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Hematopoietic damage prior to PBSCT and its influence on hematopoietic recovery

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Abstract

Background and Objective. **Patients with malignancies receive chemotherapy to induce tumor remission which could damage hematopoiesis and adversely influence hematopoietic reconstitution after transplantation. In the present study we used a long term culture (LTBMC) system and clonogenic assays to evaluate the marrow damage in patients selected to receive peripheral blood stem cell transplantation (PBSCT).**

Design and Methods. **Thirty-five patients – 20 with breast cancer (BC), 9 with non-Hodgkin's lymphoma (NHL) and 6 with Hodgkin's disease (HD) – were included. Bone marrow aspiration was performed one day prior to the initiation of the conditioning therapy. CFU-GM were cultured in methylcellulose with PHA-LCM. Delta assays of plastic adherent progenitor cells (PD) were performed according to Gordon's method. LTBMC were established for 5 weeks.**

Results. **CFU-GM from all patient groups were lower than from normal BM ($p<0.05$). In contrast, immature progenitor cells (PD) were not decreased. The total number of CFU-GM produced by LTBMC patients was significantly reduced ($p<0.05$). The adherent layer from patients was often qualitatively different. In order to know whether the hematopoietic damage could affect hematopoietic reconstitution, we correlated culture data with time taken to reach peripheral cell counts. A negative correlation (r=-0.71) was found between percentage of stromal layer and time taken to reach 20×10^9 platelets/L ($t_{plat}= 20\times3$-0.08% stromal layer).**

Interpretation and Conclusions. **We can conclude that prior to PBSCT, hematopoietic function is impaired at both the level of committed progenitor cells and that of BM stroma. This damage could influence platelet recovery.**

Key words: hematopoietic damage, clonogenic assays, long term bone marrow cultures, platelet recovery, plastic Delta assays

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Autologous peripheral blood stem cell transplantation results in trilineage recovery within two weeks. However, in some cases after transplantation there is a delayed or incomplete peripheral blood count recovery[1-3] and some studies have also pointed to a prolonged impairment of hematopoiesis after transplantation.[4,5] The number of progenitor cells infused is important because it correlates with the rate of hematopoietic reconstitution,[6-10] but effective hemopoiesis is the result of the interplay between hemopoietic progenitor cells and supporting stroma.[11]

Long term bone marrow cultures (LTBMC) are employed as an *in vitro* model for *in vivo* hematopoiesis. This experimental model allows detection of most primitive hematopoietic stem cells with long-term reconstituting potential and the study of the bone marrow microenvironment.[12,13]

Although, obviously, conditioning regimens can damage hematopoiesis, studying the influence of this damage on hematopoietic recovery is problematic because establishing cell cultures a short time after transplantation is very difficult. The chemo and/or radiotherapeutic schemes used for the treatment of hematologic malignancies can also damage stroma and hematopoietic progenitor cells.[14,15] This effect may be particularly deleterious for hematopoietic engraftment, but as far as we know no study has been performed analyzing the influence on hematopoietic damage previous to PBSCT and hematopoietic recovery after transplantation. This pre-transplant information may be relevant in further clinical decision making.

In the present study we used a long term culture (LTC) system and clonogenic assays to evaluate the marrow damage in patients selected to receive peripheral blood stem cell transplantation (PBSCT) and to study the influence of this damage on hematopoietic recovery.

Design and Methods

Patients

Thirty-five patients selected for PBSCT were included in the study. The patients had been diagnosed as follows: 20 with breast cancer (BC), 9 with non-

Hodgkin's lymphoma (NHL) and 6 with Hodgkin's disease (HD). Clinical data and treatment received before the conditioning regimen for transplantation are shown in Table 1. Median age was 45 years and the male/female ratio was 9/26. Chemotherapy regimens included CAF or superCAF (cyclophosphamide + adriamycin + 5Fu) for BC patients (20% received both treatments), CHOP or VECOP-B for NHL and MOPP/ABV or ABVD for HD as first line chemotherapy. When patients with lymphoma (HD and NHL) did not reach a complete remission or a good partial remission, salvage therapy with mini-BEAM (BCNU 60 mg/m^2 $\times$1, Ara-C 200 mg/m^2 $\times$4, VP16 75 mg/m^2 $\times$4, melphalan 30 mg/m^2 $\times$1) or ESAP (cisplatin 25 mg/m^2 $\times$4, VP16 40 mg/m^2 $\times$4 and Ara-C 2 g/m^2 $\times$1) was used. Conditioning regimens were BEAM (BCNU 300 mg/m^2 $\times$1, VP16 400 mg/m^2 $\times$4, Ara-C 400 mg/m^2 $\times$4 and melphalan 100 mg/m^2 $\times$1) for HD and NHL and cyclophosphamide 1.5 g/m^2 $\times$4 + carboplatin 200 m/m^2 $\times$4 and thiotepa 125 mg/m^2 $\times$4 in BC patients.

