Granulocyte Colony-Stimulating Factor Receptors in Human Acute Myelocytic Leukemia

By Leo M. Budel, Ivo P. Touw, Ruud Dewel, and Bob Löwenberg

The binding of granulocyte colony-stimulating factor (G-CSF) to normal and human acute myeloid leukemia (AML) cells was investigated with radiolabeled recombinant human G-CSF (rhG-CSF). In all 14 cases of primary AML specific receptors for G-CSF were demonstrated on purified blast cells. The average numbers of G-CSF receptors ranged from very low to 428 receptors per cell. Normal granulocytes showed G-CSF binding sites on their surface at higher densities (703 to 1.296 sites per cell). G-CSF receptors appeared to be of a single affinity type with a dissociation constant (kd) ranging between 214 and 378 pmol/L for AML blasts and 406 to 648 pmol/L for granulocytes. In 12 of 14 cases, including those with relatively low specific binding, G-CSF was a potent inducer of DNA synthesis of blasts in vitro; therefore, apparently relatively few receptors are required to permit activation of AML cell growth. However, in two cases cell cycling was not activated in response to G-CSF despite G-CSF receptor availability. The results show that G-CSF receptors of high affinity are frequently expressed on the blasts of human AML, but their presence may not be a strict indicator of the proliferative responsiveness of the cells to G-CSF.

MATERIALS AND METHODS

Patients and preparation of AML cells, normal BM cells, and granulocytes. AML was diagnosed according to the criteria of the French-American-British (FAB) committee, and BM (all cases except cases 4 and 9) or PB (4 and 9) was taken from the patients at diagnosis before any treatment was administered in all 14 cases. The percentage of blasts in BM specimens before purification is shown in Table I. The leukemic cells were separated from BM or PB after bovine serum albumin density gradient (BSA) of Ficoll-Isoopaque centrifugation and subsequent removal of E rosette-forming cells. In addition, AML cell preparations used in the binding experiments, proliferative assays, and colony assay were also depleted of monocytic cells after plastic adherence. This resulted in cell preparations with a purity of blasts determined by morphology that was always more than 98%. Viability of the cells after thawing was always more than 95%. Normal BM was obtained by posterior iliac crest puncture from hematologically normal adults and subsequently separated over Ficoll-Isoopaque density gradient to isolate the mononuclear cell fraction. White blood cells (WBCs) were separated from the blood of healthy subjects after sedimentation in 0.1% methylcellulose. Granulocytes were obtained as the sedimented cell fraction after Ficoll-Isoopaque centrifugation of WBCs and consisted of 90% to 95% neutrophils, 2% to 6% eosinophils, and 3% to 5% monocytes/lymphocytes.

Recombinant human G-CSF (rhG-CSF) and radiiodination. rhG-CSF (E. coli-derived, molecular weight [mol wt] 18,800 daltons) was prepared and purified at Amgen (Thousand Oaks, CA). Purified rhG-CSF was radiolabeled according to the method described by Bolton and Hunter. Five hundred microcuries Bolton-Hunter reagent (Amersham Laboratories, Amersham, England) dissolved in benzene was dried under a stream of N2 in the packing vial at 22°C. The vial was then cooled to 0°C and 3 μg rhG-CSF in 15 μL borate buffer (0.1 mol/L, pH 8.2) was applied. The reaction was permitted to proceed for 60 minutes on ice and was quenched by adding 100 μL glycine (0.2 mol/L in borate buffer). After 5 minutes on ice, 100 μL gelatin (0.25%) was added to aid recovery of the reactant from the vial. The labeled rhG-CSF was separated from the reactant by chromatography on a Sepharose column (Pierce Chemical Co., Rockford, IL) equilibrated with phosphate-buffered saline (PBS) containing 0.1% gelatin and 0.01% Tween 20. Radiolabeling of rhG-CSF resulted in a protein that retained biologic activity (Table 2), had a specific binding capacity of 50% to 60%, and was 85% to 90% trichloracetic acid insoluble. 

