Protective Effects of Tumor Necrosis Factor on Murine Hematopoiesis during Cycle-specific Cytotoxic Chemotherapy

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ABSTRACT

Tumor necrosis factor (TNF) is a pleiotropic cytokine which exerts a wide range of effects when administered in vivo. Using a murine model, we have investigated the effect of pretreatment with 1 µg (2.6 x 10^6 units) per mouse of recombinant murine TNF-α on hematopoietic recovery following administration of cyclophosphamide, 5-fluorouracil, methotrexate, or vinblastine. TNF pretreatment results in enhanced regeneration of circulating neutrophils and hematopoietic progenitors, as measured by in vivo and in vitro assays, in animals given cycle-specific chemotherapeutic agents. The results may suggest that TNF affects cycle kinetics in hematopoietic progenitor cell populations, thus making these cells less prone to the cytotoxic effects of the chemotherapeutic agents. As myeloablation is a frequent and often critical side effect following cancer treatment, these findings may have clinical implications.

INTRODUCTION

Several pleiotropic biological response modifiers have been investigated for their ability to confer protection against the potentially fatal side effects of cytoreductive therapy. Pretreatment with IL-1, γ-interferon, and TNF-α has been shown to confer radioprotection in rodent models (1-5), and IL-1 has further been shown to enhance hematopoietic recovery in animals when administered after cytotoxic drugs (6-8). IL-1 may act as an inducer of mitotic activity in hematopoietic progenitor populations (9), whereas the mechanisms underlying the TNF-mediated protective effects have remained obscure.

TNF was first identified as an endogenous tumor necrosis-inducing agent in mice sequentially challenged with Bacillus Calmette-Guérin and endotoxin (10). The factor has henceforth been associated with a diverse range of effects, which include growth inhibition of a number of neoplastic cell lines, as well as growth-promoting properties on some nonmalignant cell populations (11, 12). TNF has been found to activate polymorphonuclear cells in vitro (13) and enhance cell-mediated cytotoxicity in monocytes (14). The factor has further been shown to induce cachexia and shock-like states in laboratory animals (15).

Hematopoietic cells are responsive to the effects of TNF. In vitro, the factor inhibits colony formation by hematopoietic progenitors (16-19). On the other hand, TNF induces production of hematopoietic growth factors such as IL-1, CSF-1 (M-CSF), G-CSF, and IL-6 (20-26). The net effect of these apparently opposing actions of TNF on in vivo hematopoiesis after administration of chemotherapy has hitherto not been evaluated.

Others have presented evidence strongly suggesting that TNF and IL-1 act by distinctly different mechanisms when used prior to cytoreductive radiotherapy in animal models (2). This, together with previous reports from this laboratory which demonstrate the ability of TNF pretreatment to enhance hematopoietic recovery in irradiated mice, constitutes the rationale for the present study. Herein, we report that TNF confers protection and enhanced recovery of murine in vivo hematopoiesis when administered prior to cycle-specific cytotoxic agents. Four antitumor drugs, with different mechanisms of action and with a recognized ability to induce moderate to marked leukopenia when administered to humans (27), were used in the study.

MATERIALS AND METHODS

Mice. BALB/c mice were purchased from The Jackson Laboratory, Bar Harbor, ME, and were maintained in a laminar air-flow room under specific-pathogen-free conditions. Experiments were initiated when the mice were 12 to 16 wk old. The animals were randomized into groups of 10, each of which was administered a single chemotherapeutic agent with or without TNF pretreatment.

Factors. rmTNF-α (specific activity, 2.6 x 10^7 units/mg) was a gift from Dr. M. A. Palladino, Genentech, Inc., San Francisco, CA. Recombinant murine GM-CSF was kindly supplied by Dr. S. Gillis, Immunex Corp., Seattle, WA.

Drug Treatment. rmTNF was diluted in Earle’s balanced salts solution buffered to pH 7.2 with 10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and containing bovine serum albumin (100 µg/ml) as carrier. Mice (10 per group) received i.v. injections of either 1 µg (2.6 x 10^4 units) per mouse or an equal volume (200 µl) of diluent.

Twenty-four h after pretreatment, the mice received CTX (200 mg/kg; Bristol-Myers Oncology Division, Syracuse, NY), 5-FU (150 mg/kg; SoloPak Laboratories, Franklin Park, IL), MTX (150 mg/kg; Lederle Parenterals, Inc., Carolina, PR), or VB (2.5 mg/kg; QUAD Pharmaceuticals, Indianapolis, IN) as i.v. bolus infusions. The doses administered were based on previous experiments as the maximum compatible with survival in the BALB/c mouse.

