Long-term bone marrow culture data are the most powerful predictor of peripheral blood progenitor cell mobilization in healthy donors

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Background and Objectives. There is wide interindividual variation in progenitor cell mobilization. The present study was aimed to analyze steady state hematopoiesis in healthy donors and its influence on hematopoietic progenitor cell (HPC) mobilization.

Design and Methods. Bone marrow (BM) was aspirated from 72 healthy donors prior to administration of recombinant human granuloyte colony-stimulating factor (G-CSF). Analyses of CD34+ cells and semisolid cultures as well as long-term cultures were performed from BM or leukapheresis products.

Results. Male donors showed a higher number of BFU-E (p=0.007) and committed progenitors (p=0.05), a better stromal layer (p=0.02), and higher long-term bone marrow culture (LT-BMC) counts (p<0.05) when compared to those in female donors. When correlating the culture pattern of the BM with the data from the leukapheresis products, we observed that the number of the immature progenitors in BM correlated significantly with both the number of CD34+ cells and CFU-GM in the first leukapheresis. Univariate analysis revealed that the following variables had a beneficial impact on the number of CD34+ cells: male sex, body weight >73 Kg, G-CSF schedule and results of LT-BMC, although in the multivariate analysis only the number of CFU-GM obtained after LT-BMC showed a significant influence (p<0.001).

Interpretation and Conclusions. These results confirm the interindividual variation in HPC mobilization among healthy subjects, with LT-BMC counts being the most reliable predictor, expressing the behavior of the immature progenitors and their relationship with the microenvironment.

Key words: healthy donors, long-term bone marrow cultures, CD34+ cells, hematopoietic progenitor cells, mobilization.

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Peripheral blood progenitor stem cells (PBPSC) obtained after granulocyte-colony stimulating factor (G-CSF) administration are being increasingly used for autologous and allogeneic transplantation.[1] Allogeneic transplantation using peripheral blood instead of bone marrow stem cells is associated with faster neutrophil and platelet recovery, without a higher incidence of acute graft-versus-host disease (GVHD).[2,3] These events are probably due to a higher number of hematopoietic progenitor cells (HPC).[3,4] Glimm *et al.*[5] have also reported that PBPSC contain a higher number of short-term repopulating hematopoietic cells (STRC) that engraft sequentially in NOD/SCID-, β2 microglobulin-null mice. However, several studies indicate substantial interindividual variation in HPC mobilization among healthy donors.[6-8] Some donor characteristics such as age, sex and weight, as well as apheresis volume or G-CSF schedule have been identified as variables influencing CD34+ cell collection,[8-13] but there are several studies showing that there are poor mobilizers even among young, healthy donors.[8] As far as we know, the *in vitro* behavior of immature progenitor cells in healthy individuals has not been extensively analyzed by long-term bone marrow cultures (LT-BMC). Although cell cultures (especially LT-BMC) are not routinely performed in transplantation centers, the results of such assays are important for understanding the behavior of different types of progenitors and the mobilization pattern, which is more important from theoretical and intellectual points of view than it is of practical use.

The aim of the present study was to analyze healthy donors in order to ascertain whether there are individual characteristics influencing steady state hematopoiesis behavior. We also wanted to analyze whether hematopoietic parameters

could influence the final yield of leukapheresis products. For these reasons, the percentages of CD34+ cells, committed HPC and immature HPC (assessed by LT-BMC) were evaluated in bone marrow cells prior to mobilization and their influence on leukapheresis yields was assessed.

Design and Methods

Donors

Bone marrow cells and leukapheresis products from 72 healthy donors, considered for allogeneic hematopoietic stem cell transplantation were analyzed. The donors' characteristics are shown in Table 1. Their median age was 41 years (range 11 to 72) with a male/female ratio of 44/28. After informed consent had been obtained, a bone marrow aspiration was performed in all donors before starting recombinant human G-CSF. Donors received G-CSF at a dose of 5 μg/kg/day (19% of cases) or 5 μg/kg/12h (81% of cases) over at least four days, until the apheresis procedures had been completed according to the policy of our center; both protocols were used sequentially and each donor received the dose active at that moment. The collection of peripheral blood stem cells was started on the fifth day of G-CSF administration. The blood volume processed was two to three times the donor's total blood volume. Donors underwent 1 to 3 leukaphereses (median 1). Procedures were performed using a continuous flow cell separator (CS-3000 plus, Baxter Healthcare corp. Deerfield, USA or COBESpectra, COBE Inc. Colorado, USA). All patients were included in this study protocol after signed consent had been obtained according to the ethics committee of our institution. Flow-cytometry studies and cell cultures were performed in all healthy donors.

