The Efficacy of Recombinant Thrombopoietin in Murine and Nonhuman Primate Models for Radiation-Induced Myelosuppression and Stem Cell Transplantation

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ABSTRACT

Radiation-induced pancytopenia proved to be a suitable model system in mice and rhesus monkeys for studying thrombopoietin (TPO) target cell range and efficacy. TPO was highly effective in rhesus monkeys exposed to the mid-lethal dose of 5 Gy (300 kV x-rays) TBI, a model in which it alleviated thrombocytopenia, promoted red cell reconstitution, accelerated reconstitution of immature CD34$^+$ bone marrow cells, and potentiated the response to growth factors such as GM-CSF and G-CSF. In contrast to the results in the 5 Gy TBI model, TPO was ineffective following transplantation of limited numbers of autologous bone marrow or highly purified stem cells in monkeys conditioned with 8 Gy TBI. In the 5 Gy model, a single dose of TPO augmented by GM-CSF 24 h after TBI was effective in preventing thrombocytopenia. The strong erythropoietic stimulation may result in iron depletion, and TPO treatment should be accompanied by monitoring of iron status. This preclinical evaluation thus identified TPO as a potential major therapeutic agent for counteracting radiation-induced pancytopenia and demonstrated pronounced stimulatory effects on the reconstitution of immature CD34$^+$ hemopoietic cells with multilineage potential. The latter observation explains the potentiation of the hematopoietic responses to G-CSF and GM-CSF when administered concomitantly. It also predicts the effective use of TPO to accelerate reconstitution of immature hematopoietic cells as well as possible synergistic effects in vivo with various other growth factors acting on immature stem cells and their direct lineage-committed progeny. The finding that a single dose of TPO might be sufficient for a clinically significant response emphasizes its potency and is of practical relevance.

The heterogeneity of the TPO response encountered in the various models used for evaluation points to multiple mechanisms operating on the TPO response and heterogeneity of its target cells. Mechanistic mouse studies made apparent that the response of multilineage cells shortly after TBI to a single administration of TPO is quantitatively more important for optimal efficacy than the lineage-restricted response obtained at later intervals after TBI and emphasized the importance of a relatively high dose of TPO to overcome initial c-mpl-mediated clearance. Further elucidation of mechanisms determining efficacy might very well result in a further improvement, e.g., following transplantation of limited numbers of stem cells. Adverse effects of TPO administration to myelosuppressed or stem cell transplanted experimental animals were not observed. *Stem Cells 1998;16:375-386*

INTRODUCTION

The identification of thrombopoietin (TPO) [1-4] as the major regulator of thrombocyte production [5, 6] has resulted in novel insights in the regulation of immature hemopoietic cell differentiation [7-9] and has potentially provided a therapeutic approach for counteracting thrombocytopenic states, in particular those associated with intensive cytoreductive treatment of malignancies. The pharmaceutical development

of TPO for such applications requires demonstration of efficacy in experimental animal models, either as a single agent or in conjunction with other cytokines. In view of the generally complex receptor distribution patterns of growth factors [10-12], interactions resulting from concurrent administration of the growth factors are difficult to predict by any other approach than detailed experimental animal in vivo studies.

Myelosuppression is a serious complication of current chemotherapy regimens, resulting in life-threatening neutropenia and thrombocytopenia and hampering full deployment of anticancer therapy. Both G-CSF and GM-CSF treatment have become established therapeutics [13-17] for alleviating cytopenia, in particular the neutropenia resulting from intensive cytoreductive treatment [18]. Although G-CSF and GM-CSF are grossly similar in pharmaceutical profile [18, 19], GM-CSF has advantages in that it also stimulates megakaryocytopoiesis and monocyte differentiation [20, 21]. The beneficial effects of both GM-CSF and G-CSF on neutropenia following cytoreductive treatment are in most studies restricted to an earlier recovery of approximately five days, less in dose-intensified chemotherapy [15, 17, 22], on a total median neutropenia of about 20-25 days. It is therefore of considerable importance to select combinations of growth factors that provide optimal costimulatory efficacy. Optimal co-stimulation may result in particular from growth factors that promote reconstitution of immature multilineage cells.

