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ORIGINAL ARTICLE

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Dose schedule of recombinant murine thrombopoietin prior to myelosuppressive and myeloablative therapy in mice

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Abstract *Purpose*: Thrombopoietin is being investigated as a therapeutic agent for platelet recovery following myelosuppressive therapy. Little information is available, however, on the optimal dose of this drug or the timing of its administration. To develop these data, a series of studies were conducted to examine the effects that time of dosing has on the efficacy and safety of recombinant full-length murine thrombopoietin in murine myelosuppression and murine myeloablation models. *Methods*: For the myelosuppression model, mice were exposed to 500 rad whole-body irradiation in a cesium irradiator and received an intraperitoneal dose of 1.2 mg carboplatin at time 0. For the myeloablation model, mice were exposed to 900 to 950 rad of whole-body irradiation at time 0. *Results*: Significant increases in the number of platelets and red and white blood cells were observed by day 10 in mice that had received a single intravenous bolus dose of recombinant murine thrombopoietin from 2 h before until 4 h after myelosuppressive therapy compared to those had received myelosuppressive therapy alone. In the myeloablation studies, mice treated with 900 rad of whole-body irradiation alone had a mortality rate of 50% compared to 0% for mice that had received recombinant murine thrombopoietin 2 h prior to whole-body irradiation. When the whole-body irradiation dose was increased to 950 rad, the mortality rate of the control mice was 83% compared to 25% for mice that had received recombinant murine thrombopoietin 2 h prior to whole-body irradiation. Dosing with recombinant murine thrombopoietin 7 days prior to whole-body irradiation resulted in a mortality rate greater than or equal to that of control mice. *Conclusions*: These data suggest that pretreatment with thrombopoietin can dramatically affect recovery from myelosuppressive and myeloablative therapy. Therefore, the timing of thrombopoietin administration in relation to the therapy may be critical to the drug's safety and efficacy.

Key words Thrombopoietin · Platelets · Myelosuppression · Myeloablation · Megakaryocytopoiesis

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Introduction

Thrombopoietin (TPO), the ligand for the cytokine receptor c-Mpl, was cloned and characterized by several groups in 1994 [1, 2, 3]. TPO has been shown to regulate platelet production via c-Mpl binding, leading to increases in megakaryocyte numbers and maturation [4, 5]. The plasma levels of TPO are regulated mainly through binding to c-Mpl on platelets and bone marrow cells [4, 5]. Recent in vitro studies have indicated that TPO also acts on early progenitor cells and appears to have multilineage activities [6, 7, 8]. Administration of pharmacological doses of TPO to normal mice and nonhuman primates results in dose-dependent increases in platelet numbers that exceed the increases produced by other cytokines [1, 2, 9, 10]. These observations have led to the clinical development of TPO as a therapeutic agent to counteract the thrombocytopenic effects of cytoreductive therapies such as chemotherapy/radiation treatment for cancer. Single intravenous (i.v.) doses of recombinant full-length human thrombopoietin (rhTPO) have resulted in significant increases in platelet counts in prechemotherapy cancer patients. However, the effects of rhTPO in alleviating the myelosuppressive effects of cancer therapies have been somewhat disappointing [11, 12, 13, 14].

In contrast to the clinical experience to date, TPO has been demonstrated to be efficacious in animal models. In normal experimental animals, the response to TPO appears to be confined to the megakaryocytic lineage. However, in myelosuppressed mice and rhesus monkeys,

TPO has been shown to prevent thrombocytopenia, to accelerate platelet and red blood cell reconstitution, to alleviate neutropenia, and to promote recovery of immature bone cells [9, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24]. These multilineage effects of TPO in myelosuppressive models are consistent with the presence of TPO receptors on immature hematopoietic cells [25]. In several of these previous models, TPO was administered daily during the pancytopenic phase and resulted in supranormal platelet counts [9, 15, 18, 20]. Other groups then reported that a single dose of TPO administered 24 h after whole-body irradiation (WBI) is as effective as daily dosing in alleviating the thrombocytopenia [22, 25, 26, 27, 28, 29], thereby reducing the need for platelet transfusions and accelerating recovery in myelosuppressed nonhuman primates. A delay of more than 4 h in the administration of TPO after myelosuppressive treatment reduces its multilineage effect [28, 29, 30]. Platelet numbers still increase significantly, however, when TPO is administered from 24 h before until 24 h after myelosuppressive therapy.

