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# $CD34^+$ cell subsets and long-term culture colony-forming cells evaluated on both autologous and normal bone marrow stroma predict long-term hematopoietic engraftment in patients undergoing autologous peripheral blood stem cell transplantation

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***Objective.*** **The aim of this study was to evaluate which $CD34^+$ cell subset contained in leukapheresis products could be regarded as the most predictive of long-term hematopoietic recovery after autologous peripheral blood stem cell transplantation (auto-PBSCT).**

***Materials and Methods.*** **Based on data from 34 patients with hematologic malignancies, doses of $CD34^+$ cells and $CD34^+$ cell subsets, defined by the expression of HLA-DR, CD38, CD117 (c-kit/R), CD123 ($\alpha$ subunit of IL-3/R), CD133 (AC133), and CD90 (Thy-1) antigens, were correlated with the number of short-term (i.e., colony-forming cells [CFC]) and long-term culture CFC (LTC-CFC) (generated at week 5 of culture) and with the kinetics of hematopoietic engraftment following auto-PBSCT. The capacity of autologous stroma (AS), normal human bone marrow stroma, and M2-10B4 murine cell line to sustain $CD34^+$ cell growth was comparatively evaluated in the LTC assay.**

***Results.*** **Our data demonstrated that some of the most primitive progenitor subsets ($CD34^+CD117^-HLA\text{-}DR^-$, and $CD34^+CD38^+HLA\text{-}DR^-$) showed the strongest correlation with LTC-CFC numbers generated within the AS, whereas no significant correlation was noted using normal bone marrow stroma. Multivariate analysis showed that the only CD34 cell subset independently associated with long-term (3 to 6 months) platelet engraftment after auto-bone marrow transplantation was the $CD34^+CD117^-HLA\text{-}DR^-$ phenotype; long-term erythrocyte engraftment was correlated with $CD34^+CD38^+HLA\text{-}DR^-$ cell content. The latter further influenced platelet engraftment in the first 3 months after auto-PBSCT. The most predictive parameters for neutrophil engraftment were $CD34^+CD38^+HLA\text{-}DR^-$ cell subtype and the total LTC-CFC quantity infused.**

***Conclusions.*** **These data further support the hypothesis that the type of stromal feeders influences the frequency of LTC-CFC, possibly because they differ in their ability to interact with distinct subsets of hematopoietic stem cells. Furthermore, as the use of AS in LTC assay can mimic in vitro the human bone marrow microenvironment, it can be speculated that this culture system could be a useful means to study the kinetics of recovery of bone marrow stroma following chemotherapy and PBSCT. From these results, it can be concluded that some $CD34^+$ cell subsets appear to be more reliable predictors of long-term hematopoietic recovery rates than total $CD34^+$ cell quantity.** **© 2001 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

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Long-term culture (LTC) of bone marrow (BM) cells have been used as an in vitro model of hematopoiesis to study the interactions among early progenitors, stroma, and regulating factors [1–4]. Different stromal layers have been used in LTC assays, and recent data have shown that the type of stromal feeder used affects the frequency and maintenance of long-term culture colony-forming cells (LTC-CFC) [5]. Transformed murine and human engineered feeder cell lines, such as M2-10B4 [6], MS5 [7], S17 [8], and FBMD-1 [9] secreting supplementary growth factors, are widely used in the LTC assay, even if their ability to support the growth of hematopoietic stem cells (HSC) was found to differ from cell line to cell line [8–10]. Some authors showed that human primary BM cultures [11,12] are able to generate stromal layers that are more efficient in supporting HSC growth than the mouse-derived feeders, even if a regular and reliable provision of normal BM may present practical difficulties to many laboratories. Alternative methods are represented by the use of either cryopreserved normal BM [13] or preformed stroma and autologous stroma (AS) [14]. The use of stroma-dependent LTC has demonstrated the importance of the adherent layer as a reservoir of the most primitive stem cells and that a direct contact between stromal and hematopoietic cells is critical for stem cell growth and differentiation [15–17].

Although $CD34^+$ cells expressing little or no CD38 and lacking HLA-DR antigen define a primitive subpopulation of progenitors cells in fetal liver, leukapheresis products (LP) [18,19], cord blood, and BM [20,21], at the present time it is not known which progenitor cell subsets are optimally predictive of short- and long-term engraftment after autologous peripheral blood stem cell transplantation (auto-PBSCT) [22–28]. Most published reports have focused on the analysis of early post-transplant multilineage recovery [29–31], while the influence of $CD34^+$ subsets and LTC-CFC cells on long-term engraftment has not yet been clearly defined [32]. Interestingly, absent stromal layer development was found to be associated with poor hematologic recovery after bone marrow transplantation (BMT) [33].

The aim of this study was to evaluate which $CD34^+$ cell subsets contained in LP could be regarded as the most predictive of long-term hemopoietic recovery after auto-PBSCT. To reach this goal, we correlated $CD34^+$ cell subsets with the numbers of both CFC and LTC-CFC generated within allogeneic normal BM stroma (NBMS), M2-10B4 cell line, and AS layers after 5-week culture of selected $CD34^+$ cells obtained from LP of 34 patients with hematologic malignancies undergoing auto-PBSCT. In addition, the different $CD34^+$ cell subsets defined by highly sensitive flow cytometry were tentatively correlated with the number of LTC-CFC obtained from the adherent and nonadherent (i.e., supernatant) fractions collected after 5 weeks of culture. Finally, the total $CD34^+$ cell quantity and doses of $CD34^+$ cell subsets and LTC-CFC infused were correlated with the kinetics of hemopoietic recovery over a 6-month follow-up period after auto-PBSCT.

## Materials and methods

*Patients and PBSC collection*

PBSC were obtained from 34 patients (19 men and 15 women; median age 44 years, range 25 to 58) in remission: 24 with non-Hodgkin's lymphoma (NHL), 5 Hodgkin disease (HD), and 5 multiple myeloma (MM).