Peripheral blood stem cells were mobilized in steady state using 5 µg/kg of G-CSF; When an appropriate number of progenitor cells was not reached a second mobilization using 10 µg/kg of G-CSF was performed. The characteristics of the infused cells are shown in Table 2. Patients reached more than 0.5$\times$10^9/L granulocytes in a median of 11 days (8-21) and 20$\times$10^9/L platelets in 12 days (9-19).

Control group

Forty-eight normal subjects who gave informed consent were used as controls; 34 were patients who had undrgone orthopedic or plastic surgery, and 14 were normal donors for allogeneic BMT (Table 1).

Methods

Marrow processing. In patients, bone marrow aspiration was performed one day prior to the initiation of the conditioning regimen for transplantation. After informed consent, BM was obtained from the posterior iliac crest or via a sternal puncture. All experiments were performed with fresh BM cells.

For clonogenic CFU-GM and delta assays, mononuclear cells (MNC) were separated by centrifugation on a Ficoll Hypaque gradient (Lymphoprep™, Niergaard Co., Oslo, Norway) (d=1077 g/mL). Interface cells were washed and resuspended on Iscove's modified Dulbecco's medium (IMDM) supplemented with FCS. For LTC, cells were obtained by gravity sedimentation using a solution of 0.1% methylcellulose. The cells remaining in suspension were washed with IMDM-FCS.[16]

Clonogenic assays. CFU-GM were cultured as previously described.[17] Briefly, 2$\times$10^5 mononuclear cells /mL in IMDM were plated on 35 mm Petri dishes in 0.9% methylcellulose containing 10% PHA-leukocyte conditioned medium, 10% bovine serum albumin and 10% human AB serum. Cultures were incubated at 37ºC in a fully humidified atmosphere with 5% CO_2 and scored on day 14 under an inverted microscope. Colonies were considered when aggregates with more than 40 cells and cluster aggregates with 4 to 40 cells were observed.

Delta assays of plastic adherent progenitor cells (PΔ) were performed according Gordon's method.[18] One per 10^7 MNC were placed in 25 cm^2 plastic tissue culture flasks and incubated for 2 h. The non-adherent cells were removed. Adherent cells were incubated for a further 7 days in IMDM with 10% FCS and HS. Afterwards non-adherent cells were pelleted and assayed for CFU-GM. The score of these cultures was performed after 14 and 21 days of incubation. Results were expressed as the total number of CFU-GM obtained after clonogenic assays.

Long-term BM cultures. Long-term BM cultures (LTBMC) were established using Gartner and Kaplan's method with slight modificatons.[16] Briefly, 2$\times$10^6 cells/mL were inoculated in tissue culture flasks in LTBMC medium (350 mOsm/kg) supplemented with 10% preselected FCS (Biowhittaker), 10% horse serum (HS) (Labclinics) and 5$\times$10^{-7} M hydrocortisone sodium succinate (Sigma). The cultures were incubated in a humidified atmosphere with 5% CO_2 in air at 33ºC for five weeks. At weekly intervals before refeeding, the stromal layer formation was studied under an inverted microscope. The degree of confluence, the presence of adipocytes and

Table 1. Clinical characteristics of patients and controls.

	Patients	*Chemotherapy*	*Controls*
n	35		48
Age, median (range)	45 (14-64)		36 (18-60)
Sex, male/female	9/26		33/15
Diagnosis			
BC	20	CAF and/or superCAF	OS 34
NHL	9	CHOP / VECOP-B	BMd 14
HD	6	MOPP-ABV/ABVD	

BC: breast cancer; NHL: non-Hodgkin's lymphomas; HD: Hogkin's disease; OS: orthopedic surgery; BMd: allogeneic bone marrow donors.