Three randomly selected mice per group were bleb by the tail vein at each subsequent time point for determinations of leukocyte, differential leukocyte, platelet, and total blood cell counts.

Culture medium. IMDM was purchased from Gibco (Grand Island, NY) and was supplemented with bicarbonate (3.024 g/liter) and monothioglycerol (7.5 x 10^-5 mol). A single lot of FCS (Hazelton Research Products, Inc., Lenexa, KS) was used throughout the study.

In Vitro Progenitor Cell (CFU-C) Assays. At specific time points postchemotherapy, three randomly selected mice from each group were killed by carbon dioxide asphyxiation. Pooled femoral bone marrow cells and pooled spleen cells from each group were washed twice in IMDM with 15% FCS and counted. A defined number of cells in 1 ml of IMDM supplemented with 15% FCS and 0.36% agarose (SeaPlaque; FMC, Rockland, ME) were plated into 35-mm Petri dishes containing GM-CSF as test stimulus. The CSF was pretitrated and used in a concentration supporting optimal (plateau) levels of colony formation, which was enumerated after 7 days of incubation at 37°C in a fully

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2 Fellow of the Norwegian Cancer Society. To whom requests for reprints should be addressed, at the Clinical Pharmacology Unit, Department of Pharmacology and Toxicology, University of Bergen, N-5021 Bergen, Norway.

3 The abbreviations used are: IL-1, interleukin 1; IL-6, interleukin 6; CSF-1, macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; CTX, cyclophosphamide; 5-FU, 5-fluorouracil; MTX, methotrexate; VB, vinblastine; CFU-C, colony-forming unit assayed in culture; HPP-CFU-C, high proliferative potential colony-forming unit assayed in culture; CFU-S, spleen colony-forming unit; CFU-GM, granulocyte/macrophage colony-forming unit; CFU, colony-forming unit; TNF, tumor necrosis factor; rmTNF, recombinant murine tumor necrosis factor; IMDM, Iscove's modified Dulbecco's medium; FCS, fetal calf serum.

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huminidified atmosphere of 5% CO₂ in air. For HPP-CFU-C assays, defined numbers of cells were plated in IMDM with 20% FCS and 0.36% agarose in double-layered cultures (28), with underlayers consisting of IMDM with 20% FCS, 0.5% agarose, 10% S637 conditioned medium (29), and 1000 units/ml of recombinant human CSF-1 as test stimuli. After 10 days of incubation at low oxygen tension (5% O₂:5% CO₂:90% N₂), colonies with diameters >0.5 mm were scored as HPP-CFU-C.

CFU-S Assay. Cell preparations were assayed for CFU-S using the method of Till and McCulloch (30). Briefly, defined numbers of bone marrow or spleen cells were injected i.v. into lethally irradiated (8.5 Gy) syngeneic BALB/c mice. Spleens were harvested 12 days postirradiation/injection, and colonies were enumerated after overnight immersion in Bouin’s fixative.

Statistical Calculations. Statistical computations were performed using Student’s t test for two means.

RESULTS

At Days 7 and 14 postchemotherapy, three randomly selected mice from each group were bled for determinations of peripheral blood cell counts, the results of which are shown in Table 1. In the animals given MTX, the total white cell, neutrophil, and total blood cell (i.e., RBC) counts were significantly higher in the animals pretreated with TNF. In the 5-FU-treated groups, neutrophil counts were significantly higher in the TNF-pretreated animals, whereas no significant differences were apparent within the other groups (Table 1).

On Day 7, three randomly selected mice per group were killed and had their spleens and femoral bone marrows removed for in vitro and in vivo progenitor assays. The results of the CFU-GM assays are shown in Fig. 1. With the exception of the bone marrow-derived progenitors in the MTX-treated groups, TNF pretreatment resulted in significant differences in the numbers of CFU-GM progenitors as compared with controls. In the bone marrows of the groups given CTX, TNF pretreatment reduced the number of CFU-GM, whereas it induced an increase in progenitors in the animals receiving the other chemotherapeutic agents (Fig. 1).

