Effect of the Chimeric Soluble Granulocyte Colony-stimulating Factor Receptor on the Proliferation of Leukemic Blast Cells from Patients with Acute Myeloblastic Leukemia

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ABSTRACT

The biological roles of the soluble granulocyte colony-stimulating factor (G-CSF) receptor, which arises as a result of alternative RNA splicing, are as yet unknown. In this study, we examined the in vitro effect of a chimeric protein composed of the extracellular region of a murine G-CSF receptor and the human IgG1 Fc region because a human natural soluble G-CSF receptor was not available. First, we found that this chimeric soluble G-CSF receptor could inhibit the biological activity of G-CSF on normal bone marrow colony formation. Because G-CSF also plays an important role in the proliferation of leukemic blast cells, we next examined the effect of the soluble G-CSF receptor on leukemic blast colony formation in 10 acute myeloblastic leukemia cases. Although G-CSF stimulated the proliferation of leukemic progenitor cells to form leukemic blast colonies, the chimeric soluble G-CSF receptor completely inhibited this stimulatory effect. Furthermore, the chimeric soluble G-CSF receptor also inhibited spontaneous leukemic blast colony formation in two cases. Because a high concentration of G-CSF was observed in the supernatants of leukemic blast cells from these two cases, it seems likely that the soluble G-CSF receptor cut off the autocrine growth mechanism of leukemic blast cells mediated by G-CSF. These findings suggest the possibility that the soluble G-CSF receptor could be used in a clinical application for acute myeloblastic leukemia patients in the future.

INTRODUCTION

G-CSF is a cytokine that regulates the proliferation and differentiation of neutrophilic lineage cells (1). These biological activities of G-CSF are mediated by specific receptors on the surface of responsive cells. The wild-type receptor for G-CSF is 813 amino acids in length and has an extracellular region, a transmembrane region, and an intracellular region. However, a further four different G-CSF receptor isoforms arising from alternative RNA splicing have been isolated that are all identical in their extracellular regions but differ in their downstream sequences (2, 3). One of them is of a soluble form lacking intracellular region. On the other hand, G-CSF is also known to play an important role in the proliferation of leukemic blast cells from patients with AML (4–6). In some AML cases, leukemic blast cells are found to produce G-CSF themselves, which suggests the presence of an autocrine growth mechanism mediated by G-CSF (7). In this study, we examined the effect of the soluble G-CSF receptor on the proliferation of leukemic blast cells.

RESULTS

Effect of the Soluble G-CSF Receptor on the Biological Activity of G-CSF

Leukemic Blast Cells. Peripheral blood specimens from 10 different AML patients with highly leukemic blood cell counts were sampled at the time of diagnosis. Mononuclear cells were obtained by density gradient centrifugation, and contaminating T-lymphocytes and monocyte/macrophages were depleted by rosetting with sheep erythrocytes and repeated adherence to a plastic surface (4–6). All of the samples contained at least 97% leukemic blast cells by morphology.

Soluble G-CSF Receptor. Because a human natural soluble G-CSF receptor is not available, we used a chimeric protein composed of the extracellular region of a murine G-CSF receptor and the human IgG1 Fc region, which was kindly provided by Drs. N. Nishi and H. Ohashi (Kirin Brewery Co., Ltd., Tokyo, Japan; Ref. 8).

Hematopoietic Factors. Human recombinant G-CSF and GM-CSF were furnished by Kirin Brewery Co., Ltd. Human recombinant TNF-α was obtained from the Dainippon Pharmaceuticals Co., Ltd. (Tokyo, Japan).

Leukemic Blast Colony Assay. Details of the leukemic blast colony assay have been reported previously (4–6). Briefly, leukemic blast cells were plated in plastic 96-microwell plates in α medium (Life Technologies, Inc., Grand Island, NY) containing 0.88% methylcellulose and 10% FCS (Flow Laboratories, McLean, VA). Each well, containing 2 × 10⁶ cells in 0.1 ml of medium with appropriate stimulators, was incubated for 6 days in a moist atmosphere of 5% CO₂ in air at 37°C, and compact colonies containing more than 20 cells were counted using an inverted microscope. Individual colonies were selected, and the cells were placed directly on slides. After air drying and fixation in absolute methanol, these were stained with Giemsa solution. The cells within the colonies were similar in morphology to the patients’ leukemic blast cells.

Measurement of G-CSF Production from Leukemic Blast Cells. Leukemic blast cells were cultured at 10⁶/ml with or without 100 units/ml of TNF-α in RPMI 1640 (Life Technologies, Inc.) containing 10% FCS for 24 h. The production of G-CSF in cell-free culture supernatants was determined using a double-sandwich ELISA kit, which was purchased from R&D Systems, Minneapolis, MN. The ELISA protocol was followed in accordance with the manufacturer’s guidelines.

