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An epirubicin/paclitaxel combination mobilizes large amounts of hematopoietic progenitor cells in patients with metastatic breast cancer showing optimal response to the same chemotherapy regimen

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ABSTRACT

Background and Obiectives. **Based on our preliminary experience, we have further evaluated the capacity of the paclitaxel/epirubicin combination (at the dose of 175 and 90 mg/m², respectively) plus G-CSF to mobilize hematopoietic progenitors into the circulation.**

Design and Methods. **The study was conducted in a homogeneous cohort of 25 stage IV breast cancer patients showing response to three cycles of the same chemotherapy regimen and who were included in a high-dose chemotherapy program.**

Results. **In most cases (68%) more than 5×10^6 $CD34^+$ cells/kg b.w. (the threshold fixed in our study) were collected by a single leukapheresis, 28% and 4% of patients requiring 2 and 3 procedures, respectively. Based on the $CD34^+$ cell count in the peripheral blood, most of the leukaphereses (53%) were performed on day 11 after chemotherapy. More than 50 $CD34^+$ cells/mL along with a preleukapheresis WBC count between 10 and 20×10^9/L predicted that only a single harvest would be required in 100% of cases. The evaluation of the clonogenic potential of collected cells showed that a large number of committed colony-forming cells (CFCs) and more primitive long-term culture-initiating cell (LTC-IC) hematopoietic progenitors were present in 20 harvests studied.**

Interpretations and Conclusions. **These data demonstrate that the epirubicin/paclitaxel combination followed by G-CSF, besides being a very active regimen in MBC, is effective in releasing large amounts of progenitor cells into the circulation which can then be safely employed to support myeloablative regimens.**

Key words: epirubicin, paclitaxel, high dose chemotherapy, peripheral blood progenitors, apheresis

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Autologous progenitor cells collected from the peripheral blood (PB) by leukapheresis are used to support hematopoiesis after high-dose chemotherapy (HDC) because of their capacity to produce a much faster hematopoietic recovery than bone marrow cells.[1,2]

An adequate number of progenitor cells capable of guaranteeing short- and long-term hematopoiesis can be harvested from the PB after treatment with myelosuppressive chemotherapy plus growth factor.[3] Moreover, mobilization with disease-oriented chemotherapy is recommended in patients with chemo-sensitive neoplasms since the greater efficacy of mobilization is combined with the antitumor effect of the drug.

HDC in combination with the transplantation of autologous hematopoietic progenitor cells results in both a high frequency of objective response and a longer duration of response compared to that obtained by standard-dose chemotherapy in metastatic breast cancer (MBC).[4,5]

The efficacy of this approach is mainly dependent on the possibility of treating women who have shown a response to conventional chemotherapy regimens.[5] As a matter of fact, most autotransplant trials in MBC have been used either as a *consolidation* modality or in an attempt to convert partial remissions in responding patients into complete remissions. In this scenario, an ideal first-line regimen for patients with stage IV breast cancer should have, first the ability to induce high response rates and, second, the capacity to mobilize sufficient numbers of hematopoietic progenitors.

The combination of paclitaxel and anthracyclines fulfils both requirements. The capacity of this chemotherapy regimen to induce high response rates as first-line treatment for MBC patients has been documented by several studies.[6,7] In addition, preliminary reports show that the combination is also effective in mobilizing stem cells into the circulation.[8,9]

Based on these pieces of evidences we designed a prospective clinical trial of HDC with circulating

progenitor cell support in MBC patients showing complete or partial response to 3 cycles of paclitaxel (175 mg/m^2) and epirubicin (90 mg/m^2). A 4th cycle of the same combination was given to collect peripheral blood progenitor cells (PBPC) to be reinfused following myeloablation. Data on mobilization of CD34$^+$ cells and clonogenic progenitors, tumor cell contamination, timing of harvesting and engraftment kinetics of transplanted patients are reported.

Design and Methods

Patient population and PBPC harvesting

Twenty-five patients in complete or partial response following 3 cycles of paclitaxel (175 mg/m^2) and epirubicin (90 mg/m^2) given for MBC and elegible for a HDC program entered a phase III randomized trial at our Institution (see below). No patient had received prior treatment for advanced disease. Patients received an additional 4th cycle of the same chemotherapy (followed by G-CSF 5 µg/kg/d s.c.) for a further therapeutic intent and in the aim of mobilizing large amounts of progenitor cells into the circulation.

