short report
Haematologica 1995; 80:150-154

GRANULOCYTE COLONY-STIMULATING FACTOR (G-CSF) ADMINISTRATION INCREASES PMN CD32 (FcRII) EXPRESSION AND FcR-RELATED FUNCTIONS

Giovanni Carulli, Sistina Minnucci, Antonio Azzarà, Claudia Angiolini, Silverio Sbrana, Francesco Caracciolo, Fabio Ambrogi

Unit of Hematology, Medical Clinic 1, University of Pisa, Italy

ABSTRACT

The phenotypical and functional properties of circulating neutrophils from ten patients suffering from intermediate- and high-grade non-Hodgkin lymphoma were investigated before and after rhG-CSF administration (5 μg/kg/day subcutaneously for 5 days). The following parameters were studied: flow cytometry evaluation of surface CD32, CD16, CD11b and CD18 by means of a whole blood method; whole blood phagocytosis by means of a flow cytometric assay; whole blood chemiluminescence using opsonized zymosan as a stimulus. A significant increase in the expression of surface CD32 was detected in all patients, while CD11b expression was found to be increased in only four of them. CD16 and CD18 expression did not change. A significant enhancement of phagocytosis and phagocytosis-associated chemiluminescence was also observed. These results show that rhG-CSF administration can increase both FcRII expression and FcR-related functions.

Key words: CD32, FcRII, neutrophils, rhG-CSF

G-CSF has been shown to shorten the duration of neutropenia after cytotoxic chemotherapy and to increase the number of peripheral neutrophils in various conditions of neutropenia.[1] Its administration can cause variable changes in the expression of surface receptors involved in neutrophil functions[2–4] but, at the present time, a definite neutrophil phenotype after G-CSF therapy has not been observed. G-CSF also acts as a chemoattractant for neutrophils *in vitro*.[5]

Limited evidence has been provided so far about the *in vitro* effects of rhG-CSF on the phagocytic and the microbicidal functions of neutrophils[6] and, to the best of our knowledge, only one report about the *in vivo* effects of G-CSF on neutrophil phagocytosis and phagocytosis-associated respiratory burst had been published at the time of our study.[7]

We therefore evaluated the effects of rhG-CSF administration on phagocytosis and phagocytosis-associated chemiluminescence of neutrophils from patients with non-Hodgkin's lymphoma undergoing chemotherapy. A simultaneous evaluation of surface CD16 (FcRIII), CD11B (CR3), CD18 and CD32 (FcRII) was carried out by flow cytometry. Evaluation of CD32 appeared to be of particular interest because little information is available about the effects of G-CSF on the expression of this important effector of phagocytosis and phagocytosis-related respiratory burst.

Materials and Methods

Subjects studied

In the period from December 1993 to May 1994 we evaluated ten patients affected by intermediate- and high-grade non-Hodgkin's lymphoma (Table 1) undergoing chemotherapy with a modified (14-day interval between courses) Promice-CytaBom regimen.[8] The study was

Correspondence: Giovanni Carulli, M.D, Unit of Hematology, Medical Clinic 1, University of Pisa, via Roma 67, 56100 Pisa, Italy. Tel. international +050.592110. Fax international +50.502617.
Received September 22, 1994; accepted January 23, 1995.

carried out during the interval when the cytotoxic agents were not given. RhG-CSF was administered subcutaneously for five days at a dosage of 5 μg/kg/day. Blood samples stabilized in EDTA and in sodic heparin (10 U/mL) were drawn by cubital venipuncture before and 24h after completing rhG-CSF administration. Peripheral blood cell analysis included hemoglobin, hematocrit, MCV, MCHC, leukocyte count with a standard differential, platelet and erythrocyte counts. Blood smears were also observed by means of light microscopy after May-Grünwald-Giemsa staining.