CFU-G Assay. The CFU-G assay was performed using the colony formation technique in methylcellulose culture as described previously (9, 10). Normal bone marrow cells were obtained from an informed volunteer.

MATERIALS AND METHODS

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RESULTS

Effect of the Soluble G-CSF Receptor on the Biological Activity of G-CSF

First, we examined the effect of the soluble G-CSF receptor on normal bone marrow colony formation. A substantial number of CFU-G colonies was formed in the presence of 10 ng/ml of G-CSF. When serial concentrations of the chimeric soluble G-CSF receptor were added, these stimulatory effects began to decline at the concentration of 1 μg/ml, and completely vanished at the concentration of 50 μg/ml (Fig. 1). On the other hand, human IgG could not affect these colony formations (data not shown). These results indicate that the soluble G-CSF receptor was able to antagonize the biological activity of G-CSF on normal bone marrow colony formation in a concentration-dependent manner. In contrast, the biological activity of GM-CSF was not inhibited by the chimeric soluble G-CSF receptor (Fig. 1).

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2 To whom requests for reprints should be addressed. Phone: 81-92-642-5230; Fax: 81-92-642-5247.

3 The abbreviations used are: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; AML, acute myeloblastic leukemia; TNF-α, tumor necrosis factor α; CFU-G, granulocyte colony-forming unit.
EFFECT OF SOLUBLE G-CSF RECEPTOR ON AML CELLS

Effect of the Soluble G-CSF Receptor on the Proliferation of Leukemic Blast Cells. Next, we examined the effect of the soluble G-CSF receptor on the proliferation of leukemic blast cells from 10 AML patients. The leukemic blast colony formation was substantially stimulated by 10 ng/ml of G-CSF in 7 of the 10 cases, although the responsiveness was quite different among these cases (Table 1). When 50 μg/ml of the chimeric soluble G-CSF receptor was added, the stimulatory effects of G-CSF on leukemic blast colony formation were completely inhibited in all of the cases examined. Furthermore, in four cases, leukemic blast colonies were formed without any exogenous stimulator, yet the chimeric soluble G-CSF receptor was also able to inhibit spontaneous colony formation in two of these cases (cases 8 and 9).

Concentration of G-CSF in the Condition Medium of Leukemic Blast Cells. To clarify the mechanism involved in the inhibitory effect of the soluble G-CSF receptor on spontaneous leukemic blast colony formation, we determined the concentration of G-CSF in the supernatants of leukemic blast cells. In 5 of the 10 cases, the spontaneous production of G-CSF was observed, and it was increased in the presence of TNF-α (Table 2). A high concentration of G-CSF was found in the condition medium of leukemic blast cells obtained from cases 8 and 9, in which the chimeric soluble G-CSF receptor had inhibited spontaneous leukemic blast colony formation.

DISCUSSION

Soluble receptors for various cytokines are known to be created either by alternative RNA splicing or by proteolytic cleavage of the extracellular region (11). The biological roles of these soluble receptors are quite different among the cytokines. For example, soluble receptors for growth hormone can act as binding proteins to stabilize ligands in the extracellular space (12), whereas soluble receptor α chains for interleukin 6 can act as agonists by initiating the assembly of the β subunits into a signal-transducing unit (13). In contrast, the soluble receptors for epidermal growth factor can act as antagonists by competing with membrane-bound receptors for ligand binding (14). However, it remains unknown whether the soluble G-CSF receptor, which arises as a result of alternative RNA splicing, can modulate the activity of G-CSF positively or negatively.

First, we examined the effect of the soluble G-CSF receptor on normal bone marrow colony formation. Because a human soluble G-CSF receptor was not available, we used a chimeric protein composed of the extracellular region of a murine G-CSF receptor and the human IgG1 Fc region. It is known that human G-CSF can act through the murine G-CSF receptor, because the murine G-CSF receptor is highly homologous with the human G-CSF receptor, with an overall homology of 72.5% at the nucleotide level and 62.5% at the amino acid level (2, 15). As shown in Fig. 1, this chimeric soluble G-CSF receptor completely inhibited the biological activity of G-CSF on CFU-G colony formation but did not affect the biological activity of GM-CSF. In addition, it was reported that G-CSF binding to the soluble receptor monomer occurs with high affinity and that the binding of G-CSF enhances the soluble receptor dimerization (16). These findings indicate that the soluble G-CSF receptor works as an antagonist by competing with membrane-bound receptors for G-CSF binding.

