THROMBOPOIETIN

A PRECLINICAL EVALUATION

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EEN PREKLINISCHE EVALUATIE

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Ik zei tegen de amandelboom:
"Spreek me van God"
En de amandelboom bloeide.

voor Aad

CONTENTS

ABBREVIATIONS

ADP	adenosine diphosphate
APTT	activated partial thromboplastin time
BFU-E	burst-forming unit-erythroid
BFU-Meg	burst-forming unit-megakaryocyte
BM	bone marrow
BSA	bovine serum albumin
CD	cluster of differentiation
CFU-E	colony-forming unit-erythrocyte
CFU-Meg	colony-forming unit-megakaryocyte
CFU-S-13	colony-forming unit-spleen day 13
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony stimulating factor
GM-CFU	granulocyte/macrophage colony forming unit
GM-CSF	granulocyte/macrophage colony stimulating factor
HFN	HHBS containing FCS and sodium azide
HHBS	Hanks' buffered Hepes solution
HIV	human immunodeficiency virus
HLA	human lymphocyte antigen
IL	interleukin
IP	intraperitoneally
ITP	idiopathic thrombocytopenic purpura
IV	intravenously
kD	kilo dalton
KL	kit ligand
LDH	lactate dehydrogenase
LIF	leukemia inhibitory factor
mAb	monoclonal antibody
MDS	myelodysplastic syndrome
MEM	minimum essential medium
MGDF	megakaryocyte growth and development factor
mpl	myeloproliferative leukemia
MRA	marrow repopulating ability
mRNA	messenger ribonucleic acid
PB	peripheral blood

PBS	phosphate buffered saline
PT	prothrombin time
RT-PCR	reverse transcriptase polymerase chain reaction
SC	subcutaneously
SCF	stem cell factor
SD	standard deviation
SIV	simian immunodeficiency virus
STLV	simian T-lymphotrophic virus
TBI	total body irradiation
TGF	transforming growth factor
TNC	total nucleated cells
TNF	tumor necrosis factor
TPO	thrombopoietin

CHAPTER 1

Introduction

1.1 HEMATOPOIESIS

Peripheral blood cells are in majority short lived and exert a whole spectrum of actions, ranging from the transport of oxygen and carbon dioxide to the production of highly specific immunoglobulins targeted at antigens. The system is very adaptive and substantially increased numbers of cells can be produced after, for example, major losses of blood, or in response to infections.

Peripheral blood cells originate from a small population of bone marrow progenitor cells, together approximately 1.5 % of all bone marrow cells, which are morphologically nearly identical and share the expression of the CD34 antigen. (1) These cells all derive from an even smaller population of hemopoietic stem cells, which have the potential to self renew and are multipotent. (2) Most of the stem cells in the bone marrow do not actively participate in blood cell formation but remain in a quiescent state. The process from the hemopoietic stem cell to mature peripheral blood cells and several specific tissue cells, termed hematopoiesis, takes approximately 20 to 30 cell divisions, through which cells become increasingly more specialized. This whole process is tightly controlled by hormone like proteins, the hemopoietic growth factors or cytokines, in combination with environmental influences conducted by stromal cells and direct cell-cell contact. (2,3) Many cytokines have become known in the last 20 years, and new cytokines and cytokine receptors are still being identified.

Immature cells in the bone marrow are positive for the CD34 antigen. This transmembrane glycoprotein was identified in 1984. (1) It is expressed on early hemopoietic progenitor cells (1) and on vascular endothelium in almost any organ. (4) Its function in hematopoiesis is as yet unknown. (5) Since CD34 expressed on endothelial cells has a function in leukocyte adhesion during inflammation, where it serves as the ligand for the adhesion molecule L-selectin on lymphocytes (6), it has been proposed that CD34 expressed on hemopoietic progenitor cells plays a role in progenitor cell adhesion in the bone marrow. However, mice deficient in CD34 expression have a normal hematopoietic system and do not display defective adhesion of hemopoietic progenitor cells and a stromal cell line in vitro. (7) The only abnormality found in these mice is an impairment of eosinophil accumulation in the lung after inhalation of an allergen. (7)

Although CD34 has apparently no functional significance for hemopoietic stem cells, its unique distribution pattern makes it a useful marker to select for immature cells, including stem cells. Transplantation experiments with CD34 negative cells showed that long term repopulating cells, which contain the presumed stem cells, are all CD34 positive, since depleting such cells from a bone marrow graft results in severe impairment of engraftment in experimental animals. (8,9) Several monoclonal antibodies against different epitopes of the CD34 antigen have been widely used for experimental applications such as cell sorting. (5,10)

1.2 MEGAKARYOCYTOPOIESIS

The production of blood platelets from the hemopoietic stem cell through megakaryocyte precursors is termed megakaryocytopoiesis. Platelets are anucleate cells circulating in the peripheral blood that play an active part in hemostasis. Normal platelet counts in humans are 200-400 x 10^9/L and platelet life span is approximately 8 days. Assuming a total blood volume of 5 liter (so a total platelet mass of approximately 1500 x 10^9), in a steady state situation each day the large number of 10^{11} new platelets are formed. This probably is an underestimate because it does not account for the bone marrow pool of platelets, which has not been quantified. Platelets are released from megakaryocytes, rare cells in the bone marrow, although they compensate for small numbers by large size. This large size is due to the last phase of megakaryocyte production in which no further mitoses take place but instead the complex process of endoreplication occurs, during which the nuclei duplicate repeatedly, but the cells do not divide. Megakaryocytes are the product of their more immature progenitor cells which ultimately are all derived from the hemopoietic stem cell. (11-13)

The process of megakaryocyte production and platelet formation has long been difficult to study *in vitro*, because of the low frequency of megakaryocytes in the bone marrow and the lack of a proper identifation of early stages of megakaryocyte progenitors, which are difficult to recognize morphologically. Furthermore, *in vitro* analysis has been hampered by the lack of a purified cytokine that regulates megakaryocytopoiesis. A specific regulator for megakaryocytopoieses has been postulated as far back as 1958 (14), and could indeed be demonstrated in serum and urine of thrombocytopenic patients and animals. (15,16) Injection of this serum in normal experimental animals induced a thrombocytosis that was inversely related to the degree of thrombocytopenia in the donor. However, it proved to be very difficult to purify this substance, due to its minute concentrations.

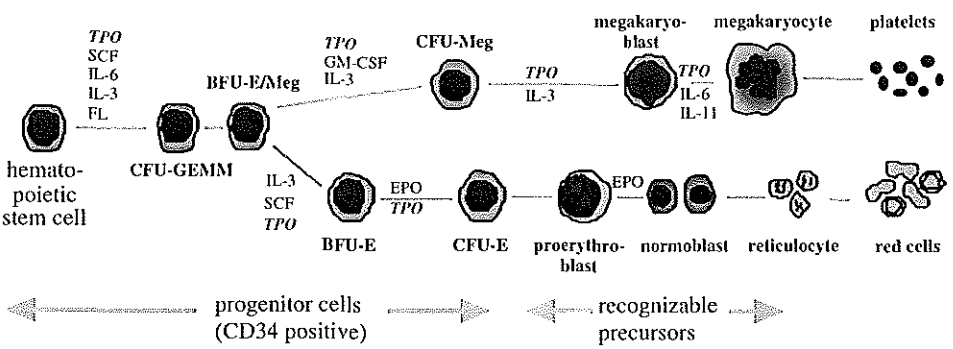

Figure 1. Schematic representation of hematopoietic stem cell differentiation into platelets and red blood cells

Assays to study megakaryocytopoiesis have been developed using serum from thrombocytopenic or aplastic donors. (17-19) Cell were grown on various semisolid or viscous media and interlaboratory comparison and standardization of the assays was difficult. However, the results were encouraging in that gradually the process of megakaryocyte development was clarified. The nomenclature for this process is similar to that used for describing the proliferation and maturation of the erythroid lineage (Fig 1). In fact, many similarities exist between the erythrocytic and megakaryocytic lineage. The two lineages share common transcription factors (20,21) and both express several surface antigens that are similar. (22,23) It has been postulated that a common precursor cell exists (24,25), and BFU-E colonies often contain megakaryocytes. (26)
The first cell in the megakaryocytic lineage that is identified by colony assays has been labeled burst forming unit-megakaryocyte (BFU-Meg). (27-29) Human colonies derived from this cell type require 21 days in culture to develop and are composed of multiple clusters of megakaryocytic cells which often number in the hundreds. This cellular entitiy is the precursor of the colony forming unit megakaryocyte (CFU-Meg), which is more mature, and gives rise to single cluster colonies in 10 to 12 days. (28,29) Early and late stages of CFU-meg differ in the number of cells per colony. Not identifiable by colony assays, but present in bone marrow as phenotypically or morphologically recognizable cells are the megakaryoblast and the megakaryocytes themselves. (30) These cells have lost the capacity to proliferate. Megakaryoblasts express the membrane markers GpIb and GpIIb/IIIa on the cell surface (31-33) and start endoreplication (34-36), a process unique to the megakaryocyte lineage. Megakaryocytes are large polyploid cells (37) which in the last stage of development develop demarkation membranes and complete cytoplasmic maturation. As a final step, functional circulating platelets are released from proplatelets formed in the demarcation membrane area of megakaryocytes. (38-42)

1.2.1 CYTOKINES INVOLVED IN MEGAKARYOCYTOPOIESIS

The cytokines influencing hematopoiesis can be subdivided in different classes, early acting cytokines, lineage restricted cytokines that act relatively late in the development of their mature end cell, and a pleiotropic class of cytokines, which influence more than one lineage or act at other stages in the development of blood cells as well. (3) Apart from such an empirical classification, one may also classify the growth factors based on their genetic relationship and/or growth factor receptor type. The latter classification would seem to be the more appropriate, since the target cell range of the growth factors and the resulting biological effects are strictly determined by the receptor distribution. Some growth factor receptors have a broad distribution pattern and, consequently the action of the ligand is pleiotropic, others have a more restricted receptor distribution and are as a consequence highly specialized.

In megakaryocytopoiesis, it is thought that a Meg-CSF stimulates the early proliferative events, and a maturation factor enhances colony formation by stimulating cytoplasmic maturation. (43,44) Growth factors that in the past two decades have been shown to affect megakaryocytopoiesis are IL-1α (45,46), IL-3 (47-50), IL-6 (51-53), IL-11 (54,55), GM-CSF (48-50,56), EPO (57-60), SCF (61,62), LIF (63,64) and Oncostatin M. (65) Although, for example, IL-3, SCF and GM-CSF stimulate megakaryocyte progenitor proliferation, and IL-6, IL-11, LIF and EPO influence megakarycyte maturation, none of those cytokines were demonstrated to be similar to the megakaryocytopoietic activity found in aplastic sera. The identification of thrombopoietin and the cloning of its gene filled the gap of a physiologic regulator of platelet production.

1.3 THROMBOPOIETIN

The existence of a cytokine specific for the megakaryocyte lineage had been postulated for decades. In 1958 Kelemen and coworkers first proposed the term thrombopoietin, on the analogy of erythropoietin, for the protein present in the plasma and urine of thrombocytopenic animals. (14) Although many cytokines were shown to influence megakaryocytopoiesis, none of them had all the attributes of the activity found in plasma from thrombocytopenic subjects, and complied with the characteristics of a physiologic regulator of platelet production. Efforts to purify the substance were initially unsuccessful. The discovery of Francoise Wendling and coworkers of a murine retrovirus that led to myeloproliferative leukemia (MPLV; myeloproliferative leukemia virus) in mice (1986)(66) and the subsequent cloning of a cellular homolog c-*mpl* that proved to be an orphan cytokine receptor (1992)(67), were of decisive importance for the discovery of the nature of thrombopoietin. The cellular variant of this virus, the oncogene c-*mpl*, was found to be expressed throughout the megakaryocytic lineage in the bone marrow, while antisense deoxynucleotides to c-*mpl* blocked CFU-meg colony growth (68), suggesting an important role in the regulation of megakaryocytopoiesis. Several groups cloned the gene encoding the ligand for this receptor simultaneously (69-73), and named it either thrombopoietin, megakaryocyte growth and development factor or megapoietin. In this thesis only thrombopoietin (TPO) will be used to refer to the recombinant *mpl* ligand. Mass production of the recombinant protein enabled an early start of preclinical experiments.

1.3.1 STRUCTURE OF TPO

Thrombopoietin is a glycoprotein, with a highly conserved amino acid sequence. (70) The gene for human thrombopoietin is located on chromosome 3q27-28 and consists of 6 exons. (74-76) Human TPO consists of 332 amino acids, and is in structure closely

related to erythropoietin (23% amino acid identity and 50% similarity) (Fig 2). (70,74) Its functional domain is the amino terminal part of 153 amino acids (70), where four highly conserved cysteines form two disulfide bridges which are both required for functional activity. (77) The amino and carboxy terminal regions are separated by a potential Arg-Arg cleavage site at position 153 that is conserved among species. (69,70,74) The carboxy terminal domain contains six potential N-linked glycosylation sites (69,70) and probably has a function in stabilization of the molecule.

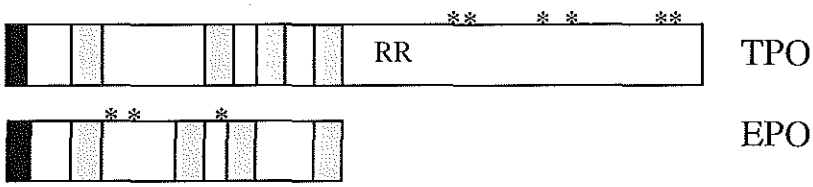

Figure 2. Schematic structures of TPO and EPO. The black boxes represent secretory leader sequences, the shaded boxes α helical segments, *: N-linked glycosylation sites, RR: two arginine residues at position 153-154.

The predicted molecular weight for the non glycosylated molecule is 35-38 kD (69,74), whereas immunoprecipitation of native material from the liver revealed a kD of 68 to 85 (74), indicating that indeed the molecule is heavily glycosylated. However, native TPO from plasma sources is smaller than 60 kD, varying from 18 to 36.7 in various species. (70-73,78) TPO isolated from rat hepatoma cell supernatants is also heterogenous in size, ranging from 17-43 kD. (79)

Isoforms of a protein can be created through alternative splicing or proteolysis. Alternatively spliced isoforms of TPO are found in the kidney, termed TPO2, (74), fetal liver (80), and mouse bone marrow. (77) The different forms of TPO arise from the use of alternative splice acceptor sites. However, agonist activity was not observed with the variant form of TPO in the Ba/F3-mpl proliferation assay (74,77), and immunoprecipitation experiments suggest that TPO2 is poorly secreted. (74)

Selective proteolysis of TPO by purified thrombin has been described as well (81), which also may account for some of the naturally occuring variants of TPO. This mechanism could play a role in the degradation of the molecule.

TPO mRNA has been demonstrated in fetal and adult liver and the kidney, which are the major places of thrombopoietin production. (69,70,76) The TPO producing cells in the liver are the hepatocytes. (79,82) Furthermore, TPO mRNA has been demonstrated in skeletal muscle (69,79,83,84), spleen (76,79,84), bone marrow stromal cells (82,85,86), brain and intestine. (79,84)

1.3.2 REGULATION OF TPO LEVELS

Levels of the humoral physiologic regulator of platelet production need to be adjusted according to the need for new thrombocytes, resulting in an inverse correlation between thrombocyte counts and cytokine levels. Long before the gene for TPO was cloned, it was demonstrated that the thrombopoietic activity present in serum, plasma and urine from thrombocytopenic animals and patients was inversely related to platelet counts. (16,87,88)

Increased levels of a protein can be achieved either by an increase in production rate or by a decrease in clearance or degradation, resulting in an increased half life. Upregulation of TPO mRNA levels as a measure of increased production, has been investigated in the liver, kidney and spleen of animals made thrombocytopenic by anti-platelet antiserum or chemotherapy (84,89-91), and in thrombocytopenic c-*mpl* -/- mice (92) that display high levels of TPO. (93) An upregulation of TPO mRNA has however not been found (84,89,90,93), with the possible exeption of an increase of bone marrow TPO transcription. (91) The significance of the latter observation is not clear, since the authors did not take into account the altered bone marrow cellularity after cytoreductive treatment.

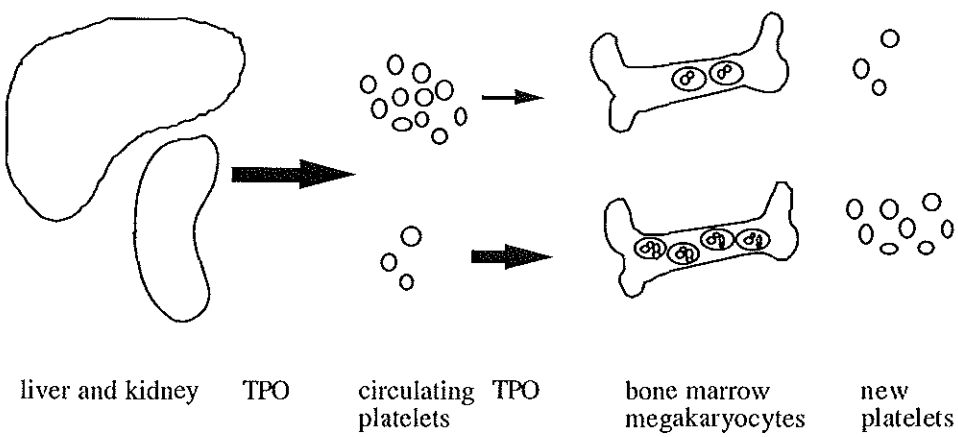

Figure 3. Schematic diagram of the regulation of TPO levels. The liver and kidney produce constant amounts of TPO. In steady state hematopoiesis (upper part of the figure) circulating platelets bind most of the TPO and the resulting plasma levels are sufficient for the production of normal numbers of new platelets. In the case of thrombocytopenia (lower part of the figure) plasma TPO levels will rise and cause increased numbers of megakaryocyte (progenitors) and higher ploidy values, resulting in increased thrombocyte production.

It is thought that the level of TPO is mainly regulated by the total mass of platelets and megakaryocytes, through a receptor mediated clearance mechanism (Fig 3). Platelets and

megakaryocytes both express the TPO receptor, and an excess of TPO in the plasma can be bound, internalised and degraded. (92,94) Isolated mouse platelets are able to reduce TPO levels in plasma in a dose dependent way. (90,92,95) Platelets from c-*mpl* -/- mice could not bind and internalize ^{125}I-TPO, resulting in a slower clearance of injected TPO in those mice. (92)

Apart from platelets, megakaryocytes also play a role in regulating TPO levels. This can be deduced from differences in TPO levels between amegakaryocytic thrombocytopenic patients, who have high TPO levels (96,97), and patients with ITP, whose TPO levels are close to normal. (97) Also NF-E2 knockout mice, which have no thrombocytes due to a defect in cytoplasmic platelet formation in megakaryocytes, do not have elevated TPO levels (98), and the clearance time of radiolabeled TPO from the circulation is not prolonged. (99)

The current concept of a constitutive production of TPO, its plasma levels being regulated by binding to c-*mpl* on megakaryocytes and platelets and subsequent degradation after internalization, would seem to be appropriate to explain variations in TPO levels in various disease states and may serve as a useful working hypothesis to explain the pharmacokinetics of exogenous TPO.

1.3.3 RECEPTOR AND SIGNAL TRANSDUCTION

c-*mpl*

Analysis of the structure of c-*mpl* indicated that it belonged to the hemopoietic growth factor receptor superfamily (Fig 4). (67,100,101) The members of this superfamily share certain structural features in the extra-cellular domain, such as 4 cysteine residues in the N terminus, and a WSXWS motif just above the transmembrane portion. The intracytoplasmic regions of these cytokine receptors lack any known signal transduction motif such as a tyrosine, or a serine/threonine kinase domain but contains two regions of partial sequence homology, termed box 1 and box 2.(102) They are formed in a double barrel, each barrel is result of antiparallel β-sandwiches. (103) This receptor family is very large and consists of the receptors for a number of interleukins, the colony stimulating factors, erythropoietin, leukemia inhibitory factor and a few non-hemopoietic cytokines such as oncostatin M, prolactin, growth hormone and ciliary neurotrophic factor. Some of the receptors homodimerize after ligand binding, others require association of a signal transducing β-chain, which may be shared by several members within the family.

Expression of the TPO receptor has been demonstrated on cells in the megakaryocytic lineage, from precursors to the final product, thrombocytes. (68,101,104) The TPO receptor on human platelets displays a high affinity for TPO with a kD of 190 pmol/l; there are approximately 30 receptors/platelet. (105,106) In normal mouse platelets an approximate receptor affinity of 560 pmol/L was found and Scatchard analysis suggests 220 binding sites per platelet. (92)

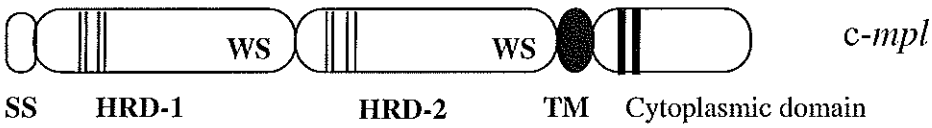

Figure 4. Schematic structure of c-*mpl*. SS = signal sequence, HRD = haemopoietin receptor domain, WS = WSXWS motif, TM = transmembrane region, |=cysteine residue and the thick black lines in the cytoplasmic domain are box 1 and 2 respectively.

TPO receptors are also found on multipotential murine cell lines (101) and on immature human and murine bone marrow cells. (68,107,108) Other cells which express the TPO receptor are endothelial cells. (68,100,109)
The TPO receptor is detected on human BFU-E colonies by RT-PCR and flow cytometry. (106) Those receptors are few in number but display a high affinity. Cross competition with erythropoietin could not be demonstrated in assays using Ba/F3 cells expressing the TPO receptor. (106) These results suggest that the stimulatory effects of TPO on red blood cell recovery after myelosuppressive therapy *in vivo* and to enhance BFU-E generation *in vitro* (as discussed later) are directly mediated by c-*mpl* on erythroid progenitor cells.

Signal transduction

Binding of TPO to c-*mpl* leads to its homodimerization (110), which initiates downstream activation of the receptor. (101,111) The cytoplasmic domains of receptors transduce signals along pathways of successive second messengers ultimately culminating in gene activation and transcription.
Deletion analysis of the intracellular domain of c-*mpl* showed that the 63 amino acids proximal to the transmembrane domain, containing box 1 and box 2 motifs, are responsible for proliferation, whereas the C-terminal domain was not necessary for this activity. (112,113) This induction of proliferation by the membrane proximal part of the receptor is mediated through activation of the JAK-STAT pathway. (112) The tyrosine kinases involved in signal transduction of the TPO receptor are JAK2 and Tyk2. (112,114-118) The STAT proteins activated by TPO were shown to be STAT1, STAT3 and STAT5. (112,116-118)
In similar experiments the C-terminal 33 amino acids were shown to be necessary for differentiation and not for proliferation. (119) The C-terminal part of the receptor is necessary Shc phosphorylation and the association of Shc with Grb2. (112,114,119,120) Thus activation of components of the Ras signalling cascade, initiated by interaction of Shc with c-*mpl*, may play a decisive role in differentiation signals emanating from the receptor. (112,119)

Platelet function

Expression of the TPO receptor on a-nuclear cells such as thrombocytes might indicate that TPO serves functions beyond the maturation of the megakaryocyte lineage. One of these functions is regulation of TPO levels, but another function could be modulation of hemostatic properties of platelets, through receptor mediated influences on platelet aggregation.

An effect of TPO on thrombocyte aggregation in platelet rich plasma or whole blood has indeed been demonstrated by several groups. On itself TPO does not influence aggregation of platelets, but when added to other agonists such as ADP, EPI, fibrinogen and thrombin, it rendered platelets more sensitive to aggregation. Addition of soluble c-*mpl* inhibited this priming effect, indicating a direct effect of TPO through receptor mediated mechanisms. This receptor-mediated effect of TPO on platelet aggregation is also suggested by the tyrosine phoshorylation of several platelet proteins, including the 85 kD subunit of phosphatidylinositol 3-kinase (PI 3-K) upon treatment of platelets with TPO. (121-124) The precise mechanism of TPO enhanced aggregation of platelets, and the physiologic implications of these observations are as yet not clear. It is important to note that thrombotic events after the administration of TPO have not been reported in specific animal models. (125,126)

1.3.4 IN VITRO ACTIVITY OF TPO

Contrary to the idea that megakaryocytopoiesis needed a Meg-CSF, which acts on progenitors inducing their proliferation, and a maturation factor, inducing platelet formation (28,44,127-129), TPO fulfilled the postulate of both the proliferation and maturation inductive activity. TPO influences cells at several maturation stages of megakaryocytopoiesis, and was shown to increase megakaryocyte size, polyploidization, expression of differentiation markers and formation of CFU-Mk on its own. (69-71,130-134) Using limiting dilution assays or single cell cultures, it was demonstrated that accessory cells were not necessary to obtain this effect (134-136), and serum free culture assays showed that it was independent of other factors present in serum. (133,134,136-138)

Adding TPO to BM megakaryocytes did not induce secretion of other cytokines such as IL-1 α and β, GM-CSF, IL-6, G-CSF, TNFα and TGF-β 1 or 2. (133) The expression of adhesion molecules on megakaryocytes did not change when TPO was added to the cultures. Furthermore, antibodies neutralizing IL-3, GM-CSF, IL-1β and IL11 did not influence TPO activity. (133)

Apart from prominently stimulating in vitro megakaryocytopoiesis, TPO also stimulates multilineage outgrowth of early murine (107,133,135,139-142) and human (136,143-145) hematopoietic progenitor cells, limited in the presence of TPO alone, but greatly in synergy with SCF and IL-3. Also, TPO suppresses apoptosis of immature cells. (146-149)

Other cells on which TPO was shown to be effective are cells from the erytroid lineage. Adding TPO to EPO containing cultures results in increased numbers of BFU-E. (106,136,145,150-152) This was not fully unexpected, since the erythroid and megakaryocyte progenitor cells probably have a common bipotential precursor (24-26), and they share a number of transcription factors (20,21) and cell surface proteins.(22,23)

1.3.5 IN VIVO ACTIVITY OF TPO

In the initial studies TPO proved to be a potent stimulator of in vivo thrombocytopoiesis in mice during rebound thrombocytosis. (70) IP injection into normal mice resulted in a fourfold increase in circulating platelet levels after 7 days. (131) This was very well illustrated by an enlarged buffy coat in hematocrit tubes, existing primarily of platelets. (69) Concurrently, an increased number of megakaryocytes with higher ploidy values was found in bone marrow and spleen. (131) Evidence exists that under certain conditions TPO, when applied in vivo will affect not only megakaryocytopoiesis and platelet levels but also erythropoiesis and immature cells.
Another approach to evaluate the function of TPO in vivo was to generate mice deficient for the receptor c-*mpl* (93,153-155), or for TPO. (153,156) c-*Mpl* -/- mice show an 85% decrease in the number of megakaryocytes and circulating platelet levels and also show increased concentrations of circulating TPO. (93) Peripheral blood cell counts of other hematologic lineages were not affected. (93,153) Interestingly, in both c-*mpl* -/- and TPO -/- mice the levels of granulocyte, erythroid and multilineage progenitor cells in the bone marrow were reduced, which might indicate that TPO plays a physiolocigal role in the maintainance of immature multilineage cells. (153,154)

1.4 RATIONALE OF THE STUDY

Thrombocytopenia is a major adverse effect of current chemotherapy regimens. Also after allogeneic and autologous bone marrow or stem cell transplantation, prolonged periods of thrombocytopenia may present prominent problems. This results in considerable morbidity, the need of intensive platelet transfusion support as hemorrhagic profylaxis, and in case of refractoriness to transfusions, mortality. Thrombopoietin, if effective, could reduce the duration and severity of thrombocytopenias.
The in vitro activities and the first in vivo effects of TPO in mice were encouraging. However, to define possible therapeutic applications, a critical evaluation in preclinical studies is required to define dose and dose schedule for optimal efficacy, predict interactions with other growth factors and detect possible adverse effects.
In this study, rhesus monkeys were used as a preclinical model to evaluate the biological effects and define possible therapeutic applications of recombinant TPO in conditions

that may be clinically relevant. TPO was administered to animals subjected to 5 Gy total body irradiation to evaluate its efficacy in mitigating the thrombocytopenia induced by cytoreductive therapy. Five Gy TBI results in approximately two log stem cell kill and a period of three weeks of profound pancytopenia. Placebo treated monkeys need on average 2 to 3 thrombocyte transfusions to prevent spontaneous bleeding. A combination of TPO and the myeloid growth factor G-CSF was also tested, since it is not unlikely that TPO will be combined with this cytokine that is already in clinical use. According to our experiences the initial dose schedules appeared supraoptimal, so that a substantial dose reduction of TPO could be achieved. In an extension of the study a single dose of TPO was administered, in combination with another registered myeloid growth factor GM-CSF. To evaluate the efficacy after bone marrow transplantation, TPO was administered to rhesus monkeys subjected to 8 Gy TBI and autologous purified stem cell transplantation, alone and in combination with G-CSF. To elucidate the background of the multilineage effect of TPO, and to adjust dose and dose schedule to achieve a maximum effect, additional experiments were carried out in myelosuppressed mice.

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CHAPTER 2

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

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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

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Abstract

Simultaneous treatment with human thrombopoietin (TPO) and granulocyte colony-stimulating factor (G-CSF) was evaluated in a placebo-controlled rhesus monkey study using 5 Gy total body irradiation (TBI) to induce 3 weeks of pancytopenia. Daily administration of TPO (10 μg/kg/day injected subcutaneously [sc] days 1–21 after TBI) promoted platelet and reticulocyte recovery, resulting in less profound nadirs and a rapid recovery to normal levels. Platelet transfusions were not required in these animals, in contrast to controls, and hemoglobin levels stabilized rapidly. TPO treatment did not influence neutrophil counts. G-CSF (5 μg/kg/day sc days 1–21) stimulated neutrophil regeneration and had no effect on platelet levels. Simultaneous treatment with TPO and G-CSF was as effective as treatment with TPO alone in preventing thrombocytopenia, although with the former regimen platelet levels did not rise to the supranormal levels seen with the latter. 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 did 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: The number of bone marrow $CD34^+$ cells was greater by two orders of magnitude in TPO-treated animals than in controls in the second week of treatment, whereas G-CSF by itself had no influence. In the third week after TBI an elevation of LDH1 values was observed in TPO-treated monkeys concurrent with normoblastosis; both of these findings were attributed to rapid erythropoiesis. TPO had no effect on hemostasis parameters. Adverse TPO and/or G-CSF effects were not observed. This study demonstrates that simultaneous TPO and G-CSF treatment after cytoreductive treatment prevents thrombocytopenia, accelerates platelet and red cell reconstitution, alleviates neutropenia, and promotes the recovery of immature bone marrow cells. The effect on $CD34^+$ GM progenitor cells may explain the augmented G-CSF responses in TPO-treated monkeys; it also suggests that TPO may become a key growth factor in the design of treatment regimens to accelerate both immature bone marrow and mature blood cell reconstitution after cytoreductive therapy.

