidenced in 78% of solid tumor xenografts, with broad tumor growth inhibition but less tumor regression. AZD2171 is currently being investigated in open Phase I trials in children and adolescents with refractory and recurrent solid tumors or acute myeloblastic leukemia (ccr.nci.nih.gov/trials), as well as in children with recurrent or progressive CNS tumors (Pediatric Brain Tumor Consortium Study PBTC-020).

7. Metronomic chemotherapy

Another strategy to target the tumor endothelium is by administering low doses of conventional chemotherapeutic agents in a combined schedule, known as low-dose metronomic chemotherapy [83]. This concept has arisen from several observations: first, conventional cytotoxic drugs have antiangiogenic effects that could contribute to their antitumor efficacy [84]; second, these effects could be optimized by administering these drugs in small doses on a frequent uninterrupted schedule for prolonged periods; and third, conventional chemotherapy administered at more toxic 'maximum tolerated doses' requires drug-free periods between treatment cycles, which could in fact counteract the potential for sustained antiangiogenic effects [20,83]. The promise of metronomic chemotherapy includes the antiproliferative effects of certain cytotoxic agents on endothelial cells as well as their reduced toxicity, where clinical benefit may be realized especially when combined with other antiangiogenic agents and/or conventional maximally tolerated doses of chemotherapy. Metronomic therapy is similar to the various maintenance chemotherapy regimens that are already being used in pediatric protocols and have demonstrated antiangiogenic effects [83]. The potential antiangiogenic effect of metronomic chemotherapy can also be boosted by combination with a targeted antiangiogenic agent.

In adult oncology, metronomic chemotherapy with low-dose oral drugs such as cyclophosphamide and methotrexate has been evaluated in clinical trials in combination with bevacizumab, with particularly encouraging results reported in metastatic or advanced breast cancer [54,55] as well as recurrent ovarian cancer (reviewed in [85]). Antiangiogenic (low-dose or

metronomic) chemotherapy has been tested in several pediatric malignancies with demonstrated activity in some cases (reviewed in [86,87]). In a study by Sterba and colleagues, 22 patients with relapsed solid tumors were treated with the COMBAT (combined oral maintenance biodifferentiating and antiangiogenic therapy) protocol using celecoxib, 13-*cis*-retinoic acid and cycles of metronomic temozolomide and low-dose etoposide [88]. Treatment was well tolerated by patients who had received prior intensive therapy, and nine of the 14 patients assessable for response demonstrated treatment benefit with prolonged disease stabilization or response. In another study by Stempak and co-workers, 32 patients with recurrent pediatric solid tumors were treated with celecoxib in combination with vinblatine or cyclophosphamide [89]. Four patients (13%) had durable stable disease (28 – 78 weeks), although no complete or partial responses were observed. Recently, a metronomic regimen associating carboplatin–vincristin–fluvastatin with thalidomide was evaluated in a Phase II trial for children with brain stem glioma [90]. Among the nine patients included, all presented a significant reduction in tumor volume and an increase in survival at 24 months, with acceptable toxicity.

Hopefully, future clinical trials will continue to support the potential promise of this therapeutic strategy, especially when integrated in combined strategies with antiangiogenic drugs. The measurement of CECs and CEPs could be useful in evaluating the optimal 'low dose' of a given metronomic chemotherapy regimen.

8. Long-term effects of angiogenesis inhibitors

One of the major hurdles to overcome is the question of long-term effects of antiangiogenic therapies in a pediatric population. Recently, careful analysis of the vasculature of several normal organs of mice treated with VEGFR TKI revealed significant capillary regression in various tissues including pancreatic islets, thyroid, adrenal cortex, pituitary, choroid, and small intestine villi [91]. Regression was dose-dependent and most capillaries grew back after cessation of treatment. Potentially life-threatening events (arterial thrombotic events and gastrointestinal perforation) have occurred in a small number of adult patients, although in young children and adults free of cardiovascular risk factors, these side effects may be rare. However, because of the growing organism of a child or an adolescent, toxicities unknown to this day may play a more important role in the future, such as skeletal complication or CNS maturation defects, as well as other processes that may in fact be angiogenesis-dependent.