Because timing appears to be key to preserving TPO's multilineage effects, we sought to determine the maximum therapeutic window for administering recombinant full-length murine TPO (rmTPO) before myelosuppressive and myeloablative therapies were administered. Neelis et al. have performed similar experiments using a radiation-only model [29]. We chose to determine the effects of TPO in a model more relevant to the cytoreductive therapies involving both radiation and chemotherapy. Another factor was concern that pretreatment with TPO might sensitize the immature hematopoietic cells to cytoreductive therapies, similar to the activity that was observed with other growth factors such as granulocyte-macrophage colony stimulating factor (GM-CSF) and with granulocyte colony stimulating factor (G-CSF) [31, 32, 33, 34, 35]. Therefore, our studies were designed to determine the safety and efficacy of dosing mice with rmTPO prior to administration of myelosuppressive therapy. These data may be important in determining the therapeutic window for dosing TPO in future clinical trials.

Materials and methods

Animals

C57BL/6 female mice, approximately 6 to 8 weeks old, were obtained from Charles River Laboratories (Hollister, Calif.). Environmental controls were set to maintain a temperature of 19–25 °C, a relative humidity of 50 ± 20%, and a 12-h light/dark cycle. The animals were group-housed until randomization. There were no known contaminants in the food or water that would be expected to interfere with the objectives of this study. Housing, experiments, and all other conditions were approved by an ethics review committee in accordance with legal regulations at Genentech.

Experimental design

For the myelosuppression model, mice were exposed to 500 rad WBI in a cesium irradiator (J.L. Shepherd and Associates, San Fernando, Calif.) followed by an intraperitoneal (i.p.) dose of 1.2 mg carboplatin (CT) at time 0. For the myeloablation model, mice were exposed to 900 or 950 rad WBI at time 0. For each data point, a random experimental group of at least three mice was used. All peripheral blood cell parameters were collected for individual mice. Following the high-dose WBI in the myeloablative studies, mice were monitored closely and moribund mice were killed and considered a mortality in the analysis.

Test material

CT (Sigma, St. Louis, Mo.) was dissolved in sterile saline to a concentration of 12 mg/ml and administered i.p. in a volume of 0.1 ml. The dose of CT was 1.2 mg/mouse. RmTPO was produced by Chinese hamster ovary cells [1] (Genentech) as a clear solution of 0.44 mg/ml in 10 m*M* Tris, 0.15 *M* NaCl, 0.01% Tween 20, pH 7.4, and was administered i.v. in a volume of 0.1 ml. The dose of rmTPO was 0.1 μg/mouse (5 μg/kg based on a mean body weight of approximately 20 g at the start of the study), which had been efficacious in our earlier mouse studies [36, 37]. The window for administering a single i.v. bolus dose of rmTPO was evaluated first from –72 h until + 24 h and then from –7 days until + 1 day in relation to the myelosuppressive treatment. Multiple dosing regimens of rmTPO were also examined in both the myelosuppressive and myeloablative models.

Hematologic examinations

After CO_2 anesthesia (inhalation to effect) the mice used in the myelosuppression model were killed by cervical dislocation and 0.5 ml blood was collected by cardiac puncture in EDTA tubes on days 10 and 14 following the myelosuppressive treatment. For the myeloablation model, when survival was an endpoint, mice were bled by retro-orbital puncture, alternating the eye that was bled at time-points from 10 to 21 days following the myeloablative treatment. Complete blood cell counts were measured for both models using a System 9000 hematology analyzer (Serono Diagnostics, Allentown, Pa.).

Statistics

The data are presented as means ± SD with the assumption of a normal distribution. The significance of the difference in blood cell counts in all groups compared to control was determined using analysis of variance followed by Fisher's protected least significant difference test. Statistical analysis of survival times in all groups compared to control was done with the Kaplan-Meier test followed by the Logrank (Mantel-Cox) test. Data were considered significant in all cases when $P < 0.05$. All statistical analyses were done using Statview (Abacus Concepts, Berkeley, Calif.).

Results

Myelosuppressive studies

Based on previous studies (data not shown) and work by Neelis et al. [29], it was determined that the mice exhibited a platelet and red blood cell (RBC) nadir between days 10 and 14 following myelosuppressive treatment. White blood cells (WBC) had a much earlier nadir, but WBC counts were still near their nadir on day 10. Therefore, we decided that days 10 and 14 would be optimal times to examine the efficacy of TPO in these models.

