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ORIGINAL ARTICLE

Mobilization of CD34$^+$CD38$^-$ hematopoietic stem cells after priming in acute myeloid leukemia

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Abstract

AIM: To evaluate quantitatively and qualitatively the different CD34$^+$ cell subsets after priming by chemotherapy granulocyte colony-stimulating factor (± G-CSF) in patients with acute myeloid leukemia.

METHODS: Peripheral blood and bone marrow samples were harvested in 8 acute myeloid leukemia patients during and after induction chemotherapy. The CD34/CD38 cell profile was analyzed by multi-parameter flow cytometry. Adhesion profile was made using CXC chemokine receptor 4 (CXCR4) (CD184), VLA-4 (CD49d/CD29) and CD47.

RESULTS: Chemotherapy ± G-CSF mobilized immature cells (CD34$^+$CD38$^-$ population), while the more mature cells (CD34$^+$CD38^{low} and CD34$^+$CD38$^+$ populations) decreased progressively after treatment. Circulating CD34$^+$ cells tended to be more sensitive to chemotherapy after priming with G-CSF. CD34$^+$ cell mobilization was correlated with a gradual increase in CXCR4 and CD47 expression, suggesting a role in cell protection and the capacity of homing back to the marrow.

CONCLUSION: Chemotherapy ± G-CSF mobilizes into the circulation CD34$^+$ bone marrow cells, of which, the immature CD34$^+$CD38$^-$ cell population. Further manipulations of these interactions may be a means with which to control the trafficking of leukemia stem cells to improve patients' outcomes.

Key words: Acute myeloid leukemia; Leukemia stem cell; Immunophenotype; Priming; Timed sequential chemotherapy

Core tip: Timed sequential chemotherapy and priming with hematopoietic growth factors have been recently used in the treatment of acute myeloid leukemia in order to mobilize more leukemic cells in the cell cycle and therefore improve the cytotoxic effect of chemotherapy. In this paper, we looked the impact of this type of treatment in a small series of patients on the mobilization of different subsets of CD34$^+$ cells involving "bulk" leukemic cells and more "immature" leukemic cells.

Plesa A, Chelghoum Y, Mattei E, Labussière H, Elhamri M, Cannas G, Morisset S, Tagoug I, Michallet M, Dumontet C, Thomas X. Mobilization of CD34$^+$CD38$^-$ hematopoietic stem cells after priming in acute myeloid leukemia. *World J Stem Cells* 2013; 5(4): 196-204 Available from: URL: http://www.wjgnet.com/1948-0210/full/v5/i4/196.htm DOI: http://dx.doi.org/10.4252/wjsc.v5.i4.196

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder that originates from leukemia stem cells (LSCs) with the ability to generate an excessive amount of malignant myeloid blasts. Despite therapeutic advances in younger adults, relapses remain a major issue[1]. Chemotherapeutic regimens markedly reduce tumor burden, but only target the "bulk", non-clonogenic cells and spare LSCs, which allow for recrudescence of leukemia[2]. Interactions of the leukemic cells with the bone marrow (BM) microenvironment via specific receptor and adhesion molecules, such as the CXC chemokine receptor 4 (CXCR4)-stromal cell-derived factor-1 (SDF-1) axis, are in part responsible for chemotherapy resistance. AMLs with high CXCR4 cell surface expression and therefore a high tendency of stromal protection have been shown to have a poor prognosis[3,4].

The cell cycle is a critical regulator of the processes of cell proliferation and growth. One strategy to increase the cytotoxicity of cycle-dependent antileukemic agents is to enter more leukemic cells into the cell cycle. Timed sequential chemotherapy (TSC) is based on the findings that the initial cytoreductive drug induces the remaining malignant cell cohort to enter a proliferative state at a predictable time following drug administration[5]. Combination with hematopoietic growth factors (HGFs) has also been developed to enhance the efficacy of cytotoxic agents[6,7]. Granulocyte colony-stimulating factor (G-CSF) stimulates very immature progenitors and cleaves SDF-1[8], inducing stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating its receptor CXCR4[9]. Such an approach could theoretically target the self-renewal machinery of LSCs by inducing the quiescent LSCs into the cycle and circulation. However, a better understanding of those mechanisms is warranted.

Basically, low numbers of immature hematopoietic cells are released into the peripheral blood (PB). Treatments, including HGFs and/or TSC DNA-damaging agents, known as priming, can cause a marked increase in hematopoietic stem cell mobilization[10]. The aim of the present study was to investigate BM and PB leukemic blasts in the CD34 versus CD38 bidimensional space in order to evaluate quantitatively and qualitatively the different CD34$^+$ cell subsets during and after priming by TSC ± G-CSF as induction chemotherapy in adult AML patients.

MATERIALS AND METHODS

Patients and treatments

PB and BM samples were procured from a total of 8 AML patients (Table 1). All studied patients were treated according to the Acute Leukemia French Association (ALFA)-0702 trial[11]. Diagnosis was morphologically proven according to the French-American-British classification[12]. The study protocol was approved by the Human Ethics Committee of our institution and was conducted in accordance with the Declaration of Helsinki. All patients gave written informed consent prior to registration on the study. This trial was registered at www.clinicaltrials.gov as No. NCT00932412. All patients received TSC induction and 5 of them were also primed by G-CSF (filgrastim). Morphological complete remission (CR)[13] and risk classification, based on cytogenetics and molecular marker analyses, were defined as previously described[7,14].

AML cells and flow cytometry analyses

PB and BM samples were harvested in all patients at different times: at diagnosis (T0), at the end of the first sequence of chemotherapy (4 d) (T4), at the beginning of the second sequence of chemotherapy (8 d) (T8), at the end of chemotherapy (10 d) (T10), during aplasia (15 d) (T15), and at the time of cell recovery (between 28 and 35 d) (TR).

