review
Haematologica 1996; 81:77-92

PERIPHERAL BLOOD STEM CELLS IN ACUTE MYELOID LEUKEMIA: BIOLOGY AND CLINICAL APPLICATIONS

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Clinical application of circulating stem cells for autologous transplantation is steadily expanding.[1] It has become increasingly clear that mobilized peripheral blood progenitor cells (PBSC) induce faster hematopoietic recovery, fewer febrile days, lower transfusion requirement and shorter hospitalization than bone marrow (BM)-derived cells.[2,3] More recently, rapid and sustained engraftment has also been reported using granulocyte colony-stimulating factor (G-CSF)-mobilized allogeneic PBSC following myeloablative therapy.[4]

In contrast to solid tumors and many hematological malignancies, PBSC transplantation is not widely used for acute myeloblastic leukemia (AML) patients. In this setting there are still unanswered questions such as the role of autologous stem cell transplantation in post-remission therapy, as well as major issues concerning PBSC mobilization and collection: the expression of CD34 antigen on leukemic stem cells as compared to their normal counterparts, the biologic significance of $CD34^+$ AML, the response of leukemic cells to CSFs used to optimize PBSC harvest, the potential contamination of PBSC grafts by residual AML cells and the role of *ex-vivo* purging of leukemic cells.

This review analyzes the most recent advances in this field, addressing clinical and biological issues relevant to the use of autologous PBSC for AML patients.

Growth factor receptor expression and response of leukemic cells to human CSFs

The CD34 antigen is a 105-120 KD glycoprotein expressed on the cell surface of hematopoietic progenitors and stem cells, but it is not expressed on late hematopoietic cells or on many tumor cells.[1] $CD34^+$ cells are responsible for the self-renewal and the expansion of the large majority of AML. It has recently been shown that most of the clonogenic cells in AML derive from the $CD34^+$ cell fraction as opposed to $CD34^-$ cells.[5,6] Moreover, $CD34^+$ cells co-expressing differentiation markers (CD33, CD38) have a reduced proliferative potential since *in vitro* they give rise to small colonies unable to originate secondary clones. This phenomenon is likely the expression of a limited self-renewal potential.[6] Lapidot *et al.* provided the most convincing evidence of the stem cell role of $CD34^+$ cells in AML by showing that only the $CD34^+/CD38^-$ cell fraction was capable of generating acute leukemia when transplanted into SCID mice.[7]

These observations indicate the relevance of defining the growth and receptor expression pattern of leukemic $CD34^+$ cells, their response to CSFs as well as their kinetic status compared to their normal counterparts. Among the different cytokines involved in the regulation of hemopoiesis, a key role in the pathogenesis of the leukemic growth is probably played by stem cell factor (SCF), interleukin 3 (IL-3), granulocyte-macrophage CSF (GM-CSF) and G-CSF.[8-12]

SCF receptor (c-kit) is expressed by the vast majority of AML.[8,9] Both high and low affinity receptors have been demonstrated (Table 1). *C-kit* shares structural similarities with the receptors for M-CSF and PDGF. A linear correlation between the percentage of $CD34^+$ cells and *c-kit* expression has been documented, thus indicat-

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Ackowledgements: preparation of this manuscript was supported by a grant from Dompé Biotec SpA and Amgen Italia SpA, Milano.
Received September 22, 1995; accepted November 27, 1995.

ing that CD34+ AML express high levels of *c-kit*. In adult patients, the presence of a high number of CD34+ cells has been shown to correlate with a bad prognosis.

C-kit activation plays a foundamental role in the regulation of the early phases of CD34+ cell stimulation. The interaction of SCF with its ligand exerts a modest proliferative stimulus on immature quiescent cells and up-regulates the expression of receptors for other growth factors. While in normal hematopoiesis this triggers myeloid differentiation, in AML it may activate self-renewal and expansion of the leukemic population.[11-14]

High affinity receptors for GM-CSF and IL-3 (Table 1) are expressed by nearly all AML, irrespective of the FAB subtype.[15,16] IL-3, GM-CSF (and IL-5) receptors consist of an α subunit (ligand specific) and a shared β subunit. While the α subunit has a low affinity for the ligand and alone is incapable of transducing the signal, the association of the two subunits gives rise to a functioning high affinity receptor which is a type I receptor devoid of endogenous tyrosine-kinase activity. β chain activation induces several tyrosine-kinases like Fyn, Lyn, Fps, Jaks, which transduce a signal common to IL-3 and GM-CSF,[17] whereas α subunit activation induces ligand specific pathways. In the majority of AML cases IL-3 and GM-CSF induce the proliferation of CD34+, although to variable extents. Correlations have been observed between responses to different factors but no significant additive effects have been noted.

Exposure of leukemic cells to GM-CSF or IL-3 *in vitro* can give rise to a generation of mature cells. However, the persistence of blast cells capable of secondary leukemic colony formation indicates that the differentiation potential of IL-3 and GM-CSF is negligible and that they are unable to abolish the self-renewal of the leukemic population.[18-20] SCF synergizes with IL-3 and GM-CSF in inducing large clones primarily composed of undifferentiated cells.

Table 1. High affinity receptors for hematopoietic growth factors in AML.

