Wound healing signals mediated by Rho/ROCK activation in response to radiotherapy and consequences fot treatmeny of late damage within normal tissues

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Alla mia grande famiglia

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ABBREVIATION LIST

α-SMA α-Smooth Muscle Actin

BLM Bleomycin

CTGF Connective Tissue Growth Factor

ECM Extracellular Matrix

EMT Epithelial to Mesenchymal Transition

FGF Fibroblast Growth Factor

Gy Gray

kDa KiloDalton

IL Interleukine

IR Irradiation

IPF Idiopathic Pulmonary Fibrosis

MMPs Metalloproteinases

RT Radiation Therapy

RIPI Radiation Induced Pulmonary Injury

RILI Radiation Induced Lung Injury

TIMPs Tissue Inhibitor of Metalloproteinases

TGF-β Trasforming Growth Factor β

TNF-α Tumor Necrosis Factor α

WT Wild type

CONTENTS

SUMMARY

Radiotherapy is the second most important treatment modality after surgery in the treatment of cancer. Recent technical advancements, such as intensity-modulated radiation therapy (IMRT) or image-guided radiation therapy (IGRT), combined with new targeted drugs have significant promise for therapeutic outcome. However radiation treatment could result in disabling normal tissue injury and in the development of progressive fibrosis in a subset of sensitive patients and in long-term cancer survivors. The main feature of tissue fibrosis is excessive accumulation of abnormal and cross-linked collagen mainly composed of fibrillar and immature extracellular matrix (ECM) components.

The organs that can be affected by this phenomenon are liver, skin, intestine, kidneys and lungs. From a clinical point of view, fibrosis can be seen as an irreversible condition, without solution. We and others recently showed that beside the activation of the canonical TGF-β/Smad pathway, other intracellular signaling cascades including the Rho/ROCK pathway are switched on in fibrotic tissues. Interestingly, the Rho/ROCK pathway seems differentially activated in radiation-induced intestinal fibrosis, thereby providing a rationale for a specific, targeted anti-fibrotic strategy. Pharmacological inhibition of Rho using statins indeed prevent and even reverse intestinal radiation fibrosis.

In our studies, we showed the role of Statin (Pravastatin e Simvastatin) and a specific inhibitor ROCK inhibitors (Y-27632) in a mice model of pulmonary induced-fibrosis obtained by a pharmacological approach (Bleomycin – BLM). Indeed, we developed a model of lung fibrosis by complete irradiation of chest and tested Pravastatin action. In this model and in a model of radiation induced gut fibrosis, we analysed, from a immunohistological point of view, the underlying mechanisms of the antifibrotic action of Pravastatin via MMP2-TIMP2 axis. Finally, *in vitro*, we investigate by zymography the expression of Gelatinases (MMP2 and MMP9) in primary lung fibroblasts cultures exposure at the different radiation and Pravastatin doses.

In our animal model of pulmonary fibrosis, Pravastatin reverts the fibrotic process and, *in vivo* and *in vitro*, metalloproteases would appear to be in turn involved in pro-fibrolytic mechanisms induced by statin.

The multiplicity of actors involved in the pathogenesis of fibrotic lesions explains why the definition of an effective therapeutic strategy is so complex.

Researches in mechanistic processes of normal tissue damage paved the way for new therapeutic approaches. These new targets include reduction of vascular activation, inflammation and thrombosis and new molecular targets definition. Effective strategies are multiple on preclinical models, but numerous efforts have to be made to achieve the complicated goal of protection of normal tissues from the side effects of radiation therapy.

RIASSUNTO

La radioterapia è la seconda modalità di trattamento più importante dopo chirurgia nel trattamento delle neoplasie. I recenti progressi tecnici, come la terapia ad intensità modulata (IMRT) o l'image-guided radioterapia (IGRT), in combinazione con nuovi farmaci ad azione mirata come gli anticorpi monoclonali, costituiscono ulteriore garanzia di incremento dell'indice terapeutico. Tuttavia il trattamento radiante può causare un'alterazione del normale processo di riparazione e indurre lo sviluppo di un quadro di fibrosi in un sottogruppo di pazienti sensibili e nei lungo-sopravviventi al cancro. La caratteristica cardinale della fibrosi radioindotta è l'eccessivo ed anomalo accumulo di collagene composto principalmente di componenti fibrillari e immature della matrice extracellulare (ECM).

Gli organi che possono essere interessati da questo fenomeno sono fegato, pelle, intestino, reni e polmoni. Da un punto di vista clinico, la fibrosi può essere vista come una condizione irreversibile, senza soluzione. Noi ed altri recentemente abbiamo mostrato che accanto alla attivazione della via canonica TGF-β/Smad, altre vie vengono attivate nei tessuti fibrotici come la cascata di segnalazione intracellulare della via Rho/ROCK. Interessante notare che la via Rho/ROCK sembra specificatamente attivata nella radiazione indotta fibrosi intestinale, fornendo così una spiegazione razionale per una specifica, mirata strategia anti-fibrotica. L'inibizione farmacologica di Rho con le statine infatti è in grado di prevenire e addirittura invertire i fenomeni di fibrosi intestinale post-attinica.

Grazie a queste premesse, nei nostri studi, abbiamo mostrato il ruolo delle statine (Pravastatina e Simvastatina) e di uno specifico inibitore di ROCK (Y-27632) in un modello murino di fibrosi polmonare indotta ottenuto con un approccio farmacologico (bleomicina - BLM). In seguito, abbiamo sviluppato un modello di fibrosi polmonare indotta dall'irradiazione completa del torace e valutata la risposta alla somministrazione della Pravastatina. In questo modello ed in un modello di fibrosi intestinale indotto da radiazioni, abbiamo analizzato, da un punto di vista immunoistologico, i meccanismi sottostanti l'azione

antifibrotica della pravastatina e il ruolo delle metalloproteasi (MMP2 e TIMP2). Infine, *in vitro*, abbiamo indagato, mediante zimografia, l'espressione delle gelatinasi (MMP2 e MMP9) in culture primarie di fibroblasti polmonari murini esposti a differenti dosi di radiazione e pravastatina.

Nel nostro modello animale di fibrosi polmonare, la Pravastatina è in grado di rendere reversibile il processo fibrotico e le metalloproteasi parrebbero essere a loro volta coinvolte, *in vivo* and *in vitro*, nei meccanismi pro-fibrolitici indotti dal farmaco.

La molteplicità di attori coinvolti nella patogenesi delle lesioni fibrotiche spiega perché la definizione di una strategia terapeutica efficace è così complessa. Ricerche nei processi meccanicistici di danno ai tessuti normali hanno aperto la strada a nuovi approcci terapeutici. Questi nuovi obiettivi comprendono la riduzione dell' attivazione vascolare, dell'infiammazione e della trombosi, oltre alla definizione di nuovi target molecolari. Esistono molteplici ed efficaci strategie su modelli preclinici, ma numerosi sforzi devono essere fatti per raggiungere il complicato obiettivo di proteggere i tessuti normali dagli effetti collaterali della radioterapia.

RESUME

La Radiothérapie occupe la deuxième place dans la liste de traitement du cancer le plus important après chirurgie. Le progrès technique récent, comme la radiothérapie avec modulation d'intensité (IMRT) ou la radiothérapie guidée par l'image (IGRT), en combinaison avec de nouveaux médicaments à action spécifique tels que les anticorps monoclonaux, sont une garantie d'augmentation de l'index thérapeutique. Cependant, la radiothérapie peut provoquer un' altération du processus normal de réparation et d'induire le développement d'un cadre de fibrose dans un sous-ensemble de patients sensibles et dans les survivants à long terme de cancer. La principale caractéristique de la fibrose radio-induit est l'accumulation excessive et anormale de collagène composé principalement des éléments fibrillaire et immatures de la matrice extracellulaire (ECM).

Les organes qui peuvent être touchés par ce phénomène sont le foie, la peau, les intestins, les reins et les poumons. D'un point de vue clinique, la fibrose peut être considérée comme une condition irréversible, sans solution. Nous et d'autres ont récemment montré que, outre l'activation de la TGF-β/Smad canonique, d'autres voies sont activées dans les tissus fibreux tels que la cascade de signalisation intracellulaire Rho/ROCK. Fait intéressant, la façon dont Rho/ROCK semble spécifiquement activé dans la fibrose intestinale radio-induite, fournis une justification pour un stratégie anti-fibrotique ciblé. L' inhibition pharmacologique de Rho avec les statines, en fait, est en mesure de prévenir et même inverser les phénomènes de fibrose post-actinique intestinale.

Avec ces prémisses, dans nos études, nous avons montré le rôle des statines (Simvastatine et Pravastatine) et d'un inhibiteur spécifique de ROCK (Y-27632) dans un modèle murin de fibrose pulmonaire obtenue avec une approche pharmacologique (Bléomycine - BLM) . Par la suite, nous avons développé un modèle de fibrose pulmonaire induite par l'irradiation complet du thorax et évalué la réponse à l'administration de la Pravastatine. Dans ce modèle, et dans un modèle de fibrose intestinale radio-induite, nous

avons analysé, grâce à l’immunohistochimie, les mécanismes sous-jacents l'action antifibrotique de la Pravastatine et le rôle des métalloprotéases (MMP2 et TIMP2). Enfin, *in vitro*, nous avons étudié par zymographie, l'expression des gélatinases (MMP2 et MMP9) dans des cultures primaires de fibroblastes pulmonaires murins exposées à différentes doses de rayonnement et de Pravastatine.

Dans notre modèle animal de fibrose pulmonaire, la Pravastatine est capable d'inverser le processus fibrotique et les métalloprotéases semblent être impliqués à leur tour, *in vivo* et *in vitro*, dans les mécanismes pro-fibrolyse induits par le médicament.

La multiplicité des acteurs impliqués dans la physiopathologie de lésions fibrotiques explique pourquoi la mise en place d'une stratégie thérapeutique efficace est si complexe. La recherche dans les processus mécaniques de dommages aux tissus normaux ont ouvert la voie à de nouvelles approches thérapeutiques. Ces nouvelles cibles comprennent la réduction de l’inflammation, de l'activation vasculaire et de la thrombose, ainsi que la découverte de nouvelles cibles moléculaires. Il existe une variété de modèles précliniques et des stratégies efficaces, mais de nombreux efforts doivent être déployés pour atteindre l'objectif difficile de protéger les tissus normaux des effets secondaires de la radiothérapie.

PROBLEMATIC

Radiation oncology is a cornerstone of modern multidisciplinary cancer treatment. It has a place in the management of most common types of cancer, either as a single modality and organ-preserving alternative to surgery, for example, in organ-confined prostate cancer, or as an element in a sequence of treatment steps, such as in adjuvant radiotherapy after breast-conserving surgery for breast cancer[1].

Strategies to improve the outcome of radiotherapy have aimed to improve tumor control rates, thereby increasing the chances of cure in radical or adjuvant therapy and the rates of symptom response in palliative situations. Thanks to technological advances like Image Guided Radiotherapy (IGRT) and Intensity Modulation Radiation Therapy (IMRT), reduction of toxicity and late effects on healthy tissue was also partially gained. At the same time, evolution of the standard of care toward combination therapies (radiotherapy + chemotherapy + targeted drugs) does enhance the rate and severity of both acute and delayed complications at the normal tissue level[2].

The physiopathology of normal tissue injury has been extensively studied by radiation oncologist and radiation biologist. Today, normal tissue toxicity is known to be the result of treatment modalities, tumor type and patient's characteristics that include biological heterogeneity, genetic factors, co-morbidities[3]. Tissue response to radiotherapy depends upon the total dose delivered and the volume irradiated. In addition, radiation therapy is delivered fractionated and thus causes multiple and iterated cellular injuries. Radiation produces free radicals, damages to the vasculature, induces a cascade of local and systemic cytokine and chemokine expression, elicits inflammatory response, and causes death of cells. The tumor itself also leads to architectural destruction of the organ affected, releases cytokines, affects the immune system, and alters vascular permeability. Patients may have underlying medical conditions predisposing to injury (e.g., diabetes, poor underlying pulmonary function, or certain collagen vascular diseases) or have genetic susceptibility. All these factors may contribute to individual radiation sensitivity and their relative importance is difficult to assess.

Nonetheless, it is becoming clear that abnormal microenvironmental conditions exist that are sustained long after the beam is turned off, the drugs are discontinued, and the tumor is eradicated, which appear to be responsible for the perpetuation of the tissue atrophy or hypertrophy, loss of epithelial/vascular/parenchymal cells, and excessive fibrosis characteristic of late normal tissue injury after cancer therapy.

Research over the past 15 years has led to a better understanding of the underlying molecular events responsible for the development of normal tissue injury after cancer therapy[4,5,6,7].

Characterization of the signalling pathways involved in radiation-induced fibrogenesis has mostly focused on one potent fibrogenic growth factor: the transforming growth factor β1 (TGF-β1)[8] and today, inhibitors of TGF-b1 such as Pirfenidone are used in the clinic to halt progression of Idiopathic Pulmonary Fibrosis (IPF)[9] More particularly, in the context of radiation-induced fibrosis several strategies have also been used in experimenatl models and in the clinic.

Tableau III. Molécules utilisées lors d'essais de traitement de fibroses radio-induites superficielles établies.

Molécules	*Disponibilité*	*Utilisation thérapeutique*	*Inhibition de la synthèse de matrice*	*Réduction de l'inflammation*	*Référence*
Interféron γ	Non disponible	Clinique			Peter et al. [104]
Acides gras essentiels	Non disponible	Expérimentale			Hopewell et al. [64]
SOD	Non disponible	Clinique			Baillet et al. [9]
		clinique	+	+	Delanian et al. [34]
		clinique			Benyaba et al. [17]
		clinique			Perdereau et al. [102]
		expérimentale	+	+	Lefaix et al. [82]
Pentoxifylline	Disponible	Clinique		+	Werner-Wasik et al. [144]
		clinique		+	Futran et al. [54]
		expérimentale	–		Lefaix et al. [86]
Vitamine E	Disponible	Clinique			Baillet [10]
Pentoxifylline + vitamine E	Disponible	Clinique			Gottlober et al. [58]
		clinique	+	+	Delanian et al. [37]
		expérimentale	+	+	Lefaix et al. [86]

SOD : superoxyde dismutase.

Amongst these various strategies, some have been used in patients. Delanian S. et al. have shown that the combination of pentoxifylline, vitamin E and clodronate (PENTOCLO) was

useful in healing sternocostal and some mandibular osteoradionecrosis[10] Moreover, treatment combining PENTOCLO significantly improved on a clinical point of view, neurological sensorimotor symptoms of two patients with radiation-induced lumbosacral polyradiculopathy[11]. Cohen et al.[12] suggested that ACE inhibitors might be used after completion of radiation therapy, but before expression of injury, to mitigate the later development of renal failure after radiation-based hematopoietic stem cell transplantation (HSCT).

Another strategy conducted in our lab and others, was to characterize the molecular signals involved in fibrogenesis to provide new therapeutic targets[13,14,15,16,17,18,19,20,21,22]. Using molecular profiling approaches, we have shown that the activation of Rho/ROCK/CTGF pathway was controlling the fibrogenic differentiation in several organs: the gut (human radiation-induced enteropathy) [13,14,16], the heart and lungs (experimental models in C57Bl6 mice)[19]. Then we showed that it was possible to target it to prevent and reverse radiation fibrosis in the organs mentioned above using Statins[17,18,19,20,21,22,23].

Inhibitors of Rho/ROCK/CTGF pathway such as statins that inhibits Rho isoprenylation; Y-27632 an allosteric inhibitor of ROCK and monoclonal antibody against CTGF have shown anti-fibrotic properties[**Errore. L'origine riferimento non è stata trovata.**,**Errore. L'origine riferimento non è stata trovata.**,**Errore. L'origine riferimento non è stata trovata.**,**Errore. L'origine riferimento non è stata trovata.**,**Errore. L'origine riferimento non è stata trovata.**,**Errore. L'origine riferimento non è stata trovata.**,22,23,24].

My thesis work was specifically focused on the anti-fibrotic mechanisms of action of one hydrophilic statin, Pravastatin. The pleiotropic actions of statins are mediated by inhibition of the production of isoprenoid residues and subsequent modulation of posttranslational protein prenylation, including that of Rho[20]. Pre-clinical studies showed that Pravastatin inhibited the Rho/ROCK/CTGF cascade in human samples *ex vivo* and reversed intestinal radiation-induced fibrosis *in vivo*[17,21]. In addition, Pravastatin protected normal intestine and cutaneous[22,23] from radiation damage without interfering with the anticancer action of

irradiation in experimental models, both *in vitro* and *in vivo*. Other studies using Simvastatin confirmed these results[18,24]. Additional mechanism may also be involved, such as preservation of endothelial barrier function, anti-inflammatory action, modulation of platelet activation and anti-thrombotic action, antioxidant properties and may certainly contribute to the anti-fibrotic action of Pravastatin. Based on this biological rational, a phase II clinical trial supported by the French Ministry of Health (PHRC 2010) is currently conducted at IGR to assess the anti-fibrotic efficacy of Pravastatin in patients with cutaneous fibrosis after treatment for Head&Neck tumors.

My thesis work was to characterize further the anti-fibrotic action of Pravastatin from the molecular point of view. Our main hypothesis was that persistent alteration of the cell phenotype induced by irradiation depended, at least in part, upon the Rho/ROCK/CCN2 pathway in various organs. In the first part, we showed that the pathological activation of Rho/ROCK pathway was not specific of radiation-induced intestinal fibrosis but could be considered as a general mechanism as it was also observed in lungs (and heart). In this manuscript we will develop our findings in details. Briefly, we showed activation of the two important fibrogenic pathways: TGF-b/Smad and Rho/ROCK, in response to radiation-exposure *in vivo* in lungs of mice, 15 and 30 weeks post-irradiation. This fibrogenic molecular imprint was associated with long-term remodelling of pulmonary histological structures was successfully modulated using Rho/ROCK inhibitors (statins and Y-27632) that induces a normalization of fibrogenic markers.

The second part of my work focused on ECM remodelling and fibrolysis induced by Pravastatin *in vivo* and *in vitro.* To investigate this question, we used the two long-term experimental models of radiation-induced (RI) fibrosis available in our laboratory that model fibrosis in two majors dose limiting organs: the intestine and the lung. Then, we studied Gelatinase and TIMP regulations and showed that Pravastatin administered as a mitigator induces a persistent activation of the MMP2-TIMP2 axis in the mucosal and muscular

compartment of the gut. When Pravastatin was administered with curative intent the mechanism seemed different since no significant modulation of MMP2-TIMP2 was observed in the gut whereas local fibrolytic process was observed in lungs. Our results suggest that Pravastatin anti-fibrotic action is mediated by Rho/ROCK pathway inhibition and gelatinase-mediated fibrolytic induction.

In conclusion, our findings extend the biological rational for using Pravastatin to treat radiation-induced fibrosis in the patients. Pravastatin offers a safe and efficient therapeutic opportunity potentially usable either before or after radiation exposure. This approach is especially attractive in (1) the radiation oncology setting, as it does not interfere with prior anti-cancer treatment and in (2) radioprotection, as applicable to the treatment of established radiation injury, for example in the case of radiation accidents or acts of terrorism.

INTRODUCTION

PRINCIPLES OF RADIATION EFFECTS ON NORMAL TISSUES

The modern era of cancer therapy involves safe intensification of radiation, chemotherapy, and biologic adjuvants. Yet, the impact of such newer radiation technologies and normal tissue constraints have been offset in some cancers by escalation of the radiation dose and concurrent chemotherapy aimed at improving the tumor response. This has resulted in a markedly increased survivorship, which now exceeds 64% overall, and is much higher for selected malignancies, such as 87% for breast cancer and 80% for all childhood cancers[25]. Malignancies resistant to therapy required very aggressive treatment approaches often at the edge of normal tissue tolerance, or that even exceeds tolerance to some "acceptable" degree. Definition of normal tissue tolerance has evolved and led to a deeper understanding of the multiparametric inputs that influence toxicity[26]. There is abundant evidence that clinicians underestimate the frequency and severity of patients' symptoms and, therefore, the published data underestimate the true toxicity burden[27].

Patients treated with radiation may experience severe and potentially life-threatening late intestinal complications, such as fistulation, sepsis, intestinal failure, perforation, obstruction and bleeding, which required surgery in 5% of cases at 5 years[13]. Recent reports suggest that, in more than half of patients, irradiation of pelvic tumours leads to permanent gastrointestinal damage (i.e. alternating episodes of diarrhoea and constipation, abdominal pain, malabsorption, faecal urgency)[28], for which no efficient treatment is available today.

Clearly, the potential to ameliorate or prevent such normal tissue damage, or to manage and rehabilitate affected patients, requires an understanding of tissue tolerance to therapy. Because "late effects" can manifest months or years after cessation of treatment, therapeutic decisions intended to obviate such effects can be based only on the probability, not the certainty, that such effects will develop. In making such decisions, the balance between efficacy and potential for toxicity should be considered and may be influenced by host, disease, and

treatment-related risk factors. Recently, many agents have been identified that target molecular pathways that can mitigate radiation toxicity. To date, no drugs have been approved as radiation injury mitigators (RIM), defined as agents administered after irradiation but before toxicity is manifest. Movsav B et al[29] have present an algorithm to guide clinical trials for such agents in patients receiving radiotherapy or radiochemotherapy. The goal is to be able to apply such promising agents to improve the quality of life for all these patients. Patients have a central role in reported outcomes They reviewed the mechanisms of radiation injury and the clinical problem related to radiation effects, the preclinical and clinical development of candidate agents, and how to design and conduct clinical trials.

Normal tissue responses to radiation can be divided into two categories: those that occur during the first days and weeks after treatment, often called *early effects*, and those that occur months, years, or even decades after irradiation, called *late effects.* "Consequential late effects" result from the host's reaction to severe acute toxicity. Some organs are prone to late toxicity (often termed *late responding tissues*) while others are prone to acute toxicity (*early responding tissues*), although in any organ both acute and/or late effects occur. The type of effect expressed by an organ is generally a function of the tissue's renewal properties, but the clinical importance of the response typically depends on the biology of the organ. The most important parameters that control toxicity occurrence in patients are the total radiation dose and fraction size, the duration of time during which the course of radiation is delivered, the interval between radiation fractions, the rate at which the radiation is given (dose rate), the specific organ being irradiated, and the volume. As previously stated, the organizational structure and the repair or compensatory capacities of the organ influence its tolerance to partial or whole-organ irradiation.

The classic concept of a single target cell explaining the dynamic sequence of events leading to normal tissue damage has been supplanted by a more complex vision in which the

interaction of multiple cellular systems through various molecular signalling and paracrine factors occurs. Moreover, the perceived acute and late phases of adverse effects now are seen as manifestations of an ongoing sequence of events perpetuated through autocrine, paracrine, and endocrine messages that are initiated immediately after injury and persist until the clinical late effect events themselves. After irradiation, a variety of growth stimulatory and inhibitory factors are released, cell receptors are altered, and the resulting dysregulation of the tissue environment is translated ultimately into postreceptor cytoplasmic, nuclear, and interstitial events. Thus, a combination of cell death, the production of reactive oxygen species, alterations in gene expression, and the expression of both proinflammatory and profibrotic cytokines are viewed as integral in the pathogenesis of late effects (Fig.1)[30,31].

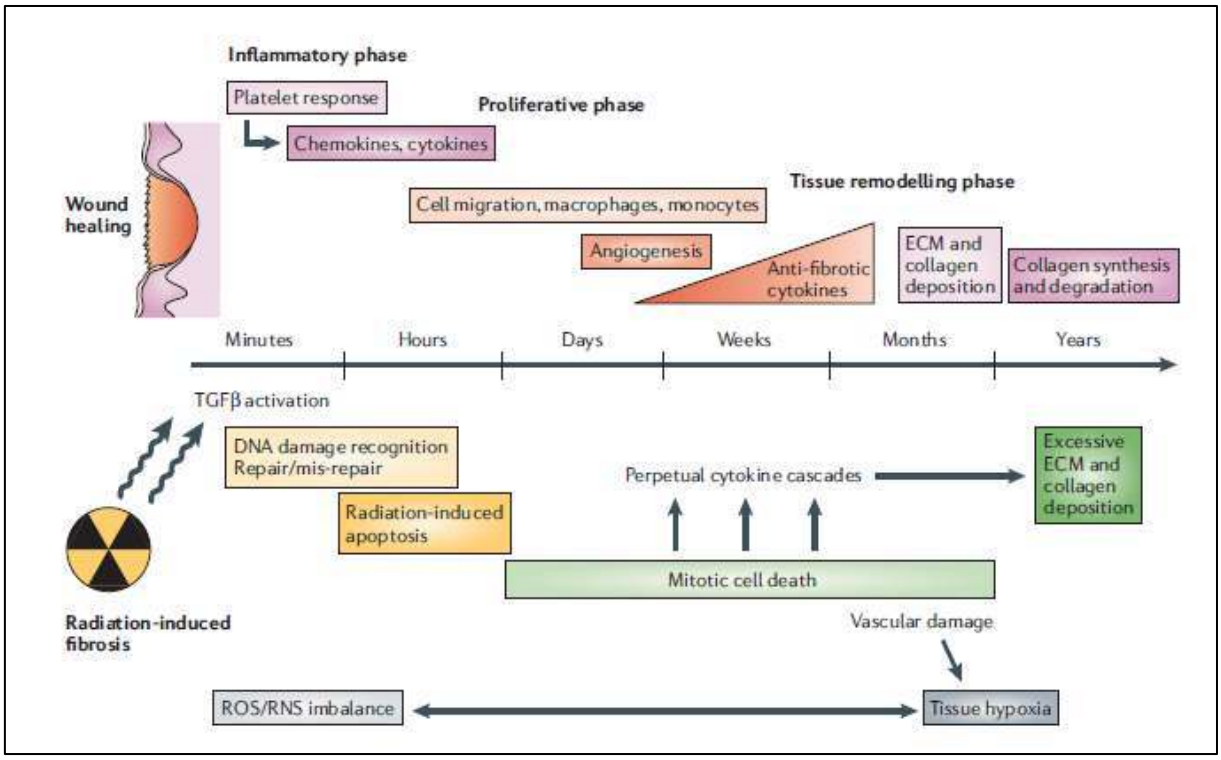


Fig. 1.: From "Preventing or reducing late side effects of radiation therapy: radiobiology meets molecular pathology. Bentzen SM. Nat Rev Cancer. 2006 Sep;6(9):702-13. Review".

Despite optimum conformation of the treatment fields to the tumour and precise treatment planning and application, the target volume in curative radiotherapy necessarily includes a substantial amount of normal tissue, for several reasons.

First, malignant tumours infiltrate microscopically into normal structures, which hence must be included into the high-dose volume as a tumour margin. Second, normal tissues within the tumour, such as soft tissue and blood vessels, are exposed to the full tumour dose. Third, normal structures in the entrance and exit channels of the radiation beam may be exposed to clinically relevant doses. Therefore, effective curative radiotherapy is unavoidably associated with an accepted risk for early and late radiation side-effects (‘adverse events’) in order to achieve adequate tumour cure rates.

