Recombinant Transforming Growth Factor β_1 and β_2 Protect Mice from Acutely Lethal Doses of 5-Fluorouracil and Doxorubicin

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Summary

Transforming growth factor β_1 (TGF-β_1) and TGF-β_2 can reversibly inhibit the proliferation of hematopoietic progenitor cells in vivo, leading us to hypothesize that such quiescent progenitors might be more resistant to high doses of cell cycle active chemotherapeutic drugs, thereby allowing dose intensification of such agents. Initial studies showed that whereas administration of TGF-β_1 or TGF-β_2 did not prevent death in normal mice treated with high doses of 5-fluorouracil (5-FU), those mice that received TGF-β_2 did exhibit the beginning of a hematologic recovery by day 11 after administration of 5-FU, and were preferentially rescued by a suboptimal number of transplanted bone marrow cells. Subsequently, it was found that the administration of TGF-β_2 protected recovering progenitor cells from high concentrations of 5-FU in vitro. This protection coincided with the finding that significantly more progenitors for colony-forming unit–culture (CFU-c) and CFU–granulocyte, erythroid, megakaryocyte, macrophage (GEMM) were removed from S-phase by TGF-β in mice undergoing hematopoietic recovery than in normal mice. Further studies showed that the administration of TGF-β protected up to 90% of these mice undergoing hematologic recovery from a rechallenge in vivo with high dose 5-FU, while survival in mice not given TGF-β was <40%. Pretreatment of mice with TGF-β_1 or TGF-β_2 also protected 70–80% of mice from lethal doses of the noncycle active chemotherapeutic drug, doxorubicin hydrochloride (DXR). These results demonstrate that TGF-β can protect mice from both the lethal hematopoietic toxicity of 5-FU, as well as the nonhematopoietic toxicity of DXR. This report thus shows that a negative regulator of hematopoiesis can be successfully used systemically to mediate chemoprotection in vivo.

Myelosuppression is the dose-limiting factor for most cancer chemotherapeutic agents (1). The resulting granulocytopenia renders many patients susceptible to opportunistic infections that can be lethal (2). Agents that protect the myeloid and lymphoid systems from the suppressive effects of radiation or chemotherapy could be beneficial because the side effects induced by a standard therapy regimen might be ameliorated, and the higher doses of therapy that could be administered could result in increased antitumor efficacy (3). Two basic approaches have been envisioned to induce chemoprotection. In one approach, positive regulators of hematopoiesis, such as IL-1, IL-6, the CSFs, and/or stem cell factor could be used to stimulate the proliferation and/or production of critical stem, progenitor, or end-stage hematopoietic cells. This approach could result in a larger pool of these cells before chemotherapy, thus allowing a more rapid recovery. Alternatively, they may be used posttreatment to restimulate the remains of the stem/progenitor compartment after myelosuppression. The validity of this type of approach is illustrated by the well-documented chemoprotective effects of IL-1 (4–7). Alternatively the number of critical stem/progenitor cells could be increased and then a potent negative regulator of hematopoiesis used to temporarily inhibit their

proliferation thereby rendering them more resistant to killing by cell cycle–active chemotherapeutic drugs. Several cytokines, including TGF-β, have been demonstrated to be potent negative regulators of hematopoiesis (8–12).

TGF-β is a 25-kD homodimeric member of a functionally related family of polypeptide growth factors that show diverse effects on growth and cell development (13–16). TGF-β exists in at least five isoforms (17), which exhibit 70–80% sequence identity (14). TGF-β_1 and/or TGF-β_2 have been shown to preferentially inhibit the proliferation and differentiation of early murine and human hematopoietic progenitors both in vitro and in vivo (18–25). Such cell cycle–arrested progenitors could be more resistant to cycle-active chemotherapeutic drugs, thereby allowing a dose intensification of such agents.

In this report we demonstrate that in vivo treatment with TGF-β_1 or TGF-β_2 protects some of the progenitors for CFU–culture (CFU-c)[1], CFU–granulocyte, erythroid, megakaryocyte, macrophage (CFU-GEMM) and high proliferative potential– colony forming cells (HPP-CFC) in normal and hyperproliferative marrow from the toxicity of 5-fluorouracil (5-FU), thereby resulting in an ability to successfully dose intensify 5-FU. We also report that recombinant r)TGF-β is effective in vivo in protecting mice from acutely toxic doses of doxorubicin hydrochloride (DXR), through effects that are at least partially nonhematological. This report thus demonstrates that negative regulation of hematopoiesis can be successfully exploited for dose intensification of chemotherapy.

Materials and Methods

Mice. Specific pathogen-free BALB/c mice at 8–12 wk of age were obtained from the animal production facility of the National Cancer Institute–Frederick Cancer Research and Development Center (Frederick, MD).

Growth Factors and Reagents. The rTGF-β_1 was a generous gift from Dr. M. Palladino, Jr. (Genentech Inc., San Francisco, CA). The rTGF-β_2 was produced in Chinese hamster ovary cells and generously supplied by Celtrix Pharmaceuticals (Santa Clara, CA). For injection, the TGF-β_1 was diluted in HBSS + 0.1% normal mouse serum (NMS) and TGF-β_2 in citrate buffer (pH 6.0) containing 1% NMS. TGF-β_1 or TGF-β_2 were administered intraperitoneally according to various protocols outlined in the figure legends. Recombinant murine IL-3 and recombinant human erythropoietin (rhEpo) were purchased from PeproTech, Inc. (Rocky Hill, NJ), and Amgen Biologicals (Thousand Oaks, CA), respectively. 5-FU (LymphMed, Rosemont, IL) was diluted in sterile distilled water and administered intravenously to the mice at the indicated doses while DXR (Sigma Chemical Co., St. Louis, MO) was diluted in HBSS immediately before intraperitoneal injection.

CFU-c and CFU-GEMM Assay. BM cells were aspirated from the paired femurs and tibias of control or experimental mice with

cold HBSS without Ca^{2+} and Mg^{2+} (washing buffer), washed twice, and resuspended in α medium (GIBCO BRL, Gaithersburg, MD) that was supplemented with 20% FCS (Biofluids, Inc., Rockville, MD), 2 × 10^{-4} mol/liter hemin (Sigma Chemical Co.), 5 × 10^{-5} mol/liter 2-mercaptoethanol (Sigma Chemical Co.), 8 U/ml Epo, 100 U/ml rhIL-3, and 0.05 mg/ml gentamicin in 0.35% Seaplaque agarose (FMC Bioproducts, Rockland, ME). 10^5 BM cells in 1 ml of this medium were plated in 35-mm Lux petri dishes (Miles Laboratories, Inc., Naperville, IL) and incubated at 37°C in 5% CO_2 for 10 d at which time the number of colonies was determined. Multipotential colonies containing granulocyte, erythroid, megakaryocyte and macrophage lineages were scored as CFU-GEMM, while monocyte (CFU-M), myeloid (CFU-G), erythroid (CFU-e, BFU-e), and myelomonocytic (CFU-GM) colonies were designed as CFU-c (26). Data are presented as the total number of CFU obtained from the paired femurs and tibias of each mouse.

