Effect of Plasma TNF-α on Filgrastim-Stimulated Hematopoiesis in Mice and Humans

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Study Objective. To delineate possible explanations for a nonmonotone hematopoiesis dose-response curve with filgrastim therapy after high-dose chemotherapy.

Design. Sequential two-phase study.

Settings. University teaching hospital and basic pharmaceutical sciences laboratory.

Subjects. Thirty-nine patients with breast cancer or melanoma and 15 normal CP-I male mice.

Interventions. Serial blood samples were obtained from patients after high-dose chemotherapy to evaluate hematopoiesis and tumor necrosis factor-α (TNF-α) concentrations. Murine hematopoiesis was induced by filgrastim with or without coadministration of lipopolysaccharide.

Measurements and Main Results. Detection of plasma TNF-α in patients corresponded to substantially slower recovery of granulocytes, erythrocytes, and platelets, and was directly proportional to the prescribed dosage of filgrastim. Lipopolysaccharide stimulated the secretion of TNF-α in mice and totally ablated filgrastim-induced granulopoiesis.

Conclusions. This in vivo evidence suggests that regulatory pathways involving endogenous cytokines may override the effect of recombinant cytokines.

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Granulocyte colony-stimulating factor (G-CSF) was originally identified as "human pluripotent hematopoietic colony-stimulating factor"; however, the current postulation is that its effects are primarily limited to cells of the neutrophil lineage. A recombinant form of G-CSF (Filgrastim; Amgen, Inc., Thousand Oaks, CA) is frequently prescribed to speed granulocyte recovery after chemotherapy and to aid other neutropenic patients. Despite extensive clinical evaluations of this cytokine, explanations for certain aspects of its pharmacology have not been provided, such as a nonmonotone dose-response curve, which was noted in one trial at very high dosages after autologous bone marrow transplantation (ABMT; Figure 1).

Little additional information is published regarding high intravenous dosages (> 20 mg/kg/day) of G-CSF after myeloablative
chemotherapy, although data suggest that additional hematopoietic effects are not seen with dosages above 20 mg/kg/day. Studies in such a setting may be pharmacologically specific, since leukocytes are thought to provide a substantial amount of the body’s clearance of G-CSF. Thus disproportionately higher systemic G-CSF concentrations are potentially achievable in these patients compared with those receiving standard chemotherapy.

Some recombinant proteins such as interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF) increase serum concentrations of endogenous cytokines such as tumor necrosis factor (TNF)-α by undetermined mechanisms. We are unaware of any published studies that evaluated the ability of high-dose G-CSF to stimulate endogenous cytokine production; however, our previous investigations in patients after transplantation identified direct associations between the degree of elevation in concentrations of cytokines such as TNF and organ toxicity. Thus given the unanticipated phenomenon of a nonmonotone hematopoietic dose-response curve after G-CSF, we hypothesized that dose-dependent stimulation of endogenous cytokines may be involved in this effect.

Several in vitro studies revealed that TNF-α can inhibit the G-CSF-induced proliferation of both normal and leukemic hematopoietic progenitors. One mechanism for this effect could involve cytokine receptor modification. Tumor necrosis factor-α down-regulates G-CSF receptor expression on human acute myeloid leukemia cells and granulocytes by a protein kinase C-dependent pathway. Conversely, it appears to potentiate GM-CSF-induced colony proliferation in vitro.

Methods

We investigated the relationship between endogenous concentrations of TNF-α and the efficacy of filgrastim after administration of high-dose chemotherapy in patients after ABMT, and in a murine model of hematopoiesis.

Patients and Treatment Protocol

Patients with breast cancer or melanoma who were undergoing high-dose chemotherapy with ABMT were treated in two sequential phase I-II studies. Forty-five received filgrastim (recombinant G-CSF from Escherichia coli; Amgen, Inc.) 4-8 µg/kg/day intravenously and 48 received regramosin (recombinant GM-CSF from Chinese hamster ovary cells; Sandov/Shering, Inc., Kenilworth, NJ) 1.2-19.2 µg/kg/day intravenously. Note that after the trial was completed the manufacturer revised the extinction coefficient for filgrastim; thus reported G-CSF dosages should be multiplied by 1.2 for equivalence to the currently marketed product.

