Evidence for a Novel In Vivo Control Mechanism of Granulopoiesis: Mature Cell-Related Control of a Regulatory Growth Factor

By Judith E. Layton, Helen Hockman, William P. Sheridan, and George Morstyn

As part of phase I/II clinical trials of granulocyte colony-stimulating factor (G-CSF), the pharmacokinetics was studied. To determine the optimal way of abrogating the neutropenia caused by melphalan, patients received G-CSF and melphalan on several schedules. The half-life (t/2) of elimination of G-CSF was in the range 1.3 to 4.2 hours and was prolonged at higher doses, suggesting that one clearance mechanism becomes saturated at doses >10 μg/kg. When a continuous subcutaneous (SC) infusion was administered for five days, a rapid reduction in serum G-CSF levels occurred during the last two days of the infusion, indicating that an additional clearance mechanism was induced. When a continuous infusion of G-CSF was administered after melphalan, serum G-CSF levels remained constant for a longer period of time but did decrease during the second phase of a biphasic neutrophil response. In another clinical trial, G-CSF was administered after high-dose chemotherapy and autologous bone marrow transplantation (ABMT). In these patients, the G-CSF levels did not decrease while the patients were neutropenic. These results show that increased neutrophil levels are associated with increased clearance of G-CSF. This may be one of the negative feedback mechanisms involved in maintaining neutrophil homeostasis in normal and disease states.

COLONY-STIMULATING factors (CSFs) are required for growth and differentiation of hematopoietic stem cells in vitro, and evidence shows that they are important in vivo for regulation of hematopoiesis. One of these CSFs, granulocyte colony-stimulating factor (G-CSF), is relatively specific for the granulocyte lineage. Neutropenia is a side effect of the cytotoxic chemotherapy frequently used in cancer treatment. G-CSF may be useful for prevention or reduction of the period of neutropenia and the associated morbidity and mortality due to infection. Recent molecular cloning of human G-CSF\(^1\) has enabled production of bacterially synthesized human G-CSF (rhG-CSF) in large quantities, making clinical studies possible.\(^2\) These early studies have confirmed that G-CSF is an effective in vivo stimulant of hematopoiesis. On the other hand, little is known about the negative regulation of granulocyte levels, although granulocytes have been reported to produce chalone\(^8\) and lactoferrin,\(^9\) which inhibit granulocyte production in vitro.

We undertook phase I/II clinical trials to determine the optimal dose and route of administration of G-CSF required to reduce the neutropenia induced either by standard-dose chemotherapy with melphalan or high-dose chemotherapy [busulfan (BU) 16 mg/kg and cyclophosphamide (Cy) 120 mg/kg] followed by autologous bone marrow transplantation (ABMT).\(^1\) As part of these trials, G-CSF serum levels were monitored and pharmacokinetics of each route of administration were determined. The results show that intravenous (IV), bolus subcutaneous (SC), and continuous SC infusion routes of administration were effective in causing elevated serum G-CSF levels. The results also suggest that neutrophil numbers regulate the level of circulating G-CSF, providing a negative feedback effect on neutrophil levels.

MATERIALS AND METHODS

Patients. The characteristics of the patients participating in the phase 1/II trial of prevention of neutropenia caused by melphalan were previously described.\(^8\) Patients with histologically proven metastatic cancer for which therapy with an alkylating agent could be indicated were eligible. The clinical aspects of the ABMT study were described previously.\(^1\) Eligible diagnostic groups for the high-dose chemotherapy and ABMT trial included acute lymphoblastic leukemia, non-Hodgkin’s lymphoma, Hodgkin’s disease, and testicular germ cell tumor. The trials met the ethical guidelines of the National Health and Medical Research Council of Australia and were approved by the Board of Medical Research and the Ethics Committee at the Royal Melbourne Hospital.