Radiation is an effective and controllable agent for inducing myelosuppression and is the most effective single agent for high-dose eradicative cancer treatment and for immunosuppression. For the preclinical evaluation of TPO, we have made use of rhesus monkeys exposed to the mid-lethal dose of 5 Gy TBI (300 kV x-rays [23]) as well as the lethal dose of 8 Gy TBI followed by an autologous transplant of highly purified stem cells. The 5 Gy TBI dose results in a profound pancytopenia for three weeks, whereas 8 Gy irradiated monkeys need a transplant to prevent protracted pancytopenia and mortality due to transfusion refractoriness. Studied parameters included, apart from blood cell counts, assessment of immature bone marrow cells as well as monitoring of adverse effects. To assess combination treatment, priority was given to those growth factors which are likely to be used clinically in conjunction with TPO, i.e., GM-CSF and G-CSF. In this summary paper, we focus on the myelosuppression data and mechanisms of action-promoting efficacy and briefly discuss the transplantation experiments. The findings in rhesus monkeys prompted us to return to more basic studies in mice to elucidate some of the relevant mechanisms. In mice, we calibrated the 5 Gy x-rays of the monkeys to 6 Gy γ-rays to accomplish a similar level of myelosuppression, in keeping with the relative biological effectiveness (RBE) of γ-rays and a similar duration of pancytopenia.

THROMBOPOIETIN AS A SINGLE AGENT IN MYELOSUPPRESSED RHESUS MONKEYS: PROMINENT THROMBOPOIETIC AND ERYTHROPOIETIC STIMULATION WITH IRON DEPLETION, LACK OF A NEUTROPHIL RESPONSE, AND ACCELERATED BONE MARROW CD34^{+} CELL RECOVERY

The effectiveness of TPO in alleviating thrombocytopenia was initially evaluated in a placebo-controlled study involving rhesus monkeys exposed to 5 Gy TBI, using supraoptimal treatment with human recombinant TPO (10 µg/kg/d, s.c., day 1-21 after TBI) [24, 25]. The TPO treatment appeared to be highly effective in preventing thrombocytopenia, with nadirs for thrombocytes on average far above 100×10^9/l; this represented a greatly accelerated recovery to normal values with no need for thrombocyte transfusions, whereas placebo controls required two to six platelet transfusions. TPO appeared to act selectively in that neutrophil regeneration was not influenced, but the red cell lineage recovery was prominently stimulated with exponential reticulocyte regeneration initiated 10 days earlier than in placebo-treated animals. The reticulocytosis was followed by a normoblastosis which occurred earlier and was more pronounced than in placebo-treated monkeys and was accompanied by elevated lactic dehydrogenase (LDH) serum levels, attributed to a rapid and partly inefficient erythropoiesis. The effect of TPO on the red cell lineage was also reflected in a less profound nadir for hemoglobin and hematocrit values than in placebo controls. Simultaneous TPO and G-CSF were as effective as TPO in preventing thrombocytopenia, although platelet levels did not rise to the supranormal levels seen with TPO alone. A similar dampening effect of G-CSF on the TPO response has been described in mice [26] and might be related to the protracted thrombocytopenia observed in stem-cell-transplanted monkeys [27] which were treated with G-CSF. The neutrophil recovery was greatly augmented compared with G-CSF treatment alone, resulting in a less profound nadir and a recovery that started much earlier, as was similarly observed for monocyte, CD11b^{+}, CD16^{+}, and CD56^{+} cell reconstitution. In addition, TPO strongly promoted the recovery of bone marrow cellularity and granulocyte/macrophage and erythroid progenitor cells, as was also reflected by a difference of two orders of magnitude with controls in bone marrow CD34^{+} cells in the second week of treatment, whereas G-CSF had no influence. In magnitude, this effect surpassed that of any other growth factor tested in the same animal model, including interleukin 3 (IL-3) and IL-6 (unpublished observations). There was no effect of TPO on hemostasis parameters, and adverse systemic effects—apart from iron depletion due to prominent erythropoiesis stimulation—were not observed for either growth factor.

The thrombopoiesis-stimulating effect of TPO was already sufficiently effective in the first week of treatment,

as was clear from the increasing platelet numbers as early as day 8, as opposed to placebo controls which did not reach similar levels before the fourth week after TBI. Also, the effect on bone marrow cellularity and immature bone marrow cells is already clear at the end of the second week of treatment. Therefore, the dose schedule of 21 consecutive days of treatment was supraoptimal, as was also evident from the supranormal platelet numbers reached in the second and third week after TBI; this is consistent with the observation in a mouse model for thrombocytopenia that a single dose of TPO given 24 h after the cytoreductive therapy is as effective as daily dosing for eight consecutive days [28]. The effect of a single injection in this model was also highly dose-dependent and shows that TPO is clearly more effective in stimulating platelet recovery than other growth factors known to stimulate platelet production, such as IL-6 [29-32], IL-11 [33, 34], IL-3 [31, 32], and IL-1 [35]. All of these cytokines stimulate platelet production, but not all are sufficiently effective for preventing thrombocytopenia at tolerable doses in view of adverse effects.