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G-CSF RECEPTORS AND AML

Table 1. Relationship Between G-CSF Receptor Positivity of AML Blasts and G-CSF Stimulability of 3H-TdR Uptake by AML Cells

<table>
<thead>
<tr>
<th>FAB Case No.</th>
<th>Blasts (%)*</th>
<th>G-CSF Receptors</th>
<th>Colonies/Clusters</th>
<th>3H-TdR Uptake (dpm x 10^-5)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sites/Cell</td>
<td>kd (pmol/L)</td>
<td>No G-CSF 300 pmol/L</td>
</tr>
<tr>
<td>M1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>96</td>
<td>108</td>
<td>263</td>
<td>0/0</td>
</tr>
<tr>
<td>2</td>
<td>87</td>
<td>—†</td>
<td>ND</td>
<td>0/0</td>
</tr>
<tr>
<td>M2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>93</td>
<td>94</td>
<td>309</td>
<td>0/0</td>
</tr>
<tr>
<td>4</td>
<td>91</td>
<td>—†</td>
<td>ND</td>
<td>0/0</td>
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<tr>
<td>5</td>
<td>58</td>
<td>—†</td>
<td>ND</td>
<td>0/5</td>
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<tr>
<td>6</td>
<td>87</td>
<td>49</td>
<td>214</td>
<td>0/0</td>
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<tr>
<td>7</td>
<td>86</td>
<td>—†</td>
<td>ND</td>
<td>89/ND</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>78</td>
<td>406</td>
<td>284</td>
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<tr>
<td>9</td>
<td>69</td>
<td>192</td>
<td>373</td>
<td>0/0</td>
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<tr>
<td>10</td>
<td>49</td>
<td>203</td>
<td>378</td>
<td>195/&gt;500</td>
</tr>
<tr>
<td>11</td>
<td>75</td>
<td>102</td>
<td>276</td>
<td>0/112</td>
</tr>
<tr>
<td>12</td>
<td>76</td>
<td>428</td>
<td>274</td>
<td>74/&gt;500</td>
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<tr>
<td>13</td>
<td>47</td>
<td>310</td>
<td>324</td>
<td>175/&gt;500</td>
</tr>
<tr>
<td>M5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>28</td>
<td>—†</td>
<td>ND</td>
<td>0/5</td>
</tr>
</tbody>
</table>

G-CSF binding to AML cells was assayed after incubation of 2 to 5 x 10^6 AML blasts with eight different concentrations of radiolabeled G-CSF (1 hour, 37°C). Mean receptor numbers per cell and kd were derived from Scatchard plots (described in legend to Fig 4). Colony and cluster formation was scored after 2 weeks. Values are the average number of colonies per clusters per 10^6 cells from duplicate experiments. Stimulation of 3H-TdR incorporation by AML blasts was determined in cultures with G-CSF. When mean dpm of stimulated cultures was significantly greater than the values of unstimulated control cultures, G-CSF responses (last column) were considered positive (+). AML cases are classified according to FAB cytology.

*Percentage of blasts in BM at diagnosis (described in Materials and Methods section).
†Specific binding was detectable (total binding exceeded nonspecific binding), but complete Scatchard analysis was not performed; in these instances, the kd was not determined.

Specific radioactivity of 125I-G-CSF. Self displacement was applied to determine the specific activity of G-CSF after labeling (Fig 1). The parallel displacement curves obtained (Fig 1A) show that the affinities of the radiolabeled and native molecules for the G-CSF receptors of granulocytes were similar and confirm that labeling had not altered the binding characteristics of G-CSF. From the slope of the line (Fig 1B), the specific radioactivity was estimated at 9.5 x 10^7 cpm/ng. This specific activity was used to determine the concentration of labeled G-CSF used in the binding experiments.

Binding of labeled G-CSF to AML blasts and granulocytes. To remove any G-CSF that might have remained present on the membrane, the blasts were washed in sodium citrate (pH 4). Before and after this acid wash, the cells were washed twice in Hanks’ balanced salt solution (HBSS). Cells (2 to 4 x 10^6) were incubated for 1 hour at 37°C in 100 μL of minimal essential medium (α-MEM) with 10% fetal calf serum (FCS) in Eppendorf tubes on ice, and centrifuged for 5 minutes at 900 × g. The pellets were snapfrozen in liquid nitrogen, and the pellet was cut off for counting in a γ-counter (Packard, Downers Grove, IL). Specific binding was determined as the difference between the amount of radioactivity bound without unlabeled G-CSF added and the amount of radioactivity bound with excess unlabeled G-CSF added. Experiments were conducted in duplicate. Receptor numbers and binding affinity were derived after analysis according to Scatchard. In calculations, the maximal binding capacity was used to correct for the free counts.

