Effects of Recombinant Human Granulocyte Colony-Stimulating Factor on Hematopoietic Progenitor Cells in Cancer Patients

By Ulrich Dührsen, Jean-Luc Villeval, Janis Boyd, George Kannourakis, George Morstyn, and Donald Metcalf

Hematopoietic progenitor cell levels were monitored in the peripheral blood and bone marrow of 30 cancer patients receiving recombinant human granulocyte-colony stimulating-factor (rG-CSF) in a phase I/II clinical trial. The absolute number of circulating progenitor cells of granulocyte-macrophage, erythroid, and megakaryocyte lineages showed a dose-related increase up to 100-fold after four days of treatment with rG-CSF and often remained elevated two days after the cessation of therapy. The relative frequency of different types of progenitor cells in peripheral blood remained unchanged. The frequency of progenitor cells in the marrow was variable after rG-CSF treatment but in most patients was slightly decreased. The responsiveness of bone marrow progenitor cells to stimulation in vitro by rG-CSF and granulocyte-macrophage colony-stimulating factor did not change significantly during rG-CSF treatment. In patients nine days after treatment with melphalan and then rG-CSF, progenitor cell levels were very low with doses of rG-CSF at or below 10 μg/kg/d, but equaled or exceeded pretreatment values when 30 or 60 μg/kg/d of rG-CSF was given.

GRANULOCYTE COLONY-stimulating factor (G-CSF) is a proliferative stimulus for precursors of neutrophils in vitro. Human G-CSF has been purified from various sources, cDNAs cloned and expressed in bacterial and mammalian cells, and the bacterially synthesized recombinant molecule (rG-CSF) found to have the same range of activities in vitro as the native hormone.

Investigations in animals have indicated that G-CSF is capable of inducing a significant increase in total granulocytes both in healthy animals and in animals with congenital or drug-induced defects of the granulocyte-macrophage system, thereby leading to enhanced resistance to microbial infections. Recent clinical trials with cancer patients receiving cytotoxic chemotherapy have shown that the same effects of G-CSF can be observed in humans.

G-CSF is a proliferative stimulus for granulocyte progenitor cells, but these are transit cells with no capacity for self-renewal. Administration of G-CSF in vivo might therefore deplete progenitor cell numbers unless other mechanisms compensate for the depletion. To monitor these possible effects of G-CSF, we analyzed progenitor cell levels in the peripheral blood and bone marrow of patients who were treated with rG-CSF in a phase I/II clinical trial reported elsewhere.

MATERIALS AND METHODS

Criteria for Eligibility and Description of Patients

The criteria for patient eligibility were reported in detail elsewhere. In brief, patients had histologic evidence of metastatic cancer with a life expectancy of at least 2 months. No chemotherapy or radiotherapy had been administered in the 6 weeks preceding treatment with rG-CSF, and radiotherapy was limited to less than 50% of the bone marrow. The type of tumor had to justify treatment with melphalan, and significant nonmalignant disease such as cardiac, respiratory, renal, or hepatic dysfunction was excluded. Pretreatment hemoglobin levels were greater than 10 g/dL, polymorphonuclear cell counts greater than 1.500/μL, and platelet counts greater than 100,000/μL. Before initiation of treatment the patients gave informed consent.

The study was performed at the Royal Melbourne Hospital under the ethical guidelines of the National Health and Medical Research Council of Australia and the Food and Drug Administration of the United States and comprised a total of 30 patients who fulfilled the aforementioned requirements (Table 1).

Properties of rG-CSF

rG-CSF was supplied by Amgen Corp, Thousand Oaks, CA. The 174-amino acid protein is a nonglycosylated single-chain polypeptide with a molecular weight of about 18.6 kilodaltons that is produced by Escherichia coli and purified via a series of chromatographic steps. The final product was formulated in an aqueous buffer as a sterile solution at a concentration of 0.25 mg/mL, and each batch was demonstrated to be biologically active and free from pyrogens before release.

Study Protocol and Drug Dosage

Each patient received two cycles of rG-CSF. The first cycle consisted of a five-day treatment period with rG-CSF alone followed by a therapy-free interval of three days' duration. The second cycle aimed at investigating the effects of rG-CSF during drug-induced myelosuppression and began on the fourth interval day. After a single intravenous (IV) injection of the alkylating agent melphalan (25 mg/m²), rG-CSF treatment continued for nine days at the same dose level as that used in the first cycle.

