Expression of Receptors for Granulocyte Colony-Stimulating Factor on Neutrophils From Patients With Severe Congenital Neutropenia and Cyclic Neutropenia

By Urte Kyas, Torsten Pietsch, and Karl Welte

We studied granulocyte colony-stimulating factor (G-CSF) binding sites on neutrophils from patients with severe congenital neutropenia (SCN; Kostmann-syndrome) and cyclic neutropenia (CN) during treatment with recombinant human (rh) G-CSF. G-CSF receptor expression was measured by scatchard analysis. Neutrophils from six healthy controls expressed between 480 and 1,210 binding sites per cell, whereas neutrophils from five SCN patients expressed increased numbers of G-CSF binding sites ranging between 2,100 and 3,900 per cell. Neutrophils from four patients with CN expressed 350 to 1,600 binding sites per cell. The affinity of rhG-CSF to its receptor was similar in patients and controls. These data suggest that SCN patients and CN patients are not defective in G-CSF receptor expression as judged by the numbers of G-CSF binding sites and binding affinity; however, we cannot exclude defects in parts of the G-CSF receptor that may be involved in the signal transduction pathway.

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MATERIALS AND METHODS

Patients. We have tested five patients with SCN and four patients with CN. The patient characteristics are listed in Table 1. All patients were diagnosed in the first year of life and had a history of at least one episode of severe bacterial infection per year requiring intravenous antibiotic treatment. There were no signs of bacterial infections at the time of receptor studies.

Isolation of neutrophils. Ten milliliters of heparinized blood (100 U/ml Heparin Novo, Novo Industrie, Mainz, Germany) was drawn 24 hours after subcutaneous G-CSF administration (before the next dose of rhG-CSF). The blood was mixed with 5 mL Haes-Ethyl-Starch (Plasmasteril, Fresenius, Oberursel, Germany) in a 15-mL glass tube and incubated for 30 minutes at room temperature. The supernatant was layered on 2 mL Ficoll-Paque (Pharmacia, Freiburg, Germany) and centrifuged at 2,000 rpm for 20 minutes. The pellet was immediately resuspended in 5 mL distilled water and 2.5 mL of a 2.7% solution of NaCl to lysate contaminating erythrocytes. Cells were washed twice with RPMI 1640. To remove bound G-CSF (from the neutrophils), cells were then resuspended for 20 seconds in sodium-citrate buffer (10 mmol/L; pH 4.0) and subsequently washed with RPMI 1640.

Iodination of recombinant human G-CSF. Three micrograms of the rhG-CSF analogue Met-Lys-rhG-CSF (kindly provided by Dr L. Souza, Amgen, Thousand Oaks, CA) was radioactively labeled by the method described by Bolton and Hunter15 using 1 mCi of iodinated Bolton-Hunter reagent (Amersham, Braunschweig, Germany). 125I-G-CSF was separated from other reaction products using a P6-DG desalting column (Biorad), equilibrated with phosphate-buffered saline, containing 0.02% Tween 20 (Biorad, Munich, Germany) and 0.01% NaCl. This procedure resulted in a specific radioactivity of 12.5 μCi/μg protein.

G-CSF binding assay. In a total volume of 500 μL RPMI 1640 containing 0.1% gelatin, 4 × 10⁶ neutrophils were incubated with various concentrations of 125I-G-CSF in the range from 100 to 30,000 pmol/L. Identical incubations were performed in the presence of 2.5 μmol/L of unlabeled rhG-CSF. The specific binding was calculated from the difference between counts per minute (cpm) in the absence and presence of unlabeled G-CSF. This mixture was incubated for 1 hour at 37°C. Aliquots of 125 μL
were then layered onto a 125-μL silicon oil cushion and centrifuged. The radioactivity in the pellet and supernatant was counted separately in a gamma counter. The dissociation constant $k_d$ and number of binding sites per cell were determined using scatchard analysis.

RESULTS

Neutrophils from five patients with SCN and four patients with CN during rhG-CSF treatment were investigated for G-CSF receptor expression. The patient characteristics including ANC at the day of receptor studies are listed in Table 1. The specific binding of $^{125}$I-G-CSF and Scatchard analysis are shown for one patient (F.R.) and one control in Fig 1.

Using this method, we detected between 480 and 1,210 G-CSF binding sites per cell with a $k_d$ of $8 \times 10^{-10}$ mol/L on neutrophils from healthy individuals ($n = 6$) (Table 2). Neutrophils from all SCN patients expressed increased numbers of G-CSF binding sites in the range from 2,100 to 3,900 per cell (Table 2). The dissociation constant between G-CSF and its receptor was comparable in both patients and healthy subjects (4.5 to $3 \times 10^{-10}$ mol/L). Neutrophils from four CN patients had 350, 500, 630, and 1,600 binding sites per cell, respectively. Neutrophils from SCN and CN patients were tested during rhG-CSF therapy (Table 1). To test the individual variability of the receptor expression in one patient (F.R.), binding studies were performed on 2 different days. The number of G-CSF binding sites on neutrophils from this patient was 3,900 per cell at the first investigation and at a second measurement 3 months later 3,200 per cell (Table 2).

