Hypoxia contributes to quiescence and chemoresistance of Leukemia Initiating Cells in B Acute Lymphocytic Leukemia

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Definition:

Haematopoietic malignancy,
Affecting B lymphoid lineages
Affecting haematopoiesis (thrombopenia, anemia, neutropenia)

Epidemiology:

1.25 / 100 000

Children: 2 to 10 years (20% of cancers)
Adult: ≥ 50 years (< 1% of cancers)
B-ALL: leukaemogenesis

Initiation
Self-renewal
ETV6-RUNX1

Additional alterations
Developmental arrest
IKZF1, PAX5, EBF1 (2/3 ALL)

Haemopoietic stem cell/lymphoid progenitor

Pro-B-cell/Pre-B-cell

Mature B cell

Additional alterations
Cooperating events
Cell cycle (RB1)
Cytokine receptor and kinase (PDGRB)
Transcriptor factor ...

Diagnosis

Adapted from Inaba et al., The Lancet, 2013

Systematic research of recurrent genetic abnormalities → Classification and stratification
<table>
<thead>
<tr>
<th>LAL</th>
<th>Anomalie</th>
<th>Incidence</th>
<th>Pronostic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hyperdiploïdie</td>
<td>&lt; 10 % chez l'adulte/30-35 % en pédiatrie</td>
<td>Favorable</td>
</tr>
<tr>
<td></td>
<td>Formes hypodiploïdes, haploïdes et quasi triploïdes</td>
<td>&lt; 5 % chez l'adulte</td>
<td></td>
</tr>
<tr>
<td>B+T</td>
<td>Réarrangements de MLL</td>
<td>80 % chez les &lt; 1 an, exceptionnel au-delà de cet âge chez l'enfant, 10 % chez l'adulte</td>
<td>Défavorable</td>
</tr>
<tr>
<td></td>
<td>Caryotypes complexes (&gt; 5 anomalies)</td>
<td>&lt; 5 % chez l'adulte</td>
<td></td>
</tr>
<tr>
<td></td>
<td>t(9;22)(q34;q11)/BCR-ABL1</td>
<td>30-40 % chez l'adulte/&lt; 5 % chez l'enfant</td>
<td>Défavorable</td>
</tr>
<tr>
<td></td>
<td>TCF3-PBX1 et TCF3-HLF</td>
<td>4 % des LAL de l'enfant et jeune adulte</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(iAMP21)</td>
<td>Enfants et adolescents principalement</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mutations/délétions du gène IKZF1</td>
<td>~ 3/4 LAL Ph+, 1/3 LAL non Ph+ / 15 % des LAL B de l'enfant</td>
<td>Défavorable</td>
</tr>
<tr>
<td></td>
<td>LAL BCR-ABL1 like</td>
<td>15 % des LAL B de l'enfant, et vraisemblablement une proportion significative chez les 15-25 ans</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Réarrangements de CRLF2</td>
<td>50 % des LAL associées au syndrome de Down et jusqu'à 50 % des LAL BCR-ABL1 like</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mutation de PAX5</td>
<td>Précoce dans la leucémogenèse B environ 30 % des LAL B</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>t(12;21)(p12;q22)/ETV6-RUNX1</td>
<td>1-3 % chez l'adulte/25 % chez l'enfant</td>
<td>Favorable</td>
</tr>
<tr>
<td></td>
<td>Délétion intragénique de ERG</td>
<td>/-3 %</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Lara et Gauthier., Hématologie, 2014
Clinical characteristics

- Age (Tolerance – different genetic abnormalities)
- Innate genetic background (Trisomy)
- Signs of central nervous system invasion
- Signs of testis invasion

Biological characteristics

- Leucocyte enumeration (Hyperleucocytosis > 30 000 / mm$^3$)
- Extended B-cell phenotyping : (CD10$^-$)
- Genetics abnormalities (BCR-ABL)