Drug administration. The early studies varied the dose, route, and timing of G-CSF administration in relation to a standard dose of melphalan (25 mg/m\(^2\)). In phase I of the trial, rhG-CSF (Amgen, Thousand Oaks, CA) was administered to groups of three patients either IV or SC for five days in the absence of cytotoxic chemotherapy. IV G-CSF was administered as a 20 to 30-minute infusion every 12 hours. SC G-CSF was administered either as daily bolus injections or as a continuous infusion by CORMED II ambulatory infusion pump (Cormed, Medina, NY). We report two different schedules of G-CSF administration. One group of patients (24 patients) was treated with G-CSF for five days (days 1 through 6) with various doses and routes of administration, followed by melphalan on day 9. A second group of patients (phase II) received a single injection of melphalan (25 mg/m\(^2\)) on day 1, followed by G-CSF (10 μg/kg continuous SC infusion) on days 2 through 18 (three patients), days 8 through 14 (three patients), or days 8 through 18 (three patients). When a continuous infusion was administered, the blood sample on the last day was taken at the time the pump was withdrawn.

Patients on the ABMT trial received BU at 16 μg/kg/day for four days (days -7, -6, -5, -4, Cy at 60 μg/kg/day for two days (day -3, -2). ABMT two days after chemotherapy (day 0), and G-CSF starting the next day (day 1). G-CSF was administered as a continuous infusion, starting at 20 μg/kg, with step-wise reductions as the neutrophil count increased.\(^1\) The starting dose was maintained until neutrophils were ≥1.0 × 10\(^9\)/L for three consecutive days.

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**G-CSF radioimmunoassay (RIA).** Serum was collected daily from all patients and more frequently in some patients to determine pharmacokinetics. Serum G-CSF levels were measured in a solid-phase RIA as described elsewhere (D. Chang, H. Hockman, and B. Altrock, manuscript in preparation). Strips of flat-bottomed wells (Immulon 2; Dynatech, Alexandria, VA) were coated with an IgG fraction of rabbit anti-G-CSF antibody. After the plates were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) G-CSF standards diluted in normal human serum, or patients' samples, were added and incubated for five hours. Monoclonal anti-G-CSF (75A) was added overnight. Binding of the monoclonal was detected with 125I-rabbit anti-mouse IgG (New England Nuclear/DuPont, Boston), which was added for two hours. Each sample was assayed in duplicate and at several dilutions, when possible. For each patient, a pretreatment serum sample was assayed, and this “background” was subtracted from subsequent samples. The sensitivity of the assay was 500 pg/mL.

**Data analysis.** The half-life ($t_1/2$) of elimination of G-CSF was calculated from the slope constant for the part of the curve that appeared to be following first-order kinetics (from 45 or 60 minutes). The slope constant was determined with a computer program by exponential regression of the concentration-time data. Analysis of variance (ANOVA) was used to determine whether there were significant differences between data values. The Tukey test was used to determine which values were different.

### RESULTS

**Pharmacokinetics of G-CSF administered IV.** The rate of clearance of G-CSF was determined in patients in phase I of the G-CSF trial who were receiving G-CSF for five days before melphalan. Three patients at each dose level were treated with 1, 3, 10, 30 or 60 $\mu$g/kg/day of G-CSF administered as a 20- to 30-minute IV infusion every 12 hours for five days. In most patients, the pharmacokinetic measurements were made after the first injection of G-CSF, but in a few patients measurements were made on later days, as indicated in Table 1. The complete data of the neutrophil levels in these patients over the course of treatment have been described elsewhere, but the neutrophil levels on the day that the pharmacokinetic data was obtained are shown in Table 1. Typical results are shown in Fig 1A through C. A single phase of G-CSF elimination was observed after the IV infusion. The peak level was dependent on the dose administered and similar to the predicted level based on distribution in the blood compartment. A plateau level was maintained for 30 to 60 minutes after the end of the infusion at doses $\geq$ 10 $\mu$g/kg. Similar results were obtained for IV doses of 1 and 60 $\mu$g/kg (results not shown). The $t_1/2$ of G-CSF at each IV dose level is shown in Table 1. The mean $t_1/2$ at the doses of 1 and 3 $\mu$g/kg was significantly shorter than the mean $t_1/2$ at 60 $\mu$g/kg, which indicates that at least one of the possible clearance mechanisms becomes saturated at doses $>3 \mu$g/kg.

