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

GERARD WAGEMAKER,[a] KAREN J. NEELIS,[a] SIMONE C.C. HARTONG,[a] ALBERTUS W. WOGNUM,[a] G. ROGER THOMAS,[b] PAUL J. FIELDER,[b] DAN L. EATON[b]

[a]Institute of Hematology, Erasmus University Rotterdam, Rotterdam, The Netherlands;
[b]Genentech Inc., South San Francisco, California, USA

Key Words. *Thrombopoietin · G-CSF · GM-CSF · Rhesus monkeys · Myelosuppression · Stem cell transplantation · Pharmacokinetics of thrombopoietin*

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

Correspondence: Dr. G. Wagemaker, Erasmus University, Institute of Hematology H Ee 1314, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

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.

A central issue of TPO treatment is prevention of bleeding as a consequence of myelosuppression.

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 40×10^9/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 10×10^9/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 100×10^9/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 (1-3 × 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 × 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 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.

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.

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

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 and IL-3 as well.

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 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 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 nonhuman 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.

ACKNOWLEDGMENTS

The authors wish to acknowledge the contributions of *Dr. F. de Sauvage* and *Dr. Jeff Gorell*, Genentech Inc., during the cloning, expression, and purification of recombinant rhesus TPO, the advice of *Drs. Mary Napier* and *Robert Cohen*, the technical assistance of *Trudy Visser*, *Hannie Busking-van der Lelie*, *Dorinde Kieboom-Pluimes*, *Eric Stefanich*, *Tauri Senn*, and *Melinda Marian*, the care of the animals of *Els van Bodegem*, *Albert Kloosterman*, and *Ron Briegoos*. This work has been partly supported by the Netherlands Cancer Foundation Koningin Wilhemina Fonds, the Dutch Organization for Scientific Research NWO, the Royal Netherlands Academy of Arts and Sciences and Contracts of the Commission of the European Communities.

[Adapted from *Wagemaker G, Neelis KJ, Hartong SCC et al*. The efficacy of recombinant TPO in murine and nonhuman primate models for myelosuppression and stem cell transplantation. THROMBOPOIETIN: FROM MOLECULE TO MEDICINE. STEM CELLS 1998;16(suppl 2):127-141.]

REFERENCES

1 de Sauvage FJ, Hass PE, Spencer SD et al. Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. Nature 1994;369:533-538.

2 Lok S, Kaushansky K, Holly RD et al. Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. Nature 1994;369:565-568.

3 Wendling F, Maraskovsky E, Debili N et al. cMpl ligand is a humoral regulator of megakaryocytopoiesis. Nature 1994;369:571-574.

4 Kaushansky K, Lok S, Holly RD et al. Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. Nature 1994;369:568-571.

5 Gurney AL, Carver-Moore K, de Sauvage FJ et al. Thrombocytopenia in c-mpl-deficient mice. Science 1994;265:1445-1447.

6 de Sauvage FJ, Carver-Moore K, Luoh SM et al. Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. J Exp Med 1996;183:651-656.

7 Methia N, Louache F, Vainchenker W et al. Oligodeoxynucleotides antisense to the proto-oncogene c-mpl specifically inhibit in vitro megakaryocytopoiesis. Blood 1993;82:1395-1401.

8 Sitnicka E, Lin N, Priestley GV et al. The effect of thrombopoietin on the proliferation and differentiation of murine hematopoietic stem cells. Blood 1996;87:4998-5005.

9 Ku H, Yonemura Y, Kaushansky K et al. Thrombopoietin, the ligand for the Mpl receptor, synergizes with steel factor and other early acting cytokines in supporting proliferation of primitive hematopoietic progenitors of mice. Blood 1996;87:4544-4551.

10 Wognum AW, van Gils FC, Wagemaker G. Flow cytometric detection of receptors for interleukin-6 on bone marrow and peripheral blood cells of humans and rhesus monkeys. Blood 1993;81:2036-2043.

11 Wognum AW, Westerman Y, Visser TP et al. Distribution of receptors for granulocyte-macrophage colony-stimulating factor on immature $CD34^+$ bone marrow cells, differentiating monomyeloid progenitors, and mature blood cell subsets. Blood 1994;84:764-774.

12 Wognum AW, Visser TP, de Jong MO et al. Differential expression of receptors for interleukin-3 on subsets of CD34-expressing hematopoietic cells of rhesus monkeys. Blood 1995;86:581-591.

13 Morstyn G, Campbell L, Souza LM et al. Effect of granulocyte colony stimulating factor on neutropenia induced by cytotoxic chemotherapy. Lancet 1988;1:667-772.