Administration of rG-CSF was by either the subcutaneous (SC) or IV route, and doses varied from 0.3 to 60 μg/kg body weight/d. In general, groups of three patients were treated with each dose and route of administration. In individual patients the dose of rG-CSF was not varied. For doses of 0.3 and 1 μg/kg, the SC administration
Bone marrow specimens were obtained by sternal aspiration on the day before initiation of the first rG-CSF cycle and after four days of treatment. Gradient centrifugation and depletion of adherent cells were performed as described for peripheral blood cells.

**Progenitor Cell Assays**

All progenitor cell assays were performed in 1-mL cultures in 35-mm plastic Petri dishes. Peripheral blood cells were usually cultured at $2 \times 10^6$ cells/dish and bone marrow cells at $5 \times 10^6$ cells/dish. The cultures were incubated in a fully humidified atmosphere of 5% CO$_2$ in air at 37°C for up to 14 days.

Granulocyte-macrophage and early erythroid progenitor cells were grown in agar cultures prepared by adding 1 vol of double-strength Iscove's modified Dulbecco's medium and 1 vol of FCS to 2 vol of 0.6% agar. Granulocyte-macrophage progenitor cells were stimulated by the addition to each dish of (a) 500 units of purified rG-CSF (specific activity, $2 \times 10^7$ U/mg) dissolved in a volume of 0.1 mL of saline, (b) 1,500 units of purified recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) (specific activity, $2 \times 10^7$ U/mg), (c) a mixture of 500 units of rG-CSF plus 1,500 units of rGM-CSF, or (d) 0.1 mL of normal saline for the detection of spontaneous colony development. (Fifty units per milliliter is defined as the concentration of a CSF stimulating the formation of half-maximal numbers of colonies in conventional agar cultures of bone marrow cells.) Since the responsiveness to GM-CSF of bone marrow cells from different individuals varied widely (see Results), a higher concentration of GM-CSF was used to ensure maximal stimulation of progenitor cells. For each stimulus, cultures were prepared in quadruplicate and duplicate dishes scored routinely after 5, 7, and 14 days of incubation by using a dissection microscope at 35× magnification. Clones of three to 40 cells were scored as clusters and clones of more than 40 cells as colonies. Cultures were then fixed with 2.5% glutaraldehyde, transferred to glass slides, and stained with Luxol–fast blue and hematoxylin for differential colony counts.

**Collection and Processing of Blood and Bone Marrow Samples**

Peripheral blood was obtained on the day preceding the first treatment cycle, after four days of rG-CSF treatment, two days after the end of the first cycle, and on the last day of the second rG-CSF course, ie, nine days after the administration of melphalan. To minimize possible interference of accessory cells with colony formation by progenitor cells, samples were fractionated by density gradient centrifugation and removal of adherent cells before culture. An aliquot of the blood sample was used to obtain a full blood count, and the remaining volume was subjected to gradient centrifugation (30 minutes at 400 g) using Ficoll-Hypaque (density, 1.077 g/mL; Pharmacia, Inc, Uppsala, Sweden). This fractionation step was routinely monitored by absolute cell counts and cytocentrifuge preparations of cells from the interface and pellet that were stained with May-Grünewald-Giemsa and then typed morphologically. The interface mononuclear cell fraction was washed, suspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS), and incubated in 90-mm plastic Petri dishes for one hour at 37°C to remove adherent cells. The proportion of nonadherent cells was determined for each specimen from cell counts before and after depletion of adherent cells and the nonadherent cell fraction used for progenitor cell assays.

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Early erythroid progenitor cells were stimulated by 2 units of recombinant erythropoietin (rEpo, supplied by Amgen) plus 0.1 mL of a preparation of human placental condition medium (HPCM) to stimulate the development of maximal numbers of colonies.\textsuperscript{21} Cultures were scored after 14 days of incubation and then fixed and stained for differential colony counts as before. Discrimination between pure erythroid and mixed erythroid colonies was based on nuclear morphology.