In study no. 1, when rmTPO was administered 48 or 72 h prior to myelosuppressive treatment (WBI/CT),

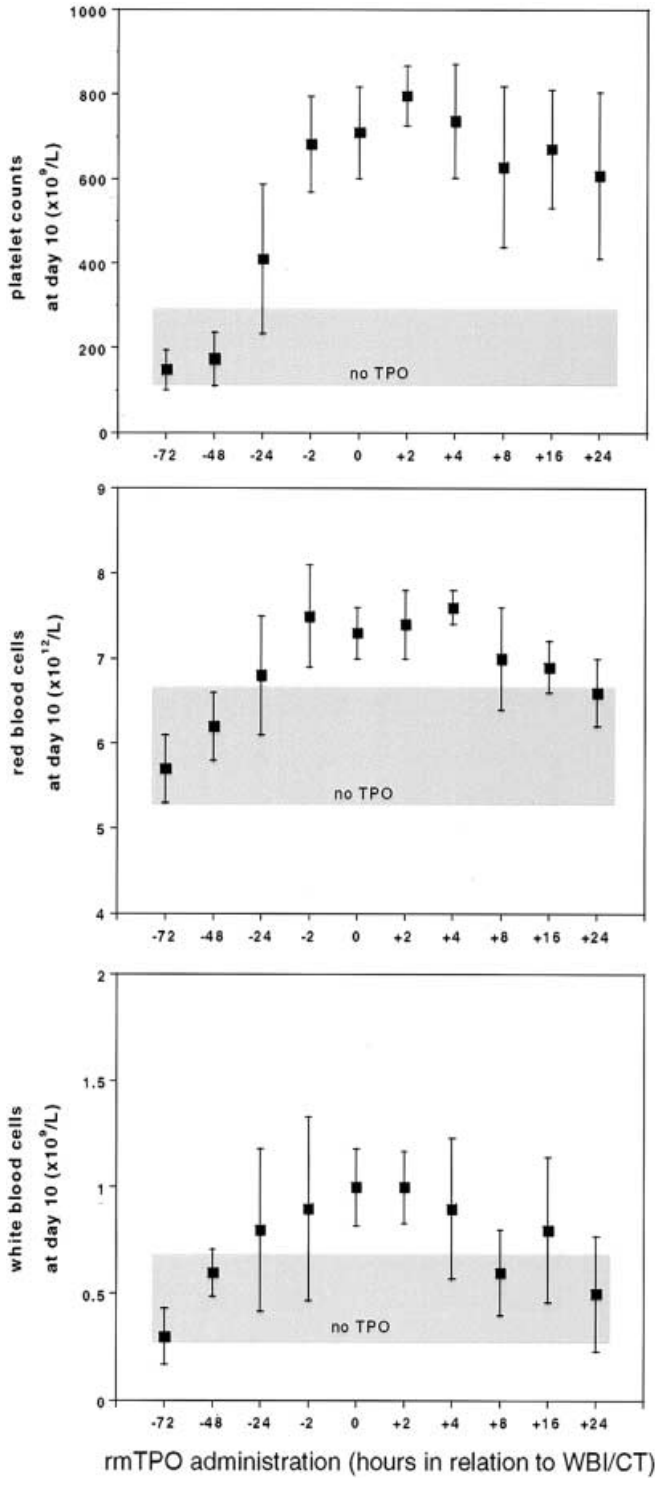

Fig. 1 The effect of the time of i.v. bolus administration of 5 μg/kg rmTPO on peripheral blood cell counts in mice 10 days after myelosuppressive treatment with 500 rad WBI plus 1.2 mg CT (WBI/CT). The data are means ± SD, $n = 8$ per time-point (*shaded area* mean ± SD of eight control mice that were exposed to WBI/CT but received no rmTPO)