	Affinity, kd (range)	*No. of receptors per cell*	*Reference*
SCF	16-158 pmol/L	200-8000	14
G-CSF	36-130 pmol/L	55-1200	16
GM-CSF	64-404 pmol/L	40-1263	15,16
IL-3	26-467 pmol/L	21-145	15,16

G-CSF receptor is expressed by nearly all AML (Table 1).[16,21] However, M2 and M3 AML appear to express the highest number of receptors. *In vitro* growth stimulation is not consistent except for M2 and M3 AML; G-CSF action is additive or synergic with that of IL-3, SCF and, to a lesser extent, with that of GM-CSF. G-CSF also induces some degree of differentiation of leukemic CD34+ cells, and the presence of the growth factor affects the formation of secondary colonies. In addition, CSF treatment seems to prevent cell death in AML.[22]

The *in vivo* use of growth factors in AML patients derives from contrasting hypotheses:

a) *use of growth factors before and during cytostatic treatment to induce the proliferation of quiescent leukemic progenitors.* The increased proliferative rate and, possibly, the intracellular accumulation of some cytotoxic drugs (i.e. Ara-CTP) should increase the fraction of cells killed.[23,24] This approach has never been tested in randomized trials specifically addressing this issue. It seems, however, to be of modest value with G-CSF or GM-CSF.[25,26] It remains to be seen if this approach would be more useful with molecules such as SCF that are particularly active on leukemic CD34 cells;

b) *use of growth factors as differentiating agents with the aim of exhausting the self-renewal potential of the leukemic progenitors.* On the basis of *in vitro* and preliminary (although still to be confirmed) *in vivo* data, G-CSF seems the most promising molecule;[27]

c) *use of growth factors for accelerating the recovery of residual normal progenitors after induction chemotherapy*. This approach has been pursued with G-CSF and GM-CSF in AML patients > 60 years of age, for whom the pancytopenia following cytotoxic treatment is particularly profound and long-lasting and

carries a relevant risk of life-threatening infections. In this setting, both G-CSF and GM-CSF given after induction chemotherapy reduce the duration of neutropenia without affecting the rate of severe infections.[28,29] Moreover, G-CSF, but not GM-CSF, appears to increase the complete remission rate. Both cytokines, however, have no impact on the survival rate. Of interest, no evidence of accelerated growth of residual leukemic cells has been observed.

All these data demonstrate how controversial the use of hemopoietic growth factors in the treatment of AML is, although the most recent results suggest the safety of G-CSF administration following induction-consolidation treatment.[29]

Stem cell kinetics in AML

The hematopoietic cell renewal process is supported by a small population of bone marrow cells termed hematopoietic stem cells. They are defined as cells capable of long-term hematopoietic reconstitution and differentiation into multiple hematopoietic lineages. It is generally held that, in the steady state, the majority of normal stem cells are dormant in the cell cycle and only a few of them supply all the hematopoietic cells at a given time. More than thirty years ago, stem cell kinetic studies[30] proposed the concept of a true resting state and coined the term G0 as the state from which stem cells randomly move to the active cell cycle.

Subsequent studies[31] confirmed Lajtha's observations by showing that brief *in vitro* exposure of bone marrow cells to highly specific radioactive thymidine does not reduce the number of multipotential progenitors. As shown in Figure 1, most normal bone marrow $CD34^+$ progenitor cells are indeed quiescent in G0. Culture of enriched human progenitors documented that they remain as single cells for as long as 2 weeks in culture and begin proliferation upon stimulation with combinations of cytokines.[32] Based on mathematical studies, stem cell function was seen as a model in which the decision to self-renew and differentiate followed a stochastic process.[33] By replating individual blast cell colonies, Till and coworkers showed that the production of secondary blast cell colonies is a self-renewal process and that the generation of secondary multilineage colonies is differentiation. Thus the self-renewal process is associated with renewed dormancy in the cell cycle while the differentiation process is characterized by continuous cell doubling.

Similar work was performed on leukemic stem cells by several authors and two fundamental antithetical models were proposed, based on the presence of quiescent progenitor cells in human leukemia.

It was postulated that leukemic progenitors were predominantly involved in rapid cell cycling as judged by measuring the proportion of S-phase cells using H^3-TdR or hydroxyurea.[34] However, this was in contrast with previous observations obtained *in vivo* by continuous infusions of ^{3}H-thymidine for 8-10 days.[35] In those experiments, 88 to 93% of the leukemic cells were labeled at the end of infusion, whereas almost all the smallest leukemic cells were not, suggesting that they were in an extended G0. Using *in vivo* pulse labeling with tritiated thymidine in AML patients, it was further shown that blasts with a high proliferative rate do not behave as a pool of normal self-main-

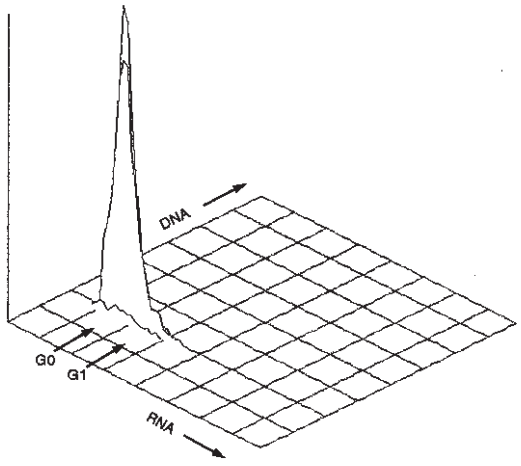

Figure 1. DNA/RNA cellular content (acridine orange) of normal enriched $CD34^+$ cells. Cell cycle measurements confirms that the majority of progenitors are quiescent (G0) with only few going into cycle (G1).

