Quantitative In Vivo Assay of Human Granulocyte Colony-Stimulating Factor Using Cyclophosphamide-Induced Neutropenic Mice

By Kunihiro Hattori, Koji Shimizu, Mayumi Takahashi, Masahiko Tamura, Masayoshi Oheda, Nakaaki Ohsawa, and Masayoshi Ono

Administration of human granulocyte colony-stimulating factor (hG-CSF) to mice with cyclophosphamide (CPA)-induced neutropenia for 4 consecutive days from the day after the CPA dosing (100 mg/kg) resulted in a dose-dependent increase in the peripheral blood neutrophil count 6 hours after the final hG-CSF injection. Within the hG-CSF dose range of 0.1 to 10 μg per mouse per day, there was a strong linear relationship (r > .9) between the logarithm of the dose and the peripheral blood neutrophil count in the treated mice. Using the same hG-CSF preparation, 38 experiments indicated that the regression lines are highly reproducible. Such an association never occurred with intact mice, and 100 mg/kg of CPA induced the highest response to hG-CSF. This linear relationship between the two variables allows us to determine the biologic potency of a test hG-CSF preparation relative to a reference standard using a parallel line assay, with a coefficient of precision of around .2. When assayed by this bioassay procedure, which we have termed CPA-mouse assay, natural hG-CSF and recombinant hG-CSF (produced by Chinese hamster ovary cells) were nearly equipotent in specific biologic activity. These results confirm the CPA-mouse assay as an especially useful assay method for quantifying the in vivo activity of hG-CSF.

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MATERIALS AND METHODS

Mice. We used 7- to 9-week-old male C57BL/6N mice (Cler Japan Inc, Tokyo, Japan) raised under specific pathogen-free (SPF) conditions. Natural hG-CSF and recombinant hG-CSF. As described previously, natural hG-CSF (nhG-CSF) was purified from medium conditioned by a squamous cell line (CHU-2) that constitutively produced CSFs. Purified recombinant hG-CSF (rhG-CSF) was prepared from medium conditioned by Chinese hamster ovary (CHO) cells transfected with vectors containing full-length cDNA for hG-CSF. The purified hG-CSFs were diluted in phosphate-buffered saline (pH 7.4) containing 1% human serum albumin (Green Cross Co, Osaka, Japan) and 0.01% Tween-20 (Nakarai Chemicals, Kyoto, Japan). The amounts of hG-CSF in the preparations were determined by high-pressure liquid chromatography (HPLC).

In vivo treatments. On day 0, mice were given intraperitoneally a single injection of cyclophosphamide (CPA; Shionogi Pharmaceuticals, Osaka, Japan). Beginning 24 hours later and for 4 consecutive days from the day after the dosing with CPA, the mice were given subcutaneously 0.2 mL of hG-CSF solution or control vehicle. The mice were bled retro-orbitally for blood cell counts.

Blood cell counts. Total leukocytes were counted using a Microcellcounter (Type CC-180A, Toa Medical Electronics, Japan). Leukocyte differentials were determined by enumerating 200 cells on Giemsa-stained smears.

Statistical analysis. Four or five mice were assayed individually for each group. (The actual numbers of mice used in each experiment are given in the table or figure legends.) The probability of significant differences between groups was determined using Student's t test or Aspin-Welch's method.

RESULTS

Effect of rhG-CSF on CPA-induced neutropenic mice. Mice given a single injection of CPA at a dose of 100 mg/kg on day 0 were given 0.2, 1, or 5 μg of rhG-CSF per mouse per day, or control vehicle for 4 days from day 1 to day 4. Blood samples for neutrophil count were obtained 6 hours after each injection and 30 hours after the injection on day 4. Pretreatment values were determined using normal mice. As shown in Fig 1, in control mice a nadir neutrophil count 50%
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Fig 1. Recovery from CPA-induced neutropenia. Mice received a single intraperitoneal injection of CPA (100 mg/kg) on day 0. Beginning 24 hours later, mice were injected subcutaneously daily for 4 days with 0.2 µg (○—○), 1 µg (▲—▲), 5 µg (△—△) of rhG-CSF, or vehicle (□—□) in 0.2 mL. Blood samples were obtained 6 hours after each injection and 30 hours after the injection on day 4. Each point is the mean of four mice with the SEM indicated by bars. *, P < .05; **, P < .01, ***, P < .001, as compared with the control mice.

below the pretreatment value occurred on days 1 to 4. In contrast, in mice given daily injections of rhG-CSF beginning 1 day after the CPA, there was a slight nadir and an accelerated, dose-dependent recovery of neutrophil levels.