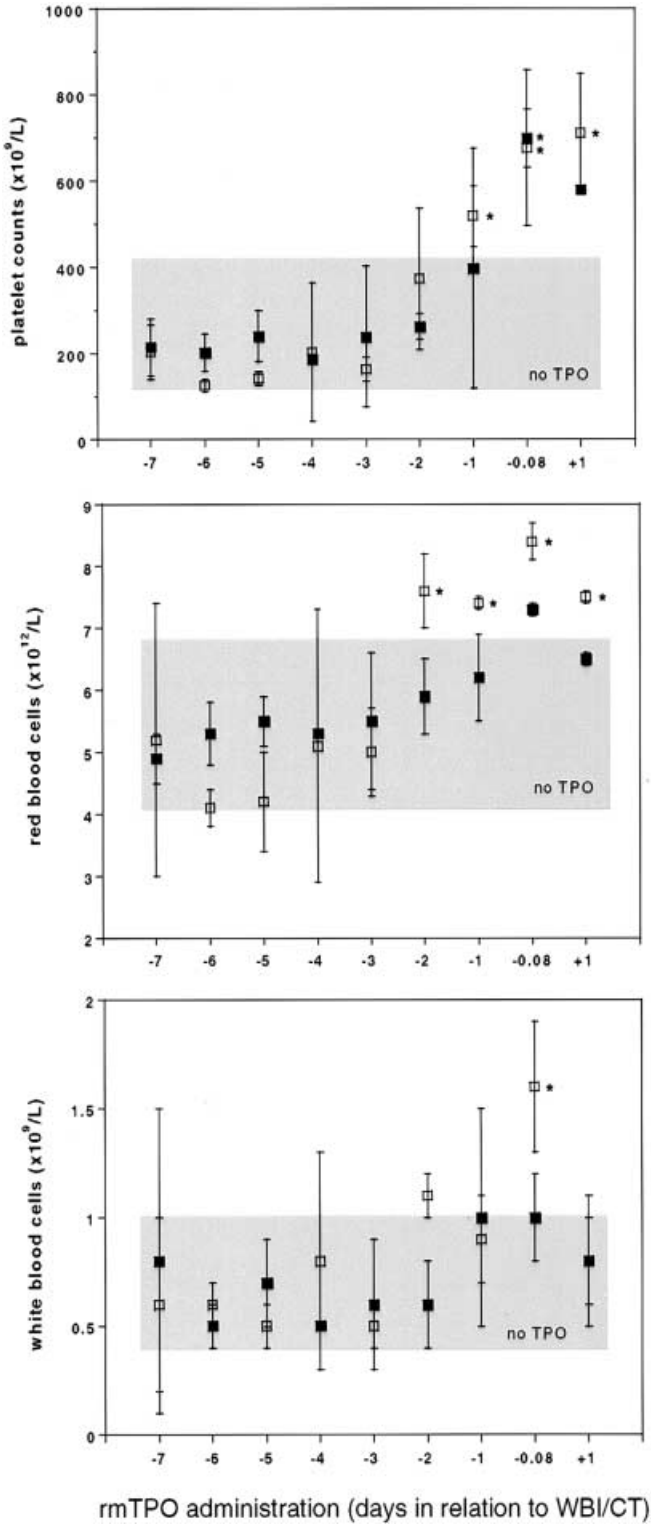

Fig. 2 The effect of the time of i.v. bolus administration of 5 μg/kg rmTPO on peripheral blood cell counts in mice after myelosuppressive treatment with 500 rad WBI plus 1.2 mg CT (WBI/CT). The data are means ± SD (*shaded area* combined mean ± SD from days 10 and 14 of control mice that were exposed to WBI/CT but received no rmTPO, *black squares* day 10, *open squares* day 14). *$P < 0.05$ vs control mice by ANOVA

there was no effect on platelet counts by day 10 compared to the control group (WBI/CT alone; Fig. 1). However, platelet numbers had increased significantly

by day 10 in the groups that received 5 μg/kg rmTPO from 24 h prior to until 24 h after WBI/CT compared to the control group (Fig. 1). By day 10, a significant difference in RBC counts was observed in the groups that had received rmTPO from 24 h prior to until 16 h after WBI/CT, compared to the control group (Fig. 1). There was also a significant difference in WBC counts by day 10 in the groups that had received rmTPO from 2 h prior to until 4 h after WBI/CT compared to the control group (Fig. 1). These results were similar to those reported by Neelis et al., which indicated reduced effectiveness in regenerating platelets and a complete loss in RBC and WBC regeneration when TPO is administered 24 h compared 2 h after treatment [29].

In study no. 2, the pretreatment of rmTPO was examined out to 7 days prior to WBI/CT. By day 14, there