Surface and intracellular antigen detection were performed by multi-parameter flow cytometry (MFC). Briefly, the CD34/CD38 cell profile was analyzed in one single tube by a multi-parameter combination using CD7, CD13, CD33, CD34, CD38, CD45 and CD19. Analyses were performed, using FACS Diva software (BD Bioscience). Instrument set-up was routinely optimized by analyzing Calibrite beads- Rainbows 8 picks beads and CST beads system. Adhesion profile was made using CXCR4 (CD184), VLA-4 (CD49d/CD29) and CD47. Cells were incubated with the appropriate combination of MoAbs (1 × 10^6 total cells per tube), washed and then analyzed by flow cytometry. The required minimal number of CD34$^+$ events was set at 20 and the total number of events ranged from 100000 to 500000. Immunoglobulin G (IgG) isotype staining was used as a negative control for both CD38 and adhesion markers expression for ratio median fluorescence intensity (rMFI) evaluation. Discriminating the apoptotic/necrotic status of each subpopulation was made possible by using fluorescein isothiocyanate (FITC)-labelled annexin V and propidium iodide (PI), as previously described[15].

The gating strategy was based on CD45^{low}/SSC total blast and CD34$^+$CD45^{low} cell populations gated from total FSC/SSC viable cells. Three populations of CD34$^+$ cells were distinguished based on differential expression of the CD34 and CD38 antigens[16]: A first cell population, which expressed a great amount of the CD34 antigen, lack of CD38 (CD34$^+$CD38$^-$) and often con-

Table 1 Characteristics at diagnosis and outcome of the 8 acute myeloid leukemia patients

Pts	Age (yr)	Sex	Cytogenetics	CD34$^+$ (%)[1]	FAB	Molecular Biology[2]	WBC (x 10^9/l)[2]	Risk-group	G-CSF priming	BM 15 d	CR	Consolidation and DFS
1	59	M	Normal	85	M5	Flt3-ITD neg CEBPA pos	55.1	Favorable intermediate	Yes	No blast	Yes	Chemotherapy 15$^+$ mo
2	35	M	Normal	85	M2	Flt3-ITD neg CEBPA pos	4.8	Favorable intermediate	Yes	No blast	Yes	Chemotherapy 16$^+$ mo
3	56	F	+8, del (20) (q11; q13)	93	M1	Flt3-ITD pos	12.6	Poor intermediate	Yes	ND	Yes	Chemotherapy 3 mo
4	19	M	Normal	67	M6	Flt3-ITD pos MLL pos	0.9	Unfavorable	Yes	ND	Yes	AlloSCT 6 mo
5	55	M	Normal	8	M1	Flt3-ITD pos NPM1 pos	147.3	Poor intermediate	Yes	ND	Yes	AlloSCT 14$^+$ mo
6	55	F	Normal	3	M5	Flt3-ITD neg Evi-1 pos	3.7	Unfavorable	No	5% blasts	Yes	AlloSCT 14$^+$ mo
7	44	M	Complex	94	M0	Flt3-ITD neg NPM1 neg	1.4	Unfavorable	No	> 5% blasts	No[3]	AlloSCT 11 mo
8	45	F	–7, +21	83	M4	Flt3-ITD neg NPM1 neg	2.4	Unfavorable	No	No blast	Yes	AlloSCT11$^+$ mo

[1]Expression of CD34 by leukemic cells; [2]Molecular biology and WBC count at diagnosis; [3]Morphological complete remission was subsequently achieved by salvage therapy combining idarubicin with high-dose cytarabine. Patients prognosis was defined according to the following classification based on cytogenetics and molecular marker analyses. Karyotype abnormalities that involved core binding factor (CBF) leukemias [*t* (16; 16) (p13; q22), inv (16) (p13; q22), or *t* (8; 21) (q22; q22)] with or without other cytogenetic abnormalities were considered favorable cytogenetics. Monosomies or deletions of chromosomes 5 and 7; abnormalities of the long arm of chromosome 3 (or Evi-1 gene mutation); 11q23 abnormality (or MLL gene mutation); or complex cytogenetic abnormalities (defined as at least five unrelated cytogenetic clones) were considered unfavorable risk factors. Other cytogenetic abnormalities and cytogenetically normal (CN)-AML were designated intermediate risk factors. Intermediate-risk cytogenetics was further subdivided into a favorable intermediate risk group [CN-AML with nucleophosmin (*NPM1*) or CCAAT/enhancer-binding protein-(*CEBPA*) mutations and no *FLT3-ITD*] and a poor intermediate risk group (other patients). Induction chemotherapy consisting of a TSC including a first sequence combining daunorubicin, 60 mg/m^2 per day Ⅳ on 1–3 d, and cytarabine, 500 mg/m^2 per day Ⅳ over the same period. The second sequence, administered after 4 d free interval, consisted of daunorubicin, 35 mg/m2 per day, Ⅳ on 8 d and 9 d, and cytarabine, 1000 mg/m^2 per 12 h on 8–10 d. Five patients received G-CSF (filgrastim) priming (5 μg/Kg per day) on 1 to 10 d. Three patients were not primed with G-CSF. AlloSCT: Allogeneic stem cell transplantation; BM: Bone marrow; CR: Morphological complete remission; DFS: Disease-free survival; F: Female; FAB: French-American-British morphological classification; M: Male; neg: Negative; pos: Positive; Pts: Patients; WBC: White blood cell; G-CSF: Granulocyte Colony-Stimulating Factor.