14 Gisselbrecht C, Prentice HG, Bacigalupo A et al. Placebo-controlled phase III trial of lenograstim in bone-marrow transplantation. Lancet 1994;343:696-700.

15 Welte K, Gabrilove J, Bronchud MH et al. Filgrastim (r-metHuG-CSF): the first 10 years. Blood 1996;88:1907-1929.

16 Antman KS, Griffin JD, Elias A et al. Effect of recombinant human granulocyte-macrophage colony-stimulating factor on chemotherapy-induced myelosuppression. N Engl J Med 1988;319:593-598.

17 Brandt SJ, Peters WP, Atwater SK et al. Effect of recombinant human granulocyte-macrophage colony-stimulating factor on hematopoietic reconstitution after high-dose chemotherapy and autologous bone marrow transplantation. N Engl J Med 1988;318:869-876.

18 Lieschke GJ, Burgess AW. Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor (2). N Engl J Med 1992;327:99-106.

19 Lieschke GJ, Burgess AW. Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor (1). N Engl J Med 1992;327:28-35.

20 Stahl CP, Winton EF, Monroe MC et al. Recombinant human granulocyte-macrophage colony-stimulating factor promotes megakaryocyte maturation in nonhuman primates. Exp Hematol 1991;19:810-816.

21 Mayer P, Lam C, Obenaus H et al. Recombinant human GM-CSF induces leukocytosis and activates peripheral blood polymorphonuclear neutrophils in nonhuman primates. Blood 1987;70:206-213.

22 Gorin NC, Coiffier B, Hayat M et al. Recombinant human granulocyte-macrophage colony-stimulating factor after high-dose chemotherapy and autologous bone marrow transplantation with unpurged and purged marrow in non-Hodgkin's lymphoma: a double-blind placebo-controlled trial. Blood 1992;80:1149-1157.

23 Zoetelief J, Wagemaker G, Broerse JJ. Dosimetry for total body irradiation of rhesus monkeys with orthovoltage x-rays. Int J Radiat Biol 1998;74:265-272.

24 Neelis KJ, Dubbelman YD, Luo Q et al. Simultaneous administration of TPO and G-CSF after cytoreductive treatment of rhesus monkeys prevents thrombocytopenia, accelerates platelet and red cell reconstitution, alleviates neutropenia and promotes the recovery of immature bone marrow cells. Exp Hematol 1997;25:1084-1093.

25 Neelis KJ, Luo Q, Thomas GR et al. Prevention of thrombocytopenia by thrombopoietin in myelosuppressed rhesus monkeys accompanied by prominent erythropoietic stimulation and iron depletion. Blood 1997;90:58-63.

26 Molineux G, Hartley CA, McElroy P et al. Megakaryocyte growth and development factor stimulates enhanced platelet recovery in mice after bone marrow transplantation. Blood 1996;8:1509-1514.

27 Neelis KJ, Dubbelman YD, Wognum AW et al. Lack of efficacy of thrombopoietin and granulocyte colony-stimulating factor after high dose total body irradiation and autologous stem cell or bone marrow transplantation in rhesus monkeys. Exp Hematol 1997;25:1094-1103.

28 Thomas GR, Thibodeaux H, Erret CJ et al. In vivo biological effects of various forms of thrombopoietin in a murine model of transient pancytopenia. STEM CELLS 1996;14(suppl 1):246-255.

29 Patchen ML, MacVittie TJ, Williams JL et al. Administration of interleukin-6 stimulates multilineage hematopoiesis and accelerates recovery from radiation-induced hematopoietic depression. Blood 1991;77:472-480.

30 Patchen ML, Fischer R, MacVittie TJ. Effects of combined administration of interleukin-6 and granulocyte colony-stimulating factor on recovery from radiation-induced hemopoietic aplasia. Exp Hematol 1993;21:338-344.

31 MacVittie TJ, Farese AM, Patchen ML et al. Therapeutic efficacy of recombinant interleukin-6 (IL-6) alone and combined with recombinant human IL-3 in a nonhuman primate model of high-dose, sublethal radiation-induced marrow aplasia. Blood 1994;84:2515-2522.

32 Winton EF, Srinivasiah J, Kim BK et al. Effect of recombinant human interleukin-6 (rhIL-6) and rhIL-3 on hematopoietic regeneration as demonstrated in a nonhuman primate chemotherapy model. Blood 1994;84:65-73.

33 Cairo MS, Plunkett JM, Schendel P et al. The combined effects of interleukin-11, stem cell factor, and granulocyte colony-stimulating factor on newborn rat hematopoiesis: significant enhancement of the absolute neutrophil count. Exp Hematol 1994;22:1118-1123.