taining cells, but rather as a normal multiplication-maturation compartment.[36] Data obtained with different techniques (labeling and cell culture methods) and the heterogeneity of study cell populations may represent the reason for these discordant results. The hypothesis that AML progenitors are characterized by a substantial number of nonproliferating or very slowly proliferating blast cells (lower RNA content)[37,38] was the rationale for different approaches to AML treatment. For instance, the combined use of cytokines and chemotherapy to recruit quiescent cells into the cell cycle, enhancing the cytotoxicity of cycle-specific agents.[39,40]

Raymakers *et al.*[41] have studied the proliferative capacity of the bone marrow fraction double stained for CD34 and CD33 in AML patients. The cloning efficacy was highly variable in different AML samples, with predominant cluster growth. Cluster and colony growth was similar between $CD34^-/CD33^+$ and $CD34^+/CD33^+$, in contrast to what is observed in normal bone marrow. The most primitive $CD34^+/CD33^-$ fraction was found in highly proliferative colony growth. When this analysis was extended to AML with a more mature phenotype (small fraction of $CD34^+/33^-$), the highly proliferative colonies deriving from the $CD34^+/33^-$ fraction were found to be disomic by *in situ* hybridization in all patients who were characterized by chromosomal abnormalities. Nevertheless, the authors could not exclude the presence of leukemic stem cells kinetically characterized by low or no proliferation under their experimental culture conditions.

A further study[42] aimed at evaluating the specific activity of SCF on enriched $CD34^+$ in suspension culture by measuring Ki67 expression and flow cytometric DNA content showed no difference in cell cycle distribution among progenitors obtained from normal bone marrow, umbilical cord blood and chronic myeloid leukemia $CD34^+$ peripheral blood stem cells.

Further investigations on the role of a family of proteins recently identified as cell cycle regulators, such as cyclin A, B, D, E and of their catalytic subunits, the cyclin-dependent kinases cdk2, cdk4, cdk6 and cdc2, may help to identify kinetic features and fine differences between normal and leukemic hemopoietic stem cells, as well as events involved in neoplastic transformation.[43]

In conclusion, the kinetic characteristics of leukemic stem cells have still not been defined, mainly because different experimental conditions allow evaluation of progenitors with different degrees of maturation and therefore with different proliferative characteristics. The heterogeneity among different leukemia subtypes should also be taken into account.

Expression of CD34 antigen in AML and $CD34^+$ leukemias: clinical and biological significance

Based on current information, there is no doubt that a substantial number of acute leukemias express the CD34 antigen on the cell membrane of blast cells. However, the incidence of such expression in AML has been found to be highly variable (25-64% of the patients examined), depending on a number of factors, as shown in Table 2.

The variability in the reported incidence of $CD34^+$ AML has also influenced the prognostic relevance of CD34 expression in AML. Most authors found a clear association between $CD34^+$ AML and a lower incidence of complete remission following induction therapy. In addition, the relapse rate was higher in AML showing positivity for the CD34 antigen compared to that of the $CD34^-$ group.[44-53] However, other authors did not confirm these results and found no significant difference in the complete remission rate or overall survival of $CD34^+$ and $CD34^-$ AML patients.[54-60]

Expression of the CD34 antigen in AML and its association with different survival rates could be due to a number of factors. First of all, the cutoff point for CD34 positivity that should be used to decided whether an AML sample is carrying this antigen. Since the proportion of $CD34^+$ cells is around 1% of normal bone marrow mononuclear cells and 0.01-0.1% of peripheral blood leukocytes, many authors have considered 5% as the optimal cutoff level for classifying $CD34^+$ AML. Nowadays, most authors agree that the cutoff point for CD34

should be 20% in order to avoid misinterpretation of the data coming from surface marker analysis. However, there is no scientific basis for considering a sample with 20% positive cells as positive, while another specimen with 19% positivity as negative, since the level of CD34 expression in a substantial number of patients is characterized by a continuous spectrum.[53] Furthermore, it must be kept in mind that the choice of a cutoff level of 5% could give rise to erroneous results, since it can be influenced by the methods used to detect antigen expression, which are characterized by different levels of sensitivity and specificity. Indirect immunofluorescence staining is more sensitive, although less specific, while the opposite is true for the direct technique. As far as the instrumentation is concerned, it must be underlined that modern flow cytometers are highly sensitive in detecting surface marker positivity with respect to microscope analysis and immunoenzymatic techniques such as APAAP, PAP, etc. In addition, whenever possible immunophenotype analysis should be preferentially performed on fresh, not cryopreserved bone marrow samples, and if this is not possible the number of blasts present in the specimen analyzed should be carefully evaluated.[61-65]

A recent report showed that CD34 antigen expression in AML samples having a marked heterogeneity in cell size was found preferentially on small leukemic cells with little or no side scatter. This feature was also associated with shorter remission duration and survival, suggesting that this morphological heterogeneity could reflect a peculiar biological behavior of AML.[66,67]

Moreover, discrimination of blast cells from residual normal nucleated cells is less likely to be obtained in AML cells by looking at light scattering properties (forward and side scatter) and expression of the CD45 antigen. For this reason, a multiparametric approach using two-three-colour analysis is strongly recommended in order to define the predominant leukemic population as well as minor pathological clones or subclones. In addition, CD34 positivity has to be evaluated solely on the blast population in order to avoid misinterpretation of the data. In fact, the percentage of blasts could vary from 30% to 99% in the bone marrow, and from 1 to 99% in the peripheral blood.