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A central issue of TPO treatment is prevention of bleeding as a consequence of myelosuppression. In monkeys, donor thrombocytes were transfused at the level of $40 \times 10^9/l$, which coincides with the first appearance of petechiae and other bleeding; this therapy was chosen to prevent undue deaths due to hemorrhages. For obvious reasons, this level is higher than used for human patients, where instructions can be given and sensitization to alloantigens should be avoided. However, as could be extrapolated from the post-irradiation drop in thrombocyte counts after the first week and the first ascending counts in the third week after TBI in placebo-treated monkeys and as demonstrated in historical controls [36-38], without transfusions the thrombocytes would have dropped to levels lower than $10 \times 10^9/l$ within two days after the first transfusion. It can thus be concluded that the TPO treatment, which prevented the decline of thrombocyte counts to levels lower than $100 \times 10^9/l$ early in the second week after irradiation, also effectively prevented the propensity for bleeding.

The TPO effect on early hematopoietic progenitor cells of different lineages was unexpected but is in line with other observations [39, 40] and consistent with the observation that its receptor is present on immature progenitor cells [7]. As megakaryocytes are capable of releasing various growth factors [41, 42], the effect observed may be an indirect consequence of stimulation of the megakaryocyte lineage. Alternatively, TPO may synergize directly with growth factors

such as kit ligand or flt-3 ligand to stimulate immature cells. Evidence for such a synergy has been provided by in vitro experiments [43-45]. The accelerated recovery of GM progenitor cells may explain the augmented response to G-CSF along the neutrophil and monocyte lineages, also reflected by CD11b positive cells. Apparently, G-CSF also stimulated the CD16/CD56 positive lineage, thought to represent natural killer cells originating in the bone marrow [46]. Since the G-CSF dose schedule was supraoptimal in this study, the augmented responses to G-CSF are best explained by increased numbers of progenitor cells along the GM lineage.

The erythroid lineage was consistently shown to be involved in the TPO response in vitro and in vivo [24, 40, 47, 48]. In normal mice and primates, TPO selectively stimulated thrombocyte production, leaving other lineages unaffected. In myelosuppression models, multilineage effects were mainly of an erythroid nature [28, 40, 49]. The effect of TPO on the red cell lineage, like its thrombopoietic effect, occurred very early after irradiation with exponential reticulocyte regeneration beginning after the first week of treatment, 10 days earlier than in placebo and G-CSF-treated controls. Reticulocytosis was followed by a normoblastosis, which was accompanied by elevated LDH serum levels, both attributed to the rapid and inefficient erythropoiesis. As a result, hemoglobin concentrations and hematocrits stabilized earlier at higher levels. However, this effect was not followed by a rapid recovery to normal values due to the development of a microcytic, hypochromic anemia. Iron depletion was directly demonstrated by measurements of total serum iron and total iron binding capacity and could be prevented by prophylactic i.m. iron before TBI or corrected by i.m. iron after TPO treatment. Based on these findings, iron status needs to be assessed before and monitored during TPO treatment. It should again be noted that the TPO administration described here was more intensive than required to normalize platelet levels and will not likely be necessary for the clinical therapy of myelosuppressed patients. It is not clear why in TPO-treated monkeys the normoblastosis followed the reticulocytosis, a pattern reverse to that which is seen with other growth factors such as IL-3 [50] and GM-CSF (unpublished observation). Since the normoblastosis was highly reduced in monkeys prophylactically supplemented with iron, the pattern observed might be related to the developing iron deficiency resulting in ineffective erythropoiesis in the course of TPO-stimulated hematopoietic reconstitution.

Likely, the iron deficiency originated from a precarious iron balance in rhesus monkeys similar to that in humans,

resulting in latent iron deficiency even before radiation exposure. This is supported by the observation that the mean corpuscular erythrocyte volume of the animals before treatment was 75-80 fl and rose to 90 fl in otherwise untreated monkeys subjected to iron supplementation. The latent iron deficiency may then be caused either by diet, malabsorption due to the pre-irradiation antibiotic regimen, parasitic infection, or some other process related to iron absorption. The iron depletion of TPO-treated monkeys is reminiscent of erythropoietin-induced iron deficiency anemia in patients with end-stage renal failure [51-53]. A similar mechanism is proposed for the iron deficiency anemia occurring with TPO. Supplementation of iron before and careful monitoring during treatment proved to be necessary for all patients receiving erythropoietin.