Flow cytometry

Cell surface markers were detected by either direct or indirect immunofluorescence using flow cytometry and monoclonal antibodies (MoAbs). A whole blood method[2] was used to avoid any *in vitro* manipulation that could activate neutrophils. The following MoAbs (1 μg/sample) were utilized: Leu 15 (PE) for Cd11b; LFA-1-β (FITC) for CD18; Leu 11a (FITC) for CD16, all from Becton Dickinson, and 2E1 (purified) for CD32, from Immunotech. The 2E1 MoAb was stained with a $F(ab')_2$-FITC-conjugated goat antimouse Ig (Dakopatts). Controls were performed using irrelevant MoAbs. Flow cytometry evaluation was determined by a FacStar flow cytometer (Becton Dickinson) and the settings of the instrument were the same for all measurements, which were carried out after live gating on neutrophils. Fluorescence intensity distribution was recorded on a 4-decade logarithmic scale, and surface expression of antigens was measured as mean fluorescence channel.

Whole blood chemiluminescence

A commercial kit (Phagolux, Bouty Diagnostici, Milan, Italy) was used as described elsewhere. Heparinized blood (0.5 μL) was transferred into vials containing 1 mL luminol (10 μM). Zymosan, which had previously been opsonized (30 min at 37°C) with autologous or homologous plasma (pool from 10 AB+ blood donors), was then added (20 μL of 5 mg/mL opsonized zymosan). Control tests were carried out without the stimulus. Chemiluminescence signals were evaluated by a Berthold LB 950 automatic luminescence analyzer and registered every minute for 30 min. Results were expressed as an integral over the total measuring time (counts/neutrophil/30 min): 40 normal subjects were used as controls.

Whole blood phagocytosis

A commercial kit (Phagotest, Orpegen, Heidelberg) was used as described elsewhere. Heparinized blood (100 μL) was incubated for 10 min at 37°C with FITC-conjugated opsonized E. coli (bacteria-to-neutrophils ratio = 30:1). Control tests were carried out at 0°C. Phagocytosis was evaluated by means of a Coulter Epics XL flow cytometer. The laser was set at the 488 nm band and run at 150 mW of power, and an electronic gate was set to include cells with granulocyte scatter characteristics. Measurements of green fluorescence included two parameters: percentage of neutrophils with ingested bacteria and mean fluorescence channel (which depends on the number of bacteria per individual neutrophil). Ten normal subjets were used as controls.

Statistics

Data were expressed as means ± SD. The data obtained after flow cytometric evaluation of surface markers were analyzed by means of the Consort 30 program (Becton Dickinson) and statistical analysis was carried out by the Kolmogorov-Smirnov two-sample test (K-S), which provides a powerful tool for the objective statistical analysis of histograms. Data were

Table 1. Patient characteristics.

Age		
	mean (yr)	54
	range	30-74
Sex		
	males	5
	females	5
Histology (Working Formulation)		
	E	3
	G	1
	H	3
	I	1
	J	2

considered to be significantly different when D > 0.1 and D/s(n) > 10 (i.e. $p < 0.05$). The statistical significance of differences between values before and after rhG-CSF treatment was determined by the Student's t-test for paired samples. Pearson's correlation coefficient was used to test the variation of CD32 expression against neutrophil counts after G-CSF administration.

Results

All patients responded to rhG-CSF treatment with an increase in neutrophil counts from $1.55\pm0.2\times10^9$/L (range 1.2-2.0) to $10.7\pm3.8\times10^9$/L (range 5.0-15.0) ($p = 0.0001$). No circulating immature myeloid cells were found. The other blood parameters did not change (data not shown).

G-CSF administration increased the expression of CD32 in all patients (Table 2), with mean fluorescence channel values of 30±16 before and 88±25 after therapy ($p = 0.0001$). The baseline values of CD32 expression by neutrophils from the patients under study were not different from those found in normal subjects (mean fluorescence channel 29±5.9; n = 10).

The increased CD32 expression after G-CSF therapy was found to be associated with a significant increase in phagocytosis (mean fluorescence channel 27±17 before, and 55±33 after G-CSF administration; $p = 0.005$).

The percentage of phagocytosing neutrophils did not change after G-CSF therapy (basal values = 86±8.8%; values after therapy = 90±8%). Patient #8 showed a basal phagocytosis value below the normal range (mean fluorescence channel = 9; normal values = 33.3±16.9, range 15-67, n = 10), and G-CSF administration was able to correct this parameter (Table 2).