Another point which deserves careful discussion is represented by the level of expression for CD34 in AML. In normal hemopoiesis, the CD34 antigen is expressed on virtually all colony forming cells (CFU) and lymphocyte progenitors of either T or B lineage. However, within the progenitor cell compartment the degree of positivity for CD34 decreases with cell differentiation (maximum for multipotent cells and minimum for unipotent cells), and disappears in morphologically identifiable bone marrow precursors. Studies performed at the *V International Workshop on Leukocyte Differentiation Antigens* (Boston, 1993) recognized three main subsets of $CD34^+$ normal bone marrow cells with CD34 antigen densities: low (2,000-5,000 binding sites per cell-ABC), medium (10,000-20,000 ABC), high intensities (25,000- 40,000 ABC). This heterogeneity in CD34 antigen expression in normal progenitors makes it difficult to use this molecule for the monitoring of minimal residual disease

Table 2. Possible explanations for the differences reported in the literature concerning the incidence of $CD34^+$ AML.

1. Cutoff levels for the discrimination of positive and negative cases
2. Detection systems employed (flow cytometry, type of flow cytometer, immunoenzymatic technique-APAAP, immunogold, PAP-immunofluorescence microscope)
3. Specimen analyzed (bone marrow, peripheral blood)
4. Percentage of leukemic cells present in the sample examined
5. Use of cryopreserved rather than fresh cells
6. Use of different CD34 antibodies recognizing distinct CD34 epitopes
7. Percentage value and level of intensity for CD34
8. Light scattering properties of CD34+ cells
9. Patients analyzed (de novo AML or secondary AML)
10. Biologic characteristics of AML cells (chromosome aberrations, gene abnormalities, immunophenotypic profile of CD34+ AML blasts)
11. Type of chemotherapy regimen employed

(MRD) in AML patients treated with chemotherapy and/or bone marrow transplantation.[68] Flow cytometry allows the recognition of a subset of CD34+ AML characterized by bright expression for CD34 (> 50,000 ABC), which could therefore be easily recognized even when present in a very low percentage (< 0.1% of nucleated cells). This subset represents about 20-30% of CD34+ AML, so the remaining AML patients should be checked for MRD by using alternative ways (strategical double or triple staining: CD34/CD56; CD34/CD65/TdT; cytogenetics, molecular biology, etc.).[68]

Another source of variability in detecting CD34+ AML is represented by the type of CD34 monoclonal antibody used for immunophenotypic analysis. It has been demonstrated that at least three distinct CD34 epitopes exist, based on their differential sensitivity to enzymatic cleavage (using neuroaminidase, chymopapain and glycoprotease), Western blotting analysis, cell reactivity studies, and cross blocking experiments.[1,69-75] So far at least twenty-two CD34 monoclonal antibodies (McAbs) have been shown to recognize the CD34 molecule, the most direct evidence being reactivity with cells transfected with CD34 cDNA and binding to CD34 glycoprotein.[72] Recently, it has been reported that a number of AML cases are positive for some CD34 McAbs and negative for others (especially if they belong to a different epitope class), confirming the necessity of using the same CD34 McAbs in order to achieve comparable results between different centres.[71,72,74]

Another point which needs to be considered when evaluating the incidence of CD34+ AML is represented by patient characteristics at diagnosis. The number of CD34+ cases is higher in secondary than in newly diagnosed AML. If we consider that both the biology and the clinical pattern of secondary AML are quite different from those of *de novo* AML, one may argue that including of both types of leukemias in a clinical setting could interfere significantly with the prognostic relevance of CD34 expression on AML blast cells. In this context, the type of treatment utilized by various authors (chemotherapy regimen, allogeneic and autologous bone marrow transplantation), which can influence the outcome of the disease, is also of some relevance.

CD34+ AML are characterized by a higher incidence of chromosomes abnormalities involving chromosomes 5 (–5, 5q–), 7 (–7.7q–), and to a lesser extent chromosomes 16 (16q), 17 (17p), 11 (trisomy 11), or multiple chromosomes at the same time, generating the so-called major karyotypic abnormalities.[44,46,48,52,57,76] Recent studies have found a close relationship between CD34 expression in AML and previous exposure to chemotherapy, radiotherapy and/or pesticides.[47] CD34+ AML are also associated with trilineage myelodysplasia, dysgranulopoiesis, and/or abnormalities of the p53 tumor suppressor gene.[77]

The correlation between CD34+ AML and FAB subtypes is illustrated in Table 3. On the other hand, most biphenotypic acute leukemias (BAL) show positivity for the CD34 antigen.

Finally, the antigenic profile of CD34+ AML is rather heterogeneous, depending essentially on the morphological subtype and to a lesser extent on the differentiation stage of the leukemic clone. The large majority of CD34+ AML coexpress a number of antigens which are not associated with cell commitment, such as HLA-DR, CD38, CD45RO, CD45RA, CD71, and IL3 receptor. In addition, some surface and cytoplasmic glycoproteins expressed by committed *myeloid* cells were found to be positive in CD34+ AML, i.e. CD33, CD13, CD117 (stem cell factor receptor), CDw116 (GM-CSF receptor), G-CSF receptor, myeloperoxidase, lysozyme[78-82] (Figures 2 and 3). The stem cell- associated antigen Thy-1 (CD90) is negative in this AML subtype, while nuclear TdT is sometimes positive. CD34+ cells also express high levels of P-glycoprotein, which is the product of the multiple drug resistance (MDR) gene.

Post-remission therapy of acute myeloid leukemia and potential role of autologous stem cell transplantation

About two thirds of previously untreated adults with primary acute myeloid leukemia enter complete remission (CR) after induction therapy based on cytarabine and an anthracy-

Table 3. Clinical and biological characteristics of CD34+ AML.