Leukaphereses were performed by a continuous blood cell separator Cobe Spectra upon recovery from the neutrophil count nadir when circulating CD34$^+$ cells exceeded 30/µL. A minimum of two blood volumes per procedure were processed as previously described.[10]

Our final target for collection was a minimum of 5×10^6 CD34$^+$ cells/kg bw as some of the harvests were going to be purged by immunomagnetic negative selection using a cocktail of 3 murine monoclonal antibodies directed against 3 membrane glycoproteins of epithelial cells (317 G5, 260 F9, and 520 C9, Baxter, Deerfield, IL, USA).[11] In the early phase of the study three patients had an additional PBPC harvest to the one(s) required to reach the planned amount of CD34$^+$ cells, which was stored as a back-up in case of graft failure following transplantation of *in vitro* purged cells. Evaluation of tumor contamination in the stem cell collections was performed by a conventional immunocytochemistry technique.[12]

Either soon after stem cell collection (arm A of the randomized study) or at the time of subsequent relapse following 2 additional cycles of paclitaxel/epirubicin combination (arm B), patients received a myeloablative treatment consisting of mitoxantrone 60 mg/m^2, cyclophosphamide 6 g/m^2 and thiotepa 500 mg/m^2. The grafts were reinfused 24 hours after completion of the HDC. The study was approved by the local Institution Review Board and written informed consent was obtained from all patients. The patients' main characteristics are listed in Table 1.

Table 1. Patient characteristics.

Number	25
Median age (range), yrs	47 (27-61)
Metastatic sites	
Skin/soft tissues	9
Visceral	10
Bone	2
Mixed	4
Previous adjuvant therapy	
Chemotherapy	9
Tamoxifen	4
Both	4
Radiotherapy	3
Disease-free interval (months)	12 (0-145)
Response to epirubicin/paclitaxel combination	
Complete	11
Partial	14

Flow cytometry

Cells (1×10^6) either from whole blood or the apheresis product were incubated for 30 min at 4°C with the phycoerythrin (PE) conjugated monoclonal antibody HPCA-2 (anti CD34) or PE-IgG1 isotype (control) (Becton Dickinson, San Josè, CA, USA). After incubation, erythrocytes were lysed in PharM Lyse lysing solution (PharMingen, San Diego, CA, USA) for 10 min at room temperature and washed with 0.1% sodium azide in phosphate-buffered saline (PBS). Without delay the samples were analyzed on a Becton-Dickinson FACScan flow cytometer equipped with a 2-W argon ion laser.

A forward-scatter versus side-scatter dot plot allowed erythrocytes and cell debris contamination to be eliminated. Quantification of CD34$^+$ cells was performed using a fluorescence-2 versus side-scatter characteristics (SSC) dot plot. Only cells with bright Fl_2 expression (absent in the isotypic control) and low granularity (SSC) were counted as CD34$^+$ cells. Using peripheral and autograft WBC count, the percentage of CD34$^+$ and the patient's actual body weight, CD34$^+$ cells/µL and CD34$^+$ cells/kg were calculated.

Cell cultures

Leukapheresis samples were processed after obtaining informed consent from patients. T-lymphocyte depletion was performed as previously described.[13] For CFCs quantification, LDT$^-$ cells were cultured in FCS-supplemented methycellulose medium containing EPO 3U/mL, SF 50 ng/mL, and 20 ng/mL of IL-3, IL-6, GM-CSF and G-CSF (Stem Cell Technologies, Vancouver, Canada). Colonies of burst-forming unit-erythroid (BFU-E), colony-forming unit-granulocyte macrophage (CFU-GM), colony-forming unit-granulocyte erythroid macrophage megakaryocyte (CFU-GEMM) were scored *in situ* by an inverted microscope after 16 days of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air. The method described by Hogge *et al*.[14] was used to detect megakaryocyte progenitor cells (CFU-Mk).

LTC-IC assay was performed by seeding an appropriate aliquot of LDT⁻ cells into cultures containing adherent irradiated murine fibroblasts engineered to produce IL-3, G-CSF and SF.[13] The LTC-IC assays were maintained at 37°C for 6 weeks, with weekly removal of half of the non-adherent cells and the replacement of half of the medium. At the end of the culture period the non-adherent cells were removed and then recombined with the adherent cells obtained after trypsinization. The cells were washed twice and an appropriate aliquot was cultured for 16 days in methylcellulose medium as described above.

