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Phenotypic changes in neutrophil granulocytes from healthy donors after G-CSF administration

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Abstract

Background and Objectives. **The aim of the present work was to analyze the immunophenotypic changes in neutrophil granulocytes (NG) and their evolution over time in 16 healthy donors who received G-CSF for stem cell mobilization for allogeneic peripheral blood stem cells (PBSC) transplantation.**

Design and Methods. **The mobilization schedule consisted of G-CSF (Filgrastim®, Amgen) 10 µg / kg, s.c. for five consecutive days. Apheresis began 16-24h after the last dose of G-CSF. Immunophenotypic analysis (CD10, CD14, CD15, CD16, CD71, CD34 and HLA-DR) of NG and measurement of G-CSF serum levels were performed before G-CSF administration (day 0), on the fifth day of G-CSF treatment and days +7 and +30 after the last dose of G-CSF.**

Results. **After G-CSF administration, peripheral blood NG presented increased expression of HLA-DR, CD34, CD14, and CD71, in addition to decreased expression of CD10 and CD15 and CD16 fluorescence mean intensity. The phenotypic changes on NG were parallel to serum G-CSF levels. All the phenotypic changes returned to baseline values by one month after the end of G-CSF treatment. No changes were found in the expression of HLA-DR on T and B-lymphocytes.**

Interpretation and Conclusions. **These immunophenotypic changes suggest that after G-CSF administration, NG from healthy donors who received G-CSF for stem cell mobilization carry transient features of immature phenotype and have increased functional activity.**

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Key words: neutrophil granulocytes, phenotype, G-CSF, healthy donors

Peripheral blood stem cells (PBSC) mobilized with G-CSF are increasingly used for allogeneic stem cell transplantation.[1-3] G-CSF combines an excellent mobilizing effect and a safe clinical profile, and donors tolerate the mobilization and apheresis procedures better than bone marrow harvest.[4] Most of the published reports have evaluated the effects of recombinant cytokines on normal hematopoietic progenitors,[5-7] and function;[8-14] phenotypes of neutrophil granulocytes (NG) after G-CSF treatment have been less studied.

Several studies have suggested that after G-CSF administration, NG express some phenotypic changes that result in an activation status, with increased adherence and phagocytosis. However, much of this information has been obtained from patients receiving G-CSF to accelerate NG recovery after chemotherapy.[15,16] Less is known about G-CSF administration to healthy volunteers receiving G-CSF for PBSC mobilization and there is scarce information on the evolution of these changes over time. The aim of the present work was to analyze the immunophenotypic changes on NG and their evolution over time in 16 healthy donors who received G-CSF for stem cell mobilization from allogeneic PBSC transplantation.

Design and Methods

Healthy donors

Sixteen healthy donors were studied before and after treatment with G-CSF (Filgrastim®, Amgen) for PBSC mobilization. Their mean±SD age was 38±10 years (range 26-54). Nine were males and seven females. All donors were thoroughly informed about the investigational nature of the procedure and gave their written consent. The protocol was approved by the Hospital Ethics Committee.

The donors received G-CSF at a dose of 10 µg/Kg by subcutaneous injection once a day for five consecutive days. Apheresis began 16-24h after the last dose. The study of surface markers of NG and measurement of serum levels of G-CSF were performed before G-CSF administration (day 0), on the fifth day of G-CSF treatment, and on days 7 and 30 after the end of G-CSF administration.