Table 2. Quality of PBSCT infusion.

	Nº of cells (mean±SD)
MNC $\times$10^8/kg	5.8±2.6
CD34$^+$ cells $\times$10^6/kg	1.9±1.2
CFU-GM $\times$10^4/kg	44.5±48

MNC: mononuclear cells. Results expressed as mean ± standard deviation.

Table 3. Clonal and LTBM cultures prior to PBSCT.

Diagnosis	*CFU-GM /10^5 cells plated*	*LTBMC CFU-GM#*	*Stromal layer (%)#*
BC	39±40°	1634±2299°	67±35
n=20	24 (1-128)	615 (0-9,183)	75 (0-100)
NHL	44±40°	1062±922°	77±20
n=9	31 (5-124)	698 (138-2,514)	80 (40-100)
HD	37±27*	1,183±966*	65±33
n=6	35 (4-73)	1,154 (96-2,155)	80 (20-100)
All patients	40±37°	,1416±1,852°	69±31
n=35	31 (1-128)	677 (0-9,183)	80 (0-100)
Control	150±180	5735±9,295	79±21
n=48	96 (2-917)	2,996(9-60,396)	80 (10-100).

*Results are expressed as mean±SD and median (range) *p< 0.05; °p<0.01. BC: breast cancer; NHL: non-Hodgkin's lymphoma; HD: Hodgkin's disease; LTBMC/CFU-GM: Progenitors obtained from long term bone marrow culture throughout the culture period. #Results obtained from 10×10^6 cells plated. %:percentage of flask surface covered by the stromal layer.*

Table 4. Immature progenitor cells analyzed by Delta assays.

Diagnosis	*Day +14*	*Day+21*
BC (n=16)	4 (0-630)	9 (0-30)
NHL (n=3)	3 (2-8)	3(2-15)
HD (n=3)	38 (37-39)	38 (37-39)
All patients (n=22)	4 (0-630)	5 (0-38)
Controls (n=7)	7 (0-15)	14 (0-15)

BC: breast cancer; NHL: non-Hodgkin's lymphomas; HD: Hodgkin's disease; Day +14 or +21: progenitors scored 14 or 21 after after culture. Results expressed as median and range. Results from 5×10^6 cells plated.

cobblestone areas of hemopoiesis were assessed. For refeeding, 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 5 weeks of culture the total 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.

Statistical analysis was carried out with the SPSSG.1 program for Apple PowerMac®. The following non-parametric tests were used: Mann-Whitney's U test for unpaired variables, Pearson's test for quantitative correlation and stepwise analysis for multiple correlation.

Results

Hematopoietic function before PBSCT

Tables 3 and 4 show the results of the parameters analyzed in clonogenic and long-term BM cultures and comparison of the results obtained in different groups of patients with the results from the control group.

Clonogenic assays. Growth was heterogeneous within each group (Table 3). The number of determined granulomonocytic progenitor cells was significantly reduced in all patient groups compared with the number from normal BM ($p<0.05$) (Table 3). By contrast, the most immature progenitor cells studied by PΔ assays did not decrease. It should be stressed that BM from the three cases of HD analyzed by PΔ assays showed a higher number of immature progenitor cells than normal BM or BM from other patient groups (Table 4).

Long term BM cultures. From week 1 to week 5 of culture the number of CFU-GM in the culture supernatant of patients was lower than that from normal BM (Figure 1) and therefore the total number of CFU-GM produced by LTBMC patients was significantly lower than that from the controls. No statistical difference was observed between the three patient groups (Table 3). Although some patients showed a decreased stromal layer confluence, these changes did not translate into a significant difference when compared with controls (Table 3).

Weekly microscopic examination showed that the adherent layer from patients was often qualitatively different from that from the controls and at week 5 no adipocytes were present in the stromal layer from 40% of patients. This alteration was more frequent in BC patients while it appeared in only one patient with HD (Figure 2).