Radiotherapy is a localised treatment. The definition of tumour and target volumes for radiotherapy is vital to its successful execution. This requires the best possible characterisation of the location and extent of tumour. Diagnostic imaging, including help and advice from diagnostic specialists, is therefore essential for radiotherapy planning.

There are three main volumes in radiotherapy planning (Fig.2, 3)

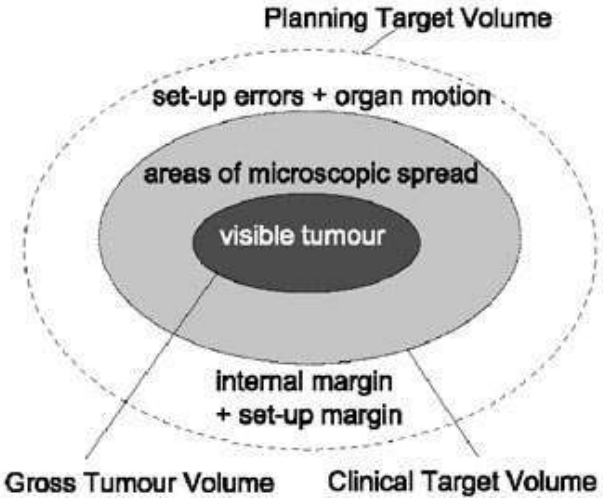


Fig.2: Volume in radiotherapy planning

First volume is the position and extent of gross tumour, i.e. what can be seen, palpated or imaged; this is known as the gross tumour volume (GTV). Developments in imaging have contributed to the definition of the GTV. The second volume contains the GTV, plus a margin for sub-clinical disease spread which therefore cannot be fully imaged; this is known as the clinical target volume (CTV). It is the most difficult because it cannot be accurately defined for an individual patient, but future developments in imaging, especially towards the molecular level, should allow more specific delineation of the CTV. The CTV is important because this volume must be adequately treated to achieve cure. The third volume, the planning target volume (PTV), allows for uncertainties in planning or treatment delivery. It is a geometric concept designed to ensure that the radiotherapy dose is actually delivered to the CTV. Radiotherapy planning must always consider critical normal tissue structures, known as organs at risk (ORs). In some specific circumstances, it is necessary to add a margin analogous to the PTV margin around an OR to ensure that the organ cannot receive a higher-than-safe dose; this gives a planning organ at risk volume. This applies to an organ such as the spinal cord, where damage to a small amount of normal tissue would produce a severe clinical manifestation. The concepts of GTV, CTV and PTV have been enormously helpful in developing modern radiotherapy.

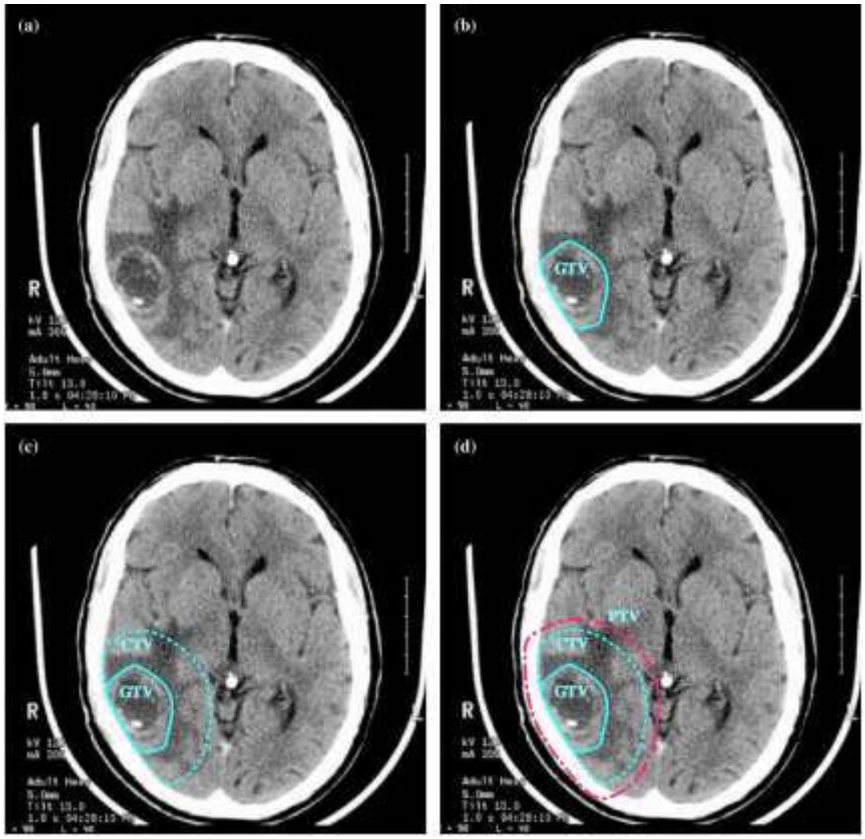


Fig. 3: from: ***Cancer Imaging 2004 Oct 21;4(2):153-61.***
Planning volumes for a patient with WHO Grade 4 glioma (glioblastoma). (a) Planning CT showing contrast-enhancing tumour. (b) The GTV is the visible tumour. (c) A margin for microscopic spread has been added to make the CTV; the margin is the same in all directions except that it is restricted by the skull. (d) The PTV has been added outside the CTV to account for uncertainties in planning and execution of treatment; this extends beyond the inner table of the skull.

Late radiation effects, in particular radiation-induced fibrosis (RIF) and atrophy, hence represent a multifaceted, orchestrated response with various components[32].

Each cellular components of an organ does respond to radiation injury with a specific dose-dependence, which, in that orchestrated response, then defines the overall dose–response for the different clinical endpoints of the entire tissue. RIF is a relatively rare, late, local consequence of high-dose RT.

It is estimated that, in pelvic tumor irradiation, most patients present digestive effects in the short term (80%) and that 5 to 10% develop late complications due to irradiation of non-tumor tissues.

Co-morbidity factors such as cardiovascular disease; preexisting collagen vascular diseases; and hypersensitivity or very rare congenital diseases are confounding factors that enhance the rate of radiation-induced fibrosis[33].

At the cellular level, fibrosis is characterized by fibroblast proliferation and differentiation. Excessive extracellular matrix deposition is also a major hallmark of the disease, amplified by the action of cytokines and growth factors leading to abnormal reactive oxygen species (ROS) and reactive nitrogen species concentrations.

All tissues affected by advanced RIF are at risk to develop radionecrosis that might be seen as to ultimate evolution of the disease. One of more severe clinical manifestations of this cellular and molecular impaired response to radiation is Osteoradionecrosis (ORN)[34].

RADIATION-INDUCED FIBROSIS

Radiation-induced fibrosis is a dynamic process, which varies both in its intensity and in its qualitative effect on the structure and function of the affected organ or tissue. Schematically, spontaneous RIF is divided into 3 histopathological phases, each of which is predominantly cellular, extracellular, or a mixture of both and is characterized by gradual worsening over several years: (1) a prefibrotic phase often asymptomatic, marked by signs of chronic inflammation, where endothelial cells play an important role; (2) a phase of organized fibrosis, characterized by a patchwork of areas of active fibrosis containing a high density of myofibroblasts in an unorganized matrix, surprisingly adjacent to poorly cellularized fibrotic areas consisting of senescent fibrocytes in a dense sclerotic matrix; and (3) a late fibroatrophic phase, with retractile fibrosis and gradual loss of parenchymal cells.

General mechanisms of wound healing and fibrosis

Acute and late normal tissue injury occurs from a complex interaction between radiation-induced death of parenchymal cells, damage to the supporting vasculature, and associated inflammatory and fibrotic reactions. Long-term depletion of tissue-specific stem cells or progenitor cells can lead to fibrosis, organ dysfunction, and necrosis[6].

A wound-healing response is often described as having three distinct phases: injury, inflammation and repair[35].

Phase I: injury

Cells acknowledge damage from radiation exposure through multiple sensor molecules and structures. Not surprisingly, chemokines and proinflammatory cytokines are highly prominent among the panoply of molecules expressed in tissues after irradiation. Perhaps less obvious is the fact that anti-inflammatory cytokines can also be increased, some of which may

participate in angiogenesis. This cytokine balance is a common feature of inflammatory responses. Cytokines, in turn, amplify further coordinated changes in additional cytokines, cell adhesion molecules, prostaglandins and leukotriene species, redox regulating enzymes and pro- and antioxidant species (manganese superoxide dismutase, inducible nitric oxide synthase, metallothionein, heme oxygenase, gamma-glutamylcysteine synthetase, and myeloperoxidase), matrix remodeling enzymes and inhibitors, plasminogen activators and inhibitors, and heat shock proteins. These components also often have mutually antagonistic aspects that allow tight control on the inflammatory and tissue-healing responses.

In many respects, the tissue responses to irradiation mimic the cytokine storms generated by many other tissue-damaging insults.

Phase II: inflammation

Once access to the site of tissue damage has been achieved, chemokine gradients recruit inflammatory cells. Neutrophils, eosinophils, lymphocytes, and macrophages are observed at sites of acute injury with cell debris and areas of necrosis cleared by phagocytes. The timing of inflammatory events may determine the role played by the inflammatory process. Early inflammation that is diminished at the later stages of disease may promote wound healing and may contribute to fibrosis. For example the early recruitment of eosinophils, neutrophils, lymphocytes, and macrophages providing inflammatory cytokines and chemokines can contribute to local TGFβ and IL-13[36].

However, following the initial insult and wave of inflammatory cells, a late-stage recruitment of inflammatory cells may assist in phagocytosis, clear cell debris, and control excessive cellular proliferation, which together may contribute to normal healing. Thus late-stage inflammation may in fact serve an anti-fibrotic role and could be required for successful resolution of wound-healing responses.

The nature of the insult or causative agent often dictates the character of the ensuing inflammatory response and the nature of the inflammatory response dramatically influences resident tissue cells and the ensuing inflammatory cells. Inflammatory cells themselves also propagate further inflammation through the secretion of chemokines, cytokines, and growth factors. Many cytokines are involved throughout a wound-healing and fibrotic response, with specific groups of genes activated in various conditions[37]. Each of these cytokines can exhibit significant pro-fibrotic activity, acting through the recruitment, activation and proliferation of fibroblasts, macrophages, and myofibroblasts.

Phase III: tissue repair and contraction

The closing phase of wound healing consists of an orchestrated cellular re-organization guided by a fibrin-rich scaffold formation, wound contraction, closure and re-epithelialization. Myofibroblast-derived collagens and α-SMA form the provisional extracellular matrix, with macrophage, platelet, and fibroblast-derived fibronectin[38] forming a fibrin scaffold. Collectively, these structures are commonly referred to as granulation tissues.

Growth factor and TGFβ-activated fibroblasts migrate along the extracellular matrix network and repair the wound. Fibroblast to myofibroblast differentiation, as discussed above, also creates stress fibers and the neo-expression of α-SMA, both of which confer the high contractile activity within myofibroblasts[35]. The attachment of myofibroblasts to the extracellular matrix at specialized sites pull the wound together, reducing the size of the lesion during the contraction phase. The degree of extracellular matrix laid down and, the quantity of activated myofibroblasts[39] determines the amount of collagen deposition. To this end, the balance of MMPs to TIMPs[40] and collagens to collagenases vary throughout the response, shifting from pro-synthesis and increased collagen deposition, towards a controlled balance, with no net increase in collagen. For successful wound healing, this balance often occurs when fibroblasts undergo apoptosis, inflammation begins to subside, and granulation tissue

recedes, leaving a collagen-rich lesion. The removal of inflammatory cells and especially α-SMA+ myofibroblasts is essential to terminate collagen deposition[41].

Collectively, the degree of inflammation, angiogenesis, and amount of extracellular matrix deposition all contribute to the net collagen deposition and ultimately whether a fibrotic lesion develops. Therapeutic intervention, interfering with fibroblast activation, proliferation or apoptosis requires a thorough understanding and appreciation of all of the phases of wound repair. Although these three phases are often presented sequentially, during chronic or repeated injury these processes function in parallel, placing significant demands on regulatory mechanisms.

THE LUNGS AND THE SMALL INTESTINE: TWO MAJOR DOSE-LIMITING ORGANS IN RADIOTHERAPY

An important challenge to modern radiation therapy is to increase the tolerance of normal tissues, in order to improve the quality of life of the patients, and to enhance local tumor control using dose escalation and/or new biological radiosensitizers[42].

The recent progress made by intensity-modulated radiation therapy (IMRT) and image-guided radiation therapy (IGRT) has reduced radiation-induced complications[43] especially in dose-limiting organs like the **intestine**[44] and **lungs** that still remains major dose limiting organs[45].

The dose effect relationship in both tumor and normal tissue in characterized by a sigmoid curve (Fig.4).

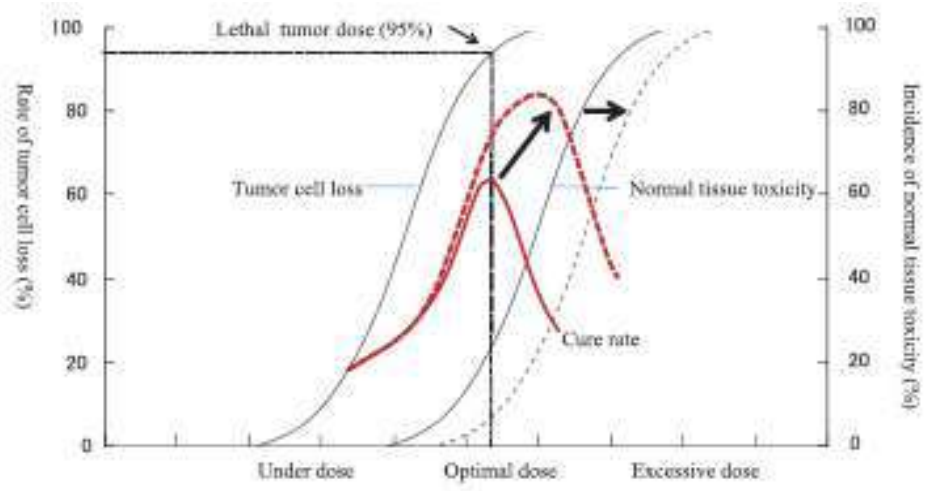


Fig. 4: Dose effect on tumor and normal tissue. *From "Ikushima H. Radiation therapy: state of the art and the future. J Med Invest. 2010 Feb;57(1-2):1-11.* Review**"**.

If we can reduce the radiation dose to the surrounding normal tissues adjacent to the tumor, by improving dose conformity, the sigmoid curve for normal tissue damage can be shifted to a higher dose area. This makes it possible to escalate the tumor dose, resulting in an improvement in the cure rate. Technical innovation in RT always aims to improve dose distribution conformity with the objective of decreasing normal tissue toxicity.

Despite technological progress, give the preferable high tumor dose is not always achievable (due the presence of organs at risk), with consequent reduction in probability of regional tumor control.

Reducing the dose delivered to the bowel is also required in prostate[46], bladder[47], and gynaecological[48] tumor treatment.

Lungs sparing is necessary not only during lung cancer treatment[49] but also in breast cancer[50], Hodgkin Lymphoma[45], and oesophagus neoplasm irradiation[51].

An alternative mechanism to reduce normal tissue toxicity is the use of radiation modifiers/protectors, agents that when present prior to or shortly after radiation exposure alter the response of normal tissues to irradiation. This approach has also been viewed as an attractive countermeasure for possible nuclear/radiological terrorism. To be useful in the radiotherapy clinic, radioprotectors should ideally have several characteristics that relate to the ability of the agent to improve the therapeutic ratio. First, the agent should be selective in protecting normal tissues from radiotherapy without protecting tumor tissue, otherwise no benefit in the therapeutic index will be realized. Second, the agent should be delivered with relative ease and with minimal toxicity. Finally, the agent should protect normal tissues that are considered sensitive such that acute or late toxicities in these tissues are either dose-limiting or responsible for a significant reduction in quality of life (i.e., mucositis, pneumonitis, myelopathy, xerostomia, proctitis, and leukencephalopathy)[52].

In effect, low and mild grade chronic gastrointestinal and lung side effects continue to influence the patient's quality of life. Acute enteritis affects most patients treated with pelvic radiotherapy.

The symptoms occur during or immediately after radiotherapy: diarrhea, abdominal pain and incontinence, with, more rarely, constipation, bleeding and discharge of mucus[53].

The epithelial damage promotes bacterial translocation and septic risks, bleeding, and reduce the absorption capacity of the digestive mucosa.

Therapeutic management associates symptomatological and support treatment. Acute enteritis resolves most of the time by itself during the weeks following the end of treatment.

Acute symptoms may be followed by a phase of evolution and a progressive worsening of the patient's clinical status. The most common symptoms are chronic recurrent episodes of diarrhoea and constipation, with violent abdominal pain. The wall thickening due to tissue fibrosis and the restriction of the intestinal lumen disrupt transit, promote stenosis and can lead to a total bowel obstruction. Ulceration and tissue necrosis can cause severe gastrointestinal bleeding, perforation of the intestinal wall and create entero-cutaneous, entero-enteric or entero-urinary fistulae.

Acute and subacute radiation pneumonitis, with late occurrence of fibrosis, are well-known risk factors for quality of life and survival of patients receiving radiotherapy to the thoracic region. Although 30 to 40% of the patients with lung cancer can benefit from radiotherapy, approximately 20% of these patients develop radiation-induced pulmonary injury.

The occurrence and severity of damage are semiquantitatively related to the volume of lung irradiated, and the dose rate of irradiation. The clinical syndrome occurs in up to about 10% of patients and consists of an acute transient phase, radiation pneumonitis, usually occurring 6 to 12 weeks after radiation therapy. Symptoms of radiation pneumonitis, including low-grade fever, congestion, dry cough, pleuritic chest pain, and a sensation of chest fullness, usually develop one to three months after completion of radiation therapy. Diagnosis is difficult, often complicated by comorbid conditions and radiation injury to adjacent structures (e.g., esophagus, pericardium). Although patients with acute pneumonitis may exhibit complete resolution of their signs and symptoms, unfortunately, the majority of them will go on to develop progressive pulmonary fibrosis; interestingly, this chronic condition also has been shown to occur in the absence of a preceding acute phase. In general, pulmonary fibrosis evolves between 6 to 24 months post-treatment, but then stabilizes after 2 years[54]. The

condition can result in a chronic pulmonary insufficiency, although this will be dependent upon the volume of lung treated, since fibrosis, like radiation pneumonitis, is characteristically restricted in its appearance to within the portal field. Where a large volume has been irradiated, the chronic insufficiency may progress to chronic cor pulmonale from the resultant pulmonary hypertension and othropnea, with associated cyanosis, hepatomegaly, or liver tenderness[54].

Concomitant chemotherapy, repeat courses of radiation, and steroid withdrawal are exacerbating factors. Because the clinical evolution of delayed toxicity is progressive and inevitable, these complications are of much concern in clinical practice and further improvement in the management of such patients is required.

THE LUNGS

Basic structure and function

Lungs, in air-breathing vertebrates, are two large organs of respiration located in the chest cavity and responsible for adding oxygen to and removing carbon dioxide from the blood. In humans each lung is encased in a thin membranous sac called the pleura, and each is connected with the trachea (windpipe) by its main bronchus (large air passageway) and with the heart by the pulmonary arteries.

Diagram in Fig.5 shows the respiratory system.

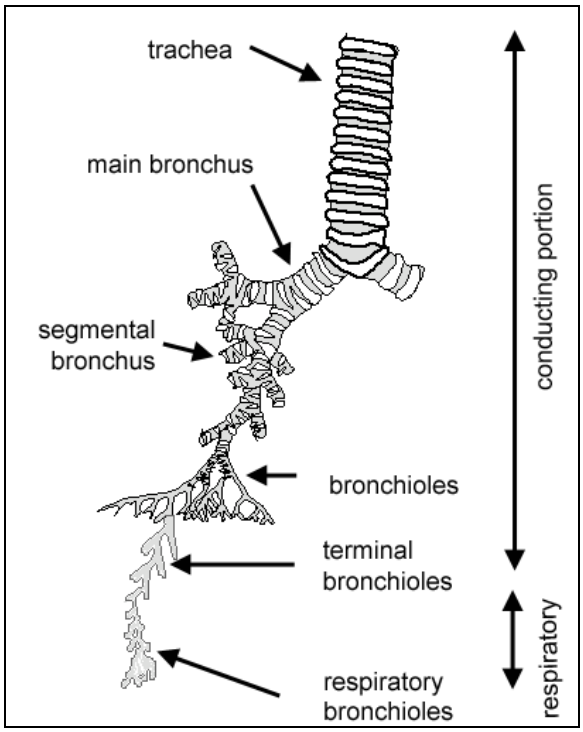


Fig.5

It can be divide into two major components:

1. Conducting portion

The main function of the conducting system is to 'condition' the inspired air to **humidify** (by serous and mucous secretions) **warm** (by underlying blood vessels) and **filter** (by particles being trapped in mucous secretions, and transported towards the throat, where the mucous is

swallowed). The upper regions of the respiratory system (in the nasal passages) are covered with respiratory mucosa, and in some regions, olfactory mucosa.

The **conduction portion** is made up of: nasal cavities, nasopharynx, larynx, trachea, bronchi and bronchioles.

2. Respiratory portion

The interphase for passive exchange of gases between the atmosphere and blood.

The **respiratory portion** is made up of: respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli. The epithelium of the alveoli (Fig.5), contains two main types of cells:

1. Type I pneumocytes: large flattened cells (95% of the total alveolar area) which present a very thin diffusion barrier for gases. They are connected to each other by tight junctions.

2. Type II pneumocytes (making up 5% of the total alveolar area, but 60% of total number of cells). These cells secrete 'surfactant' which decreases the surface tension between the thin alveolar walls, and stops alveoli collapsing during expiration.

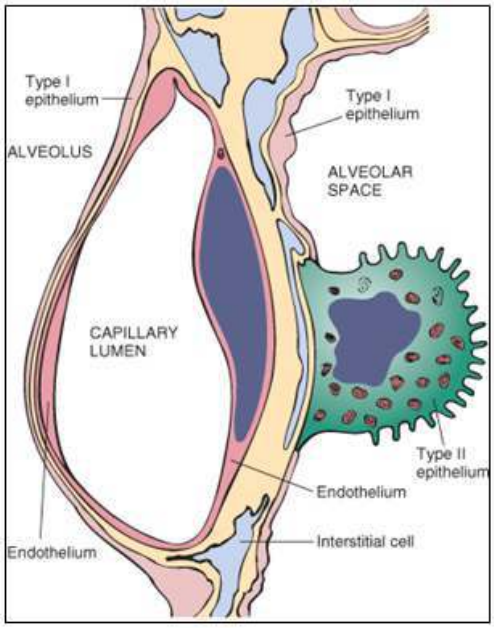


Fig.6.

Physiopathology of Radiation induced lung fibrosis

Pneumonitis and fibrosis are distinct features of the lung's response to radiation damage. The former may occur after 6 to 16 weeks and is typified by inflammation and interstitial pneumonia. The latter is a more chronic response lasting months to years with progressive obliterative fibrosis[55].

Multiple mechanisms have been identified in RT-induced fibrosis, including alveolar damage, increased reactive oxygen species (ROS) and the toxic effects of ROS on parenchymal cells, disruption of proliferation-associated transcription factors, and the influx of inflammatory cells, such as macrophages and lymphocytes[35].

Type II pneumocytes have traditionally been considered "target" cells for radiation whose loss leads to inflammation, desquamation of epithelial cells from the alveolar surface, edema, and discharge of proteinaceous material into the alveoli. More recently, the cellular response has come to be viewed more in its entirety as involving multiple cell types with the outcome being dependent on the genetically determined molecular profile that drives the wound-healing process. Similar to other tissues, the lung's response to radiation involves an inflammatory response.

After initial decreases in total cell number, neutrophils then lymphocytes infiltrate and are found elevated in bronchoalveolar lavage (BAL) for months.

Despite the evidence for dynamic changes within the BAL population, they contribute less to radiation-induced cytokine alterations than interstitial cells.

Rubin and coworkers[56] first used the cytokine "cascade" in the lung after irradiation to argue that there was no real "latent period" before the genesis of fibrosis and suggested that this was a self-sustaining continuous process.

Dysregulated pro-inflammatory and pro-fibrotic cytokines, TGF-β1, IL-6, MMPs[57] and chemokines, in addition to reduced anti-inflammatory cytokines following radiation can further exacerbate the inflammatory and wound-healing response. TGF-β1 drives procollagen

1 production by fibroblasts, myofibroblasts, and other reparative cells through the Smad transcription factor pathway in addition to controlling many other aspects of extracellular matrix homeostasis. TGF-β1 has, however, other numerous biological functions. These include inhibiting proliferation of many cell types including lymphocytes and type II pneumocytes[5].

Other cytokines have, however, been implicated in radiation-induced pulmonary injury as IL-1α, tumor necrosis factor-α (TNF-α) and interpheron-γ (INF-γ).

RT of the thoracic region can cause significant damage to radiation-sensitive alveolar regions of the lung invoking a dysregulated inflammatory cascade, rich in pro-inflammatory and pro-fibrotic mediators. Dysregulated chemokines, transcription factors, and anti-inflammatory pathways can further compound this uncontrolled response, leading to pulmonary fibrosis.

THE SMALL INTESTINE

Basic structure and function

The main functions of the small intestine are digestion, absorption of food and production of gastrointestinal hormones. The small intestine is 4-6 metres long in humans. Structure is represented in Fig.7.

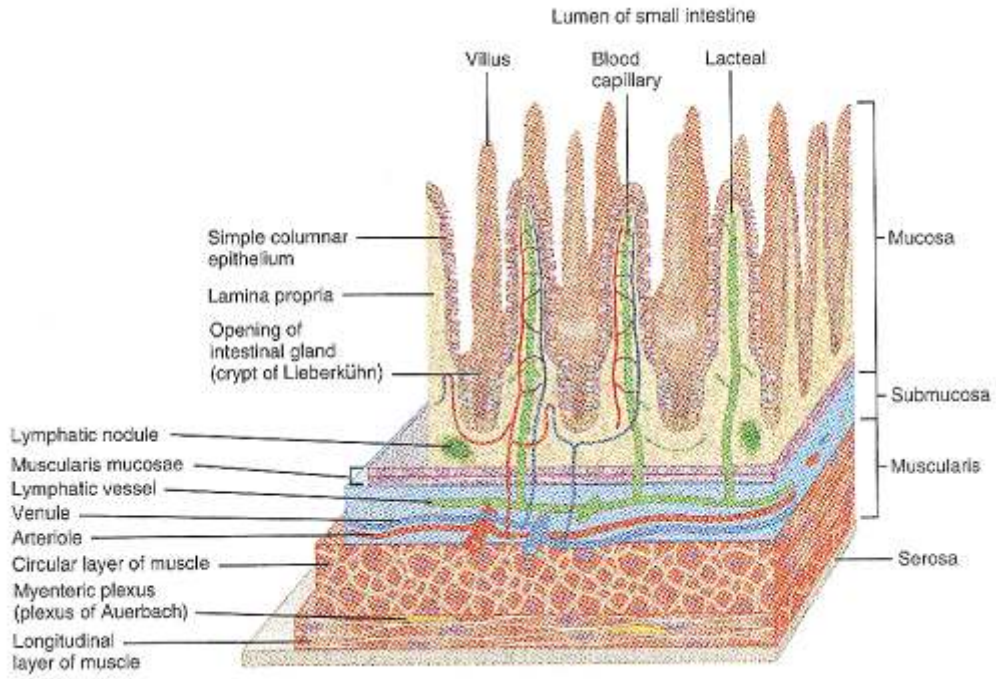


Fig. 7

To aid in digestion and absorption:

1) the small intestine secretes enzymes and has mucous producing glands. The pancreas and liver also deliver their exocrine secretions into the duodenum.