HPP-CFC Assay. HPP-CFC were detected using a modified double-layer agar technique (27). Briefly, lower layers (0.5% agarose in α medium/20% FCS containing hemin, IL-3, IL-1, and supernatant from L929 cells [source of CSF-1]) were prepared in 35-mm Lux petri dishes, and 2–8 × 10^4 cells in 0.35% agarose in α medium/20% FCS was added as a second layer. Cultures were scored after incubation for 14 d under low oxygen tension (7.5% O_2/7.5% CO_2/85% N_2). All colonies with a diameter >0.5 mm were scored as HPP-CFC.

Thymidine Suicide Assay. The proliferative status of various hematopoietic progenitors was measured by calculating the percentage of CFU-c and CFU-GEMM remaining after incubation with methyl[^{3}H]thymidine (37.0 MBq/ml, sp act 925 GBq/mmol) (Amersham Corp., Arlington Heights, IL) as previously described (28, 29). BM cells were resuspended in culture medium (α medium/20% FCS) at a concentration of 2 × 10^6 cells/ml and incubated with 100 μCi/ml of [^{3}H]TdR for 30 min at 37°C. The incubation was terminated by placing the cell suspensions on ice. CFU-c and CFU-GEMM assays were initiated immediately after washing the cells twice with cold thymidine at 100 μg/ml and plating was done in cold thymidine at 10 μg/ml.

Statistical Analysis. All statistical evaluations were performed using computer software; Instat version 2.02 or GraphPad Prisma version 1.0 (GraphPad Software, San Diego, CA). The significance of the differences between groups was evaluated by analysis of variance followed by two-tailed Student's t test. A two-way analysis of variance test was used to determine statistical significance of treatment on cell cycle. Results from survival experiments were analyzed using a log-rank nonparametric test and expressed as Kaplan-Meier survival curves.

Results

Pretreatment with rTGF-β_1 In Vivo Delays Progenitor Recovery in Mice Subsequently Treated with a Sublethal Dose of 5-FU. The results presented in Fig. 1 demonstrate that although pretreatment with rTGF-β_1 initially decreased the formation of all progenitors by day 9 after injection with a sublethal dose of 5-FU, the later recovery that occurs by the end of the rebound phase (days 13–15) exceeded the rebound levels observed for mice that received only 5-FU. This effect was significant at the p <0.001 level for both CFU-c and HPP-CFC, with CFU-GEMM numbers also showing a trend toward an increase.

Pretreatment of Normal Mice with TGF-β_1 and TGF-β_2 Does

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[1] *Abbreviations used in this paper:* BMT, BM transplant; CFU-c, CFU-culture; CFU-GEMM, CFU-granulocyte, erythroid, megakaryocyte, macrophage; DXR, doxorubicin hydrochloride; Epo, erythropoietin; 5-FU, 5-fluorouracil; HPP-CFC, high-proliferative potential–colony-forming cell; NMS, normal mouse serum; r, recombinant.
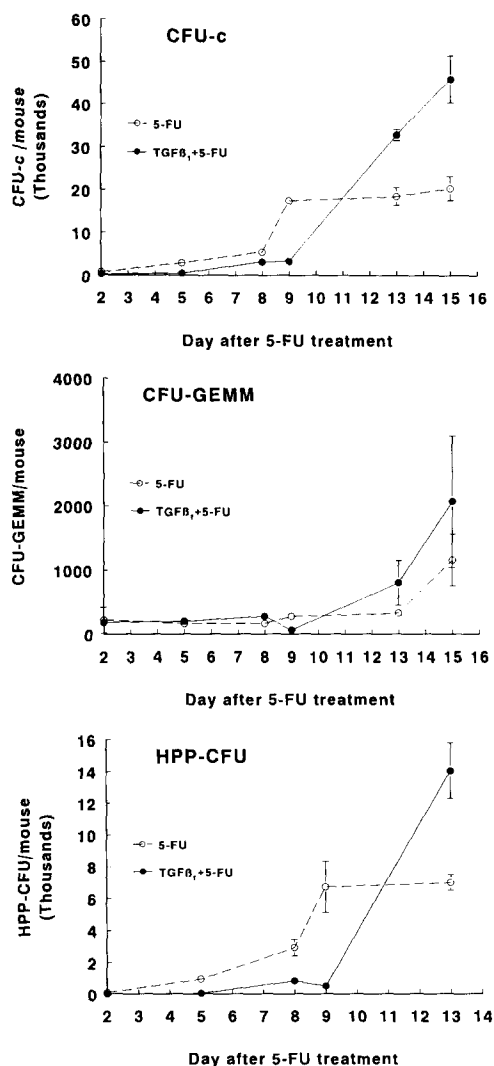
Figure 1. Effect of pretreatment with rTGF-β_1 on 5-FU–induced recovery of myeloid cells. Mice (n = 10) were injected intraperitoneally with HBSS + 0.1% NMS or 2.5 µg rTGF-β_1 twice daily on days –4–0. On day 0, all mice were injected intravenously with 5-FU (150 mg/kg). Groups of mice were killed at various times thereafter, BM collected, and colony assays performed. The data are presented as the mean ± SD for three replicates of a pooled cell population, and the total CFU/mouse (total CFU from paired femurs and tibias) was calculated based on their frequency multiplied by the total cellularity per mouse.
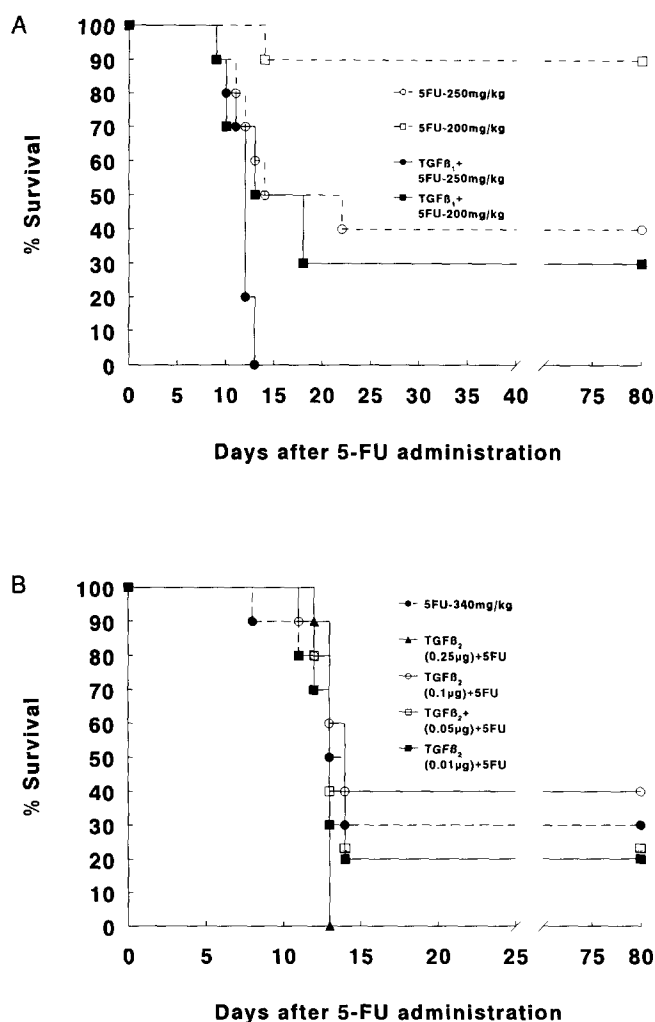
Figure 2. Effect of rTGF-β_1 and rTGF-β_2 pretreatment on 5-FU–induced lethality. A shows an experiment where mice (n = 10) were injected intraperitoneally twice a day for 5 d consecutively with the previously established optimal dose of rTGF-β_1 (2.5 µg/dose) or with diluent (HBSS + 0.1% NMS). 1–2 h after the last injection of rTGF-β_1, mice were injected with high sublethal (200 or 250 mg/kg) doses of 5-FU. B shows an experiment where mice (n = 10) were injected intraperitoneally with various dilutions of rTGF-β_2 (0.01–0.25 µg/dose) diluted in citrate buffer. Control mice were injected with diluent only. 1–2 h after last injection of rTGF-β_2 mice were injected with a lethal dose (340 mg/kg) of 5-FU. All mice were then monitored for toxicity and survival.