Late erythroid progenitor cells (colony-forming units, erythroid; CFU-E) were assayed in methylcellulose cultures by using 2 U/mL of rEpo.\textsuperscript{1} The cultures were scored after seven days of incubation by using an inverted microscope and CFU-E identified as hemoglobinized clones of eight cells or more.

Megakaryocytic colonies were grown by a plasma clot technique using 4 × 10\textsuperscript{4} peripheral blood cells or 2 × 10\textsuperscript{4} bone marrow cells per 1-mL dish.\textsuperscript{22} After 11 days of incubation the cultures were dried, labeled with a monoclonal mouse antibody against the glycoprotein IIb/IIIa complex (antibody WM 18, kindly provided by Dr M. Berndt, Sydney, Australia),\textsuperscript{22} and stained with a fluorescein-conjugated sheep antimouse immunoglobulin antibody (Silenus Laboratories, Hawthorn, Victoria, Australia). Megakaryocyte colonies were identified under the fluorescence microscope as clones of three cells or more with intense surface labeling.

**Calculation of Progenitor Cell Numbers per Blood Volume**

In most of the data, levels of progenitor cells are expressed as the number per 10\textsuperscript{9} nonadherent mononuclear cells. To calculate the rise in absolute numbers of progenitor cells induced by rG-CSF treatment (see Fig 4), the numbers of progenitor cells were corrected for changes in total mononuclear cells determined on the basis of white cell differential counts and changes in the ratio between adherent and nonadherent cells. With the exception of one patient (see Results), the cell fractions from the interface were comparable in mononuclear cell composition before and after treatment with rG-CSF. The changes in the absolute numbers of mature granulocytes and granulocyte-macrophage progenitor cells (see Fig 4) were expressed as the ratio between the absolute numbers per unit blood volume by comparing samples from individual patients before and after treatment.

**Statistical Analysis**

The t test for paired samples (one tailed) was used to compare progenitor cell numbers before and after treatment with rG-CSF.

**RESULTS**

**Fractionation of Cell Samples**

Before therapy with rG-CSF, the fraction of peripheral blood cells collected from the interface of the Ficoll gradient consisted of 95.8% ± 4.0% (range, 87% to 100%) mononuclear cells composed almost exclusively of cells with lymphocytelike or monocyte morphology and less than 5% contaminating granulocytes. In blood from patients injected with up to 30 μg/kg/d of rG-CSF, the percentage of mononuclear cells in this fraction remained virtually unchanged (after four days of rG-CSF therapy, 94.0% ± 6.8% [83% to 100%]; two days after the cessation of therapy, 96.8% ± 3.8% [88% to 100%]), although in some patients a small proportion of immature granulocytic precursors was observed in addition to the dominant lymphocytelike cells and monocytes. In blood from patients administered 60 μg/kg/d of rG-CSF, the fractionated samples from two patients exhibited the same composition as that observed with lower rG-CSF doses, but the low-density cell fraction collected from the third patient during and after rG-CSF therapy unexpectedly contained 72% to 75% mature granulocytes, band forms, and metamyelocytes. In this case, the frequencies of progenitor cells were corrected according to the actual numbers of mononuclear cells cultured.

The composition of the high-density cell fraction sampled from the pellet of the density gradient did not change significantly during therapy with rG-CSF. Neutrophilic and eosinophilic granulocytes were the major cell types (before rG-CSF treatment, 93.3% ± 7.0%; after four days of therapy, 94.1% ± 5.5%; two days after the end of treatment, 95.3% ± 3.7%), with only a minority of contaminating mononuclear cells.

Treatment with rG-CSF had no consistent influence on the ratio between adherent and nonadherent cells within the mononuclear cell fraction. In peripheral blood, the proportion of nonadherent cells was 74% ± 19% (range, 39% to 100%) before therapy as compared with 66% ± 24% (25% to 100%) during and 72% ± 23% (16% to 100%) after treatment with rG-CSF. In bone marrow, the respective pretreatment and posttreatment values were 81% ± 18% (39% to 100%) and 84% ± 15% (50% to 100%). The fluctuations in the percentage of adherent cells in samples collected at different times from the same patient did not generally exceed a two- to threefold difference, and any variability observed was not correlated with the dose of rG-CSF administered.