Key words: Thrombopoietin—Myelosuppression —Granulocyte colony-stimulating factor —Total body irradiation—Rhesus monkeys

Introduction

Thrombopoietin (TPO), the ligand for the receptor encoded by the protooncogene c-mpl, was firts identified and its gene cloned in 1994 [1–3]. Because its blood levels are inversely related to platelet counts [1–3], and because mice lacking either the receptor for TPO or the ligand are severely thrombocytopenic [4,5], TPO is considered the major regulator of platelet production. Administration of recombinant TPO to experimental animals showed it to be a potent thrombopoietic agent. In normal mice, platelet counts rose more than four-fold after a 5-day administration of TPO without affecting other blood lineages [3,6]. In normal rhesus monkeys, platelet counts increased to supranormal levels in a dose-dependent manner after TPO administration for 9 days [7]. Neither red nor white blood cell counts were affected, indicating that TPO's activity is selective. TPO has also been shown to be effective in myelosuppressed mice [6,8–10]. After a single dose of carboplatin, thrombocytopenia was counteracted by TPO in a dose-dependent manner [6]. In an extended protocol using carboplatin and irradiation as myelosuppressive therapy together with a combination of TPO and G-CSF in mice, the combined treatment fully prevented mortality associated with bleeding, although it had no effect on platelet nadir [9]. In a similar model [8], stimulation of the red cell lineage was also observed. Stimulation of progenitor cell recovery has also been reported in mice after myelosuppressive treatment. In animals treated with TPO, 10-fold higher numbers of megakaryocyte, erythroid, and granulocyte/macrophage progenitor

cells were observed in femur and spleen midway through the platelet recovery period in control animals [10]. TPO was not effective after myeloablative treatment and bone marrow transplantation [11] in a syngeneic mouse model, whereas marrow from TPO-pretreated donors in the same study showed an augmented regeneration of platelet numbers.

The pharmaceutical development of TPO to counteract the adverse effects of bone marrow suppression and thrombocytopenia in patients subjected to cytoreductive therapy requires preclinical feasibility and efficacy studies in outbred nonhuman primates. Because it is anticipated that TPO will be used in conjunction with other growth factors that accelerate white cell reconstitution, rhesus monkeys were treated with TPO and G-CSF in the present study [12–16]. The monkeys were subjected to 5 Gy (x-ray) total body irradiation (TBI) to induce myelosuppression, resulting in stem cell reduction of approximately two orders of magnitude and 3 weeks of profound pancytopenia, under full supportive care. In addition to blood cell parameters, immature bone marrow cell regeneration was monitored by analysis of $CD34^+$ cells [17] and clonogenic progenitor cell assays.

Materials and methods

Animals

For the present study, purpose-bred male rhesus monkeys (*Macaca mulatta*), each weighing 2.5 to 4.0 kilograms and aged 2 to 3 years old, were used. The monkeys were housed in groups of four to six in stainless steel cages in rooms equipped with a reverse filtered air barrier, provided with normal daylight rhythm, and conditioned to 20°C with a relative humidity of 70%. Animals were given free access to commercially available primate chow, fresh fruits, and acidified drinking water. All animals were free of intestinal parasites and seronegative for herpes B, simian T-lymphotrophic viruses (STLV), and simian immunodeficiency virus (SIV). Housing, experiments, and all other conditions were approved by an ethics committee in conformity with legal regulations in The Netherlands.

Total body irradiation

Monkeys received a single dose of 5 Gy TBI delivered by two opposing x-ray generators, operating at a tube voltage of 300 kV and a current of 10 mA. The half-layer thickness was 3 mm Cu. The focus skin distance was 0.8 m and the average dose rate 0.20 to 0.22 Gy/minute. During TBI, the animals were placed in a cylindrical polycarbonate cage that rotated slowly (3 times per minute) around its vertical axis.

Supportive care

Two weeks before TBI, the monkeys were placed in a laminar flow cabinet and their gastrointestinal tracts were selectively decontaminated by oral administration of ciprofloxacin (Bayer, Mijdrecht, The Netherlands), nystatin (Sanofi BV, Maassluis, The Netherlands), and polymyxin B (Pfizer, New York, NY). This regimen was supplemented with systemic antibiotic medication when leukocyte counts dropped below 10^9/L; in most cases the antibiotic regimen, guided by fecal bacteriograms, consisted of a combination of ticarcillin (Beecham Pharma, Amstelveen, The Netherlands) and cefuroxim (Glaxo, Zeist, The Netherlands), and it was continued until leukocyte counts rose to levels higher than 10^9/L. Dehydration and electrolyte disturbances were treated by appropriate sc injections of fluids and electrolytes. The monkeys received irradiated (15 Gy γ irradiation) platelet transfusions whenever thrombocyte counts reached values below 40×10^9/L and packed red cells whenever hematocrit levels declined to below 20%; on those occasions when both thrombocytes and hematocrits dropped to these levels, the monkeys received whole blood transfusions. The level at which thrombocytes were transfused was set at $<40\times10^9$/L because monkeys already develop a propensity to petechiae and other hemorrhages at this level, which is (also) associated with mortality at the midlethal dose of radiation used in this study[18]. The hemorrhagic diathesis of monkeys subjected to 3 to 8 Gy of x-radiation has been described and reviewed earlier [19].

Test drugs

One-milliliter vials containing 0.5 mg/mL recombinant human TPO were supplied by Genentech (South San Francisco, CA). The dose used was 10 μg/kg/day injected sc once per day from days 1 to 21 after irradiation. The daily doses were diluted to a volume of 1 mL with phosphate-buffered saline (PBS)/0.01% Tween 80. Placebo-treated monkeys were given the same volume of diluent. Recombinant human G-CSF (Neupogen, Amgen, Thousand Oaks, CA) was administered in a dose of 5 μg/kg/day injected sc once per day from days 1 to 21 after irradiation. The daily doses were diluted to a volume of 1 mL in the solution recommended by the manufacturer.

Study groups

The 5-Gy irradiated monkeys were treated in groups of three or four, and in each group the monkeys were randomly assigned the four different growth factor regimens. Four monkeys were treated with TPO alone, four with G-CSF alone, and four with a combination of the two. Four monkeys were treated with the diluent alone to serve as historic controls (n = 8).

Bone marrow aspirate

Bone marrow was aspirated while the animals were under neuroleptic anesthesia using ketalar (Apharmo, Arnhem, The Netherlands) and vetranquil (Sanofi, Maassluis, The Netherlands). Small bone marrow aspirates were obtained for analytical purposes from the shafts of the humeri using pediatric spinal needles and collected in bottles containing 2 mL Hanks' buffered Hepes solution (HHBS) with 200 IU sodium heparin/mL (Leo Pharmaceutical Products, Weesp, The Netherlands). Low-density cells were isolated using Ficoll (density 1.077) (Nycomed Pharma AS, Oslo, Norway) separation.

Colony assays

Cells were plated in 35-mm dishes (Becton Dickinson) in 1 mL α-MEM (Gibco, Gaithersburg, MD) containing 0.8% methylcellulose, 5% fetal calf serum (FCS), and additives as described earlier [20–22]. For burst-forming units-erythroid (BFU-E), cultures were supplemented with hemin (2×10^{-4} mol/L), human recombinant erythropoietin (Epo: 4 U/mL; Behring, Germany) and Kit ligand (KL: 100 ng/mL; kindly provided by Dr. S. Gillis, Immunex, Seattle, WA). For granu-

locyte/macrophage colony-forming units (GM-CFU), cultures were supplemented with recombinant human GM-CSF (5 ng/mL; Behring), recombinant rhesus monkey IL-3 (30 ng/mL), produced in B. licheniformis and purified as described previously [23,24] and KL. Low density cells were plated at 5×10^4 cells per dish in duplicate. Colony numbers represent the mean ± standard deviation of bone marrow samples of individual monkeys.

Hematological examinations

Complete blood cell counts were measured daily using a Sysmex F-800 hematology analyzer (Toa Medical Electronic, Kobe, Japan). The differential of the total nucleated cells (TNC) was determined by standard counting after May-Grünwald-Giemsa staining. For reticulocyte measurements, 5 µL ethylene diamine tetraacetic acid (EDTA) blood was diluted in 1 mL PBS/EDTA/azide and 1 mL of a thiazole orange dilution was added using thiazole in a final concentration of 0.5 µg/mL. Measurements were done on a FACScan (Becton Dickinson) and analyzed using Reticount software (Becton Dickinson).

Measurements of surface antigens

Once per week, a FACScan analysis was performed on peripheral blood (PB) and bone marrow samples on the following surface antigens: CD8, CD4, CD20, CD11b, CD56, and CD16. Directly labeled monoclonal antibodies were used for CD8, CD4, CD20, CD56, and CD16 (Leu 2a-FITC, Leu 3a-PE, Leu 16-PE, Leu 19-PE, and Leu 11a-FITC [Becton Dickinson], respectively). For CD11b, we used the monoclonal antibody MO1-FITC (Coulter Immunology, Hialeah, Florida) and for CD34, a monoclonal antibody (mAb) against human CD34 (mAb 566, kindly provided by T. Egeland, University of Oslo, Oslo, Norway) that had been fluoresceinated with fluorescein isothiocyanate (FITC; Sigma) according to standard procedures. 0.5 mL of whole blood or bone marrow were lysed in 10 mL lysing solution (8.26 g ammonium chloride/1.0 g potassium bicarbonate and 0.037 g EDTA/L) for 10 minutes at 4°C. After lysing, the cells were washed twice with HHBS containing 2% FCS and 0.05% (wt/vol) sodium azide (HFN). The cells were resuspended in 100 µL HFN containing 2% normal monkey serum to prevent aspecific binding of the monoclonal antibodies. Monoclonal antibodies were added in a volume of 5 µL and incubated for 30 minutes on ice. After two washes, the cells were measured on the flow cytometer. Ungated list mode data were collected for 10,000 events and analyzed using Lysis II software (Becton Dickinson).

Clinical chemistry

Serum concentrations of sodium, potassium, chloride, glucose, albumin, total protein, aspartate-amino transferase, alanine-amino transferase, alkaline phosphatase, lactate dehydrogenase (LDH), gamma-glutamyl transpeptidase, total bilirubin, C reactive protein, creatinine, urea, and bicarbonate were analyzed twice per week using an Elan Analyzer (Eppendorf Merck, Hamburg, Germany).

Hemostasis parameters

Plasma for measurements of fibrinogen, prothrombin time (PT), and activated partial thromboplastin time (APTT) was collected twice per week and stored at –80°C. Measurements were performed on an Automated Coagulation Laboratory-100 (ACL-100, Instrumentation Laboratory, Milan, Italy). For fibrinogen measurements a commercial kit was used (fibrinogen-test; DiaMed AG, Cressier sur Morat, Switzerland); Thromborel S (Behringwerke AG, Marburg, Germany) was used for the PT and Platelin LS (Organon Teknika, Durham, NC) for the APTT.

Platelet aggregation tests were performed before treatment and at day 21 on a whole blood lumi aggregometer (Chronolog, Havertown, PA) using the standard agonists ADP (Sigma, St. Louis, MO) at a concentration of 5×10^{-5} M, thrombin (Central Laboratory of the blood bank, Amsterdam, The Netherlands) at a concentration of 5 U/mL or collagen (Sigma) at a concentration of 5 µg/mL.

TPO levels

Plasma for measurements of TPO levels was sampled from the monkeys just before cytokine administration twice per week and stored at –80°C. A full description of the TPO enzyme-linked immunosorbent assay (ELISA) has been described elsewhere [25,26]. Briefly, ELISA plates were incubated overnight at 4°C with 2 µg/mL rabbit F(ab')2 to human IgG Fc (Jackson ImmunoResearch, West Grove, PA) and for 2 hours at room temperature with conditioned medium containing 100 ng/mL of mpl-IgG [2]. Twofold serial dilutions of samples (starting at 1:10) and standards (recombinant full-length human and/or rhesus TPO) were added to wells and incubated for 1 hour. Bound TPO was detected using biotinylated rabbit antibody to full-length human TPO (Genentech) followed by peroxidase-labeled streptavidin. The range of the assay for rhesus plasma samples was 0.32 to 10 ng/mL TPO. The assay preferentially detects active full-length TPO in both rhesus monkeys and humans, and correlates well with a bioassay using the megakaryoblastic HU-3 cell line.

Statistics

If relevant, standard deviations were calculated and are given in the text, the figures, and the table on the assumption of normal distribution. The statistical significance of differences was calculated using Fisher's exact test for categorical data, and for continuous data by one-way analysis of variance followed by Student's *t*-test.

Results

Peripheral blood cell counts

Irradiated placebo-treated monkeys showed a regeneration pattern similar to that of historic controls for all lineages. On average, the nadir for platelets was far below 40×10^9/L and its time course was influenced by platelet transfusions (Fig. 1). The control monkeys needed on average 2.7 ± 1.8 transfusions. Transfusion independence (a level above 40×10^9/L) was reached on day 18.5 ± 3.7. TPO-treated monkeys did not require platelet transfusions because they had an average thrombocyte nadir of 175×10^9/L (range $78\text{-}199\times10^9$/L). In the TPO-treated animals there was an increase in platelet levels to supranormal values (775×10^9/L to 2130×10^9/L). The recovery to normal platelet values was accelerated in these animals. Pretreatment values were reached on day 13 in TPO-treated mon-

keys and on day 32 in placebo-treated controls. The effect of TPO on platelet nadir, transfusion requirement, and recovery time (defined as the first day on which thrombocyte levels remained >300×10^9/L) was statistically significant ($p < 0.02$, $p < 0.02$, and $p < 0.001$, respectively).

TPO also showed a beneficial effect in monkeys treated simultaneously with TPO and G-CSF, with a nadir of 118×10^9/L (range 81–145×10^9/L) ($p < 0.02$). The maximum platelet levels reached in these monkeys were less pronounced than in monkeys treated with TPO alone (615–786×10^9/L) (Fig. 1), although the difference did not reach statistical significance. G-CSF treated monkeys showed a pattern similar to the placebo-treated monkeys.

Mean platelet volumes dropped over the first week to 9 to 10 fL for all groups. This was influenced by platelet transfusions and started to return to normal volumes (11.5–13.0 fL) after the third week in all but the TPO + G-CSF treated animals, where volumes stabilized at levels between 10 and 11.5 fL.

TPO also had an effect on the red blood cell lineage. Reticulocyte regeneration was initiated 10 days earlier in both TPO- and TPO + G-CSF–treated monkeys compared with placebo- and G-CSF-treated monkeys ($p < 0.001$ and $p < 0.002$, respectively). Reticulocytosis was followed by normoblastosis in the TPO- and TPO + G-CSF–treated monkeys, which also occurred 10 days earlier and was more pronounced than in the placebo-treated animals (Fig. 2). The beneficial effect of TPO on the red blood cell lineage was reflected in a less profound nadir for hemoglobin and hematocrit levels and a stabilization of those values after day 30. For G-CSF- and placebo-treated monkeys the hematocrit nadir always came close to the transfusion level of 20% and four of these eight animals were given a whole blood or packed cell transfusion.

Regeneration of neutrophilic granulocytes in TPO- and placebo-treated animals followed exactly the same pattern, with values greater than 0.5×10^6/mL at day 22 (range 20–25 days) (Fig. 1). Monkeys treated with G-CSF alone reached this value on average at day 19 (range 18–21 days), and combination-treated monkeys reached it at day 14 (range 12–15 days); this difference was statistically significant ($p < 0.005$), as was the difference between the G-CSF-treated and the placebo-treated monkeys ($p < 0.05$) Remarkably, in the monkeys treated with TPO and G-CSF the neutrophil nadir was less pronounced and of much shorter duration than in the G-CSF-treated monkeys. There were no differences in lymphocyte regeneration (data not shown). For monocytes, however, the regeneration patterns were similar to those of the neutrophils (Fig. 3).

White blood cell subsets measured by flow cytometry

A profound nadir was observed for all the surface markers tested in the peripheral blood in all animals. CD8$^+$ T cells regenerated faster than CD4$^+$ T cells, but no difference was observed between the various growth factor treatment groups (data not shown). Full recovery of CD20$^+$ B cells took longer than the observation period of 6 weeks; no significant differences were noted between the four treatment groups (data not shown). The monkeys treated with TPO and G-CSF showed enhanced recovery of CD11b$^+$ cells, reflecting the recovery of neutrophils and monocytes. All TPO-treated monkeys had a slightly enhanced recovery of CD56$^+$ and CD16$^+$ cells compared with placebo- and G-CSF-treated monkeys (Fig. 4).

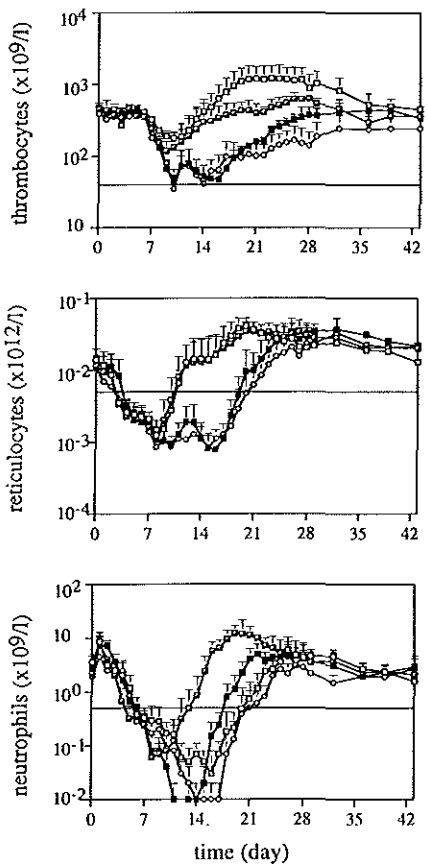

Fig. 1. Peripheral blood counts after 5-Gy TBI (day 0). Shown are changes in levels of thrombocytes (upper panel), reticulocytes (middle panel) and neutrophils (lower panel) in response to TPO (open squares,n=4), TPO and G-CSF (half-filled squares, n=4), G-CSF (black squares, n=4) and placebo treated monkeys (open circles,n=4). Data represent the arithmetic mean ± SD of the various treatment groups. Horizontal lines define the degree of cytopenia: 40×10^9/L for thrombocytes, 0.05×10^{12}/L for reticulocytes and 0.5×10^9/L for neutrophils. Vertical lines indicate SD, given in a positive direction only to avoid confusion.

Bone marrow cellularity and progenitor cell content

The monkeys treated with TPO displayed a markedly accelerated recovery of bone marrow cellularity, as shown by the bone marrow cell counts of 13 ± 23 10^6 cells/ml. aspirate observed in the animals on day 15 compared with 0.3 ± 0.2 for the placebo monkeys on the same day (Table 1). This fea-

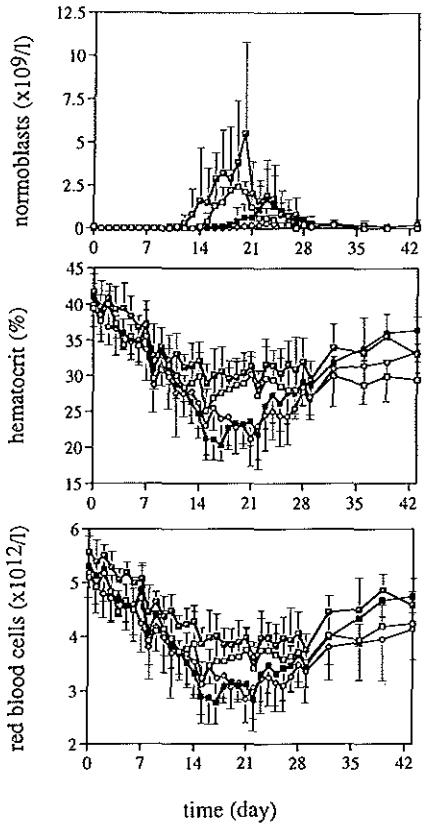

Fig. 2. Regeneration of the red blood cell lineages. Shown are normoblast (upper panel), hematocrit (middle panel), and red blood cell numbers (lower panel). See Figure 1 for an explanation of symbols. Data represent means ± SD of the various treatment groups. Vertical lines indicate SD, given in either a positive or a negative direction.

ture was also apparent in the TPO- and G-CSF-treated monkeys and could not be attributed to the G-CSF treatment. However, G-CSF treatment also improved bone marrow regeneration, as was apparent from the data obtained 1 week later. There was a decline in bone marrow cellularity in TPO and TPO- + G-CSF-treated monkeys at the end of the fourth week, probably reflecting the cessation of growth factor treatment at 3 weeks.

TPO stimulation of bone marrow recovery also occurred in bone marrow progenitor cells, i.e., GM-CFU and BFU-E, although to a greater degree in the TPO-treated monkeys than in those treated with G-CSF (Table 1). Perhaps of greater relevance, immature $CD34^+$ cells in the TPO-treated monkeys

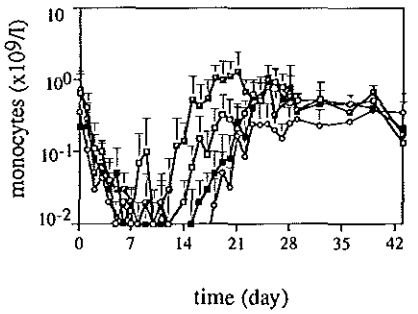

Fig. 3. Absolute numbers of monocytes after 5-Gy TBI. See Figure 1 for an explanation of symbols.

showed a 100-fold increase at 2 and 3 weeks after TBI compared with the same cells in monkeys treated with G-CSF or placebo ($p < 0.05$) (Fig. 5). In general, cellularity and progenitor cell content followed the same pattern.

Clinical chemistry parameters in serum

In most of the parameters, abnormalities were not observed. Albumin concentrations were stable at approximately 40 g/L. Electrolyte disturbances were minor during the first week after TBI because of gastrointestinal radiation damage. In addition, liver enzyme alkaline phosphatase, ALAT, and ASAT showed only minor changes. The only enzyme showing abnormalities was lactate dehydrogenase isotype 1 (LDH1). LDH1 was markedly elevated on day 3 in all treatment groups and in all TPO-treated monkeys also in the third week after TBI, in contrast to the placebo-treated controls in which it remained within the normal range. A correlation was noted between elevations in LDH1 levels and the absolute number of normoblasts appearing in the circulation (Figs. 2 and 6), suggesting that rapid (and perhaps somewhat ineffective) erythropoiesis was the source of these elevated levels. The day 3 LDH1 levels, which were elevated to the same extent in all monkeys, are most likely attributable to rapid cell disintegration shortly after irradiation.

Hemostasis parameters

No differences were found between APTT, PT, and fibrinogen levels among the various treatment groups, and fluctuation over time after irradiation did not occur (data not shown). Platelet aggregation measurements in the treatment groups did not differ from those in placebo-treated controls (data not shown).

TPO levels

TPO levels were below detection level (320 pg/mL) in all pretreatment samples. TPO levels varied from 6 to 24 ng/mL in samples taken from TPO-treated monkeys on days 3 to 21 post-TBI, after which TPO administration was stopped. These levels decreased to less than 2 ng/mL on day 24 and became undetectable on day 28 (Fig. 7). In animals treated with G-CSF and in control monkeys, low levels of TPO were detected in

samples taken on days 10, 14, and 17, concurrent with thrombocytopenia (Fig. 1); the TPO levels in these animals varied from 0.3 to 0.6 ng/mL. Obviously, the rise in TPO levels in the placebo-treated controls had been suppressed by deliberate thrombocyte transfusions in these animals whenever their platelet counts fell below 40×10^9/L.

Discussion

This study shows that TPO was highly effective in preventing thrombocytopenia following radiation-induced myelosuppression, an affect characterized by alleviation of platelet nadirs, accelerated platelet recovery, and substantial reduction in the time needed to reach normal platelet levels. TPO also stimulated red cell reconstitution and exerted no influence on neutrophilic granulocytes, monocytes, or lymphocytes. In addition, TPO markedly promoted immature $CD34^+$ bone marrow cell reconstitution, as was reflected in the increased numbers of immature progenitor cells observed in the granulocyte/macrophage and erythroid lineages. Concurrent administration of G-CSF did not enhance TPO-stimulated recovery of platelets, although platelet levels in monkeys the third week of combined G-CSF/TPO treatment tended to be lower than in the monkeys treated with only TPO during the same period. A similar dampening effect of G-CSF on the TPO response has recently been described in mice [27] and may be related to the protracted thrombopenia observed in stem cell transplanted monkeys [28], which were treated with G-CSF. Remarkably, TPO treatment considerably augmented G-CSF-stimulated neutrophil and monocyte reconstitution. Adverse systemic effects were not observed with administration of either growth factor.

The thrombopoiesis-stimulating effect of TPO was already sufficiently effective in the first week of treatment, as was shown by an increase in platelet numbers starting at day 8. In addition, the effect of TPO on bone marrow cellularity and immature bone marrow cells was already clear by the end of the second week of treatment. This suggests that a dose schedule of 21 consecutive days of treatment is excessive, as was clear from the very high platelet numbers reached in the second and third weeks after TBI. It is conceivable that a short course of TPO treatment initiated immediately after cytoreductive therapy would be sufficient to prevent thrombocytopenia. We have previously shown in a mouse model of thrombocytopenia that a single dose of TPO given 24 hours after cytoreductive therapy is as effective as daily dosing for 8 consecutive days [29]. The effect of single injections in this model was also highly dose-dependent.

TPO was clearly more effective in stimulating platelet recovery than other growth factors known to stimulate platelet production, including IL-6 [30–33], IL-11 [34,35], IL-3 [32,33], and IL-1 [36]. All of these cytokines stimulate platelet production but not sufficiently to prevent thrombocytopenia at tolerable doses, especially in view of the many side effects observed.

A central issue regarding TPO treatment is prevention of bleeding as a consequence of myelosuppression. Our policy of transfusing donor thrombocytes when platelet levels decline to 40×10^9/L is based on the finding that this level coincides with the first appearance of petechiae and other bleeding; its rationale is to prevent undue deaths resulting from hemorrhages. For obvious reasons, this level is higher than the level

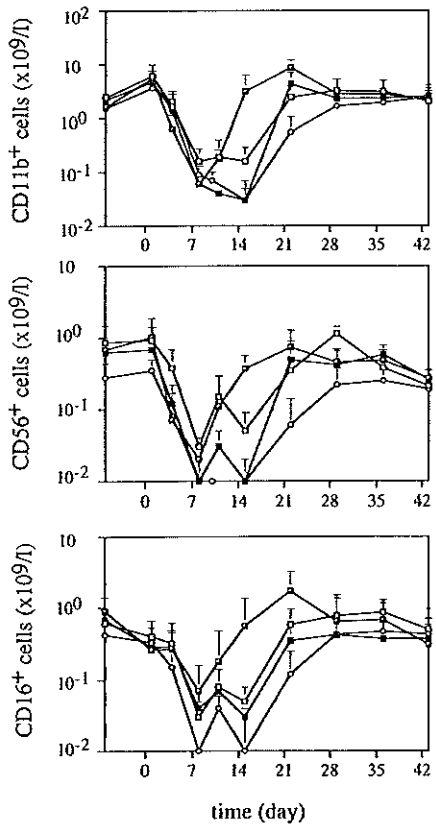

Fig. 4. Absolute number of peripheral blood cells positive for surface markers CD11b (upper panel), CD56 (middle panel), and CD16 (lower panel). See Figure 1 for an explanation of symbols.

used in human patients, to whom instructions can be given and sensitization to alloantigens should be avoided. Based of the decrease in thrombocyte counts in placebo-treated monkeys one week after TBI, and based on the fact that these levels began to increase in the third week after TBI, it can be inferred that without transfusions the thrombocyte levels would have dropped below 10×10^9/l within 2 days after the first transfusion (Fig. 1). It can thus be concluded that the TPO treatment, which prevented the decline in thrombocyte counts to levels lower than 100×10^9/L early in the second week after irradiation (Fig. 1), also effectively prevented bleeding.

An association between smaller platelets and stimulated megakaryocytopoiesis has been observed previously during experimental thrombocytopenia induced by antibody-mediated platelet consumption [37,38], and also occurs in normal

Table 1. Bone marrow cellularity ($\times 10^6$), GM-CFU numbers ($\times 10^3$), and BFU-E numbers ($\times 10^3$) per mL aspirate ± SD after TBI and growth factor treatment

		Before	Day 8	Day 15	Day 22	Day 29
Cellularity	T	65 ± 41	0.7 ± 0.3	13 ± 23	78 ± 67	29 ± 15
$\times 10^6$/mL	T+G	103 ± 65	0.5 ± 0.1	27 ± 28	136 ± 76	38 ± 19
	G	62 ± 45	0.3 ± 0.2	0.5 ± 0.3	41 ± 43	43 ± 24
	pl	43 ± 17	0.6 ± 0.5	0.3 ± 0.2	4.3 ± 2.7	13.2 ± 12
GM-CFU	T	80 ± 70	ND*	24 ± 28	103 ± 53	29 ± 17
($\times 10^3$)	T+G	137 ± 86	ND	41 ± 54	84 ± 59	24 ± 11
	G	85 ± 82	ND	ND	45 ± 52	87 ± 118
	pl	45 ± 31	ND	ND	7 ± 6	15 ± 24
BFU-E	T	32 ± 31	ND	22.5	21 ± 11	10 ± 7
($\times 10^3$)	T+G	66 ± 46	ND	21 ± 39	44 ± 46	7 ± 2
	G	39 ± 41	ND	ND	12 ± 14	32 ± 41
	pl	21 ± 14	ND	ND	2 ± 2	5 ± 7

*ND=not done because of low numbers of cells.
T: TPO treated monkeys (n = 4).
T + G: TPO + G-CSF treated monkeys (n=4).
G: G-CSF treated monkeys (n = 4)
pl: placebo treated monkeys (n = 4).

nonhuman primates [39]. The mechanism of this association is not known. The decreases in platelet volume observed in this study were similar in all treatment groups and were therefore not related either to the stimulation with pharmacological concentrations of TPO or to the circulating platelet mass. Platelet function was unaltered in that platelet aggregation measurements on the last day of growth factor administration did not differ from pretreatment measurements and did not vary among the treatment groups.

The effect of TPO on early hematopoietic progenitor cells of different lineages was unexpected, but is in line with other recent observations [10,40] and consistent with the previous finding that the receptor for TPO is present on immature progenitor cells [41]. Because megakaryocytes are capable of releasing various growth factors [42,43], the TPO effect

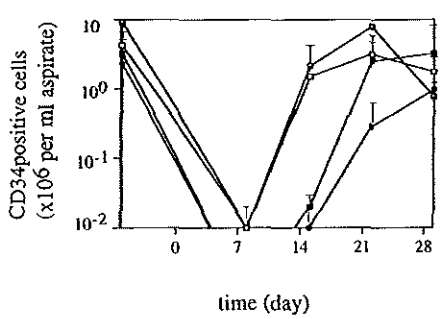

Fig. 5. Absolute number of CD34⁺ cells in the bone marrow after 5-Gy TBI. See Figure 1 for an explanation of symbols. Data represent arithmetic mean ± SD of the various treatment groups.

observed in our study 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 exists in vitro [44–46]. The accelerated recovery of progenitors cells is perhaps most clearly illustrated by the two-log increased reconstitution of immature CD34⁺ cells in the bone marrow aspirates of TPO-treated monkeys at the end of the second week of treatment. The magnitude of this effect surpassed that of any other growth factor tested in the same animal model, including IL-3 and IL-6 (manuscript in preparation). The accelerated recovery of GM progenitor cells may explain the augmented response to G-CSF in the neutrophil and monocyte lineages, also reflected by CD11b⁺ cells. Apparently G-CSF also stimulated the CD16/CD56⁺ lineage, thought to represent natural killer cells originating in the bone marrow [47]. Because the G-CSF dosage was supraoptimal, the augmented responses to G-CSF are best explained by increased numbers of progenitor cells. However, in view of the diminished platelet response in the monkeys treated simultaneously with TPO and G-CSF, we do not exclude the possibility that TPO and G-CSF also compete for a common progenitor cell.