Not Protect Mice against Lethal Doses of 5-FU. Because of the enhanced late hematopoietic recovery noted in the results presented in Fig. 1, we speculated that rTGF-β pretreatment might protect enough CFU progenitors from the toxic effects of higher doses of 5-FU that these mice might survive an otherwise lethal injection of 5-FU. However, mice that were pretreated twice daily with TGF-β_1 (2.5 µg/dose) for 5 d consecutively were actually less resistant to high doses of 5-FU (200 mg/kg 5-FU [LD10] vs. TGF-β_1 + 200 mg/kg 5-FU [LD60], p <0.01, and 250 mg/kg 5-FU [LD60] vs. TGF-β_1 + 250 mg/kg 5-FU [LD100], p <0.01; Fig. 2 A). Pretreatment with TGF-β_2 also was found to potentiate the toxicity

of high (340 mg/kg) dose [LD70] 5-FU such that no mice survived (Fig. 2 B). Lower daily doses of TGF-β_2 did not potentiate 5-FU–induced toxicity, but neither was protection induced. A series of additional studies using pretreatment with either TGF-β_1 or TGF-β_2 demonstrated only increased toxicity, not protection (data not shown).

Reversal of TGF-β_1–potentiated 5-FU–induced Toxicity by BM Transplant (BMT). Myelotoxicity as the cause of death in mice treated with TGF-β_1 and 5-FU was confirmed by pretreating mice with TGF-β_1 for 5 d consecutively before a bolus injection of high dose (250 mg/kg) 5-FU [LD50] on day 0 and subsequent syngeneic BMT (10^7 cells) on days 2,
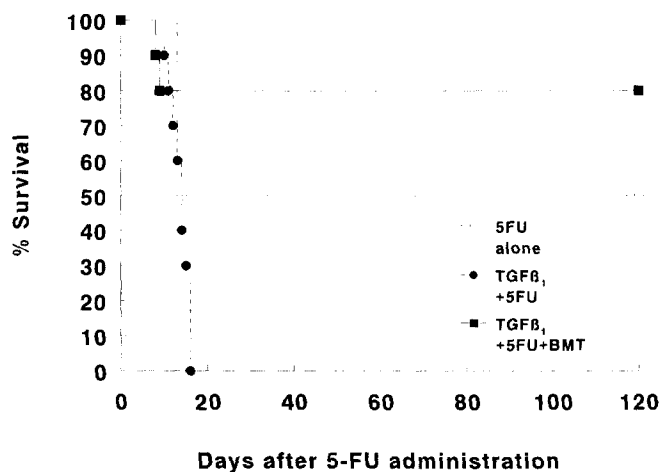
Figure 3. Effect of BM transfer on survival of mice treated with rTGF-β_1 and a high sublethal dose of 5-FU. Mice ($n = 10$) were injected intraperitoneally with (2.5 μg/dose) or rTGF-β_1 for 5 d consecutively followed by a bolus injection of 5-FU (250 mg/kg) 2 h after the last injection of rTGF-β_1. On days 2, 3, and 4 some mice were given intravenous syngeneic BMT (10^7 cells). All mice were then monitored for toxicity and survival.

3, and 4 (Fig. 3). As expected from the data shown previously in Fig. 2, none of the mice treated with both TGF-β_1 and 5-FU survived (5-FU alone [LD$_{50}$] vs. TGF-β_1 + 5-FU [LD$_{100}$], $p < 0.01$). However, 80% of the mice treated with TGF-β_1 and 5-FU followed by BMT survived (TGF-β_1 + 5-FU [LD$_{100}$] vs. TGF-β_1 + 5-FU + BMT [LD$_{20}$] $p < 0.01$), confirming that the increased toxicity of 5-FU by TGF-β_1 was predominantly hematologic.

TGF-β Protects Some Progenitors and Mature Myeloid Cells from High Doses of 5-FU. As previously noted, mice that were pretreated with TGF-β_2 for 5 d consecutively exhibited