Patients were treated according to the following chemotherapy regimen: MACOP-B followed by sequential high-dose chemotherapy (Milan protocol) for NHL, ABVD schedule for HD, and VAD regimen for MM.

$CD34^+$ cells were mobilized with chemotherapy as follows: 26 patients with high-dose cyclophosphamide (7 g/m$^2$) plus granulocyte colony-stimulating factor (G-CSF; lenograstim or filgrastim, 5 μg/kg body weight/day), and 8 patients with intermediate-dose cyclophosphamide (4 g/m$^2$) plus G-CSF (5 μg/kg body weight/day). Mean percentage of harvested $CD34^+$ cells in LP was 3.23 ± 2.28 (range 1–12.6). The conditioning regimens adopted in this study for PBSCT were mitoxantrone 60 mg/m$^2$ plus melphalan 180 mg/m$^2$ for NHL and MM, and BEAM regimen for HD. G-CSF administration started 5 days after PBSCT and continued until absolute neutrophil count was >1,000/μL for 3 consecutive days. Mean interval between the end of premobilization therapy and the mobilization regimen was 36 days (range 30–65). BM samples were collected at least 4 weeks after the last chemotherapy regimen to minimize the effect of cytotoxic drugs to the patient's BM microenvironment.

Mean number of $CD34^+$ cells infused was $6.54 \times 10^6$/kg body weight (range 2.65–11.0 $\times$ $10^6$/kg body weight). Three patients were transplanted with a $CD34^+$ cell dose $<5 \times 10^6$/kg.

Short-term engraftment was evaluated according to standard criteria (neutrophil count >500 μL and platelet count >20,000/μl on at least three consecutive analyses). Long-term recovery after auto-PBSCT was assessed by evaluating the hemoglobin (Hb) level, white blood cell count, neutrophil count, and platelet count at different time intervals (1, 3, and 6 months after PBSCT). Secondary graft failure was considered to have occurred if, on 3 consecutive days after full engraftment was documented, granulocytes decreased to <500/μL, and/or platelets to <20,000/μL. Incomplete recovery was considered for platelet count <50,000/μL, and/or neutrophil count <1,000/μL.

PBSC were collected on a Baxter CS3000 cell separator (Baxter, Milan, Italy) using a continuous collection procedure until 2.5× the patient's blood volume had been processed [34]. The target value was $4 \times 10^6$ $CD34^+$ cells/kg. LP were cryopreserved with 10% dimethylsulfoxide and autologous plasma and stored in liquid nitrogen.

*Controls.* Normal BM was obtained from proximal epiphysis of eight subjects (mean age 49 years; male/female ratio 1:1) who underwent major orthopedic surgical intervention.

*Purification of $CD34^+$ cells from LP*

Enriched $CD34^+$ cell fractions were obtained by immunomagnetic separation (Mini-MACS CD34 isolation kit; Miltenyi, Bergisch Gladbach, Germany), as previously described. A mean of $7 \times 10^7$ PBSC were separated on Lympholyte-H 1.077 g/cm$^3$ gradient (Cedarlane Laboratories Limited, Hornby, Ontario, Canada), then incubated with an hapten-conjugated CD34 monoclonal antibodies (mAbs) (QBEND-10), followed by incubation with magnetic microbeads conjugated to an anti-hapten antibody. The positive frac-

tion and unseparated cells were stained with CD34 epitope class III-reactive mAb (HPCA2-FITC). $CD34^+$ cell purity was measured by flow cytometry. Recovery, purity, and enrichment factor were calculated according to standard protocols [35].

*Flow cytometric analysis of $CD34^+$ cell subsets from LP*

Immunomagnetically selected $CD34^+$ cells were analyzed with a Facscan flow cytometer (Becton-Dickinson, San Jose, CA, USA). The instrument was calibrated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridinin chlorophyllin protein (Per-CP) beads provided by Becton-Dickinson. Data were analyzed with appropriate negative (isotypic) controls using CellQuest and Paint-a-Gate research software [36,37]. At least 20,000 cells were analyzed for each sample.

The CD34 cell population was identified using a combined approach based on multicolor analysis and evaluation of light-scattering properties of the cells [38]. Flow cytometric data were expressed as percentage of positivity (using the standard marker approach). Expression of the various antigens by $CD34^+$ cells was evaluated by direct immunofluorescence using a multiparametric approach based on the use of the following combination of mAbs: CD34 (HPCA-2)-FITC/CD38 (HB-7)-PE/HLA-DR (clone: L243)/Per-CP); CD34-FITC/IL-3/R(clone 7G3)-PE/HLA-DR/Per-CP; CD34 (HPCA-2)-FITC/AC133-PE; CD34-FITC/CD117 (c-Kit/R-clone: 95C3)-PE/HLA-DR-Per-CP; CD34-FITC/CD90-PE (Thy-1, 5E10)/HLA-DR-Per-CP. CD34, CD38, and HLA-DR mAbs were provided by Becton-Dickinson, CD90 and CD123 mAbs were purchased from Pharmingen (San Diego, CA, USA), and AC133 was purchased from Miltenyi.

Determinations for doses of the various $CD34^+$ cell subsets were calculated by calculating the percentage of total $CD34^+$ cells that expressed the appropriate markers of interest and multiplying that percentage by the CD34/kg values that had been previously determined.

