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Evaluation of thrombopoiesis kinetics by measurement of reticulated platelets and CD34+ cell subsets in patients with solid tumors following high dose chemotherapy and autologous peripheral blood progenitor cell support

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Background and Objectives. The transplantation of mobilized peripheral progenitor cells has resulted in shortening of neutrophil and platelet engraftment times following high-dose chemotherapy. Since reticulated platelet percentage (RP%) has been established as a measure of bone marrow platelet production, we performed this type of analysis on the thrombopoietic compartment during transplant-related chemotherapy.

Design and Methods. Kinetics of thrombopoiesis of 19 patients with solid tumors undergoing a single or double autologous peripheral blood progenitor cell transplant was characterized by evaluating the level of RP. The correlation between CD34+ cell subsets and the time of highest percentage of RP was also evaluated.

Results. The percentage of RP increased from day +8 after single transplant reaching the peak (3.4%) at day +10. In the group of patients receiving double transplant, the peak RP value of observed after the second transplant is not significantly different from that observed after the first transplant (3 vs 3.7%). In a subgroup of patients both the number of CD34+ cells/Kg infused and the percentage of CD34+CD61+ cell subsets correlated with the day of RP peak.

Interpretation and Conclusions. These results suggest that RP measurement is an early indicator of engraftment. Additionally, the observation that RP percentage is high at the time of platelet transfusion in 13 out of 20 cases of transfusions (the 7 cases with low RP value being transfused during the period of obligate thrombocytopenia) suggests that the evaluation of this parameter, together with the platelet count, can be used to monitor the need for platelet transfusion.

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Key words: reticulated platelets, CD34+ cell subsets, peripheral blood progenitor cell transplantation, thrombopoiesis.

High dose chemotherapy with autologous hematopoietic stem cell support has been shown to be a feasible and safe treatment modality for a variety of malignant diseases. Hematologic recovery is more rapid after infusion of peripheral blood stem cells than after bone marrow derived stem cells, with a significant shortening of the period of both neutropenia and thrombocytopenia.[1,2] One major clinical problem in autologous stem cell transplantation is the risk of late platelet engraftment which is seen in 10-20% of patients transplanted,[3,4] therefore the kinetics of bone marrow recovery with respect to platelet production must be investigated. Moreover, clinical tests of platelet homeostasis, such as the platelet count, have not consistently demonstrated utility in predicting platelet engraftment. The determination of the percentage of reticulated platelets (RP) in peripheral blood may provide a measure of thrombopoiesis,[5-8] similar to the use of erythrocyte reticulocyte count, to evaluate erythropoiesis.[5] We, therefore, optimized the methodology for quantifying reticulated platelets using the RNA fluorochrome, thiazole orange (TO), in order to evaluate the patterns of platelet recovery following high dose chemotherapy and autologous stem cell support in patients with solid tumors. Finally the correlation between CD34+ lineage-specific

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cell subset numbers in a subgroup of patients and the time of highest percentage of RP were evaluated.

Design and Methods

Patients

We studied 19 patients (18 with metastatic breast cancer (MBC), 1 with Ewing's sarcoma) receiving high dose chemotherapy with autologous peripheral blood progenitor cell transplant (PBPCT). Nine out of 18 patients with MBC received a second PBPCT. Performance status was 0 (0-1). No patient had positive bone marrow cytology. The median age of patients was 40 years (range 28-55). The conditioning regimen included thiotepa (600 mg/m^2)+L-PAM (160 mg/m^2) and, in 9 patients who received a second PBPCT, idarubicin (60 mg/m^2, 48 hours in infusion). Patients with a platelet count $<20\times10^9$/L received platelet transfusion.

The day of PBSC infusion was designated as day 0. Platelet counts were monitored from day 0, while RP was assessed from day +5 to day +13 because an obligate period (8-9 days) of thrombocytopenia is reported.[8] Thirty-five healthy subjects of the same age with no history of a coagulation defect formed the control group.