Statistical analysis

Data were stored, analyzed and reported by the package Statistica/w (StatSoft Inc., Tulsa, OK, USA). Simple linear regression and Student's t-test for unpaired data were calculated; values of *p* lower than 0.05 were considered statistically significant.

Results

Peripheral blood CD34⁺ cell mobilization and harvesting

Overall 34 leukaphereses were required to reach the target of 5×10^6 CD34⁺ cells/kg in all 25 patients. In most cases (68%) the number of CD34⁺ cells collected the first day was sufficient to reach this predefined target while 7 patients (28%) required 2 procedures and 1 (4%) underwent 3 collections.

Median WBC and circulating CD34⁺ counts on the first day of collection were 17.8×10^9/L (range 7.8-67.3) and 74.9/μL (range: 28-206), respectively. As a consequence of the variability in peripheral blood WBC counts, the total number of WBC collected varied from procedure to procedure (median 22.4×10^9, range 10-41×10^9). The median numbers of CD34⁺ cells harvested per leukapheresis and per patient were 4.7×10^6/kg/bw (range: 0.72-11.7) and 6.2 (range: 5-11.7), respectively.

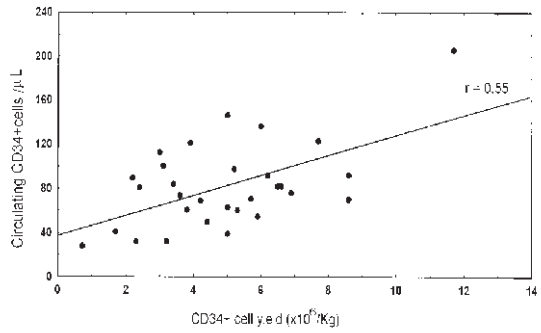

Figure 1. Correlation between the number of CD34⁺ cells / kg collected per apheresis and the preleukapheresis circulating CD34⁺ cell count.

As shown in Figure 1, a clear correlation between circulating and harvested CD34⁺ cells was observed ($p<0.0015$, r=0.55). The relationship between these parameters was even stronger when considering only the procedures performed with a WBC count ranging between 10 and 20×10^9/L (r = 0.86). Moreover, in 14 patients harvested with a WBC count ranging between 10 and 20×10^9/L, a circulating CD34⁺ cell content ≥ 50/μL (9 pts) predicted that a satisfactory harvest would be achieved in all cases, whereas for lower values of CD34⁺ cells more than 1 procedure was always required. In contrast, when collections were performed starting from a WBC value greater than 20×10^9/L, a circulating CD34⁺ cell content ≥ 50/μL was associated with an optimal harvest (5×10^6 CD34⁺ cells/Kg bw) only in 5/14 patients (35.7%).

The median time of PBPC collection was the 11th day after paclitaxel/epirubicin administration (range 10-12). The frequency of single procedures yielding more than 5×10^6 CD34⁺ cells/kg bw was 75% on day 10 (8 cases), 55% on day 11 (18 cases) and 12.5% on day 12 (8 cases). Overall, the amount of CD34⁺ cells harvested on day 10 was significantly higher than that on day 11 and 12 (p=0.049 and p=0.033, respectively).

Data on PBPC collection are summarized in Table 2. Evidence of tumor contamination by ICC was detected in only one PBPC collection (4%)

Priming of clonogenic hematopoietic progenitors

The quantification of mature (CFCs) and more primitive (LTC-IC) hematopoietic progenitors was performed on 20 leukapheresis products from 15 patients. A high number of clonogenic cells was collected by leukapheresis (Table 3). However, the amount of both CFCs and LTC-IC harvested did not correlate with either the CD34⁺ cell yield or any of the following parameters: age, previous chemo- and/or radiotherapy, duration of disease, or metastatic site.