2) The mucosa is highly folded.

a) large circular folds called plicae circulares (shown in the diagram to the right), most numerous in the upper part of the small intestine,

b) smaller folds called villi, which are finger like mucosal projections, about 1mm long.

c) the lining columnar epithelial cells have fine projections on their apical surfaces called microvilli.

Together, these folds provide a huge surface area for absorption. Between the villi there are crypts, called crypts of Lieberkuhn, which extend down to the muscularis mucosae. These crypts are short glands.

The lamina propria which underlies the epithelium has a rich vascular and lymphatic network, which absorbs the digestive products, and there is a muscularis mucosae layer immediately at the base of the crypts. The lymphatic capillaries are called lacteals, and absorb lipids. The vascular capillaries are fenestrated to aid absorption.

The muscularis externa layer contains two layers of smooth muscle, an inner circular and outer longitudinal, for continuous peristaltic activity of the small intestine. There are around 200 or so lymphoid aggregations called Peyer's patches in the mucosa.

The external serous membrane is composed of connective tissue and epithelium.

Physiopathology of Radiation induced intestinal fibrosis

Pathological changes observed in severe late intestinal lesions have been widely explored using a descriptive approach 30 years ago. The main histopathological hallmarks of radiation-induced late intestinal damages in radiotherapy patients are fibrosis associated with inflammation and vascular sclerosis and, although to a lesser extent, epithelial lesions. Bowel fibrosis is characterized by transmural accumulation of extracellular matrix within intestinal layers that induces loss of compliance, impairs intestinal function and leads to obstructive syndromes.

Initial responses to radiation in the gut are characterized by radiation-induced cytokine expression, as in other tissues[5].

Within hours of radiation exposure the rat ileal muscularis layer expresses IL-1β, TNF-α, and IL-6[58].

TGF-β1 is activated at 24 hours after irradiation and remains high throughout later responses, whereas IL-10 decreased. At this time, TGF-β1 is found in the inflammatory cells and surrounding extracellular matrix, and fibroblasts. Additional studies showed TGF-β1 association with mucosal ulceration, membrane thickening, and epithelial atypia 2 weeks after irradiation. A significant increase in the number of inflammatory cells in the mouse intestine can be observed at 24 hours after irradiation and IL-8 is upregulated at about 3 days after irradiation[58].

Long-term changes in cytokine expression in the bowel of mice after irradiation implicate TNF-α, IL-1, IL-6, and TGF-β1 in late radiation-induced bowel fibrovascular toxicity.99

Connective tissue damage and increased collagen deposition is accompanied by high expression of smooth muscle actin and increased levels of the fibrogenic growth factor connective tissue growth factor (CTGF)[16]. CTGF may be involved in radiation-induced fibrogenic differentiation in intestinal smooth muscle cells. The Rho/ROCK pathway has been shown to regulate CTGF expression and may serve as a target for intervention.[13,14,17]

Microvascular injury in both acute and chronic radiation injury to the gut has been investigated in some depth and ascribed to dysfunction of the thrombomodulin (TM)-protein C (PC) system[18]. The TM-PC system is a critical physiological anticoagulation system in which TM forms a complex with thrombin to promote anticoagulation. TM and activated PC have important anti-inflammatory properties. Cytokines such as IL-1, TNF-α, and TGF-β1, all induced after radiation, reduce the transcription of TM[5].

TM deficiency is found early postirradiation and continues to persist into chronic radiation injury, paralleling cytokine expression. This mechanism may allow persistence of the late cytokine cascade and damage.

CELLS AND MOLECULAR MEDIATORS INVOLVED IN RADIATION INDUCED FIBROSIS

Fibroblasts and Myofibroblasts

One of the most important players in fibrosis is the myofibroblast. First described by Gabbiani in 1971[59], myofibroblasts display an intermediate phenotype between fibroblasts and smooth muscle cells. Typically, they have an hyper-contractile cytosqueleton suitable to achieve wound contraction, express α-SMA and secrete large amounts of extracellular matrix proteins in particular fibrillar collagens[60]. In physiological remodeling such as during dermal wound healing, the contractile activity of myofibroblasts is terminated when the tissue is repaired; a-SMA expression then decreases and myofibroblasts disappear by apoptosis.[61]

In pathological wound healing, myofibroblast activity persists and leads to tissue deformation, which is particularly evident, for example, in hypertrophic scars that occurs in scleroderma or after burn injuries[61]

Myofibroblast-generated contractures are also characteristic of fibrosis affecting vital organs such as the liver and kidney[62,63] heart[64,65] and lung[66,67]. In addition, myofibroblast participation to the process called stromal reaction does promote cancer progression (in that case they are called CAF Carcinoma associated fibroblasts) by creating a stimulating microenvironment for epithelial tumor cells[68,69]. Recent findings showed that overload with interstitial fluids cause fibroblast-mediated stromal remodeling that facilitates tumor invasion[70].

In fibrotic lungs, myofibroblasts are key mediators of extracellular matrix deposition, structural remodeling, and destruction of alveocapillary units[66] understanding their origin would therefore be critical to understand the pathogenesis of lung fibrosis.

In intestinal fibrosis, the role of intestinal mesenchymal cells constitution and maintenance is increasingly recognized nowadays[13]. In healthy bowel, subepithelial myofibroblasts, located

in the mucosa, and smooth muscle cells, located in muscular layers, are involved in the maintenance of tissue structure and function (intestinal contractility, extracellular matrix homeostasis). In fibrotic conditions as in lungs, their role is enhanced as they are responsible for the excessive collagen secretion, impaired motility and secretion of the fibrogenic growth factors.

Several origins for myofibroblasts have been proposed that may differ depending on the affected organ and the initiating event. However, three general mechanisms can be mentioned:

I) Proliferation and activation of tissue resident fibroblasts or perivascular and vascular adventitial fibroblasts can be activated into myofibroblasts in response to specific pro-fibrotic stimuli coming from infiltrating inflammatory cells leading to a progressive evolution from quiescent fibroblasts to cells expressing a myofibroblast phenotype[71].

II) Recruitment of fibroblast precursor cells from bone marrow as a result of the local release of activated chemokines. These bone marrow precursor cells are fibrocytes, a unique cell population expressing bone marrow cellular surface markers (CD34 protein) and capable of production of extracellular matrix proteins (type I procollagen). These cells are able to migrate from the bloodstream in response to specific chemokine gradients and chemoattractant, to niche in damaged tissue to ensure physiological wound healing process or fibrogenesis[72,73].

III) Transition of epithelial cells and endothelial cells to fibroblasts, a process known as EMT which is induced by transforming growth factor β (TGF-β) and perhaps other polypeptides such as endothelin-1 (ET-1) or insulin growth factor[74,75,76].

Changes in cell phenotype between epithelial and mesenchymal states, defined as epithelial–mesenchymal (EMT) and mesenchymal–epithelial (MET) transitions, not only occurs during embryonic development or as a physiological response to injury, but is also an important

element in cancer progression and other pathologies that involve organ degeneration, such as fibrosis. At the cellular level, pathological EMTs are very similar to physiological EMTs in that they are governed by similar signaling pathways, regulators, and effector molecules[75].

Recently, Endothelial-Mesenchymal Transition (EndoMT), another type of cellular transition, has emerged as a possible mechanism in pathological fibrosis. EndoMT is a complex biological process in which endothelial cells lose their specific endothelial cell markers, such as vascular endothelial cadherin (VE cadherin), and acquire a mesenchymal or myofibroblastic phenotype initiating expression of mesenchymal cell products such as α-SMA, vimentin, and type I collagen. Besides acquisition of an activated pro-fibrogenic phenotype, these cells also become motile and are capable of migrating into surrounding tissues. Similar to EMT, EndoMT can be induced by TGF-β[77].

For instance in lungs, ionizing irradiation induces production of radical oxygen species (ROS) including superoxide, hydroxyl radical, nitric oxide and. ROS interaction with pyrimidine and purine bases nuclear DNA produces single and double strand breaks, initiation of DNA repair, communication of DNA damage through the cell cytoplasm to the mitochondrial membrane via (stress activated protein (SAP) kinases) and then translation of pro-apoptotic, BCL2 family members from nucleus to mitochondria. Then, mitochondrial membrane permeability is enhanced. Cytochrome c dissociation from cardiolipin, and cytoplasmic leakage of cytochrome c leads to activation of the caspase-3 pathway and apoptosis. Both dying and recovering cells release ROS and inflammatory cytokines including IL-1b, TNFα and TGFβ, do produce acute local tissue and systemic effects[78]. Within the irradiated tissue differences in radiosensitivity of various cell phenotypes (endothelial cells, alveolar pneumocytes, alveolar macrophages and bronchopulmonary “stem” cells) contribute to the magnitude of tissue damage.

Role of immune system, cytokines and chemokines

Contribution of T lymphocytes is most important in fibrosis. Prolonged inflammation induces a specific lymphocyte T helper (TH) polarization. Local production of specific cytokines associated with this polarization have been well investigated in fibrosis. On the one hand TH1 orientation, notably characterized by the secretion of interferon-γ, is associated with resolution of the wound healing process. On the other hand TH2 orientation, characterized by the secretion of IL-4, IL-13 and TGF-b1, triggers tissue response toward fibrosis probably mediated by the pro-fibrotic growth factor: TGF-b1[79,80,81]. Exposure of intestinal stroma to bacteria is known to induce a TH1 polarization[82], but in the lung persistent exposure to bacterial antigens reorients TH1 polarization toward a TH2 profile suggesting that chronic epithelial depletion is fibrosis-prone[83].

Macrophage infiltration into inflamed tissues has been implicated in chronic inflammation-induced lung fibrosis[84]. In addition to their roles in immune regulation, macrophages play a pivotal role in matrix regression during the recovery phase of fibrosis and in the regulation of stellate cell proliferation[85]. The phenotype of these macrophages is generally reported to match that of alternatively activated cells (M2) rather than classically activated cells (M1). M2 macrophages express immunosuppressive molecules such as IL-10 and arginase I, which suppress the induction of Th1 cells that produce the anti-fibrotic cytokine IFNγ. On the other hand, M1 macrophages express IL-1, IL-12, IL-23, and induce Th1 cell infiltration and activation. However, it remains to be established whether a particular macrophage subset with M2-type properties preferentially infiltrates into fibrotic tissues, or whether it is the pro-fibrotic microenvironment that drives macrophage polarization toward an M2 phenotype.

Pro-inflammatory mediators involved in fibrogenesis

There is a variety of pro-inflammatory chemokines have non-redundant roles of recruiting macrophages and other effector cells to the sites of inflammatory injury[86]. Chemokines, especially macrophage inflammatory protein-1α (MIP-1α, also known as CCL3) and related CC-chemokines, act as signal transducers in inflammatory injury, and perform important regulatory functions. Different activated macrophages have different behaviour related to MIP-1α secretion: M1 stimulated by LPS and IFN-γ promotes MIP-1a-generation, while IL-4 and IL-10 inhibit MIP-1a production induced by LPS or IL-1β[87].

IL-4, the archetypal type-2 cytokine, has been firmly established as a pro-fibrotic cytokine and is elevated in radiation-induced pneumonitis and pulmonary fibrosis[35]. IL-4 receptors are present on lung fibroblasts[88] with IL-4 signaling increasing extra cellular matrix proteins and collagen deposition. Surprisingly, some studies have suggested that IL-4 is superior to TGF-β1 at inducing collagen synthesis from fibroblasts[88]. Indirect mechanisms of IL-4 include its ability to promote the alternative activation of macrophages.

Finally, one of the most renowned properties of IL-4 is its ability to promote the differentiation of T cells into Th2 cells, providing a source of many type-2 cytokines in this inflammatory axis (IL-5, IL-9, IL-13, and IL-21). The Th2 cytokines interact in dramatic ways propagating wound healing and potentially pro-fibrotic responses. For example, IL-5 mobilizes, matures, and recruits eosinophils[89] and promoting TGF-β production.

IL-5 can also augment IL-13 production and increase IL-13-dependent fibrosis[90]. IL-9 can selectively recruit and activate mast cells that increasing TGFβ activity and contributing to pulmonary fibrosis[91]. Mast cells can also promote fibroblast proliferation, collagen, and MMP production, and may be involved in subepithelial fibrosis following allergen challenge. IL-21 can also amplify Th2 pulmonary responses and IL-13-associated fibrosis by upregulating IL-4/IL-13 receptor expression.

IL-13 has been identified as a key fibrogenic cytokine in many fibrotic conditions and can function independently of TGF-β[92]. IL-13 can trigger the differentiation of fibroblasts into α-smooth muscle actin (α-SMA) expressing myofibroblasts.

Investigation of the balance between pro- and anti-inflammatory events in the gut may provide important insights into the pathogenic mechanisms of radiation. An imbalance between IL-1β and IL-1 receptor antagonist is reported to be an important factor in the pathogenesis of inflammatory bowel disease (IBD) and may explain why the acute inflammatory response develops into chronic persistent inflammation in some patients.

Cytokines probably play a role in initiating and perpetuating these uncontrolled disease processes. There is, however, a remarkable paucity of information on cellular interactions in complex gut inflammatory diseases. Study by Linard et al[93] in 2004 showed in vivo that abdominal irradiation induces alterations in the expression of genes involved in acute intestinal inflammatory response and thereby modifies the balance between pro- and anti-inflammatory cytokines. They observed that increase in IL-1β, TNF-α, and IL-6 expression occurred early at 6 h after irradiation and the high levels of IL-1β and IL-6 mRNA persisted for 3 days. Increased cytokine expression and protein production were observed simultaneously. More recently, Linard et al.[94] showed in T-bet-deficient mice, an increased fibrotic response to radiation, characterized by higher TGF-β1, col3a1 expression, and collagen deposition in mucosa compared with wild-type mice. Blirando K. and co-workers[95] demonstrated that mast cells have deleterious effects on both acute and chronic radiation proctitis, possibly by limiting acute tissue neutrophil influx and by favoring phenotypic orientation of smooth muscle cells, thus making them active participants in the radiation-induced inflammatory process and dystrophy of the rectal wall.

A cascade of inflammatory events ensued, with the proinflammatory cytokines (IL-1, TNF-α, and IL-6) increasing the ability of endothelial cells, macrophages, smooth muscle cells, and

fibroblasts to secrete IL-8. The accumulation of neutrophils at the inflammatory site is known to be caused mainly by the chemotactic cytokine IL-8. Although the exact role of IL-8 in the inflammatory pathogenesis is not totally clear.

TGF-β1 is a critical profibrogenic factor that induces the synthesis and deposit of collagen and other matrix components. It appears to play a particularly prominent role in the chronic phase of injury and is consistently overexpressed in areas of the intestinal wall that have histopathological lesions. Increased TGF-β expression associated with decreased IL-10 levels characterizes a fibrotic state. The importance of this anti-inflammatory cytokine on the pathophysiology of acute radiation-induced inflammatory processes is underlined by findings that IL-10 gene knockout mice develop gastrointestinal inflammation.

In summary, inflammation and the recruitment of circulating granulocytes, lymphocytes, monocytes, macrophages, and fibrocytes, presents a continuous supply of pro- and anti-fibrotic players, vital for efficient wound repair but potentially deadly when not adequately controlled. Every step of this pathway requires negative feedback loops that evoke significant control over the entire process. An imbalance in chemokine production coupled with dysregulated cellular recruitment can result in an excess of pro-fibrotic actors likes IL-13 or TGFβ, a surplus of myofibroblasts, and can convert a normal wound-healing response into a pathological fibrotic reaction.

PRO-FIBROTIC SIGNALING PATHWAYS

The Transforming Growth Factor (TGF)-β

The transforming growth factor (TGF)-β family, including TGF-β_1, TGF-β_2, and TGF-β_3, is a group of pleiotropic secreted cytokines with a broad spectrum of biologic functions. Amongst them, TGF-β_1 is a secreted protein with many cellular functions, including cell growth, cell proliferation, cell differentiation and apoptosis. In humans, TGF-β_1 can modulate cell differentiation and proliferation in an auto- or paracrine manner[96]. The receptors including TGF-β receptor (TβR) I and TβRII are glycoproteins of 55 kDa and 70 kDa, respectively, with core polypeptides of 500-570 amino acids[97]. Smads are molecules of 42-60 kDa, with two homology domains at the amino and carboxy terminals termed as terminal Mad-homology domains MH1 and MH2[98]. Smads can be divided into three classes, receptor-regulated Smads (R-Smads), co-mediator Smads (Co-Smads) and inhibitory Smads (I-Smads). R-Smads are directly phosphorylated and activated by TβRI kinases. Smad2 and Smad3 are involved in TGF-β signaling transduction and Smad1, Smad5 and Smad8 in bone morphogenic protein signaling transduction[99]. Smad4 was termed as DPC4 (deleted in pancreatic carcinoma locus 4), which was a candidate tumor suppressor gene in chromosome 18q21 frequently subjected to mutation or deletion in pancreatic cancer[100]. Smad2/3 and Smad4 are just the factors of the signaling pathway favoring the deposit of extracellular matrix mediated by TGF-β[97]. Smad6 and Smad7 inhibit TGF-β1 signaling as negative regulators[99].

TGFβ1 is derived from most cell lineages derived from the bone marrow including T cells, macrophages, eosinophils, and neutrophils and is one of the most widely studied pro-fibrotic cytokines. The potent activity of TGFβ1 is regulated at the post-transcriptional level by a latency-associated protein (LAP), which keeps TGFβ1 in an inactive state.

Dissociation of TGFβ1 from LAP is achieved by many agents commonly found in fibrotic conditions, including cathepsins, plasmin, calpain, thromombospondin, integrin αvβ6 and MMPs. These agents trigger the release of biologically active TGFβ1[35]. Once active, it is incredibly pleiotropic with, growth and chemotactic properties: stimulating fibroblast proliferation/ inhibiting epithelial cell growth; stimulating synthesis of extracellular matrix proteins[101]; having immuno-modulatory action: recruiting inflammatory cells through MCP-1 (CCL2)[102] and suppressing T-cell responses. The various and often opposing functions of TGFβ are likely explained by its various sources and cellular targets.

Active TGFβ1 is overexpressed in the lungs of mice, with the development of severe interstitial and pleural fibrosis, consisting of excess collagen deposition, extracellular matrix proteins, fibronectin, elastin, and the presence of myofibroblasts. Inhibiting TGFβ1 activity, by interfering with SMAD-mediated signaling, significantly reduced dermal[103], renal[104] and pulmonary fibrosis[105],[106]. TGFβ-independent as well as TGFβ-1 and IL-13-combined mechanisms can contribute to wound healing and fibrosis.

The CCN family proteins

The CCN family proteins are proteins associated with the extracellular matrix (ECM) that regulate activities such as adhesion, migration, mitogenesis, differentiation and cell survival. The acronym CCN was created by P. Bork in 1993[107] and brings together the three main members of this family: CYR61 or CCN1, Connective Tissue Growth Factor (CTGF) or CCN2, and Nov or CCN3. Later, three other members of the CCN family have been identified: WISP-1 or CCN4, WISP-2 or CCN5 and WISP-3 or CCN6.

The Connective Tissue Growth Factor (CTGF/CCN2) is the most studied in the context of fibrosis, since it is responsible for the excessive extracellular matrix production, proliferation and chemotactic cell migration that are essential during fibrosis. The CTGF is identified as an

essential mediator for the maintenance of fibrosis in various pathologic conditions[108] and is expressed in different fibrotic lesions.

CTGF was originally thought to be a specific downstream mediator of the profibrotic effects of TGF-b, with a particular role in stimulating fibroblast matrix production and myofibroblast differentiation[109]. Cell surface receptors and downstream signalling pathways have not been fully determined and there is now increasing support for the notion that CTGF may not act as a classical autocrine growth factor. In addition, it is clear that CTGF is induced by a number of other pro-fibrotic mediators, including thrombin[110] and mechanical stress.

Despite the uncertainties about mechanisms of action, CTGF is an interesting and specific target in the context of a number of fibrotic disorders, including systemic sclerosis and IPF[111]. CTGF expression is increased in IPF[112] and although adenoviral overexpression induces only mild and transient fibrosis in rats[113], overexpression in mice confers susceptibility to bleomycin-induced fibrosis in the fibrosis-resistant Balb/c mouse strain[114]. Moreover, selective expression of CTGF in fibroblasts *in vivo* has recently been shown to promote systemic tissue fibrosis, including in the lung[115]. A Phase I clinical trial assessing a neutralizing antibody directed against CTGF (FG-3019; FibroGen) was recently completed[116]; the results demonstrate that this antibody is safe and well-tolerated, but require validation in a prospective, randomized, blinded study.

Our group was the first to study CCN2 expression in the context of radiation fibrosis. In human delayed radiation enteropathy we showed that TGF-β1 expression is low whereas the gene and protein levels of CCN2 are very high, suggesting occurrence of alternative stimulatory signals involved in CCN2 regulation during the chronic phase of the pathology[16]. Similar observations were made in scleroderma, in which CCN2 but not TGF-β1 expression correlated with the severity of the fibrosis[111]. This apparent paradox suggested that CCN2 was more specific of chronical phase of fibrotic diseases.

Grotendorst et al.[60] proposed that TGF-β1 was able to trigger a long-term cell response, in which its own presence was no longer required for sustained CCN2 expression. Initiation and maintenance of fibrosis seemed to depend on the specific molecular pathway.

Haydont et al.[14] showed that CCN2 alone triggered its auto-induction in fibrosis-derived cells and that TGF-β1-enhanced CCN2 autoinduction.

The results of this study showed that *1*) distinct pathways controlled CCN2 expression during initiation vs. maintenance of fibrosis, *2*) very low doses of TGF-β1 elicited potent profibrotic action in fibrosis-derived cells, *3*) CCN2 drove its autoinduction in fibrosis-derived cells, and *4*) low concentration of TGF-β1 enhanced CCN2 auto-induction in fibrosis-derived cells.

The Rho/ROCK pathway

The Ras-homologous (Rho) family of small GTPases regulates a multitude of vital cell processes such as endocytosis, cell morphology, proliferation, survival, motility, and differentiation[117,118]. Extracellular signals received by cell-matrix adhesion molecules (e.g. integrins), receptor tyrosine kinases (e.g. growth factor receptors), and cell-cell adhesion molecules (e.g. cadherins) are transmitted to Rho GTPases, which in turn activate a variety of effector proteins[119].

Rho proteins cycle between two forms: the inactive guanine diphosphate (GDP)-bound form and the active guanine triphosphate (GTP)-bound form. Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by removing the GDP and replacing it with GTP. As their name suggests, Rho GTPases possess an intrinsic hydrolytic activity that converts GTP to GDP. However, this chemical reaction is generally slow unless catalyzed by GTPase activating proteins (GAPs). GAPs have historically been classified as negative regulators of Rho proteins, but recent evidence suggests a more complex role in Rho protein regulation for GAPs. A third class of regulatory proteins, GDP-dissociation inhibitors (GDIs), may inhibit

Rho protein activation by preventing exchange of GDP for GTP or by sequestering Rho proteins in the cytoplasm.

The activity of specific Rho GTPases is tightly regulated both temporally and spatially within the cell depending on the functional state of the cell[120]. The active form of Rho GTPases binds to the cell membrane where it acts upon effector proteins.

The small GTP-binding proteins belonging to the Rho family regulate various aspects of cell shape, motility, proliferation and apoptosis[121]. ROCKs are downstream targets of RhoA, which mediate Rho-induced actin cytoskeletal changes. ROCKs consist of an amino-terminal kinase domain, followed by a mid coiled-coil-forming region containing a Rho-binding domain (RBD), and carboxy-terminal cysteine-rich domain (CRD) located within the pleckstrin homology (PH) motif. In mammalian systems, two ROCK isoforms have been identified [88]. ROCK1, which is also known as ROKβ and p160ROCK, is located on chromosome 18, and encodes a 1354 amino acid protein. ROCK2, which is also known as ROKα and sometimes referred to as Rho-kinase, is located on chromosome 2 and contains 1388 amino acids. ROCK1 and ROCK2 share an overall 65% identity in amino-acid sequence and a 92% identity in their kinase domains. Despite having similar kinase domains, ROCK1 and ROCK2 might serve different functions and could have different downstream targets[122].

Although ROCK1 and ROCK2 are ubiquitously expressed in mouse tissues from early embryonic development to adulthood, ROCK2 mRNA is highly expressed in cardiac muscle and vascular tissues. In contrast, ROCK1 is more abundantly expressed in immunological cells and has been shown to co-localize to centrosomes. But even in cells that contain both ROCK1 and ROCK2, recent findings suggest specific functions for both isoforms.

The Rho/ROCK/CTGF pathway

Previously studies conducted in our and others laboratories have shown that the activation of Rho/ROCK pathway controlled fibrogenic differentiation in human radiation-induced enteropathy and may be a specific signaling pathway in the process of chronic fibrosis[13,14,16,18,123].

Rho activation after TGF-β1 stimulation was shown by direct Rho-GTP analysis and by pharmacological inhibition approaches using Pravastatin and Y-27632. Pravastatin action is not fully specific to Rho activation since it is known to target isoprenylated protein including Ras or Rac[118]. However, the strong efficacy of Y-27632, a specific inhibitor of the ROCK, in decreasing TGF-β1-induced CCN2 expression in fibroblasts reported in our (3) and other studies, [124]confirmed the essential role of the Rho/ROCK pathway in CCN2 regulation.

Considering these data and the very low rate of side effects, inhibitors of Rho/ROCK proteins was suggested to be promising anti-fibrotic targets in a wide range of diseases, including oncological[118], neurological[125] and several cardiovascular disorders[126], and in radiation-induced fibrosis as well as others fibrotic diseases[127,128].