Mobilization and stem cell collection

All patients received more than one regimen of cytotoxic chemotherapy. PBPC were collected after a standard dose chemotherapy including granulocyte colony-stimulating factor (G-CSF) until leukapheresis was completed. Leukapheresis was performed when blood levels of CD34$^+$ cells exceeded 20×10^3/mL blood. In each leukapheresis product the number of CD34$^+$ cells was enumerated before freezing, in accordance with previously described recommendations.[9,10]

Estimation of CD34+ subsets

The identification of CD34$^+$ cells was based on the Milan protocol.[11] Briefly, CD34$^+$ cells were stained with class III anti-CD34 PE-conjugated (Immunotech, Marseille, France). Mouse IgG1 PE isotype control (Immunotech, Marseille, France) was included for determination of background staining. The positive population (SSC/FL-2) was identified in the upper left quadrant, among cells with a side scatter profile as lymphocytic cells.[12,13] The negative control was subtracted if it exceeded 0.05%. The analysis should consider not only the percentage of positive cells but also the type of antigen expression. This evaluation was performed on a minimum of 1,000 cells acquired in a CD34 SSC/FL gate. We double-stained the cells with anti-CD34 PE conjugated monoclonal antibody (moAb) plus one of the following conjugated moAbs: anti-CD38 FITC (Immunotech); anti-CD117 FITC (Caltag Laboratories, San Francisco, CA, USA); anti-HLA-DR FITC (Exalpha Corporation, Boston, USA); anti-CD33 FITC (Serotec, Oxford, England) and anti-CD61 FITC (Exalpha Corporation, Boston, USA). Mouse Ig isotype controls were included for each moAb.

Evaluation of reticulated platelets

Venous blood was collected into a tube containing ethylenediamine tetra-acetic acid (EDTA) and gently inverted to mix the blood and anticoagulant. A platelet count was measured by an automated analyzer (STK-S Coulter, Miami, FL, USA). Platelets were prepared as previously described by Ault *et al.*[5] Platelet-rich plasma was prepared by centrifugation at 120 g for 10 min. The platelets were fixed in 1% paraformaldehyde for at least 2 hours, washed twice and resuspended at 50×10^9/L in PBS containing 2 mM/L EDTA (pH 7.2). Subsequently, 100 μL of this suspension were incubated with 1 mL of the thiazole orange (TO, final concentration 90% vol/vol) (Retic-COUNT, Becton Dickinson, San José, CA, USA) at room temperature in the dark for 1 hour. Platelets were identified by a Coulter Epics XL flow cytometer (Coulter Corporation) on the basis of their characteristic size (log forward scatter) and granularity (log side scatter) and an electronic gate was drawn around the platelet cloud to exclude all non-platelet particles.[14] Ten thousand platelet events were collected. It has been recently shown that a large proportion of the TO signal of platelet is RNase insensitive and derives from other platelet compartments, such as mitochondrial DNA or dense granule nucleotides.[15,16] Therefore the TO fluorescence histogram of these gated events was analyzed by a linear gate set to capture approximately 1% of the reticulated platelet controls. When a single such marker was used to analyze a series of 35 normal samples in order to determine the variability of the technique, the average percent reticulated platelets was 0.42% (± 0.18 s.d. observed range, 0.2 to 1.0%). This standard gate was used to analyze data from all samples and measure the percentage of TO$^+$ events in this gate.[6] A normal control sample was prepared at the same time as each patient sample and run with the patient samples in order to identify possible day-to-day variability. The inter-assay CV evaluated in one patient was 8.6% and the intra-assay CV was 2.7%.

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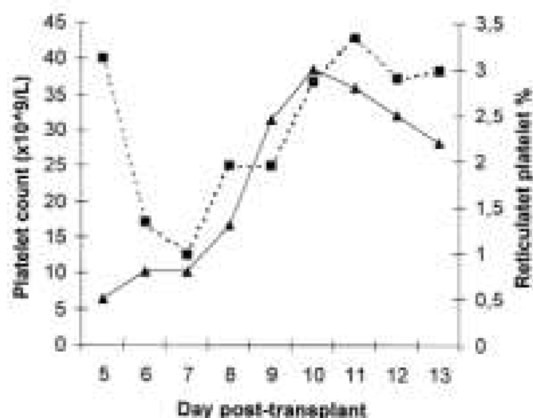

Figure 1. Median daily reticulated platelet percentage plotted against platelet count in 10 patients undergoing single PBPCT. ▲ *RP % median;* ■ *platelet count.*

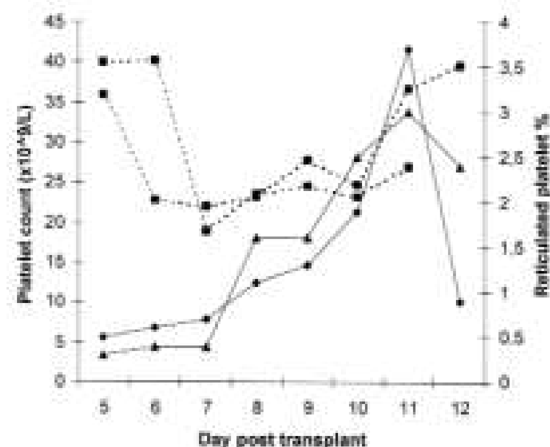

Figure 2. Median daily reticulated platelet percentage and platelet count in 9 patients undergoing double PBPCT. *First PBPCT:* ◆ *RP % median;* ● *platelet count. Second PBPCT:* ▲ *RP % median;* ■ *platelet count.*

Statistical analysis

The results are expressed as the median. Comparisons among the groups were evaluated by Student's t-test. Any p value <0.05 was considered statistically significant. A linear regression analysis was performed to identify variables influencing platelet engraftment.