LACK OF EFFICACY OF TPO AND G-CSF IN MONKEYS SUBJECTED TO HIGH-DOSE TBI AND TRANSPLANTATION OF AUTOLOGOUS, HIGHLY PURIFIED STEM CELLS

The efficacy of recombinant human TPO and recombinant human G-CSF in stimulating platelet and neutrophil recovery was evaluated in a placebo-controlled study involving transplantation of limited numbers ($1\text{-}3 \times 10^4$/kg) of highly purified autologous stem cells (CD34^{++}/RhLA-DR^{dull}) after 8 Gy TBI (x-rays) in rhesus monkeys [27]. The grafts shortened 8 Gy TBI-induced profound pancytopenia from five to six weeks to three weeks. Daily administration of TPO (10 µg/kg/d s.c., day 1-21 after TBI) did not prevent thrombocytopenia nor did it significantly stimulate regeneration to normal platelet values. With the possible exception of reticulocytes, other cell lineages were not influenced either. Simultaneous treatment with TPO and G-CSF (5 µg/kg/d, s.c., day 1-21 after TBI), tested in two monkeys, showed heterogeneous results. In one monkey, neutrophil regeneration was slightly promoted (values $>0.5 \times 10^9$/l at day 15), whereas the other monkey regenerated in the same pattern as placebo-treated controls. Platelet and reticulocyte regeneration was augmented in the monkey which regenerated its neutrophils faster, whereas the other developed protracted thrombocytopenia. G-CSF alone did not stimulate neutrophil regeneration. It did not affect the red cell lineage, and in one of three monkeys, a profound

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and long-lasting thrombocytopenia developed. To test whether the size or cellular composition of the graft was responsible for the lack of efficacy of TPO, three monkeys were transplanted with unfractionated bone marrow cells in a cell dose of 10^7/kg. TPO treatment in this setting again did not prevent thrombocytopenia either, and recovery to normal platelet values was, at best, only slightly accelerated.

In monkeys irradiated with 5 Gy TBI which did not receive a transplant, TPO (10 µg/kg/d) had a very prominent effect on platelet recovery, as reflected by much less profound platelet nadirs as well as an accelerated recovery to normal values, resulting in full prevention of thrombopenia [24]. Since the experimental conditions were calibrated in such a way that the duration of thrombocytopenia and the platelet recovery pattern were the same for the placebo-treated monkeys in both models, the different responses to TPO are most remarkable. In principle, the difference in efficacy of TPO in the stem cell transplant model and the myelosuppression model may be attributed to: A) the dose of TPO chosen; B) the difference in radiation dose which may have caused more stromal damage in the stem cell transplant model, or C) the composition of the cell populations from which hematopoietic reconstitution originated.

The dose and dose schedule of TPO were based on pilot dose-finding experiments in normal mice and rhesus monkeys and found to be supraoptimal. The dose schedule chosen was also found to be supraoptimal in the myelosuppression model in that very high levels of platelets were reached within two weeks. In addition, the TPO levels measured in the placebo controls demonstrated that full platelet reconstitution was reached in four weeks after TBI. Apparently, the elevated endogenous TPO levels, which were more than one order of magnitude lower than those obtained with exogenous TPO, were sufficient. Therefore, it is improbable that the ineffectiveness of TPO in the transplant model was attributable to the dose and dose scheduling of TPO.

The difference between 5 Gy and 8 Gy TBI may have resulted in a difference in stromal damage, thereby impairing platelet reconstitution at the higher dose of TBI. First, in the placebo control monkeys, the kinetics of platelet reconstitution were very similar, if not identical,

in both models. Second, stromal damage resulting in significantly impaired hematopoiesis has only been observed following much greater doses of radiation [54] than those used here. Nevertheless, we do not exclude the possibility that the higher radiation dose of 8 Gy resulted in significantly more damage and that the inflammatory reactions to radiation exposure might have released cytokines which counteracted the TPO efficacy in the transplant model.

The composition of the reconstituting cell populations might be a decisive determinant for a clinically relevant TPO response. In the 5 Gy myelosuppression model, the residual cells are less than two orders of magnitude depleted in stem cells and progenitor cells and remain in their normal stromal environment. It is also clear from the kinetics of the TPO response that its effectiveness is determined in the initial phase after TBI, a conclusion that has been corroborated by the diminished response to delayed administration of TPO [55] as well as by the observation in mice that a single initial i.v. injection of TPO is sufficient to prevent profound thrombocytopenia [28]. In contrast, in the 8 Gy model, there is a more than 3 log stem cell depletion, and the hematopoietic reconstitution pattern observed was dependent on infused, highly purified and progenitor-cell-depleted stem cells which needed to home into the hematopoietic sites. It is conceivable that in this situation initial hematopoietic reconstitution cannot be stimulated by TPO alone and needs to be either boosted by other growth factors or stimulated prior to TPO treatment so as to generate TPO-responsive progenitor cells. Since the TPO response was also severely diminished in the unfractionated bone marrow recipients compared with the 5 Gy myelosuppressed monkeys which had similar platelet reconstitution kinetics in the placebo control monkey, the inefficacy of TPO and G-CSF cannot be attributed to the depletion of progenitor cells but might also be due to poor engraftment of the responsive progenitor cells. Finally, these data indicate a steep decline of the TPO and G-CSF responses with increasing radiation dose, a phenomenon that has been observed for growth factors such as GM-CSF [56] and IL-3 [57] as well. So, the accelerated hematopoietic reconstitution after high-dose TBI originating from either an autologous stem cell or unfractionated bone marrow graft would seem to gain little from exogenous growth factors, possibly due to the relative lack of target cells,