Phagocytosis-associated chemiluminescence was also found to be enhanced by rhG-CSF administration (counts/neutrophil/30 min: basal values = 890±410; values after G-CSF therapy = 1,670±606 with zymosan opsonized with homologous plasma, and 1,635±612 with zymosan opsonized with autologous plasma; p vs basal values = 0.0001).

Patients #1 and 7 showed impaired chemiluminescence with both autologous and homologous plasma (Table 2), and G-CSF administration was able to bring these parameters into the normal range (n.v. 1,091±321, range 600-1,600; n = 40). CD11b expression increased after G-CSF administration in four patients only (pts #1-4). The individual responses to rhG-CSF therapy are depicted in Table 2.

The expression of CD18 and CD16 did not change significantly (mean fluorescence channel 17.4±6.2 vs 19.9±7.34 for CD18, and 34.7±23.4 vs 33.7±20.24 for CD16; p = NS). A decrease in CD16 levels was observed in patient #1 (mean fluorescence channel 68 before and 32 after G-CSF therapy). No significant correlation between neutrophil count and CD32 expression after rhG-CSF administration was found (data not shown).

Table 2. Relevant phenotypical and functional changes following rhG-CSF administration.

Case	before G-CSF				after G-CSF			
	CD32	*CD11b*	*PH*	*CL*	*CD32*	*CD11b*	*PH*	*CL*
1	17	10	19	400	99	31	21	750
2	18	18	30	690	67	34	49	1770
3	26	19	33	1500	88	41	94	2200
4	24	32	72	680	47	12	101	1650
5	26	10	29	1380	76	16	90	2000
6	24	10	30	780	99	11	75	1625
7	36	16	14	400	96	34	20	800
8	36	30	9	1270	110	37	19	2800
9	73	25	14	600	135	25	29	1500
10	21	27	23	1200	63	30	35	1650

PH: phagocytosis (mean fluorescence channel); CL: chemiluminescence (counts/neutrophil/30 min; values obtained with zymosan opsonized with homologous plasma).

Discussion

RhG-CSF administration causes a transient (up to 12h after injection) and indirect activation of neutrophils in circulation characterized by increased adherence to endothelium (with transient neutropenia)[9] and by increased surface expression of activation markers, including CD11b.[2] In a second phase (24h or more after G-CSF injection) a significant increase in CD14 (LPS receptor) and CD54 (ICAM-1) occurs, along with the appearance of surface expression of CD64 (FcRI), which is not constitutionally expressed by neutrophils.[2,4] These modifications have been explained as the consequence of an indirect effect of rhG-CSF on myeloid cells during their accelerated bone marrow transit. Activation markers (CD11b, CD13, CD45, CD67) are not affected by the cytokine during this phase,[2,4] although CD11b up-regulation has been observed in some patients during this second phase (maybe due to a different time-course of neutrophil activation in some subjects). CD16 expression can be down-regulated by rhG-CSF treatment in normal subjects,[2] in neoplastic patients subjected to chemotherapy,[4] and in neutropenic patients.[10]

Few data are available concerning CD32 alterations. Spiekermann et al. showed that these molecules are not modified.[4] Kerst et al.[2] reported a non-significant increase of FcRII expression in normal subjects after a single G-CSF injection. Elsner et al.[10] described increased FcRII expression after rhG-CSF administration in neutropenic subjects as compared to normal controls, but basal values of FcRII expression were not available due to the severe neutropenia prior to G-CSF therapy.

Our data show that CD32 expression can be enhanced by rhG-CSF administration. This molecule is not an activation marker because its expression is not affected by isolation procedures that activate neutrophils, and it cannot be up-regulated by fMLP stimulation (unpublished personal observations). Therefore an effect of G-CSF on CD32 synthesis by myeloid cells during their bone marrow transit could be hypothesized.