**Construction of dose-response curves.** rhG-CSF was given to CPA-induced (100 mg/kg) neutropenic mice for 4 days at graded dose levels of 0.005 µg per mouse per day, up to 40 µg per mouse per day (increasing in a common ratio of 2). Then peripheral blood neutrophil counts obtained 6 hours after the last hG-CSF injection were plotted against the logarithm of the dose (Fig 2). As is obvious from the graphic representation of the data, a sigmoid curve was formed with an almost linear portion over the dose range of 0.078 to 10 µg per mouse per day. A significant increase in the neutrophil count, compared with the control group, appeared at a dose level of only 0.005 µg per mouse per day (P < .05). A plateau may have been achieved by the injection of 10 µg of rhG-CSF per mouse per day, with a 70- to 80-fold rise in neutrophil levels compared with the control group. Given that the relationship between the two variables (rhG-CSF dosage and neutrophil count) is well-represented by a straight line over the indicated range, a regression line was calculated as Y = 131 + 85.2 × log X, where Y is the peripheral blood neutrophil count (×10^2 cells/µL) and X is the dose of rhG-CSF (µg per mouse per day). The correlation coefficient (r) was .963.

The lymphocyte count also tended to increase under the influence of rhG-CSF, but this change was almost negligible compared with the neutrophil count (data not shown). Thus, the neutrophil count may be reasonably regarded as representing the total leukocyte count inclusive of lymphocytes. This eliminates the need for leukocyte differential counts. The regression of rhG-CSF dosage on total leukocytes was expressed as: Y = 162 + 86.5 × log X, r = .954, where Y is the total leukocyte count (×10^2 cells/µL).

Reproducibility of the regression line. To examine the reproducibility of the regression line of dosage and total leukocyte count, the assays using the same rhG-CSF preparation were repeated 38 times. In one assay, we obtained the regression line by using 15 to 20 mice for 3 or 4 doses of rhG-CSF. Figure 3 shows the Y intersection (a) and the slope (b) of each regression line. The respective coefficients of variation of the Y intersections (the leukocyte counts induced by 1 µg of rhG-CSF per mouse per day) and the slopes were 6.1% and 14.1%.

This striking and highly reproducible linear relationship allows us to determine by parallel line assay the biologic potency of a test hG-CSF preparation relative to a reference standard. We call this the CPA-mouse assay.

**Relationship between the dose of CPA and the response to rhG-CSF.** Table 1 shows the increase of total leukocyte counts in peripheral blood by the 4-day dosing with rhG-CSF in intact mice and in mice treated with 100 mg/kg of CPA.

In intact mice, there was an rhG-CSF dose-dependent increase in leukocyte count, but the increasing ratio was much lower than it was in CPA-treated mice. This suggests that the treatment with CPA induces the higher response to rhG-CSF.

Next we examined the relationship between the dose of CPA and the response to rhG-CSF. Mice were given 50, 70, 100, 140, or 200 mg/kg of CPA. Then varying doses of rhG-CSF or control vehicle were given daily for 4 days from 1 day after the CPA treatment, and 6 hours after the last dose of rhG-CSF, the peripheral blood total leukocytes were counted. The relationship between the CPA dosage and increased peripheral blood leukocytes with increasing rhG-CSF dosage is shown in Fig 4. In the range of CPA dosage tested, the mice receiving 70 or 100 mg/kg of CPA responded most to rhG-CSF. This shows that 100 mg of CPA per kilogram is adequate for the CPA-mouse assay.

**Comparison of rhG-CSF and rhC-CSF.** The in vivo activity of these two forms of hG-CSF was determined and compared on the premise that in a CPA-mouse assay, a linear relationship exists between the logarithm of the dose of hG-CSF and the leukocyte count in peripheral blood over the dose range of approximately 0.1 to 10 µg per mouse per day. Fig 5. Computation of the data thus obtained yielded the following linear regression formulas: (rhG-CSF) Y =
induced neutropenic mice were given various doses of rhG-CSF for 4 consecutive days from the day after exposure to CPA.