34 Neben S, Turner K. The biology of interleukin 11. [Review]. STEM CELLS 1993;2:156-162.

35 Mizushima Y, Kashii T, Nakagawa K et al. Effects of granulocyte colony-stimulating factor, interleukin-1 alpha, and interleukin-6 on prolonged myelosuppression induced by nimustine hydrochloride in rats. J Immunother 1992;12:98-104.

36 Broerse JJ, Van Bekkum DW, Hollander CF et al. Mortality of monkeys after exposure to fission neutrons and the effect of autologous bone marrow transplantation. Int J Radiat Biol Relat Stud Phys Chem Med 1978;34:253-264.

37 Van Bekkum DW, De Vries MJ. Radiation Chimaeras. London: Logos Press Limited, 1967:128-130.

38 Pickering JE, Langham WH, Rambach WA. The effects from massive doses of high dose rate gamma radiation on monkeys. 1955. USAF School of Aviation Medicine Report No. 55-77.

39 Farese AM, Hunt P, Grab LB et al. Combined administration of recombinant human megakaryocyte growth and development factor and granulocyte colony-stimulating factor enhances multilineage hematopoietic reconstitution in nonhuman primates after radiation-induced marrow aplasia. J Clin Invest 1996;97:2145-2151.

40 Kaushansky K, Lin N, Grossman A et al. Thrombopoietin expands erythroid, granulocyte-macrophage, and megakaryocytic progenitor cells in normal and myelosuppressed mice. Exp Hematol 1996;24:265-269.

41 Jiang S, Levine JD, Fu Y et al. Cytokine production by primary bone marrow megakaryocytes. Blood 1994;84:4151-4156.

42 Wickenhauser C, Lorenzen J, Thiele J et al. Secretion of cytokines (interleukins-1 alpha, -3, and -6 and granulocyte-macrophage colony-stimulating factor) by normal human bone marrow megakaryocytes. Blood 1995;85:685-691.

43 Broudy VC, Lin NL, Kaushansky K. Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy in vitro. Blood 1995;85:1719-1726.

44 Deutsch V, Eldor A, Pick M et al. Stem cell factor synergizes with thrombopoietin in stimulating megakaryocytopoiesis. Exp Hematol 1995;23:824a.

45 Issarachai S, McCarty JM, Lin NL et al. Stem cell factor (c-kit ligand) stimulates murine megakaryopoiesis in vitro by acting through thrombopoietin (c-mpl ligand). Blood 1995;86:256a.

46 Robertson MJ, Ritz J. Biology and clinical relevance of human natural killer cells. Blood 1990;76:2421-2438.

47 Kobayashi M, Laver JH, Kato T et al. Recombinant human thrombopoietin (Mpl ligand) enhances proliferation of erythroid progenitors. Blood 1995;86:2494-2499.

48 Papayannopoulou T, Brice M, Farrer D et al. Insights into the cellular mechanisms of erythropoietin-thrombopoietin synergy. Exp Hematol 1996;24:660-669.

49 Kaushansky K, Broudy VC, Grossman A et al. Thrombopoietin expands erythroid progenitors, increases red cell production, and enhances erythroid recovery after myelosuppressive therapy. J Clin Invest 1995;96:1683-1687.

50 Van Gils FC, Westerman Y, van den Bos C et al. Pharmacokinetic basis for optimal hemopoietic effectiveness of homologous IL-3 administered to rhesus monkeys. Leukemia 1993;7:1602-1607.

51 Van Wyck DB. Iron deficiency in patients with dialysis-associated anemia during erythropoietin replacement therapy: strategies for assessment and management. Semin Nephrol 1989;9:21-24.

52 Van Wyck DB. Iron management during recombinant human erythropoietin therapy. Am J Kidney Dis 1989;4:9-13.

53 Grutzmacher P, Tsobanelis T, Roth P et al. Effect of recombinant human erythropoietin on iron balance in maintenance hemodialysis: theoretical considerations, clinical experience and consequences. Clin Nephrol 1992;38:S92.

54 Jenkins VK, Trentin JJ, Wolf NS. Radioresistance of the splenic hemopoietic inductive microenvironments (HIM). Radiat Res 1970;43:212a.

55 MacVittie TJ, Farese AM, Grab LB et al. Effect of delayed administration of recombinant human megakaryocyte growth and development factor on hematopoietic reconstitution in nonhuman primates following radiation induced marrow aplasia. Exp Hematol 1995;23:311a.

56 Wielenga JJ, Wagemaker G. Effectiveness of treatment of rhesus monkeys with recombinant human GM-CSF after total body irradiation and/or autologous stem cell transplantation. Exp Hematol 1989;17:484a.