Flow cytometry studies

Whole bone marrow or leukapheresis product samples were stained using a stain-and-then-lyse direct immunofluorescence technique for analysis of the proportion of CD34+ cells, as previously described.[14] Data were acquired in two consecutive steps on a FACScalibur (Becton Dickinson Biosciences, San José, USA) flow cytometer using the CellQUEST software (BDB). In the first step, a total of 20,000 events/tube were acquired, and in the second step, acquisition throughout an electronic *live-gate* drawn on CD34+ cells was performed. In this latter step, 3×10^5 events were measured with information only obtained from those events fulfilling the live-gate criteria. The Paint-A-Gate PRO software program (BDB) was used for data analysis.

Table 1. Donors' characteristics.

N	72
Age (years)	41 (11-72)
Body weight (Kg)	73 (28-108)
Number of aphereses performed	
1	n=52
2	n=16
3	n=4
Number of mononuclear cells in 1st apheresis$\times10^8$/kg	5.3 (0.3-10)
Total number of MNC in 1st apheresis$\times10^{10}$	3.9 (0.2-8.6)
Number of CD34+ cells in 1st apheresis$\times10^6$/kg	3.8 (0.8-11)
Total number of CD34+ cells in 1st apheresis$\times10^7$	28.4 (6.9-80.1)

Results expressed as median (range).

Cell cultures

Mononuclear cells from bone marrow and leukapheresis products were used for semisolid and long-term cultures using methods previously reported.[15] For clonogenic assays, mononuclear cells (MNC) were separated by centrifugation on a Ficoll Hypaque gradient (Lymphoprep™, Niergaard Co., Oslo) (d=1077 g/mL). Interface cells were washed and resuspended on IMDM supplemented with FCS. The cells for long-term cultures were obtained by gravity sedimentation using a solution of 0.1% methylcellulose. The cells remaining in suspension were washed with IMDM-FCS.

Clonogenic assays

These assays were carried out, according to previously described methodology,[16-18] in order to evaluate the committed HPC (CFU-GM, BFU-E and CFU-Mix) from bone marrow. In the leukapheresis products only CFU-GM were analyzed. We considered as CFU-GM colonies those colonies with a translucent appearance that contained granulocytes or macrophages or both. In our laboratory, colonies are considered to be more than 40 cells in an aggregate, whereas smaller numbers (5-<40 cells) are considered to be clusters. The BFU-E colonies displayed a burst configuration of three to eight closely arranged erythroid clusters (mature BFU-E) or of more than eight clusters (primitive BFU-E), both dependent on erythropoietin being added to the culture. CFU-Mix colonies usually contained an erythroid component as well as cells from two of the other myeloid lineages. CFU-Mix showed a focus of hemoglobinized red cells and, at the same time, areas of dispersed translucent cells similar to parts of the granulocyte-macrophage colonies.

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Table 2. Semi-solid and LT-BMC from steady-state bone marrow (n=72).

CFU-GM*	196±184
BFU-E*	85±13
CFU-Mix*	6±13
Total CFU-GM in LT-BMC°	10864±7345
Total cells in LT-BMC (×10⁶)°	6.3±2.3
% Stromal layer°	90±71

**Number of colonies/10⁵ cells plated. °Total number of CFU-GM and MNC obtained during the whole period of LTBMC/10×10⁶ cells plated. Results expressed as mean±SD.*

Table 3. Relationship between the final numbers of CFU-GM obtained from LT-BMC and committed progenitors in BM.