Incidence: 30-50% (*de novo* AML); 50-70% (secondary AML)
History: previous exposure to chemo-radiotherapy or pesticides
Correlation with FAB subtypes: M0,M1,M5 (70-90%); M2,M4 (20-60%); M3: 1-5%; M6, M7: 20-50%
Prognosis: poor (mean survival rates less than 12-18 months)
Cellular density for CD34: variable from case to case (range: 3,000-100,000 per blast cell)
Immunophenotypic profile: in most cases CD45+, HLA-DR+, CD38+, CD33+, CD117+, Thy1–
Chromosome abnormalities: -5, -7, 5q-, 7q-, 16q, 17p, major karyotype aberrations
Therapy: to be defined (more aggressive chemotherapy regimens?)

cline.[83] However, long-term disease-free survival occurs in a minority of cases since most subjects relapse from proliferation of occult residual leukemic cells. Following conventional consolidation treatment less than 25% of patients remain in complete remission at four years.[84,85]

In order to eradicate residual AML cells and improve disease-free survival, three approaches have been employed in the last ten years: (a) intensive postremission chemotherapy; (b) allogeneic bone marrow transplantation (BMT), and (c) myeloablative conditioning regimens followed by autologous BMT as supportive therapy.

Intensive postremission chemotherapy is essentially based on the use of high-dose cytarabine (1 to 3 g/sqm × 6 to 12 doses), either alone or in combination with other agents. Results of uncontrolled studies performed in the late '80s and early '90s (reviewed by Cassileth *et al.*[85]) indicated that intensive postremission therapy achieves long-term disease-free survival in 25-30% of patients in first CR. Mayer *et al.*[83]) recently reported a prospective study aimed at evaluating the effect of the intensity of postremission chemotherapy on survival of leukemic patients. Acute leukemia individuals in first CR were randomly treated with four courses of cytarabine at one of three doses: 100 mg/sqm

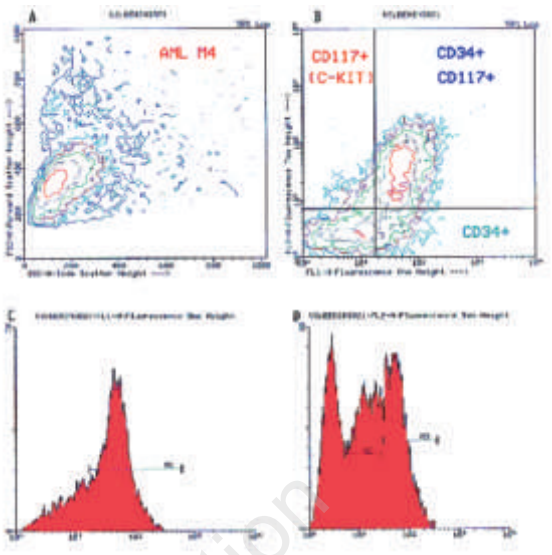

Figure 2. Dual color fluorescence analysis with a flow cytometer (CD34/FITC; c-kit-CD117-PE) in a patient with AML FAB M4. Bone marrow sample shows a blast percentage of 84%.
A) Light scattering properties of blast cells (forward scatter= cell volume; side scatter= cell granularity).
B) Contour plot diagram showing 83% of CD34+ cells, 71% of *c-kit*+ cells, and 68.5% of cells co-expressing CD34 and *c-kit*.
C) Histogram distribution of cells stained for CD34 monoclonal antibody.
D) Histogram distribution of cells labelled with CD117 monoclonal antibody.

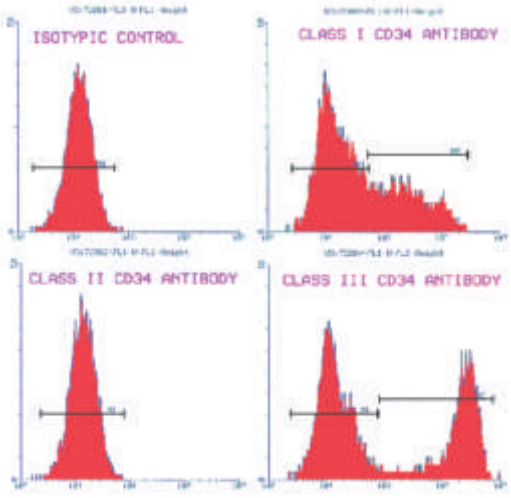

Figure 3. Discordant reactivity between different epitope class antibodies is shown in a patient with AML M1 (% blasts = 90%); 36.5% of blasts are positive for class I, 0.1% for class II, and 47% for class III. The intensity of fluorescence varied significantly from antibody to antibody even within the same epitope class.

per day for 5 days by continuous infusion; 400 mg/sqm per day for 5 days by continuous infusion, or 3 g/sqm in a 3-hour infusion every 12 hours on days 1, 3 and 5. In patients 60 years of age or younger the probability of remaining disease-free after four years correlated with the postremission cytarabine dose: 24% for the 100-mg group, 29% for the 400-mg group, and 44% for the 3-g group ($p = 0.002$). In patients older than 60 the probability of remaining disease-free after four years was 16% or less in each of the three postremission cytarabine groups, with no significant difference between groups. It should be noted that a significant proportion of AML patients achieving CR cannot proceed to intensive chemotherapy due to persistent bone marrow aplasia.