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which may already maximally proliferate due to endogenous stimuli or are otherwise not accessible for exogenous stimulation.

SINGLE-DOSE TPO ADMINISTRATION FOLLOWING MYELOSUPPRESSIVE TREATMENT: SUFFICIENT FOR PREVENTION OF THROMBOCYTOPENIA, ACCELERATED RETICULOCYTE REGENERATION, POTENTIATION OF THE RESPONSES TO G-CSF AND GM-CSF AND AUGMENTED BONE MARROW CD34+ CELL RECONSTITUTION

A third study was undertaken A) to explore the option of limiting the total dose of TPO, based on the previous studies in mice [28] which showed that a single administration of TPO might be sufficient to prevent thrombocytopenia following cytoreductive treatment, and B) to compare the concurrent administration of TPO and GM-CSF with that of TPO and G-CSF and to identify optimal growth factor therapy to counteract both neutropenia and thrombocytopenia.

Rhesus monkeys were subjected to 5 Gy TBI, inducing three weeks of profound pancytopenia, and received either 5 µg/kg of TPO i.v. at day 1 (i.e., 24 h after TBI), GM-CSF 25 µg/kg s.c. for 14 days, TPO and GM-CSF, G-CSF 10 µg/kg/d s.c. for 14 days, TPO and G-CSF, or placebo. Single i.v. dose treatment with TPO one day after TBI effectively counteracted the need for thrombocyte transfusions (provided whenever thrombocyte levels were $<40 \times 10^9$/l) and accelerated platelet reconstitution to reach normal levels two weeks earlier than placebo controls. TPO/GM-CSF was more effective than single-dose TPO alone in stimulating thrombocyte regeneration, with a less profound nadir and a further accelerated recovery to normal thrombocyte counts, as well as a slight overshoot to supranormal levels of thrombocytes. Monkeys treated with TPO/GM-CSF uniformly did not require thrombocyte transfusions, whereas those treated with GM-CSF alone needed two to three transfusions, not dissimilar to the placebo-treated monkeys, which required on average three (range two to six) transfusions. Also, reticulocyte production was stimulated by TPO and further augmented in monkeys treated with TPO/GM-CSF. TPO alone did not stimulate neutrophil regeneration, whereas GM-CSF shortened the period of neutrophils $<0.5 \times 10^9$/l approximately one week; TPO/GM-CSF treatment elevated the neutrophil nadir but did not further

accelerate recovery to normal values. TPO also augmented the neutrophil response to G-CSF, resulting in similar patterns of reconstitution following TPO/G-CSF and TPO/GM-CSF treatment. The TPO/GM-CSF treatment resulted in a significantly increased reconstitution of CD34+ bone marrow cells and progenitor cells such as GM-CFU and BFU-E.

A single i.v. dose of TPO, administered one day after TBI inducing three weeks of profound pancytopenia in placebo-treated controls, appeared thus sufficient to virtually prevent the need for thrombocyte transfusion and to accelerate thrombocyte reconstitution to normal levels by two weeks. In a clinical setting, where the transfusion criterion is generally set at a lower level of thrombocytes than is feasible in monkeys, the TPO treatment would likely have completely abolished the thrombocyte transfusion requirement at this level of myelosuppression. Furthermore, the results demonstrated that TPO/GM-CSF and TPO/G-CSF treatment display distinct response patterns among the three major peripheral blood cell types. Coadministration of TPO/GM-CSF augmented thrombocyte, red cell, and neutrophil production over either of the individual growth factors alone, whereas in the TPO/G-CSF treatment group only neutrophil reconstitution appeared to benefit from the combination of growth factors. However, the greater target cell range of the TPO/GM-CSF combination should be balanced against the slightly higher incidence of reported adverse effects of GM-CSF [15-17], whereas the slightly more rapid reconstitution of neutrophils following TPO/G-CSF administration should be weighed against the reported dampening effect of G-CSF treatment on thrombocyte recovery [24, 26, 39].