DISCUSSION

In this study, we describe G-CSF receptor expression of neutrophils from patients suffering from SCN or CN. Severe congenital neutropenia is characterized by a maturation arrest of neutrophil precursor cells to mature neutrophils at the promyelocyte level. For comparison, we also tested the receptor expression of neutrophils from patients with cyclic neutropenia. As G-CSF is the essential growth and differentiation factor for neutrophil precursors, it seemed reasonable to test whether SCN or CN patients have a defect in G-CSF production or in expression of the receptor. Recently, we have demonstrated that monocytes from SCN patients are capable of producing normal amounts of biologically active G-CSF. In addition, we found increased serum levels of biologically active G-CSF in SCN patients compared with healthy controls. In this study, we investigated the G-CSF receptor expression on neutrophils from patients with SCN or CN. The defective or decreased response of neutrophil precursors from these patients in vitro (CFU-GM assays) and in vivo suggested that neutrophil precursors or mature neutrophils from these patients might express lower receptor numbers or receptors with an altered affinity to G-CSF. Therefore, we performed binding assays with $^{125}$I-rhG-CSF on neutrophils from patients with SCN or CN. The defective or decreased receptor expression of neutrophils might be due to a decreased receptor number or affinity of the receptor. Other investigators used different analogues of recombinant G-CSF: Uzumaki et al: Ala-1, Thr-3, Tyr-4, Arg-5, and Ser-17-G-CSF with a $k_d$ of 235 pmol/L. Hanazono et al: $^{125}$I-rhG-CSF on neutrophils from healthy individuals. Using Scatchard analysis (Fig 1), increased numbers of G-CSF binding sites on neutrophils from patients with SCN as compared with healthy persons could be demonstrated (Table 2). Control subjects expressed between 480 and 1,210 G-CSF binding sites per cell with a $k_d$ of $8 \times 10^{-10}$ mol/L; neutrophils from patients with SCN expressed 2,100 to 3,900 binding sites per cell with a similar $k_d$.

This is the same range reported by Nicola et al for the binding of radiolabeled murine G-CSF on human neutrophils (600 to 1,000 receptors/cell; $k_d$: 880 pmol/L). Other investigators used different analogues of recombinant G-CSF: Uzumaki et al: Ala-1, Thr-3, Tyr-4, Arg-5, and Ser-17-G-CSF with a $k_d$ of 235 pmol/L. Hanazono et al: $^{125}$I-rhG-CSF on neutrophils from healthy individuals. Using Scatchard analysis (Fig 1), increased numbers of G-CSF binding sites on neutrophils from patients with SCN as compared with healthy persons could be demonstrated (Table 2). Control subjects expressed between 480 and 1,210 G-CSF binding sites per cell with a $k_d$ of $8 \times 10^{-10}$ mol/L; neutrophils from patients with SCN expressed 2,100 to 3,900 binding sites per cell with a similar $k_d$.

Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>ANC/μL at the Day of Study</th>
<th>Day of rhG-CSF Treatment*</th>
<th>rhG-CSF Dosage (μg/kg/d)</th>
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<tbody>
<tr>
<td>SCN</td>
<td>B.S.</td>
<td>20</td>
<td>F</td>
<td>1,168</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>T.G.</td>
<td>17</td>
<td>M</td>
<td>4,160</td>
<td>302</td>
</tr>
<tr>
<td></td>
<td>F.R.</td>
<td>20</td>
<td>M</td>
<td>2,580</td>
<td>281 (1)</td>
</tr>
<tr>
<td></td>
<td>T.S.</td>
<td>6</td>
<td>F</td>
<td>5,020</td>
<td>378 (2)</td>
</tr>
<tr>
<td></td>
<td>T.F.</td>
<td>12</td>
<td>M</td>
<td>2,304</td>
<td>607</td>
</tr>
<tr>
<td></td>
<td>E.G.</td>
<td>13</td>
<td>M</td>
<td>700</td>
<td>253</td>
</tr>
<tr>
<td></td>
<td>M.H.</td>
<td>23</td>
<td>F</td>
<td>1,950</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>T.L.</td>
<td>9</td>
<td>M</td>
<td>1,060</td>
<td>385</td>
</tr>
<tr>
<td></td>
<td>B.S.</td>
<td>36</td>
<td>M</td>
<td>2,730</td>
<td>63</td>
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</table>

*Timepoint of G-CSF receptor studies.