Fig 2. Dose-related rise in peripheral blood neutrophil levels in CPA-treated mice. CPA-induced neutropenic mice were given various doses of rhG-CSF for 4 consecutive days from the day after exposure to CPA. Blood samples were obtained 6 hours after the last dose of rhG-CSF. Each point shows the mean of five mice with the SEM indicated by bars.

\[ Y = 131 + 85.2 \cdot \log X, \quad r = 0.963 \]

Fig 3. Reproducibility of the regression line of rhG-CSF dosage and total leukocyte count. The preparation of the regression line was repeated 38 times using the same rhG-CSF solution. Each regression line was calculated as \( Y = a + b \cdot \log X \), where \( Y \) is the leukocyte count \(( \times 10^9 \text{ cells/µL} )\) and \( X \) is the dose of rhG-CSF \(( \mu g \text{ per mouse per day} )\). Each point shows the \( Y \) intersection \((a, 0)\) and the slope \((b, 0)\) of each regression line.

Table 1. Comparison of the Responsiveness to rhG-CSF in Intact Mice and CPA-Treated Mice

<table>
<thead>
<tr>
<th>Dose of rhG-CSF (µg/mouse/day)</th>
<th>Total Leukocyte Level ( \times 10^9/µL )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact Mice</td>
<td>CPA-Treated Mice</td>
</tr>
<tr>
<td>0</td>
<td>101.4 ± 7.8</td>
</tr>
<tr>
<td>0.2</td>
<td>108.4 ± 5.6</td>
</tr>
<tr>
<td>1.0</td>
<td>116.6 ± 3.7</td>
</tr>
<tr>
<td>5.0</td>
<td>133.2 ± 12.8</td>
</tr>
</tbody>
</table>

The varying doses of rhG-CSF or control vehicle were given daily for 4 days from 1 day after CPA treatment (CPA-treated mice) or nontreatment (intact mice). The peripheral blood total leukocytes were counted 6 hours after the last dose of rhG-CSF. Data show the mean ± SEM of five mice. *P < .001 as compared with each control value.

DISCUSSION

We reported on the in vivo effects of hG-CSF in normal mice in our previous study. Those animal experiments showed that hG-CSF has the following characteristic biologic effects: (1) increases the neutrophil number in peripheral blood in a few hours after administration; (2) markedly increases the number of hematopoietic stem cells (CFU-S) and all progenitor cell types (CFU-GM, CFU-Meg, BFU-E, CFU-E) in spleen; and (3) increases the mature neutrophil number in the bone marrow and spleen. Of these three actions, the first two are exclusively in vivo ones that cannot be adequately explained by the in vitro granulocyte colony-stimulating activity of hG-CSF. Some recent studies of...
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Fig 4. Relationship between the CPA dose and the rise of peripheral blood leukocyte with various doses of rhG-CSF. For 4 days the mice received daily 0.125 μg (O--O), 0.5 μg (△--△), 2 μg (Θ---Θ), or 8 μg (A----A) of rhG-CSF or vehicle per mouse per day from the day after exposure to 50, 70, 100, 140, or 200 mg/kg of CPA. Blood samples were obtained 6 hours after the last dose of rhG-CSF. The leukocyte counts in the control mice were 71.0, 54.2, 37.0, 25.0, and 12.2 (×10^9 cells/μL) in groups given 50, 70, 100, 140, and 200 mg of CPA/kg, respectively, and these control values were subtracted from the respective rhG-CSF-treated values. Each point shows the mean of four mice with the SEM indicated by bars.

Fig 5. The comparative effects of natural (O--O) and recombinant (Θ---Θ) hG-CSF on peripheral blood leukocyte levels in CPA-treated mice. The mice were injected daily for 4 days with various doses of rhG-CSF or rhG-CSF 1 day after the CPA injection. Blood samples were obtained 6 hours after the last dose of each form of hG-CSF. Each point shows the datum of an individual mouse.

The second action of G-CSF have reportedly shown that G-CSF acts in vitro on multipotent stem cells or on progenitors other than granulocytic lineage cells. But these actions do not fully explain the phenomena listed above. There are few reports concerning the first action of hG-CSF. It remains uncertain whether the phenomena occur as a consequence of a single action of hG-CSF or its multiple, independent actions. Therefore, for the in vivo activity of hG-CSF to be quantified efficiently and accurately, the preferred assay system is one that permits assessment of the above-mentioned actions "en bloc" and, at the same time, is as relevant to human clinical situations as possible. One of the clinical conditions in which hG-CSF is expected to be most useful is neutropenia caused by chemotherapy for various malignant tumors. Therefore, we developed CPA-induced neutropenic mice as a model for this human pathologic condition.

