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. Platterz

Fc receptors are important effector molecules of neutrophilic granulocytes (polymorphonuclear neutrophils [PMN]), connecting phagocytic cells with the humoral immune system. 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). FcγRII disappeared after the discontinuing 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 (eg, 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 mM/L L-glutamine (GIBCO), and 10% fetal calf serum (FCS; GIBCO), proprionate-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
and Fab fragments to FcyRII (IV.3) were purchased from
Medarex (West Lebanon, NH). For staining of FcyRII MoAb IV.3
IgG 2b; Medarex) or MoAb 2E1 (IgG 2a; Dianova, Hamburg,
Germany) were used and gave similar results. Antibody to complement
receptor III (OKM 1: IgG 2b) and irrelevant antibody
(10-3.6.2.; IgG 2a) was produced in our laboratory from the
hybridoma clones CRL 8026 and TIB 92, respectively, which were
provided by American Type Culture Collection (ATCC; Rockville,
MD). A fluorescein isothiocyanate (FITC)-conjugated goat antima-
hybridoma was used, which was a generous gift from L. Souza (Amgen,
Millwood, CA). For only experiments with 18 hours of
preincubation of PMN was rhG-CSF added in vitro at a final
concentration of 100 U/mL.

Patients. In a controlled study (GCSF8806) patients with ad-
vanced (stage III or IV) squamous cell carcinoma of the oral cavity
and pharynx received chemotherapy with 5-fluorouracil (5-FU)
(1,000 mg/m² on day 1 to 5) and cisplatinum (100 mg/m² on day 1)
before radiotherapy or surgery. Patients were prospectively random-
ized to be placed in a control arm or to receive subcutaneous (SC)
injections of open-labeled rhG-CSF (200 μg/m²/day). rhG-CSF injec-
tions were started 24 hours after each cycle of chemotherapy and
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 toxici-
ty 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, Univer-
sity of Erlanger-Nürnberg, complying with the Helsinki declara-
tion. One additional patient (M.V.) received G-CSF by compassion-
ate use because of delayed neutrophil reconstitution after
autologous bone marrow transplantation (BMT) for acute lympho-
cytic leukemia (ALL) and showed the same pattern of reactivity in
antibody-dependent cellular cytotoxicity (ADCC) and FcyRI ex-
pression as the solid 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
VP16 for acute myelogenous leukemia (AML).

Cell purification. Neutrophils were isolated as described previ-
ously.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 dextran sedimentation and the leukocyte-rich superna-
tant was layered over a discontinuous Percoll-gradient. After
centrifugation, neutrophils were collected at the interphase be-
tween two layers of 68.5% and 63% percoll and the remaining
erthrocytes were removed by hypotonic lysis. To separate neutro-
phils from cell detritus, a second Percoll-gradient centrifugation
was added during antibody incubation to inhibit nonspecific
binding to the high-affinity Fc receptor for IgG. After washing four
times in PBS + 1% bovine serum albumin (BSA), FITC-labeled
goat antimouse MoAb was added for 30 minutes at 4°C. Cells were
washed again and resuspended in PBS for analysis in EPICS
PROFILE (Coulter Corporation, Hialeah, FL). To compare results,
mean fluorescence intensity (MFI) is expressed as the
increase of fluorescence intensity compared with PMN labeled
with irrelevant antibody.

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 FcyRI and cytotoxicity. Group data are expressed as
means ± SD unless indicated otherwise.

RESULTS

In a controlled study patients with squamous cell carcino-
ma 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 granulo-
cytes (PMN), connecting phagocytic cells and the specific
immune response. G-CSF has been shown to enhance
functional activities of mature neutrophils.14 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 (FcyRI, CD64), whereas
neutrophils of control patients or of healthy donors are
either only weakly positive or even negative for this recep-
tor. To compare all patients and healthy donors, we
calculated the MFI (see Materials and Methods) of neutro-
phils 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 FcyRII (CD32) and FcyRIII (CD16) (data
not shown).

Figure 2 shows the expression of FcyRI at three different
time points of therapy. During G-CSF treatment, expres-
sion of FcyRI 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 FcyRI (MFI = 2.09).

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

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

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<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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FcyRI 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 FcyRI 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.

Figure 3 shows that freshly isolated neutrophils from G-CSF–treated patients display significantly (P = .012) higher cytotoxicity against Daudi lymphoma cells (56.73% ± 11.51% specific lysis; n = 3) compared with freshly isolated neutrophils from healthy donors (6.58% ± 4.03% specific lysis; n = 4). Cytotoxicity of freshly isolated neutrophils correlates with the expression of FcyRI (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% ± 10.87% specific lysis; n = 4) compared with PMN incubated for 18 hours without cytokine (27.50% ± 13.05% specific lysis; 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 FcyRI during G-CSF therapy, induction of FcyRI by G-CSF in vitro is very weak (MFI = 1.37 ± 0.38 vs 1.12 ± 0.10, 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 FcyRI.

Neutrophils stimulated with G-CSF in vitro kill tumor cells via FcyRI. A functional role of FcyRI expressed on PMN during G-CSF therapy is suggested, because saturat-
Fig 2. Expression of FcyRI (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.

Neutrophils were stained with MoAb 32.2 and fluorescence was determined by flow cytometry. Concentrations (10 µg/mL) of Fab fragments against FcyRII (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 FcyRII (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 FcyRII.

**DISCUSSION**

As we show here, neutrophil granulocytes from patients receiving rhG-CSF in vivo express high levels of the high-affinity receptor for IgG (FcyRI; 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 FcyRI. In vitro, FcyRI 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 FcyRI expression. G-CSF in vitro and in vivo enhances cytotoxicity of PMN, but only cells stimulated in vitro can be inhibited by blocking of FcyRII.
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 IgG 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.

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Neutrophils express the high affinity receptor for IgG (Fc gamma RI, CD64) after in vivo application of recombinant human granulocyte colony-stimulating factor

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