The results of HPP-CFU-C assays, also performed at Day 7 postchemotherapy, are shown in Fig. 2. TNF pretreatment resulted in significant increases in spleen-derived HPP-CFU-C in all the animals, whereas the differences in progenitors in the bone marrow, with the exception for the animals given 5-FU, were not significant. As in the CFU-GM assays (Fig. 1), TNF pretreatment resulted in a decrease in the number of femur-derived progenitors in the groups given CTX (Fig. 2). TNF pretreatment resulted in significant increases in spleen-derived CFU-S in all groups of mice (Fig. 3). Again with the exception of the CTX-treated groups, TNF also induced an increase in bone marrow-derived progenitors, which was statistically significant (P < 0.05) in the 5-FU-treated groups (Fig. 3).

The results of the progenitor assays are summarized in Table 2. Based on the assumption that the total CFU content per animal is adequately described by the equation CFU_total = (CFUfemur x 20) + CFUspleen (6), Table 2 gives the mean CFU per animal in the groups. TNF pretreatment generally had indifferent or detrimental effects on the Day 7 CFU content in animals treated with CTX, whereas TNF pretreatment led to 1.2- to 5.5-fold increments in mean total CFU content in the other three groups, with the most dramatic increases seen in the 5-FU- and VB-pretreated animals.

Two separate groups of mice, each consisting of 10 animals, were treated with TNF or diluent as outlined and, after 24 h, given the same dose of VB as used in the experiments described above. Three randomly selected animals per group were bled daily for determination of neutrophil counts, and the in vitro and in vivo progenitor assays were performed on Days 3, 5, and 7 postchemotherapy. In the controls, neutrophil counts reached a nadir (mean, 200 cells/mm³) at Day 4 post-VB, whereas neutrophil levels in the TNF-pretreated animals at no time points declined below 1000 cells/mm³ (Fig. 4). TNF pretreatment was also accompanied by an enhanced recovery of the mean total numbers of hematopoietic progenitors, assayed as CFU-GM, HPP-CFU-C, and CFU-S, and calculated as outlined above (Fig. 4).

DISCUSSION

The potential ability of TNF to enhance hematopoietic recovery after cytoreductive anticancer drug therapy was tested using four different chemotherapeutic agents. Of these, CTX is an alkylating agent, 5-FU and MTX are antimetabolites with cytoidal effects confined to the S phase of the cell cycle, and VB is a phase-specific inhibitor of mitosis (27). Of the four agents, only CTX is cytotoxic regardless of the cycling status in target cells, while the cytoidal effects of 5-FU, MTX, and VB are restricted to specific phases of the cell cycle. Differences in pharmacokinetic properties and modes of action among the four drugs are reflected in their dissimilar effects on the hematopoietic system in the mouse model. In addition, the drugs

Table 1 Peripheral blood cell counts in BALB/c mice treated with rmTNF-α (1 μg/mouse) or diluent as a single i.v. bolus injection 24 h prior to chemotherapy

<table>
<thead>
<tr>
<th>Days post-chemotherapy</th>
<th>Cyclophosphamide</th>
<th>5-Fluorouracil</th>
<th>Methotrexate</th>
<th>Vinblastine</th>
<th>5-FU</th>
<th>MTX</th>
<th>VB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diluent</td>
<td>TNF</td>
<td>Diluent</td>
<td>TNF</td>
<td>Diluent</td>
<td>TNF</td>
<td>Diluent</td>
</tr>
<tr>
<td>WBC (no./mm³ × 10⁹)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>7.2 ± 1.4*</td>
<td>8.3 ± 1.6</td>
<td>7.1 ± 1.0</td>
<td>7.7 ± 0.4</td>
<td>4.6 ± 1.8</td>
<td>13.7 ± 3.2</td>
<td>18.1 ± 3.4</td>
</tr>
<tr>
<td>14</td>
<td>17.1 ± 2.7</td>
<td>14.3 ± 6.6</td>
<td>17.3 ± 2.2</td>
<td>18.5 ± 4.6</td>
<td>13.1 ± 4.4</td>
<td>11.6 ± 2.1</td>
<td>13.9 ± 2.0</td>
</tr>
<tr>
<td>Neutrophils (no./mm³ × 10⁹)</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4.1 ± 0.8</td>
<td>4.9 ± 0.8</td>
<td>ND*</td>
<td>1.5 ± 0.1*</td>
<td>0.5 ± 0.4</td>
<td>2.9 ± 0.4*</td>
<td>4.6 ± 2.1</td>
</tr>
<tr>
<td>14</td>
<td>2.7 ± 1.2</td>
<td>2.4 ± 2.2</td>
<td>3.5 ± 1.8</td>
<td>3.6 ± 1.5</td>
<td>5.1 ± 2.7</td>
<td>4.1 ± 0.8</td>
<td>3.1 ± 0.9</td>
</tr>
<tr>
<td>Platelets (no./mm³ × 10⁹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>6.3 ± 1.4</td>
<td>4.9 ± 0.8</td>
<td>6.1 ± 0.8</td>
<td>8.1 ± 2.5</td>
<td>3.3 ± 2.2</td>
<td>5.8 ± 0.4</td>
<td>7.9 ± 1.3</td>
</tr>
<tr>
<td>14</td>
<td>11.1 ± 1.6</td>
<td>13.3 ± 1.9</td>
<td>27.0 ± 4.7</td>
<td>26.2 ± 3.6</td>
<td>11.1 ± 1.5</td>
<td>11.0 ± 1.5</td>
<td>12.9 ± 1.5</td>
</tr>
<tr>
<td>Total blood cells (no./mm³ × 10⁹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>7</td>
<td>9.1 ± 0.8</td>
<td>9.0 ± 0.5</td>
<td>7.8 ± 0.5</td>
<td>8.1 ± 0.4</td>
<td>9.5 ± 0.1</td>
<td>10.0 ± 0.2*</td>
<td>9.3 ± 0.5</td>
</tr>
<tr>
<td>14</td>
<td>11.1 ± 1.2</td>
<td>11.2 ± 0.2</td>
<td>9.1 ± 0.5</td>
<td>9.4 ± 0.3</td>
<td>10.5 ± 0.2</td>
<td>9.9 ± 1.1</td>
<td>9.5 ± 0.1</td>
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</tbody>
</table>