R	*p*	
CFU-GM from LT-BMC and total number of progenitors (CFU-GM, BFU-E, CFU-Mix) in BM	0.54	<0.001
CFU-GM from LT-BMC and total number of CFU-E in BM	0.48	<0.001

All the tests were performed in all patients (n=72).

Cultures were performed as previously described.[15] Plating efficiency was defined as the number of colonies produced by 10^5 plated cells.

Long-term bone marrow cultures

LTBMC were established in order to analyze the immature HPC in bone marrow and were carried out according to the method of Gartner and Kaplan with slight modifications. Briefly, 2×10^6 cells/mL were inoculated in tissue culture flasks in LTBMC medium IMDM supplemented with 10% pre-selected fetal calf serum (FCS, Biowhittaker, Belgium), 10% horse serum (HS, PAA Laboratories, GmbH, Austria) and 5×10^{-7} M hydrocortisone sodium succinate. The cultures were incubated in a humidified atmosphere with 5% CO_2 in air at 33°C for eight weeks. At weekly intervals before re-feeding, the stromal layer formation was studied under an inverted microscope. The degree of confluence and the presence of adipocytes and cobblestone areas of hematopoiesis were assessed and four stromal layer subtypes were established: (i) with all cellular components; (ii) without adipocytes; (iii) without adipocytes or cobblestone areas; and (iv) without stromal layer formation. For re-feeding, half of the supernatant was removed and replaced with fresh LTBMC medium. The non-adherent cells harvested were counted and assayed for their CFU-GM content. After 8 weeks of culture the whole supernatant was removed and the adherent layer was detached by exposure to trypsin. Cells were recovered, washed, counted and assayed for their CFU-GM content.

Long-term cultures from leukapheresis products

These assays were performed in a two-step procedure: stromal layers were established with bone marrow from a volunteer donor. When at least 70% of the flask's surface was covered by the layer, the flask was irradiated with 15 Gy in a Cobalt bomb (Theratron 780). A second inoculum to obtain hematopoiesis was constituted by leukapheresis mononuclear cells. CFU-GM progenitors were evaluated during 8 weeks, as for the one stage long-term culture.

Statistical analysis

Statistical computations were carried out with the SPSS 10.0 program. The following tests were used: Student's T-test for unpaired data, Pearson's or Spearman's test for quantitative correlation and multivariant stepwise analyses.

Results

PBPSC collection results

As shown in Table 1, only one apheresis procedure was necessary for the majority of donors (72%) and three aphereses were necessary in only four cases. The mean number of mononuclear cells and $CD34^+$ cells obtained in the first leukapheresis was $5.3\pm1.9\times10^8$/kg and $4.5\pm2.3\times10^6$/kg, respectively.

Results from bone marrow samples

Results of semisolid and long-term cultures obtained from steady state donor bone marrow samples are shown in Table 2. The sum of all committed progenitors (CFU-GM+BFU-E+CFU-Mix) obtained from 10^5 plated cells was 285±263, with CFU-GM being the most frequent progenitors and CFU-Mix the least frequent. In LT-BMC the production of CFU-GM was extremely heterogeneous, as reflected by the wide range of the number of colonies (374 to 31601 colonies/10×10^6 plated cells).

The mean percentage of $CD34^+$ cells obtained in bone marrow was 0.93%±0.5. No significant correlation between these values and the number of bone marrow progenitors (either committed, analyzed by semisolid cultures, or immature, analyzed by LTBMC) was obtained. By contrast, when the steady-state hematopoiesis was analyzed, there was a clear, positive correlation between the numbers of committed stem cells and the number of immature ones analyzed by LT-BMC (Table 3).

Certain donor characteristics were associated with differences found in the number of colonies obtained both in the semisolid cultures and the LT-BMC (Table

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Table 4. Influence of donor characteristics on the number of progenitor cells in bone marrow.