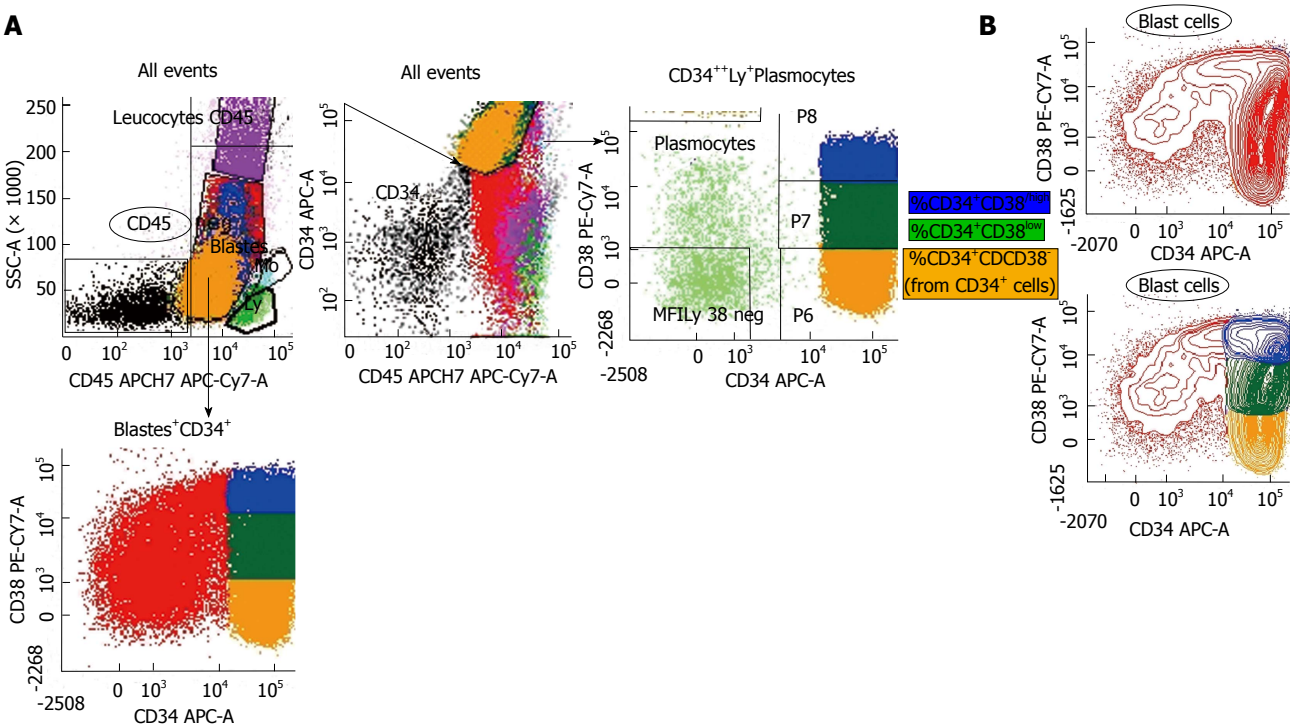

Figure 1 Multicolor flow cytometry (patient # 3): (left) Strategy used for gating the blast cell population and CD34$^+$CD38 cell subpopulations (red: immature CD45^{low} SSC blast cells, blue: CD34$^+$ cells, green: lymphocytes, blue cyan: monocytes, violet: granulocytes, black: CD45$^+$ cells (erythroblasts); CD34$^+$ cells were separated into different stem cell fractions based on their CD38 antigen expression: A first cell population expressing a great amount of the CD34 antigen and lacked of CD38 (CD34$^+$CD38$^-$), a second cell population characterized by a great amount of the CD34 antigen and by a low density of CD38 antigen (CD34$^+$CD38^{low}), and a third cell population characterized by a large density of CD38 antigen and of CD34 antigen (CD34$^+$CD38$^+$); (right) The two density plots indicate CD45low/SSC blast cells (in red) and CD34$^+$CD38 subpopulations (CD34$^+$CD38$^-$ in orange, CD34$^+$CD38^{low} in green and CD34$^+$CD38$^+$ in blue).

tained very few events requiring to be tightly clustered in a FSC/SSC and CD45/SSC plot; a second population characterized by a great amount of the CD34 antigen and by a low density of CD38 antigen (CD34$^+$CD38^{low}); and a third population characterized by a large density of CD38 and CD34 antigens (CD34$^+$CD38$^+$). Figures 1 and 2 are typical examples of an analysis. CD38 was expressed as percent positively stained cells within CD34$^+$