Informed consent was obtained from each patient. All patients had pretreatment 24-hour creatinine clearances greater than 60 ml/minute, liver function tests within 2.5 times normal limits, and serum total bilirubin less than 2 mg/dl, and received the identical myeloablative

![Figure 1](image-url)  
**Figure 1.** Effect of G-CSF dosing on hematopoiesis. Filgrastim (G-CSF) dose escalations 1-5 correspond to dosages of 4, 8, 16, 32, and 64 µg/kg/day (see Methods for additional dosage information). Regamosin (GM-CSF) dosages are 1.2, 2.4, 4.8, 9.6, and 19.2 µg/kg/day. (Left) Mean percentage marrow cellularity. (Right) Median peripheral absolute neutrophil count (ANC; cells x 10^9/l) were measured on day +15 after ABMT. Standard linear regression analysis of dose ANC data revealed a significant relationship for GM-CSF (p=0.035) but not G-CSF (p=0.801).
of lipopolysaccharide 5 mg/kg (Salmonella abortus equi; Sigma, Inc., St. Louis, MO) at the seventy-second hour to one of the filgrastin-treated groups; the other two groups were given an additional injection of PBS at that time. White blood cell concentrations were determined from tail vein blood collected before and at 48, 72, and 120 hours after the first subcutaneous injection.

The dose of lipopolysaccharide was determined by treating groups of CF-1 male mice with either 5 or 10 mg/kg. All mice that received the highest dose died within 48 hours of injection, compared with no acute or subacute mortality with the lower dose. Short-term effects of lipopolysaccharide on serum TNF-α were documented by administering lipopolysaccharide 5 mg/kg and evaluating heparinized plasma TNF-α concentrations at 30, 60, 90, 120, 130, and 1440 minutes after injection. Samples were processed immediately and stored at -70°C until analysis in approximately 6 months.

Concentrations were measured by an ELISA technique specific for murine TNF-α (R&D Systems). The standard curve for this assay was linear over the range of 23-1500 pg/ml. Data were considered acceptable if both low- and high-quality control samples were within 20% of expected concentrations. Interassay and intrassay CVs for this test were 4.7% and 5.8%, respectively.

Samples were analyzed from 39 patients receiving G-CSF and 23 receiving GM-CSF, none of which were included in our previous report of endogenous cytokines. We chose day +12 for our analysis since it was the day on which positive TNF-α detectability was most frequent based on the study in similar patients, and because most patients begin to manifest peripheral hematopoietic recovery at that time.

Marine Study

All animals were treated in accordance with institutional animal care guidelines. Three groups consisting of five CF-1 male mice (approximately 28 g) each were injected subcutaneously with either phosphate-buffered saline (PBS; controls) or filgrastin 125 pg/kg every 12 hours for 5 days. The dosages of filgrastin was assessed from literature data and a dose-finding experiment with a goal of increasing the normal white blood cell concentration by approximately 8- to 10-fold after 5 days of administration. Endogenous TNF production was stimulated by intraperitoneal administration

Immunosassay and Sampling

Tumor necrosis factor-α was measured on day +12 by enzyme-linked immunosorbent assay (ELISA) specific for the human cytokine (R&D Systems, Minneapolis, MN) from blood samples drawn in heparinized tubes between 4 A.M. and 6 A.M. Samples were immediately refrigerated, plasma was separated within 2 hours, and aliquots were stored at -70°C for approximately 6 months until analysis. The standard curve for the assay was linear over the range of 7.5-500 pg/ml. Data were considered acceptable if both low- and high-quality control samples were within 20% of expected concentrations. Interassay and intrassay CVs for this test were 4.7% and 5.8%, respectively.

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Statistics

Differences in white blood cell concentrations or numbers of transfusions were evaluated by the Mann-Whitney U test. The frequency of TNF-α detectability in patients with versus without depression in hematopoiesis was evaluated by the binomial proportions test. All statistical tests were two-sided, and p values less than 0.05 were considered significant.