This increased CD32 expression was found to be associated with enhanced phagocytosis and phagocytosis-associated chemiluminescence. Indeed, CD32 is the major effector of neutrophil phagocytosis and phagocytosis-associated respiratory burst, and therefore a link between the modified neutrophil phenotype and the enhanced functional properties displayed by neutrophils after G-CSF administration is likely to occur. In some patients, however, an additional role may be played by CD11b up-regulation. In fact, this molecule is involved in the phagocytosis of particles that are bound via FcR, and there is evidence that this activity may be due to conformational changes which might allow the role of CD11b in phagocytosis and adhesion to be distinguished.

We did not find CD16 modifications after rhG-CSF treatment with the exception of one patient. A diminished amount of this molecule has been found in some diseases, and its expression can be influenced by several factors: previous neutrophil activation and/or underlying infections, or abnormal neutrophil maturation. In addition, the kinetics of CD16 expression after G-CSF treatment has been shown to display heterogeneous down-regulation, and a statistically significant reduction in the surface expression of this molecule was obtained by Spiekermann et al.[4] with relatively high G-CSF dosages (10-20 μg/kg).

We think that the functional and phenotypical changes in neutrophils induced by rhG-CSF emphasize the role of this cytokine in regulating the defensive properties of human neutrophils. Other G-CSF effects support this view. This cytokine increases the expression of fMLP receptors and this results in an increase in fMLP-stimulated superoxide anion generation.[3] In addition, the increased expression of CD14 may lead to marked priming effects by bacterial LPS. The clinical consequences of these alterations remain to be investigated, but a positive role for G-CSF can be hypothesized in patients suffering from hematological malignancies who, like those with non-Hodgkin's lymphomas, frequently show an increased risk of infection due to several mechanisms, including neutrophil defects and neutropenia after treatment with cytostatic agents.

References

1. Lieschke GJ, Burgess AW. Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor (second of two parts). N Engl J Med 1992; 327:99-106.
2. Kerst JM, de Haas M, van der Schoot CE, et al. Recombinant granulocyte colony-stimulating factor administration in healthy volunteers: induction of immunophenotypically and functionally altered neutrophils via an effect on myeloid progenitor cells. Blood 1993; 82:3265-72.
3. Ohsaka A, Saionji K, Sato N, Mori T, Ishimoto K, Inamatsu T. Granulocyte colony-stimulating factor down-regulates the surface expression of the human leucocyte adhesion molecule-1 on human neutrophils *in vitro* and *in vivo*. Br J Haematol 1993; 84:574-80.
4. Spiekermann K, Emmendoerffer A, Elsner J, et al. Altered surface marker expression and function of G-CSF-induced neutrophils from test subjects and patients under chemotherapy. Br J Haematol 1994; 87:31-8.
5. Wang JM, Chen ZG, Colella S, et al. Chemotactic activity of recombinant human granulocyte colony-stimulating factor. Blood 1988; 72:1456-60.
6. Roilides E, Walsh TJ, Pizzo PA, Rubin M. Granulocyte colony-stimulating factor enhances the phagocytic and bactericidal activity of normal and defective human neutrophils. J Infect Dis 1991; 163:579-83.
7. Roesler J, Emmendorffer A, Elsner J, Zeidler C, Lohmann-Matthes ML, Karl W. *In vitro* functions of neutrophils induced by treatment with rh-G-CSF in severe congenital neutropenia. Eur J Haematol 1991; 46:112-8.
8. Brugiatelli M, Federico M, Gobbi PG, et al. Epidoxorubicin vs idarubicin containing regimens in intermediate and high-grade non-Hodgkin's lymphoma: preliminary results of a multicentric randomized trial. Haematologica 1993; 78:306-12.
9. Bronchud MH, Potter MR, Morgenstern G, et al. *In vitro* and *in vivo* analysis of the effects of recombinant human granulocyte colony-stimulating factor in patients. Br J Cancer 1988; 58:64-9.
10. Elsner J, Roesler J, Emmendorffer A, Zeidler C, Lohman-Matthes M-L, Welte K. Altered function and surface marker expression of neutrophils induced by rhG-CSF treatment in severe congenital neutropenia. Eur J Haematol 1992; 48:10-9.