* Mean ± SD for randomly selected groups of three mice.

† Statistically significant (P < 0.05) differences between TNF- and diluent-treated groups.

ND, Not detected.
may to a certain extent exert their cytocidal effects on different subpopulations of hematopoietic progenitors, which in turn may explain some variations in duration and magnitude of the cytopenia induced by administration of the different agents. Whereas all four drugs induce leukopenia in humans (27), this was initially not obvious after administration of VB in the murine model (Table 1). At Day 7, the TNF-pretreated animals given MTX had significantly higher WBC numbers as compared with the diluent-treated controls. Likewise, accelerated recovery of neutrophils was not observed following TNF pretreatment in the mice given CTX, but in the 5-FU- and MTX-pretreated groups, pretreatment with TNF resulted in significantly increased neutrophil levels as compared with controls at Day 7 postchemotherapy. By Day 14, all groups had recovered from the leukopenia (Table 1). In the VB-pretreated groups, significantly elevated neutrophil counts were evident at Days 0 (i.e., the day VB was administered), 1, 4, and 5 (Fig. 4). Compared with the effects seen after 5-FU and MTX (Table 1), the VB-induced neutropenia was more transient and was normalized by Day 7. The initial (Days 0 and 1) neutrophilia is probably due to a temporary demargination caused by TNF in the blood stream (31), resulting in a transient elevation of circulating neutrophils as previously described in animals (4, 32) and humans (33) after administration of TNF and endotoxin, respectively. The sustained relative neutrophilia in the TNF-pretreated animals on Days 4 to 7, when a marked decline in neutrophil levels was evident in the control animals (Fig. 4), is unlikely to be due to this effect and probably represents a sustained protective effect conferred by the factor per se.

No differences in platelet numbers were apparent among any of the groups at Days 7 and 14 (Table 1), and although the Day 7 total blood cell (i.e., RBC) number was significantly higher than controls in the TNF-pretreated animals given MTX, the difference was nevertheless small.

The results of the in vitro and in vivo CFU assays, shown in Figs. 1 to 3 and summarized in Table 2, substantiate the notion that TNF confers protection against the myeloablative effects of some anticancer drugs. Pretreatment with TNF in animals administered 5-FU, MTX, or VB resulted in an expansion of the CFU-GM population, which represents bipotential myeloid progenitor cells committed to differentiation into mature cells of the monocyte/macrophage and neutrophil cell lineages (34, 35). In the same groups of mice, TNF pretreatment expanded the HPP-CFU-C population, which constitutes an earlier population of progenitor cells capable of forming large (diameter, >0.5 mm) colonies in vitro when stimulated with a CSF species and a synergistic activity in conditioned medium from the human bladder carcinoma cell line 5637 (6, 28). Similar effects of TNF were demonstrated in the Day 12 CFU-S populations, which are relatively early multilineage progenitors with a recognized potential for both self-renewal and proliferation and ultimately differentiate into mature blood cells (36, 37). At Day 7 post-5-FU, MTX, and VB, the comparatively most impressive CFU expansions were evident in splenic tissue from TNF-pretreated animals (Figs. 1 to 3). This reflects the pivotal role of the spleen in murine in vivo hematopoietic regeneration.