*Short-term colony assays from BM and LP samples*

BM mononuclear cells (MNC) were isolated by centrifugation on Lympholyte-H gradient, washed in phosphate-buffered saline, and plated in triplicate at a density of $1 \times 10^5$ cells in 35-mm Petri dishes (Pbi, Milan, Italy) in 1.1 mL of a methylcellulose semisolid medium (Methocult H4434; Stem Cell Technologies Inc., Vancouver, Canada). Petri dishes were incubated at 37°C in a fully humidified atmosphere with 5% $CO_2$ and scored at day 14 under inverted microscope (Wilovert-Will, Wetzlar, Germany) for the presence of CFC (colony-forming unit granulocyte-macrophage [CFU-GM], burst-forming-unit erythroid [BFU-E], multilineage colony-forming unit [CFU-GEMM]). Short-term colony assays also were performed seeding $0.5 \times 10^5$ cells/mL obtained from LP, as previously described.

*BM stromal feeders*

Patient microenvironments from 41 patients (including the 34 cases investigated by multivariate analysis in this study) affected by NHL (n = 28), HL (n = 7), and MM (n = 6) were investigated by CFU-fibroblast (CFU-F) assay [39]. Briefly, $1 \times 10^6$/mL BM-MNCs were resuspended in LTC medium containing Iscove modified Dulbecco's medium (IMDM; Euroclone Ltd., Paington, UK) with preselected 12.5% fetal bovine serum, 12.5% horse serum (both sera from Stem Cell Technologies, Vancouver, Canada), 1% L-glutamine (Euroclone), and 1% penicillin-streptomycin (Euroclone), and plated on 35-mm collagenated biocoated Petri dishes (Becton-Dickinson). The medium was changed every week, and cultures were incubated at 37°C in a humidified atmosphere supplemented with 5% $CO_2$. Fibroblast aggregates with >50 cells were scored as CFU-F. All determinations were performed in duplicate and expressed as mean values. The same method was used for detecting and scoring spontaneous endothelial colonies (CFU-En), according to a previously described technique [40].

Human primary feeder layers were obtained according to a modified version of the Gartner and Kaplan method [1]. BM-MNC cells were seeded at a density of $1 \times 10^7$ in T12.5-$cm^2$ plastic tissue culture flasks (Falcon) in LTC medium supplemented with fresh $5 \times 10^{-6}$ mol/L hydrocortisone sodium succinate (Sigma, St. Louis, MO, USA). After 20 to 30 days of culture, the confluence of the stromal layers was checked under inverted microscope and assessed by a semiquantitative scoring system. The degree of confluence from the various culture systems was expressed as percentage of adherent cells covering the bottom of the flask (range 0–100%). Established autologous and normal stromal feeders, at least confluent for 70%, were irradiated at 16 Gy at the first passage of the culture. The stromal layers from 7 of 41 patients examined did not achieve the minimum level (70%) of confluence and, therefore, were not used for estimation of LTC-CFC. Thus, LTC-CFC was evaluated in only 34 patients.

The murine stromal nontransfected cell line M2-10B4 was maintained in RPMI 1640 medium (Euroclone) supplemented with 10% fetal bovine serum (Euroclone). At confluence, M2-10B4 cells were irradiated at 60 Gy. LTC-CFC assay on the M2-10B4 cell line was investigated in a smaller group of 12 patients (8 NHL, 2 HD, 2 MM). For this reason, statistical analysis between LTC-CFC numbers generated on M2-10B4 cells and $CD34^+$ cells subsets and hematopoietic engraftment after PBSCT was performed in this study.

When a confluent stromal layer was achieved, selected $CD34^+$ cells were seeded in T12.5 $cm^2$ plastic tissue culture flasks (Falcon) on the different irradiated stromal layers at a density of $3 \times 10^4$ cells in LTC-medium at 37°C and a humidified atmosphere with 5% $CO_2$. Stroma-contact cultures were fed weekly by changing half of the medium. Nonadherent cells recovered from the supernatant were assayed in short-term methylcellulose assay for the presence of committed progenitors. At week 5 of culture, both nonadherent and adherent cells, after treatment with trypsin and after stromal cell depletion, were likewise washed and separately assayed for LTC-CFC content, as described earlier.

The immunophenotypic characteristics of stromal cells were assessed using immunohistochemical technique with a wide range of mAbs [41].

*Statistical analysis*

Using the Pearson test, we first compared the percentage of positivity for the various phenotypic markers expressed by mobilized $CD34^+$ cells with the number of week 5 LTC-CFC generated within the different stromal layers. In a further analysis, the Pearson test was used to examine the relationship among the incidence of LTC-CFC generated on AS, doses of total $CD34^+$ cells and $CD34^+$ cell subsets infused, and hematologic parameters (Hb value, platelet count, white blood cell count) used to predict long-term engraftment after auto-PBSCT. The incidence of CFC present in PBSC preparations were correlated with doses of $CD34^+$ cells, $CD34^+$ cell subsets, and clinical parameters at different time intervals after PBSCT. In all cases, the confidence interval was deter-

mined to identify which parameters showed the strongest and most constant correlations. A confidence range with $p < 0.05$ was considered significant. To assess whether a certain cell dose could predict for long-term hematopoietic recovery, we calculated the mean doses of the various $CD34^+$ cell subsets transplanted in patients with normal and delayed long-term hemopoietic engraftment. Multiple regression analysis was used to determine which of the $CD34^+$ subsets or LTC-CFC numbers are the best independent predictors for the long-term engraftment.

The number of CFC, CFU-F, CFU-En, and percentage of stromal layer confluence in controls and patients groups were compared using nonparametric statistics (Wilcoxon test).