RELEVANCE OF THE REMODELLING OF EXTRACELLULAR MATRIX IN RADIATION INDUCED FIBROSIS

Extracellular Matrix (ECM)

The extracellular matrix (ECM) has a major structural role. In addition it is one an important regulators of cellular and tissue function in the body. Tight controlled ECM homeostasis is essential for development, wound healing and normal organ homeostasis, and sustained dysregulation can result in life-threatening pathological conditions[129].

The ECM is mainly composed of an intricate interlocking mesh of fibrillar and non-fibrillar collagens, elastic fibers and glycosaminoglycan (GAG)-containing non-collagenous glycoproteins (hyaluronan and proteoglycans). The interstitial matrix found in most but not all tissues consists mainly of the fibrous collagen type I, which, together with fibronectin, confers mechanical strength to tissues[130]. Although collagens are collectively the most abundant component of the ECM, the differential expression of individual interstitial ECM components underpins the specific functions of many organs and tissues. In addition to the interstitial matrix, extracellular basement membranes (BMs) are a specialized form of sheet-like ECM to which epithelial cells can anchor and which interact directly with the epithelium and endothelium. These membranes mainly consist of collagen IV, laminins, entactin (also known as nidogen) and heparan sulfate proteoglycans[130] BMs play a key role in epithelial cell function, providing cues for orientation that help to establish and maintain apicobasal polarity and cell differentiation.

The ECM serves many functions in addition to providing structural support. Macroscopically, the ECM physically segregates cells and organs and acts as a protective cushion, for example, by regulating hydrostatic pressure within tissues and organs. At the microscopic level, this highly dynamic molecular network is also capable of regulating cellular behavior through

modulation of, among other things, proliferation, cytoskeletal organization, cellular differentiation and receptor signaling[131].

The ECM structure is determined by continuous dynamic remodeling. Such remodeling is regulated by a careful balance between matrix synthesis, secretion, modification and enzymatic degradation. ECM components are degraded by matrix-degrading enzymes, including heparanase, cathepsins, hyaluronidases, matriptases, various serine and threonine proteases and the large superfamily of metzincins, which includes ADAMs (a disintegrin and metalloproteinases), ADAMTSs (ADAMs with thrombospondin motifs), and matrix metalloproteases (MMPs) and their inhibitors [tissue inhibitor of MMPs (TIMPs)][132]. The tightly controlled ECM homeostasis is sensitive to altered expression of these proteases, which, if altered for prolonged periods of time, can result in excessive ECM remodeling, as is frequently observed in fibrotic diseases[133].

Tissue fibrosis is typically characterized by excessive ECM synthesis and secretion. This increased ECM synthesis can results from overexpression of TIMPs or loss of MMP expression, and assembly and subsequent cross-linking lead to altered biochemical and biomechanical matrix properties, compromising normal tissue function and further driving disease progression resulting in incomplete matrix remodeling and irreversible fibrosis[134].

Matrix Metalloproteases (MMPs) and Tissue Inhibitors of MMPs (TIMPs)

Matrix metalloproteasis (MMPs) are proteolytic enzymes implicated in many physiological and pathological processes including embryonic development, morphogenesis, reproductive processes, bone remodeling, wound healing, cancer, arthritis, atherosclerosis[135].

MMPs are zinc and calcium-dependent enzymes able to degrade virtually all extracellular matrix (ECM) components. MMPs, by their proteolytic activity, can, on one hand, affect the adherence of cells to the extracellular matrix and, on the other hand, release both bioactive

fragments of extracellular matrix molecules and “trapped” bioactive mediators, providing signals from the microenvironment to cells allowing them to react to stimuli[136].

Currently, 28 MMPs families have been identified in vertebrates; 191 different metalloproteinases have been found in humans (Degradome database; http://degradome.uniovi.es)[137]. The latter are classified according to their substrate specificity and structural features into gelatinases (MMP2, -9), stromelysins (MMP3, -7, -10, -11), elastases (MMP12), collagenases (MMP1, -8, -13, -18), and membrane-type MMPs (MMP14,-15, -16, -17). Most MMPs are secreted as zymogens and require proteolytic activation. In vivo activation of pro-MMPs is mostly mediated through the plasminogen-plasmin cascade and by MMPs themselves[138]. Another type of MMP activation, which has been reported for MMP-2, is through the membrane-type MMP-1 (MMP-14). This process may be associated to fibrogenesis as MMP-2 degrades basement membrane type IV collagen[139] which is thought to facilitate the deposition of fibril-forming collagen. The third level of control of MMP activity is ensured by TIMPs, which are known to inhibit active MMPs at a stoichiometric ratio of 1:1[140]. Four subtypes of TIMPs (TIMP-1 to -4) have been identified so far. Whereas TIMP-1 inhibits a broad range of MMPs, TIMP-2 seems to specifically inhibit MMP-2. A controlled balance between active MMPs and TIMPs is required for maintenance of normal tissue homeostasis and imbalance in these ratios has been implicated in a number of pathological disorders, including tumour invasion[141] and metastasis [142], atherosclerosis[143], arthritis[144], emphysema[145] and fibrotic diseases[146,147,148,149].

Collagen accumulation in radiation fibrosis has been thought to be associated with a decrease in MMP activity and increased TIMP levels. Strup-Perrot et al.[149] showed that an intense ECM remodeling seems to particularly affect intestinal mucosa in radiation enteropathy. Induction of each member of the MMP family, i.e., gelatinases, stromelysin, collagenases, and membrane-type MMPs, was reported in late radiation enteritis. However, the concomitant

induction of MMP inhibitors (TIMP-1, TIMP-2, and PAI-1) counterbalances this induction of MMPs, leading to a net collagen deposition.

MMP-2 and MMP-9 and matrilysin MMP-7 are known to play a pivotal role in ECM metabolism and homeostasis of basement membrane. MMP-2 and MMP-9 have significant activity in the pathogenesis of emphysema and both can be present in fibrotic tissues[150]. MMP-2 may be associated with impaired tissue remodeling leading to pathological collagen deposition and pulmonary fibrosis, whereas MMP-9 has been linked to inflammation-induced tissue remodeling[150]. Several studies have shown that MMP-2 and MMP-9 are induced by ionizing radiation[105,149, 151,152].

TREATMENT OF RADIATION INDUCED FIBROSIS

In general, chemical/biological agents used to alter normal tissue toxicity from radiation can be broadly divided into three categories based on the timing of delivery in relation to radiation: **chemical radioprotectors, mitigators and treatment**.

Agents delivered prior to or at the time of irradiation with the intent of preventing or reducing damage to normal tissues are termed **radioprotectors.** A classic example is the use of a free radical scavenger such as amifostine. Agents delivered at the time of irradiation or after irradiation is complete, but prior to the manifestation of normal tissue toxicity, are described as **mitigators** of normal tissue injury. Examples include the use of angiotensin-converting enzyme (ACE) inhibitors. Finally, when drugs, such as Pentoxifylline plus tocopherol and statins, are delivered to ameliorate established normal tissue toxicity, they are considered **treatments** (Fig.8)[52].

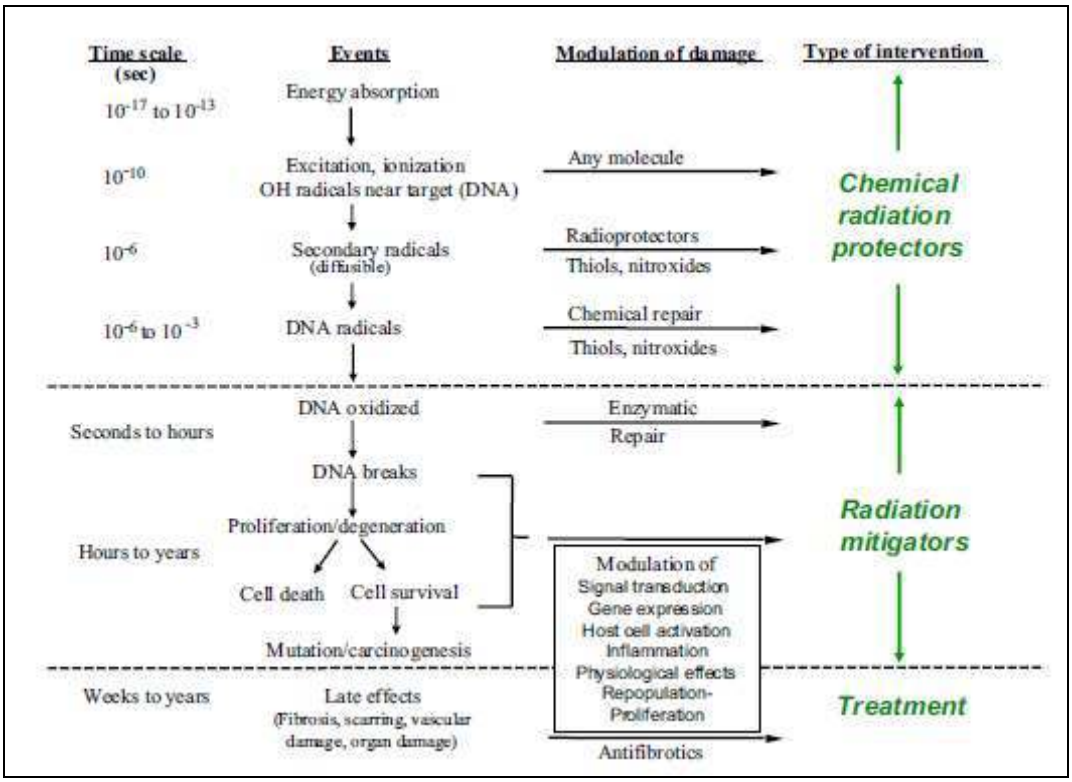


Fig. 8: Sequence of events following radiation exposure. From "Citrin D. et. al Radioprotectors and Mitigators of Radiation-Induced Normal Tissue Injury. The Oncologist 2010;15:360–371".

Anti-Inflammatory Therapies

In general, the existing experimental and clinical studies suggest that anti-inflammatory agents have only a limited role, at best, in the prophylaxis or treatment of radiation-induced normal tissue injury. Corticosteroids have been used to treat fibrosis. In vivo, dexamethasone has been used to treat RI pneumonitis, nephropathy, and liver injury in rats and appears to delay development of RI organ dysfunction[153].

Although steroids are widely used for their antiinflammatory properties in everyday clinical treatment of chronic RI, no long-term effect on the underlying fibrosis has yet been shown.

Superoxide Dismutase

Both prevention and reversal of radiation-induced fibrosis have been shown using Cu/Zn superoxide dismutase (SOD) in soft tissue radiation injury[154,155]. One of the cellular and molecular mechanisms associated with the antifibrotic action of SOD is the modulation (inhibition/reversal) of the radiation-induced myofibroblastic differentiation through repression of the profibrogenic growth factor TGF-b1[155]. Unfortunately, no approved SOD drug is currently available to patients.

Suppression of the Renin-Angiotensin System (Angiotensin Converting enzyme inhibitors and Angiotensin II receptor antagonists)

ACE inhibitors (eg, captopril and enalapril) and AII receptor antagonists (eg, losartan) are of clear benefit in the treatment and prophylaxis of experimental radiation nephropathy and experimental radiation pneumopathy.

The action of ACE inhibitors and AII blockers in limiting subsequent fibrosis requires further investigation, particularly because this latter effect may have relevance to late radiation-induced injury in other normal tissues. Clinical trials for prevention of radiation-induced

nephropathy and pneumonitis are ongoing and/or recently completed. Clinical data available suggesting stabilization of renal function in established radiation-induced nephropathy[156].

Pentoxifylline and association with tocopherol/Vit.E and clodronate

Pentoxifylline (PTX) is a methylxanthine derivative with some properties similar to caffeine but with few cardiac effects and is used to treat vascular diseases such as intermittent claudication. In vivo and in vitro studies show that PTX has 4 major properties: it increases blood viscosity and flow hence increasing tissue oxygenation and tumor radiation response, inhibits coagulation (platelet aggregation), limits wound healing (fibroblast proliferation, collagens) and tumor necrosis factor-α, reduces immunologic and inflammatory reactions (leukocytes)[157].

Pentoxifylline has been used in the treatment of radiation-induced fibrosis and soft-tissue necrosis in both experimental models and clinical trials.

The combination of pentoxifylline and vitamin E with clodronate (PENTOCLO) is useful in healing sternocostal and some mandibular ORN. Recently, Delanian and coworkers[158] showed in a randomized clinical trial that long-term PENTOCLO treatment is effective, safe, and curative for refractory ORN and induces mucosal and bone healing with significant symptom improvement. The efficacy of this combination has been reported also in others various anatomical locations (soft tissue fibrosis, uterine fibrosis); however, the associated molecular mechanism remains mostly unexplored[13]. A number of trials have been performed with tocoferol delivered during the course of radiotherapy, with the goal of reducing normal tissue toxicity (xerostomia, mucositis and pulmonary fibrosis), in many instances with promising results.

Unfortunately, the use of antioxidant vitamins, such as alpha tocopherol and beta carotene, during the course of radiotherapy was associated with evidence of poorer tumor control in randomized trials[153].

Pirfenidone

Pirfenidone is an orally available pyridine derivative that has recently received much interest in IPF in view of its anti-fibrotic, anti-inflammatory and antioxidant properties[159]. Briefly, *in vitro* pirfenidone has been shown to inhibit fibroblast proliferation and collagen synthesis. I*n vivo* it attenuates bleomycin-induced lung fibrosis when dosed either prophylactically or therapeutically and this attenuation is associated with a reduction in lung platelet derived growth factor (PDGF) and TGF-b levels. Its anti-inflammatory properties are manifested by an attenuation in TNF-α and IFN-γ levels in experimental models of inflammation[159].

The precise molecular mechanism of action remains unknown, however Pirfenidone represents a potentially important advance in IPF therapy: in fact, treatment resulted in a significant reduction in FVC decline compared with placebo in one trial at 72 weeks (CAPACITY2).

Imatinib

The tyrosine-kinase inhibitor, Imatinib mesylate, has activity against the PDGF receptor.

For over 15 years, PDGF has been known to induce procollagen production by fibroblasts *in vitro*, it may play a greater role in expanding the fibroblast accumulation at sites of injury.

Anti-fibrotic potential of this drug may reflect multiple modes of action.

Imatinib inhibits signalling pathways directly downstream of TGF-β. It is also implicated in the inhibition of two pathways recently implicated in the development of bleomycin-induced fibrosis in mice[160,161].

The groups of Daniels et Aono, in theirs preclinical studies, demonstrated that imatinib reduces collagen deposition and mesenchymal cell proliferation in the bleomycin model when dosed prophylactically, but this was not the case when imatinib was administered in a therapeutic schedule (day 14 post bleomycin onwards).[162,163].

In a recent multi-centre, randomized, placebo-controlled trial (Novartis, Switzerland) of patients with mild to moderate IPF followed for 96 weeks[164] imatinib did not affect survival or lung function.

Current inhibitors of Rho GTPase signaling

The Rho/ROCK proteins play an important role in the induction and maintenance of fibrosis. Statins and Y-27632 (respectively inhibitors of Rho and ROCK) may be an opportunity to demonstrate the implication of the Rho/ROCK pathway in the forms of radiotherapy-induced fibrosis. To inhibit the activity of these proteins is therefore an anti-fibrotic therapeutic strategy very attractive.

Statins are drugs currently used in the treatment of hypercholesterolemia. They are schematically classified into three categories according to their metabolism: simvastatin which is metabolized by CYP 3A4 predominantly (the most expressed isoform of cytochrome P450), both at the intestinal level in the liver, cerivastatin and atorvastatin exclusively linked to CYP 3A4 and fluvastatin and pravastatin metabolism does not interfere, or interferes very little, with CYP 3A4.

Although HMG-CoA reductase inhibitors are generally well-tolerated drugs, side effects can occur. The serious ones such as elevated liver transaminase levels (defined as > 3 times the upper limit of normal) or elevated creatine kinase (CK) levels (defined as > 10 times the upper limit of normal) linked to myopathy incidences occur in only 0.5% to 2% of clinical trial subjects[165].

Several clinical trials have demonstrated and confirmed the beneficial effects of statins in cardiovascular disorders, in primary and secondary prevention settings, and in asymptomatic subjects with a high cardiovascular risk. A positive role of statins has also been observed in many other diseases: cancer prevention, reduction in the frequency of fractures in patients with osteoporosis, reduction in the progression of diabetes, prevention of deep venous

thrombosis, prevention of dementia. The multiplicity of these effects, called pleiotropic effects, has no a priori relationship with the action on cholesterol.

A number of strategies have been employed to inhibit the functions of Rho proteins (reviewed in [166]). Rho GTPases are dependent on membrane localization for their activity. HMG-CoA-reductase inhibitors (statin drugs) inhibit both types of prenoid posttranslational modifications in Rho proteins. In fact, statins prevent the synthesis of cholesterol that is also involved in the synthesis of small GTP-ase. Farnesyl-PP (FPP) and geranylgeranyl-PP (GGPP) isoprenoids, which are important intermediaries in the synthesis of cholesterol, are also required for post-translational modification of small GTP-ase Rho and Ras family (Figure 9)[167].

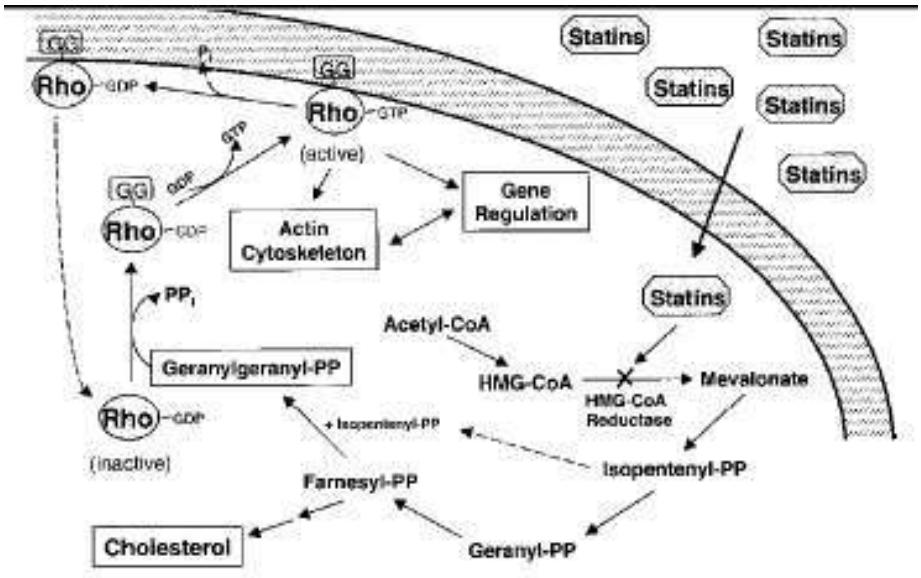


Fig. 9. From Laufs U, Liao JK. "Targeting Rho in cardiovascular disease". Circ Res. (2000) 87(7):526-528.

Studies with pravastatin showed a modulation of fibrogenic phenotype *in vitro* for limiting the expression of CTGF and synthesis of pathological extracellular matrix proteins (collagen I, III and fibronectin)[123,13]. Moreover, pravastatin inhibits the Rho/CCN2/extracellular matrix cascade in human fibrosis explants and improves radiation-induced intestinal fibrosis in rats[17].

Another potential therapeutic opportunity is to inhibit effector proteins activated by Rho GTPases. ROCK has been a popular target for kinase inhibitor development because of its important role in cell motility downstream of RhoA and RhoC. ROCK inhibitors include HA-1077 (fasudil), H-1152, and Y-27632[27]. Y-27632 blocked phosphorylation of cofilin and promoted E-cadherin expression and cell adhesion[168]. Wf-536, a more potent derivative of Y-27632, inhibited cell motility *in vitro* and angiogenesis and metastasis *in vivo*[169,170].

Inhibition of Rho-kinase by the small molecular weight inhibitor Y27632 has shown to reduce fibrotic parameters in primary hepatic stellate cells and in several animal models of liver fibrosis[171]. In addition, Bourgier C. et al.[74] have demonstrated that Y-27632 is able to reverse the fibrogenic phenotype of vascular smooth muscle cells isolated from bowel of patients with post-actinic enteritis.

These results suggest that Rho/ROCK pathway is a possible therapeutic target for the radiotherapy-induced toxicity modulation and that statins may be a new anti-fibrotic drugs.

Many studies with many others drugs and target therapies are actually ongoing or recent completed[110]. For example: Phase I clinical trial in IPF patients with GC1008 (Antibody against TGF-β); study with orally active activin-like kinase receptor-5 kinase inhibitors, SB-525334 (GlaxoSmithKline, UK); A Phase II clinical study for the treatment of skin fibrosis in systemic sclerosis with topical application of P144 (DigNA Biotech, Spain) which blocks binding of TGF-b1 to TGFbR1; Phase I clinical trial assessing a neutralizing antibody directed against CTGF (FG-3019; FibroGen, USA) etc.

All these efforts to prevent, mitigate or treat radiation-induced fibrosis are necessary to reduce this severe consequence of radiotherapy that affect quality of life of the growing number long-cancer survival.

OBJECTIVES AND STRATEGIES

Previous pre-clinical data obtained in our laboratory and others (M Hauer-Jensen laboratory) showed that statins (Pravastatin, Simvastatin, Atorvastatin) displayed anti-fibrotic action in models of radiation-induced intestinal fibrosis.

The first part of my doctoral training was to investigate if the anti-fibrotic action displayed Pravastatin and Simvastatin was restricted to the gut or could be applied to other organs. In addition we investigated the anti-fibrotic action displayed by several pharmacological inhibitors of the Rho/ROCK pathways: Simvastatin, Pravastatin and Y-27632. Therefore, we developed in mice 2 experimental models of pulmonary fibrosis. One induced by the radiomimetic drug, Bleomycin (BLM), and one by single dose of irradiation (19 Gy single dose) to the chest. Animals were treated with the various Rho and ROCK inhibitors: Simvastatin, Pravastatin and Y-27632; and we showed that all of them displayed anti-fibrotic properties in the lung. We showed that pravastatin treatment induced inhibition of RhoB, TGF-βRII and CCN2 expression suggesting that part of the anti-fibrotic action of Pravastatin could be mediated by the inhibition of these fibrogenic molecules.

Then, we investigated whether Pravastatin was able to induce a fibrolytic action. This study was performed in gut and lung samples of rodent. Modulation of the expression of MMP2 and its natural inhibitor TIMP2 was investigated by immunohistochemistry in two models of radiation-induced fibrosis (intestinal and pulmonary). Interestingly we found a different impact on fibrolysis when Pravastatin was administered preventively or curatively. In addition, alteration of gelatinase expression was studied in vitro, in cell culture of primary lung fibroblasts isolated from mC57Bl6 mice incubated with Pravastatin.

MATERIALS & METHODS

Anti-fibrotic effect of Rho/ROCK inhibitors

The potential anti-fibrotic effect triggered by Rho and ROCK pharmacological inhibitors (Pravastatin, Simvastatin and Y-27632) was assessed in the well-characterized models of radiation-induced intestinal fibrosis[17], BLM-induced lung fibrosis[172] and after thorax irradiation (19Gy).

Experimental model of gut fibrosis and drug administration

Model of intestinal fibrosis was generated by local irradiation of Wistar rat's ileum as already described[17]. Fibrosis development was monitored 15 and 26 weeks after irradiation. Briefly, ileum was irradiated locally after surgical exteriorization (19 Gy with X-ray machine operated at 225 kV and 17 mA with 0.5-mm copper-added filtration, at a dose rate of 0.98 Gy/min.). Pravastatin was administered in drinking water (30 mg/kg/day) according to the schedule shown figure 1 in both mitigation (A) and curative (B) approaches (Fig.10).

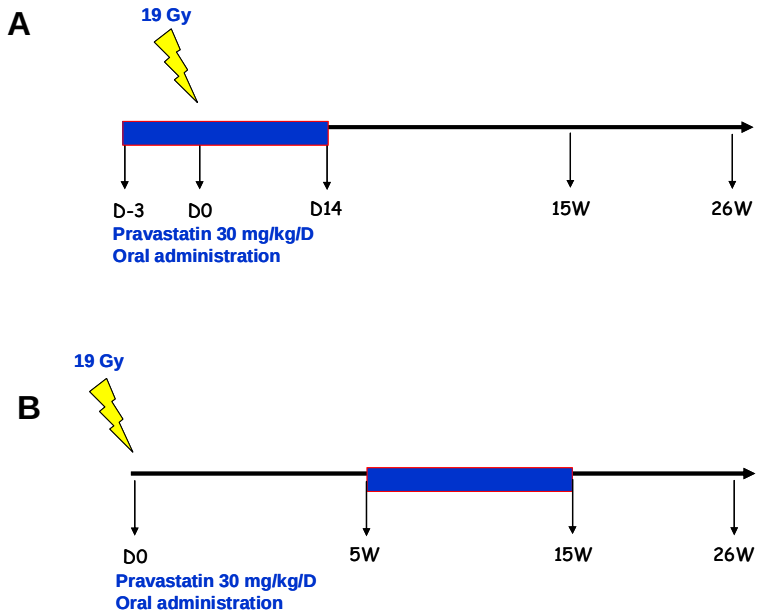


Fig 10. Schedule of gut irradiation and Pravastatin administration in rats via drinking water (30 mg/kg/day) in mitigation (**A**) and curative (**B**) approaches.

Ileal samples were washed with 0.9% sterile saline buffer, fixed in 4% formol for 24 h and paraffin embedded. Five-micrometer longitudinal sections were cut. Five animals per points were analysed with a total of forty animals.

Mice immobilization and thorax irradiation

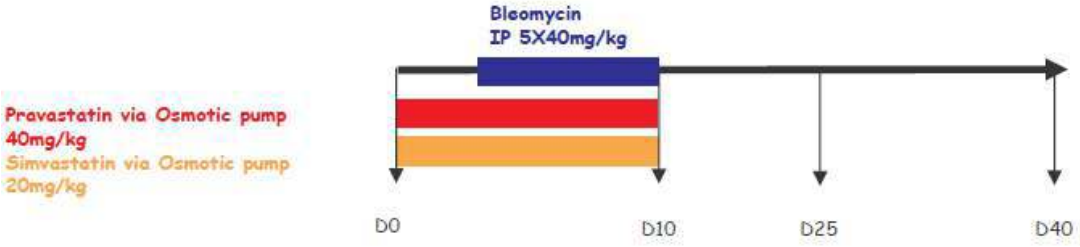
A total of 150 Female C57BL6 mice were obtained from Charles Rivers France. Experiments were conducted under the French regulations for animal experimentation (Ministry of Agriculture Act No. 87-848, 19th of October, 1987) and received ethics approval.

Using a TEM anesthesia system (Bordeaux, France), mice were anaesthetized by inhalation of an air/isoflurane (Forène, Abbott France, Rungis) mixture and irradiated with a 200kV X-ray machine operated at 15mA with 0.2mm copper filtration, providing an incident dose rate of 0.79 Gy/min. Doses of 19 Gy were given to the thorax in one fraction. To minimize unwanted off-target biological responses which may confound interpretation of thorax data, the rest of the animal was shielded with a 10mm thick lead screen.