9. Conclusion

Although the field of antiangiogenesis therapies in pediatric solid malignancies is new, it is starting. Initial studies are reporting some clinical benefit and are also reassuring clinicians as to the limited acute toxicity observed in pediatric patients compared with adults. These encouraging preliminary data motivate for further clinical evaluations of angiogenesis inhibitors in pediatric solid malignancies. Antiangiogenic drugs in pediatric clinical trials are being evaluated in end-stage refractory patients and are introduced at a time of bulk residual, relapsed or progressive disease when, in fact, their role as disease stabilizers would appear to make them ideal for use at a time of minimal residual disease, after or in combination with traditional cytotoxic therapies. Preliminary studies performed in pediatric patients with end-stage refractory disease may therefore underestimate antiangiogenic activity and should not dampen the testing of these drugs in more appropriate populations. Carefully designed studies will be critical in determining how to evaluate the role of these agents in up-front therapy, as well as how and when to use them best.

The evaluation of antiangiogenic agents and the development of antiangiogenesis therapy protocols for pediatric solid malignancies will be a critical step forward to treating children with cancer in the future. Special attention to possible long-term effects of these therapies in a pediatric population will be, however, of utmost importance. Emphasis should be placed on the development of proper surrogate markers to monitor antiangiogenic therapy in order to optimize drug activity and to provide a way of tracking therapeutic efficacy. Measurements of CECs, and more importantly CEPs, could be potentially useful and incorporation of their monitoring into future clinical trials would appear to be of great interest in pediatric solid malignancies.

10. Expert opinion

The recent clinical achievements of antiangiogenic drugs have raised some rather provocative questions as to how these drugs actually work and subsequently, how tumors could 'adapt' to these treatments [23,28]. Indeed, in both preclinical and clinical settings, escape from antiangiogenic therapy has been evidenced as inevitable with tumor growth and progression rapidly resuming after an initial but transient effect. By creating a critically hypoxic environment within tumors, antiangiogenic drugs may be 'forcing' tumor cells to adapt aggressively and perhaps even migrate to non-hypoxic locations [30]. Resistance to antiangiogenic therapy has been a disappointing awakening, summoning researchers and clinicians to quickly gain a better understanding of the conundrum in targeting tumor angiogenesis.

Although escape from antiangiogenic targeting is likely to involve multiple mechanisms, emerging mechanistic data support the proposition of two general modes of resistance: evasive resistance, consisting in an adaptation to circumvent the specific angiogenic blockade, and intrinsic or pre-existing indifference to an antiangiogenic agent (reviewed in [28]). Current experimental evidence suggests that evasive resistance includes at least four hypoxia-driven adaptive processes: i) revascularization via upregulation of alternative pro-angiogenic pathways;