TPO also affected the red cell lineage very early after irradiation, as reflected by the fact that exponential reticulocyte regeneration began after the first week of treatment, 10 days earlier than in placebo- and G-CSF-treated controls. Reticulocytosis was followed by normoblastosis, which was accompanied by elevated LDH serum levels; we attribute these last two findings to rapid and inefficient erythropoiesis. As a result, hemoglobin concentrations and hematocrit levels stabilized earlier at higher levels. However, in TPO-treated monkeys, signs of a microcytic, hypochromic anemia became apparent after the third week, suggesting depletion of iron stores caused by rapid erythropoiesis. In a separate study, the development of iron deficiency during TPO treatment was directly confirmed, as was its prevention by administration of prophy-

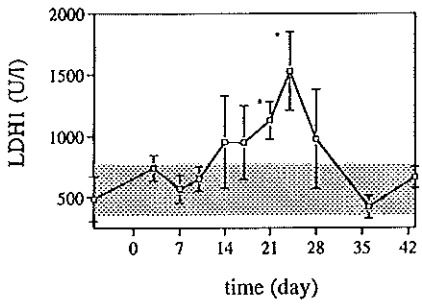

Fig. 6. Elevation of LDH1 values in TPO-treated monkeys (n=4) after 5-Gy TBI. The shaded area represents the mean ± SD of the LDH1 serum levels in 14 normal monkeys. Measurements marked with * were significantly different from normal ($p<0.05$).

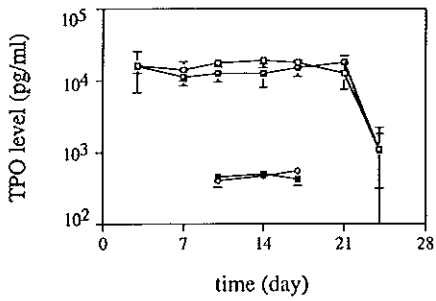

Fig. 7. TPO levels in plasma samples measured by ELISA. See Figure 1 for an explanation of symbols. Samples taken before treatment and at days 3, 7, 10, 14, 17, 21, 24 and 28. In the control and G-CSF-treated monkeys, data not shown were below detection level (320 pg/mL).

lactic intramuscular iron [48]. We infer from this that iron status needs to be assessed before, and monitored during, TPO treatment. It should be noted that the dosage of TPO used in this study was higher than required to normalize platelet levels and will likely be unnecessary for the clinical therapy of myelosuppressed patients. It is not clear why normoblastosis followed reticulocytosis in TPO-treated monkeys, a pattern that runs counter to what has been seen with other growth factors such as IL-3 [49] and GM-CSF (unpublished observation). Since normoblastosis was highly reduced in monkeys prophylactically supplemented with iron [48], the pattern observed might be related to developing iron deficiency resulting in ineffective erythropoiesis during the course of TPO-stimulated hematopoietic reconstitution. It should be noted that the onset of mild normoblastosis in placebo-treated control monkeys, which did not develop significant iron deficiency, paralleled the onset of reticulocyte regeneration.

The present study demonstrates that TPO treatment following cytoreductive treatment prevents thrombocytopenia, accelerates platelet and red cell reconstitution, and promotes the recovery of immature bone marrow cells. In addition, simultaneous G-CSF and TPO treatment alleviates neutropenia more effectively than G-CSF alone, thereby decreasing the risk of bleeding and infection, which are common complications of intensive cancer treatment. We interpret the effect of TPO on immature bone marrow cells as an indication that this growth factor may play a key role in the design of treatment regimens directed at acceleration of both immature bone marrow and mature blood cell reconstitution after cytoreductive therapy.

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CHAPTER 3

Prevention of thrombocytopenia by thrombopoietin in myelosuppressed rhesus monkeys accompanied by prominent erythropoietic stimulation and iron depletion

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Prevention of Thrombocytopenia by Thrombopoietin in Myelosuppressed Rhesus Monkeys Accompanied by Prominent Erythropoietic Stimulation and Iron Depletion

By Karen J. Neelis, Luo Qingliang, G. Roger Thomas, Bob L. Cohen, Dan L. Eaton, and Gerard Wagemaker

The effectiveness of thrombopoietin (TPO) in alleviating thrombocytopenia was evaluated in a placebo-controlled study involving rhesus monkeys exposed to 5 Gy total-body irradiation (TBI) (300-kV x-rays) to result in 3 weeks of pancytopenia. Supraoptimal treatment with human recombinant TPO (10 μg/kg/d subcutaneously, days 1 to 21 after TBI) was highly effective in preventing thrombocytopenia, with nadirs for thrombocytes, on average, far higher than 100 × 10^9/L, a greatly accelerated recovery to normal values, and no need for thrombocyte transfusions. TPO appeared to act selectively in that neutrophil regeneration was not influenced but red blood cell lineage recovery was prominently stimulated, with reticulocyte regeneration being initiated 10 days earlier than in placebo-treated animals. The reticulocytosis was followed by a normoblastosis that occurred earlier and was more pronounced than in placebo-treated monkeys. The effect of TPO on the red blood cell lineage was also reflected in a less profound nadir for hemoglobin (Hb) and hematocrit values than in placebo controls. However, this effect was not followed by a rapid recovery to normal values, due to development of a microcytic hypochromic anemia. Iron depletion was demonstrated by measurements of total serum iron and total iron-binding capacity (TIBC) and could be prevented by prophylactic intramuscular (IM) iron before TBI or corrected by IM iron after TPO treatment. Rechallenging with TPO in week 8 after TBI demonstrated a homogenous thrombocyte response similar in magnitude to the initial response, but a greatly diminished reticulocyte response. This demonstrated that the erythropoietic response to TPO administration depends on the hemopoietic state of the animal and may reflect multiple TPO target cells. It is postulated that the extremely rapid erythropoiesis due to TPO treatment in the initial regeneration phase following myelosuppression results in iron depletion by a mechanism similar to that seen following erythropoietin treatment in patients with end-stage renal failure. It is concluded that protracted TPO therapy to counteract thrombocytopenic states may result in iron depletion and that the iron status should be monitored before, during, and after TPO treatment.

THROMBOPOIETIN (TPO), the ligand for c-mpl, was identified and its gene cloned in 1994.[1-3] In vitro and in vivo evaluation showed it to be the major regulator of thrombocyte production.[4-7] In vitro experiments also demonstrated, apart from megakaryocyte colony formation,[6,8,9] effects on erythroid progenitors in synergy with erythropoietin.[10,11] In normal experimental animals, thrombocyte counts increased to supranormal values without affecting other lineages.[3,7,12-15] In several myelosuppression models, TPO has also been found to stimulate, apart from the megakaryocytic lineage, erythroid precursors,[13,14,16,17] expansion of immature bone marrow progenitor cells,[18] and, to a lesser extent granulocyte-macrophage colony-forming units.[16,17] Adverse effects have not been observed. In the present study, TPO was evaluated in a rhesus monkey model for myelosuppression to investigate its ability to alleviate thrombocytopenia. The monkeys were subjected to 5 Gy x-ray total-body irradiation (TBI) resulting in 3 weeks of profound pancytopenia. We report here that TPO, apart from greatly promoting thrombocyte regeneration after myelosuppression, also stimulated erythropoiesis, leading to iron depletion and resulting in microcytic anemia.

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MATERIALS AND METHODS

Animals. Purpose-bred male rhesus monkeys (*Macaca mulatta*) weighing 2.5 to 4.0 kg and aged 2 to 3 years were used. The monkeys were housed in groups of four to six in stainless steel cages in rooms with a reverse-filtered air barrier and normal daylight rhythm and conditioned to 20°C with a relative humidity of 70%. Animals were fed ad libitum with commercial primate chow and fresh fruits and received acidified drinking water. All animals were free of intestinal parasites and were seronegative for herpes B, simian T-lymphotrophic viruses and simian immunodeficiency virus. The housing, experiments, and all other conditions were approved by an ethics committee in accordance with legal regulations in The Netherlands.

TBI. Monkeys were irradiated with a single dose of 5 Gy TBI delivered by two opposing x-ray generators operating at a tube voltage of 300 kV and a current of 10 mA. The half-layer thickness was 3 mm Cu. The focus skin distance was 0.8 m, and the mean dose rate 0.20 to 0.22 Gy/min. During TBI, the animals were placed in a cylindrical polycarbonate cage that rotated slowly (three times per minute) around its vertical axis.

Experimental procedure. Two weeks before TBI, the monkeys were placed in a laminar-flow cabinet, and the gastrointestinal tract was selectively decontaminated by giving oral Ciproxin (Bayer, Mijdrecht, The Netherlands), nystatin (Sanofi, Maassluis, The Netherlands), and polymyxin B (Pfizer, New York, NY). This regimen was maintained until leukocyte counts exceeded 10^9/L. Systemic antibiotics were given when leukocyte counts were less than 10^9/L, in most cases as a combination of Ticarpen (Beecham Pharma, Amstelveen, The Netherlands) and Zinacef (Glaxo, Zeist, The Netherlands), as guided by fecal bacteriograms. Dehydration and electrolyte disturbances were treated by appropriate fluid and electrolyte administration subcutaneously. The monkeys received irradiated (15 Gy) thrombocyte transfusions whenever thrombocyte counts were less than 40 × 10^9/L and irradiated packed red blood cells whenever hematocrits were less than 20%, and occasionally, the monkeys received whole blood transfusions in case of simultaneous occurrence of both transfusion criteria. All monkeys were bled for diagnostic purposes, 7 mL before irradiation, 7 mL twice weekly during

the first 4 weeks, and 3 mL once weekly thereafter. Three milliliters of bone marrow was aspirated before irradiation and once weekly for 6 weeks after TBI. The total blood volume of 3-kg monkeys is estimated to be 200 mL.

Test drug. One milliliter vials containing 0.5 mg/mL recombinant full-length human TPO produced by CHO cells were supplied by Genentech Inc (South San Francisco, CA). The dose used was 10 μg/kg/d subcutaneously once daily from day 1 to day 21 after irradiation (n = 4). Three monkeys received the same dose for a second period of 7 days from day 52 to day 58 after irradiation. The daily doses were diluted to a volume of 1 mL with phosphate-buffered saline (PBS)/0.01% Tween 80. Placebo-treated monkeys were only given the same volume of diluent (n = 4).

Hematologic examinations. Complete blood cell counts were measured daily using a Sysmex F-800 hematology analyzer (Toa Medical Electronics Co, Kobe, Japan). The differential of the total nucleated cells was determined by standard counting after May-Grünwald-Giemsa staining. For reticulocyte measurements, 5 μL EDTA blood was diluted in 1 mL PBS/EDTA (5.0 mol/L)/azide (0.05% wt/vol) and 1 mL of a thiazole orange dilution was added, using thiazole in a final concentration of 0.5 μg/mL. Measurements were made on a FACScan (Becton Dickinson, San Jose, CA) and analyzed using the Reticount software (Becton Dickinson).

Iron administration. Three monkeys received iron supplementation. One monkey received 0.5 mL Imferon (Fe(III) 50 mg/mL; Fisons Pharmaceuticals, Loughborough, England) for 5 consecutive days before irradiation, and two monkeys received 3 mL in 10 days approximately 3 months after irradiation. Imferon was given intramuscularly.

Clinical chemistry. Serum levels of sodium, potassium, chloride, glucose, albumin, total protein, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase isotype 1 (LDH1), γ-glutamyl transferase, total bilirubin, C-reactive protein, creatinine, urea, and CO_2 were measured twice weekly using an Elan Analyzer (Eppendorf Merck, Hamburg, Germany). Total serum iron and total iron-binding capacity (TIBC) were determined in representative stored serum samples, and the percentage saturation was calculated.

Postmortem material. As arranged with the ethics committee for animal experiments operating at our university, all animals were euthanized at the end of the study. Most of the animals were euthanized at day 43 after irradiation by injecting 2 mL Euthasate (Apharmo, Arnhem, The Netherlands; pentobarbital 200 mg/mL) intravenously. For two of the TPO-treated monkeys, the follow-up time was prolonged to further evaluate the anemia, and for one placebo-treated monkey for other reasons. At the time of obduction, organs were fixed with 4% paraformaldehyde, and about 24 hours later the material was embedded in paraffin and slides were made stained with hematoxylin/eosin. Slides for iron staining were made from the paraffin-embedded material.

Statistics. If relevant, the mean ± SD was calculated with the assumption of a normal distribution. The significance of a difference was calculated by one-way analysis of variance followed by a Student's *t*-test.

RESULTS

Peripheral blood cell counts. TPO was highly effective in alleviating thrombocytopenia after irradiation, and recovery to normal values was significantly accelerated (Fig 1). The dose schedule of TPO was supraoptimal, as is clear from the increase to supranormal thrombocyte counts in the third week of treatment. The kinetics of the thrombocyte response strongly suggests that TPO treatment during the first week after TBI would have been sufficient for prevention of thrombocytopenia. One week after cessation of TPO

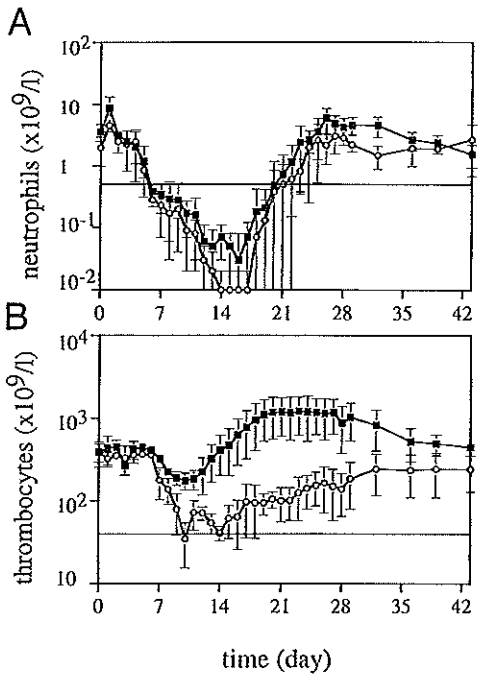

Fig 1. Neutrophil (A) and thrombocyte (B) regeneration after 5 Gy TBI for TPO-treated monkeys (■, n = 4) and placebo-treated controls (○, n = 4).

treatment, thrombocyte counts gradually returned to normal. TPO treatment was selectively effective in that neutrophil regeneration was not influenced (Fig 1), with values more than 0.5 × 10^9/L being reached at day 20 (range, 20 to 23). In addition, TPO had a prominent effect on red blood cell parameters. Reticulocyte regeneration, on average, was 10 days earlier than in placebo-treated controls, and TPO-treated monkeys developed a normoblastosis that was also earlier and much more pronounced (Fig 2). All monkeys developed anemia, partly due to diagnostic bleeding. The decrease in hemoglobin (Hb) concentration was similar in all monkeys until day 14 after TBI (Fig 3). TPO-treated monkeys showed less profound nadirs than placebo-treated controls, and Hb levels stabilized after the second week. However, the earlier stabilization of Hb levels was not reflected in an earlier recovery to normal values. Instead, TPO-treated monkeys developed a sustained anemia, whereas placebo-treated monkeys were able to recover to subnormal Hb levels at the end of the observation period (6 weeks) (Fig 3). The anemia of TPO-treated monkeys was microcytic (Fig 4) and hypochromic (Table 1) in nature. The difference in mean cell volume (MCV) kinetics is most apparent if the data set of each monkey is shown individually. The MCV of TPO-treated monkeys showed an initial increase that coincided with the reticulocyte recovery and was followed by a

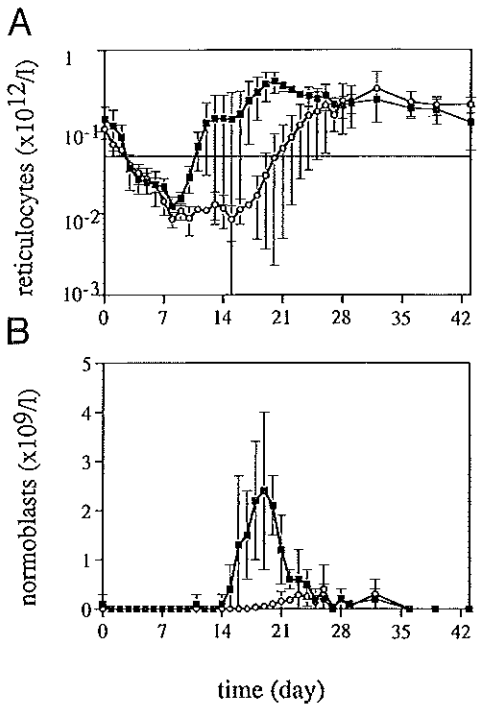

Fig 2. Reticulocyte regeneration (A) and the appearance of normoblasts (B) after 5 Gy TBI for TPO-treated monkeys (■, n = 4) and placebo-treated controls (○, n = 4).

gradual decline in the fifth and sixth weeks of treatment, whereas placebo-treated monkeys all had an increase in MCV values from the third week onward, also coinciding with reticulocyte recovery. Mean cellular Hb (MCH) was also decreased in TPO-treated monkeys in the sixth week after TBI compared with pretreatment values, whereas in placebo-treated monkeys MCH values remained constant (Table 1).

TPO rechallenge and iron supplementation. To examine the effectiveness of TPO after myelosuppression without limited iron supplies, one monkey was given intramuscular (IM) iron during the week before TBI, followed by TPO treatment. The nadir for Hb and hematocrit was similar to those of the four monkeys treated with TPO earlier, but the iron-treated monkey had a regeneration of Hb levels much earlier than the other TPO-treated monkeys and the placebo controls (Fig 3) and did not develop a microcytic anemia. Thrombocyte regeneration of the iron-treated monkey was in the range of that for the four monkeys treated earlier (Fig 5). In an extended follow-up period, two TPO-treated monkeys and the single TPO/iron-treated monkey were rechallenged with TPO in week 8 after TBI, after which a thrombocyte production response similar to that reported for normal nonhuman primates occurred[15] (Fig 5). All monkeys had a small but significant increase in reticulocyte counts, which only in the iron-treated monkey was followed by a small increase in Hb levels after an initial decline due to diagnostic bleeding. In the other two monkeys, Hb levels stabilized. Subsequent IM iron administered to the latter animals over a period of 10 days in the third month after TBI resulted in a prompt increase of Hb and hematocrit levels (Fig 6) and a correction of anemia.

Clinical parameters and transfusion requirements. The number of febrile episodes was not different between the two groups. Normal axillary body temperature in rhesus monkeys is 39°C, and fever is defined as a morning temperature more than 40°C. TPO-treated monkeys had 3.7 ± 2.9 days with fever, and placebo-treated controls 4.5 ± 3.5 days, statistically nonsignificant (P = .76).

Since iron contained in blood transfusions might confound our results, the transfusion requirement of the monkeys is also presented. TPO-treated monkeys did not receive any blood products during the experiment. All placebo-treated monkeys received several thrombocyte transfusions, which are not considered to contain significant amounts of iron. Three of four placebo-treated controls received a whole blood transfusion at days 15, 21, and 26 after TBI, respectively. This is too late in the time course to account for the difference in the Hb nadir occurring in the third week after TBI.

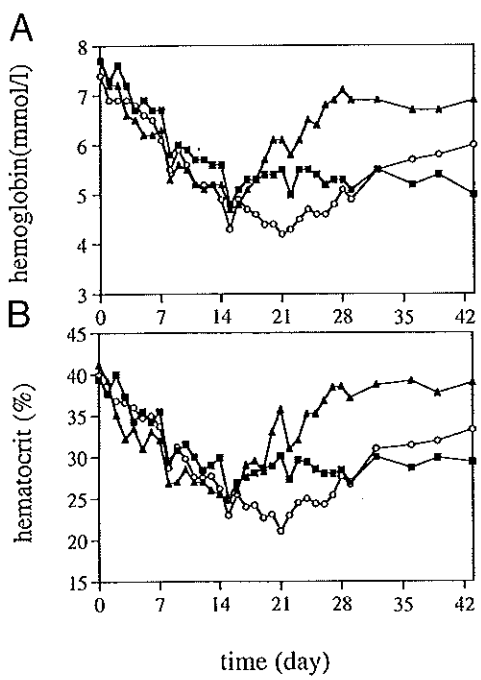

Fig 3. Hb levels (A) and hematocrit (B) after 5 Gy TBI for TPO-treated monkeys (■, n = 4), placebo-treated controls (○, n = 4), and one iron-pretreated TPO-treated monkey (▲).

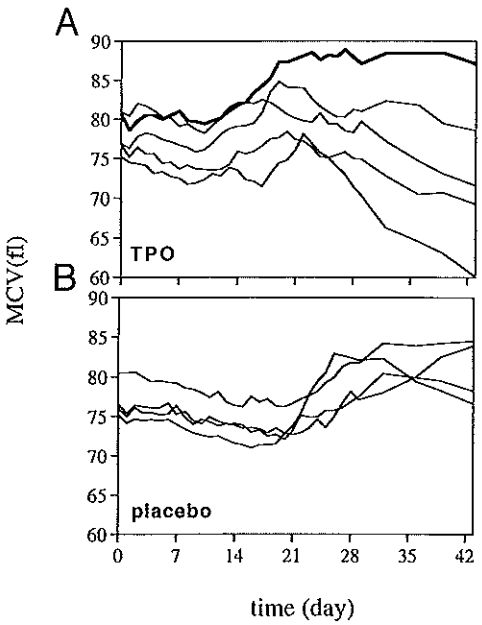

Fig 4. MCV after 5 Gy TBI for TPO-treated monkeys (A) and placebo-treated controls (B). Each line represents an individual monkey: bold line, iron-pretreated TPO-treated monkey.

Clinical chemistry. TPO-treated animals showed increased serum LDH1 levels due to the rapid but insufficient erythropoiesis. However, the iron-pretreated monkey did not display this elevation in LDH1 levels, and the normoblastosis seen in TPO-treated monkeys was also reduced in this monkey (data not shown).

Table 1. Serum Iron/TIBC, Total Serum Iron, and MCH Following TBI

Parameter	Preirradiation	Day 14	Day 28	Day 43
Serum iron/TIBC (%)				
TPO	27 ± 12	35 ± 8‡	12 ± 7	6 ± 1†‡
Placebo	31 ± 13	62 ± 11†	30 ± 19	17 ± 8
Serum iron (μmol/L)				
TPO	21 ± 8	28 ± 8‡	10 ± 5	5 ± 1†‡
Placebo	22 ± 8	41 ± 8†	21 ± 13	11 ± 4
Iron/TPO	39*	31	23	20
MCH (amol)				
TPO	1,508 ± 94	1,464 ± 141	1,427 ± 113	1,210 ± 255
Placebo	1,437 ± 61	1,393 ± 64	1,471 ± 67	1,456 ± 75

TPO-treated monkeys, n = 4; placebo-treated monkeys, n = 4; iron/TPO (monkey treated with iron before irradiation and TPO after irradiation), n = 1.

* After iron supplementation.

† Statistically significantly different from preirradiation, $P < .05$.

‡ Statistically significantly different from time-matched placebo-treated monkeys, $P < .05$.

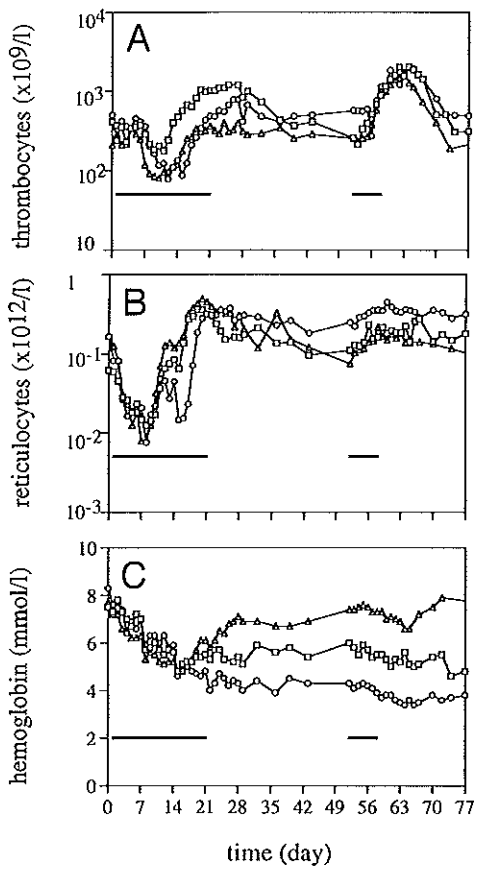

Fig 5. Effect of a rechallenge with TPO (10 μg/kg/d) from days 52 to 58 in three monkeys on thrombocytes (A), reticulocytes (B), and Hb (C). Two TPO-treated monkeys (□, ○) and one iron-pretreated, TPO-treated monkey (△). Black lines, duration of TPO treatment.

To assess the iron status of the monkeys before and during therapy, total serum iron and TIBC of representative stored serum samples were determined. Iron saturation was 27% ± 12% before irradiation, but decreased to much lower levels 6 weeks after irradiation (6% ± 1% for TPO, $P = .04$; 17% ± 8% for placebo, nonsignificant). The difference between TPO- and placebo-treated monkeys at week 6 is also significant ($P < .03$; Table 1). Serum iron followed a similar pattern in that at day 14 free serum iron was higher, probably due to diminished iron utilization in the initial period after TBI during which erythropoiesis was profoundly suppressed (Table 1). In the iron-pretreated monkey, total serum iron also decreased steadily after TBI, but not to a degree sufficiently severe to induce anemia. In TPO- and placebo-treated

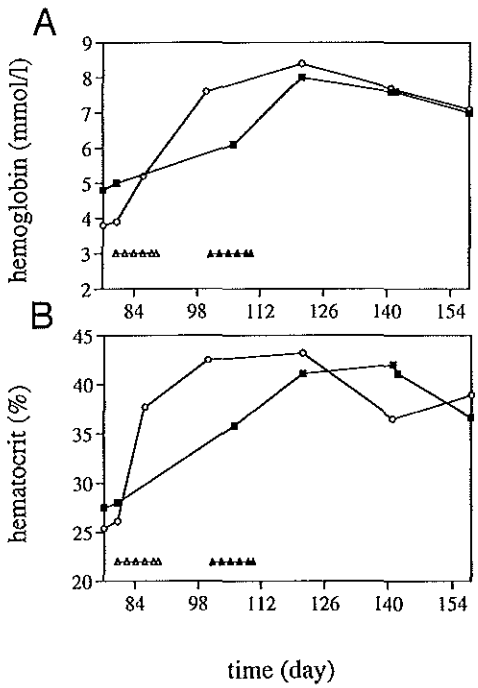

Fig 6. Effect of iron treatment in two anemic TPO-treated monkeys. (A) Hb level; (B) hematocrit. Iron was administered at the indicated time points. (△) Correspond with the monkey represented with (○), and (▲) with (■). At the indicated time points, 30 mg Fe(III) was administered.

monkeys, iron stores were not detectable in bone marrow slides stained for iron (data not shown).

DISCUSSION

TPO was effective in stimulating thrombocyte recovery after a myelosuppressive exposure to TBI and fully prevented the development of thrombocytopenia. An effect on neutrophil regeneration was not observed. TPO also prominently stimulated the erythroid lineage, an effect that occurred very early after irradiation: exponential reticulocyte regeneration started after the first week of treatment, 10 days earlier than in placebo-treated controls. The reticulocytosis was followed by a normoblastosis accompanied by elevated LDH serum levels, attributed to the rapid and probably inefficient erythropoiesis. As a result, Hb levels and hematocrits stabilized earlier at higher levels. However, in TPO-treated monkeys, signs of a microcytic hypochromic anemia became apparent after the third week, suggesting depletion of iron stores. In this study, the development of iron deficiency during TPO treatment was directly confirmed, as was its correction by IM iron treatment and prevention by prophylactic IM iron.

The kinetics of serum iron and TIBC displayed an initial increase during the 2 weeks after TBI, which is explained by a lack of iron utilization due to myelosuppression. This increase is particularly prominent in the placebo-treated monkeys, but is also statistically significant in the TPO-treated monkeys (Table 1). The smaller increase of serum iron measured on day 14 after TPO compared with the placebo control levels is attributed to the early onset of erythropoietic reconstitution in TPO-treated monkeys. The subsequent decline in serum iron and serum iron/TIBC is larger in TPO-treated monkeys, resulting in very low levels and a state of iron deficiency at the end of the observation period of 6 weeks (Table 1) in TPO-treated monkeys. It is not excluded that the iron deficiency essentially originated from a precarious iron balance in rhesus monkeys, similar to humans, resulting in latent iron deficiency already present before radiation exposure. This is more likely, since the MCV in the animals before irradiation was 75 to 80 and increased to 90 in the animal subjected to iron supplementation. The latent iron deficiency may then be caused by diet, malabsorption due to the preirradiation antibiotic regimen, or some other process related to iron absorption.

Three TPO-treated monkeys, two without iron supplementation and one provided with prophylactic IM iron before TBI, were rechallenged with TPO in week 8 after TBI after full recovery from myelosuppression, and showed a homogeneous stimulation of thrombocyte production. This indicated that (1) the effect of TPO on thrombocytes is independent of iron status, since only the iron-pretreated monkey had a demonstrated normal iron status at that time point, and (2) the variation of the TPO response following TBI should not be attributed to individual sensitivity of the animals to TPO. Rather, this should be attributed to a variation in the number of residual TPO target cells following TBI, possibly due to individual sensitivity to radiation. The rechallenge experiments also demonstrated a differential reticulocyte response to TPO early and late after TBI. Although the platelet responses were similar in magnitude, reticulocyte responses were much diminished upon rechallenge (Fig 5). This may reflect either multiple target cells of TPO and/or involvement of growth factors other than TPO in the reticulocyte response. The magnitude of reticulocytosis upon rechallenge was roughly inversely proportional to the Hb level, indicating an effect codependent on erythropoietin.

Iron supplementation to two anemic monkeys proved to be effective in treating anemia. Iron prophylaxis before TBI completely prevented microcytic anemia and Hb levels quickly returned to normal, further demonstrating the pronounced effect of TPO on red blood cell reconstitution in the initial phase after TBI.

Multilineage effects, including the erythroid lineage, of TPO have been shown in vitro and in vivo.[10,11,16,18] 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.[14,16,19] Since megakaryocytes are capable of releasing various growth factors,[20,21] the effect observed may be an indirect consequence of stimulation of the megakaryocytic lineage. This does not exclude a direct effect of TPO, since erythroid progenitors have been shown in vitro to be directly stimulated by TPO.[11] The iron depletion of TPO-

treated monkeys is reminiscent of erythropoietin-induced iron deficiency anemia in patients with end-stage renal failure.[22-24] A similar mechanism is proposed for the iron deficiency anemia occurring in the present study. Supplementation of iron before and careful monitoring during treatment proved to be necessary for all patients receiving erythropoietin. It is concluded that TPO therapy to counteract thrombocytopenic states is highly effective to prevent thrombocytopenia, and that its beneficial effect on erythroid regeneration may be hampered by iron depletion. Therefore, the iron status should be carefully monitored in case of therapeutic TPO administration.