Methods

A. Phenotypic analysis of NG

NG phenotype was analyzed by a double immunofluorescence technique and flow cytometry. Briefly,

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100 µL of whole blood were mixed with appropriate amounts of each combination of monoclonal antibodies and incubated at 22°C for 15 minutes. After incubation erythrocyte lysis was performed following the manufacturer's recommended procedure (FACS lysing solutions®, Becton Dickinson, San José, CA, USA) and washed twice in phosphate buffered saline at a pH 7.25. The NG were analyzed for the following monoclonal antibodies (MoAB): simultest™ Leuco Gate (CD45/CD14™ anti-HLe-1/LeuTM-m3™), Simultest™ control (IgG_2 FITC/IgG_2 PE); CD10 FITC; CD14 (LeuTM-M3) FITC; CD15 (LeuTM-M1) FITC ; CD16 FITC, CD71 (anti-transferrin receptor) FITC; HLA-DR PE; CD34 (anti-HPCA-2) PE; (Becton Dickinson). Cell surface membrane antigens were detected by quantitative flow cytometry using a FACScan™ (Becton Dickinson). The number of cells counted for this analysis was 15×10^3. Acquisition gates were set to exclude dead cells and aggregated material. Forward versus side scatter display was used to define granulocytes. Fluorescence was measured in log amplification after fluorescence compensation (Calibrite™ and Autocomp software from Becton Dickinson). MoAB of the same isotype and fluorochrome were used as the negative control of fluorescence. For each MoAB the percentage of positive cells as well as their mean intensity of the same Calibrite™ was used to compare the fluorescence intensity of the antigens in the studies performed before and after G-CSF administration. Results are expressed as percentages and mean intensity fluorescence. In addition the alkaline immunophosphatase (APAAP) technique[17] was used to demonstrate HLA-DR expression on NG after dextrane separation. HLA-DR expression was also measured by flow cytometry on T (CD3) and B (CD19) lymphocytes.

B. Measurement of serum levels of G-CSF

Serum G-CSF levels were analyzed by a quantitative sandwich enzyme immunoassay technique (R&R Systems, Minneapolis, USA). A MoAB specific for G-CSF was pre-coated onto a microtiter plate. One hundred microliters of standards and samples were pipetted into the wells and any G-CSF present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for G-CSF was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of G-CSF bound in the initial step. The color development was stopped and the intensity of the color was measured.

C. Statistical analysis

The t-paired test was used for evaluation of the differences between surface antigen expression on NG from healthy donors before and after G-CSF administration.

Results

G-CSF administration was well tolerated except for moderate bone pain occurring in all donors. The median peripheral WBC count on the fifth day of G-CSF administration was 40×10^9/L (range 23-62); the red blood cell and platelet counts were not modified as compared to baseline values.

The surface expression of CD10, CD14, CD15, CD71, HLA-DR, and CD34 on NG from healthy donors on day 0, day 5, day 7 and day 30 is shown in Table 1. Increased expression of HLA-DR on NG was observed, this finding being confirmed by the APAAP technique (Figure 1). Other findings at day 5 of G-CSF treatment were a significant decrease in CD10 expression and in mean fluorescence intensity expression of CD15, and CD16 and an increase in the expression of CD14, CD71 and CD34 (Figure 2). On day 7 only the expression of CD71 and CD34 remained increased with respect to day 0. Finally, on day 30 the expression of all the surface markers on NG was identical to that found at day 0 (Figure 3). No changes in the expression of HLA-DR were found in T and B-lymphocytes after G-CSF administration (data not shown).

The Calibrite™ signals of FITC and PE fluorochromes were in the same channel of fluorescence in all the measurements performed, allowing us to exclude that any differences in antigen densities were caused by different calibration between studies.

G-CSF serum levels in healthy donors are shown in Table 2. Detectable levels were present in the serum of all healthy donors before G-CSF administration, at a mean±SD of 22±7 pg/mL, range 2-69. G-CSF levels significantly increased after G-CSF administration, being 488±56 pg/mL (range 286-750), 5 days after stopping G-CSF treatment. Serum G-CSF levels on day 7 and day 30 were similar to basal levels. Thus, these levels were parallel to the peak NG count and to the phenotypic changes of NG.

Table 1. Expression of surface markers on neutrophil granulocytes from healthy donors (n=16), before and after G-CSF administration.