an enhanced toxicity (LD$_{100}$ 0.25 μg/dose and LD$_{80}$ at 0.1 μg/dose of TGF-β_2) after high dose (340 mg/kg) 5-FU [LD$_{60}$]. However, analysis of progenitor levels (Fig. 4, A and B) revealed a significant ($p < 0.001$) increase in the number of CFU-GEMM and CFU-c on days 7 and 11 in mice pretreated daily with 0.25 μg of TGF-β_2 as compared with mice treated only with 5-FU (CFU-GEMM at day 7: 4-fold increase, $p < 0.001$, and at day 11: 7.5-fold increase, $p < 0.001$; and CFU-c at day 7: 3-fold increase, $p < 0.001$, and at day 11: 12-fold increase, $p < 0.001$). Furthermore, the lower daily dose of TGF-β_2 (0.1 μg), while less efficient than a higher dose (0.25 μg) in facilitating the recovery of CFU-c at an earlier time point (day 7: 280 ± 8 vs. 620 ± 13), was equally effective to the higher dose in enhancing CFU-c at a later time point (day 11: 5010 ± 322 vs. 5365 ± 209). The 0.1 μg daily dose also was efficient in protecting CFU-GEMM on day 11 as compared with mice treated only with 5-FU (185 ± 19 vs. 40 ± 3, $p < 0.001$). This recovery of CFU by day 11 in TGF-β_2–pretreated mice was substantial in that the number of CFU-c was ~30% of normal for either dose of TGF-β_2, whereas the number of CFU-GEMM was 29 and 18% of normal for TGF-β_2 doses of 0.25 and 0.1 μg, respectively. These levels of CFU-c and CFU-GEMM were 12-fold and 7.5-fold, respectively, higher in the TGF-β–pretreated mice than in those that only received 5-FU. Similarly, an analysis of total BM cellularity (Fig. 4 C) showed that mice pretreated with TGF-β_2 exhibited some rebound by day 11 (1.5 × 10^6 and 1.6 × 10^6 cells) when given daily TGF-β_2 doses of 0.25 and 0.1 μg, respectively, as compared with 0.4 × 10^6 cells in mice treated only with 5-FU.

Mice That Are Pretreated with TGF-β_2 before High Dose 5-FU Are More Easily Reconstituted with BM Cells Than Mice Only Given 5-FU. The results presented in Fig. 4 demonstrate that pretreatment with TGF-β did protect some progenitors, suggesting such protection did not translate to ex-
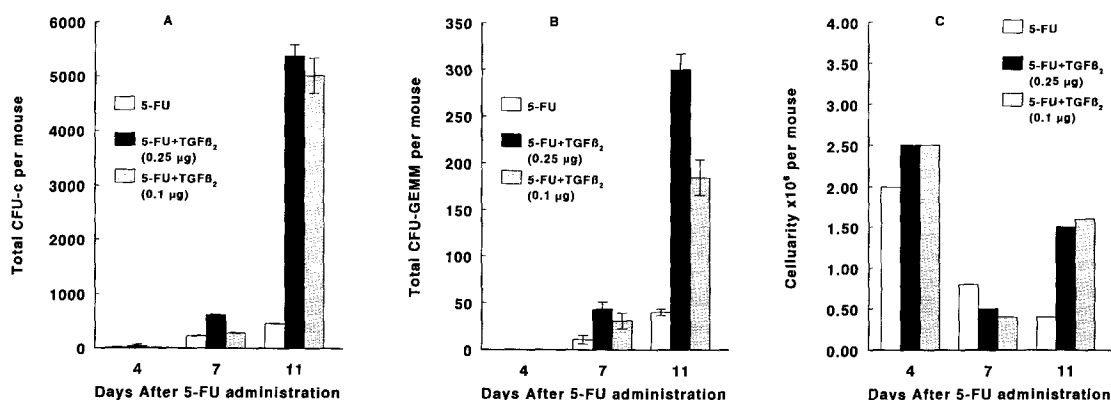

Figure 4. Effect of pretreatment with rTGF-β_2 on progenitors in mice subsequently treated with a lethal dose (340 mg/kg) of 5-FU. Mice ($n = 10$) were injected intraperitoneally with 0.25 or 0.1 μg of rTGF-β_2 twice daily on days −4–0. On day 0, 2 h after the last injection of rTGF-β_2, all mice were injected intravenously with 5-FU. Groups of mice were killed and BM was obtained at various times after the injection of 5-FU. The total number of BM cells obtained from the paired femurs and tibias of each mouse was determined before the colony assay was performed and read on day 14. The data are presented as the mean ± SD for three replicates of a pooled cell population and the total CFU/mouse was calculated based on their frequency multiplied by the total cellularity per mouse.
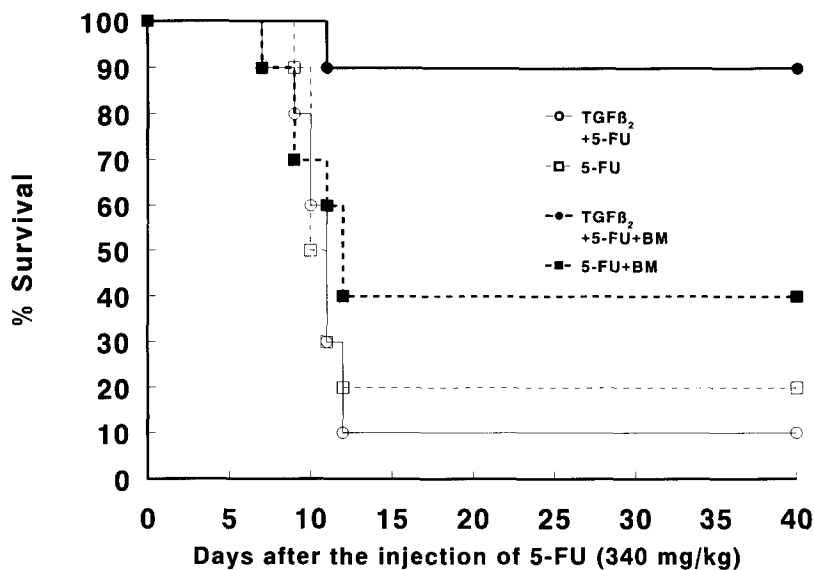
Figure 5. Effect of BMT on survival of mice treated with fTGF-β_2 and a lethal dose of 5-FU. Mice (n = 10) were injected intraperitoneally with (0.1 μg/dose) of fTGF-β_2 or diluent for 5 d consecutively followed by a bolus injection of 5-FU (340 mg/kg) 2 h after the last injection of fTGF-β_2. On days 1, 3, 6, and 9 some mice were given an intravenous transfer with a suboptimal number (10^6) of syngeneic BM cells. All mice were then monitored for toxicity and survival.

tended survival, because the number of progenitors protected fell below a critical threshold. To test this hypothesis, mice were treated with TGF-β_2 (0.1 μg/dose) or vehicle control (citrate buffer) for 5 d consecutively followed by a bolus injection of an acutely lethal [LD_{80}] dose of 5-FU (340 mg/kg). Some mice then received a suboptimal BMT (10^6) on days 1, 3, 6, and 9 (Fig. 5). Reconstitution of mice treated only with 5-FU [LD_{80}] by suboptimal BMT did not significantly increase in survival [LD_{60}] (p >0.1), whereas the group that had been pretreated with TGF-β_2 before 5-FU [LD_{90}] exhibited a significantly improved (p <0.001) survival of 90% [LD_{10}] after suboptimal BMT.