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CHAPTER 4

The efficacy of single-dose administration of thrombopoietin with co-administration of either granulocyte/macrophage or granulocyte colony-stimulating factor in myelosuppressed rhesus monkeys

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The Efficacy of Single-Dose Administration of Thrombopoietin With Coadministration of Either Granulocyte/Macrophage or Granulocyte Colony-Stimulating Factor in Myelosuppressed Rhesus Monkeys

By Karen J. Neelis, Simone C.C. Hartong, Torstein Egeland, G. Roger Thomas, Dan L. Eaton, and Gerard Wagemaker

Thrombopoietin (TPO) was evaluated for efficacy in a placebo-controlled study in rhesus monkeys with concurrent administration of either granulocyte/macrophage colony-stimulating factor (GM-CSF) or granulocyte CSF, (G-CSF). Rhesus monkeys were subjected to 5 Gy total-body irradiation (TBI), resulting in 3 weeks of profound pancytopenia, and received either TPO 5 μg/kg intravenously (IV) at day 1 (n = 4), GM-CSF 25 μg/kg subcutaneously (SC) for 14 days (n = 4), TPO and GM-CSF (n = 4), G-CSF 10 μg/kg/d SC for 14 days (n = 3), TPO and G-CSF (n = 4), or placebo (carrier, n = 4; historical controls, n = 8). Single-dose IV treatment with TPO 1 day after TBI effectively counteracted the need for thrombocyte transfusions (provided whenever thrombocyte levels were <40 × 10^9/L) and accelerated platelet reconstitution to normal levels 2 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, similar to the placebo-treated monkeys, which required, on average, three 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 neutrophil counts less than 0.5 × 10^9/L by approximately 1 week; TPO/GM-CSF treatment elevated the neutrophil nadir, but did not further accelerate recovery to normal values. TPO also augemented the neturophil response to G-CSF, resulting in similar patterns of reconstitution following TPO/G-CSF and TPO/GM-CSF treatment. TPO/GM-CSF resulted in significantly increased reconstitution of $CD34^+$ bone marrow cells and progenitor cells such as GM-CFU and BFU-E. Adverse effects of combining TPO with the CSFs were not observed. It is concluded that (1) a single IV administration of TPO is sufficient to prevent severe thrombocytopenia following myelosuppression, (2) TPO/G-CSF and TPO/GM-CSF treatment result in distinct response patterns, with TPO/GM-CSF being superior to TPO/G-CSF in stimulating thrombocyte and erythrocyte recovery while being equivalent in stimulating neutrophil recovery; and (3) TPO significantly improves the performance of CSFs in alleviating severe neutropenia.

IDENTIFICATION of thrombopoietin (TPO)[1-4] as the major regulator of thrombocyte production[5,6] has resulted in novel insights into the regulation of immature hematopoietic cell differentiation,[7-9] and has potentially provided a therapeutic approach to counteract thrombocytopenic states, particularly those associated with intensive cytoreductive treatment of malignancies. Its pharmaceutical development for the latter application requires demonstration of efficacy in experimental animal models alone and 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 approach other than detailed experimental animal in vivo studies. We have presently focused on growth factors that are likely to be used clinically with TPO, ie, granulocyte/macrophage colony-stimulating factor (GM-CSF) and granulocyte CSF (G-CSF).

In previous studies in myelosuppressed mice and rhesus monkeys, a supraoptimal dose of human TPO with or without concurrent administration of G-CSF was found to prevent thrombocytopenia, accelerate platelet and red blood cell reconstitution, alleviate neutropenia, and promote recovery of immature bone marrow cells.[13-15] The latter observation was unexpected, but was consistent with the demonstration of TPO receptors on immature hematopoietic cells[7] and with more recent reports on stimulation of immature cells by TPO.[8,9] TPO also effectively promoted the neutrophil response to G-CSF, an effect thought to be mediated by TPO-stimulated bone marrow progenitor cell expansion.

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 therapy[19-23] to alleviate the cytopenia, particularly the neutropenia resulting from intensive cytoreductive treatment.[21] Although G-CSF and GM-CSF are grossly similar in the pharmaceutical profile,[24,25] GM-CSF has advantages in that it also stimulates megakaryocytopoiesis and monocyte differentiation.[26,27] In addition, G-CSF was found to dampen thrombocyte production. This was also observed in a transplant model in rhesus monkeys,[28] as well as after TPO and G-CSF treatment of irradiated mice.[16] The beneficial effects of both GM-CSF and G-CSF on neutropenia following cytoreductive treatment are in most studies restricted to approximately a 5-day earlier recovery, or less in dose-intensified chemotherapy,[21,23,29] for a total median neutropenia of about 20 to 25 days. It is therefore of consid-

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erable importance to select combinations of growth factors that provide optimal costimulatory efficacy.

The study was undertaken (1) to explore the option of limiting the total dose of TPO, based on previous studies in mice[30] showing that a single administration of TPO might be sufficient to prevent thrombocytopenia following cytoreductive treatment; and (2) to compare concurrent administration of TPO and GM-CSF versus TPO and G-CSF, and to identify optimal growth factor therapy to counteract both neutropenia and thrombocytopenia. The study involved rhesus monkeys exposed to 5 Gy total-body irradiation (TBI), which results in a profound pancytopenia for 3 weeks, and use of an optimal dose of TPO on the first day and G-CSF or GM-CSF treatment for the first 14 consecutive days after TBI. On-study parameters included, apart from blood cell counts, assessment of immature bone marrow cells and monitoring of adverse effects.

MATERIALS AND METHODS

Animals. Purpose-bred male rhesus monkeys (*Macaca mulatta*) weighing 2.5 to 4.0 kg and aged 2 to 3 years were used. The monkeys were housed in groups of 4 to 6 in stainless steel cages in rooms with a reverse-filtered air barrier, normal daylight rhythm, and conditioned to 20°C with a relative humidity of 70%. Animals were fed ad libitum with commercial primate chow and fresh fruits and received acidified drinking water. All animals were free of intestinal parasites and were seronegative for herpes B, simian T-lymphotropic viruses and simian immunodeficiency virus. The animal housing, experiments, and all other conditions were approved by an ethics committee in conformity with legal regulations in The Netherlands.

TBI. Monkeys were irradiated with a single dose of 5 Gy TBI delivered by two opposing x-ray generators operating at a tube voltage of 300 kV and a current of 10 mA. The half-layer thickness was 3 mm Cu. The focus skin distance was 0.8 m and the average dose rate 0.20 to 0.22 Gy/min. During TBI, the animals were placed in a cylindrical polycarbonate cage that rotated slowly (three times per minute) around its vertical axis.

Supportive care. Two weeks before TBI, the monkeys were placed in a laminar-flow cabinet, and the gastrointestinal tract was selectively decontaminated by administering oral ciprofloxacin (Bayer, Mijdrecht, The Netherlands), nystatin (Sanofi, Maassluis, The Netherlands), and polymyxin B (Pfizer, New York, NY). This regimen was supplemented with systemic antibiotics, in most cases ticarcillin (Beecham Pharma, Amstelveen, The Netherlands) and cefuroxim (Glaxo, Zeist, The Netherlands), when leukocyte counts were less than 10^9/L. Guided by fecal bacteriograms, antibiotics were continued until leukocyte counts increased to more than 10^9/L. Dehydration and electrolyte disturbances were treated by appropriate fluid and electrolyte administration subcutaneously (SC). The monkeys received irradiated (15 Gy γ-irradiation) platelet transfusions whenever thrombocyte counts were less than 40×10^9/L, packed red blood cells whenever hematocrits were less than 20%, and, occasionally, whole-blood transfusions in case of coincidence of both transfusion criteria. The criterion of transfusion of thrombocytes at counts less than 40×10^9/L was chosen because monkeys already develop a propensity to petechiae and other hemorrhages at this level. These are associated with mortality at the midlethal dose of radiation used.[31-33]

Test drugs. Recombinant full-length rhesus monkey TPO produced by Chinese hamster ovary cells was supplied by Genentech Inc (South San Francisco, CA). The dose used was 0.5, 5, or 50 μg/kg IV on day 1 after TBI. The dose was diluted to a volume of 1 mL with phosphate-buffered saline (PBS)/0.01% Tween 20 before administration. Placebo-treated monkeys were only given the same volume of diluent. Recombinant human G-CSF (Neupogen; Amgen Inc, Thousand Oaks, CA) was administered at a dose of 10 μg/kg/d SC once daily during days 1 to 14 after TBI. Recombinant human GM-CSF (Leukine; Immunex Corp, Seattle, WA) was given at a dose of 25 μg/kg/d SC once daily during days 1 to 14 after TBI. The daily doses were diluted to a volume of 1 mL in the solution indicated by the suppliers.

TPO levels. Serum for measurement of TPO levels was sampled from the monkeys 24 hours after cytokine administration and stored at −20°C. A full description of the TPO enzyme-linked immunosorbent assay (ELISA) has been reported elsewhere.[34,35] Briefly, ELISA plates were incubated overnight at 4°C with 2 μg/mL rabbit F(ab')2 to human IgG Fc (Jackson ImmunoResearch, West Grove, PA) and 2 hours at room temperature with conditioned medium containing 100 ng/mL mpl-IgG.[1] Twofold serial dilutions of samples (starting at 1:10) and standards (recombinant full-length human and/or rhesus TPO) were added to wells and incubated for 1 hour. Bound TPO was detected using biotinylated rabbit antibody to full-length human TPO (Genentech), followed by peroxidase-labeled streptavidin. The range of the assay for rhesus serum samples is 0.32 to 10 ng/mL TPO. The assay preferentially detects active full-length TPO, rhesus TPO equally as well as human TPO, and correlates well with a bioassay using the megakaryoblastic HU-3 cell line.

Study groups. Monkeys were randomly assigned to the treatment groups and received either rhesus TPO IV on day 1 after TBI at a dose of 5 μg/kg (n = 4), 0.5 μg/kg (n = 2), or 50 μg/kg (n = 1), GM-CSF at a dose of 25 μg/kg/d SC from days 1 to 14 (n = 4), TPO and GM-CSF (TPO/GM-CSF, n = 4), G-CSF at a dose of 10 μg/kg/d SC (n = 3), TPO and G-CSF (TPO/G-CSF, n = 4), or placebo (n = 4; historical controls, n = 8). The TPO dose of 5 μg/kg on day 1 after TBI was administered to the monkeys that also received GM-CSF or G-CSF SC for 14 consecutive days.

Bone marrow aspirates. Bone marrow was aspirated under neuroleptic anesthesia using Ketalar (Apharmo, Arnhem, The Netherlands) and Vetranquil (Sanofi, Maassluis, The Netherlands). Small bone marrow aspirates for analytical purposes were taken from the shaft of the humerus using pediatric spinal needles and collected in bottles containing 2 mL Hanks buffered HEPES solution (HHBS) with sodium heparin 200 IU mL (Leo Pharmaceutical Products, Weesp, The Netherlands). Low-density cells were isolated using Ficoll separation (density = 1.077; Nycomed Pharma, Oslo, Norway).

Colony assays. Cells were plated in 35-mm dishes (Becton Dickinson, Leiden, The Netherlands) in 1 mL α-DMEM (GIBCO, Gaithersburg, MD) containing 0.8% methylcellulose, 5% fetal calf serum (FCS), and additives as described previously.[36,37] For burst-forming units–erythroid (BFU-E), cultures were supplemented with hemin (2×10^{-4} mol/L), human recombinant erythropoietin (4 U/ml; Behring, Germany), and Kit ligand (KL) 100 ng/mL; kindly provided by Dr S. Gillis, Immunex, Seattle, WA). For granulocyte/macrophage colony-forming units (GM-CFU), cultures were supplemented with recombinant human GM-CSF (5 ng/mL; Behring), recombinant rhesus monkey interleukin-3 (30 ng/mL) produced in *Bacillus licheniformis* and purified as described previously,[39,40] and KL. Low-density cells were plated at 5×10^4 per dish in duplicate. Colony counts were calculated per milliliter of bone marrow aspirated using the recovery of cells over the Ficoll density gradient. Colony numbers represent the mean ± SD of bone marrow samples of individual monkeys.

Hematologic examinations. Complete blood cell counts were measured daily using a Sysmex F-800 hematology analyzer (Toa Medical Electronics Co, Kobe, Japan). The differential of the nucleated cells was determined by standard counting after May-Grünwald-Giemsa staining. For reticulocyte measurements, 5 μL EDTA blood

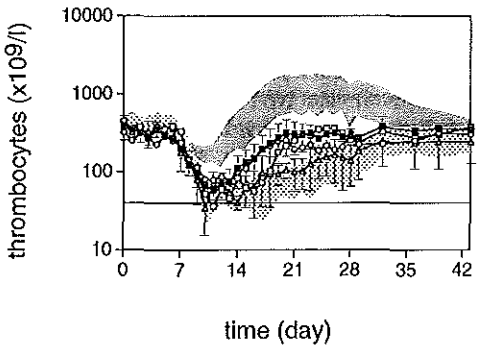

Fig 1. Thrombocyte counts after 5 Gy TBI (day 0) for monkeys treated with TPO 5 μg (■, n = 4), TPO 50 μg (□), TPO 0.5 μg (○, n = 2), and the concurrent placebo treatment (△, n = 4). The lower shaded area represents the mean ± SD of 12 control monkeys; the upper shaded area is the mean ± SD of 4 monkeys treated with human TPO for 21 days after irradiation. Data represent the arithmetic mean ± SD of the various treatment groups. The horizontal line defines the level of thrombocytopenia (40 × 10^9/L) below which thrombocyte transfusions are given.

was diluted in 1 mL PBS/EDTA/azide and 1 mL thiazole orange dilution was added, using thiazole at a final concentration of 0.5 μg/mL. Measurements were made on a FACScan (Becton Dickinson, Leiden, The Netherlands) and analyzed using the Reticount software.

Measurement of surface antigens. Once weekly, a FACScan analysis was made on peripheral blood and bone marrow samples on the following surface antigens: CD8, CD4, CD20, CD11b, CD56, CD16, and CD34. Directly labeled monoclonal antibodies (MoAbs) were used for CD8, CD4, CD20, CD56, and CD16 (Leu 2a-FITC, Leu 3a-PE, Leu 16-PE, Leu 19-PE, and Leu 11aFITC [Becton Dickinson], respectively). For CD11b, the MoAb MO1-FITC (Coulter Immunology, Hialeah, FL) was used, and for CD34, an MoAb against human CD34 (MoAb 566) that had been fluoresceinated with FITC (Sigma, St Louis, MO) according to standard procedures. Whole blood or bone marrow (0.5 mL) was lysed in 10 mL lysing solution (8.26 g ammonium chloride, 1.0 g potassium bicarbonate, and 0.037 g EDTA per liter) for 10 minutes at 4°C. After lysis, the cells were washed twice with HHBS containing 2% FCS and 0.05% (wt/vol) sodium azide (HFN). The cells were resuspended in 100 μL HFN containing 2% normal monkey serum to prevent aspecific binding of the MoAbs. MoAbs were added in a volume of 5 μL and incubated for 30 minutes on ice. After two washes, the cells were measured on the flow cytometer. Ungated list-mode data were collected for 10,000 events and analyzed using the Lysis II software (Becton Dickinson).

Statistics. Standard deviations were calculated and are presented in the text and figures on the assumption of a normal distribution. The significance of differences was calculated by Fisher's exact test for categorical data, and for continuous data by a one-way analysis of variance followed by a nonpaired Student's *t*-test.

RESULTS

Dose of rhesus monkey TPO and pattern of thrombocyte reconstitution. Based on data in normal monkeys[41] and extrapolation of doses used in mice,[30] as well as the supraoptimal dose of 10 μg/kg human TPO in a previous study,[13] a single IV dose of 5 μg/kg rhesus monkey TPO at day 1 after TBI was considered optimal. The thrombocyte regeneration of four monkeys treated in this way is shown in Fig 1 in comparison to placebo controls and the previous data on human TPO administered for 21 consecutive days at a dose of 10 μg/kg/d. The data for the four concurrent control monkeys have been included in Fig 1 in addition to the eight historical controls; significant differences were not observed in the regeneration patterns of any of the blood cell lineages between these groups of controls. The single-dose treatment was effective in that the four TPO-treated monkeys needed only two thrombocyte transfusions (one in each of two monkeys), as opposed to the four concurrent placebo controls, which needed a total of 13 transfusions (mean, 3; range, 1 to 6) since their thrombocytes had decreased to less than 40 × 10^9/L; this is statistically significant ($P < .04$; Table 1). In addition, TPO-treated monkeys displayed a clearly accelerated thrombocyte reconstitution and reached normal thrombocyte levels 2 weeks before the placebo-treated controls ($P < .03$).

To validate the choice of the dose of 5 μg/kg IV further, one monkey was treated with a 10-fold higher dose and two monkeys with a 10-fold lower dose. The results are also presented in Fig 1. Rhesus monkey TPO at the single dose of 50 μg/kg IV was effective in preventing thrombocyte transfusions, with a nadir for thrombocytes of 75 × 10^9/L at day 13. Thrombocyte reconstitution following this dose precisely coincided with the mean values for monkeys treated with the 5-μg/kg dose, on which basis we considered that the latter dose provided the maximal stimulation to be expected from a single administration of TPO 1 day after TBI. Monkeys treated with 0.5 μg/kg both had nadirs lower

Table 1. Transfusion Requirements and Blood Cell Regeneration After 5 Gy TBI and Growth Factor Treatment

Treatment	No. of Monkeys	No. of Transfusions	Thrombocytes >40 × 10^9/L (d)	Reticulocytes >1% (d)	Neutrophils >0.5 × 10^9/L (d)
TPO	4	0/0/1/1*	NA	14.2 ± 3.6*	21.5 ± 2.4
TPO + GM-CSF	4	0/0/0/0*	NA	10.5 ± 1.7*	14.5 ± 2.6*
TPO + G-CSF	4	0/0/1/0*	NA	13.2 ± 2.6*	14.2 ± 1.2*
GM-CSF	4	3/2/2/2	13.2 ± 1.7	16.2 ± 2.5*	17.7 ± 2.2*
G-CSF	3	1/2/1	13.3 ± 1.5	17.3 ± 0.6	19.7 ± 3.2
Placebo	4	3/6/3/3	19.5 ± 7.0	20.5 ± 2.1	22.5 ± 2.4

Data for individual monkeys or the mean ± SD are shown.

Abbreviation: NA, not applicable, ie, level <40 × 10^9/L not reached in the majority of monkeys.

* Statistically significantly different from placebo-treated monkeys ($P < .05$).

than 40 × 10^9/L, and needed one and two thrombocyte transfusions, respectively, providing a further validation of the dose of 5 μg/kg as being close to the minimum required for a maximal response.

TPO levels. TPO levels were measured in serum collected 24 hours after cytokine administration (day 2 after irradiation), and for some monkeys also in samples taken later in the first week after irradiation. Levels measured were 1.9 ± 0.5 ng/mL for TPO-treated monkeys and 2.5 ± 0.4 for monkeys treated with TPO and G-CSF or GM-CSF; this difference is not significant. TPO levels were not different for monkeys treated simultaneously with either TPO/G-CSF or TPO/GM-CSF. Levels at day 3 or 4 were just above the detection limit of the assay (31.2 pg/mL in a 1:10 dilution) at about 0.4 ng/mL in some monkeys, and below the detection limit in others.

Comparison of peripheral blood cell counts in monkeys treated with TPO/GM-CSF and TPO/G-CSF. In contrast to monkeys treated with TPO alone, of which two needed a single thrombocyte transfusion, monkeys treated with TPO/GM-CSF remained completely transfusion-free and showed an even more elevated nadir, with two of four monkeys never reaching thrombocyte levels less than 100 × 10^9/L.

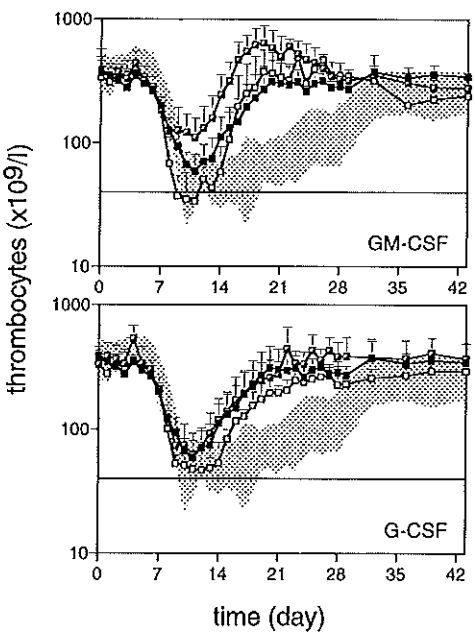

Fig 2. Thrombocyte counts after 5 Gy TBI (day 0) for monkeys treated with TPO (■, n = 4), TPO/GM-CSF (▨, n = 4), or GM-CSF (□, n = 4) in the upper panel and TPO/G-CSF (▨, n = 4) or G-CSF (□, n = 3) in the lower panel. Data represent the arithmetic mean ± SD of the various treatment groups. The horizontal line defines the level of thrombocytopenia (40 × 10^9/L) below which thrombocyte transfusions are given.

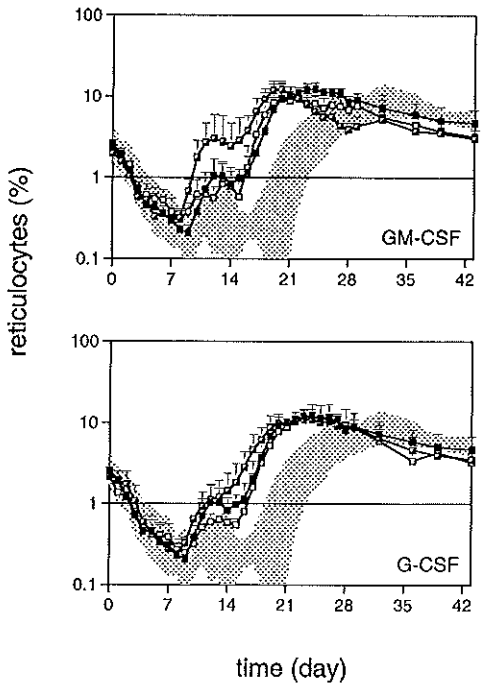

Fig 3. Reticulocyte regeneration after 5 Gy TBI (day 0). Symbols are as in Fig 2. The horizontal line defines the level of 1%, a regeneration marker.

Recovery to normal values was accelerated by 23 days compared with placebo-treated monkeys ($P < .003$), and an overshoot to supranormal values was apparent, which returned to normal values after cessation of GM-CSF treatment (Fig 2). Monkeys treated with TPO/G-CSF had a thrombocyte regeneration pattern identical to that of the monkeys treated with TPO alone, and one of the four monkeys needed a thrombocyte transfusion. Monkeys treated with GM-CSF or G-CSF alone all needed one to three transfusions. Although there was no significant difference versus the placebo-treated controls in the nadir and transfusion requirement, the subsequent thrombocyte regeneration was faster than that of the placebo controls for both G-CSF– and GM-CSF–treated monkeys (Fig 2).

Reticulocyte regeneration to values more than 1% occurred approximately 1 week earlier in TPO-treated monkeys than in placebo-treated controls ($P < .03$) and showed a biphasic response (Fig 3). Again, TPO/G-CSF was similar to TPO alone, whereas TPO/GM-CSF was more effective, further accelerating the reconstitution by 4 days. GM-CSF alone also slightly accelerated reticulocyte regeneration by 4 days ($P < .04$) and G-CSF alone by 3 days (Fig 3). This pattern was also reflected in the recovery of red blood cell counts after the initial decline, which is largely due to diag-

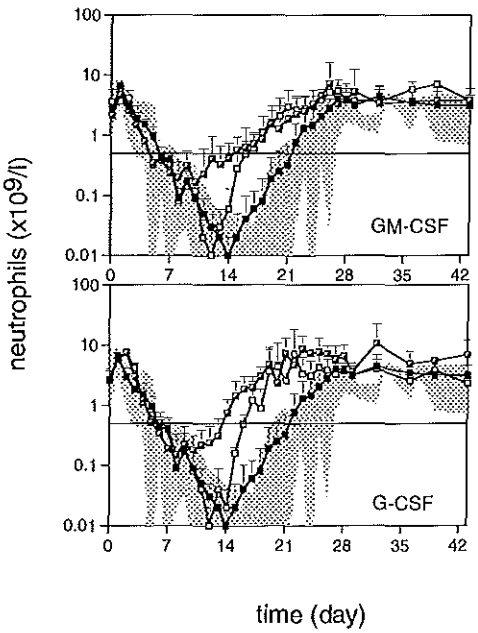

Fig 4. Neutrophil regeneration after 5 Gy TBI (day 0). Symbols are as in Fig 2. The horizontal line defines the level of 0.5 × 10^9/L.

nostic bleeding, with TPO/GM-CSF monkeys being the fastest to recover (data not shown).

Neutrophil reconstitution for TPO-treated monkeys was within the range of that for placebo-treated monkeys, with recovery to levels more than 0.5 10^9/L occurring at about day 21 (Fig 4). Both TPO/GM-CSF and TPO/G-CSF treatment elevated the neutrophil nadir, reflecting an accelerated recovery to reach levels more than 0.5 × 10^9/L 1 week earlier than TPO and placebo treatment. Recovery to normal values was slightly more pronounced in monkeys treated with TPO/G-CSF versus TPO/GM-CSF, but the difference did not reach statistical significance. Monkeys treated with G-CSF alone and GM-CSF alone had similar neutrophil recovery patterns, reaching levels more than 0.5 × 10^9/L 5 days before placebo-treated monkeys ($P < .03$, Fig 4). Treatment with CSFs resulted in a slightly earlier but not detectably less profound neutrophil nadir.

Transfusion data and regeneration parameters are summarized in Table 1, which shows that the TPO, TPO/GM-CSF, and TPO/G-CSF monkey groups are all significantly different in transfusion requirements versus the placebo controls. In addition, the TPO/GM-CSF group differed significantly from the GM-CSF group in transfusion requirement ($P < .003$), but not from the TPO-treated group ($P = .13$).

White blood cell subsets measured by flow cytometry. Flow cytometry was performed on lysed peripheral blood cell samples to assess regeneration of several subsets of white blood cells. $CD11b^+$ cells, representing granulocytes and monocytes, showed a pattern similar to neutrophil regeneration, in which both TPO/G-CSF– and TPO/GM-CSF–treated monkeys had a higher nadir and an accelerated recovery to normal values. Placebo-treated monkeys recovered to the lower range of baseline levels at day 28, TPO-treated monkeys at day 21, TPO/G-CSF–treated monkeys at day 14, and TPO/GM-CSF–treated monkeys at day 17. G-CSF-alone– and GM-CSF-alone–treated monkeys recovered similarly to TPO/GM-CSF–treated monkeys, but had a more profound nadir. The difference between TPO-alone and placebo-treated monkeys could be attributed to a more rapid monocyte regeneration (data not shown) and not to differences in neutrophil regeneration (Fig 4).

$CD8^+$ lymphocytes recovered to normal levels within the observation period of 6 weeks in all treatment groups without significant differences. All animals had subnormal levels of $CD4^+$ T lymphocytes and $CD20^+$ B lymphocytes at the end of the observation period. These levels were not significantly different between the various treatment groups (data not shown).

Bone marrow cellularity and progenitor cell content. Bone marrow aspirations were performed before TBI and once weekly thereafter. Cellularity was very low in the first week after TBI in all monkeys, not yielding sufficient numbers of cells to perform colony assays. Two weeks after irradiation, both TPO/G-CSF and TPO/GM-CSF monkeys started to show repopulation of the bone marrow. Three weeks after TBI, all treatment groups except for placebo-treated monkeys had normal bone marrow cellularity. Placebo-treated monkeys did not have near-normal cell numbers until week 4 after TBI. The stimulating effect of the single administration of 5 μg/kg TPO on bone marrow cellularity and $CD34^+$ cell and clonogenic progenitor cell recovery was considerably less prominent than reported in the previous TPO/G-CSF study using the same myelosuppression model[13] for the human TPO dose of 10 μg/kg/d administered for 21 consecutive days after TBI. This is understandable, since the total TPO dose is much lower. Since the variance of this type of data is inevitably large due to variations of bone marrow content at the puncture site and admixture with peripheral blood cells, we present only the most relevant data, ie, those obtained in the TPO/GM-CSF group of monkeys for $CD34^+$ cells at day 15 and for GM-CFU and BFU-E at day 22 after TBI (Fig 5). At day 15 after TBI, $CD34^+$ cell numbers significantly exceeded those of placebo controls in the TPO/GM-CSF ($P < .03$; Fig 5) and G-CSF ($P < .01$; not shown) treatment groups. For GM-CFU, only the TPO/GM-CSF group significantly exceeded ($P < .01$) the placebo controls by approximately 1 log at day 22 after TBI (Fig 5). The TPO/GM-CSF group was also richer in GM-CFU than animals treated with GM-CSF alone ($P < .04$), underscoring the significant impact of TPO if combined with GM-CSF. As for BFU-E, similar patterns were observed, with only the TPO/GM-CSF treatment group doing significantly ($P < .02$) better than the placebo controls (Fig 5).

Other study parameters and febrile episodes. No abnormalities were observed for most of the serum clinical chemistry. Albumin concentrations were stable at about 40 g/L,

except for GM-CSF monkeys, which developed edema (vide infra). Electrolyte disturbances due to gastrointestinal radiation damage were minor during the first week after TBI. The liver enzymes alkaline phosphatase, ALAT, and ASAT also showed only minor changes. The number of febrile episodes (axillary temperature >40°C) was 5.3 ± 3.4 days, with no significant differences between the various treatment groups, although TPO/GM-CSF and TPO/G-CSF monkeys tended to have more febrile days. There was no association between febrile episodes and C reactive protein (CRP) levels.

Adverse effects of growth factor treatment. Two of four GM-CSF–treated monkeys developed generalized edema ascribed to capillary leakage, which resolved following discontinuation of GM-CSF. In comparison to the other 26 consecutive monkeys that did not receive GM-CSF, the phenomenon proved statistically significant ($P = .02$, Fisher's exact test). None of four TPO/GM-CSF–treated monkeys developed edema; the difference with the GM-CSF–treated monkeys is statistically nonsignificant. Apart from slight local irritation at the injection site of GM-CSF and to a lesser extent at that of G-CSF, other adverse effects were not observed.

DISCUSSION

The present preclinical evaluation demonstrates that a single IV dose of TPO administered 1 day after a myelosuppressive dose of radiation that results in 3 weeks of cytopenia in placebo-treated controls is sufficient to virtually prevent the need for thrombocyte transfusions and to accelerate thrombocyte reconstitution to normal levels by 2 weeks. Furthermore, the results demonstrate that TPO/GM-CSF and TPO/G-CSF treatment display distinct response patterns among three major peripheral blood cell types. Coadministration of TPO/GM-CSF augmented thrombocyte, red blood cell, and neutrophil production over either of the individual growth factors alone, whereas in the TPO/G-CSF group only neutrophil reconstitution appeared to benefit from the combination of growth factors.

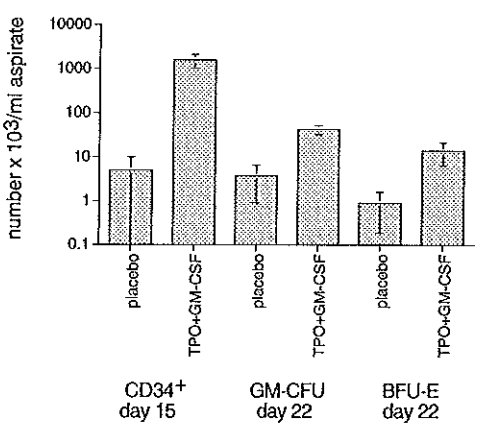

Fig 5. Bone marrow CD34⁺ cells and GM-CFU 2 and 3 weeks after 5 Gy TBI, respectively (arithmetic mean ± SE). Since the variance of this type of datum is inevitably large due to variations of bone marrow content at the puncture site and admixture with peripheral blood cells, in addition to possible variations in radiation sensitivity and exponential reconstitution, the other groups did not show significant differences versus the placebo controls.

The observation that a single IV 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 the data in mice,[39] with the kinetics of TPO-stimulated thrombocyte reconstitution in nonhuman primates indicating a very early action of TPO,[13,14] and with the decline in response when TPO administration is delayed.[42] These studies all point to a critical time phase early after TBI during which TPO has to be administered to achieve an optimal response. This could be explained by a decline in the number of bone marrow TPO-responsive target cells as a function of time after irradiation, possibly due to the absence of sufficiently high concentrations of TPO. This may be due to either prevention of physiologic death or apoptosis in the presence of TPO similar to that identified for erythropoietin and red blood cell progenitors,[43,44] or protection of TPO-responsive progenitors from radiation-induced cell death by TPO. Elucidation of such mechanisms not only will provide further insight into the physiologic function of TPO, but also will be of considerable importance to achieve the maximum clinical benefit of its therapeutic use. Although the data show that a single IV dose of 5 μg/kg 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.