A separate and more detailed study of the kinetics of CFU expansion after VB administration (Fig. 4) showed low and comparable levels of CFU-GM, HPP-CFU-C, and CFU-S in both TNF- and diluent-pretreated mice at Day 3 postchemotherapy. The populations increased over the following 4 days, with a substantially larger increase in progenitors in the TNF-pretreated

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**Table 2** Total complement of CFU in BALB/c mice treated with rmTNF-α (1 μg/mouse) or diluent as a single i.v. bolus injection 24 h prior to chemotherapy

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cyclophosphamide</th>
<th>5-Fluorouracil</th>
<th>Methotrexate</th>
<th>Vinblastine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diluent</td>
<td>TNF</td>
<td>Diluent</td>
<td>TNF</td>
</tr>
<tr>
<td>CFU-GM (× 10⁶)</td>
<td>1930</td>
<td>1080</td>
<td>86.8</td>
<td>477</td>
</tr>
<tr>
<td>HPP-CFU-C (× 10⁵)</td>
<td>116</td>
<td>129</td>
<td>34.4</td>
<td>89.8</td>
</tr>
<tr>
<td>CFU-S (× 10³)</td>
<td>256</td>
<td>130</td>
<td>69.2</td>
<td>163</td>
</tr>
</tbody>
</table>

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animals as compared with controls (Fig. 4). In addition to demonstrating the efficacy of TNF pretreatment for hematopoietic regeneration in VB-treated mice, the results may indicate a potential use for VB in experimental models where rapid increases in hematopoietic progenitor populations are desired.

Several mechanisms, acting alone or in concert, may be responsible for the TNF-conferred protective effects after chemotherapy. These include the ability of TNF to stimulate secretion of cytokines, such as IL-1 and IL-6, CSF-1, GM-CSF and G-CSF (20-26), which themselves may aid hematopoietic reconstitution in chemotherapy-challenged animals. IL-1 has previously been shown to enhance hematopoietic reconstitution in mice after administration of CTX or 5-FU in the mouse (6-8). TNF has also been shown to induce the production of mRNA for manganous Superoxide dismutase, a scavenger of Superoxide radicals (38), and may further influence the cycling status of cells (39, 40). The results presented herein can certainly not be explained by the induction of IL-1 production by TNF. Administered prior to (7) or after (5, 7) CTX, IL-1 has been reported to confer hematopoietic protection in murine models, whereas the present study shows that TNF has no or detrimental effects on hematopoietic recovery after administration of CTX (Tables 1 and 2; Figs. 1 to 3). It also seems unlikely that the observed effects are attributable to induction of CSF species or IL-6, which presumably would induce mitotic activity in target cells and make them more susceptible to the cytoidal effects of drugs like 5-FU, MTX, and VB. The results presented herein show that TNF protects hematopoietic cells when administered prior to drugs which kill cells in cycle and favor a hypothesis that TNF may affect cell cycle kinetics in these populations. This does not, however, exclude the possibility that TNF induction of scavengers or other moieties may be of importance for the observed effects.

If TNF inhibits the cell cycle in hematopoietic progenitor populations, this may further suggest an expansion of the clinical use of this cytokine. A number of investigations have shown that GM- and GM-CSF aid in the reconstitution of hematopoiesis after chemotherapy (41-44), an effect which is due to a potent and apparently specific mitogenic stimulus conveyed by the CSF cytokines to the appropriate target cells. If those cells may be switched off with respect to proliferation prior to cycle-specific chemotherapy, this would render the populations resistant to the detrimental effects of phase-specific cytotoxic agents, while regaining their potential for proliferation and differentiation under the influence of other cytokines, administered after the anticancer agents are eliminated.

In summary, TNF pretreatment results in augmentation of murine hematopoietic recovery, measured by peripheral cell counts and CFU assays, after administration of cycle-specific cytotoxic chemotherapy, but not after administration of an alkylating agent. The results suggest that TNF may induce cycle arrest in hematopoietic progenitor populations from which the cellular components of the blood originate. This effect may be explored therapeutically and could lead to the implementation of a novel strategy in the prevention of chemotherapy-induced hematological suppression in the clinical management of cancer patients.

ACKNOWLEDGMENTS

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