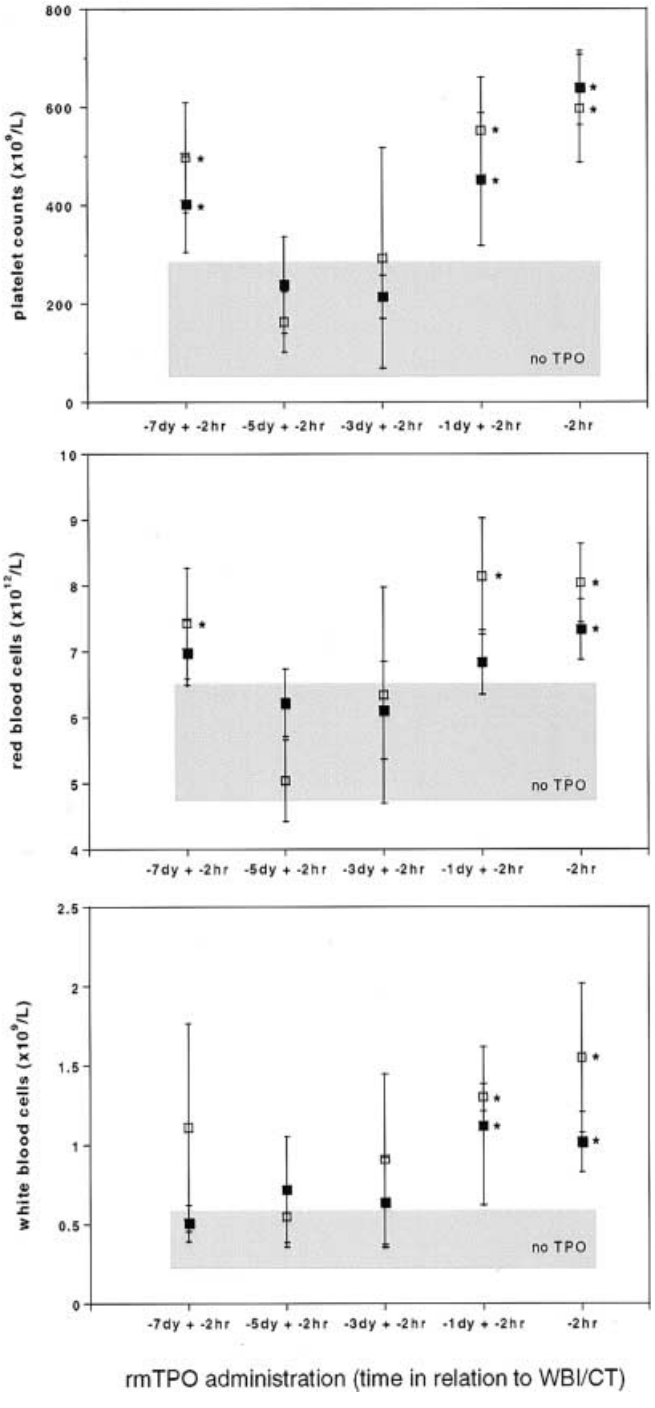

Fig. 3 The effect of the time of administration of two doses (5 μg/kg) of rmTPO on peripheral blood cell counts in mice after myelosuppressive treatment with 500 rad WBI plus 1.2 mg CT (WBI/CT). Single i.v. bolus doses of rmTPO were administered 7, 5, 3, or 1 days and 2 h prior to WBI/CT and were compared with the effect of a single dose of rmTPO administered 2 h prior to WBI/CT. The data are expressed as means ± SD (*shaded area* combined mean ± SD from days 10 and 14 of control mice that were exposed to WBI/CT but received no rmTPO, *black squares* day 10, *open squares* day 14). *$P < 0.05$ vs control mice by ANOVA

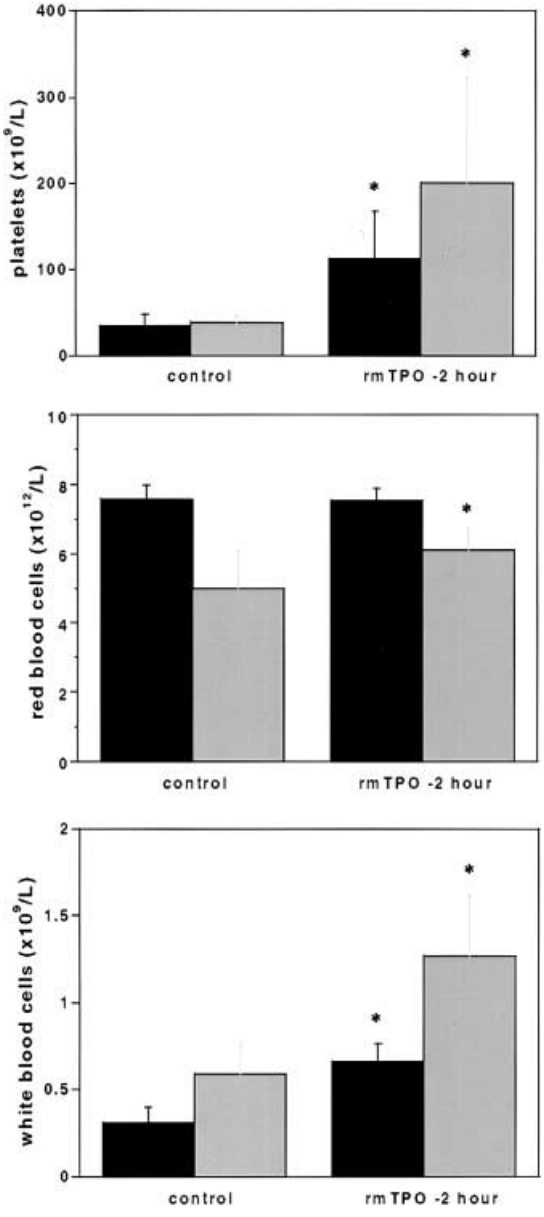

Fig. 4 Peripheral blood cell counts in mice following 900 rad WBI with or without a single i.v. bolus dose of 5 μg/kg rmTPO administered 2 h before WBI. The data are means ± SD (*black bars* day 10, *gray bars* day 14). *$P < 0.05$ vs control mice by ANOVA