Colony cultures. To estimate whether the biologic activity of radiolabeled G-CSF had been retained, normal BM cells (0.5 x 10^6) were cultured in a 1-mL mixture of Iscove’s modified Dulbecco’s medium (IMDM), 0.8% methicellulose, 30% FCS, BSA, transferrin, lecithin, sodium-selenite and 2-mercaptoethanol. Nonlabeled, labeled, and a 50%/50% mixture of nonlabeled and labeled G-CSF were added to the cultures in titrated concentrations. Clusters of more than 15 cells and colonies of more than 50 cells were scored at day 14. Each estimate is based on the data of duplicate cultures. The effect of G-CSF on AML colony formation was also determined. In these experiments, 1 x 10^6 blasts were plated per dish containing 1 mL serum-free medium in 0.9% methicellulose with and without addition of G-CSF. After 14 days, clusters of more than 15 cells and colonies of more than 50 cells were scored (Table 1). Each estimate is based on the data of duplicate cultures.

1H-thymidine 3H-TdR incorporation into AML blasts. Blasts (2 x 10^6) were cultured for 72 hours in serum-free medium with or without G-CSF. DNA synthesis of AML blasts was measured as described. All experiments were performed in triplicate, data were expressed as mean dpm, and differences of DNA synthesis between stimulated and nonstimulated cultures were analyzed for significance (P > .05, Student’s t test).
RESULTS

Radioiodination of G-CSF. Radiolabeled G-CSF had retained the ability to stimulate colony formation from normal BM cells in methylcellulose (Table 2). Fig 2A shows that competition of nonlabeled G-CSF with binding of radiolabeled G-CSF to granulocytes was dose dependent. Both specific and nonspecific binding of radiolabeled G-CSF to granulocytes using excess ligand appeared linear with cell numbers up to 5 x 10⁶ cells and the linear functions extrapolated through zero (Fig 2B). This was indicative that binding of radiolabeled G-CSF as measured was cell associated and independent of cell number. Self-displacement analysis demonstrated similar affinities of labeled and unlabeled G-CSF for granulocytes (Fig 1). From these data, it was apparent that the ¹²⁵I-G-CSF preparation permitted quantitative receptor binding studies.

Binding of radiolabeled G-CSF to granulocytes. Binding of ¹²⁵I-G-CSF to human granulocytes appeared to be time and temperature dependent (Fig 3). G-CSF was more efficiently bound at 37°C than at 0°C. After 1 hour of exposure of the cells to G-CSF at 37°C, the amount of cell-associated radioactivity began to decrease. Nonspecific binding (radioactivity bound in the presence of excess unlabeled competitor) was virtually independent of time or temperature. When granulocytes were incubated with increasing concentrations of ¹²⁵I-G-CSF (20 to 1,250 pmol/L), binding appeared to be saturable, whereas nonspecific binding increased linearly with increasing concentrations of labeled rhG-CSF and varied with cell type and cell number from 0.4% to 1.8% of total input cpm (Fig 4A). The Scatchard plot of the binding data revealed an average of 1,019 G-CSF sites per cell of high affinity (kd 405 pmol/L) (Fig 4A). G-CSF receptor analysis of granulocytes obtained from the blood of five different donors showed limited variations as regards receptor densities (703 to 1,296 sites per cell) and receptor affinity for G-CSF (kd 405 to 648 pmol/L) (Table 3).

Binding of radiolabeled G-CSF to AML blasts. Binding of ¹²⁵I-G-CSF to AML blasts was examined in 14 cases of
Table 3. Binding of $^{125}$I-G-CSF to Granulocytes

<table>
<thead>
<tr>
<th>Donor</th>
<th>Sites per Cell</th>
<th>$k_d$ (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>703</td>
<td>648</td>
</tr>
<tr>
<td>2</td>
<td>1,019</td>
<td>405</td>
</tr>
<tr>
<td>3</td>
<td>955</td>
<td>492</td>
</tr>
<tr>
<td>4</td>
<td>1,066</td>
<td>481</td>
</tr>
<tr>
<td>5</td>
<td>1,296</td>
<td>586</td>
</tr>
</tbody>
</table>

Receptor numbers (mean per cell) and $k_d$ were derived from binding experiments and subsequent analysis according to Scatchard (described in legend to Fig 4). Granulocytes were obtained from five different normal donors.

Fig 3. Binding of radiolabeled G-CSF to granulocytes as a function of time: $4 \times 10^8$ granulocytes were incubated with 300 pmol/L G-CSF at the indicated temperatures (0°C and 37°C). Total binding (○) and nonspecific binding (+) were assessed at various times of incubation. Values are means of duplicate estimations.

AML. In all cases, specific binding of $^{125}$I-G-CSF was measurable (Fig 4B, Table 1). In five of these cases, binding was too low to permit complete Scatchard analysis. In the other nine cases, Scatchard analysis revealed comparatively low numbers of G-CSF receptors (49 to 428 sites per cell).