**Pharmacokinetics of G-CSF administered SC.** One patient received G-CSF at 10 $\mu$g/kg every 24 hours from day 1 to day 5 before melphalan, and serum G-CSF levels were measured after the first SC injection. In two other patients, the serum G-CSF levels were determined after a single injection of 10 $\mu$g/kg G-CSF administered one day after melphalan. The results from these three patients are shown in Fig 1D. The peak level of G-CSF reached after about four hours was maintained for approximately six hours in two patients; the third patient showed a steady decline from the peak. At 24 hours, serum levels were $<10\%$ of the peak level. The $t_1/2$ for elimination could not be calculated because the kinetics of release of G-CSF from the SC site are unknown. Whether melphalan affected the results or whether the differences were due to individual variation is not clear.

**Pharmacokinetics of continuous SC infusions of G-CSF.** Six patients received a continuous SC infusion of G-CSF 10 $\mu$g/kg/day for five days before melphalan. During the five-day period, circulating neutrophil levels increased for the first two to three days and then reached a plateau (Fig 2A, upper panel). The serum G-CSF level of these patients is shown in the lower panel of Fig 2A. We expected a plateau to occur during the five-day period, because the rate of infusion was constant. Instead, the G-CSF levels decreased markedly over the last two days of the infusion and were significantly less than the peak level (day 3) on days 5 and 6. This substantial decline in G-CSF levels could be explained by induction of an additional clearance mechanism that was not operating during the first few days of the infusion. An alternative explanation for this finding may be that the G-CSF in the bags used with the infusion pump was being absorbed nonspecifically onto the bag and depleted from the solution. Therefore, a bag was set up in an identical manner to the patients' bags and samples were withdrawn for a six-day period for assay by RIA. The G-CSF concentration did not change (data not shown). Because the patients' infusion bags were changed every two or three days, nonspecific loss does not explain the decreasing serum level.

**Serum levels of G-CSF administered after chemotherapy.** Results from the first three ABMT patients are shown in Fig 2B. All three patients received G-CSF at 20 $\mu$g/kg/day for at least ten days. For most of this period, their

### Table 1. The $t_1/2$ of G-CSF Administered IV

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<th>G-CSF Dose ($\mu$g/kg/day)</th>
<th>Day of Treatment</th>
<th>Neutrophils ($\times 10^9$)</th>
<th>$t_1/2$ (h)</th>
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neutrophil levels were undetectable and their serum G-CSF levels remained at a plateau, with no sign of the decrease shown in Fig 2A, which suggested that an increase in neutrophil levels was required for the decrease in G-CSF serum levels to occur.

Further evidence supporting this conclusion was obtained from the patients who received a continuous G-CSF infusion (10 μg/kg/day) after a single injection of melphalan. One group received melphalan on day 1 and G-CSF on days 2 through 18 (Fig 3A), and another group received melphalan on day 1 and G-CSF on days 8 through 18 (Fig 3B). In these patients, the neutrophil response to G-CSF was biphasic (upper panels) and a decrease in the serum G-CSF level was not observed until the increase in neutrophil count occurred in the second phase of the response.

**DISCUSSION**

The major findings of our study are that IV, SC bolus, and SC infusions of G-CSF are effective at rapidly elevating G-CSF levels in the serum. Peak levels are achieved within four to six hours of an SC bolus injection, and levels >10 ng/mL are maintained for ten to 16 hours at the 10 μg/kg dose level. After a single IV injection, the peak level achieved was dose dependent and the t½ varied from 1.3 to 4.2 hours, with doses <10 μg/kg having a shorter t½ than doses ≥10 μg/kg. When G-CSF was administered as a continuous SC infusion before chemotherapy, the expected plateau of serum G-CSF was not maintained for the duration of the infusion. Instead, the G-CSF level declined rapidly over the last two days of the five-day infusion, when the neutrophil levels had risen >30 × 10⁹/L.

Our results suggest that several mechanisms may be involved in the clearance of exogenous G-CSF. The IV pharmacokinetic data establish that one mechanism is saturated at doses ≥10 mg/kg, so that at least two methods of clearance are involved after a single injection of G-CSF. In addition, a third clearance mechanism is induced when neutrophil numbers are high. This effect was observed most clearly in the patients receiving a continuous infusion of G-CSF before chemotherapy. The decrease in G-CSF levels was delayed by a single injection of melphalan and was abolished by high-dose chemotherapy before ABMT. If the increased rate of clearance of G-CSF is related to high neutrophil numbers, the t½ of G-CSF determined after a single injection would be related to the neutrophil level of the patient. Although data are insufficient to be conclusive, the data in Table 1 suggest that the t½ was reduced when neutrophil counts were elevated on the later days of treatment.