Donor characteristics	*CFU-GM (×10⁵ cells plated)*	*BFU-E (×10⁵ cells plated)*	*CFU-Mix (×10⁵ cells plated)*	*Total progenitors (×10⁵ cells plated)*	*% SL in LT-BMC (×10⁷ cells plated)*	*Components of SL (×10⁷ cells plated)*
Sex						
M	218±219	105±105	6±11	328±314	80±15	0.98±0.15
F	161±106	53±51°	6±16	219±136*	63±25*	0.82±0.39*
Body weight						
<73 kg	174±120	73±69	3±4*	248±173	73±20	0.94±0.23
>73 kg	217±230	96±108	9±17	321±326	74±22	0.89±0.32
CD34+ in BM						
<0.93	153±96	64±52	10±22	225±128	69±19	0.86±0.36
>0.93	184±199	104±107	9±16	296±304	67±29	0.76±0.43

*All the tests were performed in all patients (n=72). Results are expressed as mean±SD per 10⁵ cells plated for CFU-GM, CFU-E; CFU-Mix and total progenitors, and per 10x10⁶ cells plated for %SL. *p<0.05; °p<0.01. % SL: percentage of flask covered by the stromal layer. Components of SL: 1= all components present in the stromal layer (normality).*

4). For example, when a fixed number of cells was plated, compared to female donors, male donors had a higher number of BFU-E (p=0.007), higher number of total progenitors in semisolid cultures (p=0.05) and a better stromal layer formation (p=0.02) with more cellular components. Moreover, donors with a body weight above 73 kg produced more progenitors in culture, although differences only reached statistical significance for CFU-Mix (p=0.04). When the age of donors and percentage of CD34⁺ cells in bone marrow were analyzed, no statistical differences were found for any of the cut-off points used.

In the LTBMC studies, we observed that male donors showed higher cell counts (both CFU-GM and mononuclear cells) for values analyzed each week, and they also showed greater stroma formation with more cellular components, which reflects a better microenvironment. These differences between LTBMC from male and female donors were statistically significant (p<0.05).

Results from leukaphereses

Table 5 shows the plating efficiency and absolute numbers of CFU-GM generated both in the first and in all subsequent leukaphereses performed. A wide range of inter-individual variation was observed. The median number of CFU-GM in the first leukapheresis was 211 colonies /10⁵ cells plated (range 9-1800).

Correlation between steady state bone marrow and leukapheresis products

Relating the culture pattern of the steady state bone marrow with the data from the leukapheresis products, we observed that the number of the immature progenitors in the bone marrow (studied by counting the number of CFU-GM produced throughout the LTBMC) significantly influenced both the number of CD34⁺ cells (r=0.56; p<0.0001) (Figure 1), and, to a lesser extent, CFU-GM present in the first leukapheresis product (r=0.45; p=0.001) and in the final apheresis (r=0.40; p=0.006). By contrast, neither the committed bone marrow progenitor cells nor the percentage of CD34⁺ cells in bone marrow showed a significant relationship with the quality of the apheresis.

Table 5. Results of cell cultures from leukapheresis products.

N. of CFU-GM/10⁵ cells plated in first leukapheresis	211 (9-1800)
N. of CFU-GM/10⁵ cells plated in all the leukaphereses	262 (38-2540)
Total number of colonies CFU-GM in the 1st leukapheresis (×10⁵)	270 (7-1450)
Total number of colonies CFU-GM in all the leukaphereses (×10⁵)	360 (5-1450)

All the tests were performed in all patients (n=72). The results are expressed as the median (range).

Factors influencing the PBPSC collection

In order to ascertain their predictive value, we analyzed a series of clinical and culture assay variables. Univariate analysis revealed that the following variables had a beneficial impact on the yield of CD34⁺ cells or mononuclear cells (Table 6): male sex, body weight above 73 Kg, dose of 5 μg/kg/12h of G-CSF and a higher number of CFU-GM in LTBMC. Furthermore, male sex was associated with a lower number of leukaphereses, with differences close to statistical significance (p=0.06). In order to ensure that the donor's body weight did not condition the differences in results, the parameter was independently analyzed and produced similar results with only one new variable, sex, showing an impact on