## Results

*Immunophenotypic profile of selected $CD34^+$ cells*

Flow cytometric analysis of the selected $CD34^+$ cell population showed a mean purity of 81.3% (range 60–99.7%) and a mean recovery of 60% (range 33–81%). Multicolor analysis of purified $CD34^+$ progenitor cells obtained from PBSC is given in Table 1. In brief, the lowest mean percentage of CD34-expressing cells was found in the $CD34^+$ $CD38^-$HLA-$DR^-$ cell fraction (0.12%), as well as in the $CD34^+CD38^-$HLA-$DR^+$ (0.18%), $CD34^+CD123^-$HLA-$DR^-$ (0.32%), $CD34^+CD90^+$HLA-$DR^-$ (0.49%), and CD $34^+CD38^+$HLA-$DR^-$ (1.1%) cell subsets. The highest values were found among the following $CD34^+$ cell subsets: $CD34^+CD38^+$HLA-$DR^+$(98.5%), $CD34^+CD123^+$HLA-$DR^+$ (92.2%), $CD34^+$CD $117^-$HLA-$DR^+$ (77.3%), and $CD34^+$ $AC133^+$ (75.9%).

*Short-term colony assays of BM and LP samples*

No significant difference was found between the clonogenic potential of BM cells from patients in remission and controls. Cell morphology and colony size were comparable in the two groups. Mean values of the clonogenic potential of hematopoietic progenitors obtained from LP are given in Table 2.

*Clonogenic mesenchymal progenitors and stromal confluence assays*

With regard to the microenvironment, patients showed a significantly lower CFU-F number than controls ($p = 0.003$) (Table 2). The incidence of CFU-En found to grow spontaneously in LTC assay was higher in BM samples obtained from NHL and MM patients compared with controls (1.5 vs 0.45; $p = 0.01$) and other patient categories, as previously described by our group [41]. We also evaluated the capacity of the autologous and normal BM stromal cells to form a confluent monolayer usable as primary feeder layer in the LTC assay (Table 2). Healthy control stromal layers always reached the confluence (100%), whereas BM specimens from the patient group displayed a variable confluence degree (range 70–100%). However, in 7 (17%) of 41 samples analyzed (4 NHL, 2 HD, 1 MM), the stromal layer confluence capacity was particularly impaired (mean 35%, range 10% to 55%). Cells from these cases were not used as feeder layers in the LTC-CFC assay and, therefore, were excluded from this study.

*LTC-CFC assay*

The number of committed progenitors generated from week 1 to week 5 of culture was assessed, and the results were subdivided according to the type of stromal layer used in the LTC assay. As shown in Figure 1A, the mean numbers of CFU-GM/$10^5$ $CD34^+$ cells derived from AS (1416.2 at week 1 of culture to 251.5 at week 5) were found to be higher than those on NBMS (906.8 at week 1 of culture to 165 at week 5) and M2-10B4 cell line (732.2 at week 1 to 61.9 at week 5); however, these differences were found to be not significant, except at week 1 comparing CFU-GM on AS vs those on M2-10B4 ($p = 0.01$). The frequency of BFU-E (Fig. 1B) from the M2-10B4 cell line was lower (42 at week 1 to 0 at week 5) than that on AS (179.2 at week 1 [$p = 0.001$] to 3.2 at week 5) and NBMS (121.8 at week 1 to 0.64 at week 5). No significant differences in the incidence of CFU-GEMM among AS (21 at week 1 to 0 at week 5),

**Table 1.** Immunophenotypic profile of selected $CD34^+$ cells from apheresis products of patients with hematologic malignancies

| | $34^+/38^+/DR^+$ | $34^+/38^+/DR^-$ | $34^+/38^-/DR^-$ | $34^+/38^-/DR^+$ | $34^+/90^+/DR^+$ | $34^+/90^+/DR^-$ |
|---|---|---|---|---|---|---|
| Mean ± SD | 98.5 ± 1.1 | 1.1 ± 0.86 | 0.12 ± 0.2 | 0.18 ± 0.19 | 31.6 ± 10.7 | 0.49 ± 0.43 |
| Range | 96.8–99.9 | 0.10–3.5 | 0–0.44 | 0.03–0.36 | 11.5–46.1 | 0.05–2.0 |
| | $34^+/90^-/DR^+$ | $34^+/90^-/DR^-$ | $34^+/123^+/DR^+$ | $34^+/123^+/DR^-$ | $34^+/123^-/DR^+$ | $34^+/123^-/DR^-$ |
| Mean ± SD | 65.3 ± 11 | 2.5 ± 1.6 | 92.2 ± 7.8 | 1.8 ± 1.2 | 5.2 ± 7.6 | 0.32 ± 0.37 |
| Range | 44.8–84.1 | 0.31–5.7 | 67.6–99.4 | 0.37–4.2 | 0.19–30.2 | 0–1.6 |
| | $34^+/AC133^+$ | $34^+/AC133^-$ | $34^+/117^+/DR^+$ | $34^+/117^+/DR^-$ | $34^+/117^-/DR^+$ | $34^+/117^-/DR^-$ |
| Mean ± SD | 75.9 ± 14 | 20.9 ± 10.1 | 23.9 ± 28.1 | 0.60 ± 0.55 | 77.3 ± 28.2 | 1.6 ± 1.04 |
| Range | 47.0–93.7 | 5.5–43.9 | 3.1–92.9 | 0.04–1.8 | 17.8–97 | 0.05–3.6 |

Data are given as percentage of $CD34^+$ cells coexpressing the various phenotypic markers.

**Table 2.** Bone marrow and peripheral blood clonogenic assays in patients and controls

| Culture assay | BM patients Mean ± SD (range) | BM Controls Mean ± SD (range) | PBSC Patients Mean ± SD (range) |
|---|---|---|---|
| CFU-GM | 41 ± 18.2 (3.5–70) | 52.7 ± 14.5 (25.3–72.5) | 104 ± 87.8 (0–372) |
| BFU-E | 36.9 ± 17.1 (12–66) | 37.8 ± 18.5 (10–64) | 67.4 ± 77 (0–295) |
| CFU-GEMM | 2.5 ± 2.4 (0–10) | 2.6 ± 1.7 (0–5) | 4.02 ± 4 (0–15) |
| CFU-F | 9.1 ± 6.7 (2–24) | 16.2 ± 5.06 (5–24) | |
| CFU-En | 1.5 ± 2 (0–8) | 0.45 ± 0.93 (0–2) | |
| Percent confluence | 83.5 ± 25.7 (70–100) | 100 ± 10 (80–100) | |

CFU-GM = colony-forming unit granulocyte-macrophage per $10^5$ LP derived cells; BFU-E = burst-forming unit erythroid per $10^5$ LP derived cells; CFU-GEMM = colony-forming unit granulocyte, erythroid, monocyte, and megakaryocyte per $10^5$ LP derived cells; CFU-F = colony-forming unit fibroblast per $10^6$ BMMNC; CFU-En = colony-forming unit endothelial colony per $10^6$ BMMNC.