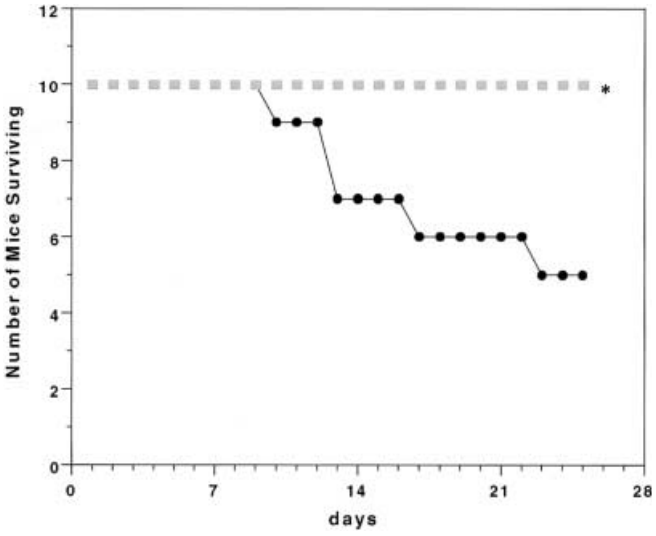

Fig. 5 Mortality of mice following 900 rad WBI with or without a single i.v. bolus dose of 5 μg/kg rmTPO administered 2 h before WBI. The data are the number of mice surviving each day following the WBI on day 1 (*black circles* control mice that received WBI alone, *gray squares* mice that received rmTPO). $*P < 0.05$ improved survival vs control by Kaplan-Meier survival statistics

was significant protection of platelet counts when rmTPO was administered from day +1 until day –1 in relation to the myelosuppressive therapy. However, when rmTPO was administered from day –3 until day –7 the platelet numbers were lower than those of the control group (Fig. 2). Similar to the results observed in study no. 1, significant protection of RBCs occurred by day 14 when rmTPO was administered from day +1 until day –2 in relation to the WBI/CT (Fig. 2). However, the WBCs were only significantly protected when rmTPO was administered 2 h before the administration of WBI/CT (Fig. 2).

Data from studies no. 1 and no. 2 suggested that 5 μg/kg rmTPO administered 2 h prior to myelosuppressive therapy significantly protected all three major blood cells in the mice compared to the control mice. The data also suggest that administration of rmTPO more than 24 h prior to myelosuppressive therapy had little effect on blood cell counts in mice. Therefore, in study no. 3 we wanted to determine whether a dose of 5 μg/kg rmTPO administered on day 7, 5, 3, or 1 prior to WBI/CT as well as 2 h prior to WBI/CT would provide additional protection of the blood cells. However, the data indicated that two doses of rmTPO did not provide protection above and beyond that of a single dose of rmTPO administered 2 h prior to WBI/CT (Fig. 3). When rmTPO was administered 7 days prior to WBI/CT in study no. 2 (Fig. 2), the mice had lower platelet counts compared to controls by day 10. In study no. 3, however, when rmTPO was administered 7 days and 2 h prior to WBI/CT, there was a significant protection of platelet counts by day 10 (Fig. 3). The additional dose of rmTPO at −2 h seemed to at least counteract the reduction in platelet counts caused by the first dose of rmTPO at −7 days.

Myeloablative studies

Following 900 rad of WBI, the control mice exhibited a platelet nadir that was decreased approximately 96% from baseline counts by day 10 (Fig. 4). This dose of radiation resulted in a 50% mortality rate (five out of ten) in the mice during the study period (Fig. 5). By day 14, the mice that had received 5 μg/kg rmTPO 2 h prior to WBI had significantly higher counts of platelets, RBCs and WBCs than the surviving control mice (WBI alone) (Fig. 4). Also, none of the mice that received rmTPO died, demonstrating significantly increased survival compared to the control mice (Fig. 5).