A central issue of TPO treatment is prevention of bleeding as a consequence of myelosuppression. Our policy to transfuse donor thrombocytes at a level of 40×10^9/L coincides with the first appearance of petechiae and other bleeding, and was chosen to prevent undue deaths due to hemorrhage. For obvious reasons, this level is higher than used for human patients where instructions can be given to the patients and sensitization to alloantigens should be avoided. However, as can be extrapolated from the postirradiation decrease in thrombocyte counts after the first week and the first ascending counts in the third week after TBI in placebo-treated monkeys, without transfusions, the thrombocytes would have decreased to levels less than 10×10^9/L within 2 days after the first transfusion (Fig 1). It can thus be concluded that TPO treatment that prevented the decline of thrombocyte counts to levels less than 40×10^9/L early in the second week after irradiation also effectively prevented the propensity to bleeding (Fig 1).

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 monkeys may reflect synergism of the two growth factors,

since the initial recovery of both thrombocytes (Fig 2) and reticulocytes (Fig 3) 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 in normal nonhuman primates,[26,45] although the results of in vivo treatment with GM-CSF on thrombocyte reconstitution after cytotoxic insult to the bone marrow have always been heterogenous[26-43] and, in retrospect, may have been codependent on variations in endogenous TPO levels. From TPO levels measured 24 hours after injection, we could not conclude that there was a major change in TPO pharmacokinetics due to coadministration of GM-CSF as a basis for the augmented recovery of thrombocytes. The same observation was made for G-CSF. Surprisingly, the 10-μg/kg/d dose (for 2 weeks) of G-CSF also appeared to stimulate thrombocyte production to a certain extent, in contrast to the 5-μg/kg/d dose (for 3 weeks), which was used in a previous study in the same nonhuman primate model,[13] but the result is consistent with that in a similar model in which 10 μg/kg/d was also used.[14] We previously observed, in the same myelosuppression model as used here, a dampening effect of G-CSF on TPO-stimulated supranormal thrombocyte production.[13] This was also seen in mice.[16] 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.[28] The reported effects of G-CSF on thrombocyte production are ambiguous: most reports mention no effects at all,[19,49-51] some a positive effect,[52] and others a negative effect.[53-55] 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.[56] We conclude provisionally that combining TPO and G-CSF treatment may have a variable, as-yet-unpredictable, and occasionally adverse outcome.

TPO augmented the neutrophil reconstitution stimulated by GM-CSF as well as G-CSF, which might be of considerable clinical significance, since in the reported clinical trials the effects of the CSFs in general have been modest,[22,23,29] similar to the results presented here in a preclinical nonhuman primate model. In particular, the neutrophil nadir appeared to be greatly improved in TPO/CSF-treated animals. G-CSF or GM-CSF treatment alone did not appreciably influence the neutrophil nadir, but accelerated regeneration afterward with a time course similar to that for clinically acceptable neutrophil counts. We attributed the advantageous effect of TPO to expansion of immature cells along multiple hematopoietic lineages,[7-9,13,57] thus making more G- or GM-CSF target cells available for myelopoiesis. From the kinetics of neutrophil reconstitution, it is also apparent that the effect originates from stimulation at an early stage after TBI (Fig 4).

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,[13,15] although this effect was not apparent in the present study. 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,[22,23] as also seen in the present study. The development of edema in two monkeys treated with GM-CSF was an unwanted adverse effect of the cytokine treatment, a rare event that has been previously reported, albeit only at higher doses of GM-CSF.[22,23,58] For as-yet-unexplained reasons, edema was not observed in monkeys treated with TPO/GM-CSF; however, this difference is not statistically significant. Apart from stimulation of thrombocyte production, a prominent feature of TPO administration is the accelerated reconstitution of immature bone marrow cells, consistent with the presence of TPO receptors and stimulatory effects in vitro.[7-9] Accelerated recovery of bone marrow cellularity, $CD34^+$ cells, and clonogenic progenitor cells such as GM-CFU and BFU-E following supraoptimal TPO treatment has been reported previously.[13,14] The present study with a much more limited TPO dose confirmed these data and demonstrated that the effect is reinforced by combining TPO with GM-CSF. This places TPO among the few growth factors that can presently be safely used clinically to promote the recovery of immature bone marrow cells in an early stage after cytoreductive therapy without notable adverse effects. As a direct implication, the future development of TPO treatment regimens in the clinic should be directed not only at optimal thrombocyte recovery but also at the most optimal reconstitution of immature bone marrow cells.

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CHAPTER 5

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

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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

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Abstract

The efficacy of recombinant human thrombopoietin (TPO) and recombinant human granulocyte colony stimulating factor (G-CSF) in stimulating platelet and neutrophil recovery was evaluated in a placebo-controlled study involving transplantation of limited numbers ($1–3\times10^4$/kg) of highly purified autologous stem cells ($CD34^{++}$/RhLA-DR^{dull}) into rhesus monkeys after the animals were subjected to 8 Gy of total body irradiation (TBI) (x-rays). The grafts shortened profound TBI-induced pancytopenia from 5 to 6 weeks to 3 weeks. Daily subcutaneous (sc) injection of TPO (10 μg/kg/day, days 1–21 after TBI) did not stimulate platelet regeneration after transplantation either alone or in combination with G-CSF (5 μg/kg/day sc, days 1–21 after TBI). G-CSF treatment failed to prevent neutropenia in the monkeys and did not stimulate recovery to normal neutrophil levels. Simultaneous administration of TPO and G-CSF did not influence the observed recovery patterns. To test the hypothesis that the limited number of cells transplanted or the subset chosen was responsible for the lack of effectiveness of TPO, three additional monkeys were transplanted with 10^7/kg unfractionated autologous bone marrow cells. Two of these animals received TPO and the other served as a control. In this setting, as well, TPO treatment did not prevent thrombocytopenia. This study demonstrates that treatment with TPO does not accelerate platelet reconstitution from transplanted stem cells after high-dose TBI. These findings contrast with the rapid TPO-stimulated platelet recovery in myelosuppression induced by 5 Gy of TBI in rhesus monkeys; we conclude from this that the clinical effectiveness of the TPO response depends on the availability of TPO target cells in the first week after TBI, that is, before endogenous TPO levels reach the saturation point. In addition, protracted isolated thrombocytopenia was observed in two G-CSF-treated monkeys, one of which also received TPO. Furthermore, TPO treatment for 7 days in the 6th week after TBI during severe thrombocytopenia in one monkey produced prompt clinical improvement and an increase in platelet counts.

Key words: Thrombopoietin—Bone marrow transplantation—Total body irradiation—$CD34^+$ stem cell subset—Rhesus monkeys

Introduction

Thrombopoietin (TPO), the ligand for the receptor encoded by the protooncogene *c-mpl*, was first identified and its gene cloned in 1994 [1–3]. Since blood TPO levels are inversely related to platelet counts [1–3], and mice lacking either the receptor for TPO or the ligand are severely thrombocytopenic [4,5], TPO is considered the major regulator of platelet production. Administration of recombinant TPO to normal experimental animals has shown it to be a potent thrombopoietic agent [2,6,7]. TPO has also been tested in several myelosuppression models in mice [6,8,9] and shown to be effective in accelerating platelet reconstitution as well as in preventing morbidity caused by bleeding. TPO was not effective after myeloablative treatment and bone marrow transplantation (BMT) in a syngeneic mouse model, whereas marrow from donors pretreated with TPO showed augmented regeneration of platelet numbers [10].

One of the likely clinical uses of TPO will be as a stimulator of hematopoietic reconstitution following autologous or allogeneic BMT. It is hoped that TPO will accelerate early reconstitution as well as counteract protracted thrombocytopenia, which is especially common in patients subjected to prior chemotherapy for leukemia. The use and efficacy of granulocyte colony-stimulating factor (G-CSF) as a single growth factor to stimulate neutrophil recovery has been described extensively in several animal models and has been confirmed by clinical trials [11–15].

Preclinical evaluation of TPO and other growth factors requires testing in large animal models such as rhesus monkeys. In the present study we examined the simultaneous administration of TPO and G-CSF to assess their synergistic efficacy and possible stimulatory and/or adverse interactions. The setting chosen involved transplantation of limited numbers of the immature RhLA-DR^{dull} subset of autologous $CD34^+$

[16,17] cells following 8 Gy of TBI in rhesus monkeys. A stem cell graft of this small size after high-dose TBI allows investigators to determine the lower limit of residual hematopoietic stem cell numbers, which permit stimulation of blood cell formation by hematopoietic growth factors [18], as well as to test for the capacity of a growth factor to accelerate peripheral blood cell reconstitution from immature hematopoietic stem cells. The dose of TPO was chosen based on previous dose finding studies in mice and experiments in normal monkeys [19], and because it proved highly effective in preventing thrombocytopenia after radiation-induced myelosuppression in rhesus monkeys [20]. The latter study also showed that TPO treatment augmented the G-CSF response and accelerated immature bone marrow cell recovery. In the present study we attempted to extend these previous findings and to further define the potential efficacy of TPO therapy in the clinical setting.

Materials and methods

Animals

Purpose-bred male rhesus monkeys (*Macaca mulatta*), weighing 2.5 to 4.0 kilograms each and aged 2 to 3 years were used in this study. The monkeys were housed in groups of 4 to 6 in stainless steel cages in rooms equipped with a reverse-filtered air barrier, provided with normal daylight rhythm, and conditioned to 20°C with a relative humidity of 70%. Animals were allowed free access to commercially available primate chow, fresh fruits, and acidified drinking water. All animals were free of intestinal parasites and seronegative for herpes B, simian T-lymphotrophic viruses (STLV), and simian immunodeficiency virus (SIV). Housing, experiments, and all other conditions were approved by an ethics committee in accordance with legal regulations in The Netherlands.

Total body irradiation

After aspiration of bone marrow, monkeys were irradiated with a single 8-Gy dose TBI delivered by two opposing x-ray generators operating at a tube voltage of 300 kV and a current of 10 mA. The half-layer thickness was 3 mm Cu. The focus skin distance was 0.8 m and the average dose rate 0.20 to 0.22 Gy/minute. During TBI, the animals were placed in a cylindrical polycarbonate cage which rotated slowly (three times/minute) around its vertical axis.

Supportive care

Two weeks before TBI, the monkeys were placed in a laminar flow cabinet and their gastrointestinal tracts were selectively decontaminated by administration of oral ciprofloxacin (Bayer, Mijdrecht, The Netherlands), nystatin (Sanofi BV, Maassluis, The Netherlands), and polymyxin B (Pfizer, New York, NY). When leukocyte counts dropped below 10^9/L, this regimen was supplemented with systemic antibiotic medications, in most cases a combination of ticarcillin (Beecham Pharma, Amstelveen, The Netherlands) and cefuroxim (Glaxo, Zeist, The Netherlands); this antibiotic regimen, which was guided by fecal bacteriograms, was continued until leukocyte counts rose to levels >10^9/L. Dehydration and electrolyte disturbances were treated by appropriate sc fluid and electrolyte injection. The monkeys received irradiated (15 Gy) platelet transfusions whenever platelet counts reached values below 40×10^9/L and irradiated packed red cells whenever hematocrit levels declined below 20%; in those instances in which platelet counts and hematocrit levels were found to be simultaneously decreased, whole blood transfusions were administered.

Test drugs

One-milliliter vials containing 0.5 mg/mL recombinant human TPO were supplied by Genentech Inc. (South San Francisco, CA). The dosage used was 10 μg/kg/day, administered by sc injection once per day from day 1 to day 21 postirradiation. The daily doses were diluted to a volume of 1 mL with phosphate-buffered saline (PBS)/0.01%Tween 80. Placebo-treated monkeys were given the same volume of diluent. Recombinant human G-CSF (Neupogen, Amgen, Thousand Oaks, CA) was administered at a dosage of 5 μg/kg/day sc, once per day from day 1 to day 21 postirradiation. The daily doses were diluted to a volume of 1 mL in the solution recommended by the manufacturer.

Study groups

The monkeys were treated sequentially at intervals of 1 week with a break every 4 to 5 weeks to thoroughly clean and decontaminate the isolation facility; the study was completed in a total of 7 months. Animals were randomly assigned to study groups. Results from one historic control that did not receive growth factors are included in the graphs. Four monkeys were treated with TPO alone, two simultaneously with TPO and G-CSF, three with G-CSF alone, and two with placebo. Two monkeys that did not receive a transplant served as irradiation controls. Three additional monkeys were transplanted with unfractionated bone marrow grafts; two of these received TPO, and the other served as a control.

Bone marrow aspirates

Bone marrow was aspirated while the animals were under anesthesia using ketalar (Apharmo, Arnhem, The Netherlands) and vetranquil (Sanofi, Maassluis, The Netherlands). For transplantation, bone marrow was aspirated from the shaft of both femurs into bottles containing 10 mL Hanks' buffered Hepes solution (HHBS) and 250 IU sodium heparin/mL (Leo Pharmaceutical Products, Weesp, The Netherlands). Erythrocytes contained in the aspirate were returned to the monkey after two washings with 0.9% NaCl and filtration to remove bone fragments, either before irradiation or as irradiated (15 Gy) blood product after TBI. Small bone marrow aspirates were obtained for analysis from the shafts of the humeri using pediatric spinal needles. Low-density cells were isolated using Ficoll (density 1.077; Nycomed Pharma AS, Oslo, Norway) separation.

Colony assays

Cells were plated in 35-mm dishes (Becton Dickinson, San Jose, CA) in 1-mL enriched Dulbecco's medium (Gibco, Gaithersburg, MD) containing 0.8% methylcellulose, 5% fetal calf serum (FCS), and additives, as described previously [21-23]. For burst-forming units-erythroid (BFU-E), cultures were supplemented with hemin (2×10^{-4} mol/L), human

recombinant erythropoietin (Epo; 4 U/mL; Behringwerke AG, Marburg am Lahn, Germany) and Kit ligand (KL; 100 ng/mL; kindly provided by Dr. S. Gillis, Immunex, Seattle, WA). For granulocyte/macrophage colony forming-units (GM-CFU), cultures were supplemented with recombinant human GM-CSF (5 ng/mL; Behring), recombinant rhesus monkey IL-3 (30 ng/mL) produced by *B. licheniformis* and purified as described previously [24,25], and KL. Low-density cells were plated at 5×10^4 cells per dish and sorted cell fractions at 10^3 per dish in duplicate. Colony numbers represent the mean ± standard deviation of bone marrow samples from individual monkeys.

Stem cell isolation and transplantation

To enrich for progenitor cells, buffy-coat cells were subjected to a discontinuous bovine serum albumin density gradient [26,27]. Low-density cells were collected and $CD34^+$ cells isolated by immunomagnetic separation using an IgG2A antibody against CD34 (mAb 561; from G. Gaudernack and T. Egeland, Rikshospitalet, Oslo, Norway) that was noncovalently linked to rat anti-mouse IgG2A beads (Dynal, Oslo, Norway). $CD34^+$ cells devoid of the anti-CD34 antibody were recovered using a polyclonal antibody against the Fab part of the anti-CD34 antibody (Detachebead, Dynal). Purified cells were incubated with a monoclonal antibody (mAb) against human CD34 (mAb 566; from G. Gaudernack and T. Egeland) that had been conjugated with fluorescein isothiocyanate (FITC; Sigma, St. Louis, MO) and simultaneously with a phycoerythrin (PE)-conjugated mAb against human HLA-DR that reacts with rhesus monkey RhLA-DR antigens (Becton Dickinson, San Jose, CA). Control cells were stained with each mAb separately to allow proper adjustment of fluorescence compensation. Cell sorting was performed using a FACS Vantage flow cytometer (Becton Dickinson) with the argon laser set at 488 nm (100 mW). $CD34^{bright}$/$RhLA\text{-}DR^{dull}$ cells [17] were sorted in the normal C mode and reanalyzed for purity. The sorted cells were transplanted at a dose of 1.4 to 3×10^4/kg body weight and returned to the monkey on the same day.

Hematological examinations

Complete blood cell counts were measured daily using a Sysmex F-800 hematology analyzer (Toa Medical Electronics, Kobe, Japan). The differential of the total number of nucleated cells (TNCs) was determined by standard counting after May-Grünwald-Giemsa staining. For reticulocyte measurements, 5 µL ethylene diamine tetraacetic acid (EDTA) blood was diluted in 1 mL PBS/EDTA (0.5 M)/azide (0.05% wt/vol), and 1 mL of a thiazole orange dilution was added at a final concentration of 0.5 µg/mL. Measurements were done on a FACScan (Becton Dickinson) and analyzed using Reticount software (Becton Dickinson).

Measurements of surface antigens

Once per week FACScan analysis was performed on PB and BM samples on the following surface antigens: CD8, CD4, CD20, CD11b, CD56, and CD16. Directly labeled monoclonal antibodies were used for CD8, CD4, CD20, CD56, and CD16 (Leu 2a-FITC, Leu 3a-PE, Leu 16-PE, Leu 19-PE, and Leu 11a-FITC (Becton Dickinson), respectively. CD11b antigens were detected by the FITC-conjugated monoclonal antibody MO1 (Coulter Immunology, Hialeah, Florida). Red blood cells were removed by incubation of 0.5 mL whole blood or bone marrow in 10 mL lysing solution (8.26 g ammonium chloride/1.0 g potassium bicarbonate, and 0.037 g EDTA/L) for 10 minutes at 4°C. After lysing, cells were washed twice with HHBS containing 2% FCS (vol/vol) and 0.05% (wt/vol) sodium azide (HFN). The cells were resuspended in 100 µL HFN containing 2% (vol/vol) normal monkey serum to prevent nonspecific binding of the monoclonal antibodies. Monoclonal antibodies were added in a volume of 5 µL and incubated for 30 minutes on ice. After two washes, the cells were measured by flow cytometry. Ungated list mode data were collected for 10,000 events and analyzed using Lysis II software (Becton Dickinson).

Clinical chemistry

Serum concentrations of sodium, potassium, chloride, glucose, albumin, total protein, aspartate-amino transferase, alanine-amino transferase, alkaline phosphatase, lactate dehydrogenase (LDH), gamma-glutamyl transpeptidase, total bilirubin, C reactive protein, creatinine, urea, and bicarbonate were analyzed twice per week using an Elan Analyzer (Eppendorf Merck, Hamburg, Germany).

TPO levels

Plasma for measurements of levels of TPO was sampled from the monkeys just before cytokine administration twice per week and stored at -80°C. A full description of the TPO enzyme-linked immunosorbent assay (ELISA) is given elsewhere [28,29]. Briefly, ELISA plates were incubated overnight at 4°C with 2 µg/mL rabbit F(ab')2 to human IgG Fc (Jackson ImmunoResearch, West Grove, PA) and 2 hours at room temperature with conditioned medium containing 100 ng/mL of mpl-IgG [3]. Twofold serial dilutions of samples (starting at 1:10) and standards (recombinant full length human and/or rhesus TPO) were added to wells and incubated for 1 hour. Bound TPO was detected using biotinylated rabbit antibody to full-length human TPO (Genentech), followed by peroxidase-labeled streptavidin. The range of the assay for rhesus plasma samples is 0.32 to 10 ng/mL TPO. The assay preferentially detects active full-length TPO with equal accuracy in rhesus monkeys and humans, and correlated well with a bioassay using the megakaryoblastic HU-3 cell line.

Hemostasis parameters

Plasma for measurements of fibrinogen, prothrombin time (PT), and activated partial thromboplastin time (APTT) was collected twice per week and stored at -80°C. Measurements were done on an Automated Coagulation Laboratory-100 (ACL-100; Instrumentation Laboratory, Milano, Italy). A commercially available kit was used for fibrinogen measurements (fibrinogen-test, DiaMed AG, Cressier sur Morat, Switzerland), thromborel S (Behringwerke AG, Marburg, Germany) was used for the PT, and platelin LS (Organon Teknika Corporation, Durham, NC) for the APTT. Platelet aggregation tests were done before treatment and at day 21 using a whole blood lumi aggregometer (Chronolog, Havertown, PA) using 5×10^{-5} M ADP (Sigma, St. Louis, MO), 5 U/mL thrombin (Central Laboratory of the Blood Bank, Amsterdam, the Netherlands), or 5 µg/mL collagen (Sigma).

Table 1. Regeneration parameters after 8-Gy TBI and stem cell transplantation

Cell fraction	Number of monkeys	Growth factor	Reticulocytes >1% at day	Neutrophils > 0.5 10^9/mL at day	Plt > 40 10^9/mL at day
CD34^{++}/RhLADR^{dull}	4	TPO	20 ± 2	21 ± 2	21 ± 5
	2	TPO + G-CSF	23;17	25;15	32;15
	3	G-CSF	21 ± 2	19 ± 3	57;24;18
	3	placebo	23 ± 0	23 ± 2	24 ± 1
None	2	none	27;27	32;30	37;34
Unfractionated	2	TPO	11;19	17;20	15;17
Unfractionated	1	placebo	20	24	24

Statistics

If relevant, standard deviations were calculated and are given in the text, the figures, and the table on the assumption of a normal distribution. The statistical significance of differences was calculated by Fisher's exact test for categorical data, and by one-way analysis of variance followed by a Student's *t* test for continuous data.

Results

Characterization of stem cell transplants

To assess the efficiency and enrichment potential of the selection procedure used for stem cell isolation, cell numbers were counted for each cell fraction, colony assays were performed, and a FACScan analysis was done for CD34 antigen expression. Total cell recovery for the CD34 selection step using the Dynal immunomagnetic beads was 76.4 ± 15%, with an average of 8.6 ± 3.5% of the cells in the positive fraction. Calculated as percentages of the aspirated bone marrow cells, CD34^{++}/RhLA-DR^{dull} cells represented 0.03% of the total cell number. Recovery of GM-CFU numbers during the CD34 selection stage was 50.6 ± 24.1%, with 41.9 ± 23% of the GM-CFUs recovered in the CD34^{+} cell fraction. Recovery of BFU-E was 82.8 ± 52.5% during the CD34 selection step, with 56.6 ± 38.9% found in the positive cell fraction. Recovery of the CD34^{++}/RhLA-DR^{dull} fraction, compared with the CD34^{+} cell fraction of GM-CFU and BFU-E, was 8.1 ± 5.9% and 1.3 ± 1.6%, respectively, reflecting a depletion of more mature progenitor cells, which are segregable from immature cells on the basis of HLA-DR expression [17] in this subset. FACScan analysis revealed a recovery of CD34^{+} cells of 38.0 ± 15.8%, with 24.9 ± 13% found in the positive cell fraction, and a purity of CD34^{+} cells of 43 ± 18%. The CD34^{++}/RhLA-DR^{dull} cells represented 3.4 ± 2.2% of the CD34^{+} cell fraction. The cell dose transplanted was on average 2.7×10^4/kg (range $1.4–3\times10^4$/kg).

Hematopoietic reconstitution

Based on previous transplantations in this setting [30], the reticulocyte regeneration was found to be the best parameter to quantify the engraftment potential of a BMT. Because TPO has been found to influence reticulocyte regeneration after myelosuppression [20], this parameter is less reliable in TPO-treated monkeys, but in placebo- and G-CSF-treated monkeys regeneration was very homogeneous, i.e., 21.7 ± 1.7 days after TBI (n=6). The monkey transplanted with 10^7/kg unfractionated bone marrow cells and treated with placebo had greater than 1% reticulocytes at day 20, similar to historic controls [30]. Comparison of the regeneration times of the unfractionated transplants and those of the stem cell transplants yielded an enrichment in repopulating capacity of approximately 300-fold for the latter. TBI without transplant resulted in a 5- to 6-week period of pancytopenia. A CD34^{++}/RhLA-DR^{dull} transplant without growth factor treatment resulted in a three week period of profound pancytopenia (Table 1). All monkeys were dependent on platelet transfusions and antibiotic treatment during the recovery phase.

Peripheral blood cell counts

Platelet regeneration after bone marrow transplantation was not influenced by TPO treatment. The nadir for platelets was not alleviated by TPO, and the time needed for normalization of those values was not shortened (Fig. 1). TPO-treated monkeys needed 3 to 6 transfusions compared with 4 to 5 for placebo-treated animals. Of monkeys treated with TPO and G-CSF (n=2), one needed only 2 transfusions and reached a transfusion-free platelet level at day 15, whereas the other required 10 transfusions and did not reach transfusion independence until day 32. In G-CSF-treated monkeys (n=3), the data were also heterogeneous. Two monkeys showed a regeneration pattern for platelets similar to that of placebo-treated controls, whereas the other developed protracted thrombocytopenia, requiring 22 transfusions and reaching transfusion independence on day 57 after irradiation. The monkeys with G-CSF-associated protracted thrombopenia will be discussed separately (*vide infra*).

Reticulocyte regeneration, defined as the day on which the percentage of reticulocytes exceeded 1%, occurred 3 days earlier in TPO-treated monkeys than in placebo-treated controls, although this difference was not statistically significant. G-CSF had no effect on reticulocyte regeneration, and for TPO plus G-CSF results were variable (Table 1). Neutrophilic granulocytes regenerated in placebo-treated controls and TPO-treated monkeys in a similar way (Fig. 1). The recovery of neutropenia in monkeys treated simultaneously with TPO and G-CSF (n=2) was also heterogenous. The first one had neutrophil levels higher than 0.5×10^9/L by day 25 and the other by day 15. For the three G-CSF-treated monkeys, these levels were attained on days 17, 22, and 19, respectively.

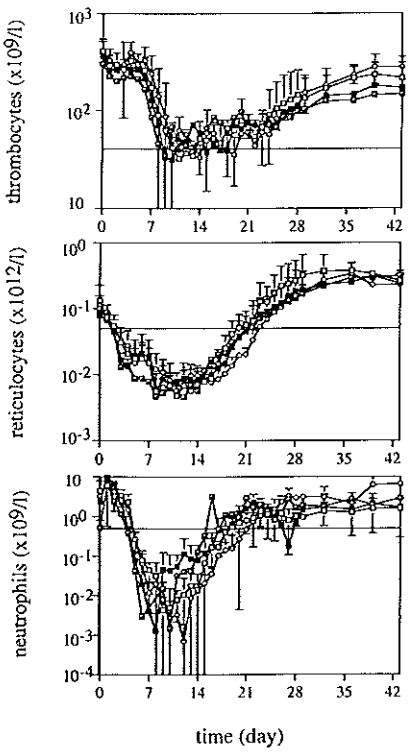

Fig. 1. Mean peripheral blood counts after 8-Gy TBI and stem cell transplantation. Thrombocytes (upper panel), reticulocytes (middle panel), and neutrophils (lower panel) for TPO (open squares, n=4), TPO and G-CSF (half filled squares, n=2), G-CSF (black squares, n=3), and placebo-treated monkeys (open circles, n=3). Horizontal lines define degree of cytopenia, 40×10^9/L for thrombocytes, 0.05×10^{12}/L for reticulocytes and 0.5×10^9/L for neutrophils.

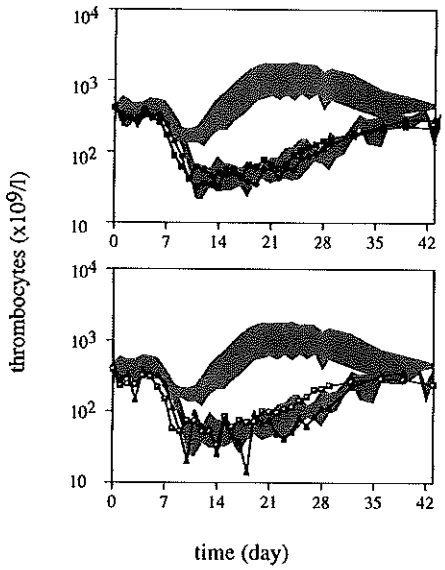

Fig. 2. Comparison of the effect of TPO following transplantation of purified stem cells and unfractionated bone marrow. In the upper panel the mean thrombocyte counts of 4 TPO-treated stem cell-transplanted monkeys (filled squares) and 3 placebo-treated stem cell-transplanted monkeys (filled circles) are plotted. The lower panel displays the mean of two TPO-treated unfractionated marrow transplanted monkeys (half-filled squares) and the unfractionated marrow-transplanted control monkey (filled triangles). The dose and route of TPO were the same for all monkeys (10μg/kg/day, sc, days 1–21). The shaded areas represent means ± SDs of thrombocyte levels of 5-Gy irradiated monkeys after TBI; the lower shaded area represents 8 placebo-treated controls, the upper one 4 TPO-treated monkeys.

Unfractionated bone marrow transplants

To examine the effect of size and cellular composition of the graft on TPO-stimulated platelet regeneration, three monkeys received an autologous unfractionated bone marrow transplant at a cell dose of 10^7/kg body weight. Two of these monkeys received TPO at the same dose as the stem cell-transplanted monkeys and one of these received placebo treatment. TPO treatment did not prevent the platelet nadir and did not significantly stimulate recovery toward normal values (Fig. 2). Days to reticulocyte regeneration >1% were 11, 19, and 20, respectively, and days to neutrophil regeneration 17, 20, and 24, respectively, in these three animals (Table 1). In Figure 2, the mean platelet regeneration in the unfractionated transplants is compared with that in the stem cell transplants, illustrating the similarity of response to TPO between the two groups. Figure 2 also displays the TPO response in the 5-Gy irradiated monkeys [20], showing that the transplants resulted in platelet reconstitution at a level similar to that in the placebo controls following myelosuppression induced by 5-Gy of irradiation, and illustrating the virtual lack of a TPO response in the transplanted monkeys.

White blood cell subsets measured by flow cytometry

A profound nadir was observed for all surface markers tested in the peripheral blood. Regeneration patterns did not differ between different treatment groups. The number of $CD20^+$ cells did not recover to pretreatment levels during the observation period (6 weeks) and neither did the number of $CD4^+$ cells. $CD11b^+$ cells followed the same pattern as the neutrophils shown in Figure 1. $CD8^+$ cell numbers reached pretreatment levels during the sixth week.

Table 2. Bone marrow cellularities ($\times 10^5$), GM-CFU numbers ($\times 10^3$), and BFU-E numbers ($\times 10^3$) ± SD per mL aspirate after 8-Gy TBI and stem cell transplantation

	Treatment	Before	Week 1	Week 2	Week 3	Week 4
Cellularity $\times 10^5$/mL	T	39 ± 7	0.1 ± 0.2	0.4 ± 0.3	3.9 ± 3.4	28.7 ± 48.9
	T + G	26;53	0.02;0.03	0.14;1.0	0.8;ND	5.2;6.5
	G	22 ± 5	0.2 ± 0.2	0.6 ± 0.5	14.3 ± 20.7	4.1 ± 4.8
	pl	20;23	0.14;0.0	0.26 ± 0.0	1.7;1.5	19 ± 8
GM-CFU $\times 10^3$	T	70 ± 32	ND	ND	3 ± 4	36 ± 72
	T + G	45;12	ND	ND	0;ND	0;2
	G	28 ± 10	ND	ND	15 ± 25	0.3 ± 0.1
	pl	39;54	ND	ND	0.3;0.1	6;5
BFU-E $\times 10^3$	T	17 ± 6	ND	ND	0.9 ± 1.7	17 ± 30
	T + G	16;26	ND	ND	0;ND	0;0.6
	G	9 ± 0.4	ND	ND	5.6 ± 9.7	0.5 ± 0.6
	pl	9;11	ND	ND	0;0	0.7;2

ND = not done because of low cell numbers; TPO: TPO-treated monkeys (n=4); T + G: TPO- + G-CSF-treated monkeys (n=2); G: G-CSF-treated monkeys (n=3), pl: placebo-treated monkeys (n=2).