	CD10 %	*CD14* %	*CD15* M.I	*CD71* %	*HLA-DR* %	*CD34* %
A. Day 0	74±4*	2±0.4*	3289±306*	0.5±0.1*	2±0.2*	0.3±0.1*
B. Day 5	3±2*	53±8*	1232±187*	22±6*	6.5±1*	4.4±1.4*
C. Day 7	58±10	2±0.4	2982±477*	2±0.4°	4±1	4.4 ±1.6°
D. Day 30	55±7	2±0.4	3011±448	0.8±0.3	2±0.5	1±0.1

Results expressed as mean ± standard deviation. MI= mean intensity.
**p=0.0001 A vs B. °p=0.001 A vs C*

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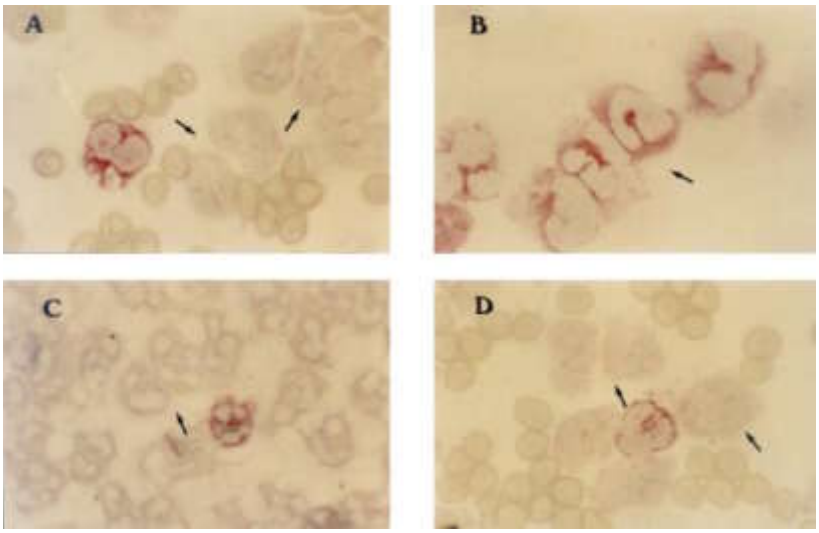

Figure 1. HLA-DR negative expression on neutrophil granulocytes (arrows) and positive expression on a monocyte as measured by the APAAP technique on day 0 before G-CSF administration (A); HLA-DR positive expression on the neutrophil granulocytes (arrow) on day 5 of G-CSF treatment (B); on day 7 of G-CSF administration neutrophil granulocytes only show occasional HLA-DR expression (C); none of the neutrophil granulocytes expressed HLA-Dr (arrows) (only expressed on monocytes) (D).

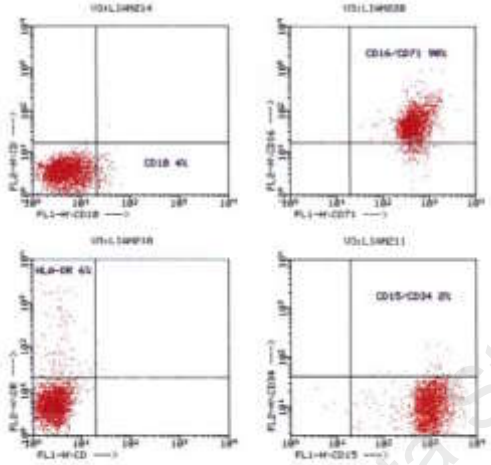

Figure 2. The surface antigen expression on 100% of neutrophil granulocytes by flow cytometry on the fifth day of G-CSF treatment of a healthy donor. These is a great decrease of CD10 (4%), a great increase of CD71 (90%) and a small increment of HLA-DR (6%) and CD34 (2%). Mean expression of surface markers expressed in percentages is shown in Table 1.