Results

Human Study

Thirty-nine patients (mean age 40 yrs, range 30-57 yrs) who received filgrastim were evaluable. Five received high-dose chemotherapy for the treatment of myeloma, and the rest for breast cancer. Plasma TNF-α was detectable in 12 (31%) of the evaluable patients treated with filgrastim. 5 (23%) of 22 receiving dosages of 16 μg/kg/day or less, 2 (25%) of 8 receiving 32 μg/kg/day, and 5 (56%) of 9 receiving 64
Table 1. Effect of Endogenous Plasma TNF-α on Hematopoietic Recovery after ABMT

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Day +12 WBC (cells × 10⁹/mL)</th>
<th>Platelets (U)</th>
<th>RBC (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>1.8 (0.8–2.3) *</td>
<td>25 (18–35) *</td>
<td>22 (18–26) *</td>
</tr>
<tr>
<td>G-CSF</td>
<td>2.0 (0.8–3.1) *</td>
<td>25 (18–35) *</td>
<td>22 (18–26) *</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>3.0 (2.1–5.8) *</td>
<td>25 (18–35) *</td>
<td>22 (18–26) *</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>2.2 (1.0–3.1) *</td>
<td>25 (18–35) *</td>
<td>22 (18–26) *</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>2.4 (1.0–2.6) *</td>
<td>25 (18–35) *</td>
<td>22 (18–26) *</td>
</tr>
</tbody>
</table>

*Thirty-six patients receiving filgrastim (G-CSF) and 22 receiving regranostim (GM-CSF) were available for analysis. Values shown are medians (interquartile range). Platelets and red blood cells represent transfusion requirements and relate inversely to hematopoietic recovery. Nonparametric statistical analysis was performed using the Mann-Whitney U test for unpaired group comparisons.

p = 0.016.
p = 0.006.
p = 0.01.
p = 0.2.

Table 2. Multivariate Analysis of TNF-α Detectability and a Quadratic Model for Filgrastim Dosage on Efficiency in 36 Patients

<table>
<thead>
<tr>
<th>Therapy</th>
<th>WBC (cells × 10⁹/mL)</th>
<th>Platelets (U)</th>
<th>RBC (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B²</td>
<td>0.21</td>
<td>0.30</td>
<td>0.23</td>
</tr>
<tr>
<td>Coefficient dose</td>
<td>3.6 × 10⁻⁵</td>
<td>8.4 × 10⁻⁵</td>
<td>2.6 × 10⁻⁵</td>
</tr>
<tr>
<td>(p dose)</td>
<td>(0.07)</td>
<td>(0.27)</td>
<td>(0.5)</td>
</tr>
<tr>
<td>Coefficient TNF-α²</td>
<td>−4.0 × 10⁻⁷</td>
<td>−4.2 × 10⁻⁷</td>
<td>−3.6 × 10⁻⁷</td>
</tr>
<tr>
<td>(p dose)</td>
<td>(0.04)</td>
<td>(0.05)</td>
<td>(0.6)</td>
</tr>
<tr>
<td>Coefficient TNF-α</td>
<td>4.5 × 10⁻⁶</td>
<td>8.2 × 10⁻⁶</td>
<td>1.3 × 10⁻⁶</td>
</tr>
<tr>
<td>(p TNF-α)</td>
<td>(0.17)</td>
<td>(0.001)</td>
<td>(0.004)</td>
</tr>
</tbody>
</table>

The logistic regression relationship can be described as follows: \log \text{WBC}_{\text{U}} = (A \text{ TNF-}\alpha + B \text{ dose} + C \text{ dose}² + D \text{ dose}³)/E. The sensitivity of the assay we used (7.5 pg/ml) was noted.

The association of serum TNF-α with degree of myeloid reconstitution was evaluated by segregating patients into groups based on the presence of detectable serum TNF-α concentrations (Table 1). Twelve patients with and 24 without detectable TNF-α tolerated the prescribed course of filgrastim therapy and had all data available. Those with detectable TNF-α had a slower white blood cell recovery than those with undetectable levels. Associations were even stronger between endogenous TNF-α and both platelet and red blood cell transfusion requirements; that is, requirement for transfusion products was higher in patients with detectable TNF-α.

Multivariate analysis was performed to investigate the interaction further among filgrastim dosage, endogenous TNF-α, and white blood cell recovery (Table 2). Both increased dosage and measurable TNF-α corresponded to slower white blood cell recovery but the effect was statistically significant only for the former.