In Vivo Administration of TGF-β_1 or TGF-β_2 Protects Regenerating Progenitor Cells from 5-FU In Vitro. The results presented in Figs. 1, 4, and 5 demonstrate that TGF-β spares some critical short-term repopulating progenitors in mice from the acutely toxic effects of 5-FU, suggesting that these cells

are more resistant to direct killing by 5-FU. This hypothesis was confirmed by treating mice with fTGF-β_1 (2.5 μg/mouse twice daily) (experiment 1) or fTGF-β_2 (0.1 μg/mouse once a day) (experiment 2) on days 7-11 of the subsequent hyperproliferative recovery phase that follows a sublethal dose (150 mg/kg) of 5-FU. Progenitors for CFU-c (Fig. 6 *A*) and CFU-GEMM (Fig. 6 *B*) obtained from mice that had received fTGF-β_1 or TGF-β_2 in addition to 5-FU were more resistant to the direct toxic effects of 5-FU in vitro. Specifically the percent decrease in total CFU-c number after exposure to 10 μg/ml or 25 μg/ml 5-FU in vitro was 70 and 93%,

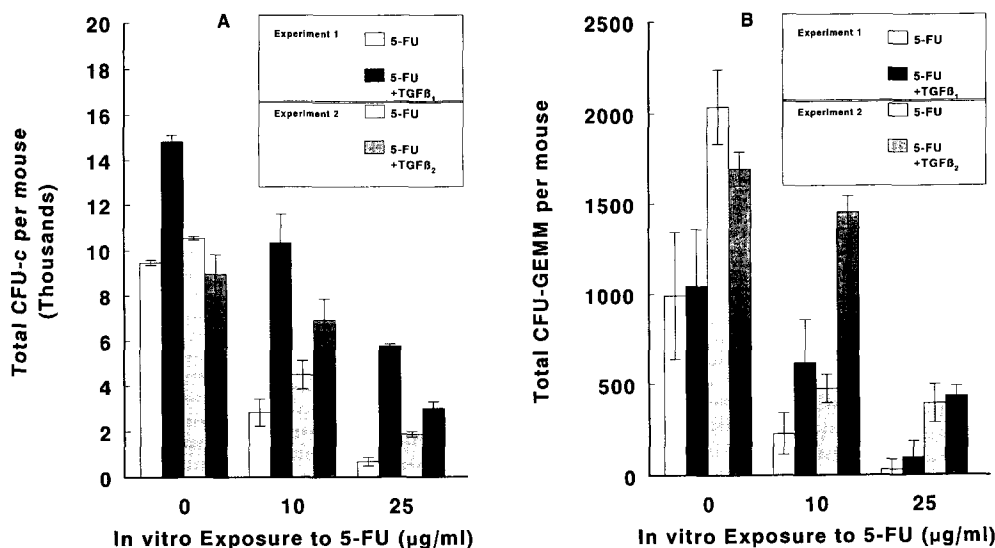**Figure 6.** Effect of fTGF-β_1 and fTGF-β_2 administration in vivo on subsequent sensitivity of progenitor cells to 5-FU in vitro. Mice (n = 5) were injected intravenously on day 0 with 150 mg/kg of 5-FU. In experiment 1, some mice received 2.5 μg/mouse of fTGF-β_1, whereas in experiment 2, some mice received 0.1 μg/mouse of fTGF-β_2, twice daily on days 7-11 and once in the morning of day 12. 2 h after the last injection of fTGF-β, all mice were killed and the BM was harvested. BM samples from each group were then treated for 2 h in vitro with media only, 10 μg/ml of 5-FU and 25 μg/ml of 5-FU. The cells were then washed, recounted, and plated in triplicate for CFU-formation. The data are presented as the mean ± SD for three replicates of a pooled cell population and the total CFU/mouse was calculated based on obtained frequency multiplied by the total cellularity per mouse obtained after in vitro treatment.

Table 1. *Effect of rTGF-β₂ Administration In Vivo on Subsequent Sensitivity of HPP-CFC Progenitor Cells to 5-FU In Vitro*

In vivo treatment	In vitro treatment	Number of HPP-CFC recovered	Percent decrease from control mice (media only)	Fold increase in HPP-CFC formation*
5-FU	Media only	576 ± 77	N/A‡	N/A
	5-FU (10 μg/ml)	132 ± 46	77	N/A
	5-FU (25 μg/ml)	115 ± 40	80	N/A
5-FU rTGF-β₂	Media only	682 ± 48	N/A	0.2
	5-FU (10 μg/ml)	544 ± 91	20	3.1
	5-FU (25 μg/ml)	398 ± 99	42	2.5

Mice (n = 5) were injected intravenously on day 0 with 150 mg/kg of 5-FU. Mice received 0.1 μg of rTGF-β₂, BID on days 7–11, and once on the morning of day 12. 2 h after the last injection of rTGF-β₂, all mice were killed and the BM cells were harvested. BM samples from each group were then treated for 2 h in vitro with media only, 10 μg/ml of 5-FU, and 25 μg/ml of 5-FU. The cells were then washed, recounted, and plated in triplicate for HPP-CFC formation. The data are presented as the mean ± SD for three replicates of a pooled cell population and the total CFU/mouse was calculated based on obtained frequency multiplied by the total cellularity per paired femurs and tibias obtained after in vitro treatment. This is a representative experiment of two performed.
* Value indicates fold increase in HPP-CFC formation in mice treated in vivo with 5-FU (150 mg/kg) and rTGF-β₂ compared with 5-FU alone.
‡ N/A, not applicable.

respectively, in mice that had not received rTGF-β₁ in vivo, but only 30 and 61%, respectively, in those that had received rTGF-β₁. Mice receiving TGF-β₂ also were more resistant to 5-FU in vitro (23 and 67% decrease at doses of 10 and 25 μg/ml) as compared with a decrease of 57 and 82% for mice that had not received TGF-β₂. Similar effects were observed for both TGF-β₁ and TGF-β₂ on progenitors for CFU-GEMM.