Bone marrow cellularity and progenitor cell content

The bone marrow cellularity of the aspirates used for transplantation was 30.9 ± 11×10^6/mL and served as a control value. In all treatment groups, cellularity at days 8 and 15 was too low to allow performance of colony assays (Table 2). G-CSF-treated monkeys showed greater marrow cellularity at day 22 but with wide variations. At day 28 no differences were observed between the treatment groups. The progenitor cell content of the bone marrow, expressed as colonies per milliliter of aspirate, was also subject to wide variation and showed no trend favoring a growth factor regimen.

TPO levels

Pretreatment TPO levels were below detection level (320 pg/mL) in all but one sample, varied from 2438 to 25,143 pg/mL in samples taken from TPO-treated monkeys from day 3 to day 21 after TBI, and became very low again at days 24 and 28 (Fig. 3). In animals treated with G-CSF alone or placebo, elevated levels were measured in samples taken at days 7, 10, 14, 17, 21, and 28, consistent with the thrombocytopenia present in those animals at those times (Fig. 1). These endogenous TPO levels varied from 314 to 1627 pg/mL, which was in the low range compared with TPO-treated monkeys, but clearly elevated compared with baseline values. These elevated TPO levels should probably be considered relatively moderate elevations because the animals in this group were given platelet transfusions whenever their platelet levels dropped below 40×10^9/L.

Clinical chemistry and hemostasis parameters

Abnormalities were not observed for most parameters. Minor electrolyte disturbances were observed during the first week after TBI because of gastrointestinal radiation damage. Some monkeys had a short period of hypalbuminemia. No differences were found between APTT, PT, and fibrinogen levels among the different treatment groups, and these levels did not fluctuate during the postirradiation period. Platelet aggregation tests scheduled for day 21 after TBI were not always feasible because of the low number of platelets; the tests done in 6 animals revealed no differences attributable to growth factor treatment (data not shown).

G-CSF associated thrombocytopenia

Two monkeys developed protracted thrombocytopenia. One had been treated with G-CSF alone, the other with TPO and G-CSF. Platelet counts for those monkeys are shown in a separate graph (Fig. 4). The clinical condition of the G-CSF-treated thrombocytopenic monkey became very severe, including dependence on almost daily transfusions and abundant petechiae and other hemorrhages. At this stage, local regulations demanded either euthanasia or emergency treatment. We elected to kill one monkey and treat the other with TPO (10 µg/kg/day sc, from day 37 to day 43). After a lag phase of

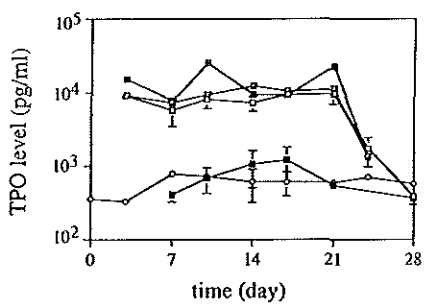

Fig. 3. TPO levels in plasma measured by ELISA. For explanation of symbols see Figure 1. The shaded squares represent TPO-treated unfractionated bone marrow transplants. Samples were taken before treatment and at days 3, 7, 10, 14, 17, 21, 24, and 28. Data not shown were below detection level (320 pg/mL).

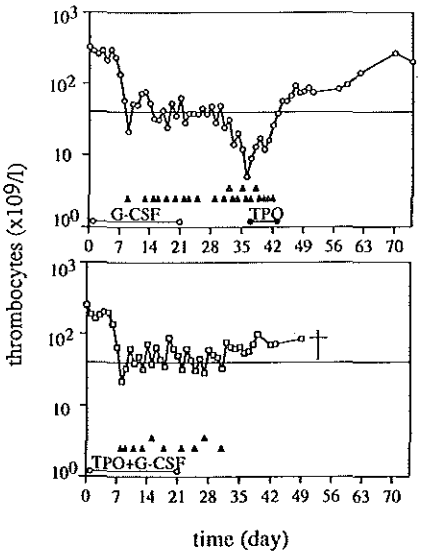

Fig. 4. Thrombocyte counts in the monkey treated with G-CSF alone (upper panel), and of the monkey treated with TPO and G-CSF (lower panel) that developed protracted thrombopenia. The black line indicates the level of 40×10^9/L thrombocytes under which transfusions were given. The black arrows indicate the transfusions given to the monkeys, the closed ones represent thrombocyte transfusions, and the open ones whole blood transfusions.

approximately 1 week, platelet counts rose sharply, increased slowly thereafter, and reached normal values by the tenth week after irradiation (Fig. 4). The petechiae and hematomas resolved within days.

Discussion

In this study TPO and G-CSF were combined to evaluate their effects after transplantation of highly purified stem cell concentrates into irradiated rhesus monkeys after TBI at a dose of 8 Gy. TPO alone did not prevent thrombocytopenia and did not 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 (tested in two monkeys) showed variable results. In one monkey, neutrophil regeneration was enhanced ($>0.5\times10^9$/L at day 15) whereas in the other it regenerated at the same rate as that observed in placebo-treated controls. Platelet and reticulocyte regeneration was augmented in the monkey whose neutrophils regenerated faster, whereas the other monkey developed protracted thrombocytopenia. Treatment with G-CSF alone did not stimulate neutrophil regeneration or affect the red cell lineage, and in one of three monkeys 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 at a cell dose of 10^7/kg. TPO treatment in this setting again did not prevent thrombocytopenia, and recovery to normal platelet values was only slighuly accelerated.

In monkeys that were irradiated with 5 Gy TBI without receiving a transplant, TPO (10 μg/kg/day) exerted a highly stimulatory 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 thrombocytopenia in these animals [20] (Fig. 2). Since 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 a significant feature. In principle, the difference between the efficacy of TPO in the stem cell transplant model and in the myelosuppression model may be attributed to the dose of TPO chosen, the difference in radiation dose, which may have caused more stromal damage in the stem cell transplant model, or the composition of the cell populations in which hematopoietic reconstitution originated.

The dose and dose schedule of TPO were based on pilot dose-finding experiments in normal mice and rhesus monkeys [19] and were 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 2 weeks. Apparently, the elevated endogenous TPO levels measured in the placebo-treated controls (Fig. 3), which were more than one order of magnitude lower than those obtained with exogenous TPO, were sufficient for full platelet reconstitution. Therefore, it is unlikely that the ineffectiveness of TPO in the transplant model was attributable to the dose or dose schedule used.

It is equally unlikely that the difference between 5 Gy and 8 Gy TBI resulted in significantly increased stromal damage after the latter dose, and hence impaired platelet reconstitution. This is because, in the placebo control monkeys, the kinetics of platelet reconstitution were very similar, if not identical, in both models. Furthermore, stromal damage resulting in significantly impaired hematopoiesis has only been observed following much greater doses of radiation [31] than those used in this study.

The composition of the reconstituting cell populations is most likely the decisive determinant of a clinically relevant response to TPO. In the 5-Gy myelosuppression model, the residual cells were depleted of stem cells and progenitor cells by less than 2 orders of magnitude and remained in their normal stromal environment. It is also clear from the kinetics of the TPO response in this model that its effectiveness was determined in the initial phase after TBI, a conclusion that has been corroborated by the diminished response to delayed administration of TPO [32] as well as by the observation made in mice that a single initial iv injection of TPO was sufficient to prevent profound thrombocytopenia [33]. In the 8 Gy model, on the other hand, there was a greater 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, but needs to be either boosted by other growth factors or stimulated before TPO treatment to generate TPO-responsive progenitor cells. Since the response to TPO was also severely diminished in the unfractionated bone marrow recipients compared with the 5-Gy myelosuppressed monkeys, which had platelet reconstitution kinetics similar to the placebo-treated control monkey, the inefficacy of TPO and G-CSF cannot be attributed solely to the depletion of progenitor cells; it may also be a result of poor engraftment of the responsive progenitor cells. Finally, our results indicate a steep decline in TPO and G-CSF responses with increasing radiation doses, a phenomenon that has also been observed for growth factors such as GM-CSF [34] and IL-3 [35]. Thus, although it is possible to accelerate hematopoietic reconstitution after high-dose TBI by giving either a stem cell graft or unfractionated bone marrow, exogenous growth factors add little to the reconstitution because of the relative lack of progenitor cells [18,34].

There is a strong and justified tendency in treating a majority of patients to replace bone marrow transplantation with cytokine-mobilized peripheral blood stem cell transplantation, because the latter have significant advantages in terms of stem cell procurement and the number of cells that can be harvested. As a consequence of the rapid hematopoietic reconstitution observed in recipients following transplantation of mobilized peripheral blood cells, it is difficult to justify the use of growth factor stimulation to further accelerate blood cell production. Indeed, thrombocyte regeneration may well be complete within approximately 2 weeks [36–38], making it unlikely that growth factors such as TPO will significantly enhance this response. Therefore, the present data apply only to clinical conditions in which limited numbers of immature hematopoietic cells are available for transplantation. In spite of advances made, the relative lack of these cells will continue to be observed in a minority of autologous transplantation cases after intensive chemotherapy or in gene transfer protocols. This lack of stem cells will also continue to be seen following allogeneic transplantation, which requires more stem cells than used in autologous recipients because of the histocompatibility barriers and because the number of donor cells available for procurement, especially when matched unrelated donors are involved, is necessarily limited. Similarly, allogeneic transplantation following suboptimal immunosuppression, such as occurs in whole body radiation accident victims and in some of pediatric recipients treated for hereditary deficiencies, may result in significant loss of stem cells through rejection. For those cases it remains necessary to develop growth factor treatment regimens that both accelerate short-term hematopoietic regeneration and promote sustained reconstitution. The present study shows that this cannot be easily accomplished.

Our finding that two of five G-CSF-treated monkeys developed protracted thrombocytopenia, one despite treatment with TPO, suggests that in situations in which limited numbers of cells are available for hematopoietic reconstitution, G-CSF treatment may prove detrimental to platelet reconstitution. The association in our study between protracted thrombocytopenia and G-CSF was statistically significant ($p < 0.03$, Fisher's exact test) when the results are compared with those obtained in 24 monkeys exposed to similar doses of TBI and similar numbers of transplanted $CD34^+$ cells (including the 6 monkeys not treated with G-CSF in the present study), in which protracted thrombocytopenia was not observed (manuscript in preparation). Thrombocytopenia is an occasionally reported side effect of G-CSF treatment [11,39,40]. Although delayed thrombocyte recovery, as well as dampening of TPO-stimulated thrombocyte production by G-CSF treatment, has been observed in recent animal studies [41,42], no animal model has been developed thusfar for the study of protracted isolated thrombocytopenia after BMT. A model exists in normal dogs treated with canine GM-CSF in which consistent thrombocytopenia develops [43,44], but its mechanism may be different from that of post-BMT thrombocytopenia. We took advantage of the G-CSF-associated, isolated thrombocytopenia to examine the effect of TPO treatment for 7 days (10 μg/kg/day sc) in one of the monkeys, using the other, less severely thrombocytopenic monkey as a control. After 1 day of TPO treatment, the severely thrombocytopenic monkey's clinical condition improved, with a rapid disappearance of petechiae and hematomas. Platelet counts started to rise after a week and the monkey became transfusion independent 6 days after the start of TPO treatment. The kinetics of the increase in thrombocytes was similar to that seen in unirradiated TPO-treated monkeys [7]: After discontinuation of TPO, the further rise in platelet counts slowed down and an additional 3 weeks elapsed before normal values were restored. Although this case report remains anecdotal, the pattern observed suggests that TPO will be beneficial in protracted isolated thrombocytopenia following stem cell transplantation and/or BMT.

The present study demonstrates that, after high-dose total body irradiation and transplantation of limited numbers of bone marrow cells or highly purified stem cells, TPO and G-CSF are ineffective in accelerating platelet and neutrophil reconstitution, respectively, although they both enhance recovery from radiation-induced myelosuppression. These findings may be explained by the composition of the cell populations in which hematopoietic reconstitution originates; they may also indicate that TPO and G-CSF targets in transplanted BM are either few in number or difficult to transplant because of poor engraftment, which became especially apparent in this study after transplantation of the limited cell numbers used. The study further defines the limitations of effective growth factor therapy following intensive cytoreductive treatment.

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CHAPTER 6

A single dose of thrombopoietin shortly after myelosuppressive total body irradiation prevents pancytopenia in mice by promoting short-term multilineage spleen-repopulating cells at the transient expense of bone marrow-repopulating cells

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A Single Dose of Thrombopoietin Shortly After Myelosuppressive Total Body Irradiation Prevents Pancytopenia in Mice by Promoting Short-Term Multilineage Spleen-Repopulating Cells at the Transient Expense of Bone Marrow–Repopulating Cells

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Thrombopoietin (TPO) has been used in preclinical myelosuppression models to evaluate the effect on hematopoietic reconstitution. Here we report the importance of dose and dose scheduling for multilineage reconstitution after myelosuppressive total body irradiation (TBI) in mice. After 6 Gy TBI, a dose of 0.3 μg TPO/mouse (12 μg/kg) intraperitoneally (IP), 0 to 4 hours after TBI, prevented the severe thrombopenia observed in control mice, and in addition stimulated red and white blood cell regeneration. Time course studies showed 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 data showed that IP injection resulted in maximum plasma levels 2 hours 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 intravenous 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 is consistent with a threshold level of TPO required to overcome initial c-*mpl*-mediated clearance and to reach sufficient plasma levels for a maximum hematopoietic response. In mice exposed to fractionated TBI (3 × 3 Gy, 24 hours apart), IP administration of 0.3 μg TPO 2 hours after each fraction completely prevented the severe thrombopenia and anemia that occurred in control mice. Using short-term transplantation assays, ie, colony-forming unit–spleen (CFU-S) day 13 (CFU-S-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 was replenished during long-term reconstitution followed for a period of 3 months. Apart from demonstrating again that MRA cells and CFU-S-13 are separate functional entities, the data thus showed 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 shows that TPO is able to intervene in mechanisms that 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.

THROMBOPOIETIN (TPO), the ligand for the cytokine receptor c-*mpl*, has been cloned and characterized in 1994[1-3] and shown to be the physiological regulator of platelet production by generating mice deficient for either TPO or c-mpl.[4,5] Administration of pharmacological doses of TPO to normal mice and nonhuman primates resulted in dose-dependent increases in platelets, far exceeding that observed after administration of other growth factors.[1,2,6,7] These observations have led to the pharmaceutical development of TPO as a therapeutic to counteract thrombopenic states, such as those resulting from myelosuppression due to cancer treatment. In myelosuppression models, TPO effectively alleviated the nadir for platelets and accelerated recovery to normal values.[6,8-11] In several of those models, daily administration during the pancytopenic phase resulted in an overshoot in platelet counts to supranormal values.[6,8,10,11] Although in normal experimental animals the response to TPO was dominant along the megakaryocytic lineage, in myelosuppression models multilineage effects have been shown such as stimulation of erythroid recovery,[8,11-14] acceleration of immature progenitor cell reconstitution in bone marrow,[8,15] and augmentation of the responses to granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF).[8,16] We and others have shown that a single dose of TPO administered 24 hours after total body irradiation (TBI) was as effective in alleviating the nadir for thrombocytes as daily dosing,[13,16-18] thereby reducing the need for thrombocyte transfusions in myelosuppressed nonhuman primates and accelerating recovery to normal platelet counts. The effects on red cell regeneration and immature bone marrow progenitor cells were retained with single dosing of TPO.[13,16] These results showed that the more conventional dose schedules that have come into use for growth factors such as GM-CSF and G-CSF were less appropriate for TPO.

Careful design of growth factor treatment protocols requires preclinical experiments to gain insight into the mechanisms of action and in the optimal dose and dose schedule to achieve maximum therapeutic benefit and reductions in the occurrence of side effects, as well as to prevent unnecessary treatment, thereby reducing costs. Because a delay in the administration of TPO after cytoreductive treatment proved to be detrimental to its efficacy,[18,19] we examined in detail the time dependence of TPO efficacy in the initial 12 hours after myelosuppressive TBI.

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with special emphasis on the response of immature bone marrow cells and their progeny, to reveal mechanisms of TPO action on immature repopulating cells important for efficacy.

MATERIALS AND METHODS

Animals. Female (C57BL × CBA)F1 (BCBA) mice, approximately 12 weeks of age, were bred at the Experimental Animal Facility of Erasmus University (Rotterdam, The Netherlands) and maintained under specific pathogen-free conditions. Housing, experiments, and all other conditions were approved by an ethical committee in accordance with legal regulations in The Netherlands.

Experimental design. TBI was administered at day 0 using two opposing ^{137}Cs sources (Gammacell 40; Atomic Energy of Canada, Ottawa, Canada) at a dose rate between 0.92 and 0.94 Gy/min as described.[20] Doses used were 6 Gy for single dose irradiation and a fractionated dose of 9 Gy in three equal doses with 24-hour intervals. Mice were bled only once; for each data point a random experimental group of three mice was killed. All parameters were collected for individual mice.

Test drug. Recombinant full length murine TPO produced by Chinese hamster ovary cells (Genentech Inc, South San Francisco, CA) was used throughout the experiments, diluted in phosphate-buffered saline/0.01% Tween 20, and administered intraperitoneally (IP) or intravenously (IV) in a volume of 0.5 mL. The dose of TPO used was 0.3 μg/mouse (12 μg/kg, based on a mean body weight of 25 g at the time of irradiation) unless otherwise indicated. We have previously shown that this dose was effective in a similar model for myelosuppression.[13]

Hematologic examinations. After ether-anesthesia, the mice were bled by retro-orbital puncture and killed by cervical dislocation. Blood was collected in EDTA tubes. Complete blood cell counts were measured using a Sysmex F-800 hematology analyzer (Toa Medical Electronics Co, LTD, Kobe, Japan).

Phenotypic analysis of white blood cells. For phenotypic analysis blood was collected in EDTA tubes. Samples from three mice that received the same treatment were pooled to yield sufficient numbers of cells. Red blood cells were removed by incubating whole blood in lysing solution (8.26 g ammonium chloride/1.0 g potassium bicarbonate and 0.037 g EDTA/L) for 10 minutes at 4°C. After lysing, cells were washed twice with Hanks' buffered Hepes solution (HHBS) containing 0.5% (vol/vol) bovine serum albumin (BSA; Sigma, St Louis, MO), 0.05% (wt/vol) sodium azide, and 0.45% (wt/vol) glucose (Merck, Darmstadt, Germany) (HBN). The cells were resuspended in 50 μL HBN containing 4% (vol/vol) normal mouse serum to prevent nonspecific binding of the monoclonal antibodies (MoAbs). To detect neutrophilic granulocytes and monocytes the MoAb ER-MP20 (rat IgG_{2a})[21] was added in a volume of 50 μL. ER-MP20 bright cells are monocytes (corresponding with Mac-1–positive cells[22]) and cells staining intermediate with ER-MP20 are granulocytes (corresponding with Gr-1–positive cells[22]). To detect lymphocytes the anti-CD4 MoAb YTS 191 and the anti-CD8 MoAb YTS 169[23] (a kind gift from Dr H. Waldmann, Department of Pathology, Cambridge University, UK) were added at a concentration of 2 μg/mL. Cells and MoAbs were incubated for 30 minutes on ice. After two washes the cells were incubated with a fluorescein-labeled goat-anti-rat MoAb. After another two washes, the cells were labeled with propidium iodide and measured by flow cytometry. Ungated list mode data were collected for 10,000 events and analyzed using Lysis II software (Becton Dickinson, Mountain View, CA).

TPO levels. Data for characterization plasma TPO pharmacokinetics were generated at Genentech Inc as previously described.[24] In short, mice were injected IP with ^{125}I-rmTPO either with a single dose of 0.9 μg/mouse (36 μg/kg) or with three doses of 0.3 μg/mouse (12 μg/kg) separated by 24 hours. Citrated blood was collected immediately after dosing and at intervals thereafter (n = 3 mice per time point), centrifuged at 2,950*g* for 10 minutes, plasma obtained, and TCA-precipitable radioactivity determined. Pharmacokinetic parameters were estimated after converting trichloroacetic acid–precipitable cpm/mL and fitting the data of concentration versus time to a two-compartment model with first order absorption using nonlinear least-squares regression analysis (WIN-NONLIN; Statistical Consultants, Lexington, KY). Area under the concentration time curves, maximum concentration, terminal half-lives, and clearance (mL/h/kg) were calculated using coefficients and exponents obtained from the model fits.

Colony assays. Serum-free methylcellulose cultures were used in this study.[25-27] Appropriate numbers of bone marrow cells were suspended in Dulbecco's modified Eagle's medium obtained from GIBCO (Life Technologies LTD, Paisley, Scotland) supplemented with the amino acids L-alanine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, and L-proline (Sigma); vitamin B_{12}, biotin, Na-pyruvate, glucose, $NaHCO_3$, and antibiotics (penicillin and streptomycin) at an osmolarity of 300 mOsm/L; supplemented with 1% BSA (Fraction V, Sigma), 2×10^{-6} mol/L iron-saturated human transferrin (Intergen Company, New York, NY), 10^{-7} mol/L Na_2SeO_3 (Merck), 10^{-4} mol/L β-mercapto-ethanol (Merck), linoleic acid (Merck), and cholesterol (Sigma), both at a final concentration of 1.5×10^{-5} mol/L and 10^{-3} g/L nucleosides (cytidine, adenosine, uridine, guanosine, 2'-deoxycytidine, 2'deoxyadenosine, thymidine, and 2'deoxyguanosine obtained from Sigma) and 0.8% methylcellulose (Methocel A4M Premium Grade; Dow Chemical Co, Barendrecht, The Netherlands). The cultures were plated in 35-mm Falcon 1008 Petri dishes (Becton Dickinson Labware) in 1-mL aliquots.[25]

Granulocyte/macrophage colony formation was stimulated by a saturating concentration of macrophage colony-stimulating factor (M-CSF) purified from pregnant mouse uteri extract as described before,[28,29] supplemented with 100 ng/mL murine stem cell factor (SCF; Immunex Corporation, Seattle, WA) and 10 ng/mL murine interleukin-3 (IL-3; R&D, Minneapolis, MN). Granulocyte/macrophage colony-forming unit (GM-CFU) colonies were counted after 7 days of culture. Burst-forming unit–erythroid (BFU-E) growth was stimulated by 100 ng/mL SCF and 4 U/mL human erythropoietin (EPO; Behringwerke, Marburg, Germany), titrated to an optimal concentration. Colonies were counted after 10 days of culture. The culture medium of the erythroid progenitors also contained hemine (bovine, type I; Sigma) at a concentration of 2×10^{-4} mol/L.

Megakaryocyte progenitor cells (CFU-Meg) were cultured in 0.375% agar cultures. Colony formation was stimulated by 100 ng/mL SCF, 10 ng/mL IL-3, and 10 ng/mL murine TPO (Genentech Inc). After 10 days, the cultures were dried, stained for acetylcholinesterase-positive cells, and counted.[30-32] All cultures were grown in duplicate at 37°C in a fully humidified atmosphere with 10% CO_2 in the air. Colony numbers are expressed as a number per femur or per spleen and represent the mean ± SD of individual mice.

The spleen colony assay. This assay was performed as described by Till and McCulloch.[33] Briefly, mice were injected with one fifth of the cell content of a femur in HHBS one day after TBI. Thirteen days later, mice were killed, and spleens were excised and fixed in Tellyesniczky's solution (64% ethanol, 5% acetic acid, and 2% formaldehyde) in H_2O. Colony numbers were expressed as a number per donor femur ± SD. The cells giving rise to the spleen colonies were designated as day 13 CFU-spleen (CFU-S), shortly CFU-S-13.

Marrow repopulating ability (MRA). Bone marrow from mice irradiated with 9 Gy TBI (in 3 equal fractions, each separated by 24 hours) was collected 24 hours after the last fraction of TBI, and 10 lethally irradiated recipient mice were injected with the cellular content of one femur of control mice or mice treated with 0.3 μg of TPO 2 hours after each fraction of TBI. After 13 days the bone marrow of recipient

mice were assayed for the presence of GM-CFU.[34] MRA is expressed as the number of GM-CFU per recipient femur. Data from two independent experiments with similar results were pooled. At the time points of 1 and 3 months and for normal mice, the standard number of 10^5 bone marrow cells was injected.

Statistics. SDs were calculated and are given in the text and the figures on the assumption of a normal distribution. The significance of a difference was calculated by one-way analysis of variance followed by a nonpaired Student's *t*-test using StatView (Abacus Concepts Inc, Berkeley, CA). The SD of CFU-S-13 was calculated on the assumption that crude colony counts are Poisson distributed. Differences in repopulating abilities were evaluated using Fisher's exact test. All colony assays were performed in duplicate for individual mice. The results of the colony assays are expressed as the mean ± SD per femur or spleen for at least three mice per group.

RESULTS

Efficacy of TPO after 6 Gy TBI. Exposure of mice to a TBI dose of 6 Gy resulted in severe bone marrow suppression and impaired blood cell production for a period of about 3 weeks. The nadir for thrombocytes occurred around day 10 (Fig 1). A single dose of TPO administered 24 hours after TBI was effective in alleviating the thrombocyte nadir. Platelet counts 10 days after irradiation were 465 ± 142 × 10^9/L (n = 15) compared with 144 ± 62 × 10^9/L for control mice (n = 14), (Fig 1 and Table 1). TPO injected 2 hours after exposure was significantly more effective, thrombocyte levels 10 days after TBI being 739 ± 165 × 10^9/L (n = 15, $P < .0001$). White blood cell regeneration was not influenced by TPO administered 24 hours after TBI; counts at day 10 were 0.4 ± 0.1 × 10^9/L, similar to 0.4 ± 0.1 × 10^9/L for control mice (Table 1). However, if TPO was administered 2 hours after TBI, white blood cell counts 10 days after TBI were 1.1 ± 0.4 × 10^9/L. For red blood cells the results were: 7.2 ± 0.5 × 10^{12}/L for control mice, 7.5 ± 0.5 × 10^{12}/L for the 24-hour mice, and 9.0 ± 0.6 × 10^{12}/L for mice treated 2 hours after TBI (Table 1). Clearly, administration earlier than 24 hours after TBI resulted in an accelerated multilineage peripheral blood reconstitution in contrast to the lineage dominant effect of TPO when administered 24 hours after TBI. As is well known from previous observations in mice and nonhuman primates,[9,15] the multilineage effects originated from accelerated reconstitution of progenitor cells along the neutrophil, erythroid, and megakaryocytic lineages measured in bone marrow of mice 7 days after irradiation. Femoral GM-CFU, BFU-E, and CFU-Meg reached consistently higher numbers in the treatment group compared with the placebo controls. (Fig 2). The results in the 24-hour treatment group were in between those of the 2-hour and control groups, although the differences were significant only for GM-CFU due to the large variance as a result of exponential reconstitution of progenitor cells at this time interval after TBI. The higher number of femoral GM-CFU in the 24-hour treatment group compared with control numbers was not reflected in accelerated leukocyte regeneration 10 days after TBI (Fig 3), which is consistent with our previous observation in rhesus monkeys that the TPO effect on GM progenitors may

Table 1. Major Peripheral Blood Cell Counts 10 Days After 6 Gy TBI and IP TPO Administration

Time Relative to TBI	TPO Dose (μg)	n	Red Blood Cells (×10^{12}/L)	White Blood Cells (×10^9/L)	Platelets (×10^9/L)
—	—	14	7.2 ± 0.5	0.4 ± 0.1	144 ± 62
+24h	0.3	15	7.5 ± 0.6	0.4 ± 0.1	465 ± 142*
+2h	0.3	15	9.0 ± 0.6*†	1.1 ± 0.4*†	739 ± 165*†
−2h	0.3	3	9.0 ± 0.2*†	0.7 ± 0.2*†	533 ± 55*‡
−2h	30.0	3	9.0 ± 0.3*†	0.8 ± 0.2*†	718 ± 86*†
Normal mice	—	19	10.2 ± 0.6	5.3 ± 1.9	1,123 ± 89

*Significantly different from control mice.
†Significantly different from mice treated 24 hours after TBI.
‡Significantly different from mice treated with 30 μg TPO 2 hours before TBI ($P = .03$).

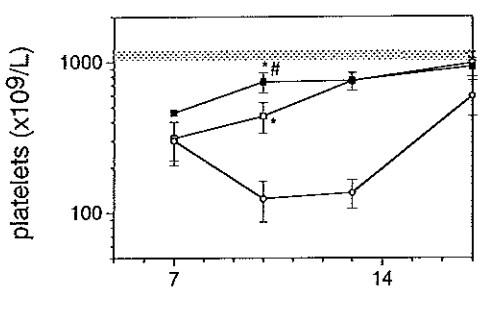


Fig 1. Regeneration pattern of platelets in mice irradiated with 6 Gy TBI and treated with 0.3 μg/mouse of TPO 24 hours after TBI (□), 2 hours after TBI (■), or placebo (○), data from one representative experiment, n = 3 per data point. The shaded area represents the mean platelet counts ±SD of 19 normal mice (1123 ± 89). *$P < .0001$ compared with control mice; #$P < .0001$ compared with mice treated 24 hours after TBI.

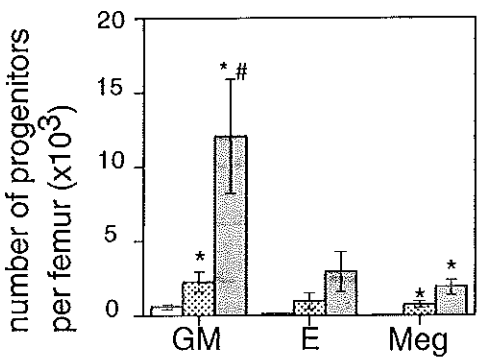


Fig 2. The effect of 0.3 μg TPO 24 hours or 2 hours after TBI on the regeneration of bone marrow progenitors after 6 Gy TBI in mice. GM, GM-CFU; E, BFU-E; and Meg, CFU-Meg per femur at day 7 after TBI (means ± SE). The open bar (□) represents control mice (n = 6), the lightly shaded bar (▨) represents mice treated with TPO 24 hours after TBI (n = 6), and the dark shaded bar (▩) represents mice treated with TPO 2 hours after TBI (n = 6). *Significant compared with control mice ($P < .03$), #significant compared with mice treated with TPO 24 hours after irradiation ($P < .03$).

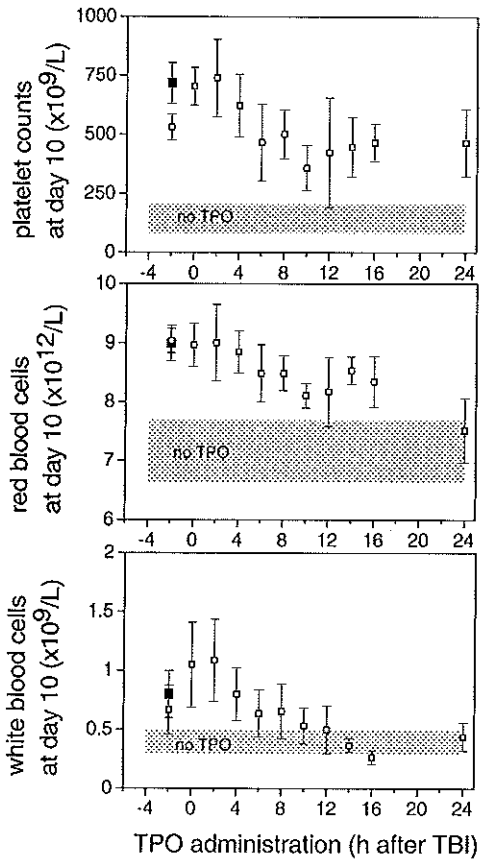


Fig 3. The effect of the time of administration of 0.3 μg TPO IP on peripheral blood cell counts 10 days after 6 Gy TBI. Values are means ± SD. Shaded areas represent the mean ± SD of 14 control mice. The closed squares (■) are counts from mice treated with 30 μg of mTPO 2 hours before irradiation.

require administration of G- or GM-CSF for peripheral blood manifestation.[8,16]

To establish the optimal time point for TPO administration, mice were injected IP with a single dose of TPO at various intervals before or after 6 Gy TBI. Based on Fig 1, the time dependence of the effect of TPO treatment was evaluated 10 days after TBI. The effect of TPO was consistently optimal if TPO was administered IP 0 or 2 hours after TBI (Fig 3). Progressively lower counts were observed when TPO was injected at later time points; platelet counts at day 10 after TBI did not significantly differ between mice treated 6 hours or later after TBI, whereas the difference between later groups and the 0- and 2-hour groups were highly significant ($P < .003$).