When we analyzed the relationship between the stroma and the production of progenitors we observed that there was a positive correlation between

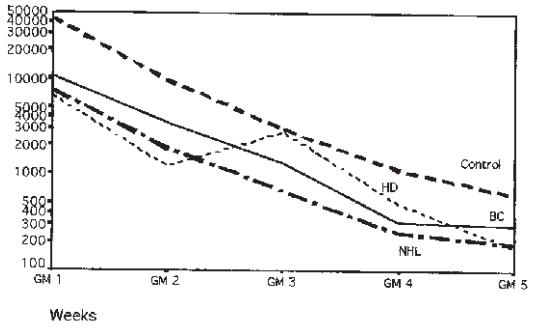

Figure 1. CFU-GM from long term marrow cultures. Evolution during five weeks of the culture. *BC: breast cancer; NHL: non-Hodgkin's lymphomas; HD: Hodgkin's disease.*

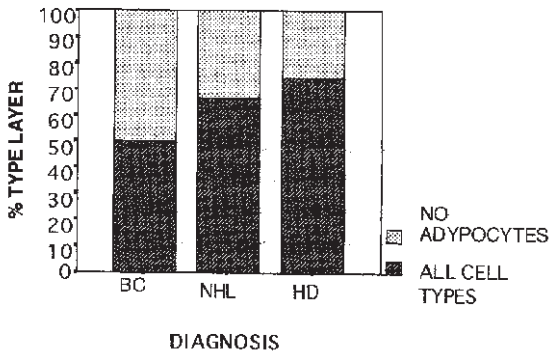

Figure 2. Morphologic appearance of stromal layers in different patient groups. *BC: breast cancer; NHL: non-Hodgkin's lymphomas; HD: Hodgkin's disease.*

the stromal layer confluence and CFU-GM production in HD patients (r= 0.78), but no correlation was found with the other patient groups.

Relationship between chemotherapy received and hematopoietic damage prior to PBSCT

No relationship was observed between the number of cycles of chemotherapy received and the hematopoietic parameters analyzed in cell cultures. However patients who received both cycles (CAF + super-CAFin BC) or salvage therapy (mini-BEAM or ESAP in lymphoma patients) showed decreased stromal layer confluence ($p<0.05$) and a different cell composition of the BM microenvironment ($p=0.005$) (Table 5).

Correlation between culture parameters and hematopoietic recovery after PBSCT

The number of CFU-GM/10^5 cells plated, the total number of CFU-GM produced during 5 weeks in LTBMC and the confluence of stromal layer at 5 weeks were tested for correlation with the engraftment assessed by: time to reach 0.5×10^9 granulocytes/L and 20×10^9 platelets/L. None of these parameters correlated with the time necessary to reach $>5\times10^5$/L granulocytes. A negative correlation (r = –0.71) was, however, found between the percentage of stromal layer and the time to reach 20×10^9 platelets/L with the following equation: t_{plat}= 20×3-0.08% stromal layer (Figure 3), t_{plat} being the time required to reach more than 20×10^6/L platelets.

Correlation between stromal damage prior to PBSCT and CD34⁺ cell mobilization

To ascertain whether stromal damage occurring prior to PBSCT could condition a lower CD34⁺ mobilization we related culture parameters with the number of CD34⁺ cells mobilized and used for transplantation. No relationship was observed (correlation between percentage of stromal layer and CD34⁺ cells: r=.006; p=.97)

Table 5. Relationship between chemotherapy and stroma formation.

*Salvage therapy**	*% stromal layer*	*Type of SL*
No	79±19 80 (40-100)	1±0 1 (1-2)
Yes	47±30° 40 (20-80)	2±1° 2 (1-2)

*Results expressed as mean±SD and median (range). Type of SL: type of stromal layer formed (see text). *Patients who received BEAM or ESAP after first line therapy and patients who received super-CAF after CAF; °p<0.05 when compared with normal bone marrow.*

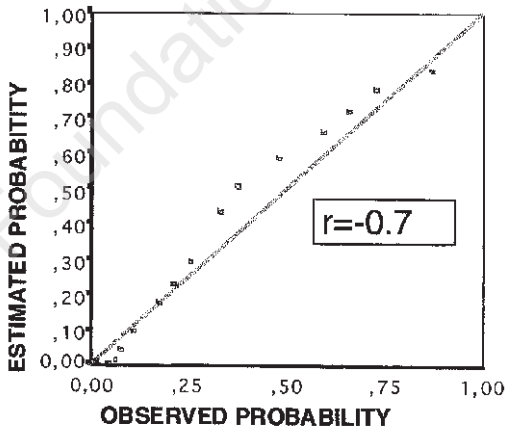

Figure 3. Scattergram: probability of reaching $>20\times10^9$/L platelets.