Experimental models of lung fibrosis and drug administration

In the experimental model of **BLM-induced lung fibrosis**, mice were divided in 7 groups: sham, Bleomycin, Prava-Bleo, Simva-Bleo, Bleo-Prava, Bleo-Simva, Bleo-Y group. Pravastatin and Y-27632 were hydrosoluble drugs, simvastatin was diluted in DMSO at 10% and all drugs were administered *via* 2 weeks Osmotic pump (Alzet, n°1002) implanted sub-cutaneously in a preventive (A) and curative (B) approach (Fig. 11). At day 40, lungs were collected for histology.

A



B

Fig 11. Schedule of BLM-induced lung fibrosis and Rho/ROCK inhibitors administration in mice via osmotic pump in a preventive (**A**) and curative (**B**) approach.

In the **radiation-induced fibrosis model**, after a single dose of 19 Gy to the thorax, pravastatin was administered in the drinking water between the 5th and the 15th week post irradiation (Fig. 12). Lungs were collected 15 and 26 weeks post-irradiation using the same procedure.

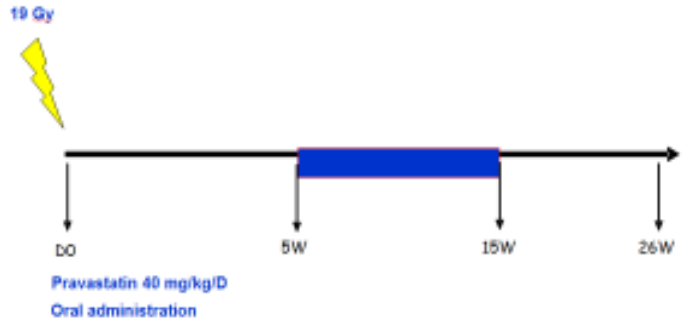


Fig.12. Schedule of lung irradiation and Pravastatin oral administration in mice in a curative approach.

Histological examination and immunohistochemistry

Gut and lungs are collected for histology and immunohistochemistry. Organs were fixed in Finefix (Milestone medical, Italy), paraffin embedded and cut into 4 µm sections.

Lung sections were stained with Hematoxilin-Eosin-Saffranin (HES) and examined using conventional light microscopy. Expression of TGF-BRII, RhoB, and CCN2 deposition were studied by immunohistochemistry.

Moreover, immunohistochemistry for MMP2 and TIMP2 was performed for all models of radiation-induced fibrosis (gut and lung) as previously described[152].

Primary antibodies were used at the following dilutions: TGF-BRII 1:100 (Santa Cruz H-70:sc-28565), RhoB 1:100 (Santa Cruz C-5:sc-8048), CCN2 1:50 (Santa Cruz L-20:sc-14939), mouse anti-human MMP2 monoclonal antibody (cat. #42-5D11 Millipore; respectively 1:200 and 1:100 for intestine and for lung) and mouse anti-human TIMP2 monoclonal antibody (cat. #67-4H11 Millipore; respectively 1:1000 and 1:100 for intestine and for lung) (table 1).

<table>
<tr><th></th><th colspan="2">LUNG</th><th colspan="2">INTESTIN</th></tr>
<tr><th></th><th>Ab primary</th><th>Ab secondary</th><th>Ab primary</th><th>Ab secondary</th></tr>
<tr><td>MMP2 monoclonal antibody</td><td>1:100</td><td rowspan="5">HRP-conjugated secondary antibody 1:5000</td><td>1:200</td><td rowspan="2">HRP-conjugated secondary antibody 1:5000</td></tr>
<tr><td>TIMP2 monoclonal antibody</td><td>1:100</td><td>1:1000</td></tr>
<tr><td>TGF-BRII</td><td>1:100</td></tr>
<tr><td>RhoB</td><td>1:100</td></tr>
<tr><td>CCN2</td><td>1:50</td></tr>
</table>

Table 1.

Sections were incubated with corresponding HRP-conjugated secondary antibody (GE Healthcare Life Sciences; diluted at 1/5000 in TBST containing 2% BSA). Endogenous peroxidases were blocked by incubation with 0.1% H_2O_2 in PBS for 10 min. Colour development in immunoperoxidase staining was performed with 3,3'-diaminobenzidine

enhanced liquid substrate system (DAB) and sections were counterstained using Mayer's haematoxylin (Fluka Chemie, Buchs, Switzerland). Images were acquired using a Leica microscope equiped with a JVC color video camera coupled to an imaging analysis system (Histolab software, Microvision, France). Omission of primary antibodies was used as a negative control.

Cells

Primary lung fibroblasts were isolated from 2-8 week-old C57BL/6 and sub-cultured in Dulbecco's modified Eagle's medium GlutaMax (DMEM GlutaMax, GIBCO Invitrogen) supplemented with 20% fetal bovine serum (FBS; PAA Laboratories GmbH - Austria), 1% penicillin-streptomycin (Sigma-Aldrich), and 1% Hepes (GIBCO Invitrogen). The fibroblasts were passaged at sub-confluency and were used between the 3rd and 6rd passage. Cells were trypsinized (trypsin-EDTA; GBCO Invitrogen, 0.05% trypsin 0.53 mM EDTA) and resuspended in serum-free DMEM GlutaMax containing Pravastatin (500-1000 µM, final concentration) 30 minutes before irradiation. Cells were irradiated at: 2Gy, 8Gy and 16 Gy γ-irradiation with Linear Accelerator (Precise Elekta, dose rate of 1,243 cGy/UM). Culture conditioned medium and fibroblast were collected 4-6-24 and 48 hours post-irradiation and frozen in 80°C until analysis.

Protein isolation and gelatin zymography

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (Roche). Zymography was performed as previously described on cells lysed and colture conditioned medium [ref]. Briefly, samples were separated by electrophoresis on 80 g/L SDS-polyacrylamide gels copolymerized with 1 g/L gelatin (Type A from porcine skin; Sigma-Aldrich, Milan, Italy). Gels were washed in 2,5% Triton X-100, incubated in a buffer containing 50 mmol/L Tris–HCl (pH 7.8), 5 mmol/L CaCl2·2H2O, 0.005% Brij 35, 0.02%

Azide and 1 µmol $ZnCl_2$, stained with 5 g/L Coomassie blue in 300 mL/L isopropanol/100 mL/L acetic acid, and destained in a 300 mL/L methanol/100 mL/L acetic acid solution. Gelatinolytic bands appeared as clear zones against the blue background. Gelatinases were identified by their molecular weight and densitometric analysis were performed.

RESULTS

In order to investigate the anti-fibrotic efficacy triggered by Rho/ROCK inhibition, pulmonary fibrosis was modeled using chronic injection of Bleomycin and single dose RX-irradiation.

Pharmacological inhibitors of the Rho/ROCK pathway prevent and reverse lung fibrosis.

Mice were treated with various Rho (*i.e.* pravastatin and simvastatin) and ROCK (*i.e.* Y-27632) inhibitors using both mitigating and curative approaches according to the schedule shown in materials & methods (Fig.11 A e B).

Survival rates were monitored according to the various treatments and histopathological examinations were performed. In the Bleomycin model, curative administration of Pravastatin and Y-27632 (from day 25 to day 40) partially rescued bleomycin-induced mortality, whereas Simvastatin administration only delayed mortality (Fig. 13B). Bleomycin administration induced typical subpleural and intra-parachymatous fibrotic lesions with dense extracellular matrix deposition (saffron-orange staining), associated with remodeled vessels surrounded by intense inflammatory infiltration (Fig. 13C). Interestingly, a significant improvement of lung structure was observed in the animals treated with the 3 drugs. Thin fibrotic lesions were still observed in sub-pleural area but the overall pulmonary structure was normal. Pravastatin induced the same anti-fibrotic action in the model of radiation-induced lung fibrosis (RX, 19 Gy).

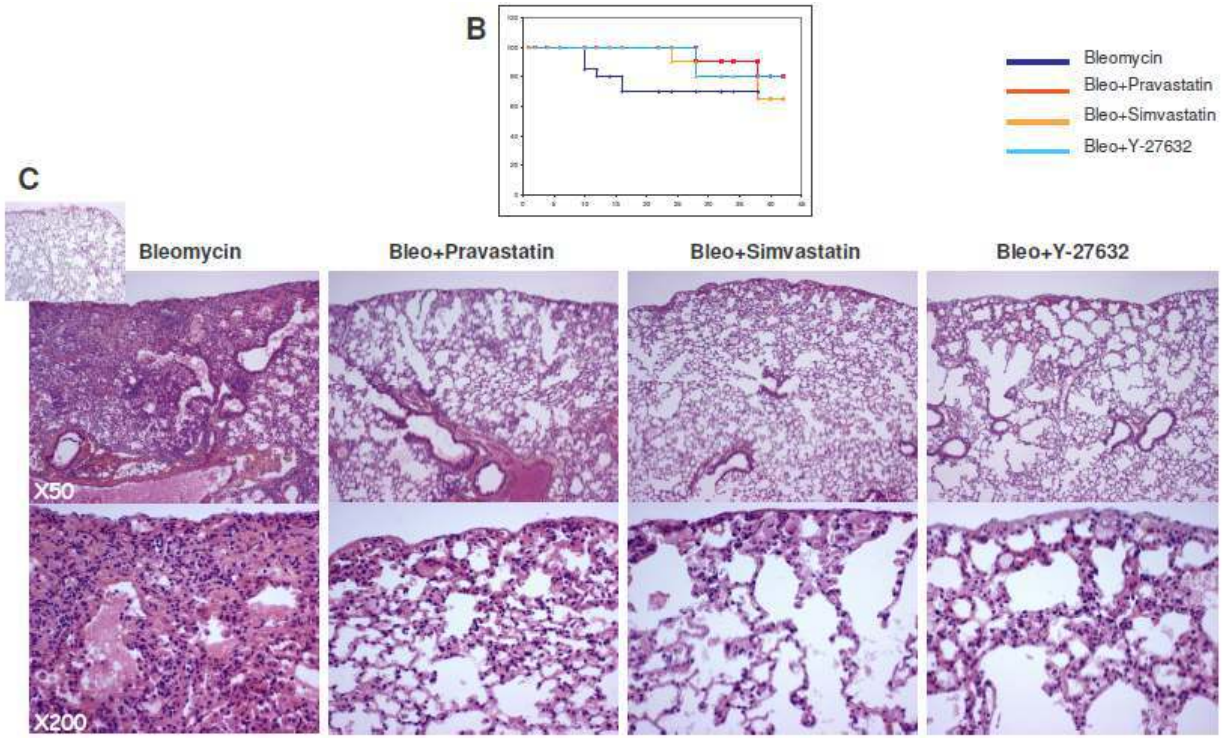


Fig. 13: **Reversion of bleomycin-induced pulmonary fibrosis. B.** Monitoring of the survival rates. In the Bleomycin model, curative administration of Pravastatin and Y-27632 partially rescued bleomycin-induced mortality, whereas Simvastatin administration only delayed mortality.
C. BLM induced widespread subpleural thickening and marked fibrotic lesions with dense extracellular matrix deposition (saffron-orange staining), associated with oedema of alveolar septa. Intra-parachymatous fibrotic remodeling surrounding vessels was observed with intense infiltration of inflammatory cells including macrophages, lymphocytes and neutrophils, leading to abnormal lung architecture. The 3 drugs used in the animals reduced oedema of alveolar walls and thickening of subpleura. Small fibrotic lesions were still observed in sub-pleural area but the overall pulmonary structure was normal.

Prophylactic administration of Pravastatin and Simvatatin also gave positive results preventing from the development of Bleomycin-induced lung fibrosis (Fig. 14 C). Mortality was delayed but the overall survival rate did not improve (Fig. 14 B).

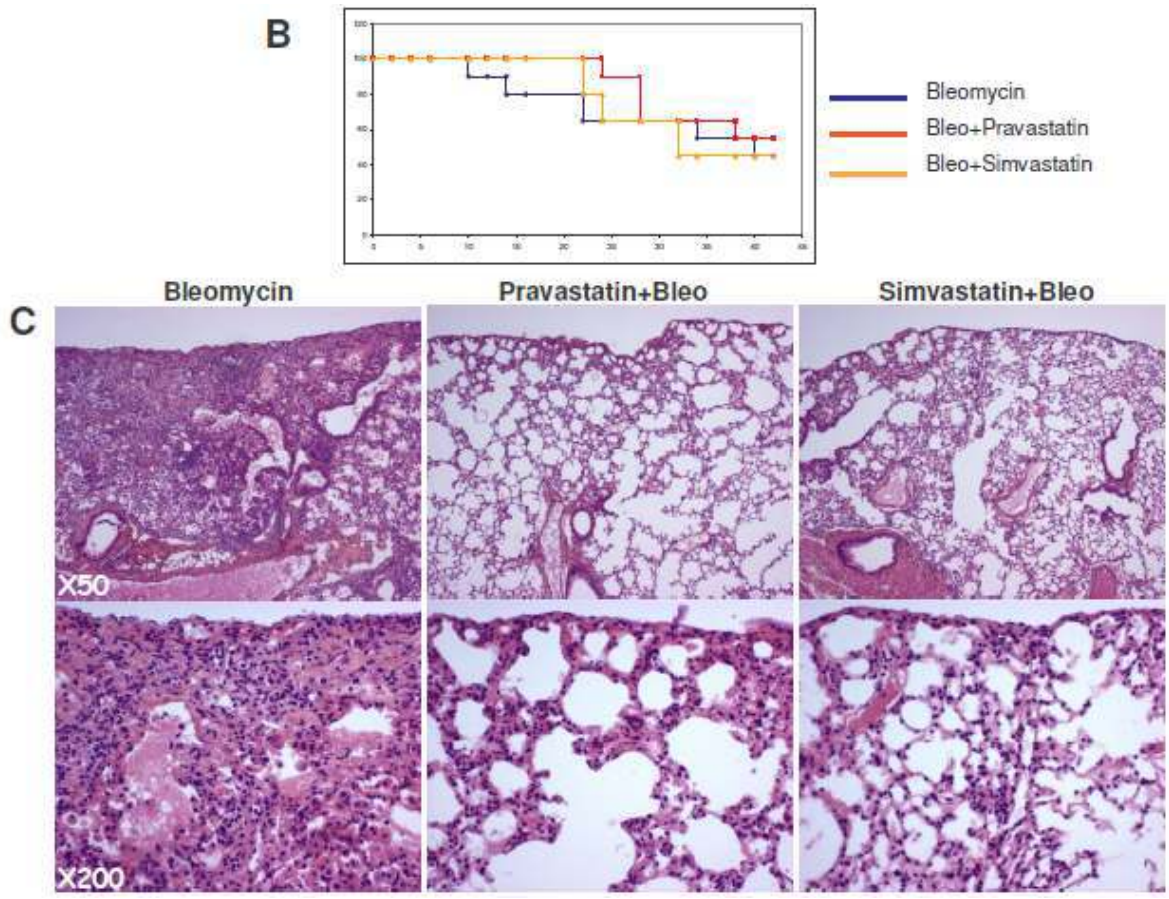


Fig. 14: **Prevention of bleomycin-induced pulmonary fibrosis. B.** Mortality was delayed but the overall survival rate did not improve.
C. Preventive and concomitant administration of Pravastatin and Simvatatin gave positive results preventing from the development of Bleomycin-induced lung fibrosis.

No intra-parenchymatous fibrotic lesions were observed in the animals long-term-treated with Pravastatin (from week 5 to week 15 post-irradiation) (Fig. 15).

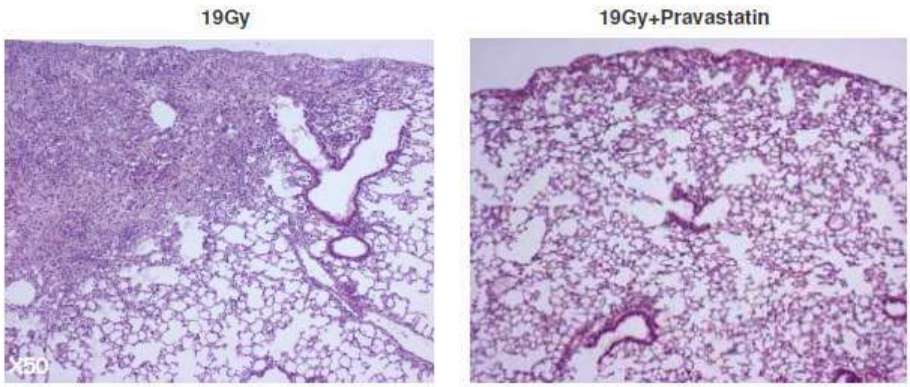


Fig. 15: Reversion of radiation-induced pulmonary fibrosis. Pravastatin induced the same anti-fibrotic action in the model of radiation-induced lung fibrosis (RX, 19 Gy). No intra-parenchymatous fibrotic lesions in animals long-term-treated with Pravastatin (from week 5 to week 15 post-irradiation).

In order to understand the molecular basis of the fibrolytic action triggered by Pravastatin, we investigated *in situ* by immunohistochemistry the downstream effect of pravastatin inhibition on RhoB, TGF-βRII and CCN2 expression 15 weeks after irradiation in lungs of mice irradiated and treated or not with Pravastatin (Fig. 16)**.**

Pravastatin modulates the fibrogenic cascade involving TGF-βRII and Rho in the lungs.

In non-irradiated lungs, type I pneumocytes did not stained for RhoB whereas few microvessels and macrophages were positive. TGF-βRII staining was stronger and located in alveolar septa and macrophages. CCN2 was not expressed in normal lung (Fig.16).

The irradiated group exhibited dense sub-pleural fibrotic lesions; the *interstitium* was invaded by extracellular matrix and massive infiltration of myofibroblasts, polynuclear cells and macrophages. Within these remodelled zones, a black-brown RhoB staining was observed in all interstitial cells and at the plasma membrane of smooth muscle cells surrounding bronchi. TGF-βRII staining was even more intense in interstitial cells and may also appear in the extracellular space suggesting the release of a soluble fraction of the receptor in irradiated lungs. The cytoplasm of bronchial epithelia cells and peri-bronchial smooth muscle cells also stained for TGF-βRII. CCN2 deposition was associated with extracellular matrix deposition within the fibrotic area, bronchial epithelial cells as well as vessels also stained for CCN2.

Interestingly, Pravastatin-treament normalized the expression of the three markers: RhoB expression being restricted to the peri-vascular area, TGF-βRII to alveolar septa and slight TGF-βRII and CCN2 staining in type II pneumocytes, confirming the involvement of Rho/ROCK/CTGF pathway in lung fibrosis (Fig. 16).

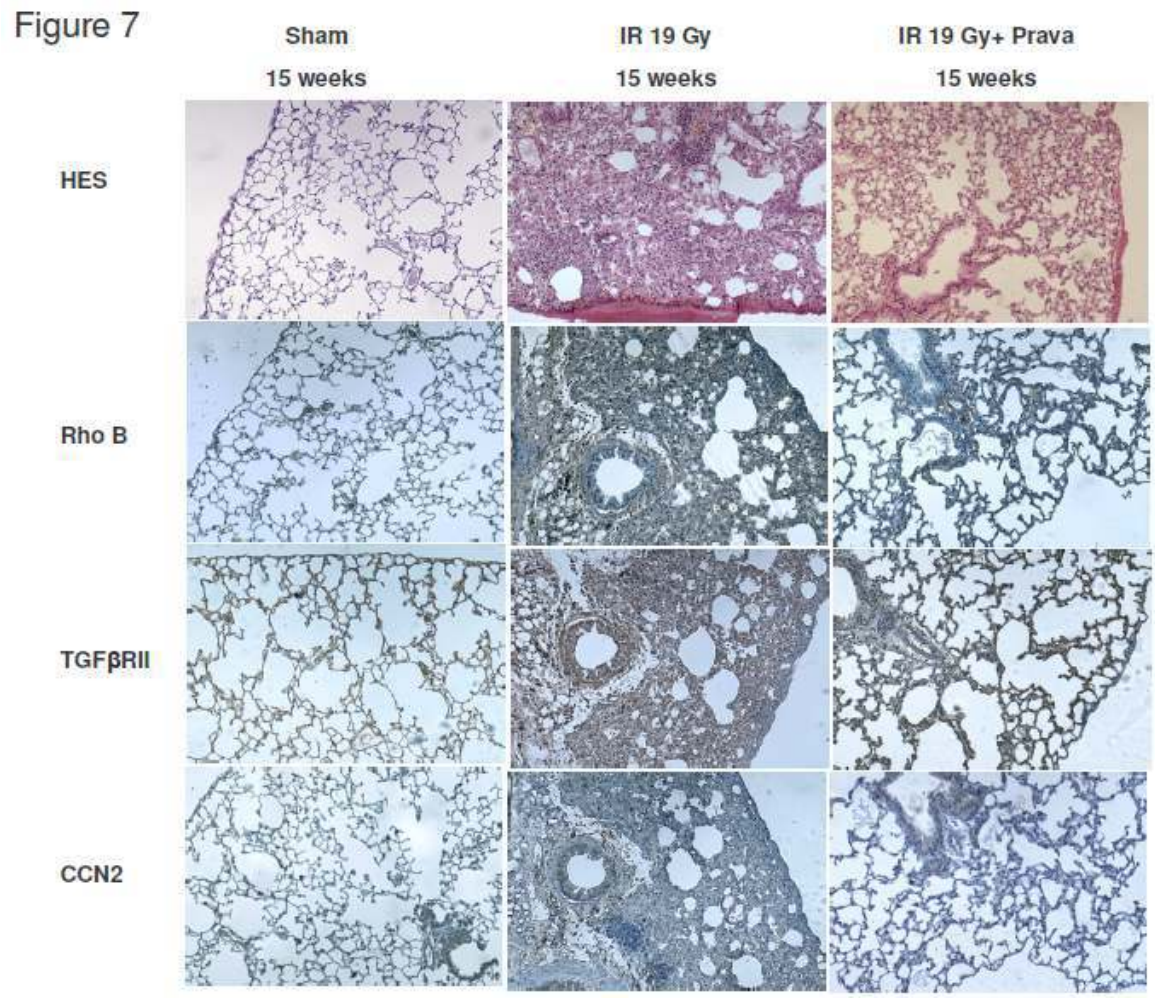
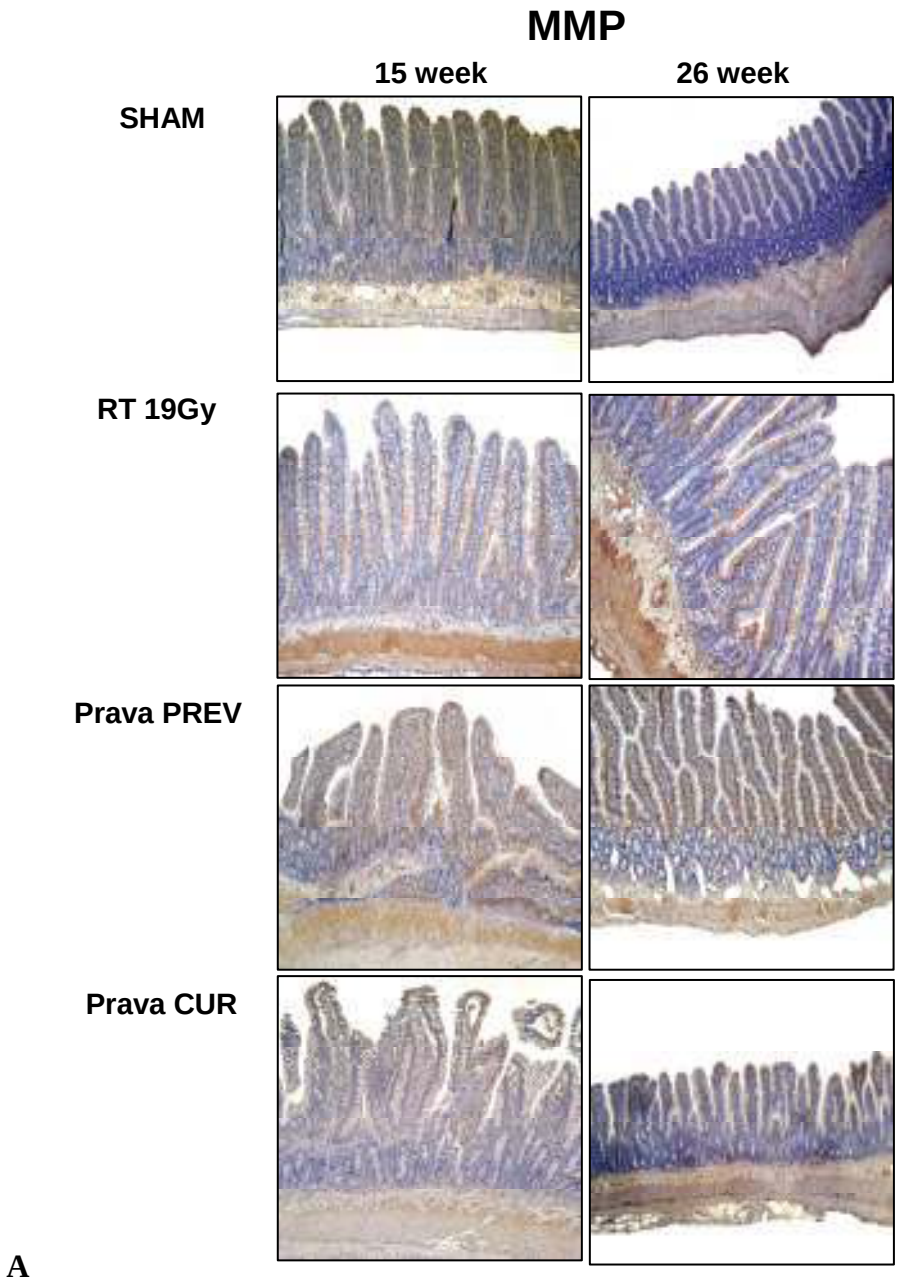


Fig. 16: Normalization of RhoB, TGFBRII and CCN2 in lungs after pravastatin treatment. Histological analysis of pulmonary tissue by HES and immunostaining of Rho B, TGFBRII and CCN2 in sham, 19 Gy irradiated group (IR 19Gy) and pravastatin treated group (IR 19 Gy + Prava). Original magnification X 100.

Moreover, we questioned whether the anti-fibrotic action of Pravastatin was associated with MMP-mediated fibrolysis on extracellular matrix. As fibrotic tissues are know to be composed of fibrillar collagens, we specifically investigated the modulation of gelatinases, especially MMP2 and its natural inhibitor TIMP2, in two models of radiation-induced fibrosis: in the gut and the lung.

Preventive and curative anti-fibrotic action of pravastatin involved distinct fibrolytic mechanism in irradiated gut.