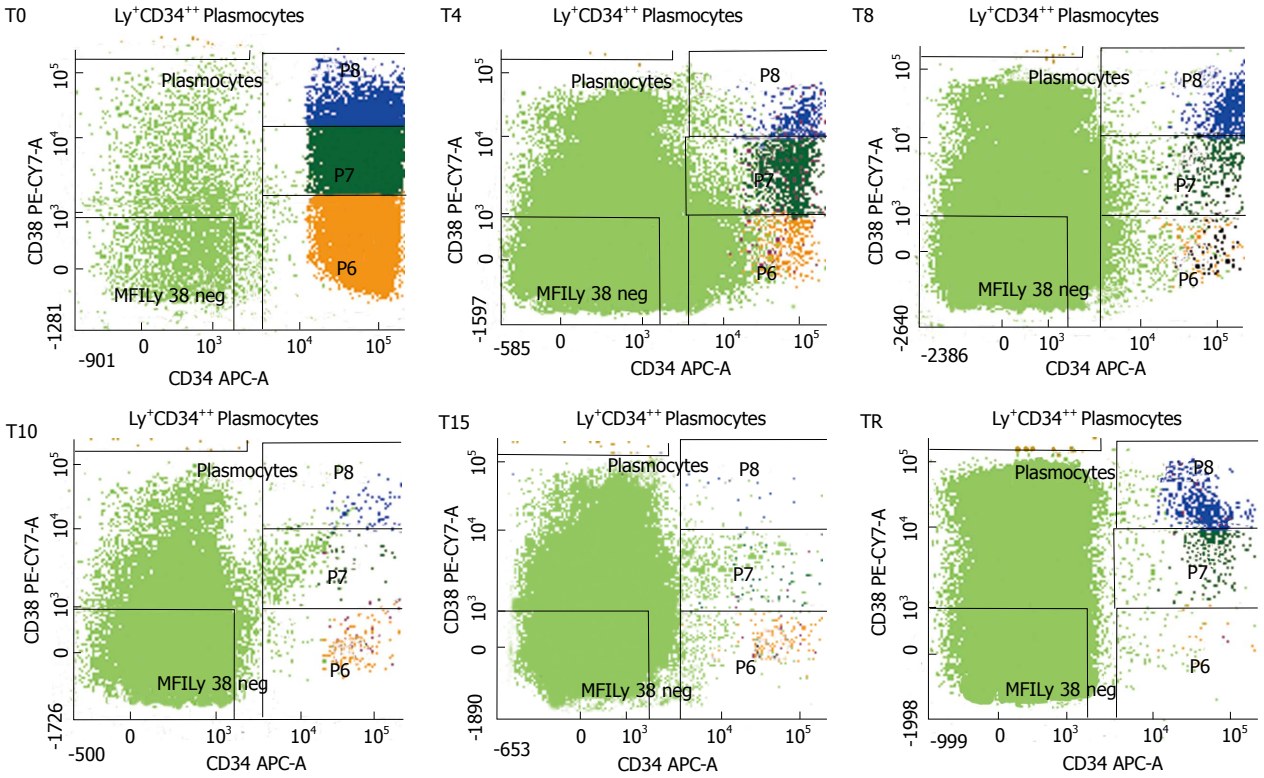

Figure 2 Characterization of the different stem cell fractions. A typical example of analysis (patient 3) performed at different times: at diagnosis (T0), at the end of the first sequence of chemotherapy (4 d) (T4), at the beginning of the second sequence of chemotherapy (8 d) (T8), at the end of chemotherapy (10 d) (T10), during aplasia (15 d) (T15), and at the time of cell recovery (TR). CD34$^+$ cells were separated into different stem cell fractions based on their CD38 antigen expression: A first cell population expressing a great amount of the CD34 antigen and lack of CD38 (CD34$^+$CD38$^-$); a second cell population characterized by a great amount of the CD34 antigen and by a low density of CD38 antigen (CD34$^+$CD38^{low}); and a third cell population characterized by a large density of CD38 antigen and of CD34 antigen (CD34$^+$CD38$^+$).

populations as well as intensity of fluorescence signal quantified as rMFICD38 from CD34$^+$ gated cells and from CD45^{low}/SSC total blast cells. The adhesion markers CXCR4 and VLA4 were also expressed as rMFI. Regarding CD47 expression, a normalized rMFI was used and defined as followed: (median expression of CD47 in blasts-median expression of IgG1 in blasts)/(median expression of CD47 in lymphocytes-median expression of IgG1 in lymphocytes).

Statistical analysis

Descriptive statistical values included median, mean ± SD and range. For most of the analyses, BM cell samples from the 8 patients were pooled as well as all PB cell samples from the same 8 patients. Statistical significance was assessed using the ANOVA test, assuming equal variance for comparison of 2 groups. Correlations between the expressions of cell surface antigens were given by the linear Pearson test.

RESULTS

Expression of CD34 during and after chemotherapy

At T0, the average proportion of CD34$^+$ cells in BM was 32.06% ± 28.96% (median, 21.47%) and 15.33% ± 23.89% (2.84%) in PB. At T0, most CD34$^+$ cells corresponded to the "bulk" leukemia cell population. The CD34$^+$ cell population decreased progressively in BM (4.6% ± 9.54%; 0.31% at T15) corresponding to the decrease in leukemic blasts after chemotherapy, and increased again lightly at the time of cell recovery (1.25% ± 0.84; 1.22%), corresponding mainly to the emergence of normal progenitor cells. In PB, a nadir of CD34$^+$ cells was observed at T15 (0.51% ± 0.87%; 0.15%), while the percentage of CD34$^+$ cells remained low at TR (0.10% ± 0.07%; 0.16%), confirming undetectable levels of immature cells in PB.

Expression of CD38 antigen on CD34$^+$ cells

Three populations of CD34$^+$ cells were distinguished on differential expression of the CD38 antigen. The CD34$^+$CD38$^-$ population appeared in a restricted light-scattering region. When considering CD34 and CD45 expressions, these cells projected initially mainly in the CD34$^+$CD45$^{low/+}$, and then appeared in the CD34$^+$CD45$^{-/low}$ area after cell mobilization. The CD34$^+$CD38^{low} cells were mainly found in the CD34$^+$CD45$^{low/+}$ area, but also involved the CD34$^+$CD45$^{-/low}$ area. With respect to light-scattering properties, the cell population indicated by CD34$^+$CD38$^+$ was more heterogeneous but projected almost exclusively in the CD34$^+$CD45$^{low/+}$ area. At T0, the smallest popula-

Table 2 Circulating CD34$^+$ cell subsets before, during and after intensive chemotherapy