**Frequencies of Blood Progenitor Cells**

Early progenitor cells. The frequency of circulating day 14 colony-forming cells (CFCs) (presumptively less mature progenitors) responsive to stimulation by a mixture of rG-CSF and rGM-CSF rose significantly (\textit{P} < .01) during treatment with rG-CSF (Fig 1). In most patients, this effect was still demonstrable two days after discontinuation of rG-CSF treatment. In cultures stimulated by either rG-CSF or rGM-CSF, the levels of G-CSF-responsive and GM-CSF-responsive progenitor cells both rose to a similar extent following rG-CSF therapy. With all doses of rG-CSF administered, granulocyte, granulocyte-macrophage, macrophage, and eosinophil CFCs showed, overall, no significant change in relative frequencies (Fig 2).

Megakaryocyte colony formation was monitored with cells from three patients treated with 3 or 10 μg/kg/d of rG-CSF SC or 30 μg/kg/d IV, respectively. In the patient receiving the lower SC dose, circulating megakaryocyte CFCs rose from 1.5 per 10\textsuperscript{5} mononuclear nonadherent cells before therapy to 8.5 during r-G-CSF administration and 9.5 after the cessation of therapy. The respective values in the other SC-treated patients were 7, 66.5, and 41.5. In the patient receiving r-G-CSF IV, treatment induced a rise in the frequency of megakaryocyte progenitor cells from 4.5 to 26 per 10\textsuperscript{5} cells (no samples were analyzed after withdrawal of r-G-CSF therapy).

Cultures stimulated by rEpo plus HPCM consistently showed a comparable rise in the number of hemoglobinized day 14 CFCs following the administration of r-G-CSF.
Differential colony counts indicated that the increase affected both pure erythroid and mixed erythroid colonies (Fig 2). These latter colonies did not vary significantly in morphology before and after treatment with rG-CSF and contained predominantly granulocytic and less frequently macrophage or eosinophilic elements in addition to erythroid cells. The characteristic feature of erythroid progenitors in blood, with a higher proportion of mixed colonies than in cultures of marrow, did not change in the major increase following treatment with rG-CSF.

The increased levels of progenitor cells detected in peripheral blood after therapy with rG-CSF might have been due either to increased numbers of circulating CFCs or an altered proportion of accessory cells with inhibitory or enhancing effects on colony formation in vitro. To exclude the latter possibility, mixing experiments were performed by using pretreatment and posttreatment blood samples from a patient treated with 10 µg/kg/d of rG-CSF IV; squares, data from a patient treated with 3 µg/kg/d SC. Abbreviations: G-CFC, granulocyte colony-forming cells; GM, granulocyte-macrophage; M, macrophage; Eo, eosinophil; E, pure erythroid; Mix, mixed erythroid. G-, GM-, M-, and Eo-CFCs were enumerated in cultures stimulated by 500 units of rG-CSF plus 1,500 units of rGM-CSF, and E- and Mix-CFCs were enumerated in cultures stimulated by 2 units of rEpo plus 10% HPCM.

Late granulocyte and erythroid progenitors both showed a remarkable increase in numbers following rG-CSF administration (Fig 3). The presence of significant numbers of CFU-E was particularly striking since this type of progenitor cell was usually undetectable in the blood before treatment. These peripheral blood CFU-E were usually larger and frequently less intensely hemoglobinized than were the CFU-E.
Fig 3. Frequency of day 5 cluster-forming cells (top panels) and CFU-E (bottom panels) in peripheral blood (closed symbols, solid lines) and bone marrow (open symbols, broken lines) before, during, and after treatment with different doses of rG-CSF. Each panel contains data from three patients (circles, squares, triangles) treated at the same dose level of rG-CSF. Asterisks denote patients with previous chemotherapy and/or radiotherapy. E developing in bone marrow cultures, thus suggesting a slightly earlier maturational stage.

**Dose-response relationship.** The administration of rG-CSF was followed by a dose-dependent increase in the numbers of neutrophilic granulocytes in peripheral blood. We compared the effect of individual doses of rG-CSF on mature neutrophils with that on day 14 CFCs stimulated by a combination of rG-CSF and rGM-CSF.