NBMS (16 at week 1 to 0.4 at week 5), and M2-10B4 (5 at week 1 to 0 at week 5) were found (Fig. 1C). Figure 1D shows the variation of total CFC number during the 5 weeks of culture of the LTC assay.

With regard to the total number of LTC-CFC after 5 weeks of culture, the highest value was seen on AS compared with both NBMS and M2-10B4, but these differences were not significantly different.

At week 5 of culture, we considered separately the number of LTC-CFC generated from nonadherent and adherent fractions. The mean values obtained from the adherent fraction were significantly lower than the values from the nonadherent fraction. The highest number of LTC-CFC from the adherent fraction was seen on AS, whereas lower values were documented in LTC-CFC on NBMS and, above all, on the M2-10B4 cell line ($p$ values are given in Table 3).

Data collected from a smaller series of 12 patients showed a good reproducibility of our LTC-IC culture system. In fact, if the same CD34 sample was seeded onto three identically derived stromal support layers, the 5-week CFC activity on the three identically derived stromal layers was similar ($p$ = NS).

Furthermore, purified $CD34^+$ cell preparations obtained from different positive selection procedures were grown in triplicate on several AS preformed layers. Results were found to be comparable in all culture systems (CV < 7%; $p$ = NS).

In three experiments, purified $CD34^+$ cells from healthy subjects were grown onto AS and data were compared with

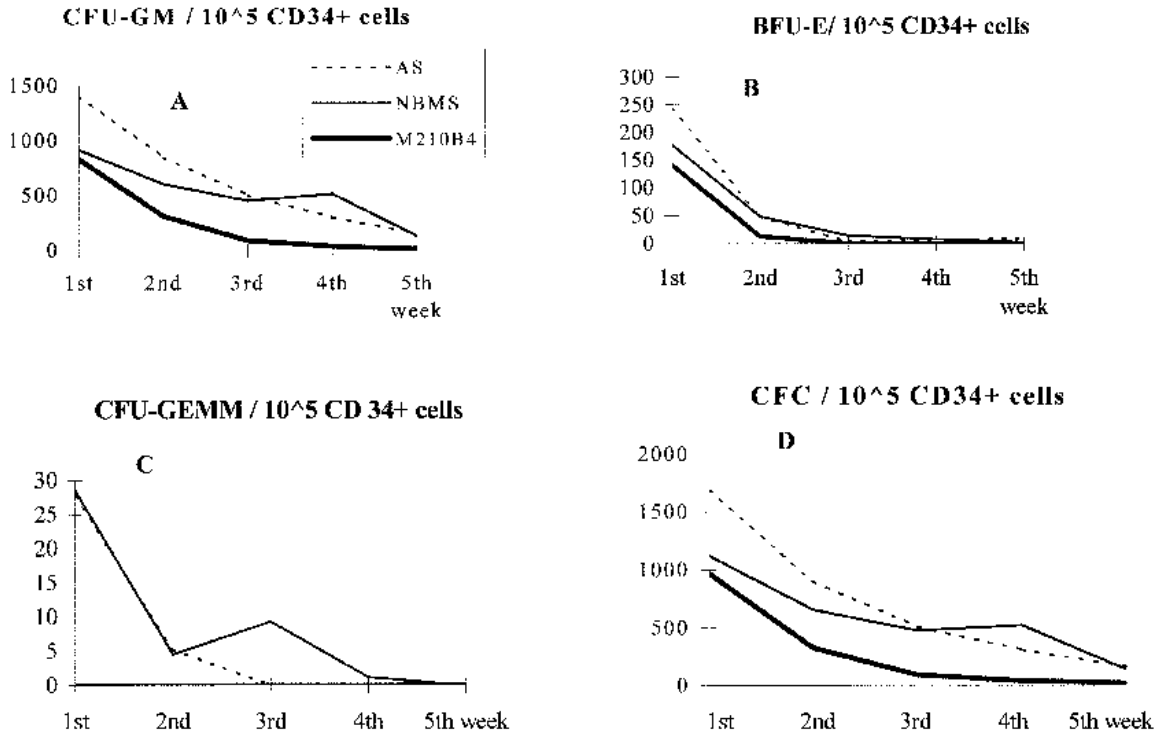


**Figure 1.** LTC-CFC assay. Mean numbers of CFU-GM/$10^5$ $CD34^+$ cells **(A)**, BFU-E/$10^5$ $CD34^+$cells **(B)**, and CFU-GEMM/$10^5$ $CD34^+$ cells **(C)** generated at week 5 of culture on the different stromal layers. **(D)** Total number of CFC/$10^5$ $CD34^+$ cells. Broken line = AS (autologous stroma); thin solid line = NBMS (normal bone marrow stroma); thick solid line = M2-10B4 line.

allogeneic NBMS and M2-10 B4 cell line. LTC-CFC number was found to be higher on AS than on allogeneic NBMS and cell line (data not shown).

*Correlation between CD34$^+$ cell subsets, CFC from LP, and LTC-CFC from different stromal feeders*

A strong correlation between the total number of CD34$^+$ cells and of CFU-GM contained in the LP ($p = 0.0001$) was found. The incidence of CFU-GM from LP was correlated with the CD34$^+$AC133$^+$ cell subset ($p = 0.03$) and inversely with CD38$^+$DR$^-$ ($p = 0.048$) (Table 4). No strict associations between CFC and other CD34 cell subsets were noted in our study.