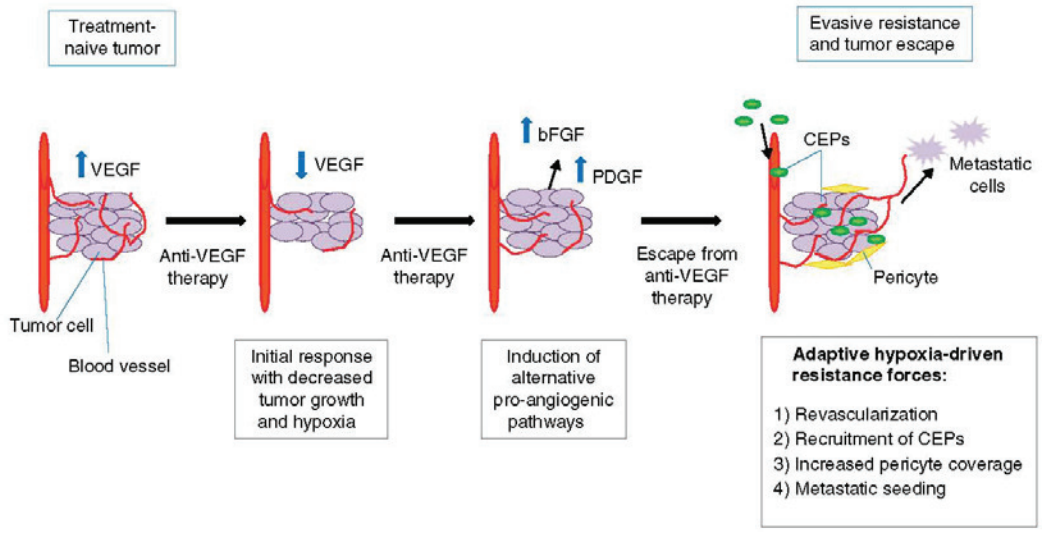

Figure 3. Mechanisms of resistance to antiangiogenic therapy. Resistance and escape from antiangiogenic targeting is likely to involve multiple mechanisms. For evasive resistance, mechanistic hypotheses include at least four hypoxia-driven adaptive processes: first, revascularization via upregulation of alternative pro-angiogenic pathways; second, recruitment of bone marrow-derived progenitor cells for reinitiation of tumor angiogenesis and growth; third, protection of tumor vasculature by recruiting pro-inflammatory cells or by increasing pericyte coverage; and finally, enhancement of metastatic seeding.

ii) recruitment of bone marrow-derived progenitor cells for reinitiation of tumor angiogenesis and growth; iii) protection of tumor vasculature by recruiting pro-inflammatory cells or by increasing pericyte coverage; and iv) enhancement of metastatic seeding (**Figure 3**). Interestingly, two of these four major mechanisms implicate bone marrow-derived progenitor cells, i.e., possibly CEPs, which could subsequently be monitored and targeted if clearly evidenced to play a role in this mode of resistance.

One hypothesis invoked to increase the efficacy of antiangiogenic therapies and possibly circumvent resistance mechanisms stems from pioneering studies evidencing that tumor repopulation and regrowth could be driven by potent CEP-mediated revascularization induced by conventional cytotoxic therapies [18,19]. Using VDAs in several experimental models, authors evidenced therapy-induced vasculogenic 'rebounds' characterized by a reactive systemic host response with the mobilization of bone marrow-derived circulating cells, including CEPs, which homed to the vasculature of treated tumors and promoted tumor neovascularization and subsequent regrowth [18]. Recent evidence supports that cytotoxic therapies display similar effects [19]. Moreover, preclinical data are providing substantial proof that therapy-induced, and perhaps hypoxia-driven, vasculogenic rebounds with tumor cell repopulation could be slowed or blocked by administering an antiangiogenic agent during the drug-free periods in between successive cycles of chemotherapy [19,92]. The mechanistic insights evidenced by these data suggest that antiangiogenic therapies could increase the efficacy of conventional cytotoxic chemotherapy by targeting the CEPs mobilized by these very same cytotoxic agents [20].

Of major importance, cytotoxic chemotherapy is very often accompanied by hematopoietic growth factor support using recombinant GCSF to accelerate recovery from myelosuppression by mobilizing hematopoietic stem cell progenitors from the bone marrow. However, as mentioned previously, there is evidence that GCSF also mobilizes CEPs [51,93]. Consequently, chemotherapy-induced vasculogenic rebounds could be greatly amplified and possibly sustained by co-administration of GCSF, thereby adversely promoting tumor vasculogenesis and increasing the risk of relapse. These findings may urge caution when using GCSF in patients with residual tumors.

Overall, as evidenced by current research, CEPs appear to play a critical role in three majors tumor angiogenesis escape mechanism: i) resistance to standard chemotherapy through cytotoxic-induced vasculogenic rebounds, perhaps aided by GCSF; ii) resistance to antiangiogenic agents through hypoxia-induced CEP mobilization; and iii) promotion of metastatic seeding and progression via neovascularization at metastatic sites.

We believe that antiangiogenic strategies are promising for patients with pediatric malignancies. However, questions regarding optimal combined strategy scheduling and resistance to antiangiogenic agents will soon concern these populations as well. It is also becoming clear that the impact of CEPs in tumor vascularization cannot be neglected and understanding the

molecular mechanisms responsible for the acute mobilization, homing and retention within tumors of CEPs could clearly assist in understanding some of the key issues of resistance and metastatic dissemination. The development of strategies targeting CEP in order to prevent these from incorporating regions of tumor neovascularization is likely to be a new challenge. Monitoring of CECs and CEPs could help to elucidate these mechanisms, and perhaps even help to uncover new targets for antiangiogenic agents. Hopefully, future clinical trials will include the evaluation of these cells in pediatric patients and will be instrumental for their validation as biomarkers of antiangiogenic treatments so as to improve clinical strategies.

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Declaration of interest

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