![Fig 1. Binding of $^{125}$I-rhG-CSF to neutrophils. For 1 hour at 37°C, 8 × 10⁶ cells/mL were incubated with different concentrations of $^{125}$I-rhG-CSF. Binding was determined in the absence and presence of 2.5 μmol/L unlabeled rhG-CSF. The binding parameters were determined by Scatchard analysis. (A) Specific binding; (B) Scatchard analysis. (---), Control; (----), F.R.](image-url)
Tyr-Prol-Tyr–G-CSF with a kd of 250 pmol/L. The higher affinity of these G-CSF analogues might be due to the modification of the molecule. In addition, the techniques for radioactive labeling G-CSF were different in these investigations (eg, lactoperoxidase) compared with our technique (Bolton-Hunter). In our study we wanted to show that the kd of G-CSF receptors is not altered in SCN patients as compared with healthy persons. The small number of individuals tested by all investigators does not allow statistical statements about the “normal” kd and “normal” number of receptors.

The increased numbers of G-CSF binding sites on neutrophils from SCN patients could be a result of a feedback regulation mechanism via other cytokines released due to the maturation arrest of neutrophil precursors. During rhG-CSF treatment, a partial maturation arrest at the level of promyelocytes is still present in these patients. A second hypothesis for the increased number of G-CSF binding sites is that rhG-CSF treatment might upregulate the number of G-CSF binding sites as has been shown in vitro for other cytokines such as interleukin-2. However, neutrophils from patients with CN seem to have normal numbers of G-CSF binding sites even during rhG-CSF treatment. Neutrophils from CN patients might have changing numbers of G-CSF binding sites depending on the timepoint within the cycle. On the other hand, the “normal” receptor numbers on neutrophils from CN patients might be explained by the complete maturation of neutrophil precursors in these patients and, therefore, no need to overcome a maturation arrest with an increased receptor number as seen in SCN patients.

From this data, we conclude that there is no defect in G-CSF receptor expression on neutrophils from patients with SCN and that the G-CSF receptor is able to bind rhG-CSF with the same affinity as shown for control subjects. However, from this data we cannot exclude that the intracellular portion of the G-CSF receptor might be defective by point mutation or alternative splicing mechanisms of mRNA. Indeed, recently published data about the human G-CSF receptor demonstrated that there are at least three different G-CSF receptor molecules, depending on alternative splicing of the RNA. Nothing is known about the quality of signal transduction of these various G-CSF receptor molecules or about the second messenger pathways activated by binding of G-CSF to these receptors. In patients with SCN, the G-CSF receptor remains to be biochemically characterized. Our preliminary results showed that the gene for the G-CSF receptor is not grossly altered as judged by Southern blot analysis of DNA from patients with SCN (Larson A, Kyas U, Pietsch T, Welte K: unpublished observations, July 1990).

In conclusion, we have demonstrated that the defective response to G-CSF in vitro and in vivo in SCN and CN patients is not due to low G-CSF receptor numbers or binding affinity of G-CSF to its receptor. However, from this data we cannot exclude that neutrophil precursors (eg, promyelocytes) might have decreased receptor numbers even during rhG-CSF therapy.

NOTE ADDED IN PROOF

The transmembrane part of the G-CSF receptor from SCN patients seems not to be altered compared with receptors from healthy donors as could be shown in sequencing analysis.

ACKNOWLEDGMENT

We thank Frau Petra Beenken for excellent technical assistance, Larry Souza for helpful discussion, and Frau Angela Schober for secretarial help.

REFERENCES


<table>
<thead>
<tr>
<th>Patient</th>
<th>G-CSF Binding Sites per Cell</th>
<th>Dissociation Constant (×10^11 mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T.G.</td>
<td>2,100</td>
<td>4.5</td>
</tr>
<tr>
<td>F.R. (1)*</td>
<td>3,900</td>
<td>10</td>
</tr>
<tr>
<td>F.R. (2)</td>
<td>3,200</td>
<td>7.6</td>
</tr>
<tr>
<td>T.S.</td>
<td>2,500</td>
<td>6</td>
</tr>
<tr>
<td>T.F.</td>
<td>3,800</td>
<td>18</td>
</tr>
<tr>
<td>CN E.G.</td>
<td>630</td>
<td>14</td>
</tr>
<tr>
<td>M.H.</td>
<td>350</td>
<td>7</td>
</tr>
<tr>
<td>T.L.</td>
<td>500</td>
<td>5</td>
</tr>
<tr>
<td>B.K.</td>
<td>1,600</td>
<td>12</td>
</tr>
<tr>
<td>Controls</td>
<td>480-1,210</td>
<td>8-20</td>
</tr>
</tbody>
</table>

*Patient F.R. was tested twice. The second measurement (2) was performed 3 months after the first (1).


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