Time	CD34 *vs* CD38		
	CD34$^+$CD38$^-$	CD34$^+$CD38^{low}	CD34$^+$CD38$^+$
At diagnosis (T0)			
Median	7.32%	35.45%	56.74%
Mean ± SD	15.40% ± 19.21%	32.35% ± 20.41%	53.81% ± 35.37%
At 4 d (T4)			
Median	12.82%	39.15%	37.30%
Mean ± SD	21.17% ± 22.68%	34.01% ± 23.08%	45.11% ± 37.48%
At 8 d (T8)			
Median	15.99%	26.21%	36.81%
Mean ± SD	30.14% ± 29.51%	30.28% ± 20.87%	39.06% ± 32.98%
At 10 d (T10)			
Median	41.35%	22.99%	18.90%
Mean ± SD	48.18% ± 26.79%	27.86% ± 18.65%	24.29% ± 22.73%
At 15 d (T15)			
Median	73.56%	26.18%	3.93%
Mean ± SD	66.42% ± 20.18%	27.33% ± 9.26%	8.05% ± 12.24%
At recovery (TR)			
Median	5.37%	32.76%	63.42%
Mean ± SD	10.63% ± 13.81%	29.53% ± 11.23%	61.79% ± 18.39%

TR: Time of cell recovery.

tion (CD34$^+$CD38$^-$) lacked the CD38 antigen, while the larger population (CD34$^+$CD38$^+$) expressed the greatest amount of the CD38 antigen. The average proportions of the CD34$^+$CD38$^-$, CD34$^+$CD38^{low} and CD34$^+$CD38$^+$ populations in BM and PB were 3.48% ± 5.66%, 12.62% ± 7.31%, 84.65% ± 11.91%, 15.40% ± 19.21%, 32.35% ± 20.41% and 53.81% ± 35.37%, respectively. Chemotherapy ± priming with G-CSF mobilized immature cells (CD34$^+$CD38$^-$ population), while the more mature cells expressing CD38 (CD34$^+$CD38^{low} and CD34$^+$CD38$^+$ populations) decreased progressively after treatment. At T15, the average proportions of the CD34$^+$CD38$^-$, CD34$^+$CD38^{low} and CD34$^+$CD38$^+$ cell populations in PB were 66.42% ± 20.18%, 27.33% ± 9.26%, and 8.05% ± 12.24%, respectively. In BM, the CD34$^+$CD38$^-$ and CD34$^+$CD38^{low} populations were increased at T15, while the CD34$^+$CD38$^+$ population was decreased: 22.02% ± 17.11%, 48.96% ± 16.20%, and 29.91% ± 26.69%, respectively. Proportions of CD34CD38 cell fractions returned to baseline at TR (Table 2).

Effect of priming with G-CSF on CD34$^+$ cell populations

CD34$^+$ cells in PB tended to be more sensitive to chemotherapy after priming with G-CSF (mean ± SD: 0.21% ± 0.18%; median: 0.17% at T10) than without G-CSF (5.17% ± 8.47%; 0.31%). The evolution of the three subsets of CD34$^+$ cell populations over time were confirmed when considering the absolute number of cells. The CD34$^+$CD38$^{+/low}$ cell population, containing the leukemia bulky cell population, followed the evolution of white blood cell count with a massive decrease during and after chemotherapy corresponding to cell lysis and a slight increase at the time of evaluation corresponding to cell recovery. At T15, the absolute number of the more mature cell population (CD34$^+$CD38$^+$) tended to be lower after priming with G-CSF (5.1 × 10^6/l ± 4.1) than without