Evaluation of posttransplantation leukocyte recovery curves for individual patients identified a cohort that had transient reductions in white blood cell reconstitution (Figure 2). Patients who experienced a significant fall in myelopoiesis (decline in white blood cells of > 100 cells/mm³ on 2 consecutive days or decrease of > 200 cells/mm³ over 24 hrs) between days +8 and +14 were more likely to have detectable plasma TNF-α on day +12 (7/13, 54%) than those with steadily increasing white blood cell levels (3/23, 13%; binomial proportions test p = 0.01). No such relationship existed in the 23 patients treated with regranostim.
Murine Study

Administration of filgrastim alone for 5 days resulted in approximately an 8-fold increase in white blood cell concentrations (Figure 3). Intraperitoneal administration of lipopolysaccharide produced rapid increases in plasma TNF-α that peaked above 1000 pg/ml (mean 1400 pg/ml) at 90 minutes in all mice. Plasma TNF-α was not detected in any mouse treated with PBS alone. Lipopolysaccharide administration resulted in transient decreases in white blood cells by approximately 50%, with levels returning to baseline within 24 hours (data not shown). Administration of lipopolysaccharide to animals treated with filgrastim essentially negated the positive hematopoietic effect of the recombinant cytokine (Figure 3). Leukocyte concentrations at the end of the 5-day experiment in the latter group were indistinguishable from those of control animals not given filgrastim and 10-fold below the group that received filgrastim without lipopolysaccharide.

Discussion

Clinical medicine has admittedly tended to oversimplify the integration of cytokines into the hematopoietic process. The rationale behind this approach is multifaceted, but can be traced to general lack of comprehensive preclinical evaluations of some new cytokines, as well as to the potential for species specificity in the pharmacologic effects of recombinant proteins. We found a biologic effect that was evident in early human trials of a recombinant cytokine, and suggest a hypothesis which may explain this phenomenon. The animal study concurred with this reasoning.

Our results reveal that increased endogenous plasma concentrations of TNF-α correspond to inhibition of G-CSF activity in vivo. Reductions in white blood cells that occurred in association with TNF-α elevations in patients after high-dose chemotherapy and ABMT were reminiscent of the short-term decline in levels that was described accompanying the termination of a prolonged colony-stimulating factor infusion and was not explained by other causes such as infections or myelosuppressive ancillary drug therapy.

Data from the murine model also suggest a causative role for TNF-α in attenuation of granulopoiesis induced by filgrastim; however, the biologic activity of the substance we used to stimulate endogenous TNF-α (lipopolysaccharide) may also induce the secretion of other cytokines. In addition, not all patients with severely declining white blood cell levels had detectable TNF-α in their blood. Plasma concentrations of many cytokines such as IL-6 and macrophage (M)-CSF are frequently elevated in conjunction with TNF-α concentrations after ABMT. Thus, a direct role for TNF-α, although strongly suggested, cannot be firmly concluded from these data.

Studies should be conducted to substantiate the specificity of TNF for these effects. These could include modulation of TNF by therapeutic

Figure 2. Effect of TNF-α on white blood cells in patients receiving filgrastim after ABMT. (Left) Example of white blood cell response in two individuals treated with filgrastim. Day +12 TNF-α was detectable in the patient with a drop in levels between days 10 and 13, whereas it was not detectable in the patient with steadily increasing levels. (Right) Median daily change in white blood cells. Solid bars represent patients classified as having a fall in myelopoiesis. Day +12 TNF-α was detectable in 7 of these 13 patients. Hatched bars represent patients classified as having steadily increasing white blood cells. Tumor necrosis factor-α was detectable in 3 of these 25 patients (p<0.01 binomial proportions test).
blockade of its receptors with monoclonal antibodies, or evaluation of this phenomenon in a murine TNF receptor knockout model. The fact that G-CSF receptors on granulocytes and human acute myeloid leukemia cells are downregulated by a protein kinase C-dependent pathway offers a plausible mechanism for our results, if such a pathway is also operative in developing cells. Taking these data and published in vitro evidence into account, the argument for TNF-α being the likely candidate as the direct effector attenuating G-CSF activity is strong; however, further studies should investigate the factor’s role in this setting.

The endogenous concentrations of TNF-α that potentially led to inhibition of G-CSF activity in our patients were very low, below 40 pg/ml in all observations. In contrast, lipopolysaccharide induced nanogram quantities of endogenous TNF-α. In vitro studies also typically use at least nanogram quantities of TNF-α. Whereas the low concentrations we measured may partly reflect rapid degradation of the cytokines in plasma, differences of these orders of magnitude suggest that the in vivo mechanism is sensitive to lower quantities of TNF-α than those traditionally required in vitro. This may be explained by differences in the duration of exposure, but also could be due to the fact that most in vitro work focuses on the neutrophil effects of G-CSF, whereas our results suggest that other targets of G-CSF that lead to multilineage proliferation are most sensitive to low quantities of TNF-α.