Evolution Characteristics : Response to treatment

- Initial corticoresistance
- Chemoresistance (MRD level)
ALL Treatment: Polychemotherapy

Long (≈3 years) in 3 phases preceded by 1 week of corticotherapy

**Induction: ≈ 1 month**
- Anthracyclines (Daunorubicine)
- Corticoïds (MéthylPrednisolone)
- Vincristine
- L-Asparaginase
- Alkylating agents

**Consolidation: ≈ 7 months**
- Methotrexate
- Cytarabine (Ara C)
- Cyclophosphamide
- Other induction therapy

**Maintenance: ≈ 2-3 years**
- 6-mercaptopurine
- Methotrexate
- Vincristine
- Corticoïds

Allograft
Adult
Results

Kaplan-Meier analysis of survival for 2852 children enrolled in 15 consecutive studies from 1962 to 2007

Adapted from Pui and Evans, Semin Hematol., 2013
Clonal relationship of diagnosis and relapse samples in ALL

Mullighan et al., Science, 2008

61 paediatric patients

Relapses: Leukaemia Initiating Cells persistence
Leukemia initiating cells (LIC)

- Malignant cells that initiate leukaemia

- That kept/reacquire HSC properties
  - Self-renewal
  - Differentiation (partial – tumoral heterogeneity)
  - Homing
  - Protection mechanisms

Leukaemic transformation model
Adapted of Rosen and Jordan, Science, 2009
Relapses are due to residual LIC

Aguirre-Ghiso, Nat Rev Cancer., 2007

Relapses: Leukaemia Initiating Cells in dormancy state
Tumor mass dormancy

% Proliferation = % Apoptosis

Angiogenic dormancy

- Dormant micrometastasis
- Pro-angiogenic factors: Ras → VEGF, Ras → TSP, Low O₂
- Anti-angiogenic factors: p38 → VEGF, p38 → TSP, p53 → TSP

Growing micrometastasis

Angiogenic switch
Exogenous angiogenic ‘spike’

Immunosurveillance

- Dormant micrometastasis
- Immunity coordinated by CD8⁺ T cells and memory T cells
- Humoral response

Growing micrometastasis

Cellular escape mechanisms
Immunosuppression?

Cellular dormancy

- Quiescent solitary tumour cell
- Microenvironment-dependent
- Evasion of the immune system by quiescent tumour cells

Adapted from Aguirre-Ghiso, Nat Rev Cancer., 2007
Cellular dormancy = quiescence

Reversibility of the G0 state of the cell cycle
Adapted from Rodgers et al., Nature, 2014

- Cell cycle arrest with low metabolic rate
- Quiescence = Reversibility ≠ senescence
- Poorly characterized (rare)
- Crucial to maintaining the HSC pool (preservation of key functions)
- Protection mechanism of the HSC
### Identification of quiescent cells

<table>
<thead>
<tr>
<th>Markers</th>
<th>Effects</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>EdU</td>
<td>No S phase incorporation</td>
<td>Nucleic acid analogue</td>
</tr>
<tr>
<td>DiD</td>
<td>Retention</td>
<td>Membrane Lipophilic marker</td>
</tr>
<tr>
<td>Ki67</td>
<td>Absence</td>
<td>Proliferation marker</td>
</tr>
<tr>
<td>Pyronine Y</td>
<td>Low</td>
<td>RNA marker</td>
</tr>
<tr>
<td>Rhodamine 123</td>
<td>Low</td>
<td>Mitochondrial activity marker</td>
</tr>
</tbody>
</table>
Control of stem cell quiescence

Regulation of murine HSC quiescence exit.
Adapted from Yamada et al., Cell Cycle, 2013
BM micro-environment modulates quiescence: HSC

HSC localisation in the bone marrow
adapted from Trumpp et al., Nat Rev Immunol., 2010

The role of hypoxia in the maintenance of hematopoietic stem cells.
Cipolleschi MG et al, Blood. 1993
BM micro-environment modulates quiescence: LIC hypothesis

LIC localisation in the bone marrow niche?

adapted from Trumpp et al., Nat Rev Immunol., 2010
Role of the BM micro-environment in quiescence: What is known about ALL

Model for osteopontin-induced dormancy of ALL at the endosteum

*Adapted from Boyerinas et al., Blood, 2013*
A BM environmental factor: $O_2$
Why hypoxia?