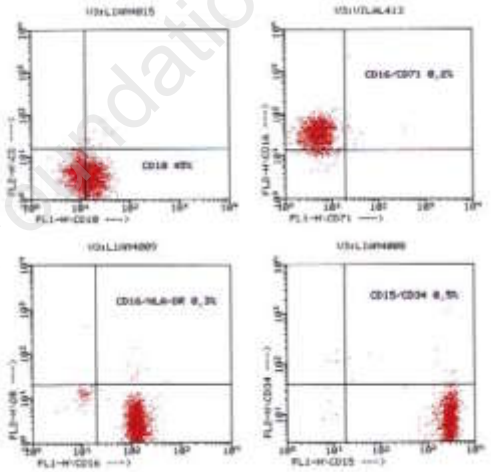

Figure 3. The surface antigen expression on 100% neutrophil granulocytes by flow cytometry 30 days after the end of G-CSF administration shows a return to normal antigenic expression of CD10, CD 71, HLA-DR and CD34.

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Discussion

The use of G-CSF treatment for stem cell mobilization in healthy donors for allogeneic transplantation provides a unique opportunity to study the effect of this cytokine on normal hematopoiesis *in vivo*. The impact of G-CSF treatment on mobilization of $CD34^+$ cells, as well as the effect of this cytokine on peripheral blood cell counts is well-studied.[18-24] However, studies on phenotypic changes of NG and their evolution on NG over time in healthy donors receiving G-CSF are scarce.[25,26] In our study surface antigens on NG were serially studied by flow cytometry in 16 healthy donors who received G-CSF for PBSC mobilization. The most outstanding findings in this study were the marked increase of HLA-DR and CD34 expression on NG after G-CSF administration. The expression of HLA-DR on T and B-lymphocytes did not change after G-CSF administration. Such a finding was reported in another study on a patient with chronic idiopathic neutropenia[15] and one on patients with acute lymphoblastic leukemia receiving G-CSF for neutropenia secondary to intensification chemotherapy.[16] To our knowledge, these features have not been previously described on NG of healthy donors after G-CSF treatment. The expression of HLA-DR and CD34, together with the decrease in CD10 and in the mean intensity of fluorescence of CD15 and CD16 on NG would suggest the presence of NG with

Table 2. G-CSF serum levels before and after G-CSF administration in healthy donors (n=16).

	Serum G-CSF levels (pg/mL)	*P-value*
A. Day 0	22±7	<0.0001
B. Day 5	488±56	<0.0001
C. Day 7	10±3	NS
D. Day 30	39±16	

Results expressed as mean ± standard deviation. NS= not significant.

features of immaturity, because these surface antigens are expressed on mature NG.[17] In addition, the higher CD71 expression seems to indicate that these NG expressed surface markers with a high degree of proliferation. Similarly to other authors, who observed an increase of CD14 expression in cultures of cord blood and bone marrow mononuclear cells stimulated with G-CSF,[13,14] we found a simultaneous increase in the expression of the activation marker CD14 on NG after G-CSF administration. CD14 is the receptor for the lipopolysaccharide complexed to lipopolysaccharide binding protein and it may be important for achieving efficient response to infections caused by Gram-negative bacteria.[13]

Several studies have analyzed the immediate activating effects on circulating NG after G-CSF administration.[24,25] In our patients, we demonstrated that most of the changes found after the administration of this cytokine disappeared seven days after the last dose of G-CSF; one month later, the phenotypic profile of NG was identical to that found prior to G-CSF administration. In addition, there was a relation between these changes and serum levels of G-CSF. Changes in antigenic expression on NG over time have been previously published and a rapid return to normal antigenic profile has also been observed.[25,26]

Although the short-term effects of G-CSF administration are known, there is some concern about long-term effects of the administration of this cytokine to healthy donors. Our study demonstrates that, although after G-CSF treatment NG carry some features of immaturity, there is a rapid return to normal phenotypic profile, and that these changes parallel the serum G-CSF levels.

Contributions and Acknowledgments

MAZ and JMR designed the study and wrote the paper. AUI, CM, EF and EM followed the donors clinically. MAZ and RA analyzed the phenotypic changes of NG. XF measured the serum levels of G-CSF.

The criteria for the order in which the names of the authors appear are based on their contribution to the design, analysis, and interpretation of data and execution of the study.

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Disclosures

Conflict of interest: none

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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