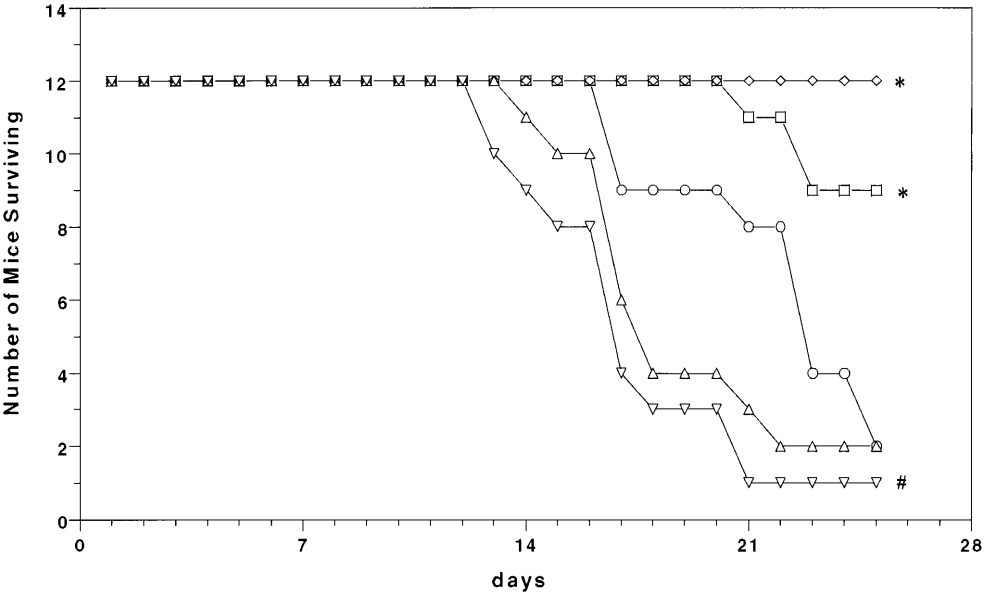

Fig. 6 Mortality of mice following 950 rad WBI with or without a single i.v. bolus dose of 5 μg/kg rmTPO administered at different time-points in relation to WBI. The data are the number of mice surviving each day following the WBI on day 1 (*circles* control mice that received WBI alone; *squares* mice that received rmTPO at –2 h; *diamonds* mice that received rmTPO at −2 h, and on days 4 and 10; *triangles* mice that received rmTPO on day –7 and at –2 h; *inverted triangles* mice that received rmTPO on day –7, at –2 h, and on days 4 and 10). $*P < 0.05$ improved survival vs control; $^{\#}P < 0.05$ decreased survival vs control; Kaplan-Meier survival statistics

When the dose of radiation was increased to 950 rad, the mortality rate increased to 83% (10 out of 12 mice) and was significantly higher than the group that had received 5 μg/kg rmTPO 2 h prior to WBI (25%, 3 out of 12 mice; Fig. 6). The blood cell counts appeared to recover faster in the mice that had received rmTPO at –2 h, and these mice had significantly higher platelet counts by day 14 compared to control mice (Table 1). When rmTPO (5 μg/kg) was administered to the mice 2 h prior to WBI as well as on days 4 and 10 following WBI, survival was significantly increased (0% mortality) compared to the control group (83%) (Fig. 6). The blood cell counts also recovered faster and all three lineages were significantly higher than in the control group by day 21 (Table 1). Importantly, the mice that had received rmTPO 7 days prior to WBI did not tolerate the radiation and had an 83% to 92% mortality rate (10/12 and 11/12) regardless of any additional rmTPO administration (Fig. 6). Additional doses were not able to counter the increased sensitivity, as was the case in our myelosuppression study no. 3 (Fig. 3).

Discussion

Many current therapies for cancer treatment are cytoreductive; thus their use can be limited because they cause thrombocytopenia in patients. TPO is a potential therapy that may help counteract the reduction in platelet numbers that results from myelosuppressive therapies. The data reported here suggest that a single i.v. bolus dose of 5 μg/kg rmTPO is effective in preventing pancytopenia in mice due to either radiation or radiation plus chemotherapy treatment. However, the dose scheduling of TPO administration appears to be most important in realizing its multilineage effect. In addition, it appears that there were no adverse effects by administering rmTPO intravenously 24 h before until 24 h after myelosuppressive therapy in mice. These results are consistent with those of previous studies, which suggest that rmTPO administered shortly after cytoreductive therapy prevents pancytopenia in mice [29]. The administration of rmTPO more than 24 h before myelosuppressive therapy had little effect on blood cell counts.

The results from the myeloablative model we used in these studies are similar to those of the myelosuppression studies. They suggest that a single i.v. bolus dose of 5 μg/kg rmTPO administered 2 h prior to WBI provides significant protection of blood cells and survival of the animals (compared to other rmTPO regimens tested) at radiation exposures $\leq$900 rad. Following exposure to >900 rad of radiation, additional injections of rmTPO after exposure appear to be needed for recovery of blood cells and increased survival of the mice.