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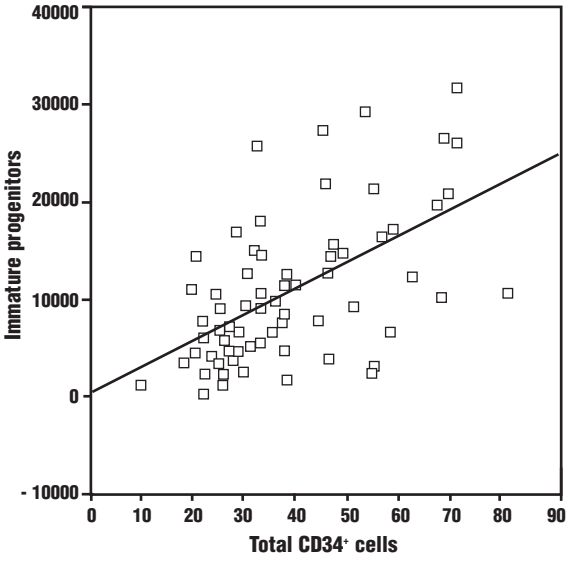

Figure 1. Correlation between the number of immature progenitors (CFU-GM produced throughout the long-term culture) and CD34$^+$ cells.

CFU-GM yields (p=0.017). When a multivariate analysis was performed in order to assess which variables could have an independent impact on CD34$^+$ cell mobilization, only the total number of CFU-GM generated after LTBMC showed a significant influence (p<0.001). This significance was maintained when the body donor's weight was considered.

Discussion

Long-term cultures are used to analyze primitive hematopoietic stem cells and their relationship with the bone marrow microenvironment[19,20] while clonogenic assays are used to analyze committed hematopoietic progenitor cells. Taken together, both types of assay offer a comprehensive measurement of marrow function.

Peripheral blood hematopoietic progenitor cells have been successfully employed in autologous and allogeneic transplantation and their use has been associated with a significantly faster hematologic recovery than that afforded by bone marrow progenitor cells. This effect is probably related to the fact that the leukapheresis product contains several fold more progenitor cells than does bone marrow[4] but also because higher numbers of STRC are present in mobilized peripheral blood.[5] However, it has frequently been reported that a wide interindividual variation exists in the characteristics of peripheral blood progenitor mobilizations among healthy donors.[6–13] This is why continuous studies are carried out on steady-state hematopoiesis in healthy donors. In the present work we analyzed how different parameters of steady-state hematopoiesis, as well as other individual characteristics (such age or weight) or the G-CSF schedule, may influence final leukapheresis yields. Regarding the behavior of bone marrow progenitors, a great degree of variability in the

Table 6. Factors influencing progenitor cell mobilization (univariate analysis).

	CD34+ cell in) 1st apheresis (×10^7)	*Total n° of CD34+cells (×10^7)*	*MNC number in 1st apheresis (×10^{10})*	*Total number of MNC (×10^{10})*	*CFU-GM in 1st apheresis (×10^6)*	*Total n. of CFU-GM (×10^6)*	*Apheresis number*	*Total n. of CFU-GM in LT-BMC*
Sex								
Male	36.4±18.9	41.3±17.5	3.8±1.5	4.3±1.7	48±40	52±39	1.2±0.5	12424±7799
Female	26.5±13.9*	33.4±11.5*	3.8±1.6	5.3±2.5	31±40	46±44	1.5±0.7	8448±5924
Age								
<42	30.7 ±16.9	35.2±15.6	3.5±1.5	4.2±1.8*	48±42	54±42	1.2±0.4	11079±7177
>42	34.3±18.5	41.1±15.8	4.1±1.6	5.2±2.2	36±39	46±40	1.5±0.7	10638±7617
Body weight								
<73	29.3±17.0	35.5±13.1	3.4±1.2*	4.7±2.3	35±44	46±45	1.5±0.7	9557±7237
>73	36.0±18.0	41.1±18.0	4.2±1.7	4.7±1.9	47±37	54±37	1.2±0.1*	12172±7318
G-CSF								
5	30.9±18.8	36.1±14.9	4.0±2.1	4.6±1.9	27±32	31±31*	1.5±0.8	N.V.
5/12h	31.4±15.6	36.1±15.3	3.6±1.5	4.3±2.0	50±40	60±41	1.2±0.4	
%CD34$^+$								
<0.93	27.1±13.7	29.5±11.9	3.8±2.3	4.2±2.5	46±42	51±39	1.3±0.6	8652±3925
In BM								
>0.93	39.3±21.7	40.3±20.3	4.0±1.1	4.2±1.2	57±49	57±48	1.1±0.5	10701±9217