Oxygenation of murine calvaria.
Spencer et al., Nature, 2014

Human bone marrow $[O_2] \approx 0\%$
after 10 cellular layers
Chow et al, Biophysical Journal, 2001
Regulation of quiescence through HIF1α in normoxia and hypoxia

*Forristal et al., Blood, 2013*
Role in energy metabolism

**Effect of hypoxia on the energetic metabolism**

adapted from Takubo et al., Cell Stem Cell, 2013
Objectives of the present study

Development of an *in vitro/in vivo* model for exploring the relationships between hypoxia and ALL LIC

Role of low [O$_2$] on self-renewal and quiescence of ALL cells

Role of quiescence in chemoresistance

**Potential Clinical benefit**

To explore mechanisms of persistence of residual LIC in ALL

Potential role in relapse
A BM environmental factor: $O_2$

What do we know? Previous results

- **CD34$^+$ ex vivo**
  - **HSC**
  - **Progenitors**

- **CD34$^+$ in vitro**
  - 20% $O_2$: Exhaustion
  - 3% $O_2$: Maintenance
  - 1% $O_2$: Expansion
  - 0.1% $O_2$: Accumulation of quiescent cells

- Ivanovic et al. (Stem Cells, 2005)
- Ivanovic et al. (Br J Haematol, 2000; Exp Hematol, 2002)
- Hermitte et al. (Stem Cells, 2006)
Human cell line: NALM6 (Pre-B)

- **Origin**: 19 year-old man in relapse; peripheral blood (1976)

- **Immunophenotype**: CD3⁻, HLA-DR⁺, CD10⁺, CD19⁺, HLA-DR⁺, CD34⁻, cyCD79a⁺, CD37⁻, CD80⁻, CD138⁺, sm/cy IgG⁻, cy IgM⁺, sm IgM⁻

- **Genetic**: closed to diploid caryotype
  
  46 (43-47)<2n>XY
  
  t(5 ; 12) (q33.2 ; p13.2) (ETV6 – PDGFRB)

Primary cells from patients:

- **Samples heterogeneity** (Dif genetic Abnormalities...)

- **In vitro** culture difficulties

- Transplant difficulties
Study of modification of the cell cycle according to Oxygen concentration

Study of LIC survival after in vitro exposure to 5-FU

Survival study of LIC after in vivo 5-FU exposure (Flow cytometer)

Localisation study of LIC after in vivo 5-FU exposure (Histology)

1- Study of modification of the cell cycle according to Oxygen concentration

Ki67
Phospho-pRB
EdU

NALM6

In vitro:
CFC assay
CRC ability

In vivo:
Xenograft in NSG

NALM6 (Primary cells)

Human Cells
Cleaved Caspase -3
Ki67
Pimonidazole

Chimerism
Ki67
Annexin V
Secondary transplants

Methods
1- Study of modification of the cell cycle according to oxygen concentration

NALM6 : *in vitro* part 1
Conclusion: NALM6 cells survive at 0.1% (low mortality) with low proliferation.