The observation that a single i.v. dose of TPO shortly after intensive cytoreductive treatment is sufficient to significantly alleviate the course of thrombocytopenia is of considerable practical and clinical importance. The finding is consistent with data in mice [28], with the kinetics of TPO-stimulated thrombocyte reconstitution in nonhuman primates [24, 39], and with the decline in response when TPO administration was delayed [55]. These studies all point to a critical time phase early after TBI during which TPO has to be administered to achieve an optimal response. The most obvious explanation is a decline in bone marrow TPO-responsive target cells as a function of time, possibly due to the absence

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of sufficiently high concentrations of TPO. This may either be due to a physiological death function or apoptosis similar to that identified for erythropoietin-deprived red cell progenitor cells [58, 59] or to protection of TPO-responsive progenitors from radiation-induced cell death. Elucidation of such mechanisms will not only provide further insight into the physiological function of TPO, but will also be of considerable importance to achieve the maximum clinical benefit of its therapeutic use. Although these data reveal that a single i.v. dose of 5 µg/kg of TPO is sufficient to prevent thrombocytopenia at the level of myelosuppression chosen, this finding does not preclude that clinical cytoreductive therapy requires a more intensive TPO treatment regimen.

The administration of TPO/GM-CSF proved to be superior to all other growth factors or combinations of growth factors studied for stimulating thrombocyte reconstitution. GM-CSF alone did not influence the thrombocyte nadir or significantly reduce the need for thrombocyte transfusions. However, it was as effective as the single administration of TPO alone in stimulating post-nadir thrombocyte production. The augmented thrombocyte reconstitution of the TPO/GM-CSF-treated monkeys may reflect synergism of the two growth factors, since the initial recovery of both thrombocytes and reticulocytes in the TPO/GM-CSF group exceeded the sum of those in the monkeys treated with either of the growth factors alone. A stimulatory action of GM-CSF on megakaryocytes resulting in increased thrombocyte production has been described before [20, 60], although the results of in vivo treatment with GM-CSF on thrombocyte reconstitution have always been heterogenous [20, 60-62], and, in retrospect, may have been codependent on variations in endogenous thrombopoietin levels.

Surprisingly, the 10 µg/kg/d dose (for two weeks) of G-CSF also appeared to stimulate thrombocyte production to a certain extent, in contrast to the 5 µg/kg/d dose (for three weeks) which in a previous study was used in the same non-human primate model [24]. The reported effects of G-CSF on thrombocyte production are not unambiguous, most reports mentioning no effects at all [13, 63-65], some a positive effect [66], and others a negative effect [67-69]. The observations in nonhuman primates follow essentially the same pattern. We observed earlier in the same myelosuppression

model as used here a dampening effect of G-CSF on TPO-stimulated supranormal thrombocyte production [24]. This was also seen in mice [26]. Meanwhile, in a transplant model in rhesus monkeys involving 8 Gy TBI followed by infusion of highly purified stem cells, protracted thrombocytopenia significantly related to G-CSF treatment was encountered in a few cases [27]. The causes of the variable reaction of thrombocytes to G-CSF treatment are not elucidated and may be governed by complex mechanisms; it should be noted that G-CSF receptors are present on thrombocytes [70] as well as on megakaryocytes, overexpression of G-CSF tending to decrease the ploidy of megakaryocytes. We concluded provisionally that combining TPO and G-CSF treatment may have a variable, as yet unpredictable, and occasionally, adverse outcome.

MECHANISMS OF TPO ACTION IN MYELOSUPPRESSED MICE

The importance of dose and dose scheduling for multilineage reconstitution after myelosuppressive TBI was further evaluated in mice [71]. After 6 Gy TBI, a dose of 0.3 µg TPO/mouse (12 µg/kg) i.p., zero to four h after TBI, prevented the severe thrombocytopenia observed in control mice and in addition stimulated red and white blood cell regeneration. Time course studies revealed a gradual decline in efficacy after an optimum within the first hours after TBI, accompanied by a replacement of the multilineage effects by lineage-dominant thrombopoietic stimulation. Pharmacokinetic (PK) data demonstrated that i.p. injection resulted in maximum plasma levels two h after administration. On the basis of the data we inferred that a substantial level of TPO was required at a critical time interval after TBI to induce multilineage stimulation of residual bone marrow cells. A more precise estimate of the effect of dose and dose timing was provided by i.v. administration of TPO, which showed an optimum immediately after TBI and a sharp decline in efficacy between a dose of 0.1 µg/mouse (4 µg/kg; plasma level 60 ng/ml), which was fully effective, and a dose of 0.03 µg/mouse (1.2 µg/kg; plasma level 20 ng/ml), which was largely ineffective. This observation is interpreted as evidence for a threshold level of TPO required for optimal efficacy. It was previously demonstrated [28, 78] that i.v. injection of ^{125}I-rmTPO into mice results in an initial sharp decline in plasma levels, followed by steady-state clearance approximately three hours after i.v. injection [78]. A lower dose does not influence the terminal half life [28]. After i.v. bolus injection, the initial rapid decline in plasma TPO levels is due to binding of TPO to c-mpl on platelets and on cells in the spleen [78], whereas the slower terminal decline is likely related to uptake and clearance by c-mpl on platelets, spleen cells, and megakaryocytes, as well as non-specific mechanisms [78, 79]. The initial binding and uptake