*All the tests were performed in all patients (n=72). Results expressed as mean±SD; *p<0.05; N.V.: not valuable. Total n° of CFU-GM in LTBMC: total number of CFU-GM obtained during the whole period of LTBMC/10×10^6 cells plated.*

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plating efficiency and number of immature progenitors in bone marrow samples from healthy donors was observed, showing that there is considerably interindividual variability in hematopoeisis. Interestingly, differences were particularly striking between sexes with males showing a higher production of all types of progenitors and, moreover, a better stroma confluence, and hence a better microenvironment. As far as we know, this feature (the influence of donor sex on the number of all hematopoietic progenitors and stroma formation) has not been previously reported. However a higher proportion of erythropoietic precursors in men has been related to the stimulatory effect of androgens.[21]

The second goal of the present study was to analyze whether steady-state hematopoiesis could influence the yields of leukapheresis. We found that the only parameter which correlated with the final number of progenitor cells obtained in leukapheresis was the number of CFU-GM during LTBMC. It is well known that these progenitors reflect the number of immature hematopoietic progenitors in bone marrow, which, theoretically, will be mobilized into the peripheral blood after G-CSF administration. Our results confirm, using a multivariate approach, those published recently by Carlo-Stella *et al.*[22] who showed that steady state marrow LTC-IC had a clear relationship with the $CD34^+$ and CFU-GM cell yields in leukapheresis after G-CSF mobilization.

In previous studies it was observed that some donor characteristics influenced peripheral blood progenitor cell mobilization.[7,10-12,23-25] Data from the Spanish Registry[8] showed that age (above vs below 38 years) and G-CSF schedule (once vs twice a day) are the most powerful predictors of $CD34^+$cell mobilization. However, poor mobilizers have been observed in all age groups.[8] In the present analysis the clinical factors that influenced the peripheral blood mobilization were: age, male sex, G-CSF schedule, higher body weight and a higher percentage of $CD34^+$ cells in the bone marrow. However, these influences on mobilization were only observed using univariate analysis. The influence of age has been observed by other authors;[8] in fact, older donors show a poorer apheresis yield. It should be noted that among our group only 5 donors were older than 60 years. When a multivariate analysis was performed, the only variable that retained independent predictive value was the number of immature progenitors, as detected by LT-BMC cultures. Previous analyses[26] in the autologous transplantation setting have shown the relevance of immature progenitors on peripheral blood cell recovery. In the present study these results were not confirmed in the allogeneic setting. This could be due to the fact that a second apheresis was performed when not enough cells were obtained with the first apheresis and this could have disguised the real impact of the immature progenitor cells.

Taken together, our results confirm the interindividual variation in progenitor cell mobilization among healthy subjects. Some individual donor characteristics (sex, G-CSF schedule) can influence cell yields after mobilization. However, the only independent parameter that influences mobilization is the production of CFU-GM in LTBMC which reflects the behavior of the immature progenitors and their relationship with the microenvironment. Although this kind of analysis is of no clinical value because of the long period that is required for their results, our analysis shows that there are poor mobilizers, even among young donors, because of an inefficiency in mobilizing hematopoiesis. These endogenous differences in hematopoiesis and their possible causes are of great interest for further analysis and point to new ways to overcome such problems in healthy donors.

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NLH, CP and MCC were responsible for the conception of the study and interpretation of results. NLH and CP performed the statistical analysis and wrote the manuscript. All authors critically revised the paper and give the final approval for its submission. The order in which the names of the authors appear is based on their contribution to the study. JFSM and MCC, as heads of department and laboratory, are cited last. The authors declare that they have no potential conflict of interest.

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