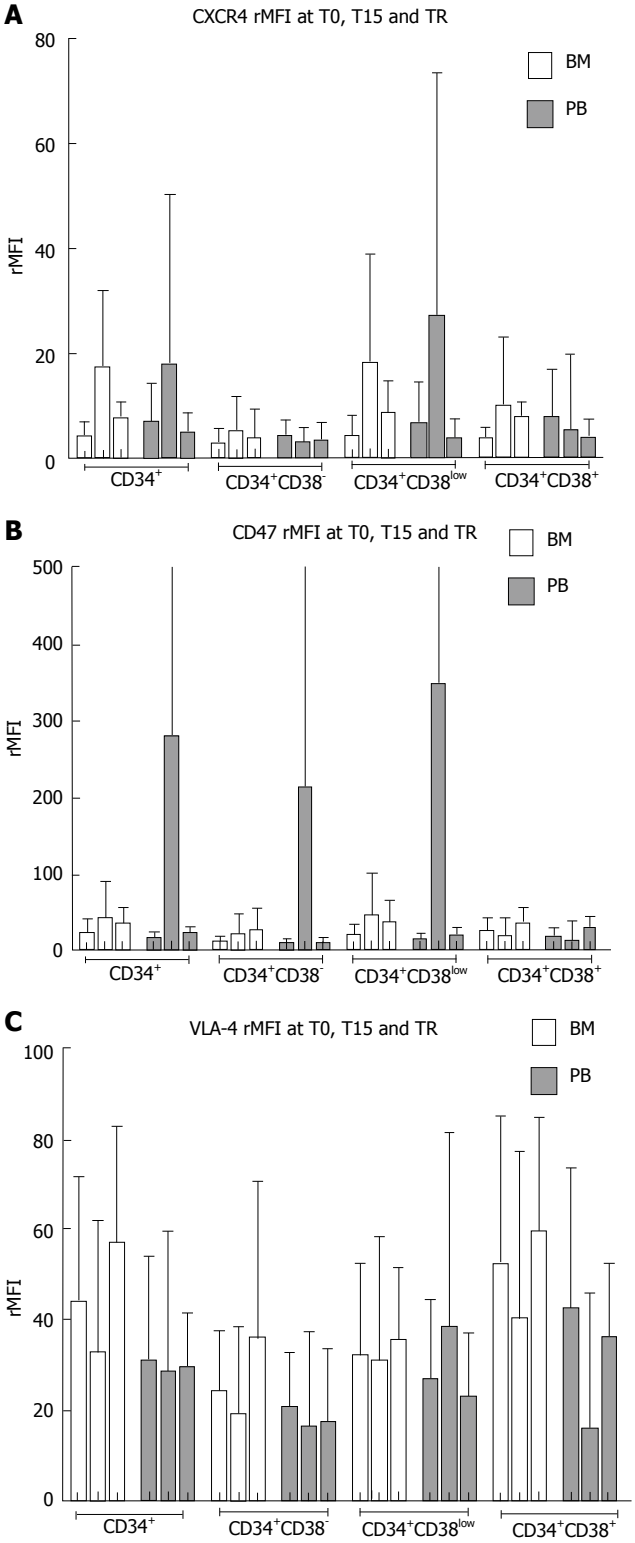

Figure 3 Evolution of CXCR4, CD47, and VLA-4 ratio median fluorescence intensity on CD34$^+$ cells and CD34+CD38 cell sub-populations in bone marrow and peripheral blood. A: CXCR4, B: CD47, C: VLA-4. PB: Peripheral blood; BM: Bone marrow; rMFI: Ratio median fluorescence intensity.

G-CSF (19.3 × 10^6/l ± 15.2), suggesting a lytic impact of priming on leukemic cells. Reversely, the most immature subset (CD34$^+$CD38$^-$) increased progressively during and after chemotherapy corresponding to mobilization

Table 3 Mean percentage of viable, apoptotic and necrotic cells in peripheral blood and bone marrow samples at T0, T15 and time of cell recovery

Cell population	T0 (BM)	T0 (PB)	T15 (BM)	T15 (PB)	TR (BM)	TR (PB)
Immature cells						
Viable cells	75.5 ± 17.6	52.0 ± 21.2	18.5 ± 3.5	14.5 ± 12.0	37.0 ± 16.9	34.0 ± 14.1
Apoptotic cells	17.5 ± 17.6	19.5 ± 21.9	33.0 ± 7.0	27.5 ± 23.3	34.5 ± 13.4	24.5 ± 7.7
Necrotic cells	3.0 ± 1.4	28.0 ± 0.0	45.0 ± 11.3	54.0 ± 38.1	25.0 ± 2.8	38.5 ± 24.7
$CD34^+$ cells						
Viable cells	77.5 ± 20.5	59.5 ± 33.2	34.0 ± 19.7	24.5 ± 3.5	65.0 ± 25.4	29.0 ± 19.7
Apoptotic cells	20.5 ± 20.5	34.5 ± 28.9	31.0 ± 29.6	38.0 ± 15.5	32.0 ± 22.6	44.0 ± 1.4
Necrotic cells	2.5 ± 0.7	5.0 ± 2.8	35.0 ± 8.4	34.0 ± 21.2	3.0 ± 2.8	24.0 ± 19.7
$CD34^+CD38^-$ population						
Viable cells	68.0 ± 19.7	59.5 ± 34.6	52.5 ± 24.7	21.5 ± 10.6	51	32.5 ± 30.4
Apoptotic cells	27.5 ± 24.7	38.0 ± 33.9	33.5 ± 23.3	60.0 ± 2.8	47	52.5 ± 16.2
Necrotic cells	0.5 ± 0.7	2.0 ± 1.4	13.5 ± 2.1	18.0 ± 14.1	1	22.5 ± 0.7
$CD34^+CD38^{low}$ population						
Viable cells	72.5 ± 26.1	48.0 ± 43.8	15.5 ± 6.3	15.0 ± 7.0	33.5 ± 2.1	30
Apoptotic cells	25.5 ± 26.1	45.0 ± 38.1	34.5 ± 28.9	38.5 ± 20.5	54.0 ± 5.6	49
Necrotic cells	2.0 ± 1.4	7.0 ± 5.6	49.5 ± 33.2	46.5 ± 27.5	12.5 ± 3.5	18
$CD34^+CD38^+$ population						
Viable cells	78.0 ± 19.7	70.5 ± 30.4	21.5 ± 7.7	27.5 ± 6.3	67.0 ± 25.4	44.5 ± 36.0
Apoptotic cells	19.0 ± 19.7	23.5 ± 27.5	26.0 ± 26.8	20.5 ± 20.5	31.0 ± 24.0	30.5 ± 0.7
Necrotic cells	2.0 ± 0.0	5.0 ± 2.8	53.0 ± 18.3	42.0 ± 43.8	1.5 ± 0.7	28.0 ± 39.5

BM: Bone marrow; PB: Peripheral blood; TR: Time of cell recovery.

with the highest absolute count for the $CD34^+CD38^-$ cell subset in PB at T15 (179.4×10^6/l ± 79.7) and decreased thereafter suggesting partial lysis, migration to BM and/or differentiation through the $CD34^+$ $CD38^+$ cell pool. When considering the $CD34^+CD38^-$ population, the increase in cell percentage between T0 and T15 was 24 fold higher after priming with G-CSF than without priming with G-CSF, suggesting a higher level of cell mobilization.