of TPO to c-mpl is concentration-dependent and becomes saturated at higher doses leading to greater plasma TPO levels [80]. This relationship between TPO pharmacokinetics and c-mpl levels has recently been studied in normal mice, which demonstrated that an i.v. dose of approximately 6 µg/kg (~0.15 µg/mouse) was needed to obtain an occupancy of 50% of c-mpl sites. Doses greater than 6 µg/kg began to saturate this specific clearance mechanism, whereas lower doses failed to reach 50% receptor occupancy [80]. This is consistent with a threshold level of TPO needed to overcome initial c-mpl-mediated clearance and to result in sufficient plasma TPO levels to achieve a maximal hemopoietic response. Doses larger than 6 µg/kg (0.15 µg/mouse) may not provide any greater efficacy. These data are consistent with demonstrating that the hematopoietic recoveries after i.v. doses of 0.3 and 0.1 µg/mouse (12 and 4 µg/kg respectively) were not different, whereas a dose of 0.03 µg/mouse (1.2 µg/kg) was suboptimal in preventing myelosuppression. Based on the previous i.v. PK data comparing TPO plasma levels in c-mpl knockout and normal mice [78], it was inferred that 40% of the exogenous TPO binds to c-mpl on platelets, and 60% is available as free TPO in the plasma. Assuming a plasma volume of 1 ml in a 25-g mouse, the suboptimal dose of 0.03 µg/mouse results in a maximum level of 20 ng/ml and the effective dose of 0.1 µg/mouse in 60 ng/ml. Consequently, the minimum effective or threshold plasma level is in between those levels.

Fractionation of the dose of radiation and appropriate dosing of TPO was thought to amplify the TPO effect on immature cells to enable short-term transplantation assays. Such an approach would also be more representative of clinically used radiation regimens and of the protracted nature of cytoreductive treatment by means of chemotherapy. Also after fractionated TBI (3×3 Gy, 24 h apart), the optimal dose and dose-scheduling of TPO derived from the 6 Gy experiments, i.e., 0.3 µg/mouse, two h after each TBI fraction, prevented thrombocytopenia and promoted erythrocyte and leukocyte reconstitution. Using short-term transplantation assays, i.e., colony-forming unit-spleen (CFU-S) day 13 and the more immature cells with marrow repopulating ability (MRA), it could be shown that TPO promoted CFU-S-13 and transiently depleted MRA. The initial depletion of MRA in response to TPO appeared to be replenished during long-term reconstitution followed for a period of three months. Apart from demonstrating again that MRA cells and CFU-S-13 are separate functional entities, these data thus revealed that TPO promotes short-term multilineage repopulating cells at the expense of more immature ancestral cells, thereby preventing pancytopenia. The short time interval available after TBI to exert these effects demonstrates that TPO is able to intervene in mechanisms which result in functional depletion

of its multilineage target cells shortly after TBI and emphasizes the requirement of dose scheduling of TPO in keeping with these mechanisms to obtain optimal clinical efficacy.

The mechanism by which TPO makes multilineage cells available for accelerated hemopoietic reconstitution remains to be further elucidated, as is their apparent functional depletion as a function of time following TBI in the absence of TPO. Multilineage TPO responsiveness declined sharply as a function of time after TBI, leaving a lineage-dominant thrombopoietic response when administered 24 h after TBI. This indicates that in the absence of TPO, multilineage TPO-responsive cells are rapidly depleted or become inaccessible. The loss of the multilineage TPO response may have diverse and complex causes, which include apoptosis or radiation-induced cell death, differentiation along other hemopoietic lineages, inhibition mediated by cytokines produced in response to radiation injury, or inaccessibility of the immature cells for TPO due to stromal reactions to radiation. TPO has been shown to prevent apoptosis of immature hemopoietic cells [72], and this might be a prime candidate mechanism to explain the short time interval available for optimally effective TPO intervention. In vitro, TPO does not seem to confer a proliferative response to immature hemopoietic cells but does so strongly in the presence of suitable other factors, e.g., kit-ligand [8, 47, 73-77]. The mechanisms involved might therefore include activation of one or more cofactors required for the strong proliferative response observed. We also do not exclude the possibility that the effect of TPO on multilineage cells is augmented by the release of various cytokines by megakaryocytes [41, 42] subsequent to stimulation by TPO, although the time frame observed makes such a mechanism unlikely.