Figure 4 demonstrates that the capacity of r-G-CSF to elevate the levels of mature blood granulocytes was dose related. Both for the SC and the IV routes of delivery, maximal effects were observed at 10 µg/kg/d of r-G-CSF, with no further increase at higher IV doses. Before treatment with r-G-CSF the average calculated number of circulating day 14 CFCs was 8.2 ± 7.7 x 10^4/L of blood. For the SC route of administration the increase in calculated total blood progenitor cell numbers showed a similar dose dependency to the rise in mature neutrophils, pronounced increases being observed at the 3- and 10-µg/kg dose levels (P < .01). In contrast, no clear-cut dose-response relationship was observed after IV infusion of r-G-CSF, and the lowest doses of IV administered r-G-CSF proved efficient in inducing a considerable increase in the numbers of circulating CFCs (P < .01). While the maximal rise in neutrophil numbers did not exceed an 11-fold increase after four days of r-G-CSF therapy, blood progenitor cell numbers increased up to 100-fold. In patients treated with 10 µg/kg/d of r-G-CSF or higher, the calculated numbers of blood progenitor cells ranged from 1 to 5 x 10^6/L.

Circulating erythroid and megakaryocyte progenitor cells showed the same increase in number as their counterparts in the granulocyte-macrophage lineage. In contrast, the numbers of mature erythrocytes and platelets did not change significantly during r-G-CSF therapy.

**Colonies formation in unstimulated cultures.** Unstimulated agar cultures prepared from blood cells before the initiation of r-G-CSF therapy frequently showed the formation of low numbers of clusters (usually macrophage) and colonies (mainly granulocytic). In blood cultures from some patients treated with high doses of r-G-CSF SC or IV (10 to 60 µg/kg/d) the frequency of spontaneously developing colonies increased five- to 20-fold after therapy, but again granulocytic colonies were by far the most frequent colony type.
Frequencies of Bone Marrow Progenitor Cells

In contrast to the consistent changes in the number of peripheral blood CFCs, the behavior of the marrow progenitor cells was more variable. In 70% of patients, treatment with rG-CSF was associated with a decrease in the number of day 14 CFCs, which affected both the granulocyte-macrophage and erythroid lineages (Figs 1 to 3). Likewise, megakaryocyte progenitor cell levels dropped to 30% or 67% of pretreatment values in two patients receiving 10 or 30 μg/kg/d of r-G-CSF, respectively. There was no consistent correlation between a history of prior cytotoxic treatment or irradiation and the observed reduction in the frequency of bone marrow CFCs after r-G-CSF treatment (Fig 1). While late granulocyte-macrophage progenitor cells tended to show the same changes during r-G-CSF therapy as their more immature precursors (decrease in number in 74% of patients), an increase in frequency was more common for CFU-E (61% of patients, Fig 3). Spontaneous colony and cluster formation in unstimulated bone marrow cultures did not change noticeably during treatment with r-G-CSF.

In Vitro Responsiveness of Progenitor Cells to r-G-CSF and rGM-CSF

To investigate whether treatment with r-G-CSF induced changes in the responsiveness of progenitor cells to CSFs, bone marrow cells obtained from six patients before and after four days of r-G-CSF treatment were stimulated in agar cultures with serial twofold dilutions of known amounts of r-G-CSF or rGM-CSF. The CSF concentrations stimulating the development of half-maximal numbers of colonies were determined graphically (effective concentration, 50% [EC50]) and used to characterize the responsiveness of the marrow cells.

Untreated bone marrow cells from different patients showed little variation in responsiveness to r-G-CSF, which is illustrated by similar EC50 values before r-G-CSF therapy (Table 2). In contrast, responsiveness to rGM-CSF varied widely from patient to patient, in general associated with a more shallow CSF titration curve. In individual patients, however, the EC50 values both for r-G-CSF and rGM-CSF were in the same range before and after the administration of r-G-CSF, which indicates that a significant change in the responsiveness of the progenitor cells to the CSFs had not occurred (Table 2). In addition, the shape of the dose-response curves remained un influenced by r-G-CSF therapy.