Expression of CXCR4 and adhesion molecules on $CD34^+$ cell subsets

Figure 3 summarizes the evolution of CXCR4 (Figure 3A), CD47 (Figure 3B) and VLA-4 (Figure 3C) rMFI on $CD34^+$ cells and on $CD34^+$ CD38 cell sub-populations in BM and PB during the induction course. CXCR4 and CD47 rMFI on $CD34^+$ BM and PB cells increased with chemotherapy ± priming by G-CSF, while VLA-4 remained stable. The evolution of CXCR4 and CD47 expression was correlated in both BM ($r = 0.64$) and PB ($r = 0.55$). From T0 to T15, CD47 expression increased 10 fold more on PB $CD34^+$ cells than on BM cells with a mean expression of 280.2 *vs* 43.6, while increased expression of CXCR4 was similar. When considering $CD34^+$ cell subsets according to CD38 expression, CD47 significantly increased at T15 mainly in PB for the $CD34^+CD38^-$ and $CD34^+CD38^{low}$ populations, while mean rMFI did not change for the $CD34^+CD38^+$ population. CXCR4 significantly increased at T15 in both PB and BM mainly for the $CD34^+CD38^{low}$ cell population, while mean rMFI did not change for the $CD34^+CD38^-$ and $CD34^+CD38^+$ populations.

Identification of apoptotic and necrotic cells

The fluorescence parameters allowed characterization of necrotic (PI^+ annexin V-$FITC^+$ cells), apoptotic (PI^- annexin V-$FITC^+$ cells) and viable cells (PI^- annexin V-$FITC^-$ cells) (Table 3). The evolution over time of mean proportions of apoptotic and necrotic cells was similar in BM and PB for all cell subsets: a decrease of viable cells was noted after chemotherapy, while a massive increase in apoptotic and necrotic cell populations was observed (Figure 4). However, the percentage of cells with apoptotic/necrotic status tended to be higher in PB. At T15, the percentage of necrotic cells in both PB and BM was higher in the more mature cell populations ($CD34^+CD38^{low}$and $CD34^+CD38^+$) than in the immature $CD34^+CD38^-$ population. However, survival tended to be promoted by G-CSF, as indicated by a decrease of annexin V-$FITC^+$ cells in patients primed with G-CSF. The percentage of apoptotic cells did not differ among patients primed with G-CSF and those not primed: 51% ± 21% *vs* 48% ± 21% in the whole $CD34^+$ cell population. Although not statistically significant, the percentage of necrotic cells tended to be lower in patients primed with G-CSF: 12% ± 6% *vs* 27% ± 21% in the whole $CD34^+$ cell population ($P = 0.2$).

DISCUSSION

The pursuit of the best chemotherapy regimen for AML continues in an attempt to improve CR proportions and long-term survival. The LSC model has implications for the development of new therapeutic strategies. LSCs respond to depletion of the leukemia cell mass that occurs when anti-proliferative drugs are administered. It is therefore suggested that one way to eliminate dormant LSCs is to find the window in which they cycle and then kill them[17]. Priming may modulate cell cycle kinetics of AML blasts and render them more susceptible to phase-specific agents. This has been shown *in vitro*[18] and *in vivo*

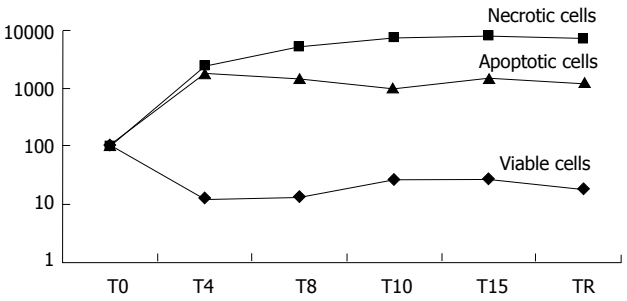

Figure 4 Kinetics of viable, apoptotic and necrotic cells in peripheral blood according to the different times of study (patient 8). Values were given comparatively to those observed at the time of diagnosis, assuming that the observed value at T0 corresponded to 100%.

in leukemic mice[19], and more recently in clinical trials for younger adults with AML[6,7].

Our study showed that CD34$^+$ cells were significantly affected by chemotherapy in both BM and PB. However, treatment mainly impacted on the more mature cell populations, as demonstrated by the decrease of the CD34$^+$CD38$^{+/low}$ cell population corresponding to "bulk" leukemic blasts. LSCs represent only a small fraction of malignant cells[20] and are believed to be restricted to the Lin$^-$CD34$^+$CD38$^-$ fraction[21,22], which was insignificant at T0 in most of our patients. However, this cell subset was able to proliferate and to egress from BM to PB after chemotherapy ± G-CSF, and partly differentiates through the more mature CD34$^+$CD38$^{+/low}$ or CD34$^+$CD38$^+$ cell phenotypes, before returning to its initial value after leukocyte recovery. However, LSCs can arise from the malignant transformation of a normal stem cell that has accumulated oncogenic insults over time, or from a more differentiated cell that develops the capability for continual self-renewal[23]. The phenotype of LSCs is therefore heterogeneous and can vary even within a single sample. Some AML have LSCs exclusively in the CD34$^-$ fraction[24]. Similarly, CD34$^+$CD38$^+$ fraction of certain AML samples contains all or at least most LSCs[25]. Furthermore, CD38 is reversibly expressed on CD34$^+$ repopulating cells between negative and low levels[26]. Characteristics that are relevant to therapy may then differ based on the origin of the malignant cell with sensitive AMLs derived from more differentiated stem cells and resistant AMLs derived from earlier stages.