Since the range of stimulation by TPO was not lineage-specific, it was hypothesized that the effect was mediated by stimulation of multilineage cells. Following transplantation of bone marrow into lethally irradiated recipients, the number of CFU-S-13 is a measure for relatively immature repopulating stem cells [81], associated with the initial, short-term wave of hemopoietic reconstitution, which lasts for several months [82]. By this assay it was shown that the multilineage effect of TPO administered two h after TBI is mediated through stimulation of these immature cells and is already manifest 24 h after the last fraction of TBI. The number of secondary in vitro clonogenic progenitors in the bone marrow of such recipients is a measure of the MRA of the graft, the primary cells being closely associated with those that provide sustained hemopoiesis following bone marrow transplantation [83]. MRA, measured by enumeration of GM-CFU numbers in the bone marrow of mice injected with cells from TPO-treated mice, appeared to be one to two orders of magnitude less than in control mice. The increase of CFU-S-13 and the concomitant decrease of cells with MRA most probably indicate recruitment of multilineage short-term repopulating cells from a more immature ancestral population. This is more conceivable from the magnitude of this effect (14-fold for CFU-S), which suggests three to four cell doublings. Since these occurred during the three days which elapsed from the first TPO administration to the time of the measurement, 24 h after the last fraction of TBI, this would be in close agreement with the doubling time established previously for such immature cell populations during hemopoietic reconstitution [59]. The decline in marrow repopulating cells with the concomitant increase of spleen repopulating cells could also be considered as a shift in homing pattern among these immature cells. To date, there is no evidence to suggest that such a shift may occur without cell divisions, whereas the ancestral position of the marrow repopulating cells relative to the spleen colony-forming cells has been well documented [83-85]. To date, the effect of TPO treatment on long-term repopulating cells has not been quantitatively documented. Recently, it was reported that transplantation of bone marrow from TPO-treated, 3.5 Gy-irradiated donor mice facilitated the 90-day survival of the recipient mice [86]. However, this survival effect had already become established at the short-term hematopoietic reconstitution parameter of 30-day survival (already in practice within 17 days), closely associated [82] to the CFU-S-13. Using such an experimental design to establish TPO effects on long-term repopulating cells would require a genetic marker able to distinguish between donor and recipient cells along multiple hemopoietic lineages. TPO treatment shortly after much higher ("supralethal") doses of TBI promoted survival and prevented bleeding [87].

The depletion of MRA cells in the TPO-treated mice, measured 24 h after TBI, appeared to be transient. Peripheral blood cell regeneration one and three months after three fractions of 3 Gy was not different in mice treated with TPO compared to controls and neither were the femoral and spleen in vitro colony-forming cell numbers. Assessment of the MRA at the same time intervals also did not reveal differences between the TPO-treated mice and the placebo group. We interpret these observations as replenishment of the MRA cells from a more ancestral cell population with, by definition, long-term repopulating ability, consistent with a model in which MRA cells are a transitory population intermediate to stem cells with long-term repopulating ability and the spleen repopulating cells measured by the CFU-S-13 assay.

In vivo studies on TPO efficacy have yielded various results; in normal animals, usually only a platelet response was obtained [88-94], whereas in myelosuppressed animals multilineage responses appeared to be the prevailing pattern [24, 25, 28, 39, 40, 86, 95-97]; following transplantation of limited

numbers of stem cells, no response was obtained at all [27]. On the basis of this heterogeneity, it can be assumed that the response to exogenous TPO is determined by multiple factors. We already pointed out the importance of cotreatment with G-CSF or GM-CSF to make the TPO effect on GM-CFU reconstitution manifest in neutrophil numbers in the peripheral blood. This study in mice also identified time relative to myelosuppression and dose of exogenous TPO as pivotal factors. In addition, the difference between normal and myelosuppressed animals indicated that the TPO response of immature cells might be dependent on the presence or activation of one or more cofactors. As already pointed out, TPO by itself does not induce in vitro a proliferative response in immature bone marrow cells, but does so strongly in synergy with, e.g., kit-ligand [8, 47, 73-77]. Identification of the cofactor(s) which operate in irradiated or otherwise myelosuppressed animals to generate a proliferative response to TPO administration might therefore be highly relevant as well to improve the clinical TPO response.

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