Elevations in endogenous cytokines such as TNF-α, IL-6, and M-CSF were associated with increased erythrocyte or platelet transfusion requirements after ABMT. In this study the need for both platelet and erythrocyte transfusions was significantly greater in filgrastim-treated patients who had detectable plasma TNF-α. This suggests that previously noted relationships between endogenous cytokines and platelet transfusion requirements may not be primarily due to direct effect of these cytokines, but instead to their interaction with filgrastim. Increases in transfusion requirements associated with attenuation of filgrastim activity by TNF-α are highly significant and not well explained by dosage effects.

The substantial impact of TNF-α on erythrocyte and platelet recovery after ABMT in patients treated with filgrastim, but not recombinant, is evidence that a major in vivo effect of G-CSF lies in its capacity to synergize with other cytokines, producing multilineage or early progenitor cell proliferation. In vitro data suggest that the G-CSF receptor is present on cells from the myeloblast to the mature neutrophil but not on any erythroid or megakaryocytic lineage cells. However, G-CSF was originally isolated as a plasminogen activator. Synergy between G-CSF and IL-1, IL-3, IL-6, or c-kit ligand in the stimulation of early progenitors has been demonstrated in vitro.

Treatment of patients receiving high-dose chemotherapy with filgrastim and peripheral blood progenitor cells induced by filgrastim dramatically improved platelet and erythrocyte transfusion requirements after ABMT. This in vivo effect could be explained by an indirect mechanism (e.g., G-CSF inducing production of simulators of other lineages), but is best interpreted in light of in vitro data by G-CSF acting in synergy with other cytokines to stimulate early progenitors. In any event, these results indicate that this cytokine has, either directly or indirectly, a major role in regulating a broad spectrum of hematopoiesis. Picogram concentrations of circulating TNF-α appear potentially to provide a very sensitive mechanism to modulate this pathway.

The selective advantage of the G-CSF-TNF-α interaction is not clear. Published data suggest that GM-CSF is not involved in basal hematopoiesis and extenuate the possibility that it is the primary regulator of this process, whereas GM-CSF, M-CSF, IL-6, or other cytokines may be crucial in acute situations such as defense.

Figure 3. Effect of lipopolysaccharide on filgrastim induced granulopoiesis. Male CBA mice were treated with filgrastim 125 mcg/kg subcutaneously twice daily or placebo for 5 days. After 72 hours they received intraperitoneal injections of either lipopolysaccharide 5 mg/kg in PBS or PBS alone. Values shown are mean ± SD from the five animals treated in each group.
against microorganisms. It is plausible that the interaction of TNF-α and G-CSF is one of many required to tailor hematopoietic response during different types of infections. The capacity of G-CSF to reduce TNF-α production after lipopolysaccharide administration and to modulate glucose metabolic response to endotoxin also may play a role in response to infection.

A better understanding of such interactions is crucial for designing clinical trials. Our results suggest that attempts to stimulate neutrophil proliferation with filgrastim in patients with gram-negative sepsis might fail due to high TNF-α concentrations. Similarly, induction of TNF-α, IL-6, and M-CSF by moderate doses of GM-CSF may explain the unimpressive clinical results of combining recombinant G-CSF and GM-CSF despite known in vitro synergy.

Suppression of endogenous TNF-α might improve response to recombinant or endogenous G-CSF in some patients. Utilization of ciprofloxacin, a known inhibitor of in vitro TNF production, after myelosuppressive chemotherapy was associated with accelerated hematopoietic recovery, although the relationship to in vivo TNF-α suppression has not been evaluated in this setting. Alternatively, simultaneous infusion of G-CSF and monoclonal antibodies to TNF-α may improve efficacy in some patients. Clinically relevant reductions in transfusion requirements after chemotherapy are plausible, given the strong association between endogenous TNF-α and platelet recovery. Recent work in our laboratory showed that endogenous TNF-α concentrations as low as 1 pg/ml relate to increased platelet requirements in patients receiving G-CSF and peripheral blood progenitor cells after ABMT (data not shown). Since very low concentrations of TNF-α are effective in blocking G-CSF activity, perhaps a therapeutically meaningful effect could be achieved with modest doses of antibodies.

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References

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TNF/G-CSF INTERACTION