With regard to LTC-CFC in vitro assay, the strongest and most constant correlations were observed between CD34$^+$CD117$^-$HLA-DR$^-$ ($p = 0.016$) and CD34$^+$CD38$^+$ HLA-DR$^-$ ($p = 0.02$) progenitor cell subsets and LTC-CFC number, generated within the AS at week 5 of culture. No significant correlations were found between CD34$^+$ cell subsets and LTC-CFC evaluated on NBMS.

With regard to the correlations between the clonogenic potential of CD34$^+$ progenitors obtained from the adherent and nonadherent (i.e., supernatant) fractions collected after week 5 of culture of the LTC assays and the different CD34$^+$ cell subsets, we found that the CD34$^+$CD38$^+$DR$^-$ ($p = 0.006$) and the CD34$^+$CD117$^-$HLA-DR$^-$ ($p = 0.016$) cell subtypes showed a strong correlation with the adherent fraction generated within AS. No correlation were found with the non adherent fraction (Table 4).

*Correlation between doses of CD34$^+$ cells, CD34$^+$ cell subsets, CFU-GM colonies, LTC-CFC infused, and kinetics of engraftment after auto-PBSCT*

We correlated doses of CD34$^+$ cells and CD34$^+$ cell subsets infused with the incidence of LP-derived CFU-GM and LTC-CFC, and the kinetics of engraftment in the late post-transplant period (3 to 6 months after PBSCT). These data were compared with the following hematologic parameters: Hb level, and neutrophil, leukocyte, and platelet counts at 1, 3, and 6 months after auto-PBSCT.

With regard to clinical data, short-term engraftment occurred in all patients examined in this study (range 9–12 days after PBSCT). For long-term engraftment after PBSCT, 30 of the 34 patients achieved a neutrophil count >1,000/μL, and 29 achieved a platelet count >50,000/μL over a 6-month follow-up period. Within this patient group, two patients with NHL occasionally required platelet and red blood cell transfusions 2 to 6 months after PBSCT. Loss of platelet engraftment occurred in the same 4 patients who experienced a transitory loss of neutrophil engraftment. All five patients who presented a late loss of neutrophil and/or platelet engraftment received a number of CD34$^+$ cells ($6.21 \times 10^6$/kg) comparable to that infused into patients who achieved good long-term hematopoietic engraftment ($6.87 \times 10^6$/kg CD34$^+$ cells) (Table 5).

No significant correlation was found between the total CD34$^+$ cell quantity and clinical parameters.

A positive correlation between the number of transplanted LTC-CFC tested on AS and platelet count 1 month after auto-PBSCT was noted ($p = 0.048$) (data not shown). Higher correlations were found between LTC-CFC tested on AS and platelet values at 3 to 6 months post-transplantation ($p = 0.004$). No significant correlation was found between doses of LTC-CFC on NBMS and hematologic parameters (Table 4).

With regard to CD34$^+$ cell subset analysis, complete and durable platelet engraftment was correlated with CD34$^+$ CD117$^-$DR$^-$ ($p = 0.04$) and CD34$^+$AC133$^-$ ($p = 0.027$) cell subsets (Table 5). CD34$^+$CD38$^+$DR$^-$ cells also showed a direct correlation with both leukocyte ($p = 0.025$) and neutrophil count ($p = 0.023$) 3 to 6 months after PBSCT; CD34$^+$AC133$^-$ cell doses were correlated with long-term (3 to 6 months) leukocyte ($p = 0.03$) and neutrophil ($p = 0.02$) engraftment; and CD34$^+$CD123$^+$DR$^-$ correlated only with neutrophil count ($p = 0.038$).

Table 5 shows that the numbers of CD34$^+$CD117$^-$DR$^-$, CD34$^+$CD38$^+$DR$^-$, and CD34$^+$CD123$^+$DR$^-$ cells/kg infused were significantly lower in the group of patients (n = 5) who had delayed or long-lasting loss of engraftment from month 3 to 6 after PBSCT. In contrast, all patients who had received higher doses of these cell subtypes experienced complete and durable platelet and neutrophil engraftment after PBSCT.

Multivariate analysis showed that the only CD34 cell subset independently associated with long-term (3 to 6 month) platelet engraftment after auto-BMT was the CD34$^+$ CD117$^-$HLA-DR$^-$ phenotype ($b = 0.63$, $p = 0.039$). A significant association between the CD34$^+$CD38$^+$HLA-DR$^-$ cell content and platelet engraftment also was found, but this relation reached a significant level only within 3 months after auto-PBSCT ($b = 0.49$, $p = 0.011$). In contrast, the only parameter predictive for long-term erythrocyte engraftment was the CD34$^+$CD38$^+$HLA-DR$^-$ cell content ($b = 0.45$, $p = 0.023$). In the first 3 months after auto-PBSCT, both CD34$^+$CD38$^+$HLA-DR$^-$ cell number ($b = 0.77$, $p < 0.001$) and the total LTC-CFC quantity infused ($b = 0.51$, $p = 0.011$) were associated with neutrophil engraftment. In contrast, no correlation was found between CD34$^+$ cell quantity and long-term hemopoietic engraftment.

**Table 3.** Adherent and nonadherent output of CFC at week 5 of LTC

| Stroma type | Nonadherent fraction | Adherent fraction | Total |
|---|---|---|---|
| AS ($p = 0.002$) | 179.6 | 71.8 | 251.4 |
| NBMS ($p = 0.003$) | 129.2 | 35.8 | 165 |
| M210B4 ($p = 0.001$) | 49.4 | 7.36 | 61.9 |

*p* Values in parentheses are relative to the comparison between the nonadherent and adherent fractions.