Engraftment kinetics after myeloablation

Eighteen out of 25 patients underwent HDC with hemopoietic progenitor support receiving a median number of 5.4×10^6 CD34⁺ cell/kg bw (range: 2.9-7.6). Thirteen patients received a purged graft. All patients showed hemopoietic engraftment (ANC > 10^9/L and PLT >20×10^9/L) within 16 and 14 days, respectively. No correlation was found between the number of either CD34⁺ cells and any type of CFCs, or LTC-IC reinfused and neutrophil or platelet recovery. No long term graft failure was documented at a median follow-up of one year.

Discussion

Previous reports from our institution[8] and from Bengala *et al*.[9] have demonstrated that the combination of epirubicin and paclitaxel is capable of mobilizing large amounts of PBPC. This observation is of relevant clinical interest as these compounds are very

Table 2. Summary of the harvesting results in 25 MBC patients

Patient	*Day of collection*	*WBC at collection (x10⁹/L)*	*CD34⁺ cells (μL PB)*	*WBC collected (x10⁹/L)*	*CD34⁺ cells collected (x10⁶/kg)*
1	+10	14.9	82.0	25.0	6.5
2	+10	19.3	206.0	33.7	11.7
3	+11	13.4	60.3	16.0	5.3
4	+12	67.3	ND	41.0	7.1
5	+11	28.3	70.7	15.5	5.7
6	+11	10.7	54.6	21.7	5.9
7	+11	50.5	136.0	25.9	6.0
8	+11	17.9	63.0	22.3	5.0
9	+10	12.3	70.1	30.0	8.6
10	+11	25.3	76.0	33.0	6.9
11	+11	24.4	61.0	32.0	3.8
	+12	43.7	100.5	32.7	3.1
12	+10	16.6	49.8	32.0	4.4
	+11	40.4	121.0	76.0	3.9
13	+11	7.8	39.0	21.7	5.0
14	+11	8.0	73.8	15.0	3.6
	+12	13.8	ND	15.0	3.2
15	+11	25.6	89.6	17.5	2.2
	+12	30.0	69.0	22.0	4.2
16	+11	24.9	92.1	38.8	8.6
17	+11	16.8	97.4	24.6	5.2
18	+10	10.5	122.8	18.0	7.7
19	+11	29.2	146.0	19.0	5.0
20	+10	16.4	82.0	22.4	6.6
21	+11	17.8	41.0	13.0	1.7
	+12	36.5	84.0	40.9	3.4
22	+10	10.2	28.0	10.0	0.72
	+11	24.2	ND	26.0	1.8
	+12	40.3	ND	41.0	2.7
23	+11	26.1	80.9	22.8	2.4
	+12	56.3	112.6	28.0	3.0
24	+11	15.4	32.3	17.0	3.2
	+12	18.9	32.1	16.2	2.3
25	+10	35.2	91.5	27.7	6.2
Median (range)	11 (10-12)	17.8 (7.8-67.3)	74.9 (28-206)	22.4 (10-41)	4.7 (0.72-11.7)

Table 3. Progenitor content per kg of body weight in 20 leukapheresis products from 15 MBC patients.

Progenitor type	*No.*	*Median x 10⁴/kg*	*Range x 10⁴/kg*
CD34+	20	5.4	3.1-11.7
CFC	20	132.0	64-501
CFU-GM	20	94.0	32-341
iBFU-E°	20	68.0	10-341
CFU-GEMM	20	6.0	0.1-17
CFU-Mk	9	11.0	5-37
LTC-IC§	17	1.2	0.02-9.2

°iBFU-E = immature BFU-E (> 8 clusters); §LTC-IC: calculated dividing the total number of secondary CFC by 20, based on previous limiting dilution analysis (Ponchio, 1997).

efficient as induction chemotherapy before high-dose regimens in previously untreated MBC. However, the data presented in the two papers cited above lacked conclusive information that could be transferred to everyday clinical practice. In fact, neither study mimicked the most frequent transplantation situations either because collections were performed too early (after the first or second cycle of induction chemotherapy) to identify responding patients[8] or because too many patients had been exposed to multiple courses of chemotherapy before harvesting,[9] which made large yields hard to get.

In the present study we have explored the mobilization capacity of paclitaxel and epirubicin in a homogeneous cohort of patients with MBC, who are the ideal candidates for an HDC program. All patients received the same treatment (in terms of drugs, dose and number of courses) and showed optimal response to first-line chemotherapy, the prerequisite for a subsequent HDC approach. The paclitaxel/epirubicin combination is highly effective as a mobilizing regimen since more than 5×10^6 CD34⁺ cells/kg were easily collected from all MBC patients.