Results

Kinetics of RP

In the control group, composed of 35 healthy individuals, the median RP percentage was 0.4% (range 0.2-1%). Kinetics of RP was evaluated in twenty-eight courses of high dose chemotherapy. In 10 patients receiving single PBPCT, the median platelet count nadir was 12.35×10^9/L (range 5.1-17.3×10^9/L) and occurred at median day +7 (range 5-9). The median time to platelet recovery (>20×10^9/L) was day +11 (range 10-13). The behavior of the platelet count is reported in Figure 1.

The median RP percentage increased (1.65%; range: 1.1-3.1) on day +8 (range 5-9) reaching the median peak value (3.4%; range: 1.7-10.4) on day +10 (range 9-12), as shown in Figure 1. Eight patients required platelet support; platelet transfusions were given when the median platelet count and median RP percentage were 14×10^9/L (range 5.1-17.3×10^9/L) and 1.2% (range 0.4-3.1), respectively. In five out of these patients the RP percentage was >1%. Nine patients received a double course of high dose chemotherapy with BCS. After the first transplant, the median platelet count nadir was 16×10^9/L (range 7.1-17×10^9/L) and occurred at median day +7 (range 6-9) (Figure 2). The same median value of platelet count nadir range (9.1-18×10^9/L) was observed after the second transplant at median day +7.5 (range 7-10) (Figure 2). The median time to platelet recovery was 11 days (range 10-13) after the first transplant and 10 days (range 9-12) after the second one. After the first transplant the median RP percentage increased to 1.3% (range: 1.1-1.9) at median day +8 (range 5-10). The median peak value of RP occurred on day +11 (range 10-12) with a RP percentage of 3.7% (range 1.7-6.9) (Figure 2). After the second transplant the median RP percentage started to increase on day +9 (range 8-9) with a value of 1.45% (range 1.1-2.4). The median peak RP value was reached on day +11 (range 8-12) with a median percentage of 3% (range 2.2-9.5). The difference between the peaks of RP percentage observed after the first and second transplant was not statistically significant (p>0.05) (Figure 2).

After the first transplant 7 patients required platelet support when the median RP percentage was 1.1% (range: 0.3-1.9); in 4 out of these patients RP percentage was ≥1.1%. After the second transplant 5 patients required platelet support and presented a median RP percentage of 1.4% (range: 0.4-2.2); in 4 out of these patients RP percentage was ≥1.2%. All the other patients transfused were in the period of obligate thrombocytopenia.

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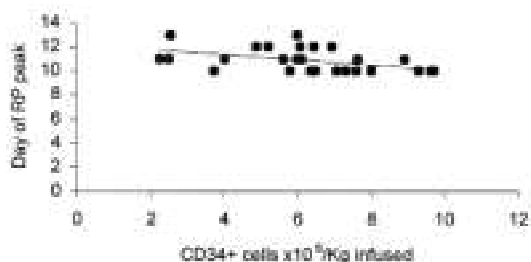

Figure 3. Correlation between the number of CD34+ cells × 10^6/Kg infused and day of RP peak.

Table 1. Median percentage and range of CD34+ cell subsets in 6 patients.

Subset of CD34+ cells	*Median % (range)*
CD34+CD38-	44.75 (14.3-56.9)
CD34+CD117+	97.35 (91.4-99.7)
CD34+HLA-DR+	95.4 (85.9-96.9)
CD34+CD33+	23 (9-39.9)
CD3+CD61+	14 (2.8-21)

Correlation between RP and CD34+ cell subsets

Patients undergoing a single transplant received a median of 7.01×10^6 CD34+ cells/kg (range 2.24-9.7), which was not significantly different from the dose infused to the patients receiving a double transplant: 5.98×10^6 (range: 2.5-8.9) and 6.4×10^6 (range: 2.5-9.2) in the first and second transplant, respectively. The number of CD34+ cells/kg reinfused correlated with the day of RP peak (r= -0.4353; p=0.02) (Figure 3).