In addition, mice that received 5-FU plus TGF-β₁ or TGF-β₂ exhibited a considerably greater number of all types of CFU at the time of marrow harvest on day 12 than did mice that received only 5-FU. This effect, combined with the enhanced resistance to 5-FU in vitro, resulted in a 3.7-fold (TGF-β₁) and 1.5-fold (TGF-β₂) increase in the number of CFU-c and a 2.7-fold (TGF-β₁) and 3.1-fold (TGF-β₂) increase in the number of CFU-GEMM that remained after

Table 2. *Effects of rTGF-β₂ on Cycling of Progenitor Cells in Mice Previously Treated with a Sublethal Dose of 5-FU*

Progenitor type	Experimental treatment	Total no. of progenitor cells in S-phase	Percent reduction in S-phase cells by TGFβ₂
CFU-c	5-FU	19,787	–
	5-FU + TGFβ₂	400	>99
	Diluent	11,836	–
	Diluent + TGFβ₂	8,433	29
CFU-GEMM	5-FU	4,667	–
	5-FU + TGFβ₂	1,467	69
	Diluent	1,563	–
	Diluent + TGFβ₂	613	61

Donor mice (n = 3) were injected intravenously with 5-FU (150 mg/kg). From day 9 to 13 mice were treated intraperitoneally with rTGF-β₂ (0.1 μg/dose) or citrate buffer (diluent). 2 h after the last injection of rTGF-β₂, all mice were killed and BM cells were harvested. For cell cycling analysis of various progenitors, BM cells (2 × 10⁶/ml) were cultured in the presence of [³H]TdR (100 μCi/ml) or (cold) TdR (control) for 30 min at 37°C. After treatment, BM cells (10⁵) were plated for colony forming assays. The total number of progenitor cells in S-phase was calculated by subtracting the mean number of colonies obtained after in vitro treatment with [³H]TdR from those treated with (cold) TdR, and multiplied by the total cellularity of paired donors' femurs and tibias.

exposure to 10 µg/ml of 5-FU in vitro for mice treated with 5-FU plus TGF-β vs. 5-FU alone, with similar effects noted at an in vitro concentration of 25 µg/ml 5-FU for TGF-β_1 or TGF-β_2. This protection by TGF-β also extends to the more primitive HPP-CFC progenitors since the number surviving after exposure to 10 µg/ml or 25 µg/ml 5-FU in vitro was increased 4.1-fold and 3.5-fold, respectively, in mice that had received TGF-β_2 in vivo as compared with those that did not receive TGF-β_2 (Table 1). These results demonstrate that TGF-β renders various progenitor cells more resistant to direct killing by 5-FU.

Treatment of Mice with TGF-β_2 during the Post-5FU Hyperproliferative Phase Decreases Cycling of CFU Progenitors. The experiment shown in Table 2 demonstrates that TGF-β_2 treatment during the hyperproliferative phase of hematologic recovery after 150 mg/kg 5-FU reduces the total number of CFU-c progenitors in S-phase by 19,387 (>99%) and thereby protects them from killing by high sp act radioactive thymidine. In contrast, in normal mice, which have fewer total CFU-c progenitors in S-phase, the administration of TGF-β_2 reduces the total number of CFU-c in S-phase by 3,403, or only 29%. The results for CFU-GEMM progenitors were
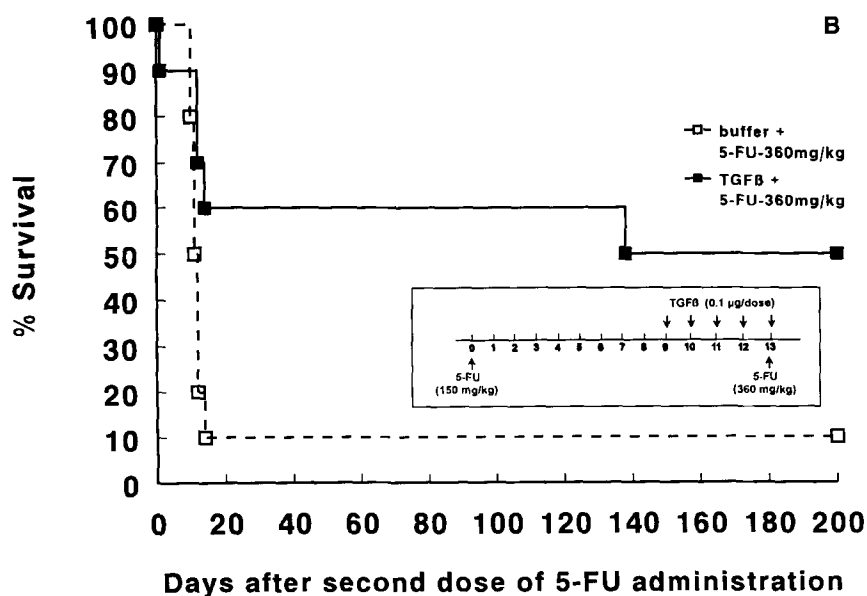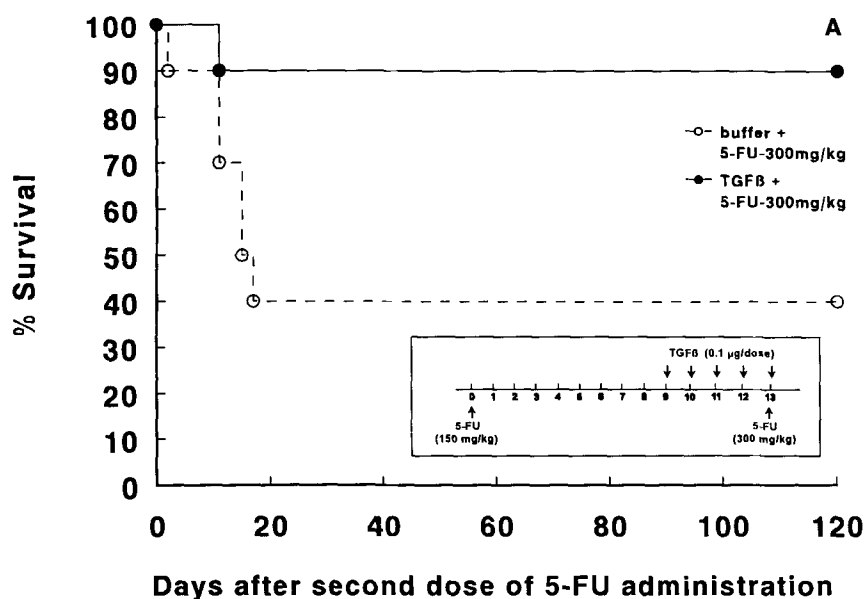

Figure 7. Effect of rTGF-β_2 administered during the late hyperproliferative phase of mice rendered leukopenic by 5-FU on subsequent sensitivity of progenitor cells to 5-FU in vivo. Mice ($n = 10$) were injected intravenously on day 0 with 150 mg/kg of 5-FU. On days 9–13, mice were treated twice a day with rTGF-β_2 (0.1 µg/dose) or with diluent only. On day 13, 2 h after last injection, a second high dose of 5-FU (A, 300 mg/kg; or B, 360 mg/kg) were administered. All mice were then monitored for toxicity and survival.

similar with TGF-β_2 reducing the total number by 3,200 in mice recovering from 5-FU, but only by 950 in normal mice (p <0.01).