Which is their cell cycle status?
Culture at 0.1% O₂ induces cell cycle arrest in G₀

Ki67:
- Nuclear protein associated to hétérochromatin
- Role ??
  DNA organisation ? rRNA synthesis ?
- Required for cell proliferation

Conclusion: NALM6 cells become quiescent in severe hypoxia

Molecular actors ?
Conclusion: quiescent NALM6 cells express dephosphorylated pRb

Do these cells remain undivided from day 3 to day 7? EdU assays
EdU (analog of thymidine):
- Incorporated in DNA during S phase

Incorporated EdU is detected using AlexaFluor488 azide

20% O₂

Control

EdU : 1 µM

3 days

48h et 96h

Primary culture

0.1% O₂

Control

EdU : 1 µM

Click-IT™ Alexa Fluor® azide

Incorporated EdU

Primary culture
Culture at 0.1% O₂ induces G₀/G₁ cell cycle arrest of a subset of NALM6 cells.

Conclusion:
13% of NALM-6 cells did not enter in S phase after 4 days of exposure to hypoxia.

Is this arrest related to chemotherapeutic agents resistance?
Protocol of NALM6 Primary culture

- **20% O₂**
  - 3 days of conditioning
  - Control
  - Drug

- **0.1% O₂**
  - 4 days of treatment
  - Control
  - Drug

- Question marks in the diagrams indicate an unknown outcome.
Culture at 0.1% O\textsubscript{2} contributes to NALM6 chemoresistance

Chemoresistance during culture (n = 3)

At 1 and 3%, the results are similar to those at 20%

Do these residual viable cells behave as LIC?
2- Study of LIC survival after *in vitro* exposure to 5-FU

NALM6 : *In vitro* part 2
Protocol of primary and secondary culture

5-FU (5-Fluoro-Uracil) : target S phase

*In vitro* assay to detect:
- clonogenic cells
- LIC, by repopulating liquid culture

Primary culture

- 20% O\(_2\)
  - 3 days of conditioning
  - 4 days
  - Control
  - 5-FU : 25 µg/ml

- 0.1% O\(_2\)
  - Control
  - 5-FU : 25 µg/ml

Functional analysis of residual cells

- Colony assay
  - Secondary culture at 20% of O\(_2\)
  - Transplantation
Conclusion: NALM6 is heterogeneous. After 7 days in hypoxia, CFU-L and CRC are maintained. Hypoxia protects CFU-L and CRC from 4 days of 5-FU exposure.

What would happen in vivo?
Conclusions:
Control conditions: no significant difference
5-FU conditions:
At 20%, all LIC are killed by 5-FU
Primary culture at 0.1% maintains LIC
NALM6 culture at 0.1% $O_2$ leads to:

- Proliferation slowing down without mortality increase
- G0 arrest of a limited proportion of cells (75% are still cycling after 3 days)
- Resistance to several drugs used in ALL
- Resistance to 5-FU of rare quiescent LIC able to
  - Repopulate secondary liquid cultures \textit{in vitro}
  - Engraft leukemia into mice
3- Survival study of LIC after *in vivo* 5-FU exposure (Flow cytometry)

NALM6: *In vivo* assay 1
Patient: *In vivo* assay *(Experimental bias)*
Protocol for NSG:

Chimerism tracking by intrafemoral withdraw every 15 days

- Busulfan conditioning (20mg/kg)
- Transplantation of 250 000 primary cells (IF) or 10 000 NALM6 cells (IV)
- Chimerism > 50% : Intra-peritoneal 5-FU treatment (150mg/kg)
- Sacrifice DX+3
  - Bone marrow

Flow cytometry analysis of *in vivo* chemoresistant cells
in vivo experiments

Residual human cells FACS analysis (NALM6 and primary cells from patients)

• phenotype:
  - Human: HLA-DR; hCD45;
  - Leukaemic B: hCD10; hCD19;
  - Stemness: hCD34;

• Cell cycle: Ki67

• Secondary transplant.
Profiles of 3 tested samples

Secondary transplants of Phi+ patient cells

Serial transplant is lethal
BM residual human cells are mainly alive and quiescent

Proportion of Ki67 negative human cells in femur

**NALM6**

(n = 2; 8 mice)

**Patients (Secondary recipients)**

(n = 1; 3 mice)
NALM6: 5-FU treatment of xenografted mice increases the percentage of quiescent cells among residual BM cells.