Allogeneic bone marrow transplantation offers many advantages, including the graft-versus-leukemia effect; however, the availability of a histocompatible sibling donor is restricted to approximately 25% of potential candidates. Published studies report disease-free survival rates at four years ranging from 45 to 58%.[86-88] These figures should be considered with caution since they are biased by the exclusion of patients who relapsed before allogeneic BMT. The new approach recently described by Aversa *et al.*,[89] i.e. a strong immunosuppressive and myeloablative conditioning regimen followed by transplantation of a large number of haploidentical stem cells depleted of T lymphocytes, may open new perspectives for allogeneic bone marrow transplantation in AML patients. In this setting, the mobilization and collection of allogeneic PBSC is crucial to overcoming HLA-disparity.[89]

Pilot studies on the use of autologous BMT as postremission therapy[90,91] indicate that disease-free survival at four years is on the order of 50% (i.e. comparable to that of allogeneic BMT). Autologous BMT has the advantage of lower procedure-related mortality than allogeneic BMT (approximately 10-15%), but involves a high risk of leukemic relapse (about one half treatment failures). A recent trial by Zittoun *et al.*[92] showed that both autologous and allogeneic BMT performed in first CR resulted in a significantly better disease-free survival than intensive consolidation chemotherapy. The projected rate at four years was 55% for allogeneic BMT, 48% for autologous BMT and 30% for intensive chemotherapy with no differences between allogeneic and autologous BMT.

Taken together, these results demonstrate the potential benefit of autologous stem cell transplantation for leukemic patients. However, the high incidence of leukemic relapse and delayed hematological recovery after ABMT have prompted several authors to investigate the use of mobilized PBSC.

$CD34^+$ mobilized hematopoietic cells for support of intensive postremission chemotherapy of AML

As stated above, autologous transplantation of mobilized hematopoietic progenitor cells has been shown to reconstitute hematopoiesis more efficiently than BM-derived grafts.[2] Moreover, early studies have failed to detect neoplastic cells in the peripheral blood of AML patients during the early recovery phase following remission induction/consolidation chemotherapy.[93,94] Based on these reports, several investigators have addressed the question of whether the use of PBSC might result in a more rapid engraftment and a lower risk of relapse in AML patients. Relevant issues include the level of malignant cell contamination, the threshold dose of hematopoietic precursors (i.e. CFU-GM and $CD34^+$ cells), the optimal timing for stem cell collection and the potential benefit derived from the use of selected cytokines to improve PBSC harvest.

To *et al.*[93] studied leukemia-associated cytogenetic abnormalities in myeloid colonies derived from early remission PBSC. At a sensitivity level of 2:100 cells, they were not able to detect the t(8;21) in 293 samples examined. Recently, the more sensitive *nested* reverse transcriptase polymerase chain reaction (RT-PCR) was used to monitor minimal residual disease in the BM and peripheral blood of leukemic patients considered in remission by morphologic analysis.[95] In that study, the authors found no differences between PBSC collections and simultaneous

BM harvests. However, the degree of leukemic contamination may have been different among the PCR-positive groups because the two-step PCR is highly sensitive but not quantitative.

The issue of leukemia-free autograft was recently underscored by gene-marking studies showing that residual contaminating AML cells contribute to relapse when reinfused into patients.[96] In this regard, leukemic recurrence remains the most frequent cause of treatment failure in AML patients[97,98] and preliminary non-randomized clinical studies have not reported any advantage for ABMT over PBSC[99-101] in terms of disease-free survival and overall survival rate (Table 4). Moreover, it could be argued that the interval between complete remission and myeloablative therapy may be shorter for PBSC patients, since the exclusion rate is higher for ABMT patients.[101] Thus, randomized studies are warranted to rule out selection bias. Reinfusion of PBSC markedly shortened the period of marrow aplasia compared to purged and unpurged ABMT[99-102] and reduced morbidity and resource utilization (Table 4). In particular, To *et al.*[102] demonstrated an advantage of 11 and 19 days in the median time to achieve 0.5×10^9 neutrophils/L and 50×10^9 platelets/L, respectively, in favor of PBSC, whereas two other studies[99,100] showed a more rapid neutrophil engraftment (28 days and 12 days, respectively) but not a highly significant faster platelet recovery. Most likely the acceleration of hematopoietic reconstitution derives from the reinfusion of a higher number of early pluripotent precursors and large amounts of committed progenitor cells which require less time to reach maturation.[103]

Early studies in acute leukemia indicated that an optimal CFU-GM dose of 50×10^4/kg body weight is required for complete and sustained reconstitution of BM function.[104] Other reports[99-101] and our own preliminary experience (Tables 4 and 5) have demonstrated similar results with lower CFU-GM numbers. These differences are probably related to different assay methods, whereas the more reproducible evaluation of progenitor cells by flow cytometry has suggested a threshold dose of 2×10^6 $CD34^+$ cells/kg.[105] Interestingly, the number of progenitor cells infused is only indirectly related to long-term engraftment, suggesting that additional measurements of $CD34^+$ cell fraction subsets may be helpful in predicting sustained recovery of hematopoiesis. Moreover, a transient secondary fall in neutrophil and platelet count has been described[104] during the 3rd-8th weeks after transplantation, indicating a time lag between exhaustion of the committed progenitor cell pool, which is responsible for early engraftment, and expansion of the more immature pluripotent stem cell compartment.

Lastly, the timing of BM harvest or PBSC collection in the autologous setting plays an important role in the quality of the autograft. It has been clearly established that the amount and the length of previous therapy affects the number of circulating $CD34^+$ cells;[106] however, reinfusion of PBSC collected in the recovery

Table 4. Reported studies on autologous stem cell transplantation in AML patients. PBSC indicates peripheral stem cell transplantation, while ABMT refers to autologous bone marrow transplantation.