The apheresis products contained a high number of both primitive and mature hematopoietic progenitors. Previous data from a large series of PBPC transplants[15] showed that the transfusion of $>20\times10^4$ CFU-GM/kg was predictive of a rapid and safe hematopoietic recovery; we were able to collect on average three times more CFU-GM from our patients. Although in an animal model more immature progenitor cells seem to be responsible for early engraftment,[16] very little is known about the minimum number of LTC-IC required for a safe transplantation in humans.[17] Our data on this subset of early progenitors do not contribute to clarify this issue as the time of hemopoietic engraftment did not correlate with the number of LTC-IC reinfused. We are currently evaluating the correlation between the total number of LTC-IC collected in a larger cohort of patients after various mobilization regimens and their short- and long-term engraftment potential. Quite unexpectedly, possibly due to the small number of samples evaluated, we did not find any correlations between the number of CD34⁺ cells collected and the amount of any type of clonogenic progenitors.

Since the threshold dose of CD34⁺ cells required for safe hematopoietic recovery following myeloablation is $2\text{-}2.5\times10^6$ CD34⁺ cells/kg,[18,19] the possibility of harvesting higher amounts of hemopoietic progenitors by a single mobilization is important in view of i) the increasing interest in the repetitive HDC strategy in MBC,[20] and ii) the foreseeable application of purging procedures, which might be associated with a significant loss of hematopoietic progenitors on contaminated grafts.[21,22] When either purged or unpurged paclitaxel/epirubicin mobilized PBPC were transplanted after myeloablation, a rapid and complete hematopoietic recovery was observed in all patients, and no long-term sequels were found at a median

follow up of one year.

In our series the tempo for PBPC collection was highly predictable. Based on circulating CD34+ cell count, all harvestings were performed from day 10 to day 12 following chemotherapy. In most cases a single leukapheresis was sufficient for an optimal CD34+ cell yield. Previous reports have shown that WBC, MNC and platelet count in the peripheral blood do not correlate with PBPC yield, circulating CD34+ cells being the only independent significant predictor.[23] In keeping with these results, we found a significant correlation between circulating and harvested CD34+ cells. However, early mobilization (circulating CD34+ cells > 50/μL) along with a WBC count between 10 and 20×10^9/L resulted in larger stem cell collections, while PBPC harvesting was less satisfactory in the presence of a WBC count > 20×10^9/L in spite of adequate number of CD34+ cells circulating in the blood. This phenomenon, previously reported by others,[24] can be related to the fact that pre-apheretic CD34+ cell number may not reflect the intra-procedure count which turns out to be higher in those patients with a starting low (but rapidly increasing) WBC count. Furthermore, the release of CD34+ cells (but not of neutrophils) from the bone marrow may reach a saturation point in the late stages of the mobilization process when a high peripheral WBC count is observed. When this occurs, dilution of CD34+ cells in the bulk of WBC might hamper the efficiency of the harvesting device.

Finally, although the PBPC collection was performed only in those patients who had achieved a complete or very good partial response, the detection of tumor cells in the graft by ICC showed a much lower percentage of contaminated samples than that in previous reports.[25,26] This result is probably related to an *in vivo* pharmacologic purging achieved by the 4 cycles of paclitaxel/epirubicin combination given before PBPC collection.[27]

In conclusion, our study demonstrates the possibility of mobilizing and harvesting large amounts of hematopoietic progenitors, capable of safely reconstituting myeloablated patients, by means of a combination of conventional doses of paclitaxel and epirubicin. It also gives information on the timing of PBPC collections and the quality of the graft. This may help to make a single leukapheresis feasible in the vast majority of cases with evident advantages in terms of costs and patient compliance.

Contributions and Acknowledgments

CZ, PP, LP were responsible for the conception of the study and wrote the paper. NG and CZ carried out the flow cytometric analyses and the stem cell processing. LP and NG did the cell culture experiments. AL performed the tumor contamination studies. GADP, AZ, LP followed the patients clinically. CP and LT performed the leukapheresis procedures. All authors critically revised the manuscript.

The order in which authors appears is based on the amount of work engaged in the study except LS and GRDC who are the last names since they are chiefs of the Institutions in which the study was performed.

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