RAPID COMMUNICATION

Neutrophils Express the High Affinity Receptor for IgG (FcγRI, CD64) After In Vivo Application of Recombinant Human Granulocyte Colony-Stimulating Factor

By R. Repp, Th. Valerius, A. Sendler, M. Gramatzki, H. Iro, J. R. Kalden, and E. Platzer

Fc receptors are important effector molecules of neutrophilic granulocytes (polymorphonuclear neutrophils [PMN]), connecting phagocytic cells with the humoral immune response. Neutrophils from healthy donors express the low-affinity receptors for IgG FcγRII (CD32) and FcγRIII (CD16), but not the high-affinity receptor FcγRI (CD64). The latter has been found on neutrophils from patients with certain bacterial infections and can be induced in vitro after incubation with interferon-γ. We show here that neutrophils strongly express FcγRI after in vivo application of recombinant human granulocyte colony-stimulating factor (rhG-CSF). PMN from patients receiving rhG-CSF displayed higher cytotoxicity against Daudi lymphoma cells in vitro compared with control patients and with healthy donors. Fab fragments against FcγRII (monoclonal antibody [MoAb] IV.3) inhibited neutrophil-mediated cytotoxicity of healthy donors but not of patients during rhG-CSF therapy. Therefore, expression of Fc receptors by PMN was investigated by flow cytometry and the mean fluorescence intensity (MFI) was compared. After staining with MoAb 32.2 against FcγRI, the median MFI of neutrophils from G-CSF patients (median, 4.78; range, 2.40 to 8.50; n = 5) was significantly higher (P = .002 and P = .001, respectively) than the median MFI of patients not receiving G-CSF (median, 1.23; range, 1.01 to 1.58; n = 6) and the median MFI of healthy donors (median, 1.04; range, 0.67 to 1.12; n = 6). FcγRI disappeared after the discontinuation of the G-CSF injections, but was reinduced during the next treatment cycle with rhG-CSF. The high expression of FcγRI during rhG-CSF therapy correlated with enhanced cytotoxicity. In vitro incubation with rhG-CSF also enhances cytotoxicity, but only minor increments in FcγRII expression were observed. Thus, during in vivo application of rhG-CSF neutrophils acquire an additional potent receptor for mediating tumor cell killing in vitro by induction of the high-affinity receptor for IgG (FcγRI, CD64).

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The main function of neutrophilic granulocytes is to protect the body from invading microorganisms like bacteria and fungi. Consequently, the disappearance of neutrophils (e.g., as a consequence of chemotherapy for malignant diseases) can lead to severe, life-threatening infections. Detection and characterization of several hematopoietic growth factors that were found to regulate proliferation, commitment, differentiation, and functional activity of blood cells has fundamentally increased our knowledge of hematopoiesis. Recent data support the idea that treatment-related morbidity and mortality of cancer patients can be reduced by the clinical use of these growth factors as an adjunct to chemotherapy. Among the most promising of these substances is recombinant human granulocyte colony-stimulating factor (rhG-CSF) because it selectively promotes the proliferation and differentiation of neutrophilic progenitors and activates mature neutrophils in many aspects.

Receptors for the constant part of the heavy chain of IgG are important effector molecules on myeloid cells, connecting phagocytic cells with the humoral immune system. Three different receptors are known, which are recognized by different monoclonal antibodies (MoAbs). Only the high-affinity Fc receptor (FcγRI, CD64) binds monomeric IgG. In contrast, the low-affinity receptors FcγRII (CD32) and FcγRIII (CD16) only bind aggregated IgG. Freshly isolated neutrophils from healthy donors exclusively express FcγRII and FcγRIII, but FcγRI has been found on neutrophils from patients with streptococcal pharyngitis and on polymorphonuclear neutrophils (PMN) from patients with leukocyte adhesion deficiency. In vitro, FcγRI can be induced on neutrophils by interferon-γ (IFN-γ) incubation and neutrophils are able to kill extracellular targets via FcγRI after induction of this receptor by incubation with IFN-γ. In a controlled phase II/III trial, patients with squamous cell carcinoma of the oral cavity and pharynx were treated with chemotherapy and randomized for subcutaneous G-CSF injections (manuscript in preparation). Neutrophils isolated from the peripheral blood of patients during G-CSF therapy were more cytotoxic in an antibody-dependent cellular cytotoxicity (ADCC) assay against Daudi lymphoma cells than neutrophils from control patients or neutrophils from healthy donors. Our studies show that neutrophils from rhG-CSF–treated patients express high quantities of FcγRI during in vivo administration of rhG-CSF, which correlates to their enhanced cytotoxic capabilities. A functional role of FcγRI appears likely, because cytotoxicity of neutrophils without FcγRI (healthy donors) but not neutrophils with FcγRI (G-CSF–treated patients) can be completely inhibited by Fab fragments against FcγRII (IV.3).