Resistance may in part be provoked by cell adherence to the stromal environment. Inhibition of the CXCR4-SDF-1 axis induces mobilization of cells into circulation and enhances anti-leukemic effects of chemotherapy[27]. CXCR4 plays a dominant role in cell-trafficking, as confirmed here by up-regulation after CD34$^+$ cells egress to the circulation. This was observed for all PB CD34$^+$ cells. However, the more mature CD34$^+$ cells mobilized earlier than immature CD34$^+$ cells, suggesting different degrees of sensitivity. CXCR4 expression favors the enrichment of a non-cycling population of AML cells, which represent dormant leukemia progenitors serving as a reservoir for minimal residual disease. G-CSF results in a decreased expression of SDF-1 in the BM, resulting in premature release of immature cells[9]. This was in accordance with our results showing an increased expression of CXCR4 on immature CD34$^+$ cells after reaching the PB circulation. The peak number of labeled cells was shown after 72 h preventing an early homing back to the BM[28]. CXCR4 may contribute to immature cell clearance from the blood observed after T15 by directing cells again to the BM.

Many other proteins, including the VLA-4 integrins, are regarded as essential for AML cell adhesion to stromal cells and their protection from drug-induced apoptosis[29], but only slight variations in their expression level were noted during and after therapy, suggesting a minor involvement in immature CD34$^+$ cell trafficking.

Mobilizing treatments cause CD47 to be transiently up-regulated on progenitors just prior to and during their migratory phase[30]. This was confirmed by our study, showing an increased expression of CD47 most particularly in the circulating CD34$^+$CD38$^-$ cell subgroup after chemotherapy ± G-CSF. Overexpression of CD47 on AML cells is known to increase their pathogenicity by allowing them to evade macrophage phagocytosis[31,32]. This could explain the strong correlation found between the evolution of CD47 expression and that of CXCR4 on immature CD34$^+$ cells.

Circulating CD34$^+$ cells tended to be more sensitive to chemotherapy after priming with G-CSF. However, the impact of priming mainly concerned the more mature CD34$^+$ cells containing "bulk" leukemic cells, while it was limited on immature CD34$^+$ cells susceptible to contain leukemia-initiating cells. All CD34$^+$ annexin V$^+$ cell populations were involved in the apoptotic/necrotic process, which concerned a larger proportion of PB cells than BM cells. Cell survival seemed to be promoted by G-CSF, as indicated by a decrease in annexin V$^+$ cells. At T15, the necrotic process involved preferentially the more mature cell subsets (CD34$^+$CD38^{low} and CD34$^+$CD38$^+$ populations), suggesting a higher protection of CD34$^+$CD38$^-$ cells from the action of cytotoxic drugs.

Despite disappointing results regarding priming of the most immature CD34$^+$ cells, our study represents a first step towards further explorations. Mobilization of LSCs is a concept that is presently being revisited by novel targeted therapies. Inhibition of CXCR4, based on the ability of the CXCR4 antagonist plerixafor to push LSCs out of their BM niches, induces the rapid mobilization of stem cells from BM to PB[33], and has been shown to sensitize leukemic blasts to chemotherapy[27,34-36]. A mobilization of up to 80% leukemic cells, including the more primitive CD34$^+$CD38$^-$ cell subset, was observed when HGF was followed by anti-CXCR4[37]. Accompanied by chemotherapy, this might lead to a better eradication of LSCs. From that perspective, plerixafor is currently being tested by French centers for the mobilization of dormant LSCs. Consequently, new paradigms must be devised for evaluating the therapeutic agents. For instance, clinical trial design can use intermediate end points such as time

to progression following the administration of an agent that can target LSCs.

COMMENTS

Background

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder that originates from leukemia stem cells (LSCs) with the ability to generate an excessive amount of malignant myeloid blasts. Interactions of the leukemic cells with the bone marrow (BM) microenvironment via specific receptor and adhesion molecules, such as the CXC chemokine receptor 4 (CXCR4)-stromal cell-derived factor-1 (SDF-1) axis, are in part responsible for chemotherapy resistance. AMLs with high CXCR4 cell surface expression and therefore a high tendency of stromal protection have been shown to have a poor prognosis.

Research frontiers

The cell cycle is a critical regulator of the processes of cell proliferation and growth. One strategy to increase the cytotoxicity of cycle-dependent antileukemic agents is to enter more leukemic cells into cell cycle. Timed sequential chemotherapy is based on the findings that the initial cytoreductive drug induces the remaining malignant cell cohort to enter a proliferative state at a predictable time following drug administration. Combination with hematopoietic growth factors (HGFs) has also been developed to enhance the efficacy of cytotoxic agents.

Innovations and breakthroughs

The study represents a first step towards further explorations. Mobilization of LSCs is a concept that is presently being revisited by novel targeted therapies. Inhibition of CXCR4, based on the ability of the CXCR4 antagonist plerixafor to push LSCs out of their BM niches, induces the rapid mobilization of stem cells from BM to PB and has been shown to sensitize leukemic blasts to chemotherapy. A mobilization of up to 80% leukemic cells, including the more primitive $CD34^+CD38^-$ cell subset, was observed when HGF was followed by anti-CXCR4. Accompanied by chemotherapy, this might lead to a better eradication of LSCs. From that perspective, plerixafor is currently being tested by French centers for the mobilization of dormant LSCs. Consequently, new paradigms must be devised for evaluating the therapeutic agents. For instance, clinical trial design can use intermediate end points such as time to progression following the administration of an agent that can target LSCs.

Applications

The study was to evaluate quantitatively and qualitatively the different $CD34^+$ cell subsets after priming by chemotherapy and granulocyte colony-stimulating factor (± G-CSF) in patients with acute myeloid leukemia.

Peer review

The authors studied the mobilization of $CD34^+$ bone marrow stem cells into the circulation in AML patients. The results show that $CD34^+$ cells are more sensitive to chemotherapy after priming with GM-CSF associated with an increase in CXCR4 and CD47 expression.

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