Fig 4. Binding of radiolabeled G-CSF to granulocytes (A) and AML blasts (B): $4 \times 10^8$ granulocytes (donor 2, Table 3) or blasts (AML case 8, Table 1) were incubated with increasing amounts of radiolabeled G-CSF for 1 hour at 37°C. Nonspecific binding was determined in the presence of excess unlabeled G-CSF. Specific binding (+) and nonspecific binding (○) data are plotted (left). Each point is the mean of two estimates. Scatchard plots of these data (right): bound/free ratio (BF).

The apparent dissociation constant ranged between 214 and 373 pmol/L (mean 299 ± 53 pmol/L ± SD), which is similar to the $k_d$ of G-CSF receptors on granulocytes (mean 522 ± 95 pmol/L ± SD). On the average, the cases of M4 cytology expressed greater numbers of G-CSF receptors (mean 274 ± 129 sites per cell ± SD) than did M1 and M2 cases (positive receptor expression but no Scatchard analysis possible in cases 2, 4, 5 and 7; mean 84 ± 31 sites per cell ± SD in the remaining three cases). However, the small number of cases tested do not allow any conclusions regarding the relationship between cytology and receptor expression.

In vitro: Comparison with receptor data. Although thymidine uptake generally provides a more sensitive assay of proliferative activity of AML cells than the colony assay, we also determined the in vitro response of the cells in colony culture. In five cases (cases 7, 10, 11, 12, and 14), a significant increase in colony numbers was noted when G-CSF had been added (Table 1). The stimulative effects of rhG-CSF on DNA synthesis of AML cells was determined in serum-free culture and the response of the cells as compared with growth factor receptor expression (Table 1). A significant increase in tritiated thymidine uptake was observed in most cases (12 of 14). In two cases (cases 5 and 6), DNA synthesis could not be activated, although G-CSF receptors were clearly identified on the cells. Receptor density and affinity of the cells from the two nonresponders (case 5, positive specific binding; case 6, 49 sites per cell, $k_d$ 214 pmol/L) were comparable with those of the responding cases.

DISCUSSION

G-CSF receptors were demonstrated on the blast cells of all 14 patients with AML included in this study. In 5 of 14 cases, estimation of G-CSF receptor numbers was not possible owing to low binding, although significant specific binding was evident. In the other nine cases, the estimated receptor density ranged between 49 and 428 sites per cell. That the cells from most cases, including three of the five patients with low specific binding, showed increased DNA synthesis on stimulation with G-CSF established that only minimal numbers of receptors per cell are required to evoke a proliferative response to G-CSF. G-CSF receptors demonstrated on the (immature) AML blasts were all of high...
affinity with a dissociation constant (kd 212 to 378 pmol/L), which was slightly less than that of G-CSF receptors on granulocytes (kd 405 to 648 pmol/L). How these properties compare with those of normal immature progenitor cells remains unresolved as long as it is impossible to analyze great numbers of purified marrow precursors. On the other hand, the density of G-CSF receptors on granulocytes (703 to 1,296 sites per cell) appeared greater than that on AML blasts. A similar phenomenon (ie, more receptors on granulocytes than on AML blasts, has previously been reported for the GM-CSF receptor.25,26

We showed in this and in a previous study9 that 3HTdR uptake provides a more sensitive assay for the mitogenic response of AML cells than the colony assay. Indeed, in several cases, a significant increase in DNA synthesis was observed, whereas no effect could be demonstrated in the colony assay. Therefore, we related the receptor data to 3H-TdR uptake as parameter of response (Table 1, last column). G-CSF was able to stimulate DNA synthesis of the blasts from most cases (Table 1). The expression of G-CSF receptors did not predict unequivocally whether these cells would enter active cell cycling in response to G-CSF. In cases 5 and 6, G-CSF was incapable of inducing DNA synthesis, although the blasts of these patients clearly expressed G-CSF receptors on their membranes. The unresponsiveness of AML cells to CSF despite receptor availability has also been reported for IL-3 and GM-CSF.26 Why AML cells of some patients express G-CSF receptors, but cannot respond to stimulation by the ligand is unclear. Although in 12 of 14 cases the 72-hour period proved sufficient to detect an increase of DNA synthesis, in the remaining two cases an undetectable response of only a small subpopulation of cells may have occurred. The receptors of the cells of these patients similar to those of granulocytes may be functional for other purposes so that they cannot elicit proliferation. Investigations along the lines of signal transduction may clarify mechanisms that determine the inability of the cells of cases of AML to proliferate in reaction to G-CSF stimulation.

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