MATERIALS AND METHODS

Media and reagents RF10* was prepared from RPMI 1640 (Seromed, Berlin, Germany) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (GIBCO, Paisley, Scotland), 4 mmol/L D-glutamine (GIBCO), and 10% fetal calf serum (FCS; GIBCO). Prophosphate-buffered saline (PBS)-Dulbecco (Ca2+ and Mg2+-free) was purchased from Seromed. Heparin (preservative free) was bought from Nordmark (Uetersen, Germany). Dextran T 500 was obtained from Pharmacia (Uppsala, Sweden). Isotonic Percoll

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advanced (stage I11 or IV) squamous cell carcinoma of the oral cavity were discontinued when the absolute neutrophil count exceeded 10,000/µL. The aim of the study was to evaluate the use of rhG-CSF in alleviating chemotherapy-induced bone marrow toxicity and its clinical consequences. All patients included in this study gave informed consent and the protocol of the study was approved by the Institutional Review Board of the Medical Faculty, University of Erlanger-Nürnberg, complying with the Helsinki declaration. One additional patient (M.V.) received G-CSF by compassionate use because of delayed neutrophil reconstitution after autologous bone marrow transplantation (BMT) for acute lymphocytic leukemia (ALL) and showed the same pattern of reactivity in antibody-dependent cellular cytotoxicity (ADCC) and FcRII expression as the total tumor patients.

An additional control patient (M.Z.), not receiving rhG-CSF, was studied for neutrophil Fc receptor expression during recovery from myeloablative therapy consisting of daunorubicin, ara-c, and VP-16 for acute myelogenous leukemia (AML).

Cell purification. Neutrophils were isolated as described previously.1 Briefly, 10 to 40 mL of peripheral blood was drawn into 2 mL of preservative-free heparin. Most of the erythrocytes were removed by hypotonic lysis. To separate neutrophils from cell detritus, a second Percoll-gradient centrifugation was performed. After washing, targets were adjusted to 100,000 cells in RF10*. Samples of 50 µL of the neutrophil suspension (100,000 cells) were added to 100 µL of RF10* in round-bottom microtiter plates. For experiments with freshly isolated PMN, assays were started directly by addition of 50 µL of the target cell suspension (5,000 cells) and release of label was measured after 3 hours. For experiments with G-CSF stimulation of PMN in vitro, targets were added after 18 hours of incubation and again 11Cr release was measured after 31 hours.

For experiments with Fab fragments to FcRII (IV.3), a concentration of 10 µg/mL was used, which gave complete inhibition of cytotoxicity mediated by FcRII-negative neutrophils when titrated from 0.32 to 10 µg/mL. Fab fragments were added to neutrophils 30 minutes before the start of the cytotoxicity assay. Data were analyzed using the formula:

\[
\text{Specific Lysis} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Maximum cpm} - \text{Spontaneous cpm}} \times 100
\]

Statistical analysis. Significance of difference of MFI and of specific lysis was analyzed by Student t-test. Pearson’s correlation coefficient were calculated to assess the correlation between expression of FcRI and cytotoxicity. Group data are expressed as means ± SD unless indicated otherwise.