On the basis of the above findings, it may be possible to use the soluble G-CSF receptor as a new therapeutic tool for AML patients, because G-CSF can also stimulate the proliferation of leukemic blast cells (4–6). To gauge this possibility, we examined the effect of the soluble G-CSF receptor on leukemic blast colony formation. Whereas G-CSF stimulated the proliferation of leukemic progenitor cells to form leukemic blast colonies, the chimeric soluble G-CSF receptor completely inhibited this stimulatory effect of G-CSF. Leukemic blast cells are considered to be constantly stimulated by G-CSF in vivo, because substantial concentrations of G-CSF have been observed in the condition medium of normal bone marrow stromal cells.4 Soluble G-CSF receptor may be effective in inhibiting this constant growth stimulation of leukemic blast cells by G-CSF.

On the other hand, the growth mechanism of leukemic blast cells in vivo is thought to be very complicated. Previously, we reported that

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Table 1 Effect of the chimeric soluble G-CSF receptor on leukemic blast colony formation

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Medium</th>
<th>G-CSF</th>
<th>Soluble G-CSFR</th>
<th>G-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (M1)</td>
<td>0</td>
<td>48±6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 (M2)</td>
<td>0</td>
<td>20±0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 (M3)</td>
<td>7±1</td>
<td>43±5</td>
<td>6±2</td>
<td>7±2</td>
</tr>
<tr>
<td>4 (M4)</td>
<td>0</td>
<td>18±5</td>
<td>0</td>
<td>0</td>
</tr>
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<td>5 (M5)</td>
<td>0</td>
<td>60±4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6 (M6)</td>
<td>0</td>
<td>25±4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7 (M7)</td>
<td>47±6</td>
<td>73±7</td>
<td>16±4</td>
<td>14±5</td>
</tr>
<tr>
<td>8 (M8)</td>
<td>83±9</td>
<td>134±5</td>
<td>57±4</td>
<td>59±10</td>
</tr>
<tr>
<td>9 (M9)</td>
<td>26±9</td>
<td>27±11</td>
<td>26±7</td>
<td>31±3</td>
</tr>
</tbody>
</table>

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* Numbers of leukemic blast colonies are shown as means ± SD per 10⁷ cells as determined from triplicate plates.

50 μg/ml.

10 ng/ml.

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Table 2 Concentration of G-CSF in the condition medium of leukemic blast cells

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Concentration of G-CSF (pg/ml)</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;10</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>&lt;10</td>
<td>&lt;10</td>
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<td>5</td>
<td>&lt;10</td>
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<td>6</td>
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<td>40</td>
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<tr>
<td>8</td>
<td>630</td>
<td>1200</td>
</tr>
<tr>
<td>9</td>
<td>101</td>
<td>480</td>
</tr>
<tr>
<td>10</td>
<td>14</td>
<td>43</td>
</tr>
</tbody>
</table>

* Mean values of triplicate cultures, with SD always <10%.

# 100 units/ml.

leukemic blast cells produced various cytokines, including G-CSF, in some AML cases (7, 17). In addition, TNF-α, which is secreted by normal bone marrow stromal cells and/or some leukemic blast cells (7), can stimulate leukemic blast cells to produce other cytokines (18). These phenomena suggest the presence of an autocrine or paracrine growth mechanism in vivo. When we examined the concentration of G-CSF in the supernatants of leukemic blast cells with or without TNF-α, a relatively high concentration was observed in two cases (cases 8 and 9). Because the chimeric soluble G-CSF receptor inhibited the spontaneous leukemic blast colony formation in these two cases, the soluble G-CSF receptor may be able to cut off the autocrine growth mechanism of leukemic blast cells mediated by G-CSF. However, even in these cases, the spontaneous leukemic blast colony formation was not completely inhibited, although we had added a high dose of the chimeric soluble G-CSF receptor, which was expected to completely inhibit the activity of G-CSF secreted from leukemic blast cells. This finding suggests that the soluble G-CSF receptor is not a potent inhibitor for the autocrine growth of leukemic blast cells, because some other autocrine growth mechanism, e.g., mediation by GM-CSF, may also be present.

In this report, we used a chimeric protein composed of the extracellular region of a murine G-CSF receptor and the human IgG1 Fc region, instead of the natural soluble human G-CSF receptor. Because this chimeric soluble G-CSF receptor may have different effects on human cells than the natural soluble human G-CSF receptor, further studies using the natural soluble receptor are essential to analyzing the possibility of a clinical application. The organ toxicities caused by administration of the natural soluble G-CSF receptor may be mild, because the natural soluble receptor arising from alternative RNA splicing is not an artificial chemical reagent but a physiological substance. The soluble G-CSF receptor may provide a further clinical application for AML patients in the future.

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