Treatment of Mice with TGF-β_2 during the 5-FU–induced Bone Marrow Hyperproliferative Phase Protects Against High Doses of 5-FU. Because TGF-β protects recovering marrow progenitor cells from both 5-FU and [^{3}H]thymidine, studies were performed to determine whether mice could be protected against a subsequent higher dose of 5-FU in vivo. Therefore, TGF-β_2 was administered on days 9–13 (late recovery phase) after initial exposure to 150 mg/kg 5-FU, and these mice were then rechallenged with high doses (300 or 360 mg/kg) of 5-FU (Fig. 7). Mice pretreated with low dose 5-FU plus TGF-β_2, followed by high doses of 5-FU (300 mg/kg [LD$_{60}$]) (Fig. 7 *A*) and 360 mg/kg [LD$_{90}$] (Fig. 7 *B*) exhibited an increased survival rate of 90%, p <0.05 and 60%, p <0.05, respectively, as compared with the mice that did not receive the TGF-β_2 treatment (Fig. 7, *A* and *B*). This protective effect was lost if the TGF-β_2 was administered on days 5–8 (early recovery phase) after 150 mg/kg 5-FU (data not shown).

TGF-β_1 and TGF-β_2 Protect Mice against Lethal Doses of DXR. The effects of rTGF-β on lethality induced by high doses of the anthracycline chemotherapeutic drug DXR also were studied. Surprisingly, as the data from a representative experiment show, pretreatment with TGF-β_1 (Fig. 8 *A*) or TGF-β_2 (Fig. 8 *B*) for 4 d protected mice in a dose-dependent manner from an acutely lethal dose (16 mg/kg) of DXR (TGF-β_1: 2.5 μg/day, 80% survival, p <0.01; TGF-β_1: 0.5 μg twice daily, 20% survival, p <0.01; and TGF-β_2: 0.5 μg twice daily, 70% survival, p <0.01). Because DXR is only moderately myelosuppressive in mice, we also investigated whether the mechanism of TGF-β-mediated protection was exclusively hematopoietic (Fig. 8 *C*). It is interesting to note that the mortality of mice treated with a lethal dose of DXR was not reversed by a subsequent BM transfer (10^7 cells injected 24 h after DXR administration), suggesting that the mechanism of TGF-β protection is at least partially nonhematopoietic.

Discussion

Myelosuppression is a major dose-limiting toxicity of many widely used cancer chemotherapeutic drugs (1, 2). Both positive and negative regulators of hematopoiesis have been proposed as potential chemoprotective agents (12). Positive regulators of hematopoiesis (e.g., CSFs, IL-1, Epo, and stem cell factor) are known to be useful in this regard (9, 12), while the application of negative regulators (e.g., TGF-β) of hematopoiesis as potential protective agents is not yet clear (30). Negative regulators might temporarily protect vital hematopoietic stem/progenitor cells in the BM by inhibiting their ability to cycle during and shortly after the delivery of chemotherapy thereby allowing either acute or chronic dose intensification (31). This hypothesis is supported by reports that TGF-βs can reversibly inhibit the proliferation of CFU-S (25) and primitive hematopoietic progenitors in vitro (32), and arrest a variety of cells in middle to late G$_1$ phase of the cell
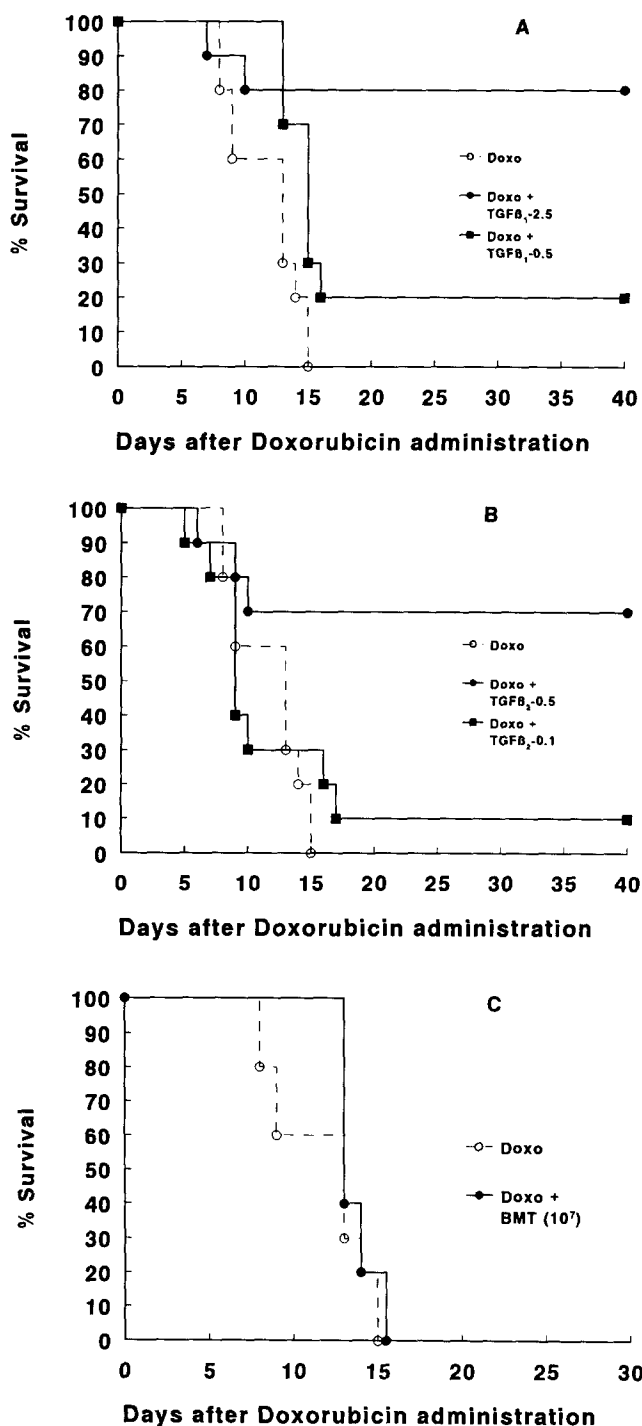

Figure 8. Chemoprotective effects of rTGF-β_1 and TGF-β_2 for DXR. Groups of mice (n = 10) were injected intraperitoneally twice a day for days: −4–0 with various doses of rTGF-β_1 (*A*, 0.5–2.5 μg/dose) or rTGF-β_2 (*B*, 0.1–0.5 μg/dose). Control groups were injected with diluents. 1–2 h after the last TGF-β injection mice were challenged with a single intraperitoneal injection (14 mg/kg) of DXR. Additionally mice injected with DXR only were also given a single injection of BM cells (10^7) (*C*). All mice were then monitored for toxicity and survival.

cycle (33–35). The investigation of TGF-β as a protective agent stems from observations that TGF-β_1 in the BM microenvironment may play a role in the regulation of hematopoiesis (36), TGF-β_1 and TGF-β_2 potently inhibit the proliferation of murine progenitor cells (11, 19), and that TGF-βs synergize in vitro with GM-CSF to increase murine granulopoiesis (36) and the number of CFU-GM (37, 38). Overall, these findings suggest that TGF-βs alter both proliferation and differentiation of hematopoietic progenitor cells.