When G-CSF was administered after an injection of melphalan, the neutrophil response was biphasic, unlike the response in patients who received G-CSF alone. This effect is probably due to the myelosuppressive action of melphalan, and the first peak is probably due to release of neutrophils that were relatively mature at the start of treatment whereas the second peak probably resulted from neutrophils produced as the BM recovered. A significant reduction in G-CSF serum levels was only observed during the second phase of the neutrophil response, indicating that extensive proliferation in the BM may be involved.

A possible explanation for the increased clearance is neutrophil receptor-mediated endocytosis and degradation. Our calculations based on receptor numbers and binding kinetics of mature neutrophils indicate that this mechanism is unlikely to account for all of the large decrease in serum G-CSF observed. However, because several assumptions are necessary to make these calculations, this conclusion may be incorrect. Immature neutrophils may also contribute to the clearance. Bartocci et al suggested that clearance of physio-
Fig 2. G-CSF and neutrophil levels in patients receiving a continuous infusion of G-CSF. (A) Six patients from the phase I/II trial who received G-CSF at 10 μg/kg/day for five days before melphalan. (B) Three patients from the ABMT trial who received 20 μg/kg/day for at least ten days after high-dose chemotherapy. Data are mean ± SE. Error bars are not shown where they were smaller than the size of the data point. (A) ANOVA indicated that the mean G-CSF levels on days 2 through 6 are significantly different (P = .003). The G-CSF levels on days 5 and 6 were significantly lower than the peak on day 3 (Tukey test, significance level .05).

Fig 3. G-CSF and neutrophil levels in patients receiving a continuous infusion of 10 μg/kg/day after an injection of melphalan (day 1). G-CSF was administered to three patients from day 2 to day 18 (A). Three patients received G-CSF from day 8 to day 14 and three patients received G-CSF from day 8 to day 18. Data from these latter two groups are combined in B. (n = 6 for days 2 through 14; n = 3 for days 15 through 18). Each point is the mean ± SE. Where error bars are not shown, only two values were available (days 7 and 8) or the error bars were too small (days 16 through 18). ANOVA showed that the mean G-CSF levels were significantly different: (A) days 3 through 18, P = .0001, (B) days 9 through 18, P = .004. (A) The G-CSF levels on days 12 through 18 were significantly lower than the peak on day 3 (Tukey test, significance level .05). (B) The G-CSF levels on days 14 through 16 were significantly lower than the peak on day 12 (Tukey test, significance level .05).
logic concentrations of CSF-1 could be accounted for by receptor-mediated internalization by macrophages. They showed that most of the CSF-1 administered to mice was cleared by the kidney, but the urinary CSF-1 was largely degraded and not immunoreactive. We tested urine samples from the patients receiving 60 μg/kg G-CSF by IV injection and could not detect any G-CSF; however, because the sensitivity of the assay was reduced in urine, levels <10 ng/mL would not have been detected. Apart from receptor-mediated elimination, nonreceptor-mediated clearance mechanisms may be induced, including enzymatic degradation in the liver and release of proteolytic enzymes by mature neutrophils. Antibodies to G-CSF have not been detected (J. Layton, unpublished observations, April 1988).

Although most normal subjects do not have detectable serum G-CSF levels, a minority of sera were positive (five of 51, J. Layton, unpublished observations, June 1988) and the highest of those were similar to the levels reached in the clinical trials. In addition, we observed high levels of G-CSF (>100 ng/mL) in patients with infections (J. Layton, unpublished observations, August 1988). Thus, the levels achieved in the trials are similar to those induced by natural stimuli and the rate of clearance of G-CSF observed in the trial patients should be applicable to normal situations. The exact mechanisms of clearance require more investigation, but the downregulation of G-CSF associated with elevated neutrophil levels is probably an important mechanism in homeostatic control of neutrophil levels, especially in disease states.

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