A similar time dependence was observed for white and red blood cell counts. For the white blood cells, TPO administration 10 hours or later after TBI resulted in counts not significantly different from control mice while being significantly lower than the 0- and 2-hour treatment groups. The effect on white blood cell regeneration could be attributed to differences in neutrophilic granulocytes and monocytes. Flow cytometric analysis of peripheral blood cells 10 days after irradiation performed in two experiments revealed a major increase in the numbers of ER-MP20–positive granulocytes and monocytes in the TPO treated mice, whereas the number of CD4/CD8-positive T cells was similar in TPO treated mice compared with controls (data not shown), thus showing the myeloid nature of the white blood cell response. Also for red blood cells a gradual decline in effectiveness of TPO with later administration of TPO was observed. We noted that there was no difference between the effects of TPO administration at 0 hours and at 2 hours after TBI, whereas TPO administration 2 hours before TBI resulted in consistently lower levels of platelets and white blood cells at day 10. The hypothesis that pharmacological levels of TPO in the first hours after TBI are required was initially evaluated by administration of a very high dose of TPO before TBI to examine whether an efficacy could be reached similar to that obtained by TPO early after irradiation. Administration of 30 μg TPO IP 2 hours before TBI was as effective as 0.3 μg IP 2 hours after TBI (Table 1 and Fig 3). The dose of 0.3 μg/mouse IP 2 hours before irradiation was significantly less effective compared with the 30-μg dose in alleviating the thrombocyte nadir ($P = .03$) (Table 1 and Fig 3).

The improved efficacy of TPO when administered early and the decline in the efficacy at time points later than 4 hours after TBI led us to speculate that relatively high levels of TPO in the first 2 hours after administration would be of decisive importance for its efficacy. A pharmacokinetic analysis of plasma levels after IP injection of 0.3 μg TPO revealed that peak levels of 29 ng/mL were reached 2 to 2.5 hours after administration, with a terminal half-life of 35 hours and a clearance of 27 mL/h/kg. After the maximum value, there was an initial steep decline followed by a slower wash-out phase (Fig 4). On the basis of these data, in combination with those of Fig 3, we postulated that the multilineage efficacy of TPO was dependent on a threshold level of TPO within the first few hours after TBI. The time course and the plasma level required were accurately assessed using IV administration of TPO (Table 2 and Fig 5). IV administration also showed that early treatment (0 and 2 hours after TBI) was considerably more effective in stimulation of platelet level regeneration than treatment 24 hours after TBI. The multilineage effects seen with early IP treatment were also observed with early IV administration, and were similarly lost if TPO was administered 24 hours after TBI (Table 2). Lowering the dose of TPO to 0.1 μg/mouse did not affect the results, but at 0.03 μg/mouse or lower efficacy was lost, thus demonstrating a threshold TPO level required to achieve optimal multilineage efficacy (Fig 5).

Efficacy of TPO after fractionated irradiation. To enable assessment of immature hematopoietic cells by short-term transplantation assays, which is technically not feasible shortly after 6 Gy TBI due to the very low frequency of residual immature cells, we amplified the TPO response by adjusting the model by fractionation of a total dose of 9 Gy TBI in three equal doses separated by 24 hours. This regimen induced a slightly

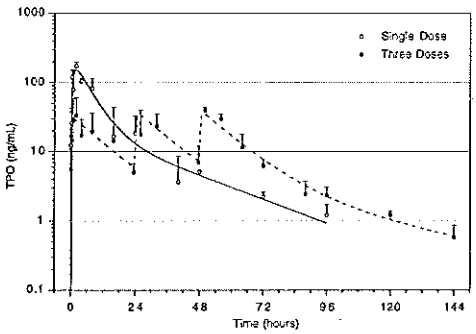


Fig 4. Plasma level of TPO after IP administration of 3 doses of 0.3 μg/mouse separated by 24 hours each (dotted lines), and of a single dose of 0.9 μg/mouse (solid line). For explanation, see text.

more profound pancytopenia than the single dose of 6 Gy TBI (Fig 6). Fractionated TBI would also more closely resemble the protracted cytotoxic insult administered to chemotherapy patients. Groups of mice received either no TPO, or were treated with 0.9 μg TPO 2 hours after the last fraction of TBI, 0.3 μg TPO 2 hours after each fraction of TBI, or 0.9 μg TPO 2 hours before the first fraction of TBI. Pharmacokinetic analysis of repetitive IP administration (three doses of 0.3 μg/mouse, each separated by 24 hours) did not reveal accumulation of TPO plasma levels. The dose of 0.9 μg/mouse resulted in higher peak plasma levels (151 ng/mL) without affecting the terminal half-life or clearance (Fig 4). TPO administered 2 hours after each fraction of radiation completely prevented thrombopenia (Fig 6 and Table 3). This schedule did not prevent the severe reduction in neutrophils, but accelerated their recovery to normal values. Platelet counts and red and white blood cells were significantly lower in the group treated with 0.9 μg TPO 2 hours after the last fraction compared with 0.3 μg TPO 2 hours after each fraction. Administration of TPO 2 hours before the first fraction in a dose of 0.9 μg/mouse was largely ineffective. The effect of TPO on blood cell regeneration was also reflected at the level of progenitors of different blood cell lineages (Fig 7). Placebo mice and mice treated with 0.9 μg/mouse 2 hours before TBI displayed very low levels of progenitors even at day 13 after TBI, before progenitor cell reconstitution resulted in a large overshoot, especially manifest in the spleen 3 weeks after TBI. In contrast, the schedule optimal for blood cell regeneration (2 hours after each fraction) also led to a rapid normalization of progenitor cells in the spleen without the characteristic large overshoot in the placebo controls ($P = .01$ at day 17) and in the mice treated with the suboptimal TPO dose schedule. In the fractionated radiation model, administration of 30 μg of TPO 2 hours before the first radiation fraction was as effective as TPO administered 2 hours after each dose of radiation (Table 3 and Fig 6).

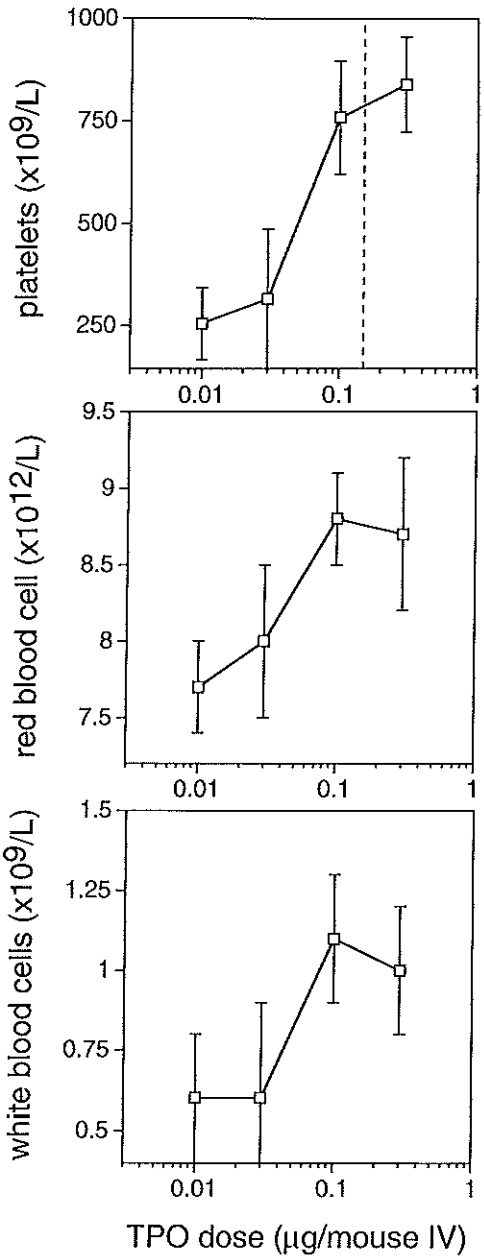
Table 2. Major Peripheral Blood Cell Counts 10 Days After 6 Gy TBI and IV TPO Administration at Different Timepoints After TBI

Time Relative to TBI	TPO Dose (μg)	n	Red Blood Cells ($\times 10^{12}$/L)	White Blood Cells ($\times 10^{9}$/L)	Platelets ($\times 10^{9}$/L)
–	–	14	7.2 ± 0.5†	0.4 ± 0.1†	144 ± 62†
0	0.3	12	8.7 ± 0.5*	1.0 ± 0.2*	838 ± 116*
2	0.3	6	8.8 ± 0.5*	1.0 ± 0.1*	765 ± 119*
4	0.3	6	8.2 ± 0.5*	0.7 ± 0.1*†	658 ± 140*†
6	0.3	6	8.3 ± 0.3*	0.5 ± 0.1*†	691 ± 77*†
8	0.3	6	7.8 ± 0.3*†	0.5 ± 0.1†	504 ± 88*†
24	0.3	3	7.5 ± 0.2†	0.3 ± 0.1†	424 ± 143*†
Normal mice	—	19	10.2 ± 0.6	5.3 ± 1.9	1,123 ± 89

*Significantly different from control mice.
†Significantly different from the 0-hour treatment group.

Because the progenitor cell data of irradiated TPO-treated mice in this fractionated TBI regimen indicated a prominent effect of TPO on immature bone marrow BM cells, CFU-S-13 were enumerated, the MRA of bone marrow of the TPO-treated mice assessed, and progenitor cells assayed 24 hours after the last TBI fraction (Table 4). The numbers of CFU-S-13 of mice treated with TPO 2 hours after each dose of irradiation were approximately 14-fold those of the placebo control mice (Table 4). The progenitor cell content of the bone marrow 24 hours after TBI was also significantly increased (Table 4), GM-CFU numbers per femur approximately 4-fold, BFU-E numbers 65-fold, and CFU-Meg 20-fold those of placebo control mice, which grossly corresponds to the efficacy of TPO treatment along those lineages. The MRA of TPO-treated mice, defined as secondary GM-CFU in the bone marrow of lethally irradiated recipients, was more than one order of magnitude reduced in the TPO-treated mice compared with placebo-treated controls (Fig 8). This result showed that the TPO-stimulated increase of CFU-S-13 and progenitor cells occurred at the expense of the more immature MRA cells, which were proportionally depleted. Although day 13, which is the time of the peripheral blood count nadirs at which white blood cells were undetectable in both groups of lethally irradiated recipients, is not the most suitable time interval to study the impact of this shift among the immature bone marrow cells on peripheral blood cell reconstitution, the difference in repopulating ability was also clearly reflected in the erythrocyte as well as the thrombocyte counts of the lethally irradiated recipients used for the MRA assay (measured in one of the two experiments from which the upper panel of Fig 8 was derived). The 10 recipients of bone marrow from TPO-treated mice reached erythrocyte counts all exceeding 4.4×10^{12}/L (4.7 ± 0.3), and all but 1 reached thrombocyte counts of more than 10×10^{9}/L, as opposed to the 8 (of 10) surviving recipients of bone marrow from placebo-treated mice that had erythrocyte counts of 3.4 ± 0.9 × 10^{12}/L and thrombocyte counts of 5.5 ± 2.8 × 10^{9}/L. These differences are highly significant ($P = .002$ and $P = .006$, respectively; Fisher's exact test), thus illustrating the importance of spleen-repopulating cells in early reconstitution of peripheral blood counts in mice. Because a depletion of immature stem cells, such as measured by the MRA assay, could be potentially deleterious in the clinical setting, the effect of TPO on bone marrow progenitor cells was also evaluated after a more prolonged interval. Peripheral blood count and progenitor cell content of both spleen and femurs were evaluated 1 and 3 months after the 3 × 3 Gy irradiation protocol. Peripheral blood counts did not differ between TPO- or placebo-treated mice at 1 and 3 months, and also the femoral and spleen content of GM-CFU, BFU-E, and CFU-Meg was not different between

both groups at either time point (data not shown). At these time intervals, we also measured the MRA of the bone marrow of the TPO- versus placebo-treated mice (Fig 8). MRA cells were similar in both groups of mice at 1 month after irradiation



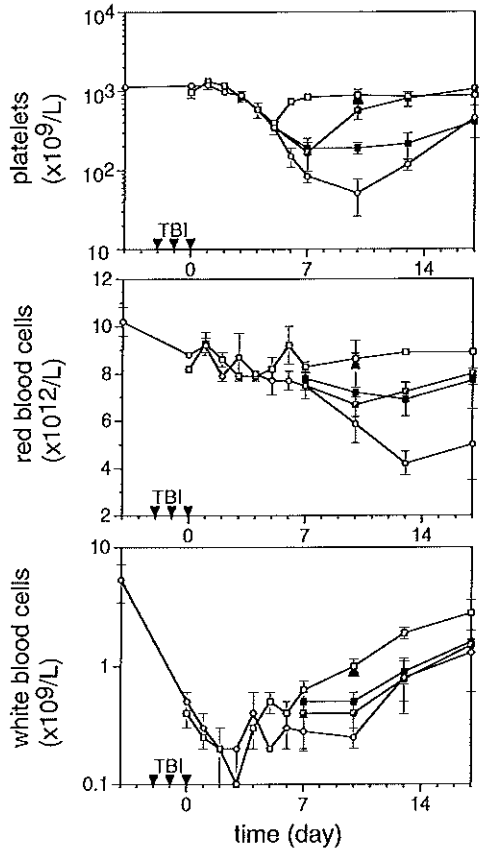


Fig 6. The effect of TPO on hematopoietic regeneration after 9 Gy TBI in mice. The mice were irradiated with three fractions (▼) of 3 Gy with 24-hour intervals. In the upper panel platelet regeneration is depicted, in the middle panel red blood cell regeneration is shown, and in the lower panel white blood cell regeneration is shown. (□), mice treated with three doses of 0.3 µg/mouse of mTPO IP 2 hours after each fraction of TBI; (◪), mice treated with 0.9 µg IP 2 hours after the last fraction of TBI (day 0); (■), mice treated with 0.9 µg IP 2 hours before the first fraction of TBI; (○), control mice. Data are given as means ± SD, three mice per data point per group. The closed triangles (▲) are counts from mice treated with 30 µg of mTPO IP 2 hours before irradiation (n = 3).

although still depleted compared with normal levels. At 3 months after irradiation, MRA cells in both groups had returned to subnormal levels, again without a difference between the recipient groups. On this basis, we concluded that the initial

Fig 5. Dose-response curves for the multilineage effect of TPO administered IV immediately after 6 Gy TBI. Peripheral blood cell counts 10 days after 6 Gy TBI. Values are means ± SD, n = 6 for the doses of 0.01, 0.03, and 0.1 µg and n = 12 for the dose 0.3 µg/mouse. The vertical line in the upper panel represents the dose level of 0.15 µg/mouse, which results in 50% saturation of *c-mpl* on platelets. The placebo control levels coincide with the level at which the horizontal axis is set.

Table 3. Major Peripheral Blood Cell Counts 10 Days After 9 Gy TBI (3 × 3 Gy, 24 Hours Apart) and IP TPO Administration

Time Relative to TBI	TPO Dose (μg)	n	Red Blood Cells ($\times 10^{12}$/L)	White Blood Cells ($\times 10^{9}$/L)	Platelets ($\times 10^{9}$/L)
—	—	9	5.8 ± 0.8	0.26 ± 0.05	51 ± 25
3× + 2h	0.3	6	8.6 ± 0.2*	0.93 ± 0.23*	883 ± 163*
+2h	0.9	3	6.6 ± 0.7	0.43 ± 0.06*	527 ± 135*
−2h	0.9	3	7.2 ± 0.2*	0.47 ± 0.06*†	215 ± 80*†
−2h	30.0	3	8.4 ± 1.0*	0.90 ± 0.10*	792 ± 157*
Normal mice	—	19	10.2 ± 0.6	5.3 ± 1.9	1,123 ± 89

3× + 2h, three doses of 0.3 μg TPO 2 hours after each dose of TBI; +2h, 0.9 μg TPO 2 hours after the last dose of TBI; −2h, 0.9 or 30 μg TPO 2 hours before the first dose of TBI.

*Significantly different from control mice.

†Significantly different from mice treated with 30 μg TPO 2 hours before TBI.

depletion of MRA of TPO-treated mice became replenished during long-term hematopoietic reconstitution from cells with, by definition, long-term repopulating ability. The protracted nature of MRA reconstitution, which has not been documented before, is noteworthy.

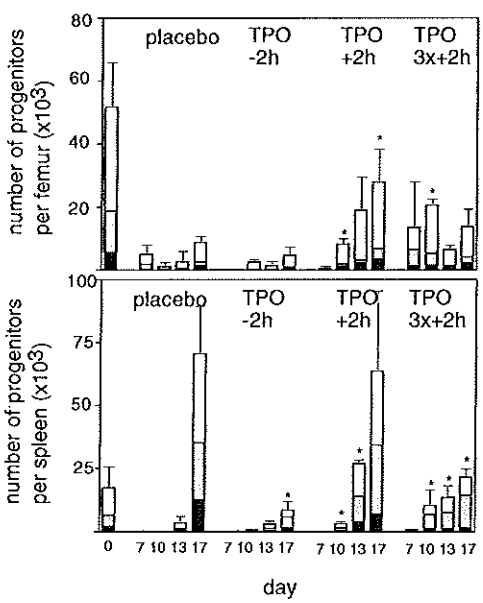


Fig 7. Regeneration of progenitor cells in bone marrow (upper panel) and spleen (lower panel) at 7, 10, 13, and 17 days after the last fraction of TBI in mice irradiated with three fractions of 3 Gy with 24 hour intervals. (■), GM-CFU per femur or spleen; (▨), BFU-E; (□), CFU-Megs per femur or spleen. Placebo, control mice; TPO-2h, mice treated with 0.9 μg TPO 2 hours before the first fraction of TBI; TPO+2h, mice treated with 0.9 μg 2 hours after the last fraction of TBI; TPO3x+2h, mice treated with three doses of 0.3 μg/mouse 2 hours after each fraction of TBI, three mice per data point. SDs were calculated for the sum of the individual colonies per mouse. *Significantly different from time matched controls ($P < .05$), **($P < .005$).

Table 4. Effects of TPO Treatment After 9 Gy TBI (3 × 3 Gy, 24 Hours Apart) on CFU-S-13 and Progenitor Cell Content of Bone Marrow 24 Hours After the Last Dose of TBI in Mice Treated With TPO IP 2 Hours After Each Dose of TBI Versus Control

Treatment	CFU-S-13 (Colonies per Femur)	GM-CFU/ Femur	BFU-E/ Femur	CFU-Meg/ Femur
Placebo	2 ± 2	597 ± 229	8.3 ± 3	22 ± 10
TPO 3 × 0.3	27 ± 6*	2026 ± 131*	558 ± 282*	430 ± 135*

*Significantly different from placebo.

DISCUSSION

The present analysis shows that a single administration of TPO is capable of counteracting radiation-induced pancytopenia mediated through its effect on immature multilineage repopulating cells and their direct progeny, thereby promoting peripheral blood reconstitution of thrombocytes, erythrocytes, and granulocytes. Multilineage efficacy was critically dependent on time relative to TBI and a relatively high dose of TPO. In particular, the first few hours after TBI appeared to be important to elicit the multilineage response to TPO. Mechanisms involved included prevention of depletion of multilineage cells during the first 24 hours after TBI, a preferential stimulation of spleen-repopulating cells with short-term peripheral blood reconstituting ability at the transient expense of marrow-repopulating cells, and a threshold dose of TPO to overcome initial *c-mpl*–mediated clearance.

The mechanism by which TPO makes multilineage cells available for accelerated hematopoietic reconstitution remains to be further elucidated, as does their apparent functional depletion as a function of time after 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 hours 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 hematopoietic 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 hematopoietic cells[35] 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 confer a proliferative response to immature hematopoietic cells, but does so strongly in the presence of suitable other factors, eg, Kit ligand.[36–42] The mechanisms involved might therefore also include activation of one or more cofactors required for the strong proliferative response observed in the present study. We also do not exclude that the effect of TPO on multilineage cells is augmented by the release of various cytokines by megakaryocytes[43,44] subsequent to stimulation by TPO, although the time frame observed makes such a mechanism not likely.

The lack of a leukocyte response 10 days after 6 Gy TBI in the presence of significantly more GM-CFU at 7 days in the 24-hour treatment group is noteworthy. The neutrophil response to TPO has been variable throughout the reported studies.[6,9,11,45,46] We observed in a myelosuppression model in

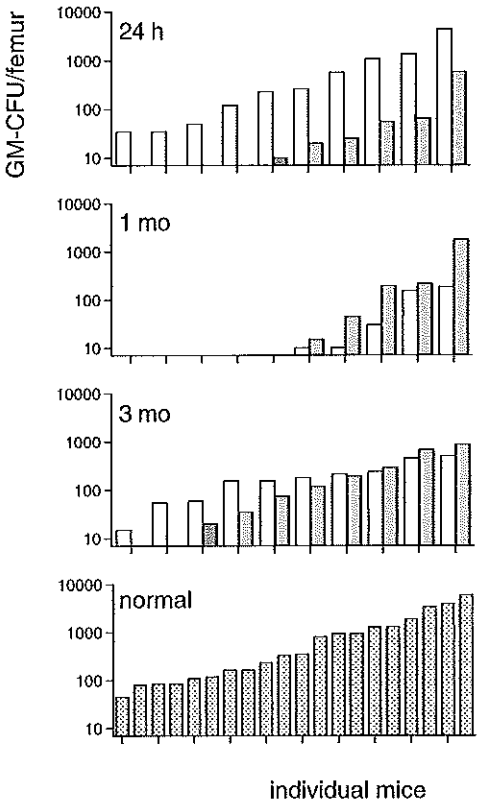


Fig 8. Marrow repopulating ability: GM-CFU, ranked in ascending order, on day 13 in femurs of recipients of bone marrow of mice treated with 0.3 μg TPO IP 2 hours after each fraction of 3 Gy TBI (▩) in comparison with those of recipients of control marrow (□) of mice that did not receive TPO. Upper panel: a total of 25 mice were injected with bone marrow (the content of 1 femur, 24 hours after TBI) of TPO-treated mice of which only 5 mice had more than 10 GM-CFU per femur, and 17 (surviving of 20 injected) mice with bone marrow of placebo-treated mice of which 10 had more than 10 GM-CFU per femur. Results from two experiments. This difference is highly significant ($P = .01$, Fisher's exact test). Upper middle panel: 10 mice were injected with the standard 10^5 bone marrow cells from each of the groups 1 month after TBI, of which in each recipient group 9 mice survived and 5 mice had more than 10 GM-CFU per femur. Lower middle panel: 10 mice were injected with the standard 10^5 bone marrow cells from each of the groups 3 months after TBI, of which in the recipient group of the TPO-treated mice 1 mouse died before day 13 and 1 mouse had less than 10 GM-CFU/femur. Lower panel: 20 mice were injected with the standard 10^5 bone marrow cells from normal, untreated donors for comparison.

rhesus monkeys that TPO-stimulated GM-CFU reconstitution was not reflected in an increase in peripheral blood granulocytes which, however, could be brought out by coadministration of G- or GM-CSF.[8,16] Thus, without exogenous CSFs, the myeloid progenitor cell effect of TPO after myelosuppression may remain unnoticed at the peripheral blood cell level, to which the short survival of circulating neutrophils might also contribute. These observations are fully consistent with the mouse data presented here. It is not inconceivable that some myelosuppression regimens may result in sufficiently high endogenous CSF levels for a significant neutrophil response to exogenous TPO alone, as has been observed in some studies.[9,11,45]

To establish the TPO dose level needed, a dose titration was performed using IV administration immediately after irradiation. The doses of 0.3 and 0.1 μg/mouse (12 and 4 μg/kg, respectively) gave identical results, whereas 0.03 (1.2 μg/kg) and 0.01 μg/mouse were largely ineffective. This observation is interpreted as evidence for a threshold level of TPO required for optimal efficacy. It was previously shown[13,24] that IV injection of ^{125}I-rmTPO into mice results in an initial sharp decline in plasma levels, followed by steady state clearance approximately 3 hours after IV injection.[24] A lower dose does not influence the terminal half-life.[13] After IV 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,[24] whereas the slower terminal decline is likely related to uptake and clearance by c-*mpl* on platelets, spleen cells, and megakaryocytes, as well as nonspecific mechanisms.[24,47] 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.[48] This relationship between TPO pharmacokinetics and c-*mpl* levels has recently been studied in normal mice, which showed that an IV dose of approximately 6 μg/kg (≈0.15 μg/mouse) was needed to obtain an occupancy of 50% of c-*mpl* sites (indicated in Fig 5). Doses greater than 6 μg/kg began to saturate this specific clearance mechanism, whereas lower doses failed to reach 50% receptor occupancy.[48] 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 hematopoietic response. Doses larger than 6 μg/kg (0.15 μg/mouse) may not provide any greater efficacy. These data are consistent with the findings presented in Fig 5, showing that the hematopoietic recoveries after IV 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 IV pharmacokinetic data comparing TPO plasma levels in c-*mpl* knockout and normal mice,[24] 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 hours apart), the optimal dose and dose scheduling of TPO derived from the 6 Gy experiments, ie, 0.3 μg/mouse, 2 hours after each TBI fraction, prevented thrombopenia and promoted erythrocyte and leukocyte reconstitution.

We noted that also the femoral and spleen progenitor cells (Fig 7) normalized rapidly and lacked the late overshoot in the spleen characteristic of the placebo controls. Because the range of stimulation by TPO was not lineage specific, we postulated that the effect was mediated by stimulation of multilineage cells. After transplantation of bone marrow into lethally irradiated recipients, the number of CFU-S-13 is a measure for relatively immature repopulating stem cells,[49] associated with the initial, short-term wave of hematopoietic reconstitution, which lasts for several months.[50] By this assay it was shown that the multilineage effect of TPO administered 2 hours after TBI is mediated through stimulation of these immature cells and is already manifest 24 hours 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 hematopoiesis after bone marrow transplantation.[34] MRA, measured by enumeration of GM-CFU numbers in the bone marrow of mice injected with cells from TPO treated mice, was one to two orders of magnitude less than that in control mice. The increase of CFU-S-13 and the concomitant decrease of cells with MRA most likely indicates recruitment of multilineage short-term repopulating cells from a more immature ancestral population. This is the more conceivable from the magnitude of this effect (14-fold for CFU-S), which suggests three to four cell doublings. Because these occurred during the 3 days that elapsed from the first TPO administration to the time of the measurement, 24 hours after the last fraction of TBI, this would be in close agreement with the doubling time established previously[27] for such immature cell populations during hematopoietic reconstitution. 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.[34,51,52]

The depletion of MRA cells in the TPO-treated mice, measured 24 hours after TBI, was transient. Peripheral blood cell regeneration 1 and 3 months after three fractions of 3 Gy was not different in mice treated with TPO compared with 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 show 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. 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.[53] However, this survival effect had become established already at the short-term hematopoietic reconstitution parameter of 30-day survival (in practice already within 17 days), closely associated[50] to the CFU-S-13 assay used in the present study. Using such an experimental design to establish TPO effects on long-term repopulating cells would require a genetic marker capable of distinguishing between donor and recipient cells along multiple hematopoietic lineages.

In addition, we showed that TPO is effective if administered at a very high dose shortly before myelosuppressive TBI. The dose used, 30 μg/mouse (1.2 mg/kg), suprasaturates the c-*mpl*–mediated clearance mechanism (which makes pharmacokinetic measurements futile) and is not recommended for clinical use. We did not so far establish empirically the minimum effective dose required for prophylactic TPO administration, but such a dose could be derived on the assumption of maintaining plasma TPO levels of approximately 60 ng/mL in the first hours after TBI as calculated above. However, the observation has relevance for the further development of efficacious dose and dose scheduling regimens, especially in conjunction with chemotherapy, as well as in the area of radiation protection. The present study did not address propensity to hemorrhage and prevention of mortality, but rather was directed at mechanisms important to optimize efficacy. The benefit of TPO treatment shortly after much higher ("supralethal") doses of TBI on survival and prevention of bleeding will be published separately.[54]

In vivo studies on TPO efficacy have yielded various results, in that in normal animals usually only a platelet response was obtained,[6,7,55-59] whereas in myelosuppressed animals multilineage responses was the prevailing pattern,[8,9,11-13,15,16,53,60,61] and after transplantation of limited numbers of stem cells no response was obtained at all.[62] 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. The present study also identifies time relative to myelosuppression and dose of exogenous TPO as pivotal factors. In addition, the difference between normal and myelosuppressed animals indicates 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, eg, Kit ligand.[36-42] 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.

Irrespective of the mechanisms involved, the optimal efficacy of TPO if administered within 2 hours after cytoreductive treatment places emphasis on the importance of dose and dose scheduling in clinical protocols of cancer treatment and/or after bone marrow transplantation. Indeed, the initial clinical experience did not demonstrate a major effect similar to those observed in the experiments described here.[63-66] It is concluded that dosing similar to what has become conventional based on the G-CSF and GM-CSF experience is not optimal for TPO and that its efficacy can be substantially improved by achieving relatively high TPO levels shortly after cytoreductive treatment. This was attributable to the dual target cell nature of the TPO response, of which the important multilineage component is functionally depleted shortly after radiation exposure, and to the

identified threshold plasma level of TPO required to overcome its initial c-*mpl*–mediated clearance.

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CHAPTER 7

General Discussion

7.1 INTRODUCTION

The cloning of the gene for thrombopoietin and the subsequent production of various forms of the protein have led to extensive research directed at the physiological role, mechanism(s) of action, regulatory mechanisms and potential clinical applications of TPO. The experimental work described in this thesis supports the hypothesis that the cytokine regulating platelet production might have therapeutic applications in counteracting induced thrombocytopenic conditions. In preclinical experiments in rhesus monkeys, the efficacy of TPO after myelosuppression and after bone marrow transplantation was investigated, and in studies with myelosuppressed mice the administration schedule was improved and the cellular basis of the multilineage effects further elucidated.

Evaluation of TPO in a myelosuppression model is clinically relevant since many oncology patients become thrombocytopenic as an adverse effect of anti-cancer treatment. This may lead to complications such as bleeding and often results in treatment interruptions which are unfavorable for probability of cure. (1-5) Prevention of thrombocytopenia therefore would reduce morbidity and decrease the number of thrombocyte transfusions needed. This is also beneficial in view of the occurrence of allosensitization in response to platelet transfusions which should be avoided, especially if an allogeneic bone marrow or stem cell transplantation is contemplated, and in view of infection risks related to the use of blood products. Since thrombocyte transfusions account for a considerable part of treatment costs, thrombopoietin therapy might also reduce those.