AS = autologous stroma; NBMS = normal bone marrow stroma.

**Table 4.** Correlation between number of CFU-GM, LTC-CFC, $CD34^+$ cells, and $CD34^+$ cell subsets in PBSC preparations from 34 patients with hematologic malignancies in relation to long-term engraftment

| | | | | | Hematologic parameter 3–6 months after PBSCT | | | |
|---|---|---|---|---|---|---|---|---|
| | $CD34^+$ | $CD34^+/CD117^-/HLA\text{-}DR^-$ | $CD34^+/CD38^+/HLA\text{-}DR^-$ | $CD34^+/AC133^+$ | Hb (g/dL) | WBC | Neut | Plt |
| CFU-GM | $r = 0.83$<br>$p = 0.0001$ | NS | $r = 0.44$<br>$p = 0.048$ | $r = 0.54$<br>$p = 0.03$ | NS | NS | NS | NS |
| Total number | | | | | | | | |
| LTC-CFC* (week 5) | NS | $r = 0.89$<br>$p = 0.016$ | $r = 0.68$<br>$p = 0.02$ | NS | NS | NS | NS | $r = 0.78$<br>$p = 0.004$ |
| Adherent fraction | | | | | | | | |
| LTC-CFC* (week 5) | NS | $r = 0.89$<br>$p = 0.016$ | $r = 0.79$<br>$p = 0.006$ | NS | ND | ND | ND | ND |
| Nonadherent fraction | | | | | | | | |
| LTC-CFC* (week 5) | NS | NS | NS | NS | ND | ND | ND | ND |
| NBMS LTC-CFC | NS | NS | NS | NS | ND | ND | ND | ND |

*LTC-CFC evaluated on autologous stroma.

Hb = hemoglobin; NBMS = normal bone marrow stroma; nd = not done; Neut = neutrophil count; Plt = platelet count; WBC = white blood cell count.

## Discussion

In this article, we assessed the predictive value of the total quantity of $CD34^+$ cells and of the various $CD34^+$ cell subsets obtained from LP for the long-term hemopoietic recovery after auto-PBSCT in patients with hematologic malignancies in remission phase. To obtain this goal, we also tested the ability of selected $CD34^+$ progenitors obtained from LP to produce progeny over long time spans by LTC assay on different stromal cell feeder layers. Because it has been postulated that BM microenvironment could play a role in HSC engraftment and considering that our LTC system was based on the use of both human primary autologous and normal BM stroma, we also examined the clonogenic potential of BM mesenchymal cells in the pretransplant period. Our data showed that cells from patients with hematologic malignancies could have a lower CFU-F incidence than that of normal BM, and occasionally a slower and reduced stromal layer formation capacity, compared with that of the control group, thus confirming previous reports [42]. However, these alterations do not necessarily lead to altered architecture of BM microenvironment or defective functional support for in vitro hematopoiesis, as further supported by our data from LTC assay on AS that exhibited a normal supportive capacity for stem cells. Previous studies documented that either chemotherapy or autologous and allogeneic stem cell transplantation could damage the BM microenvironment [43–46], and these alterations were found to be correlated with a very poor hematopoietic recovery after BMT [33]. However, based on our results, to obtain an efficient stromal layer from these patients, we would recommend collecting BM aspirates as far as possible from the time of administration of the cytotoxic drugs. This could

**Table 5.** Correlation between doses of $CD34^+$ cells, $CD34^+$ cell subsets, and long-term hemopoietic engraftment after PBSCT

| | | | | | Patients (n = 5) who had delayed long-term engraftment | | | Patients (n = 29) who had normal long-term engraftment | |
|---|---|---|---|---|---|---|---|---|---|
| | Hematologic parameter 3–6 months after PBSCT | | | | Cell number transplanted/kg ($\times 10^4$) | | | Cell number transplanted/kg ($\times 10^4$) | |
| | Hb | WBC | Neut | Plt | Mean | Range | *p* Value | Mean | Range |
| $CD34^+$ cells | NS | NS | NS | NS | 621 | 265–1100 | NS | 687 | 410–1004 |
| $CD34^+/CD117^-/HLA\text{-}DR^-$ | NS | NS | NS | $r = 0.54$<br>$p = 0.04$ | 3.4 | 0.3–8 | 0.006 | 27 | 7–134 |
| $CD34^+/CD38^+/HLA\text{-}DR^-$ | NS | $r = 0.63$<br>$p = 0.025$ | $r = 0.61$<br>$p = 0.023$ | NS<br>NS | 2.9 | 0.6–9 | 0.049 | 9 | 4–23 |
| $CD34^+/AC133^-$ | NS | $r = 0.67$<br>$p = 0.03$ | $r = 0.71$<br>$p = 0.02$ | $r = 0.69$<br>$p = 0.027$ | 119.8 | 81–159 | NS | 252 | 33–318 |
| $CD34^+/CD123^+/HLA\text{-}DR^-$ | NS | NS | $r = 0.54$<br>$p = 0.038$ | NS | 8 | 5–18 | 0.042 | 13 | 4–37 |

Data are subdivided according to whether or not patients achieved good hematologic recovery 3–6 months after autologous transplant.

The two groups of patients with normal or delayed long-term engraftment were compared using Wilcoxon's test.

AS = autologous stroma; NBMS = normal bone marrow stroma.

minimize the short-lasting negative effect of chemotherapy on the BM microenvironment.

With regard to the capacity of different stromal cell layers (AS, NBMS, and M2-10B4) to sustain the proliferation of selected $CD34^+$ progenitors in the LTC-CFC assay, we observed a higher number of CFC generated within the AS and NBMS than those on M2-10B4 line, thus suggesting a specific release of BM human stroma-derived cytokines capable of inducing the differentiation and proliferation of progenitor cells. These data confirm previous findings indicating that the type of stromal feeder influences the frequency and maintenance of LTC-IC in different pathologies [5] and may suggest that certain cell types do not provide an ideal environment for stem cell growth [5,8,47].