	PBSC/ABMT	*PBSC/ABMT*	*PBSC/ABMT*
Reference #	99	102	100
Pts	28/683	38/13°	20/23*
CFU-GM reinfused ($x10^4$/Kg)	NR	86.6/12.1**	2.3/0.1 (0.2-4.1/0-1)
Median time to:			
$> 0.5 \times 10^9$ PMN/L	15.5/27 (9-60/9-389)	11/22 (9-17/12-35)	14/42 NR
$> 50 \times 10^9$ PLT/L	58.5/50 (11-713/10-700)	13.5/32 (9-NA/21-NA)	30/46 NR
PLT transfusion	NR	5.4/8.8**	NR
Days on antibiotics	NR	9.3/14.3**	NR
Hospitalization (days)	NR	27.5/35.1**	45/73
Relapse rate	57%/48%	NR	NR
DFS	39%/42%	NR	35%/51%

° AML pts = 19 in PBSC group and 1 in ABMT group.
** Comparison was made with 23 pts receiving purged marrow.*
*** Results expressed as the mean.*
Abbreviations: NR, not reported; NA, not achieved; DFS, disease free survival; PMN, neutrophil; PLT, platelet.

Table 5. Experience of the Institute of Hematology "Seragnoli", Bologna on autologous stem cell transplantation in AML patients. The results are expressed as median (range) and refer to AML (n=7) and RAEB-T (n=2) patients in I CR. PBSC collections were carried out following consolidation treatment. PMN and PLT recovery was recorded as such when the PB count was > 0.5 and 20 x 10^9/L, respectively.

Apheresis products

Pts	PBSC collections	MNC (10^8/Kg)	CFU-GM (10^4/Kg)	CD34+ (10^6/Kg)
9	3 (2-3)	7 (2.8-11.6)	11.8 (2.8-78.2)	7 (3.1-17.5)

Hematological reconstitution

Pts	day to PMN Hospital recovery	day to PLT recovery	PLT transfusion	RBC transfusion discharge
8	14 (11-34)	18 (10-NR)	2.5 (0->10)	4 (1-9) 18 (14-38)

Abbreviations: MNC, mononuclear cells; RBC, red blood cells.

phase of induction therapy has resulted in a higher relapse rate[101,107] indicating that returning a larger quantity of cells to patients without careful analysis of minimal residual disease may increase the probability of transplantation of leukemic cells. Thus, at least one consolidation cycle of treatment should be performed in the case of stem cell mobilization to take advantage of *in vivo* purging, coupled with a low number of apheresis procedures. To this end, it has been shown[108] that administration of G-CSF during the recovery phase of consolidation chemotherapy in acute leukemia patients increased the peak level of CFU-GM and CFU-MIX by 5.8 and 4.3 times, respectively, compared to cycles were G-CSF was not used, and significantly prolonged the period of mobilization of stem cells. Although the role of cytokine treatment in AML patients is still under evaluation, preliminary data from a large cohort of leukemic patients suggest that G-CSF administration does not affect either the remission or relapse rate,[109,110] whereas a protective effect regarding relapse was shown in a randomized study.[29]

In practice, leukapheresis sessions should be started after a careful evaluation of $CD34^+$ cells in the peripheral blood by flow cytometry, at time of hematopoietic recovery from transient myelosuppression.

When adequate mobilization of progenitors ($CD34^+$ cells > 10-15/μL) occurs, daily leukapheresis should be performed until the collection of a minimum number of 2×10^6/kg $CD34^+$ cells. The leukapheresis products should be evaluated for the presence of residual contaminating leukemic cells by immunophenotyping, karyotypic analysis and RT-PCR-based molecular analysis in those samples deriving from patients who had shown a specific phenotypic and/or molecular marker at diagnosis.

Moreover, because the content of circulating progenitors is generally low (< 1% of the mononuclear cell fraction), blood cell separator efficiency must be optimized. Collection efficiency (CE) is the percentage of cells entering the system that are eventually collected:

$$CE\ (\%) = \frac{\text{No. harvested cells}}{\text{No. of cells in preapheresis blood unit volume}} \times \text{processed blood volume}$$

Acceptable CE should not be lower than 50%. CE is a useful parameter for evaluating blood cell separator effectiveness in harvesting PBSC, independently of the patient's clinical condition.

Purging in AML

Considering the possibility of relapse from minimal residual disease (MRD) derived from autologous graft, several investigators addressed the issue of *ex-vivo* purging of leukemic cells prior to stem cell reinfusion. Using the Brown Norway myelocytic leukemia rat model, it has been shown that injection of 25 leukemic cells induces leukemia in 50% of recipients.[111] By applying the same mathematical model to humans, it has been suggested that reinfusion of 10,000 residual leukemic cells may result in a relapse rate as high as 50%.[111] More recently, the role of residual tumor cells in clinical relapse after autograft was indicated by a clinical study involving 114 B-cell lymphoma patients with t(14;18) who received autologous marrow treated with a combination of monoclonal antibod-

ies directed against B-cell associated antigens plus complement.[112] Following purging, no lymphoma cells could be detected by PCR amplification of the bcl-2 gene in the marrow of 57 patients. Disease-free survival was increased in these individuals with respect to that of patients whose marrow contained detectable tumor cells. Moreover, the ability to remove lymphoma cells was the most important prognostic factor for predicting relapse (39% versus 5% of purged patients after a median follow-up of 23 months).[112] Lastly, genetic marking of marrow cells with the neomycin-resistance gene has provided the formal proof that reinfusion of residual leukemic cells in AML patients contributes to a recurrence of the disease.[113] Taken together, these findings demonstrate the need for effective *ex-vivo* treatments to improve the outcome of autologous transplantation.