As shown in Fig 1, an abrupt decrease in the peripheral blood neutrophil count occurred after a single dose of CPA, a potent anticancer drug, in mice. When hG-CSF was given to these mice for 4 consecutive days from the day after the CPA exposure, a marked recovery from neutropenia occurred with a quick return to the pre-CPA levels of CFU-GM and CFU-S in the spleen. It was particularly significant that a linear relationship existed between the neutrophil count (or total leukocyte count) in the peripheral blood, as determined 6 hours after the last dose of hG-CSF, and the logarithm of the dose (0.1 to 10 μg per mouse per day), as shown in Fig 2. This correlation between the two variables was striking (r > .9) and highly reproducible. Thus, it allows us to establish the in vivo quantitative assay for hG-CSF (the CPA-mouse assay).

In our preliminary studies, the measurements yielded by this assay method were closely correlated with those yielded by the in vitro colony-forming assay using mouse marrow cells or by the proliferation assay using NFS-60 cells (data not shown).

One factor influencing the precision and sensitivity of this assay is the CPA dosage. As shown in Table 1, mice treated with CPA induced higher response to hG-CSF. The mechanism of the rising responsiveness to hG-CSF was unclear. It may be that in intact mice, the hematopoiesis maintains a balance. The CPA treatment breaks down that balance and the regulation of response to exogenous hG-CSF, which in turn increases the capacity for greater hematopoiesis in the neutrophil lineage. Another possibility is that the treatment induces several endogenous factors, and the synergistic effect of exogenous hG-CSF with these factors results in a marked increase in the neutrophil level in peripheral blood. There was an optimum dose of CPA inducing the highest response
to hG-CSF: mice receiving 70 or 100 mg/kg of CPA were most sensitive to the rhG-CSF (Fig 4). Higher doses of CPA may severely suppress hematopoiesis and reduce the progenitor cells of neutrophil-lineage affected by hG-CSF, resulting in lower responsiveness to hG-CSF. Further, the lower the dosage of CPA, the lower were the coefficients of correlation for the regression line in Fig 4 or in our other experiments. Thus, for the CPA-mouse assay, we usually use 100 mg of CPA per kilogram to prepare the neutropenic mice.

This report does not refer to the influence of strain, age of mice, or time of blood collection after final hG-CSF injection, but these factors also affect the CPA-mouse assay.

By examining the neutrophil levels in bone marrow and spleen of CPA-treated mice (in preparation), we confirmed that the CPA-mouse assay mainly quantifies two major biologic effects of hG-CSF: the effect of stimulating neutrophil generation (the third action of hG-CSF observed in normal mice) and the effect of mobilizing neutrophils from bone marrow into the peripheral blood (the first action observed in normal mice).

Bacterial endotoxins,18-20 glucocorticosteroids,18 and catecholamines21 are known to raise blood neutrophil levels, while the factors that stimulate or facilitate neutrophil-colony formation in vitro, such as multi-CSF22-24 or GM-CSF,25-27 are thought to stimulate neutrophil generation in vivo. However, we have already found that these factors, as assayed by the CPA-mouse assay method, were virtually ineffective compared with hG-CSF (K. Hattori et al, manuscript in preparation).

We also reported in our previous study28 that in mice infected with Pseudomonas aeruginosa, Serratia marcescens, Escherichia coli, Staphylococcus aureus, or Candida albicans 4 days after being given CPA, hG-CSF administration for 4 consecutive days from the day after the exposure to CPA was markedly anti-infective. This showed that elevated neutrophil levels on day 4 of hG-CSF administration can be a reliable indicator of the effectiveness of hG-CSF.

In summary, the CPA-mouse assay, with its advantages of accuracy, reproducibility, and simplicity, provides a useful means of quantifying the in vivo activities of hG-CSF. In addition, this assay system will certainly prove useful in evaluating the effects of other cytokines or the combinations of these factors with hG-CSF on neutrophil generation, and in elucidating the mechanisms underlying the proliferation and maturation of cells of the neutrophil series in hematopoietic tissues, as well as those whereby mature neutrophils are mobilized from the bone marrow pool into the peripheral blood.


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