Representative immunohistochemical staining patterns of MMP2 and TIMP2 in samples are shown in figures 17 A and B. In Sham-irradiated animals no MMP2 nor TIMP2 immunostaining was found, whereas irradiation induced a significant enhancement of MMP2 staining at the 15th and 26th week post-irradiation. Staining was mainly found in the muscularis propria with induction in the vessels of the submucosa and in 7-8% of mucosa's epithelial cells.



A

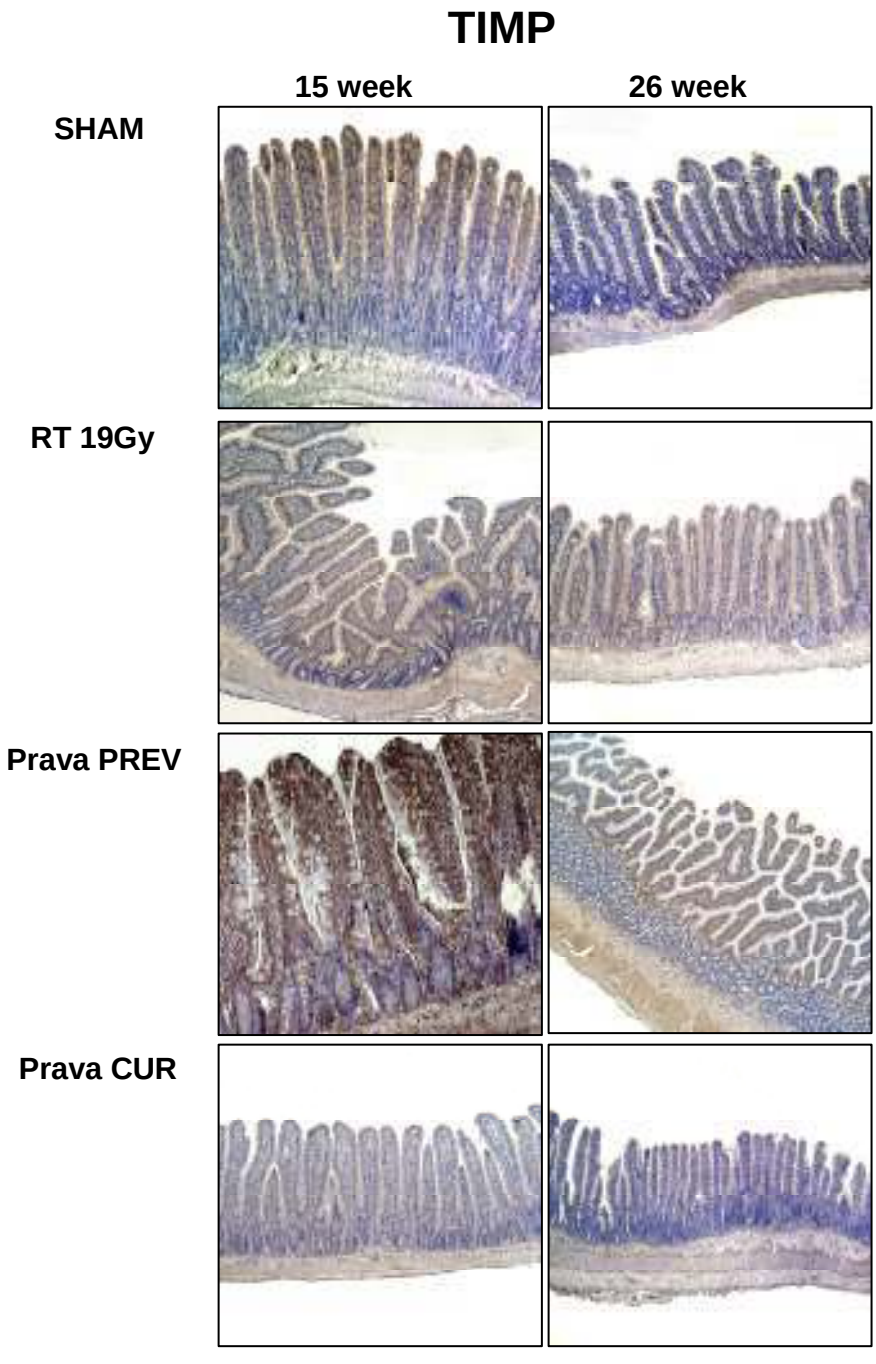


Fig. 17. A) Pravastatin trigger fibrolytic mechanisms in irradiated gut. In control samples, MMP2 staining was not detected. Expression and activity of metalloproteinase 2 significantly increase in pravastatin-preventing treatment group where a strong increase in MMP2 staining was found. In this group, the balance being in favor of extra-cellular matrix degradation particularly at 15 weeks.
B) In Pravastatin preventing-treated group, TIMP2 staining also increased in particular in the epithelial cell of the villi and in muscular layer, but seems weakest, suggesting a balance in favour of an ongoing fibrolytic process. Instead, there isn't a substantial induction in MMP2/TIMP2 expression in curative group.

Interestingly, when animals were treated with pravastatin as mitigator, a strong increase in MMP2 immunostaining was found in each intestinal layer. In the ileal mucosa, MMP2 was detected in 30% of epithelial cell of the villi and in the lamina propria, especially in sub-

epithelial myofibroblasts and in inflammatory cells as well as in smooth muscle cells of the muscularis propria (Fig. 18).

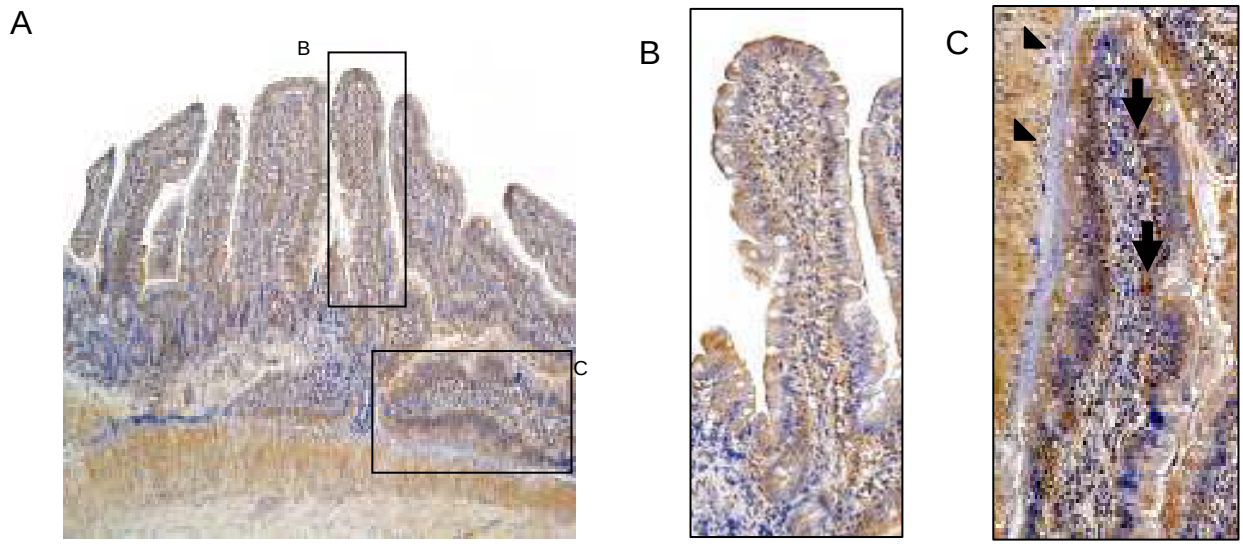
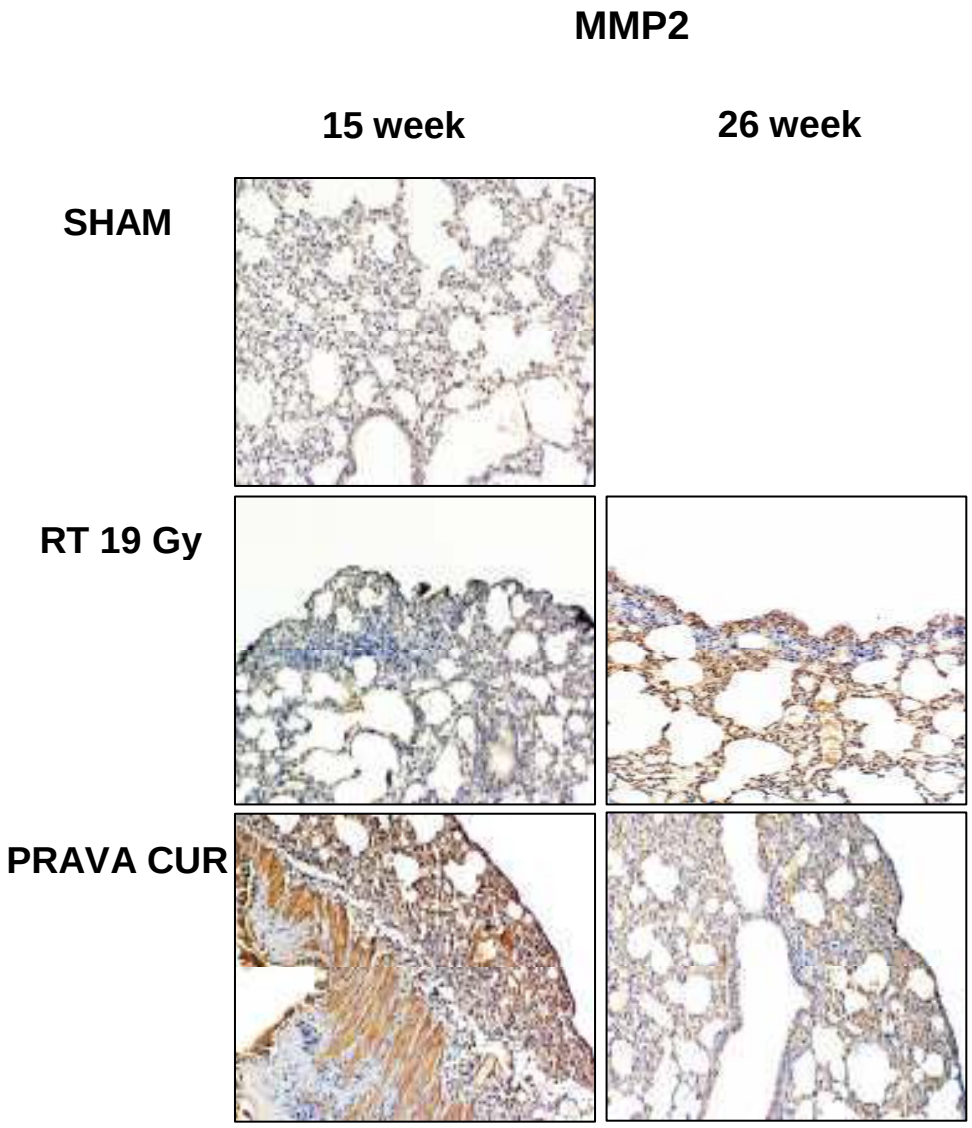


Fig. 18. A) Intestinal MMP2 immunohistochemical staining in Pravastatin-preventing treated group. A strong increase in MMP2 staining was found in each layer of the bowel. Original magnification, ×10.
B) and C) MMP-2 immunolocalization in villi. Irradiated gut treated with Pravastatin as a mitigator shows a strong increase in MMP2 expression in epithelial cells (arrows) and smooth muscle cells of the muscularis propria **(**arrowhead).

TIMP2 staining also increased, in particular in the epithelial cell of the villi, in muscular layer and, more rarely, in inflammatory cells, but was weakest than MMP2 staining, suggesting a balance in favour of an ongoing fibrolytic process. When pravastatin was administered in a curative way, no alteration of MMP2 and TIMP2 immunostaining was seen than sham-irradiated animals, suggesting that fibrolysis occurs earlier or that another mechanism was involved.

Fibrolysis mediated by MMP2 is involved in the firsts phases of Pravastatin mechanisms to reverse lung fibrosis

Representative immunohistochemical staining patterns of MMP2 and TIMP2 in samples are shown in Fig. 19 A and B.



A

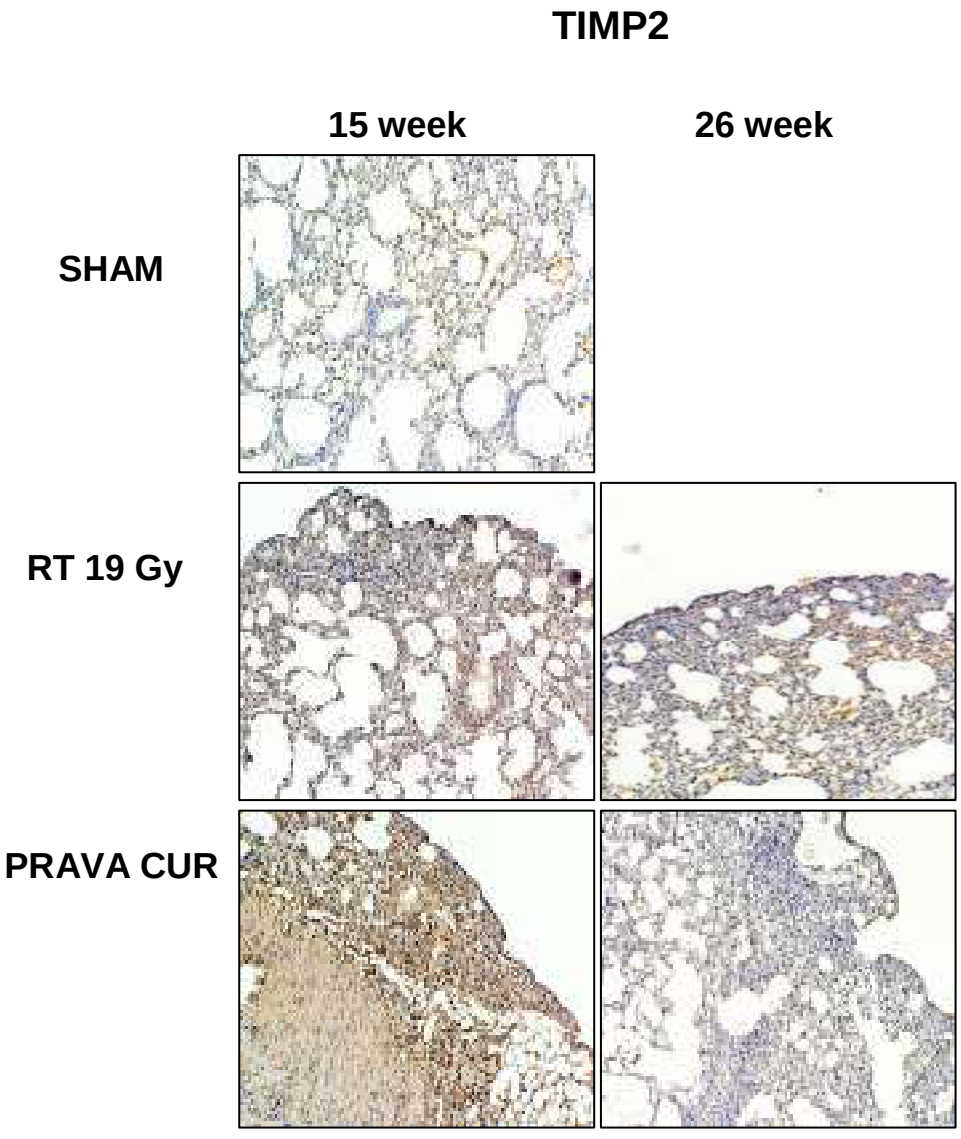


Fig. 19. A) and B) Fibrolysis mediated by Pravastatin, via MMP2, reverse lung fibrosis.
In Sham-irradiated animals no MMP2 or TIMP2 immunostaining is found. Single dose of 19 Gy induced a significant enhancement of MMP2 and TIMP2 staining 15th and 26 th week post-irradiation in smooth muscle cells, in fibroblasts and reactive bronchial epithelial cells and less frequently in squamous type I pneumocytes. In Pravastatin curative-treated group, 15th week post-irradiation, a strong increase in MMP2 immunostaining was found in smooth muscular layer of vessels and bronchi and in squamous type I pneumocytes followed by a reduction on 26th week. Regarding TIMP-2, it was found in residual sub epithelial fibroblast/myofibroblast foci and also in some reactive bronchial epithelial cells.

In Sham-irradiated animals no MMP2 or TIMP2 immunostaining was found, whereas similarly to what occurred in the gut, irradiation induced a significant enhancement of MMP2 and TIMP2 staining at the 15th and 26 th week post-irradiation in smooth muscle cells, in fibroblasts and in reactive bronchial epithelial cells, and less frequently in squamous type I

pneumocytes. The diffuse intra-alveolar fibrosis was a common finding, with an excessive collagen deposition and cell proliferation in airspaces that obliterated the alveolar spaces. The fibrotic lesions were essentially located in sub pleural areas and around large vessels with a severe distortion of the structures. Areas of active injury were characterized by fibroblastic foci and inflammatory cells as lymphocytes and plasma cells. It was common to observe sheets of macrophages within the alveolar spaces. Variable vascular injury and haemorrhage were noted in more advanced areas of fibrosis.

Interestingly, when pravastatin was administered in curative way, 15th week post-irradiation, a strong increase in MMP2 immunostaining was found in smooth muscular layer of vessels and bronchi and in squamous type I pneumocytes followed by a reduction on 26th week. Regarding TIMP-2, it was found in residual sub epithelial fibroblast/myofibroblast foci (figure 20 A and B), some of them partially occupying the alveolar spaces. TIMP-2 was also observed in some reactive bronchial epithelial cells.

Balance is in favor of fibrolysis especially in the early phase of the treatment (the first 15th week).

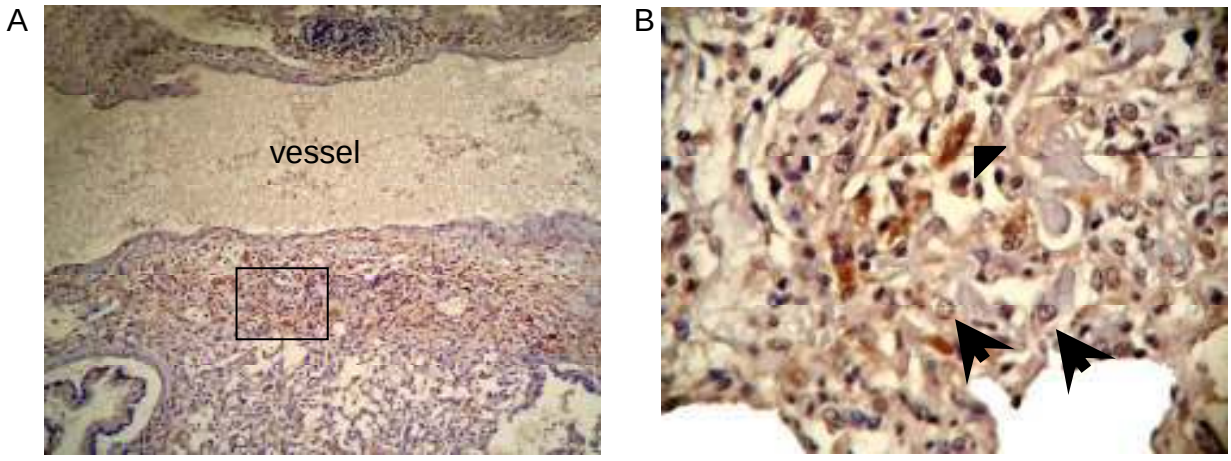


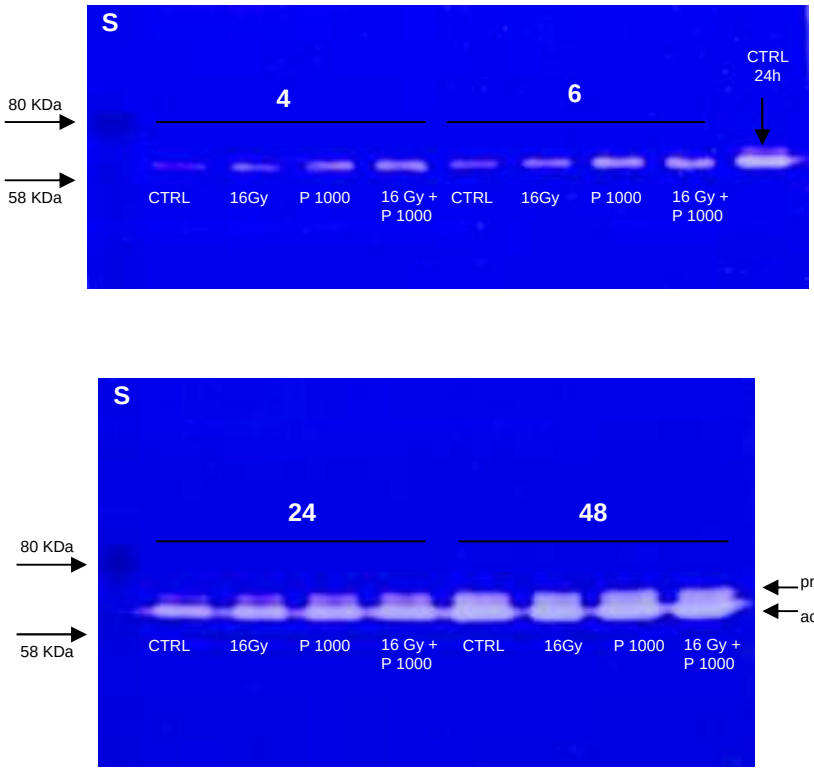
Fig. 20. A) Pulmonary TIMP-2 localization in Pravastatin curative-treated group.
Lung exhibiting immunolabeled subepithelial fibroblast/myofibroblast foci, partially occupying the alveolar spaces, stained for TIMP-2. Original magnification, ×10.
B) Positive fibroblast (arrowhead) and negative macrophages (arrows). Original magnification, ×40.

Zymography <u>preliminary</u> results: irradiation and Pravastatin induced gelatinase activity

To evaluate whether irradiation and Pravastatin could induce gelatinase activity, gelatin zymography was performed on gel culture supernatants and cells lysed. Our very preliminary results showed MMP2 expression in conditioned medium (Fig. 19): it seems to be a time-dependent MMP2-active form secretion, but most important, irradiation and Pravastatin, alone and in combination, seemed to increase this secretion, in particular at 4 and 6 h after irradiation. This induction is evident for 24 h.

Pro-MMP2 band increased from 24h after irradiation. MMP9 was not detected.

Analyses of gelatinase activity in cells lysed and after different doses of radiation (2-8-16 Gy) and drug (500 and 1000 µM) are ongoing.



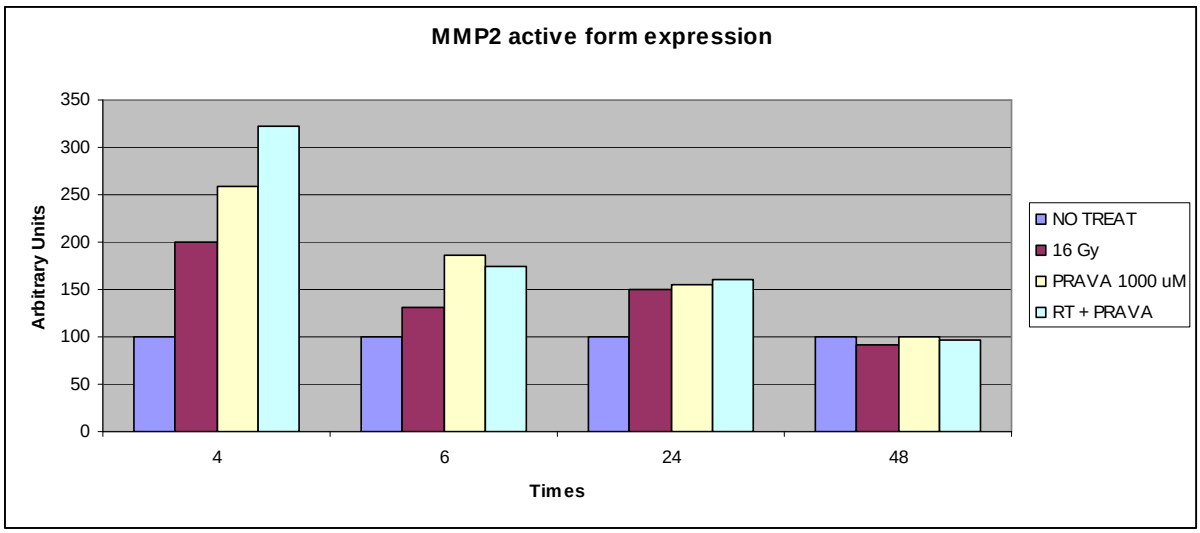


Fig. 21. Study on gelatinase activities in primary lung fibroblasts culture conditioned medium at 4-6-24 and 48h after irradiation (RT). LEGEND: S: standard; CTRL-NO TREAT: 0Gy/NO Pravastatin; P1000: Pravastatin 1000 µM.

DISCUSSION

Radiotherapy to the chest and abdomen is efficient primary treatment of several cancers with good prognosis (breast, lymphoma, gynaecological prostate etc). The long survival of patients allow the expression of delayed radiation damage. They can potentially be further exacerbated by novel treatment modalities (for example monoclonal antibodies) In that context, one of the most concerning aspects of radiation-induced toxicity is fibrosis, due to its progressive and seemingly irreversible evolution.

Moreover, in lungs, fibrosis not only occur after localized high-dose pulmonary irradiation, but are also seen subsequently to intermediate-dose whole body irradiation used, for example, as part of preconditioning regimens for bone marrow transplantation[173].

Therefore, development of pharmaceutical agents that can protect, mitigate, or treat the development of fibrosis is highly needed in radiotherapy patients and may also be applied to radioprotection purposes after nuclear or radiological terrorism or in cases of accidental radiation exposures[54,174,175].

The biochemical induction and maintenance of radiation fibrosis is a complex process that depends on continuous and integrated activation loops that involve cell differentiation and cross-talk between the various cellular components of the tissue within the matrix[17]. In this context, targeting one central pathway involved in vascular, immune, and stromal pathogenic response would provide an efficient antifibrotic strategy.

Rho proteins are small GTPases acting as molecular switches to control a wide range of cellular functions. We and others showed the important functional contribution of the Rho/ROCK pathway to radiation fibrogenesis, as pharmacological inhibition of Rho using statins and specific ROCK inhibitors prevented and reversed intestinal radiation fibrosis[17,21,18].

We extend previous findings to examine delayed injury to lungs in mice, using an experimental model of irradiation targeted specifically to the thorax; we also use a model of pulmonary fibrosis induced by the chemotherapeutic radiomimetic agent, bleomycin

(BLM)[172]. We found that for both irradiation and BLM-treated animals the use of pharmacological inhibitors of the Rho/ROCK cascade improved both the histological structure and normalized the expression of fibrogenic markers. These data suggest that Rho/ROCK activation by fibrogenic agents may be neither organ-specific nor agent-specific, but more likely a common response to the chronic wound healing (active fibrotic) process.