57 van Gils FC, Mulder AH, van den Bos C et al. Acute side effects of homologous interleukin-3 in rhesus monkeys. Am J Pathol 1993;143:1621-1633.

58 Iscove NN. The role of erythropoietin in regulation of population size and cell cycling of early and late erythroid precursors in mouse bone marrow. Cell Tissue Kinet 1977;10:323-334.

59 Wagemaker G, Visser TP. Erythropoietin-independent regeneration of erythroid progenitor cells following multiple injections of hydroxyurea. Cell Tissue Kinet 1980;13:505-517.

60 Monroy RL, Skelly RR, MacVittie TJ et al. The effect of recombinant GM-CSF on the recovery of monkeys transplanted with autologous bone marrow. Blood 1987;70:1696-1699.

61 Farese AM, Williams DE, Seiler FR et al. Combination protocols of cytokine therapy with interleukin-3 and granulocyte-macrophage colony-stimulating factor in a primate model of radiation-induced marrow aplasia. Blood 1993;82:3012-3018.

62 Nienhuis AW, Donahue RE, Karlsson S et al. Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) shortens the period of neutropenia after autologous bone marrow transplantation in a primate model. J Clin Invest 1987;80:573-577.

63 Gabrilove JL, Jakubowski A, Scher H et al. Effect of granulocyte colony-stimulating factor on neutropenia and associated morbidity due to chemotherapy for transitional-cell carcinoma of the urothelium. N Engl J Med 1988;318:1414-1422.

64 Bronchud MH, Scarffe JH, Thatcher N et al. Phase I/II study of recombinant human granulocyte colony-stimulating factor in patients receiving intensive chemotherapy for small cell lung cancer. Br J Cancer 1987;56:809-813.

65 Shimazaki C, Oku N, Uchiyama H et al. Effect of granulocyte colony-stimulating factor on hematopoietic recovery after peripheral blood progenitor cell transplantation. Bone Marrow Transplant 1994;13:271-275.

66 Meisenberg BR, Davis TA, Melaragno AJ et al. A comparison of therapeutic schedules for administering granulocyte colony-stimulating factor to nonhuman primates after high-dose chemotherapy. Blood 1992;79:2267-2272.

67 Gillio AP, Bonilla MA, Potter GK et al. Effects of recombinant human granulocyte-colony stimulating factor on hematopoietic reconstitution after autologous bone marrow transplantation in primates. Transplant Proc 1987;19:153-156.

68 Lindemann A, Herrmann F, Oster W et al. Hematologic effects of recombinant human granulocyte colony-stimulating factor in patients with malignancy. Blood 1989;74:2644-2651.

69 Antich Rojas JL, Balaguer Munoz H, Girona Llobera E et al. Transient thrombocytopenia after the administration of granulocyte colony-stimulating factor in a patient with acute agranulocytosis. Sangre 1993;38:155-156.

70 Shimoda K, Okamura S, Harada N et al. Identification of a functional receptor for granulocyte colony-stimulating factor on platelets. J Clin Invest 1993;91:1310-1313.

71 Neelis KJ, Visser TP, Dimjati W et al. A single dose of thrombopoietin early after myelosuppressive total body irradiation prevents pancytopenia by promoting short-term multilineage spleen repopulating cells at the transient expense of bone marrow repopulating cells. Blood 1998;92:1586-1597.

72 Borge OJ, Ramsfjell V, Veiby OP et al. Thrombopoietin, but not erythropoietin promotes viability and inhibits apoptosis of multipotent murine hematopoietic progenitor cells in vitro. Blood 1996;88:2859-2870.

73 Ramsfjell V, Borge OJ, Cui L et al. Thrombopoietin directly and potently stimulate multilineage growth and progenitor cell expansion from primitive ($CD34^+CD38^-$) human bone marrow progenitor cells. J Immunol 1997;158:5169-5177.

74 Banu N, Wang JF, Deng B et al. Modulation of megakaryocytopoiesis by thrombopoietin: the c-Mpl ligand. Blood 1995;86:1331-1338.

75 Zeigler FC, de Sauvage F, Widmer HR et al. In vitro megakaryocytopoietic and thrombopoietic activity of c-mpl ligand (TPO) on purified murine hematopoietic stem cells. Blood 1994;84:4045-4052.

76 Young JC, Bruno E, Luens KM et al. Thrombopoietin stimulates megakaryocytopoiesis, myelopoiesis, and expansion of $CD34^+$ progenitor cells from single $CD34^+Thy\text{-}1^+Lin^-$ primitive progenitor cells. Blood 1996;88:1619-1631.