RESULTS

In a controlled study patients with squamous cell carcinoma of the pharynx and the oral cavity were treated with chemotherapy and were randomized for subcutaneous G-CSF injections beginning 24 hours after each cycle of chemotherapy (see Materials and Methods). Fc receptors are important effector molecules of neutrophilic granulocytes (PMN), connecting phagocytic cells and the specific immune response. G-CSF has been shown to enhance functional activities of mature neutrophils.34 Because many functions of granulocytes are mediated by Fc receptors, their expression was investigated. Figure 1 shows the results of a representative experiment showing that neutrophils examined during rhG-CSF treatment strongly express the high-affinity receptor of IgG (FcRy1, CD64), whereas neutrophils of control patients or of healthy donors are either only weakly positive or even negative for this receptor. To compare all patients and healthy donors, we calculated the MFI (see Materials and Methods) of neutrophils labeled with MoAb 32.2 for flow cytometry. Table 1 shows that the median MFI of neutrophils from G-CSF patients (4.78) was significantly higher (P = .002 and P = .001, respectively) than the median MFI of patients not receiving G-CSF (1.23) and the median MFI of healthy donors (1.04). No consistent difference was found in the expression of FcRyRII (CD32) and FcRyRII (CD16) (data not shown).

Figure 2 show the expression of FcRy1 at three different time points of therapy. During G-CSF treatment, expression of FcRy1 was high and decreased to the level of control patients as measured 6 days after discontinuing G-CSF injections. After reinstalling G-CSF therapy, high levels were induced again.
G-CSF treatment is known to enhance proliferation in the neutrophil lineage. Similar conditions occur during spontaneous recovery from severely myelotoxic chemotherapy. Therefore, for comparison we studied neutrophils from a patient (M.Z.) recovering from myeloablative consolidation therapy for AML (see Materials and Methods). On the day of investigation the leucocyte count was 2,800/µL with 8% neutrophils. Of these neutrophils 74.3% stained positive for FcγRI (MFI = 2.09).

FcγRI, induced on neutrophils by in vitro incubation with IFN-γ, has been shown to be a trigger molecule for neutrophil cytotoxicity. Therefore, expression of FcγRI was compared with the cytotoxic capability of neutrophils.

**Figure 3** shows that freshly isolated neutrophils from G-CSF-treated patients display significantly \((P = .012)\) higher cytotoxicity against Daudi lymphoma cells \((56.73\% \pm 11.51\% \text{ specific lysis}; n = 3)\) compared with freshly isolated neutrophils from healthy donors \((6.58\% \pm 4.03\% \text{ specific lysis}; n = 4)\). Cytotoxicity of freshly isolated neutrophils correlates with the expression of FcγRI \((r = .953; P < .001)\). In vitro incubation of neutrophils with G-CSF for 18 hours also results in an significantly \((P = .001)\) increased cytotoxicity \((62.00\% \pm 10.87\% \text{ specific lysis}; n = 4)\) compared with PMN incubated for 18 hours without cytokine \((27.50\% \pm 13.05\% \text{ specific lysis}; \text{not shown in the figure})\). Thus, in vitro incubation with rhG-CSF enhanced neutrophil ADCC activity to a similar extent as G-CSF treatment in vivo. In contrast to the high expression of FcγRI during G-CSF therapy, induction of FcγRI by G-CSF in vitro is very weak \((MFI = 1.37 \pm 0.38 \text{ vs } 1.12 \pm 0.10, \text{G-CSF or medium controls, respectively}; n = 4)\) and does not correlate to the enhanced cytotoxicity. These results demonstrate that G-CSF stimulates neutrophil cytotoxicity in vivo and in vitro, but only in vivo application is related to enhanced expression of FcγRI.