Our previous studies showed that CCN2 regulation depends upon low levels of TGF-β1 and Rho/ROCK pathway activation in mesenchymal cells isolated from radiation enteropathy patients[14,108,15].

Similarly, delayed TGF-βRII, RhoB and CCN2 activation were also shown in irradiated lung. Interestingly, the functional consequences of CCN2 overexpression reported in our model are consistent with the pathological modifications reported in transgenic mice over-expressing CCN2 in lung (as reviewed in [176]).

The lung transgenic mice provided clear evidence that CCN2 overexpression impaired formation of the alveolar and vascular network associated with fibrosis in and around alveolar septa, bronchi and vessels[177]. In addition, our results suggest the involvement of RhoB to transactivate CCN2 and trigger radiation-induced lung fibrosis.

Rho proteins are small GTPases acting as molecular switches to control cell adhesion, formation of stress fibers, and cellular contractility through the reorganization of actin-based cytoskeletal structures. These functions are accomplished specifically *via* their effectors, the ROCKs[178] and our previous expression profiling studies highlighted RhoA, B and ROCK-1 as contributors to radiation-induced fibrogenesis and maintenance of the fibrogenic phenotype [ref. INTRO]. Therefore we pharmacologically targeted Rho and ROCKs activation using statins (pravastatin and simvastatin)[179] and Y-27632[180].

These inhibitory drugs displayed anti-fibrotic properties and improved delayed pulmonary radiation injury and bleomycin-induced lung fibrosis. As shown by Shimizu[172], Williams[181] and Horowitz[182], pharmacological inhibition of ROCK by statins or other selective inhibitors

reduces experimental radiation-induced lung fibrosis. Inhibition of ROCKs probably contributes to some of the cholesterol-independent beneficial effects of statin therapy. However, statin and selective ROCKs inhibitors display different genomic targets[183] suggesting possible synergistic or additive properties and opening novel options for combined treatments.

With the recent development of RhoB and ROCKs-knockout mice, further dissection of the relevant signaling pathway is now possible. Targeted deletion of ROCK-1 protects mice from pressure overload and inhibits the development of reactive fibrosis in the heart[184]. Furthermore RhoB deletion altered cell response to TGF-β1 signals[185]. As RhoB is also known to control tumour radiosensitivity[186,187], it is possible that specific inhibition of RhoB could trigger a differential beneficial effect, protecting normal tissue from radiation damage and sensitizing tumours.

Another means by which Rho proteins are involved in radiation fibrosis is through regulation of the extracellular matrix (ECM) by modifying matrix metalloproteinase activity[188189,190].

We therefore hypothesized that delayed-radiation fibrosis is a potentially reversible/preventable process involving MMPs. We examined the underlying mechanisms of the antifibrotic action of Pravastatin using a murine experimental model of irradiation targeted the thorax and a previous rat model of gut irradiation. We also studied *in vitro* the effects of Pravastatin on gelatinases expression after irradiation.

In vivo, we found that Pravastatin treatment increased the expression of MMP2 and TIMP2 in two different models of radiation-induced fibrosis. In gut, preventive antifibrotic action of statin stimulated gelatinase activity and fibrolytic process occured, in particular, 15 weeks after IR. Whereas, no change in MMP/TIMP expression was found in Pravastatin-curative treated group.

In the lung, enhanced expression of MMP2 was evident in mice treated with statin, in this case only in the first phases after treatment (15 weeks after IR).

As previously described by Strup-Perrot[149], a marked upregulation of collagen and enzymes was involved in ECM remodeling in late radiation enteritis, showing an induction of each member of the MMP family, i.e., gelatinases, stromelysin, collagenases, and membrane-type MMPs. The concomitant induction of MMP inhibitors (TIMP1, TIMP2, and PAI-1) counterbalances this induction of MMPs, leading to a net collagen deposition. Collagen accumulation in radiation fibrosis has been thought to be associated with a decrease in MMP activity and increased TIMP levels.

In our study, statin contributed to reverse this tendency: the drug administered before and during irradiation induced MMP2 secretion in epithelial cell of the villi and in the lamina propria, especially in sub-epithelial myofibroblasts and in inflammatory cells as well as in smooth muscle cells of the muscularis propria. In lungs, when Pravastatin was administered in curative way, 15th week post-irradiation, a strong increase in MMP2 immunostaining was found in smooth muscular layer of vessels and bronchi and in squamous type I pneumocytes followed by a reduction on 26th week.

There are some reports showing that statins could decrease the expression of several MMPs, including MMP2, *in vitro*[191,192] as well as *in vivo*[193,194].

However, to our knowledge, a decreased activation of MMP2 in response to statins has not been reported so far. MMP2 is synthesized as an inactive zymogen form, and must undergo a proteolytical cleavage of its prodomain in order to become catalytically active.

Taras D. et al. showed that Pravastatin treatment resulted in a significantly reduced activation of pro-MMP2[195]. Since only the active form of MMP2 is proteolytically active, this could be a significant finding. The mechanism of activation of pro-MMP2 involves the formation of a complex with TIMP2[196]. We found that Pravastatin treatment enhanced the

immumohistochemical expression of TIMP2. This is likely one of possible mechanisms for the increased processing of pro-MMP2 to active MMP2.

Moreover, differences between our and others findings was probably do to specific experimental model used and timing of Pravastatin administration and effect evaluation.

MMPs can be both profibrotic and antifibrotic[197]. MMPs are profibrotic by releasing and activating profibrotic growth factors (eg,TGF-β1), which in turn activate migration, proliferation, and survival of fibroblasts and myofibroblasts[39,198,199].

Statins had anti-fibrotic effects characterized by a dose-dependent decrease in the level of a fibrosis-related growth factor (CTGF)[200]. TGF-β1 has been described as the main CTGF inducer; thus, one intriguing point of our previously results was that the CTGF-sustained expression during the late fibrotic phase could be partly independent of TGF-β1[16].

In our model expression of MMP2 and TIMP2 could be independent of TGF-β1 et more related to Rho/ROCK/CTGF pathway.

MMPs can also be anti-fibrotic by degrading extracellular matrix to prevent its excessive accumulation in the tissue[201].

Continuous upregulation of MMP2 may play a dual role: on the one hand to enhance matrix remodelling, mainly in advanced stages, and on the other hand to inhibit fibroblast proliferation.

In lungs of mice treated with Pravastatin, rare fibroblast focus, characterized by a distinct cluster of fibroblasts and/or myofibroblasts, are evident within the alveolar wall. As in Idiopathic Pulmonary Fibrosis[202], in our lung fibrosis model, TIMP2 was found almost exclusively associated with fibroblast foci. Airway fibroblasts and myofibroblasts are a primary source of ECM proteins, including fibronectin, in subepithelial fibrosis linked to airway remodeling.

As previously mentioned, although MMP inhibition is the main function of TIMPs, paradoxically, TIMP2 might be influencing an enhanced activation of this enzyme through its

binding to pro-MMP2. This feature may be of important physiological significance in modulating the cell surface activation of pro-MMP2. Such a mechanism should depend on the molar equilibrium between the enzyme and the inhibitor. Pravastatin seems to induce these profibrolytic signals.

In conclusion ours studies shows a radiation-induced regulation of two important fibrogenic pathways (*i.e.* TGF-β/Smad and Rho/ROCK) in lungs and support the potential therapeutic importance of Rho/ROCK inhibition to treat radiation and bleomycin-induced pulmonary delayed injury and fibrosis. Furthermore our experiments show a possible mechanism by which Pravastatin reduces *in vivo* lung and gut post-actinic fibrosis. Our immunohistochemical findings showed a possible mechanisms through Pravastatin reverse balance of pro- and anti-fibrotic molecules. Using these reliable models of radiation-induced fibrosis, we demonstrated a significant protective effect of Pravastatin on intestinal and pulmonary radiation injury.

PERSPECTIVES

Our hypothesis is that the Rho/ROCK pathway may be a central mechanism in radiation-induced fibrosis response and form an effective therapeutic target to increase the therapeutic index of radiation therapy.

We have show that Rho/ROCK pathway appears involved in control of development and maintenance of radiation-induced intestinal fibrosis[17,21,108,13]. It plays also a role in cardiac and pulmonary fibrosis[19]. The mechanisms involved in pathological response to radiation injury are complex and to elucidate completely their part in this form of fibrotic response, more efforts are necessary.

I - Role of the Rho/ROCK pathway in the development and maintenance of radiation-induced fibrosis.

To show the involvement of the Rho/ROCK/CTGF cascade in the development and maintenance of radiation-induced fibrosis, we have developed a genetic approach based on Rhoβ knockdown mice and the use of primary cells isolated from Rhoβ deficient mice. Two models of pulmonary fibrosis will be generated by thoracic irradiation and chronic Bleomycin injection. These models will allow us to extend the study of the involvement of the way Rho/ROCK in the lungs. In parallel and to ensure a future transfer in clinical pharmacology, it will necessary to test new pharmacological molecules.

A first model of pulmonary fibrosis in RhoB deficient mice inducted by a single radiation dose of 20 Gy are actually in course of characterization. First evidences at the immunohistochemical level, show an important reduction of lung inflammatory response in $Rho\beta^{-/-}$ lung mice compared to WT mice (C57BL6).

II-Role of the EMT in the development and maintenance of radiation-induced fibrosis

The EMT is a phenomenon of phenotypic differentiation during which epithelial cells lose their epithelial signatures and acquire characteristics of mesenchymal cells. The molecular

characteristics of the EMT include: deregulation of adhesion molecules, increased expression of MMPs leading to matrix remodeling, activation of the family that controls Rac/Rho/cdc42 remodeling of the cytoskeleton[203,204]. If all of these molecular characteristics have been described in radiation-induced fibrosis[15,16,149] the direct contribution of EMT to our knowledge has never been studied and will be explored in models of pulmonary fibrosis described above.

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ENCLOSED

Modulation of the Rho/ROCK Pathway in Heart and Lung after Thorax Irradiation Reveals Targets to Improve Normal Tissue Toxicity

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Abstract: The medical options available to prevent or treat radiation-induced injury are scarce and developing effective countermeasures is still an open research field. In addition, more than half of cancer patients are treated with radiation therapy, which displays a high antitumor efficacy but can cause, albeit rarely, disabling long-term toxicities including radiation fibrosis. Progress has been made in the definition of molecular pathways associated with normal tissue toxicity that suggest potentially effective therapeutic targets. Targeting the Rho/ROCK pathway seems a promising anti-fibrotic approach, at least in the gut; the current study was performed to assess whether this target was relevant to the prevention and/or treatment of injury to the main thoracic organs, namely heart and lungs.

First, we showed activation of two important fibrogenic pathways (Smad and Rho/ROCK) in response to radiation-exposure to adult cardiomyocytes; we extended these observations *in vivo* to the heart and lungs of mice, 15 and 30 weeks post-irradiation. We correlated this fibrogenic molecular imprint with alteration of heart physiology and long-term remodelling of pulmonary and cardiac histological structures. Lastly, cardiac and pulmonary radiation injury and bleomycin-induced pulmonary fibrosis were successfully modulated using Rho/ROCK inhibitors (statins and Y-27632) and this was associated with a normalization of fibrogenic markers.

In conclusion, the present paper shows for the first time, activation of Rho/ROCK and Smad pathways in pulmonary and cardiac radiation-induced delayed injury. Our findings thereby reveal a safe and efficient therapeutic opportunity for the abrogation of late thoracic radiation injury, potentially usable either before or after radiation exposure; this approach is especially attractive in (i) the radiation oncology setting, as it does not interfere with prior anti-cancer treatment and in (ii) radioprotection, as applicable to the treatment of established radiation injury, for example in the case of radiation accidents or acts of terrorism.

Keywords: Fibrosis, radiation therapy, cardiac toxicity, pulmonary fibrosis, Rho/ROCK, Smad, Statins, ROCK inhibition.

INTRODUCTION

Development of effective and safe radio-protective drugs a key goal in the fields of radioprotection and radiotherapy. The threat of nuclear and radiological attacks has grown recently and only few drugs are available to treat the resulting radiation injury. In parallel, radiotherapy is the second most important treatment modality after surgery in the treatment of cancer and over 60% of cancer patients are treated with radiation therapy in France, with over 50% completely cured. Recent technical progress, such as 3D-conformal or intensity-modulated radiation therapy, combined with new targeted drugs have significant promise for therapeutic outcome, but could result in disabling normal tissue injury in a subset of sensitive patients and in long-term cancer survivors. Therefore, one great challenge of modern radiation protection and radiation therapy is the development of individualized treatment regimes [1] by i) early prediction of individual radiosensitivity and outcome; and ii) protecting normal tissues from radiation injury by increasing its tolerance or treating the cellular and molecular defects that disturb tissue homeostasis and interfere with proper wound healing. The mechanisms involved in delayed normal tissue toxicity involve the activation of cytokine/growth factor cascades resulting in a non-controlled 'wound healing'-type response. When vital organs like the heart, lung or intestine are affected [2], the most concerning aspect of these late

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complications is radiation fibrosis which is a progressive pathology with what has seemed an irreversible evolution. Thus, the development of curative anti-fibrotic strategies is nowadays highly anticipated by both patients and physicians [3, 4].

We and others recently showed that beside the activation of the canonical TGF-β/Smad pathway, other intracellular signaling cascades including the Rho/ROCK pathway are switched on in fibrotic tissues. Interestingly, the Rho/ROCK pathway seems differentially activated in radiation-induced intestinal fibrosis, thereby providing a rationale for a specific, targeted anti-fibrotic strategy [5]. Pharmacological inhibition of Rho using statins indeed prevent and even reverse intestinal radiation fibrosis [6-8]. Rho small GTPases and their downstream effectors, ROCKs, are key regulators of cell motility, controlling the dynamics of the actin cytoskeleton that trigger changes in cell morphology *via* myosin phosphorylation [9] and may elicit the fibrogenic changes in cell phenotype. Our main hypothesis is that persistent alteration of the cell phenotype induced by irradiation depends at least in part upon the Rho/ROCK/CCN2 pathway in various organs; the present study examined this precept in heart and lungs to address this hypothesis. Therefore, we investigated acute and delayed radiation-induced Rho/ROCK and Smad pathway activation in primary cultures of cardiomyocytes and *in vivo* in lungs and heart of mice, 15 and 30 weeks after whole thorax irradiation. As activation of the Rho/ROCK/CCN2 cascade was seen, we validated its therapeutic relevance by studying the prophylactic and curative anti-fibrotic efficacy of various Rho and ROCK inhibitors in experimental models of lung fibrosis. The data suggest that targeting the Rho/ROCK pathway is effective in preventing and treating delayed radiation-induced injury, including fibrosis in various organs.

MATERIALS AND METHODS

Animals and Experimental Procedures

A total of 150 Female C57BL6 mice were obtained from Charles Rivers France. Experiments were conducted under the French regulations for animal experimentation (Ministry of Agriculture Act No. 87-848, 19th of October, 1987) and received ethics approval.

Primary Cardiomyocyte Isolation and *in vitro* Irradiation

Cardiomyocytes (CM) were isolated from C57B6 (8-12 week old) mouse ventricular tissue using the Cellutron method (~0.6 million cells per heart). The procedure was optimized for our use accordingly: 1) Hearts were minced into pieces and digested at 37°C in Cellutron enzymatic solution diluted two-fold to preserve CM structure and function; 2) Incubation-time was reduced to 12 minutes and cell suspension collected every 12min. The digestion procedure was repeated four times. 3) CM were pelleted for 10 minutes in the incubator, plated onto Collagen type I-coated (5 µg/cm2, Becton) culture plates and subcultured in FGM medium (Lonza). After plating, adherent CM were irradiated *in vitro* at 16 Gy (RX, 250 KeV). Alteration of the Rho/ROCK and Smad pathways were studied by immunofluorescence and Western-blot 4 and 24h after irradiation.

Protein Isolation and Western Blotting

Cells and tissue were lysed in RIPA buffer containing protease and phosphatase inhibitors (Roche). Immunodetection by Western-blot was performed by electrophoresis of proteins in a 12% or 4-12% tris-HCL SDS-PAGE, transferred to PVDF membranes (Biorad). Equal loading of proteins was confirmed by Ponceau red staining of membranes after immunoblotting. Membranes were blocked with TBS-Tween 0,1%- BSA 5%(sigma) and incubated with primary antibodies included mouse polyclonal anti-Rho ABC (Pierce 89854; diluted at 1/500); rabbit polyclonal anti-TGF beta RII (Santa Cruz H-70:sc-28565, diluted at 1/250); mouse monoclonal anti-smad 4 (Santa Cruz B-8:sc-7966, diluted at 1/500); rabbit polyclonal anti-smad 2/3 (Cell signalling: 4087, diluted at 1/500); sheep polyclonal anti-smad 2/3 P (Santa Cruz : sc-11769, diluted at 1/500); rabbit polyclonal anti- CCN2 (Abcam: ab6992, diluted at 1/250); mouse polyclonal anti-α-actin sarcomeric (SIGMA, diluted at 1/250). After washing in TBS-T, membranes were incubated with corresponding HRP secondary antibody conjugated (GE Healthcare Life Sciences; diluted at 1/5000 in TBST containing 2% BSA). Reactive proteins were visualized by chemiluminescence detection system. Incubation with rabbit monoclonal beta actin antibody was performed to normalize the chemiluminescence levels and exposure times.

Animal Immobilization and Thorax Irradiation

Using a TEM anesthesia system (Bordeaux, France), mice were anaesthetized by inhalation of an air/isoflurane (Forène, Abbott France, Rungis) mixture and irradiated with a 200kV X-ray machine operated at 15mA with 0.2mm copper filtration, providing an incident dose rate of 0.79 Gy/min. Doses of 16 and 19Gy were given to the thorax in one fraction. To minimize unwanted off-target biological responses which may confound interpretation of thorax data, the rest of the animal was shielded with a 10mm thick lead screen.

Echocardiography

A 14MHz transducer ultrasonographic system (Aplio, Toshiba, Japan) was used to monitor heart physiological parameters in anesthetized mice (isofluorane anesthesia, 0.75% to 1.0% in oxygen with spontaneous ventilation) using Transthoracic M-Mode echocardiography, 15 and 30 weeks post-irradiation. Body temperature of the mice was maintained using a heating pad. The left ventricle (LV) was imaged in parasternal long-axis views to obtain different measurements of LV. LV diameters at end-diastole (LVEDD) and end-systole (LVESD) were measured according to the American Society of Echocardiography leading edge method [10]. LV fractional shortening (LVFS; %) was calculated by the Teichholz method [11] as follows: LVFS = [(LVEDd-LVDs)/LVDd] X 100 and LV ejection fraction (LVEF; %).

Histological Examination and Immunohistochemistry

One week after hemodynamic studies, the lungs and heart were collected for histology and immunohistochemistry. Organs were fixed in Finefix (Milestone medical, Italy), paraffin embedded and cut into 4 µm sections. Sections were

stained with Hematoxilin-Eosin-Saffranin (HES) and examined using conventional light microscopy.

Expression of TGF-BRII, RhoB, and CCN2 deposition were studied by immunohistochemistry as previously described [12]. Primary antibodies were used at the following dilutions: TGF-BRII 1:100 (Santa Cruz H-70:sc-28565, RhoB 1:100 (Santa Cruz C-5:sc-8048) and CCN2 1:50 (Santa Cruz L-20:sc-14939). Sections were incubated with corresponding HRP-conjugated secondary antibody (GE Healthcare Life Sciences; diluted at 1/5000 in TBST containing 2% BSA). Endogenous peroxidases were blocked by incubation with 0.1% H_2O_2 in PBS for 10 min. Colour development in immunoperoxidase staining was performed with 3,3'-diaminobenzidine enhanced liquid substrate system (DAB) and sections were counterstained using Mayer's haematoxylin (Fluka Chemie, Buchs, Switzerland). Images were acquired using a Leica microscope equiped with a JVC color video camera coupled to an imaging analysis system (Histolab software, Microvision, France). In the lung, tissue lesions were scored as fibrotic in each of the subpleural, vascular and intraparenchymal areas. Inflammatory infiltrates were also scored. In the heart, the score related to fibrosis, occurrence of stress fibers, necrotic areas, cardiomyocyte hypertrophy and pericarditis.

Experimental Model of Fibrosis and Drug Administration

The potential anti-fibrotic effect triggered by Rho and ROCK pharmacological inhibitors (Pravastatin, Simvastatin and Y-27632) was assessed in the well-characterized model of BLM-induced lung fibrosis and after thorax irradiation (19Gy). Intra-peritoneal injection of BLM (5X40Mg/kg) was performed every other day. Fibrosis occurrence was monitored 40 days after initiation of BLM administration. Animals were divided in 7 groups: sham, Bleomycin, Prava-Bleo, Simva-Bleo, Bleo-Prava, Bleo-Simva, Bleo-Y group according to the schemes shown Fig. (**4**). Pravastatin and Y-27632 were hydrosoluble drugs, simvastatin was diluted in DMSO at 10% and all drugs were administered *via* 2 weeks Osmotic pump (Alzet, n°1002) implanted sub-cutaneously. At day 40, lungs were collected for histology, fixed in Finefix (Milestone medical, Italy), paraffin embedded and cut into 4 µm sections. Sections were stained with Hematoxylin-Eosin-Saffranin (HES) and examined using conventional light microscopy. In the radiation-induced model, pravastatin was administered in the drinking water between the 5th and the 15th week post irradiation. Lungs and heart were collected 15 weeks post-irradiation using the same procedure.

Statistical Analysis

Data were expressed as the mean ± SEM and analyzed using the t-test.

RESULTS

Irradiation Induces Sequential Activation of Smad and Rho Pathways and Cytoskeletal Remodeling in Primary Cardiomyocytes

The balance between TGF-β1/Smad and Rho/ROCK pathway signalling to mediate activation of the fibrogenic

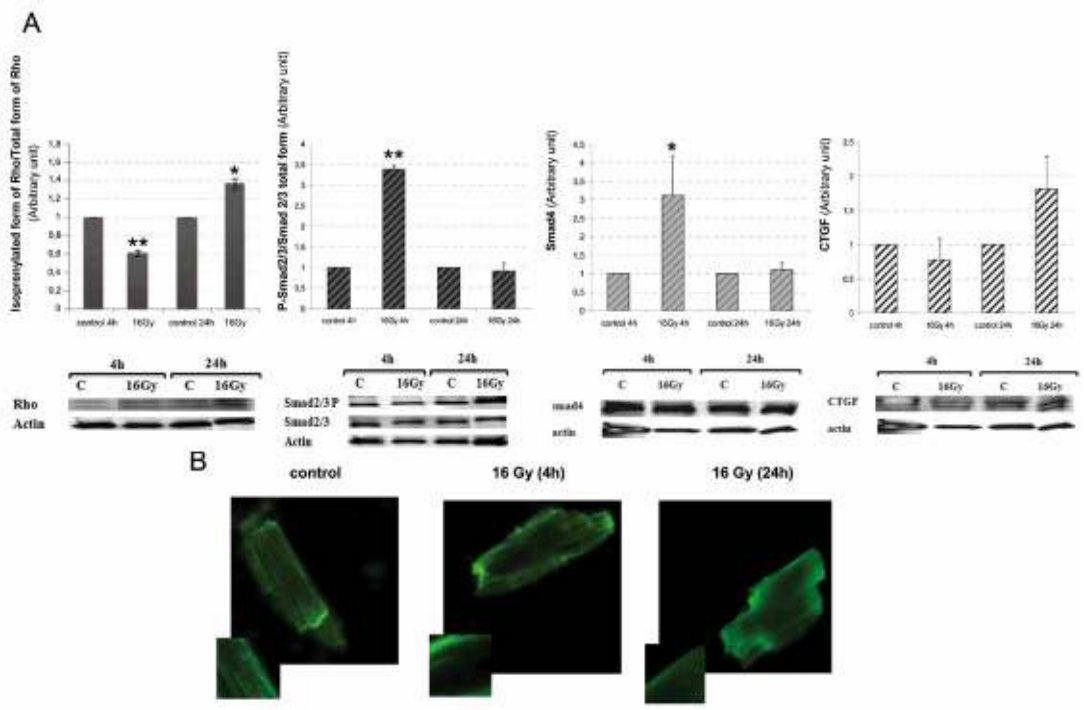

Fig. (1). Effect of irradiation and involvement of Smad and Rho pathways in adult mice cardiomyocytes. **A.** Western blotting and quantification of RhoB, Smad4, phospho-Smad2/3 and CCN2 expression in cardiomyocytes sham-irradiated and 4h and 24h after 16Gy-radiation. An equal amount of samples (40 µg protein) was loaded and actin is shown as loading control. **B.** Imunolabelling of sarcomeric α-actin in cardiomyocytes freshly isolated and *in vitro* irradiated at 16Gy.

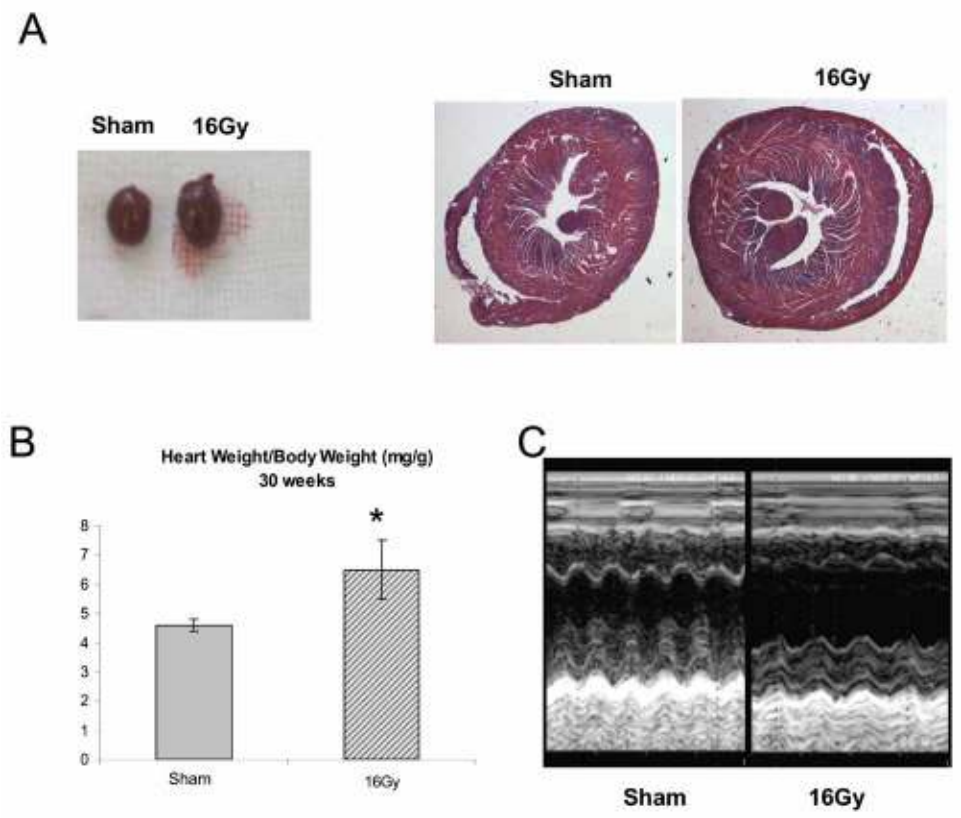

Fig. (2). Radiation-induced cardiac hypertrophy. A. Pictures of whole excised heart and HES staining sampled from sham and irradiated mice (16Gy). **B.** Heart weight/body weight ratios (mg/g) of irradiated mice was increased compared with sham, as indicated. *,p<0.05. Total heart weight was indexed to tibia length. **C.** Representative M-Mode echocardiograms obtained from 16Gy irradiated mice or sham.

mediator CCN2 was investigated by Western blotting in primary cardiomyocytes. Irradiation at 16Gy induced early phosphorylation of Smad2/3 and increased expression of Smad4, indicative of Smad pathway activation 4h post-irradiation (Fig. **1A**). This was followed by radiation-induced increase of the isoprenylated Rho form 24h after irradiation, indicative of Rho activation. Subsequent CCN2 production occurs 24h post-irradiation (Fig. **1A**). IF studies showed a radiation-induced remodelling of the central network of α- sarcomeric actin which is the main structural protein of cardiac muscle (Fig. **1B**). Alteration of actin network in irradiated CM was visible as early as 4h after irradiation and persists at 24h and consistent with Rho/ROCK alteration.