77 Tanimukai S, Kimura T, Sakabe H et al. Recombinant human c-Mpl ligand (thrombopoietin) not only acts on megakaryocyte

progenitors, but also on erythroid and multipotential progenitors in vitro. Exp Hematol 1997;25:1025-1033.

78 Fielder PJ, Gurney AL, Stefanich E et al. Regulation of thrombopoietin levels by c-mpl-mediated binding to platelets. Blood 1996;87:2154-2161.

79 Stefanich E, Senn T, Widmer R et al. Metabolism of thrombopoietin (TPO) in vivo: determination of the binding dynamics for TPO in mice. Blood 1997;89:4063-4070.

80 Marian M, Mallett P, Senn T et al. Dose-dependent kinetics of rmTPO in mice: influence of receptor-mediated clearance. Blood 1996;88:2166a.

81 Magli MC, Iscove NN, Odartchenko N. Transient nature of early haematopoietic spleen colonies. Nature 1982;295:527-529.

82 van der Loo JC, van den Bos C, Baert MR et al. Stable multilineage hematopoietic chimerism in alpha-thalassemic mice induced by a bone marrow subpopulation that excludes the majority of day-12 spleen colony-forming units. Blood 1994;83:1769-1777.

83 Bertoncello I, Hodgson GS, Bradley TR. Multiparameter analysis of transplantable hemopoietic stem cells: I. The separation and enrichment of stem cells homing to marrow and spleen on the basis of rhodamine-123 fluorescence. Exp Hematol 1985;13:999-1006.

84 van Zant G. Studies of hematopoietic stem cells spared by 5-fluorouracil. J Exp Med 1984;159:679-690.

85 Ploemacher RE, Brons RH. Separation of CFU-S from primitive cells responsible for reconstitution of the bone marrow hemopoietic stem cell compartment following irradiation: evidence for a pre-CFU-S cell. Exp Hematol 1989;17:263-266.

86 Shibuya K, Akahori H, Takahashi K et al. Multilineage hematopoietic recovery by a single injection of pegylated recombinant human megakaryocyte growth and development factor in myelosuppressed mice. Blood 1998;91:37-45.

87 Mouthon MA, Van der Meeren A, Gaugler MH et al. Thrombopoietin promotes hematopoietic recovery and survival after high dose whole body irradiation. Int J Rad Onc Biol Physics 1998 (in press).

88 Ulich TR, del Castillo J, Yin S et al. Megakaryocyte growth and development factor ameliorates carboplatin-induced thrombocytopenia in mice. Blood 1995;86:971-976.

89 Farese AM, Hunt P, Boone T et al. Recombinant human megakaryocyte growth and development factor stimulates thrombocytopoiesis in normal nonhuman primates. Blood 1995;86:54-59.

90 Kabaya K, Akahori H, Shibuya K et al. In vivo effects of pegylated recombinant human megakaryocyte growth and development factor on hematopoiesis in normal mice. STEM CELLS 1996;14:651-660.

91 Harker LA, Hunt P, Marzec UM et al. Regulation of platelet production and function by megakaryocyte growth and development factor in nonhuman primates. Blood 1996;87:1833-1844.

92 Andrews RG, Winkler A, Myerson D et al. Recombinant human ligand for MPL, megakaryocyte growth and development factor (MGDF), stimulates thrombopoiesis in vivo in normal and myelosuppressed baboons. STEM CELLS 1996;14:661-677.

93 Arnold JT, Daw NC, Stenberg PE et al. A single injection of pegylated murine megakaryocyte growth and development factor (MGDF) into mice is sufficient to produce a profound stimulation of megakaryocyte frequency, size, and ploidization. Blood 1997;89:823-833.

94 Daw NC, Arnold JT, Abushullaih BA et al. A single intravenous dose of murine megakaryocyte growth and development factor potently stimulates platelet production, challenging the necessity for daily administration. Blood 1998;91:466-474.

95 Neelis KJ, Hartong SCC, Egeland T et al. The efficacy of single-dose administration of thrombopoietin with coadministration of either granulocyte/macrophage or granulocyte colony-stimulating factor in myelosuppressed rhesus monkeys. Blood 1997;90:2565-2573.

96 Hokom MM, Lacey D, Kinstler OB et al. Pegylated megakaryocyte growth and development factor abrogates the lethal thrombocytopenia associated with carboplatin and irradiation in mice. Blood 1995;86:4486-4492.

97 Grossmann A, Lenox J, Deisher TA et al. Synergistic effects of thrombopoietin and granulocyte colony-stimulating factor on neutrophil recovery in myelosuppressed mice. Blood 1996;88:3363-3370.