Comparison between the expression of the various immunophenotypic markers (HLA-DR, CD38, CD117 [c-kit/R], CD123 ($\alpha$ subunit of IL-3/R), AC133, and CD90) on $CD34^+$ cells and the in vitro incidence of LTC-CFC from LP showed that some of the more primitive $CD34^+$ cell subtypes, such as $CD34^+CD117^-DR^-$ and $CD34^+/38^+$HLA-$DR^-$ phenotypes) positively correlated with week 5 LTC-CFC numbers generated within the AS. No correlation was found between $CD34^+$ cell subsets and week 5 LTC-CFC numbers from NBMS. These results further support the hypothesis that distinct stromal cell subtypes could in vitro interact selectively with different subsets of stem/progenitor cells, and that the various stromal cell feeders used in this study have a different ability to support in vitro stem cell growth, thus confirming previous results [48,49].

In this study, we also studied the differences in the clonogenic potential of selected $CD34^+$ progenitor cells obtained from the adherent and nonadherent (i.e., supernatant) fractions collected after week 5 of culture of the various LTC assays. Our data confirm that some $CD34^+$ subpopulations, such as $CD34^+CD38^+$HLA-$DR^-$ and $CD34^+CD117^-$ HLA-$DR^-$ cells, have a prevalence to proliferate in close contact to human stroma layers and particularly to AS.

Based on our data and the fact that the homing process by HSC is rather specific, the use of AS in the LTC assay can be proposed in some selected cases to better investigate the in vivo interactions between HPC and BM stroma in patients undergoing BMT or PBSCT and for assessment of the kinetics of recovery of patient BM microenvironment after chemotherapy and/or PBSCT [50]. In contrast, NBMS had a prevalent capacity to select in vitro more committed $CD34^+$ progenitor cells cultured in LTC assay. Our culture system also stressed the importance of assessing the progenitor content of the adherent layer, in line with two recent studies [51,52] that investigated the interaction between BM stroma and HSC by an expanding family of proteins, referred to as connexins. Our results could be explained by the occurrence of allogeneic mismatch between the selected $CD34^+$ cells and the type of stromal cells used in LTC assay. Sugiura et al. [53] recently provided further evidence that formation of cobblestone colonies under major histocompatibility complex (MHC)-mismatched stromal cells significantly decreased compared with MHC-matched stromal cells. This was related to MHC class I molecule mismatch [53].

With regard to clinical data, until now no clear correlations between the incidence of transplanted LTC-IC, doses of $CD34^+$ cell subsets infused, and long-term hematopoietic recovery after PBSCT were found [20,22,28,29], even if it is expected that LTC-IC would contribute more to later than early reconstitution following transplant [54]. Several authors found a positive correlation between the presence of certain $CD34^+$ cell subpopulations and the incidence of LTC-IC in apheresis products [55], which is in accordance with our own data showing that complete and durable platelet engraftment after auto-PBSCT was found to be influenced by $CD34^+CD117^-DR^-$ cell content, whereas the only $CD34^+$ cell subset independently associated with long-term neutrophil and erythrocyte recovery was the $CD34^+CD38^+$ HLA-$DR^-$ phenotype. These results suggest that stem cell subpopulations defined by negativity for CD117 and HLA-DR and positivity for CD38 antigen appeared to be more reliable predictors of long-term hematopoietic recovery rates than total $CD34^+$ cell dose infused, thus supporting the validity of our approach in this context. These $CD34^+$ cell subsets were able to identify patients who experienced delayed engraftment 3 to 6 months after PBSCT. Interestingly, the same two $CD34^+$ cell subsets showed the strongest correlation with LTC-CFC grown on AS, thus confirming the biologic role played by these progenitor cell subsets. Multivariate analysis also showed that in the first 3 months after auto-PBSCT, better neutrophil recovery was correlated with the number of transplanted LTC-CFC generated on AS. These in vitro data make a testable prediction that using NBMS or AS cells in LTC assay would improve the in vivo analyses of stem cell recovery after PBSCT. However, the patients examined in this study were mobilized with chemotherapy plus G-CSF. As a result, these conclusions may not apply to PBSC mobilized with G-CSF alone.

At this point, it should be said that our functional and phenotypic analyses were performed on selected $CD34^+$ cells. From a theoretical point of view, this could represent a bias of the study; however, some considerations must be made. First, it should be kept in mind that using both Ficoll and Percoll density gradients to establish culture assays causes a considerable loss of $CD34^+$ cells [56,57]. Second, multicolor flow cytometric analysis of LP-derived $CD34^+$ cell subsets is far from standardized, especially for samples having low percentages of $CD34^+$ cells. Based on these data, we thought it noteworthy to estimate the presence of the various $CD34^+$ cell subsets on purified $CD34^+$ cells, to minimize these methodologic problems. Selective loss of progenitor subsets following $CD34^+$ cell enrichment procedures has been documented in a few reports [58–60], raising the possibility that our analysis cannot predict the composition of the various progenitor cell subpopulations present in the whole LP.

In conclusion, these data further support the hypothesis that the type of stromal feeders influences the frequency of LTC-CFC, and that the use of human stromal layers could be more suitable for an in vitro study that more strictly analyzes the in vivo engraftment process following autologous PB-SCT. Furthermore, the $CD34^+$ cell subsets defined by CD117, CD38, and HLA-DR appear to be more reliable predictors of long-term hematopoietic recovery rates than total $CD34^+$ cell quantity. These data may help to predict the repopulation capacity of PBSC, especially when relatively low numbers of $CD34^+$ cells/kg ($<3 \times 10^6$/kg) are reinfused.

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