The present studies were initiated to determine whether transient inhibition of CFU progenitor proliferation by TGF-β could be exploited to protect bone marrow stem/progenitor cells from destruction by chemotherapeutic drugs, thereby allowing dose intensification of chemotherapy. Initial experiments confirmed that pretreatment with TGF-β_1 potentiated the rebound that follows administration of 150 mg/kg 5-FU, a regimen widely used to increase the number of cycling progenitor cells (39–42). However, there was also an initial delay in hematopoietic recovery of TGF-β–pretreated mice suggesting a lower proliferative response of any protected stem/progenitor cells to the positive regulatory signals that are produced physiologically in response to myelosuppression. This effect could be due to an inhibition by TGF-β of stem/progenitor cell entry into cell cycle. This point may be important since stem and progenitor cells are heterogeneous, with some in G_0, some slowly entering the cycle (long lag of G_1 phase), and some in cycle (43). Despite the early inhibitory effect of rTGF-β_1 on hematopoietic progenitors, the later enhanced effect suggests that the apparent inhibition of proliferation also resulted in protection of some progenitors from 5-FU–mediated toxicity.

In spite of the ability of TGF-β to allow an enhanced rebound after 5-FU, pretreatment with either TGF-β_1 or TGF-β_2 failed to protect normal mice against high sublethal or lethal doses of 5-FU, and actually rendered them more susceptible to lethality by 5-FU. This increased toxicity was hematological since subsequent BM transfer largely reversed the effect, suggesting that temporary inhibition of the function of a critical short-term repopulating subset of progenitor cells might prevent a timely recovery in 5-FU–treated mice, allowing the development of septicemia and death from infection. This hypothesis was supported by data showing that there was a significant increase in the number of total BM cells, CFU-c, and CFU-GEMM in mice treated with TGF-β plus high dose 5-FU vs. those treated with high dose 5-FU alone, although all mice in both groups died by around day 15. Hematologic protection by TGF-β was confirmed when mice pretreated with TGF-β_2 before high dose 5-FU (340 mg/kg) were salvaged by a suboptimal number of bone marrow cells, suggesting that TGF-β did protect some progenitors from 5-FU–induced toxicity in normal mice, but their number and/or function was inadequate to completely protect mice during the critical time period when septicemia develops.

It is important to note that TGF-β does not potentiate the cell cycle–dependent toxic effects of 5-FU since progenitor cells obtained from mice given a sublethal dose of 5-FU followed by TGF-β were more abundant and more resistant

to in vitro treatment with increasing doses of 5-FU. Thus, TGF-β altered the number of BM progenitors, and also their physiological status, suggesting that the effectiveness of TGF-β as a chemoprotective agent might be at least partially dependent on the cycling status of progenitors. This was confirmed by studies showing that TGF-β treatment dramatically reduced the number of CFU progenitors in S-phase and prevented their killing by high sp act [^{3}H]thymidine, an effect that has been reported by Cashman et al. (32) in vitro for human progenitors in long-term culture-adherent assays, and by Migdalska et al. (25) for in vivo treatment of recovering marrow in mice. This effect was significantly more pronounced in mice recovering from 5-FU than in normal mice, and was greater for CFU-c than CFU-GEMM progenitors, supporting the hypothesis that the protection of the mature progenitors is especially critical for protection during the critical 7–10-d period after subsequent exposure to high dose 5-FU. These results speak most directly to protection of short-term marrow repopulating activity (STMRA). However, the survival curves do extend to 200 d (Fig. 7 B) suggesting that such protection also extends to the long-term marrow repopulating cells (LTMRC) reportedly spared by 5-FU (44).

Thus, protection of existing progenitors in normal mice by TGF-β is not sufficient and there is a requirement for an increased number of progenitors for successful chemoprotection. For optimal protection against cycle-dependent chemotherapeutic agents, stem/progenitor cells needed to be positively stimulated (e.g., with a low sublethal dose of 5-FU) to cause an increase in proliferation before exposure to TGF-β. Therefore, more progenitors are cycle-arrested by TGF-β, and more can quickly reenter cycle after administration of cell cycle–dependent chemotherapeutic agents.

DXR is a DNA intercalating agent that blocks topoisomerase II and is myelosuppressive in humans (1, 45). In confirmation of our earlier preliminary findings (46), we have shown that, unlike for 5-FU, the repeated preadministration of TGF-β_1 or TGF-β_2 to unstimulated mice protects some mice from lethal doses of DXR. Although myelosuppression is one of the dose-limiting toxicities associated with the clinical use of DXR, cardio- and intestinal toxicity are also evident (47, 48), whereas liver tissue is relatively resistant (47). In mice, the cardioprotective drug ICRF-187 (ADR-529) is able to decrease DXR-related toxicity in mice (49). TGF-β_1 also has been found to ameliorate TNF-induced cardiotoxicity when given before or immediately after ischemic injury (50), and to inhibit the proliferation of intestinal epithelial cells suggesting that it might protect against cell cycle–dependent gut toxicity of DXR (25, 48). In our studies, the acutely toxic effects of DXR in mice were not reversed by BMT suggesting that TGF-β–mediated protection against DXR is at least partially nonhematologic. Thus, the cell cycle–dependent protective effects of TGF-βs are not limited to the hematopoietic system since TGF-β_3 has been shown to decrease proliferation of epithelial cells thereby decreasing 5-FU–induced mucositis (51).

In summary, we have demonstrated that TGF-β can protect mice against lethal doses of 5-FU and DXR. TGF-β_3 has been reported to also protect against in vitro toxicity by

4-hydroxycyclophosphamide (52), and TGF-β_2 may have some protective effects against cyclophosphamide in vivo (46). Overall, these results suggest a possible wide spectrum of action by TGF-β as a chemoprotective agent, possibly through several different mechanisms. Our results suggest that treatment with TGF-β before chemotherapy protects some stem/progenitor cells and increases the number that might then be available for restimulation by positive hematopoietic regulators after chemotherapy. Alternatively, pretreatment with positive regulators such as the CSFs, IL-1, IL-3, or SCF followed by TGF-β might also be useful for increasing the number of regenerating progenitor cells and then protecting them from subsequent chemotherapeutic insult. Ultimately, the combined use of positive and negative regulators of hematopoiesis might allow the delivery of more dose-intensive chemotherapy with decreased time to recovery, and lead to increased antitumor effects in patients with tumors that are partially responsive to chemotherapy.

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