Among the many purging methods proposed for the elimination of MRD,[114] cyclophosphamide (Cy) derivatives are the most widely used agents in AML patients, since preclinical models demonstrated that these compounds were able to eliminate residual BM neoplastic cells in the Brown Norway rat system.[115] The main mechanism of action of Cy active metabolites is based upon marked inhibition of leukemic progenitor cell (CFU-L) growth[115] while sparing normal primitive hematopoietic cells.[116] Furthermore, these alkylating agents seem to induce apoptotic death of leukemic cells[117] as well as the activation of immune mechanisms capable of controlling leukemic cell proliferation.[118] Combinations of 4-hydroperoxycyclophosphamide (4-HC) or nitrogen mustard and VP-16 have also been proposed to increase the selective toxicity of pharmacologic purging towards neoplastic cells.[119] Several monoclonal antibodies that recognize tumor-associated or cell-differentiation antigens not expressed by primitive cells responsible for hematopoietic engraftment have been selected for clinical trials after *in vitro* studies demonstrated a high purging efficacy with the use of complement, toxins and radioactive molecules. However, the heterogeneity of antigen expression on neoplastic cells and the lack of tumor-specific determinants may greatly affect the efficiency of antibody-based strategies for depletion of leukemic cells. In this context, the combination of two purging techniques has been explored with promising results.[120] Other approaches include the use of photoactive compounds which sensitize leukemic cells and specifically damage their cell membranes upon exposure to light,[121] and biological methods based on the different proliferative patterns of leukemic cells and their normal counterparts when cultured *ex-vivo* for several days in the presence of stromal cells.[122]

Clinical trials

Clinical retrospective data supporting the beneficial effect of purging have been progressively accumulating. The Baltimore team provided indirect evidence in favor of purging by correlating effective CFU-GM colony elimination with a significant decrease in relapse.[123] Furthermore, the same authors associated the sensitivity to 4-HC of CFU-L grown in remission with the posttransplant outcome.[124] The 3 most recent surveys of the *Leukemia Working Party of the EBMT group* have consistently reported lower relapse rates following reinfusion of BM purged with mafosfamide,[91,125,126] especially in patients transplanted within 6 months of CR and in slow remitters (> 40 days to achievement of CR), two patient populations considered at high risk of disease recurrence. In fact, the proportion of patients relapsing in the purged and unpurged groups was 29±5% vs 50±4%, respectively, following a conditioning regimen that included total body irradiation ($p < 0.0001$). More striking differences were found when considering only those patients autografted early after CR (16±6% vs 60±6%) and patients with an interval from diagnosis to CR greater than 40 days (20±8% vs 61±6%). Gulati *et al.* and Laporte *et al.*[127, 128] reported disease free survival which approximated 60% in AML patients in I CR reinfused with autologous marrow treated with 4-HC and VP-16 and mafosfamide. In the same paper by the Paris group,[151] it was suggested that the higher the initial content of BM CFU-GM, the lower the risk of transplant related mortality and the higher the chance of curing the disease.

Despite these results in favor of *ex-vivo* elimination of contaminating leukemic cells, this procedure is not routinely performed in the majority of transplant centers. The main reasons might be: 1) the delay in hematological recovery after reinfusion of purged autografts; 2) the increase in the cost of ABMT; 3) the need for technical training and, most of all, 4) the lack of results derived from prospective clinical studies demonstrating the effects of purging. The feasibility of such trials is rather limited due to the high number of patients needed to obtain adequate statistical power.

So far, no data are available on purging protocols for PBSC collections; however, there are several reasons for proposing purging strategies for autograft of circulating autologous stem cells. Unlike solid tumors and malignant lymphomas, acute leukemias easily involve PB. Moreover, the number of hematopoietic progenitor cells (and possibly leukemic precursors) in PB autotransplants is usually at least 10 times higher than that of ABMT. Finally, as discussed above, the interval between CR and autotransplant may be shorter for PBSC patients, who may be thus considered *high risk patients* for relapse. Critical issues for designing experimental studies in this setting would include the proper assessment of MRD before and after purging, the establishment of reproducible technical protocols (cell concentration, RBC contamination, etc.), and careful evaluation of the toxicity of purging agents on PB progenitors, since the kinetic status of circulating stem cells following mobilization protocols (especially if CSFs are used) may be different from BM stem cells.[129]

Conclusions

Autologous BMT has been widely used as consolidation therapy in AML patients in first or second remission; however, delayed hematopoietic engraftment occurs in a substantial proportion of patients resulting in significant morbidity and mortality. This is mainly due to the adverse effects of prior intensive chemotherapy on BM harvest, a decrease in the normal stem cell pool in leukemic patients and, perhaps, toxic damage to the marrow microenvironment. Thus, several groups have investigated the use of circulating progenitor cells with the twofold aim of reducing transplant-related toxicity and widening the number of potential candidates for myeloablative therapy with the support of autologous stem cells.

As for hematopoietic reconstitution, previous studies have provided evidence that PBSC transplantation may offer some advantages over BM autografting. However, crucial issues such as asynchronous mobilization of normal vs leukemic cells and potential contamination of PBSC collections, timing of PBSC harvest, detection of minimal residual disease, and the role of growth factors to accelerate hematological recovery and optimize stem cell collection have not been fully addressed.

In the present paper, the latest advances in this field have been reviewed with special focus on the biology of putative leukemic stem cells; operative guidelines have also been provided for those investigators who wish to design proper clinical trials on PBSC autotransplantation in acute leukemia.

Definitive answers regarding the role of PBSC will be coming from a large European randomized trial which is currently comparing peripheral blood stem cell and BM-derived graft.

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