**Table 1. Comparison of FcγRI Expression on PMN**

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<th></th>
<th>Median MFI</th>
<th>Range</th>
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<tbody>
<tr>
<td>Healthy donors</td>
<td>1.04</td>
<td>0.67-1.12</td>
<td>6</td>
</tr>
<tr>
<td>Control patients</td>
<td>1.23</td>
<td>1.01-1.58</td>
<td>6</td>
</tr>
<tr>
<td>G-CSF-treated patients</td>
<td>4.78</td>
<td>2.40-8.50</td>
<td>5</td>
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FcγRI expression of neutrophils was analyzed by flow cytometry using MoAb 32.2. Data are presented as MFI, calculated as described in Materials and Methods. Differences of MFI of granulocytes stained for FcγRI were significant for healthy donors versus G-CSF–treated patients \((P = .023)\) and for control patients versus G-CSF–treated patients \((P = .021)\), but not for healthy donors versus control patients.
Fig 2. Expression of FcγRI (CD64) of one patient is displayed at different time points of the treatment cycle (see patients and study design). Neutrophils were stained with MoAb 32.2 and fluorescence was determined by flow cytometry. Incubation concentrations (10 μg/mL) of Fab fragments against FcγRII (IV.3) inhibited cytotoxicity of neutrophils from a healthy donor incubated with G-CSF in vitro (27.2% specific lysis vs 2.7%), whereas cytotoxicity of neutrophils from patients receiving rhG-CSF in vivo is not inhibited by Fab fragments to FcγRII (56.5% ± 11.6% vs 55.8% ± 7.7%, n = 3). Thus, during rhG-CSF administration neutrophils acquire a new mechanism of antibody-dependent cytotoxicity mediated by a receptor distinct from FcγRII.

DISCUSSION

As we show here, neutrophil granulocytes from patients receiving rhG-CSF in vivo express high levels of the high-affinity receptor for IgG (FcγRI; CD64). This receptor is not regularly expressed on neutrophils from healthy donors, but has been demonstrated on PMN isolated from patients with bacterial infections. Recently, PMN from patients with streptococcal pharyngitis, but not from patients with documented urinary tract infection, were found to stain positive. Also, PMN from patients with leukocyte adhesion deficiency expressed FcγRI. In vitro, FcγRI can be induced on neutrophils by incubation with IFN-γ, but no induction by G-CSF was reported, although an additive effect between both cytokines in vitro was recently discussed. Our results show that exogenous rhG-CSF can induce large amounts of this receptor on neutrophils in vivo, whereas in vitro incubation of PMN with G-CSF has no significant effects on FcγRI expression. G-CSF in vitro and in vivo enhances cytotoxicity of PMN, but only cells stimulated in vitro can be inhibited by blocking of FcγRII.
which is the only trigger molecule of IgG-dependent lysis of untreated PMN of healthy donors. These findings suggest different mechanisms of G-CSF action on neutrophils in vitro and in vivo.

Increased levels of endogenous G-CSF are found under several hematologic conditions and after cytoreductive therapy, as well as during bacterial infections. Therefore, it seems possible that endogenous G-CSF is involved in the expression of FcyRI on PMN, which is found during certain infections and during regeneration of hematopoiesis, as shown here by patient M.Z. The mechanism of this induction is currently investigated in our laboratory, but an indirect effect of rhG-CSF via IFN-γ seems unlikely because G-CSF (unlike other cytokines) does not induce a cytokine cascade in vivo at the dose used in this study.

FcyRI differs from low-affinity Fcy receptors in the ability to bind monomeric IgG. Although in the presence of abundant monomeric Ig its functional role in vivo remains open, it is highly active in many in vitro assays. Because neutrophils can kill a broad range of tumor cells in vitro, it is worth considering whether rhG-CSF-activated neutrophils could take part in the directed host defense against certain tumors. Because neutrophil cytotoxicity is strictly antibody dependent, the presence of specific antibodies triggering cytotoxicity in vivo is a limiting condition. While many tumor hosts may not mount an antibody response to tumor cell antigens, this could be overcome by therapeutic application of exogenous MoAbs. The possibility of using heterochimeric antibodies with specificity against both cytotoxic trigger molecules on effector cells and against tumor specific epitopes appears particularly promising. Among known trigger molecules, Fc receptors are the most important on nonlymphoid cells. Thus, the combination of rhG-CSF with heterochimeric antibodies could be another approach to biologic cancer therapy.

REFERENCES

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