Cytofluorimetric analysis of CD34+ cell subpopulations was performed in 6 patients: the results, expressed as median percentages, are shown in Table 1. The median percentage of uncommitted (CD34+CD38-) was 44.75%. More than 90% of CD34+ cells co-expressed early antigens such as CD117 and HLA-DR, whereas lineage-specific antigens CD33 and CD61 were expressed in 23% and 14% of CD34+ cells, respectively.

The percentage of the CD34+CD61+ cells correlated with the day of RP peak. The analysis showed an inverse correlation between the percentage of CD34+CD61+ cells and the day of highest percentage of RP (r= -0.8248; p= 0.0434). No other subset of CD34+ cells defined by the expression of the above described lineage antigens correlated with the day of peak RP value.

Discussion

The prolonged thrombocytopenia caused by delayed platelet engraftment represents one of major clinical problems in PBSC transplantation. Many factors can be involved, including severe post-transplant regimen-related toxicity or inadequate quantity of transplanted stem cells. Together with platelet count the mean platelet volume (MPV) represents an available parameter to evaluate the rate of platelet production.[17] It is normal or decreased in patients with reduced platet production,[18] increased when megathrombocytes appear or young platelets rise in the peripheral blood.[19] Furthermore, even if an increase of MPV can indicate accelerated thrombocyte production, platelet volume is not related to aging.[20,21] Additonally, many variables such as the modality of blood taking, temperature, interval between time of drawing blood and time of performing assays and finally the role of anticoagulants can influence the mean platelet volume measurement,[17,22] thus obliging careful interpretation of the results.[23]

The mean values of MPV observed in our patients increased compared to those of normal subjects with a peak on day +10 post-tranplant (data not shown). The increase was not linear and was not observed in all patients tested (data not shown).

Reticulated platelet count has recently been introduced as a more important parameter for accurate measurement of the rate and kinetics of thrombopoiesis.[24] In the present study we measured the level of RP in order to evaluate the kinetics of thrombopoiesis in patients with solid tumors receiving high dose chemotherapy with single or double autologous PBPCT. We demonstrate that the rise in the RP percentage, which reflects megakaryocyte engraftment, can anticipate the initial rise of platelet count. As the platelet count rises, the percentage of RP falls, according to previous reports.[7,8,25]

It is interesting to observe that the patterns of RP release during autologous bone marrow transplant (ABMT) and PBPCT are different. The peak RP percentage is higher after PBPCT than after ABMT; this difference may reflect a more homogenous population of platelet precursors harvested by the PBPC technique, which has a greater synchrony of maturation.[7]

Furthermore, the fact that the peak RP percent-

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age after a second transplant is not significantly different from that observed after a first transplant demonstrates that thrombopoietic capacity is retained despite an additional course of high dose chemotherapy.[26]

It has been demonstrated that the total number of CD34+ cells/kg infused at transplant is the most important factor influencing the time of platelet recovery.[13] The present study shows a significant correlation between the number of CD34+ cells infused and the day of peak RP percentage.

CD38 antigen is expressed on more differentiated cells and is not present on primitive progenitors that, on the contrary, show CD117 and HLA-DR antigens. High numbers of CD34+CD38- cells in the reinfused stem cell product are associated with better long-term hematopoiesis.[13] In the present work we demonstrate a significant correlation between CD34+CD61+ cell subsets and the time of peak RP percentage. Thus, a small but significant population of CD34+ expressing megakaryocyte lineage-associated cell surface antigens, giving rise to regeneration of megakaryopoiesis after transplantation, can be detected.[27] No other lineage-specific CD34+ cell subsets correlated with the time to platelet engraftment.

In conclusion, we demonstrate that the level of RP is an early indicator of engraftment after transplantation of mobilized peripheral blood progenitor cells, as previously suggested.[7,8,26] The observation that RP percentage is high at the time of platelet transfusion in 13 out of 20 cases of transfusions (the 7 patients with low RP percentage being transfused during the period of obligate thrombocytopenia) suggests that the evaluation of this parameter, together with platelet count, can be used to monitor the need for platelet transfusions in patients undergoing high dose chemotherapy.

Contributions and Acknowledgments

RC, AC, AL: conception and design, analysis and interpretation of data; CB: conception and design; PFC : final approval of the version to be published.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Carlo Balduini, who acted as an Associate Editor. The final decision to accept this paper for the publication was taken jointly by Prof. Balduini and the Editors. Manuscript received April 27, 2001; accepted August 14, 2001.

Potential implications for clinical practice

This study suggests the use of RP count in order to monitor the need for platelet transfusions in patients undergoing high dose chemotherapy.

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