Irradiation Initiates Heart Failure

Photography of whole excised heart from non-irradiated and irradiated mice (16Gy) as well as HES staining of whole heart sections showed radiation-induced cardiac hypertrophy (Fig. **2A**). These findings were confirmed by the increased ratio of heart weight/body weight in irradiated animals *vs.* sham (7.4±1mg/g and 4.7±0.2mg/g, respectively;*p<0.05) indicative of LV hypertrophy 15 weeks post-irradiation (Fig. **2B**).

Measurements of cardiac physiological parameters (Table **1**) revealed an alteration of LV systolic function after thorax irradiation as shown by the significant reduction of ejection and of shortening fraction (approximately 25±9.6 %

Table 1. Echocardiographic Assessment of 0Gy, 16 Gy and 19Gy Irradiated Mice at 15 and 30 Weeks Post Irradiation

	15 weeks post-irradiation		30 weeks post- irradiation	
	0 Gy	19 Gy	0 Gy	16 Gy
Heart rate (bpm)	534+/-50	522+/-56	487+/-63	498+/-27
LVEDD (mm)	3,5+/-0,5	3,5+/-0,3	3,2+/-0,2	3, 3+/-0,9
LVESD (mm)	1,8+/-0,3	2,4+/-0,4	1,6+/-0,4	2,2+/-0,5
Fractional Shortening (%)	48,6 +/- 2,1	31,4+/- 5,6***	49,2+/- 4,5	32.4+/-7.3**
Ejection fraction (%)	80,9+/- 2,6	60,4+/- 7,8 *	81,8+/- 5,9	62,0+/- 10,7 *

Data are presented as mean ± SE. LVEDD: left ventricular end-diastolic diameter; LVESD: left ventricular end-systolic diameter. *p≤0.05;**p≤0.01,***p≤0.001,irradiated mice *vs.* sham.

and 35±11.4 % respectively; *,p<0.05 and ***,p<0.001) 15 weeks post-19 Gy-irradiation (Fig. **2C** and Table **1**). Alteration of the ejection and shortening fraction was also seen after 16Gy irradiation but 30 weeks of latency were required to induce a significant decrease (approximately 24±13 % and 34±14.8 % respectively; *,p<0.05 and **,p<0.01). These results are consistent with progressive development of heart failure.

Irradiation Induces Delayed Activation of TGF-βRII, RhoB and CCN2 in the Heart

Immunostainings of TGF-βRII, Rho B and CCN2 were performed in heart sampled 15 weeks after thorax irradiation (Fig. **3**). A significant increase in TGF-βRII was observed at the plasma membrane of cardiomyocytes and microvascular endothelial cells. Rho B expression also increased and was

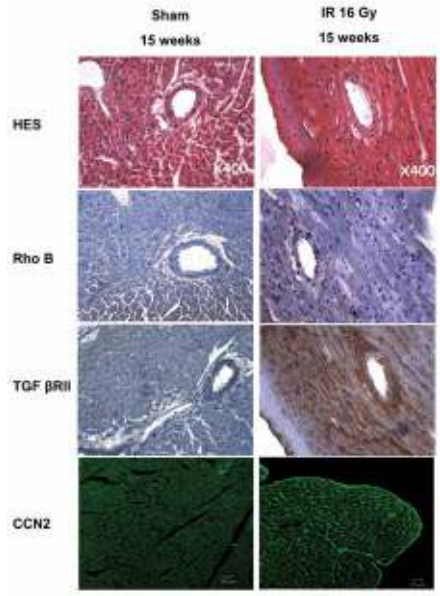

Fig. (3). Heart sections from sham and 16Gy irradiated mice 15 weeks after irradiation. Histological assessment of cardiac ventricular pathology by HES and immunostaining of Rho B, TGFBRII and CCN2 in sham or 16 Gy irradiated mice. Original magnification X 400.

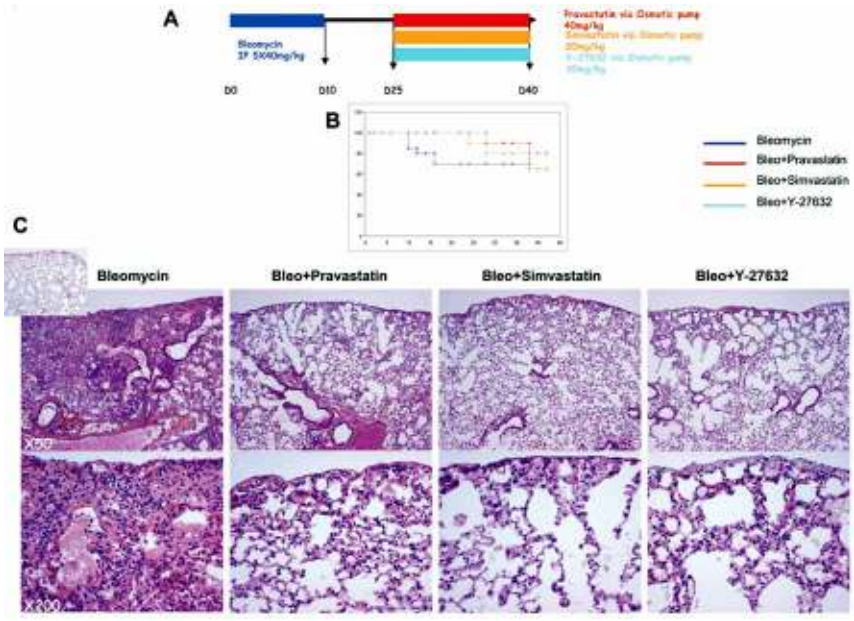

Fig. (4). Reversion of bleomycin-induced pulmonary fibrosis. A. Schematic diagram of curative approach. Animals were treated with Rho (pravastatin and simvastatin) and ROCK (Y-27632) inhibitors from 25 day to 40 day after Bleomycin intra-peritoneal administration. **B.** Monitoring of the survival rates. In the Bleomycin model, curative administration of Pravastatin and Y-27632 partially rescued bleomycin-induced mortality, whereas Simvastatin administration only delayed mortality. **C.** BLM induced widespread subpleural thickening and marked fibrotic lesions with dense extracellular matrix deposition (saffron-orange staining), associated with oedema of alveolar septa. Intra-parachymatous fibrotic remodeling surrounding vessels was observed with intense infiltration of inflammatory cells including macrophages, lymphocytes and neutrophils, leading to abnormal lung architecture. The 3 drugs used in the animals reduced oedema of alveolar walls and thickening of subpleura. Small fibrotic lesions were still observed in sub-pleural area but the overall pulmonary structure was normal.

restricted to vascular and peri-vascular areas. Similarly, increased deposition of CCN2 was found in interstitial tissue surrounding cardiomyocytes (Fig. **3**).

Pharmacological Inhibitors of the Rho/ROCK Pathway Prevent and Reverse Lung Fibrosis

In order to investigate the anti-fibrotic efficacy triggered by Rho/ROCK inhibition, pulmonary fibrosis was modeled using chronic injection of Bleomycin and single dose RX-irradiation. Animals were treated with various Rho (*i.e.* pravastatin and simvastatin) and ROCK (*i.e.* Y-27632) inhibitors using both mitigating and curative approaches according to the schedule shown Figs. (**4A, 5A** and **6A**).

Survival rates were monitored according to the various treatments and histopathological examinations were performed. In the Bleomycin model, curative administration of

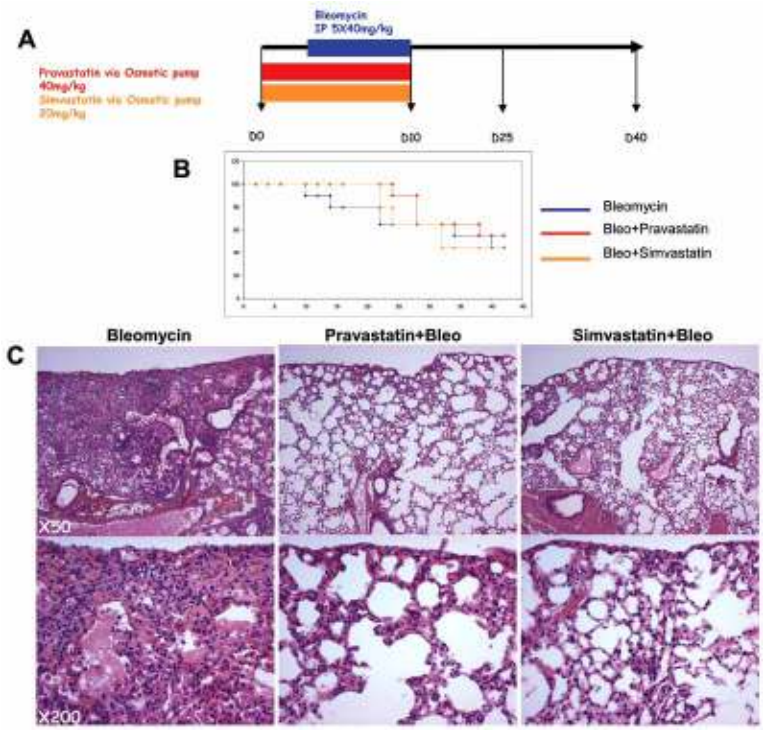

Fig. (5). Reversion of radiation-induced pulmonary fibrosis. A. and **B.** Pravastatin induced the same anti-fibrotic action in the model of radiation-induced lung fibrosis (RX, 19 Gy). No intra-parenchymatous fibrotic lesions in animals long-term-treated with Pravastatin (from week 5 to week 15 post-irradiation).

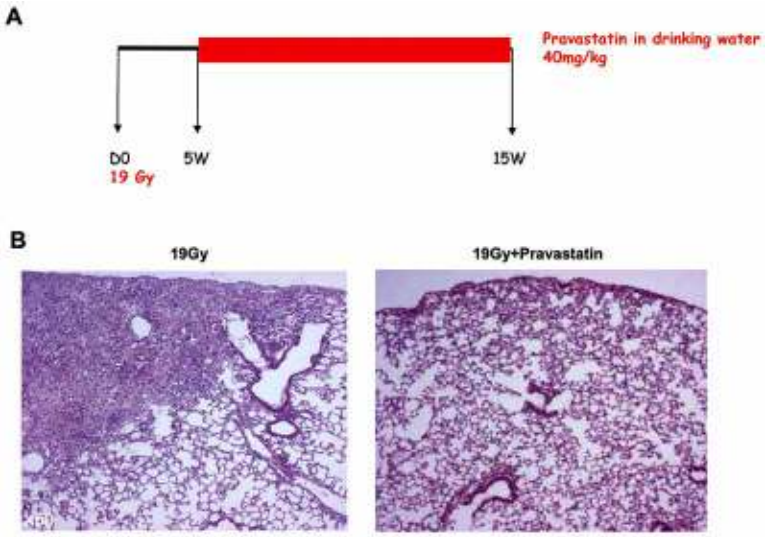

Fig. (6). Prevention of bleomycin-induced pulmonary fibrosis. A. Schematic diagram of mitigating approach. Animals were treated with Rho (pravastatin and simvastatin) and ROCK (Y-27632) inhibitors from -2 day to 10 day after Bleomycin intra-peritoneal administration. **B.** Mortality was delayed but the overall survival rate did not improve. **C.** Preventive and concomitant administration of Pravastatin and Simvatatin gave positive results preventing from the development of Bleomycin-induced lung fibrosis.

Pravastatin and Y-27632 (from day 25 to day 40) partially rescued bleomycin-induced mortality, whereas Simvastatin administration only delayed mortality (Fig. **4B**). Bleomycin administration induced typical subpleural and intra-parachymatous fibrotic lesions with dense extracellular matrix deposition (saffron-orange staining), associated with remodeled vessels surrounded by intense inflammatory infiltration (Fig. **4C**). Interestingly, a significant improvement of lung structure was observed in the animals treated with the 3 drugs. Thin fibrotic lesions were still observed in sub-pleural area but the overall pulmonary structure was normal. Pravastatin induced the same anti-fibrotic action in the model of radiation-induced lung fibrosis (RX, 19 Gy). No intra-parenchymatous fibrotic lesions were observed in the animals long-term-treated with Pravastatin (from week 5 to week 15 post-irradiation) (Figs. **5A, B**). Prophylactic administration of Pravastatin and Simvatatin also gave positive results preventing from the development of Bleomycin-induced lung fibrosis (Fig. **6C**). Mortality was delayed but the overall survival rate did not improve (Fig. **6B**).

Pravastatin Modulates the Fibrogenic Cascade Involving TGF-βRII and Rho in the Heart and Lung

We investigated *in situ* the molecular basis of the fibrolytic action triggered by pravastatin using immunohistochemistry. The downstream effect of pravastatin inhibition on RhoB, TGF-βRII and CCN2 expression were studied 15 weeks after irradiation in heart and lungs of animals irradiated and treated or not with Pravastatin (Figs. **7** and **8**).

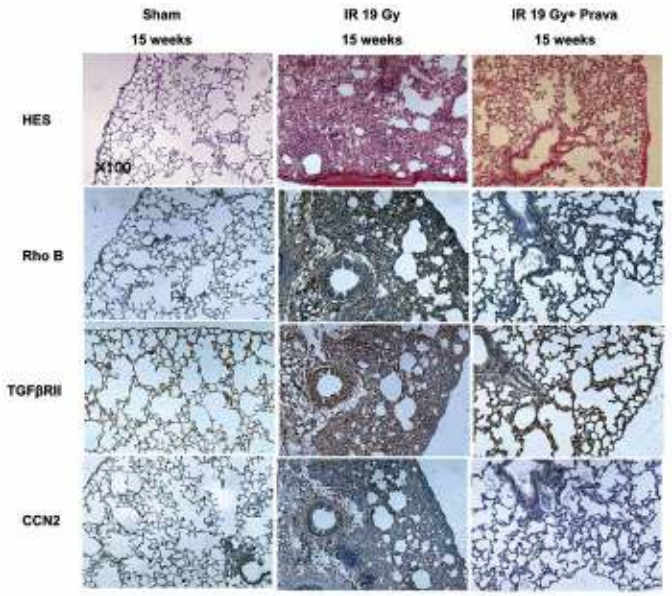

Fig. (7). Normalization of RhoB, TGFBRII and CCN2 in lungs after pravastatin treatment. Histological analysis of pulmonary tissue by HES and immunostaining of Rho B, TGFBRII and CCN2 in sham, 19 Gy irradiated group (IR 19Gy) and pravastatin treated group (IR 19 Gy + Prava). Original magnification X 100.

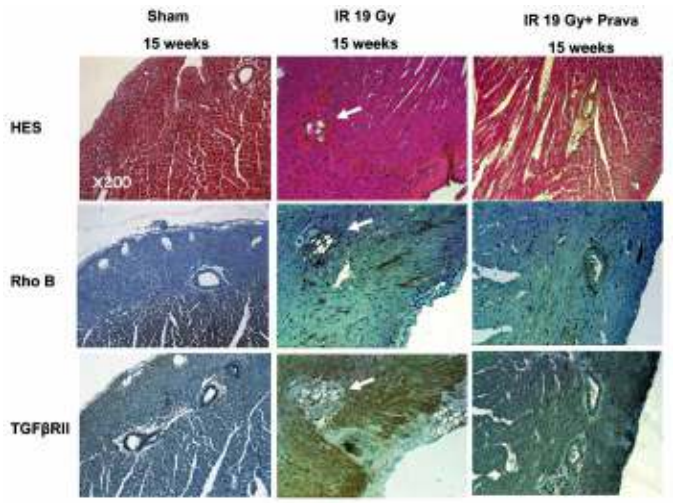

Fig. (8). Normalization of RhoB, TGFBRII in the heart after pravastatin treatment. Histological analysis of myocardial tissue by HES and immunostaining of Rho B and TGFBRII in sham, 19 Gy irradiated group (IR 19Gy) and pravastatin treated group (IR 19 Gy + Prava). Arrowheads denote regions of necrosis after a high dose of irradiation in 19 Gy irradiated group. X200 magnification for irradiated group and pravastatin treated group.

In non-irradiated lungs, type I pneumocytes did not stained for RhoB whereas few microvessels and macrophages were positive. TGF-βRII staining was stronger and located in alveolar septa and macrophages. CCN2 was not expressed in normal lung (Fig. **7**).

The irradiated group exhibited dense sub-pleural fibrotic lesions; the *interstitium* was invaded by extracellular matrix and massive infiltration of myofibroblasts, polynuclear cells and macrophages. Within these remodelled zones, a black-brown RhoB staining was observed in all interstitial cells and at the plasma membrane of smooth muscle cells surrounding bronchi. TGF-βRII staining was even more intense in interstitial cells and may also appear in the extracellular space suggesting the release of a soluble fraction of the receptor in irradiated lungs. The cytoplasm of bronchial epithelia cells and peri-bronchial smooth muscle cells also stained for TGF-βRII. CCN2 deposition was associated with extracellular matrix deposition within the fibrotic area, bronchial epithelial cells as well as vessels also stained for CCN2.

Interestingly, Pravastatin-treament normalized the expression of the three markers: RhoB expression being restricted to the peri-vascular area, TGF-βRII to alveolar septa and slight TGF-βRII and CCN2 staining in type II pneumocytes (Fig. **7**).

Increased cardiac expression of RhoB and TGF-βRII was confirmed 15 weeks after 19Gy thorax irradiation and was more intense than after 16Gy (Fig. **8**). Structurally 19 Gy-irradiation induced more damages than 16Gy with zones of tissue necrosis (shown by arrows). Interestingly, treatment with pravastatin significantly attenuated the development of myocardial fibrosis and necrosis and normalized the expression of the fibrogenic markers (Fig. **8**).

DISCUSSION

Awareness of the potential risk of late cardiac disease after exposure to radiation was raised by the analysis of mortality from cancer and non-malignant diseases among Japanese A-bomb survivors [13, 14]. In addition, radiotherapy to the chest is unavoidable in the primary treatment of several cancers with good prognosis, long survival times hence allowing many years for the expression of radiation damage. This can potentially be further exacerbated by combinations of increasingly effective novel treatment modalities for which data on long term normal tissue consequences do not exist. Portions of the heart can lie within the irradiated volume in the radiotherapy of breast cancer and mediastinal Hodgkin´s disease; for the latter, of special concern, since they are more susceptible to the late effects of radiotherapy (including tissue damage and cancers such as acute myelocytic leukemia, thyroid and breast cancers) is the irradiation of patients during childhood [15]. In addition, the lung remains a dose limiting organ of primary importance [16].

One of the most concerning aspects of radiation toxicity is fibrosis, due to its progressive and seemingly irreversible evolution. Therefore, developing therapeutic strategies to prevent and treat such radiation injury is of high priority in cancer research. We and others showed the important functional contribution of the Rho/ROCK pathway to radiation fibrogenesis, as pharmacological inhibition of Rho using statins prevented and reversed intestinal radiation fibrosis in humans [6-8]. In the present study, we extend these findings to examine delayed injury to heart and lungs in mice, using an experimental model of irradiation targeted specifically to the thorax; we also use a model of pulmonary fibrosis induced by the chemotherapeutic radiomimetic agent, bleomycin (BLM) [17]. We found that for both irradiation and BLM-treated animals, and for both heart and lung, the use of pharmacological inhibitors of the Rho/ROCK cascade improved both the histological structure and normalized the expression of fibrogenic markers. These data suggest that Rho/ROCK activation by fibrogenic agents may be neither organ-specific nor agent-specific, but more likely a common response to the chronic wound healing (active fibrotic) process.

Cardiac radiation-induced pathological changes in humans are well documented [18] and include congestive heart failure, angina pectoris, myocardial infarction, and valvular disorders. They typically occur from 1 to 25 years after irradiation, causing elevated mortality from 10 years after radiotherapy. There is evidence that older radiation techniques considerably contributed to these findings, as an overview of post-mastectomy radiotherapy for breast cancer revealed a great reduction in mortality and in fact a survival advantage for the use of modern technical approaches, such as 3D-conformal radiotherapy, for post-mastectomy radiotherapy which did not exist when older approaches were used. Initiation of cardiac pathology has been mainly attributed to vascular defect [19, 20], although their delayed occurrence suggests a predominant role for parenchymal cells including cardiomyocytes. Since cardiomyocytes are post-mitotic cells, their sensitivity to ionizing radiation was thought to be low. However, due to their high oxidative metabolism and poor antioxidant defenses [21, 22] their contribution to cardiac irradiation responses should be of primary interest. In the only *in vitr*o study published to date [23], culture of fetal cardiac myocytes isolated from rats and exposed to 8.5 Gy exhibited decreased mRNA level of TGF-β1 and other genes involved in the mitotic process. This report is not fully consistent with our observations, where exposure of murine adult cardiomyocytes to a single dose of 16 Gy induced early activation of two important fibrogenic cascades depending upon Smad2/3/4 and Rho and leading to CCN2 deposition and cytoskeletal remodeling. These discrepancies might result from differences between cell models (mouse *vs.* rat; adult *vs.* fetal) and doses (16 *vs.* 8.5Gy) used. However, our observations of radiation-induced fibrogenic pathways activation are supported by results obtained by others using pro-apoptotic stimuli, including nitric oxide, Phenylephrine [24] and Angiotensin II stimulation [25, 26] as well as mechanical stretch [27]. The functional relevance of our *in vitro* observations has been investigated further *in vivo* and we report for the first time, delayed radiation-induced cardiac hypertrophy associated with alteration of cardiac physiological parameters consistent with the development of heart failure. Cardiac hypertrophy and alteration of physiological parameters are well-described indicators of heart failure [28, 29]. Our data are consistent with recent clinical reports obtained in radiotherapy patients [15, 30] but inconsistent with results reported by Boerma

et al. in a rat model who showed a radiation-induced decreased EF [31]. Once again, the discrepancies might result from the model used (mouse *vs.* rat) and irradiation modalities (thorax *vs.* localized heart irradiation) and require further investigation. Our data correlated functional alterations with fibrogenic molecular imprints radiation-induced heart disease, co-operative interactions between delayed radiation-induced lesions and activation of intracellular fibrogenic signaling pathways *in situ* with a yet-never-described contribution for CCN2 in the heart.

Our previous studies showed that CCN2 regulation depends upon low levels of TGF-β1 and Rho/ROCK pathway activation in mesenchymal cells isolated from radiation enteropathy patients [5, 32, 33]. Similarly, delayed TGF-βRII, RhoB and CCN2 activation were also shown in the present studies in irradiated lung and heart. Interestingly, the functional consequences of CCN2 overexpression reported in our model are consistent with the pathological modifications reported in transgenic mice over-expressing CCN2 in the heart and lung (as reviewed in [34]). The heart transgenics exhibited cardiomyocyte hypertrophy and age-dependent heart disease which progressed from compensatory hypertrophy to ventricular dilatation and systolic heart failure [35]. The lung transgenic mice provided clear evidence that CCN2 overexpression impaired formation of the alveolar and vascular network associated with fibrosis in and around alveolar septa, bronchi and vessels [36]. In addition, our results suggest the involvement of RhoB to transactivate CCN2 and trigger radiation-induced heart failure and lung fibrosis. Rho proteins are small GTPases acting as molecular switches to control cell adhesion, formation of stress fibers, and cellular contractility through the reorganization of actin-based cytoskeletal structures. These functions are accomplished specifically *via* their effectors, the ROCKs [37] and our previous expression profiling studies highlighted RhoA, B and ROCK-1 as contributors to radiation-induced fibrogenesis and maintenance of the fibrogenic phenotype [38, 39]. In addition, activation of RhoB and ROCK-1 has also been described by Kajimoto et al. in ductus arteriosus smooth cells [40]. In this model, their upregulation is ROS-mediated, well-known mediators of radiation-induced damage, supporting the pathophysiological relevance of our results. Therefore we pharmacologically targeted Rho and ROCKs activation using statins (pravastatin and simvastatin) [41] and Y-27632 [42].

These inhibitory drugs displayed anti-fibrotic properties and improved delayed pulmonary and cardiac radiation injury and bleomycin-induced lung fibrosis. The results extend previous studies that demonstrate a pivotal role for Rho/ROCK in various cardiovascular diseases such as atherosclerosis, restenosis, pulmonary hypertension, cardiac hypertrophy and heart failure (as reviewed in [43]) to radiation-induced pathology. Pharmacological inhibition of ROCK by statins or other selective inhibitors led to the upregulation and activation of endothelial nitric oxide synthase and reduction of inflammation, atherosclerosis [44], injury induced by ischemia reperfusion [45] and experimental radiation-induced lung fibrosis [46]. Inhibition of ROCKs probably contributes to some of the cholesterol-independent beneficial effects of statin therapy. However, statin and selective ROCKs inhibitors display different genomic targets [47] suggesting possible synergistic or additive properties and opening novel options for combined treatments. With the recent development of RhoB and ROCKs-knockout mice, further dissection of the relevant signaling pathway is now possible. Targeted deletion of *Rock-1* protects mice from pressure overload and inhibits the development of reactive fibrosis in the heart [48]. Furthermore *rhoB* deletion altered cell response to TGF-β signals [49]. As *RhoB* is also known to control tumour radiosensitivity [50, 51], it is possible that specific inhibition of RhoB could trigger a differential beneficial effect, protecting normal tissue from radiation damage and sensitizing tumours.

In summary, the present study shows a radiation-induced regulation of two important fibrogenic pathways (*i.e.* TGF-β/Smad and Rho/ROCK) in the heart and lungs. Furthermore, it supports the potential therapeutic importance of Rho/ROCK inhibition to treat radiation and bleomycin-induced pulmonary and cardiac delayed injury and fibrosis.

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