Patients: Intrafemoral engraftment of human primary ALL cells in NSG mice

- Engraftment kinetics vary from patient to patient
- Serial transplantation (IV) evidences the presence of LIC whose phenotypic profile did not vary
- Secondary transplantation improves the mouse to mouse reproducibility kinetics of engraftment, arguing for the selection of LIC adapted to xenotransplantation
- Post 5-FU residual cells contain the quiescent LIC
BM Histological sections (femurs, dorsal and caudal vertebrae)

- Localisation of LIC before and after 5-FU treatment,
- Oxygenation and perfusion rates
4- Localisation study of LIC after *in vivo* 5-FU exposure
Protocol for NSG:

- Busulfan conditioning (20mg/kg)
- Transplantation of 10,000 NALM6 cells (IV)
- Intra-peritoneal 5-FU treatment (150mg/kg)
- Sacrifice D21

Histological analysis of BM

D-2 et D-1 → D0 → D18, D19, D20
At day 21 after transplantation, NALM6 cells aggregates are dispersed in mouse TBA and LBA
At day 21 after transplantation, NALM6 cells aggregates are dispersed in mouse TBA and LBA.

x200; ALU labeling; one representative section (n = 2 souris)
At day 21 after transplantation, apoptotic NALM6 cells are very rare in the BM

x200; cleaved Caspase 3 labelling; one representative section (n = 2 mice)
At day 21 after transplantation, quiescent NALM6 cells are very rare in the BM

Ki67 labelling; one representative section (n = 2 mice)
At day 21 after transplantation, quiescent NALM6 cells are very rare in the BM.

Ki67 labelling; one representative section (n = 2 mice)

Human cells

Proliferating cells
Leukemic aggregates dispersed in intact residual murine hematopoietic areas suggest a clonal NALM6 BM seeding and development (to be confirmed)

- Apoptotic/dead cells are very rare
- Most cells are proliferating
- Are these cell aggregates hypoxic? *Benito et al, PLoS One. 2011*

- Identifying specific BM homing/seeding sites (niches?) will require analysis of earlier engraftment time points and use of other methods.
5-FU treatment induces mice BM cytopenia

Trichrome de Masson; representative section (n = 3 mice)

Hemorragic Suffusion

Residual hematopoiesis

Cytopenia
5-FU treatment induces human and murine cells lysis

ALU labelling; one representative section (n = 3 mice)
21 days after transplantation, NALM6 cells aggregates are detected in murine femoral BM

ALU labelling; one representative section (n = 3 mice)
Most 5-FU residual cells are alive

Cleaved Caspase 3 labelling; one representative section (n = 3 mice)

Human cells

Apoptotic cells
Post 5-FU residual NALM6 cells are mostly quiescent

Ki67 labelling; one representative section (n = 3 mice)
Post 5-FU residual NALM6 cells are mostly quiescent

Ki67 labelling; one representative section (n = 3 mice)

80% of cells are human

70% of human cells are quiescent
Conclusion

- Heterogeneous BM cytopenia: are some areas (metaphyse) better protected?
- Rapid elimination of apoptotic cells
- A large percentage of 5-FU resistant NALM6 cells are quiescent
- No preferential endosteal localisation of quiescent leukemic cells
Our model will allow to continue \textit{in vitro} and \textit{in vivo} investigations on aspect of ALL biology:

- \textit{Existence of preferential Metabolic pathways}
- \textit{Existence of preferential « niches » harboring quiescent and resistant LIC}
- \textit{Mechanisms of quiescence and their relationships with LIC resistant to therapy}
- \textit{Relationships between LIC quiescence and vascularisation, perfusion and innervation of their « niches »}. 

Perspectives (1) 
Pathophysiological studies
New parameters involved in individual prognosis evaluation

innovative therapeutic approaches taking into account the results of pathophysiological studies
Thank you for your attention
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