Frequencies of Blood Progenitor Cells During Drug-Induced Myelosuppression

Nine days after melphalan treatment, patients receiving 0.3 to 3 μg r-G-CSF/kg/d had no detectable progenitors in the peripheral blood. In patients receiving 10 μg r-G-CSF/kg/d or higher, progenitor cells were detectable, and with doses of 30 and 60 μg r-G-CSF/kg/d, progenitor cell numbers equaled or slightly exceeded those in the same patients before initiation of the first treatment cycle with r-G-CSF.

DISCUSSION

On the basis of the known actions of G-CSF in vitro1,2,4,6,26 the administration of G-CSF in vivo would be expected to induce an increase in the number of mature granulocytes at the expense of the granulocytic progenitor pool. While G-CSF induced major increases in circulating granulocytes in experimental animals5-15 and patients receiving chemotherapy16-19 investigations in mice unexpectedly showed an increase in the total number of hematopoietic progenitor cells in spleen and bone marrow after G-CSF administration6,13 that not only included granulocyte-macrophage CFCs but also erythroid and megakaryocyte CFCs and pluripotent stem cells.13 Assuming that the direct actions of G-CSF in vivo are not different from those in vitro, this observation suggests the interaction of G-CSF with some other regulator or the initiation by G-CSF either of the production of other hematopoietic regulators by accessory cells or some compensatory homeostatic mechanism.

In the patients described here, the administration of r-G-CSF was followed by a pronounced increase in the number of circulating progenitor cells and, in most patients, a slight reduction in the frequency of bone marrow progenitor cells. The increase in the absolute numbers of circulating CFCs affected all types and maturational stages of progenitor cells assayed and was already demonstrable at low doses of IV-injected r-G-CSF. Although the frequency of CFCs in blood approached the frequency in bone marrow, characteristic features of the relative frequency of progenitor cell subsets in peripheral blood were retained after r-G-CSF therapy. Thus, the ratios between day 14 CFCs and day 5 clones, between eosinophil and granulocyte-macrophage colonies, and between mixed erythroid and pure erythroid colonies remained virtually unchanged. This argues against the interpretation that the increased circulating progenitor cells were simply the consequence of unselective release of CFCs from the marrow.

The mechanisms responsible for the r-G-CSF-induced increase in peripheral blood progenitor cell numbers require further investigation. Possibilities include the release of
existing marrow progenitor cells, increased production of progenitor cells from stem cells in the marrow or spleen, or a combination of these. Information on the physical nature, surface antigens, and cycling status of these cells would be useful in this context.

Following cytotoxic treatment with alkylating agents, blood CFC numbers usually show a very pronounced initial decrease of 1 week’s duration followed by a gradual increase that reaches peak levels 2 to 3 weeks after therapy.27-29 Our preliminary data suggest that treatment with high doses of rG-CSF may be capable of leading to a more rapid reappearance of progenitor cells in the circulation after chemotherapy, but this needs confirmation by analysis of control patients and a time course analysis.

The significance of the decrease in frequency of bone marrow CFCs is difficult to assess because of a lack of information on total marrow cell counts and the possibility of a variable dilution of marrow aspirates by peripheral blood. Marrow sections from rG-CSF-treated monkeys have demonstrated hypercellularity,4 thus suggesting that part of the decreased frequency of CFCs was observed in the present study might be artificial and due simply to an increased proportion of more mature granulocytes rather than to a reduction in the absolute numbers of progenitor cells. In at least some patients after rG-CSF treatment the frequency of CFCs in fractionated blood actually exceeded that in fractionated marrow, so variable dilution by peripheral blood may not account for decreases in these patients.

Successful hematopoietic reconstitution in patients receiving myeloablative treatment has been reported when using stem cells harvested from peripheral blood,30-35 a procedure that can circumvent the need for general anesthesia and allow the collection of stem cells even if the bone marrow is damaged by previous radiotherapy or infiltrated with malignant cells.31-36 Furthermore, the neutropenic period following marrow transplantation is possibly reduced after the infusion of stem and progenitor cells derived from peripheral blood.31 One of the major limitations of the technique is the scarcity of hematopoietic stem cells in the circulation.33,37 Should the behavior of pluripotent stem cells parallel that of progenitor cells, then injection of donors with rG-CSF might be a useful maneuver before the harvest of peripheral blood cells for transplantation.

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