When interpreting the blood cell data from the myeloablation studies, it is important to remember that the cell counts were obtained from surviving mice and therefore the number of mice per group varied especially

Table 1 Major peripheral blood cell counts in surviving mice on days 10, 14, 17 and 21 following 950 rad WBI with or without 5 μg/kg i.v. bolus rmTPO at various time-points in relation to WBI. Data are means ± SD. Control mice were exposed to WBI but received no rmTPO

rmTPO (time relative to WBI)	Day 10			Day 14			Day 17			Day 21		
	Platelets (10^9/l)	RBC (10^{12}/l)	WBC (10^9/l)	Platelets (10^9/l)	RBC (10^{12}/l)	WBC (10^9/l)	Platelets (10^9/l)	RBC (10^{12}/l)	WBC (10^9/l)	Platelets (10^9/l)	RBC (10^{12}/l)	WBC (10^9/l)
Control mice	56 ± 18 (n = 8)	6.6 ± 0.4 (n = 12)	0.2 ± 0.03 (n = 10)	32 ± 16 (n = 12)	3.5 ± 0.6 (n = 12)	0.3 ± 0.1 (n = 11)	39 ± 20 (n = 11)	2.1 ± 0.6 (n = 11)	0.3 ± 0.2 (n = 11)	43 ± 40 (n = 8)	1.5 ± 0.6 (n = 8)	1.5 ± 2.3 (n = 8)
–2 h	33 ± 8 (n = 9)	6.3 ± 0.6 (n = 12)	0.2 ± 0.07 (n = 7)	51 ± 25* (n = 5)	3.2 ± 0.6 (n = 12)	0.2 ± 0.1 (n = 12)	46 ± 27 (n = 12)	2.5 ± 0.6 (n = 12)	0.4 ± 0.3 (n = 12)	86 ± 71 (n = 11)	2.6 ± 1.7 (n = 11)	2.3 ± 3.0 (n = 10)
–2 h, days 4 and 10	37 ± 9 (n = 11)	6.5 ± 0.5 (n = 12)	0.2 ± 0.04 (n = 12)	49 ± 18* (n = 5)	3.4 ± 0.8 (n = 11)	0.3 ± 0.1* (n = 9)	61 ± 30* (n = 12)	2.8 ± 0.7* (n = 12)	0.4 ± 0.3 (n = 12)	120 ± 63* (n = 12)	3.2 ± 1.5* (n = 12)	6.8 ± 6.7* (n = 12)
Day –7 and –2 h	32 ± 7 (n = 12)	6.5 ± 0.5 (n = 12)	0.2 ± 0.06 (n = 4)	28 ± 11 (n = 8)	2.9 ± 0.5 (n = 8)	0.2 ± 0.1 (n = 10)	25 ± 8 (n = 8)	1.8 ± 0.5 (n = 8)	0.2 ± 0.1 (n = 7)	50 ± 47 (n = 4)	1.6 ± 1.2 (n = 4)	2.3 ± 3.7 (n = 4)
Day –7, –2 h, days 4 and 10	22 ± 9 (n = 11)	5.5 ± 1.0 (n = 12)	0.1 ± 0.07 (n = 11)	23 ± 11 (n = 8)	2.5 ± 0.6 (n = 9)	0.2 ± 0.1 (n = 9)	31 ± 9 (n = 7)	1.4 ± 0.5 (n = 7)	0.4 ± 0.2 (n = 6)	87 ± 78 (n = 2)	1.9 ± 1.6 (n = 2)	8.9 ± 12.3 (n = 2)

*$P < 0.05$ vs control mice by ANOVA

at the later time-points. This also makes it difficult to draw a correlation between the blood cell counts and survival. The majority of control mice probably would require a bone marrow transplant to avoid mortality following 950 rad WBI. However, the mice that received rmTPO 2 h prior to and on days 4 and 10 after WBI appeared to recover without the need for a bone marrow transplant. The data strongly suggest that there might be a worsening of cytoreduction in mice dosed with rmTPO 7 days prior to myeloablative radiation therapy.

The mechanisms by which TPO protects the multilineage cells from the effects of cytoreductive therapy and makes them available for hematopoietic reconstitution are still unclear. The efficacy window for TPO in the models we used appears to be a short period right before or after the chemotherapy and/or radiation treatment. The multilineage effects of TPO decline as the time of administration before cytoreductive therapy increases. TPO may protect the bone marrow environment containing these multilineage precursor cells. Alternatively, TPO may promote differentiation of the cells into a state that is better suited to survive the myelosuppressive treatment and still reconstitute the peripheral blood cells. Further studies are needed to examine what is happening in the bone marrow in order to address these issues.

The optimal efficacy of a single dose of TPO when administered 2 h prior to cytoreductive treatment emphasizes the importance of dose scheduling in the clinic when TPO is used in conjunction with cancer treatment protocols. However, more studies are needed to elucidate the mechanisms of TPO's multilineage protection from myelosuppressive treatment and increased survival following myeloablative treatment.

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