Discussion

Patients with solid tumors or hematologic malignancies receive, prior to conditioning regimens, chemotherapy and/or radiotherapy to induce tumor remission. It is well known that both therapeutic approaches can damage hematopoiesis.[14,15] This damage could adversely influence hematopoietic reconstitution after transplantation. The present study shows that prior to PBSCT, there are alterations in both hematopoietic progenitors and marrow stroma. Changes in the latter can delay platelet recovery after PBSCT.

To analyze the hematopoietic damage we used clonogenic assays (CFU-GM and PΔ) and LTBMC in order to obtain information about the hematopoietic function in both progenitor cells and stroma. Our study shows that the number of committed progenitors was reduced in all patient groups. These results

partially contrast with those of Domenech *et al*.[5] who did not observe a reduced number of CFU-GM although the number of BFU-E and CFU-MK had decreased in their study. Therefore, in accordance with our observation, it can be concluded that chemotherapy induces damage at the level of committed progenitor cells.

LTBMC studies also showed a decreased CFU-GM production , and this decrease was present throughout the study period, showing that immature progenitors were damaged by the previous treatment. This observation was common to all patient groups indicating that all the therapeutic approaches used in this study can damage the hematopoietic progenitor cells in a similar way. However, the reduction of CFU-GM production could be due to damage to BM stroma. To analyze immature progenitor cells we used plastic adherent delta assays (PΔ). It has been claimed that cells analyzed with this assay are capable of sustaining long-term hematopoiesis *in vitro*, independently of stromal function.[19] Using this approach, no differences were observed between patients and controls. Moreover, in HD patients the number of immature progenitor cells increased. One justification for the higher number of Plastic Delta progenitor cells could be that immature progenitor cells are generally more resistant to chemotherapy. When the stromal function was analyzed by the assessment of percentage of flask surface covered by the stromal layer no significant differences were observed between patients and controls, although in some patients the stromal layer did not reach confluence. Moreover, when morphologic studies were performed, 40% of patients did not have adipocytes in their stroma. Although the function of adipocytes is not well understood, their presence in LTBMC is associated with hematopoietic-productive cultures.[20] Their absence may point to abnormal stromal function.

In summary, all these data indicate that prior to PBSCT there is evidence of hematopoietic damage induced by chemotherapy. This damage affects both committed progenitor cells and BM stroma, while immature progenitor cells are better preserved.

The time required for hematologic reconstitution after PBSCT has been repeatedly correlated with the number of hematopoietic progenitor cells infused (both $CD34^+$ cells and/or CFU-GM),[6-9] but other parameters could affect it. In order to find out if the hematopoietic damage present prior to PBSCT could influence hematopoietic recovery, we have correlated several culture parameters with the time to achieve $>5\times10^5$/L granulocyte and $>20\times10^6$/L platelet counts. We observed that the percentage of stromal layer inversely correlated with the number of days to achieve more than 20×10^9 platelets/L; the larger the stromal layer the briefer the platelet engraftment. This fact together with the lack of correlation with time to achieve the appropiate number of granulocytes could have two possible explanations:

a) stromal function has a more relevant effect on megakaryocytopoiesis than on granulocytopoiesis. It must be stressed that, it has recently been shown that BM stromal thrombopoietin plays an essential role in megakaryopoiesis.[21] The alteration in the stroma cells could cause a decrease in thrombopoietin production;
b) the time required to achieve the defined granulocyte numbers after PBSCT is so short that only if the hematopoietic damage is very marked will it have a statistically significant influence on the recovery.

We can conclude from these results that prior to PBSCT, hematopoietic function is impaired at the level of both, committed progenitor cells and BM stroma. This damage may influence platelet recovery after the transplantation procedure. Further studies are necessary to ascertain the influence of these findings on long-term hematopoietic function.

Contributions and Acknowledgments

MCdC designed the study. NL, MEF and AB carried out the culture assays. LV, MDC, MLA, MVM and NG were responsible for the clinical follow-up of patients. JFSM oversaw the work and revised the manuscript. JFSM is the last name because is the head of the department in which the study was performed.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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