Severe myelosuppression as occurs with certain chemotherapy regimens, or myeloablation in the treatment of hematologic malignancies, require a hematopoietic stem cell transplant to ensure hematopoietic reconstitution. However, reconstitution following transplantation is often impaired, and protracted thrombocytopenia is a well known problem, especially in patients subjected to prior chemotherapy. (6-10) Patients would benefit from adjuvant or supportive treatment modalities to enhance blood cell reconstitution. For this reason, the efficacy of TPO to stimulate platelet regeneration was also evaluated in a model for purified stem cell transplantation.

TPO greatly promoted thrombocyte regeneration after a myelosuppressive dose of 5 Gy TBI, which results in severe pancytopenia for a period of approximately 3 weeks. TPO when administered alone also enhanced red blood cell reconstitution. Combining TPO with either of the two myeloid growth factors registered for clinical use, G-CSF or GM-CSF, was even more efficacious; the stimulating effect on platelet regeneration was maintained while TPO acted in synergy with the CSF's to enhance neutrophil regeneration. The effects outside the megakaryocytic lineage were originally not anticipated and could be demonstrated to result from accelerated multilineage BM progenitor cell regeneration. However, despite the promising results in the myelosuppression model, TPO was ineffective after 8 Gy TBI and transplantation of a highly defined stem cell subset.

A separate set of experiments was carried out in myelosuppressed mice to adjust the dose schedule of TPO in order to maximize the multilineage response. An optimal effect could be achieved if TPO was administered within the first few hours after TBI. Furthermore, the origin of the multilineage response was investigated by determining the effect of TPO on immature hematopoietic cells, i.e., day 13 spleen colony-forming cells (CFU-S-13) and cells with marrow repopulating ability (MRA).

A comprehensive model to explain both the *in vitro* and the *in vivo* results obtained with recombinant TPO is emerging. In line with expectations, TPO was shown to be the physiologic regulator of platelet production. Mice genetically engineered to be deficient for either TPO or its receptor are severely thrombocytopenic (11-13), demonstrating the importance of TPO in maintaining *in vivo* platelet levels. *In vitro,* TPO is a powerful megakaryocyte colony stimulating factor and also supports the maturation of megakaryocytes. (14-16) Injection into normal experimental animals induced a striking thrombocytosis and increased numbers of megakaryocytes in the bone marrow. (14,16-18) TPO levels appeared to be simply regulated by a c-*mpl* mediated clearance mechanism and therefore, since c-*mpl* is present on platelets, predominantly by platelet mass.

Contrary to initial expectations of lineage dominance, TPO was also shown to be an early acting cytokine. Apart from expression throughout the megakaryocytic lineage (19-21), the TPO receptor is present on multilineage hematopoietic precursor cells in both mice and humans. (21,22) Effects exerted on those early cells include suppression of apoptosis of immature cells. (23-26) Also, TPO stimulates *in vitro* colony formation from those cells in synergy with other growth factors such as SCF and IL-3. (22,27-32) This *in vitro* dependence of TPO on other cytokines for a proliferative effect on immature multilineage cells is reflected *in vivo* by the difference in effects of TPO in normal and myelosuppressed animals. In normal animals the effect is mainly thrombopoietic whereas after myelosuppression multilineage effects have been demonstrated. (this thesis and (33-36)) After myelosuppression, a variety of cytokines is upregulated and endogenously produced that could act as the required co-factors. (37-40) It is, in addition, possible that the effect of TPO on multilineage cells *in vivo* is augmented by the release of various cytokines by megakaryocytes (41,42) subsequent to stimulation by TPO. To obtain an optimal multilineage effect in mice it is important to administer TPO early after TBI in a dose sufficient to overcome initial c-*mpl* mediated clearance. The precise mechanism of action should be clarified to extrapolate these findings to an optimal clinical efficacy.

In this chapter the data presented in this thesis will be discussed against the background of the expanding literature on the effects of TPO in preclinical settings. A final paragraph will review the clinical data available to date.

7.2 EFFECTS OF TPO ADMINISTRATION IN NORMAL ANIMALS

Before a cytokine can be used in clinically relevant models, experiments in normal animals are needed to investigate the dose range needed for effectiveness, the mechanism of action and potential adverse effects. The dose of human TPO used in the experiments described in the first chapters was based on dose titration experiments carried out at Genentech in normal rhesus monkeys and rodents and was set at 10 μg/kg. This is in the range of doses used by other groups (14,16-18) resulting in four- to ten-fold increases in platelet counts in normal animals. (14,16-18) Preceding the increase in platelet numbers, an increase in the numbers of CFU-Meg in the bone marrow (17,18,34,43) and in the volume and ploidy of megakaryocytes was observed. (18,34) Although increases in the number of bone marrow BFU-E (33,34) or CFU-GM (33) have been reported, increases in peripheral blood reticulocytes, erythrocytes or leukocytes were not observed. (16-18,33,34)

Those results are consistent with the hypothesis that additional cytokines are necessary to produce mature end cells of other lineages than the megakaryocytic. In steady state bone marrow the pharmacological doses of TPO only stimulate the proliferation of megakaryocyte progenitors, the maturation of megakaryocytes and the production of platelets, resulting in thrombocytosis, whereas stimulation of more immature cells may lead to a certain expansion of bone marrow progenitors of other lineages without the production of mature blood cells.

The only adverse effect reported is the development of cross-reacting antibodies in normal dogs to human TPO. (44) This results in thrombocytopenia approximately 2 months after the start of TPO administration, accompanied by decreased numbers of bone marrow megakaryocytes. Also in sublethally irradiated dogs, sensitization to human TPO followed by thrombocytopenia has been reported. (45) Thrombocytopenia due to the development of antibodies after TPO administration has not been published for other species so far. However, patients treated with TPO should be carefully monitored, especially if TPO will be used in otherwise healthy people, such as donors of allogeneic transplants.

The presence of TPO receptors on platelets indicates that TPO might have a function beyond the maturation of thrombocytes, such as modulating hemostasis. Although activation of platelets has been observed *in vitro* (46-48), platelet aggregation only occurred in the presence of other agonists. (46-48) The physiologic meaning of this observation is not clear, and thrombotic events have not been reported in animal models. (49) Other adverse effects have not been observed, also not due to the very high thrombocyte counts induced by TPO treatment.

7.3 EFFECTS OF TPO ADMINISTRATION AFTER MYELOSUPPRESSION

In the first part of this thesis the results of TPO administration to myelosuppressed rhesus monkeys are described. A conventional growth factor dose schedule consisting of daily dosing for 21 consecutive days, starting the day after TBI, was used. Results were very promising in that thrombocyte reconstitution to normal values was accelerated by two weeks, with an improvement of the thrombocyte nadir and a complete prevention of the need for thrombocyte transfusions. The effect of TPO was not restricted to the megakaryocytic lineage. FACScan analysis revealed much greater numbers of $CD34^+$ cells in the bone marrow of TPO treated monkeys. Using progenitor cell assays of regenerating bone marrow it was shown that also GM-CFU and BFU-E recovery was accelerated. This was reflected in an enhanced red blood cell nadir and, with co-administraton of G-CSF, enhanced neutrophil reconstitution. Similar results on thrombocyte regeneration after myelosuppression have been obtained by other groups. (35,43,50,51) The effect of TPO on erythroid precursors and red blood cell regeneration after TBI has also been demonstrated in mice (34), as was the regeneration enhancement of bone marrow CFU-GM. (33) In our experiments, and in carboplatin treated mice (50), an effect of TPO alone on peripheral blood neutrophil reconstitution was not demonstrated, although in similar experiments in myelosuppressed mice and rhesus monkeys, some efficacy along this lineage was observed. (35,43,52)

The combination of TPO with G-CSF leads to various results in different settings. In our experiments, the combination of G-CSF and TPO counteracted the thrombocyte recovery induced by TPO, as was similarly reported in a chemotherapy model in baboons. (52) In normal mice, G-CSF down-modulated the effect of TPO on the ploidy of megakaryocytes. (53) However, in ^{60}Co irradiated rhesus monkeys and in mice myelosuppressed by irradiation and carboplatin this adverse effect was not seen (35,36), or even enhanced thrombocyte regeneration compared to TPO alone was reported. (51) For neutrophil regeneration the effect of the combination of TPO and G-CSF is also heterogenous. We found a synergistic or additive effect as did certain other groups (35,36,51), whereas in chemotherapy treated baboons such effects were not observed. (52) The reason for these discrepancies could relate to differences in the production of endogenous cytokines due to variations in the induction of myelosuppression, or differences in the dose of exogenously administered G-CSF.

The effects of TPO on erythrocyte regeneration and BM progenitor cell recovery after myelosuppression, fit with the concept of multilineage stimulation by TPO. The difference between the effect of TPO on hematopoiesis in normal and myelosuppressed animals likely results from the change in the cytokine environment that occurs following myelosuppressive therapy. During the pancytopenic state cytokines are produced which are normally below detection level. This could for instance explain the differential effect of TPO on erythroid cell production in normal and myelosuppressed animals. It has been demonstrated that the erythropoietic effect of TPO is dependent on the presence of EPO

(32,54,55), and the TPO receptor has been demonstrated on cultured human erythroblasts (19) and on the early erythroid progenitor cell BFU-E. (56) In normal animals, administration of TPO leads to increased numbers of erythroid progenitors, but because in steady state hematopoiesis levels of EPO are very low (39), an increase in red cell numbers is not observed. After cytoreductive treatment, the animals progressively become more anemic, which will induce a rise of EPO levels. Synergism between EPO and TPO leads to increased numbers of CFU-E and reticulocytes. The increased production of red blood cells elevates the hemoglobin nadir and, in the presence of sufficient supplies of iron, accelerates reconstitution of normal Hb levels. (Chapters 2,3 and 4) Similarly, the increase in white blood cells in response to TPO administration in myelosuppressed animals may be the result of the synergistic effect of TPO and endogenously increased other cytokines, such as G-CSF and GM-CSF.

In experimental animal models for myelosuppression, TPO was initially administered for prolonged periods of time after the cytotoxic insult, starting 24 hours after irradiation or chemotherapy. Delayed administration in rhesus monkeys and mice was significantly less effective than administration early after TBI. (57,58) It has been shown recently that a single dose of the molecule is as effective (58-61) as repetitive administration to achieve the main goal of treatment: elevate the nadir for thrombocytes to reduce the need for thrombocyte transfusions and accelerate reconstitution to normal thrombocyte counts. Also in normal mice the effects of single dose administration of TPO on platelet counts (50,61), megakaryocyte numbers, size and ploidy (62) in the bone marrow, are quite similar to those seen in animals to which repetitive doses were administered. The fact that a single dose of TPO produces a platelet response that is sustained several days explains why the dependence of the response on repetitive doses of TPO is not very striking. After a first injection of TPO additional injections will not add much to the magnitude of the platelet response.

In principle, stimulation of blood cell regeneration after myelosuppression by hematopoietic growth factors is either due to increased numbers of progenitor cells activated to produce end cells, or to accelerated cell divisions, resulting in a shorter doubling time. If growth factor stimulated production only occurs from a larger number of progenitor cells, the doubling time will be unaltered, resulting in regeneration patterns in the exponential phase of reconstitution that will be similar in both groups, be it that the growth factor stimulated curve occurs earlier. However, if cell multiplication is accelerated, the regeneration patterns will be different in the stimulated versus the placebo treated group. Thrombocyte production is a special case, since platelets are membrane fragments of cells driven by endomitosis to mature. Platelet production is therefore directly related to ploidy and size of megakaryocytes. TPO stimulates endomitosis, increasing the ploidy and size of the megakaryocytes. (14-16,63)

From the data obtained in the rhesus monkey model for myelosuppression the doubling time for the number of thrombocytes during the exponential phase of regeneration could be calculated at approximately 3 days for monkeys treated with TPO, whereas it was

more than 8 days for placebo treated monkeys (Fig 1). Apparently TPO treatment decreased the doubling time for platelets. Since doubling time is the resultant of thrombocyte production and destruction, the underlying assumption is that the life span of the produced thrombocytes is similar in the TPO stimulated and the placebo treated group. The life span of platelet produced in response to TPO treatment in normal baboons is not altered compared to unstimulated platelets. (18)

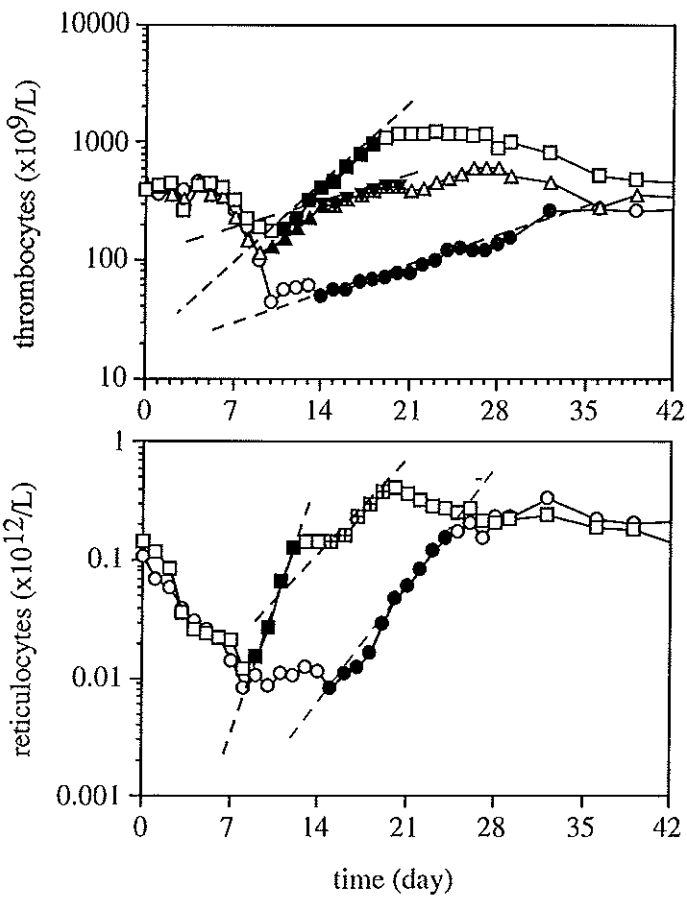

Figure 1. Analysis of regeneration patterns of thrombocytes and reticulocytes in 5 Gy irradiated rhesus monkeys. The upper panel represent thrombocyte regeneration in response to TPO (10 µg/kg/day, sc, days 1-21, n=4, squares); or in response to TPO/G-CSF (TPO 10 µg/kg/day, sc, days 1-21 and G-CSF 5 µg/kg, sc, days 1-21, n=4, triangles); or in placebo treated controls (n=12, circles). The lower panel represent reticulocyte regeneration in reponse to TPO (10 µg/kg/day, sc, days 1-21, n=4, squares); or in placebo treated controls (n=4, circles). Regression lines were calculated for the exponential phases of reconstitution, as indicated by solid symbols. This was done using the exponential curve fit analysis in Cricket Graph III (Computer Associates International Inc.) Doubling times for thrombocytes and reticulocytes were calculated using the equation $10^{a.x}=2$, in which the a.x was provided by Cricket Graph.

It should be noted that in this period thrombocyte transfusions were given to placebo treated monkeys whenever platelet counts dropped below 40 x 10^9/L (from day 10 to 29). This might have influenced the thrombocyte reconstitution pattern in two ways. The transfused platelets increase thrombocyte counts at the time of the nadir and cause the curve to be more shallow than it would have been without transfusions. The calculated platelet doubling time for placebo treated monkeys could therefore have been shorter in undisturbed regeneration. Binding of endogenously produced TPO to transfused platelets might have influenced platelet production in a more indirect way.

Extrapolation of the regression of exponential thrombocyte reconstitution of TPO and placebo treated monkeys shows an early divergence after TBI, indicating an early effect of TPO. In TPO treated normal nonhuman primates thrombocyte counts start to rise after a lag phase of 6 to 7 days. (17,18) Therefore, also from the effect on platelet nadir at day 8 after TBI it can be concluded that the effect of TPO starts early after the first administration.

Comparison of the regeneration patterns for TPO treated monkeys and monkeys treated with TPO and G-CSF displays an interesting phenomenon. The exponential phase of platelet reconstitution of the TPO/G-CSF monkeys is clearly biphasic, from day 10 to 14 the slope is similar to that of monkeys treated with TPO alone (calculated doubling time 3.4 days) and from day 14 to 19 the slope is similar to the placebo treated controls (calculated platelet doubling time 8 days). Apparently concurrent G-CSF administration alters the response of the megakaryocytic lineage to TPO. We hypothesize that the initial pattern results from a maturational effect of TPO immediately after administration on late megakaryocyte progenitors, resulting in larger megakaryocytes able to produce more thrombocytes. However, whereas in monkeys treated with TPO alone this effect is maintained through an increased production of megakaryoctye progenitors from multilineage ancestral cells, in the presence of G-CSF the balance in lineage commitment is shifted. More cells differentiate into the granulocyte-macrophage lineage, as can also be concluded from the accelerated reconstitution of neutrophils in monkeys treated with TPO/G-CSF, compared to monkeys treated with either of the growth factors alone. This apparently results in a slow-down in the release of thrombocytes because smaller number of megakaryocytes, that probably are still larger than in placebo treated animals, are produced.

The exponential curve fit for thrombocytes for TPO treated monkeys was calculated for day 11 to 18, and rendered a platelet doubling time of 2.9 days. For TPO/G-CSF treated monkeys in the first phase of regeneration (days 10 to 14) the platelet doubling time was 3.4 days, and in the second phase of regeneration (days 14 to 20) 10.0 days. For placebo treated monkeys the curve was fitted from day 14 to 32, with a doubling time of 8.1 days. For reticulocyte regeneration the exponential curve fit for TPO treated monkeys in the first phase of regeneration was calculated for day 9 to 12 with a doubling time of 0.95 days, and in the second phase for day 15 to 19 2.66 days. For placebo treated monkeys it was not possible to fit a curve in the first phase of regeneration and in the second phase (days 15 to 24) the doubling time was 2.01 days. Correlation coefficients r^2 ranged from 0.961 to 0.996 for the 7 displayed exponential curve fits.

Another explanation for the reduction in thrombocyte production would be inhibition by G-CSF. The reported effects of G-CSF on thrombocyte production have been variable, most often no effect was demonstrated (64-67), sometimes a stimulatory effect was shown (68) and occasionally inhibition. (69-71) Using flow cytometry and radioreceptor assays it has been shown that platelets express the G-CSF receptor (72), and G-CSF receptor transcripts were demonstrated in megakaryocytes. (73) Apart from a priming effect of G-CSF on *in vitro* platelet aggregation, a functional role has not yet been determined for the G-CSF receptor on platelets and megakaryocytes. A reduction in the mean ploidy of megakaryocytes was observed in mice genetically engineered to overexpress human G-CSF, and after G-CSF administration to normal mice. (53) Furthermore, G-CSF down-modulated the effect of TPO on the ploidy of megakaryocytes. Peripheral blood platelet count and the number of megakaryocytes were unaltered. (53) Taken together, it can be concluded that G-CSF might exert a suppressive effect on the maturation of megakaryocytes. However, it is difficult to explain the biphasic pattern for thrombocyte reconstitution observed in the TPO/G-CSF treated monkeys solely from inhibition by G-CSF, although it might contribute to the effect. It is not clear why G-CSF induced inhibition of megakaryocyte maturation would not occur in the first week after TBI. It could be that G-CSF is unable to inhibit megakaryocyte maturation from a certain point in development (from precursors that are present at TBI), but able to do so in 'new' precursors stimulated by TPO in which the G-CSF receptor is upregulated. Possibly, inhibition occurs through as yet unidentified factors released from neutrophils or their precursors that are stimulated by TPO and G-CSF.

Another explanation for the dampening effect of G-CSF on TPO stimulated thrombocyte regeneration could be altered pharmacokinetics of TPO due to G-CSF. Since plasma TPO levels were similar in both treatment groups at all data points measured (Chapter 2), this is unlikely. Also Harker et al (52) did not find alteration in plasma levels of either cytokine with concurrent administration of TPO and G-CSF.

The TPO stimulated regeneration of red blood cells was subjected to a similar analysis (Fig 1). Reticulocyte regeneration reflects the production of new red blood cells. A biphasic pattern for reticulocyte reconstitution is common after myelosuppression, with a small, apparently abortive rise in the second week after TBI, and an exponential increase in the third. From the lag phase between TBI and the occurrence of the first wave of reconstitution it can be concluded that the reticulocytes in the abortive rise develop from a precursor cell that is at a BFU-E stage at the time of TBI. The production time from BFU-E to mature red cells is approximately a week. Maturation of these precursor cells is dependent on EPO levels that start to rise with a decrease in hematocrit, explaining the occurrence in the second week after TBI. The second wave of reconstitution should then result from the multilineage ancestral cells responsible for sustained reconstitution.

Continuation of blood cell production after radiation insult to the bone marrow progenitors is dependent on supplementation of progenitor cells from more immature

stadia of differentiation. Population sizes of various progenitor cells in steady state bone marrow might vary, depending on the transit time from the previous stage of differentiation to the next. Therefore the effect of radiation on the number of cells at various stages of differentiation is dependent on the original number of cells in that stage, the radiation sensitivity of the cell type, the transit time required to produce new cells, and the mitotic potential.

The interruption in the regeneration curve that occurs around day 15 in both TPO and placebo treated monkeys suggests that a certain stage during erythroid cell differentiation is more affected by irradiation than later and earlier cells, causing a gap in the production of reticulocytes. This can either be due to a decisive difference in radiation sensitivity or to the size of the original population. It has been demonstrated that CFU-E cells are for 70% in S-phase and are susceptible to cell cycle specific cytotoxins. (74) It is very well conceivable that this population is also more prone to radiation induced cell death. However, since maturation of CFU-E to reticulocytes requires approximately 3 to 4 days, this mainly explains the initial drop in reticulocytes occurring after TBI. If the abortive rise result from cells at the BFU-E stage at the time of TBI, the more susceptible precursor cell should be a pre-BFU-E.

Because the first wave of reticulocyte regeneration is brief and not very pronounced in placebo treated monkeys, it is not possible to calculate a doubling time. In TPO treated monkeys the abortive rise also occurs in the second week but is greatly stimulated and contributes to the reticulocyte regeneration that occurs 10 days earlier than in placebo treated monkeys. The doubling time for this specific cell population was approximately 1 day for TPO treated monkeys in the first phase, and 2.5 days in the second phase of regeneration, whereas it is two days for placebo treated controls. The difference between placebo and TPO treated monkeys in the first phase most likely results from synergism of TPO and EPO on erythroid progenitors. Since the appearance in time is not altered compared to placebo treated monkeys, the stimulation by TPO is most probably dependent on erythropoietin, a result that is consistent with *in vitro* observations. (32,54,55) An anti-apoptotic effect of TPO (23-26) resulting in increased survival of progenitor cells might also contribute to the increased cell production.

Monkeys treated with TPO/G-CSF display a regeneration for reticulocytes that is identical to that of monkeys treated with TPO alone. (Chapter 2) Apparently concurrent G-CSF administration does not influence TPO-stimulated red blood cell regeneration. This is consistent with the absence of detectable G-CSF receptors on erythrocytes or on erythroid precursors.

In myelosuppressed mice it was demonstrated that administration of TPO early after TBI and a threshold plasma level to be reached in the first hours were critical for an optimal multilineage effect. (Chapter 6) Delayed administration causes an increasing decline in efficacy of TPO. (57,58) The presence of TPO in the first hours after radiation induced myelosuppression in mice influences hematopoietic reconstitution along several lineages,

whereas a delay in administration results in effects that are predominantly megakaryocytic/thrombopoietic. This indicates an early effect of TPO on a multilineage precursor cell that can subsequently respond to other cytokines required for proliferation and end cell production. Using short-term transplantation assays, ie colony-forming unit-spleen (CFU-S) day 13 (CFU-S-13) and the more immature cell with marrow repopulating ability (MRA), it could be shown that TPO promoted CFU-S-13 and transiently depleted MRA (Fig 2). The multilineage effect of TPO therefore most likely results from the stimulation of short term multilineage repopulating cells represented by the CFU-S-13 at the expense of more immature ancestral cells. The short time interval available after TBI to exert these effects shows that TPO is able to intervene in mechanisms that result in functional depletion of its target cells shortly after TBI.

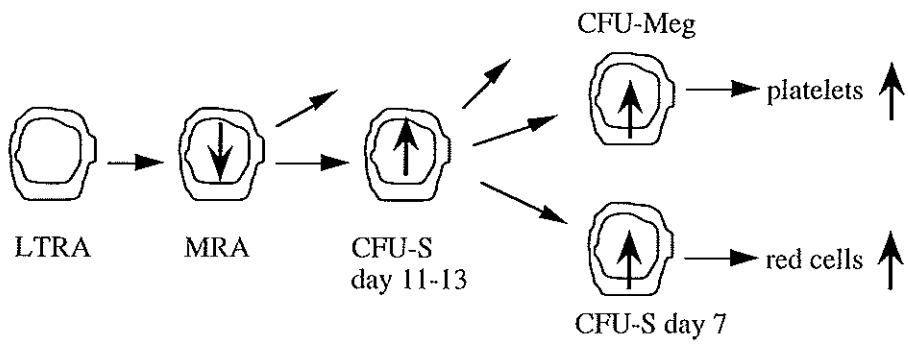

Figure 2. Schematic representation of the effects of TPO on immature murine bone marrow cells. MRA cells, following transplantation associated with sustained regeneration of the host, are transiently depleted, wheras CFU-S-13, following transplantation associated with rapid, transient regeneration, are stimulated.

The mechanisms which require almost immediate TPO availability for optimal efficacy are not fully elucidated. It is clear that c-*mpl* is expressed on developmentally early, multilineage cells, and, therefore, a direct effect of TPO is most likely. TPO enhances survival of immature cells and suppresses apoptosis (23-26), and this might explain the importance of time of administration of TPO. In the absence of TPO, multilineage cells damaged by TBI may be subject to apoptosis, whereas in its presence those cells survive, become susceptible to proliferative stimuli such as TPO and other cytokines and may contribute thereby to hematopoietic reconstitution.

Radiation-induced changes in the microenvironment might also influence the hematopoietic regeneration potential. Hematopoietic reconstitution in the bone marrow is dependent on complex interactions of hematopoietic cells with stromal cells,

extracellular macromolecules and cell adhesion molecules, together termed the hematopoietic microenvironment. It is possible that TPO modifies radiation induced (inflammatory) reactions, thereby making multilineage cells more accessible to either TPO itself or to other growth factors. However, stromal reactions to radiation and subsequent alterations thereof by cytokines, are difficult to assess *in vivo*.
It is also conceivable that TPO first induces other cytokines that subsequently act on multilineage cells to become responsive to TPO. However, induction of cytokine production takes at least several hours, which makes it unlikely that the change from a multilineage to an unilineage thrombopoietic effect of TPO in 24 hours would be mediated through such a mechanism.

7.4 EFFECTS OF TPO ADMINISTRATION AFTER BMT

After 8 Gy TBI and transplantation with limited numbers of selected bone marrow cells, 21 day administration of TPO unfortunately did not accelerate thrombocyte regeneration compared to placebo treated controls. (Chapter 5) Transplantation of larger unfractionated cell numbers was also performed, to exclude the possibility that the content of the graft was responsible for the lack of effect, but gave identical results. This lack of efficacy of TPO post-transplantation has also been observed by others. (75,76) More promising results after bone marrow or peripheral blood stem cell transplantation have been reported, but the degree of cytotoxicity in those models was more myelosuppressive than myeloablative (77,78), explaining the post-transplant effectiveness of TPO. Interestingly, transplantation of bone marrow from donor mice or rhesus monkeys that had been treated with TPO resulted in accelerated platelet and red cell reconstitution, although post transplantation TPO treatment did not further influence the thrombocyte recovery. (75,76) The mechanism might well be similar to that proposed for the results described in chapter 6, in which also TPO pretreated bone marrow (be it from myelosuppressed mice) was injected into lethally irradiated recipients, and the effect could be attributed to an increased number of multilineage spleen repopulating cells. However, the usefulness of these observations in the clinical setting is probably limited to only a small number of patients eligible for *in vivo* pretreatment and autologous bone marrow transplantation.
The absence of a beneficial effect of TPO after higher doses of cytotoxic insult to the bone marrow is a disappointing feature, that has not been fully elucidated. After myelotoxic damage hematopoietic reconstitution relies on remaining or injected stem and progenitor cells. Without growth factor treatment, endogenously produced cytokines regulate peripheral blood reconstitution, resulting in the regeneration patterns of placebo treated animals. Placebo treated monkeys transplanted with autologous bone marrow cells and control monkeys in the myelosuppression model recover normal thrombocyte counts at approximately the same time (Fig 3). This similarity is not a coincidence: the cell

number transplanted was chosen so as to provide a similar reconstitution pattern as the 5 Gy myelosuppression model. This suggests the availability of adequate numbers of bone marrow cells for platelet reconstitution in both models and makes it unlikely that the lack of effect of TPO after transplantation can be attributed to limited numbers of reconstituting cells after bone marrow transplantation. However, although placebo treated monkeys display similar thrombocyte regeneration patterns, TPO treatment was highly effective in the myelosuppression model but not at all after bone marrow transplantation.

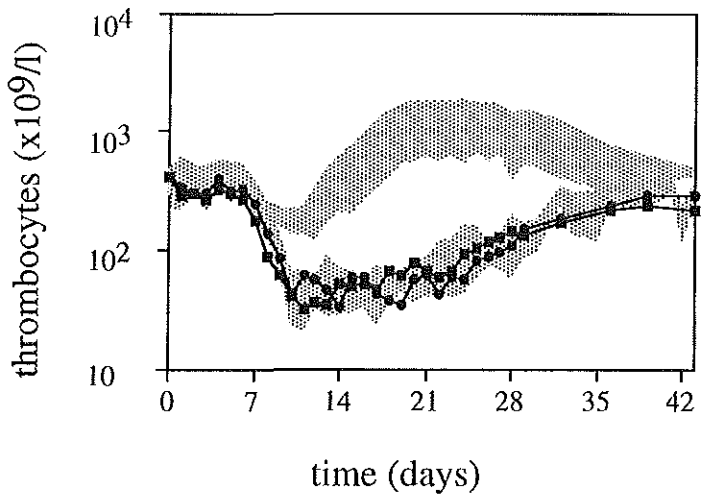

Figure 3. Comparison of the effect of TPO on thrombocyte regeneration after transplantation of purified stem cells and after myelosuppression. Mean thrombocyte counts of 8 Gy irradiated, stem cell transplanted monkeys are represented with symbols and lines; the filled squares represent 4 TPO treated monkeys, the filled circles 3 placebo treated controls. The shaded areas represent means ± SDs of thrombocyte levels of 5 Gy irradiated monkeys after TBI; the upper shaded area represents 4 TPO treated monkeys, the lower shaded area 8 placebo treated controls. The dose and route of TPO were identical for all monkeys (10 µg/kg/day, sc, days 1-21).

Exogenously added growth factors might enhance regeneration if they accelerate effective plasma or tissue levels over endogenous production of the cytokine. We demonstrated that achieving threshold plasma levels of TPO in the first hours after TBI is crucial for optimal efficacy of TPO in myelosuppressed mice. (Chapter 6) Apparently after bone marrow transplantation, endogenous levels of TPO (above detection level approximately one week after TBI in placebo treated monkeys, chapter 5) are adequate for the thrombocyte reconstitution that occurs in both placebo and TPO treated monkeys after approximately three weeks. The reason why TPO administration in the first week is ineffective in accelerating thrombocyte reconstitution is not clear.

Possibly, the administration schedule of TPO used in this BMT model, although sufficient for a prominent thrombopoietic response in myelosuppressed rhesus monkeys, was not optimal (Chapter 6) and therefore did not exert an effect on the transplanted cells. However, this is not very likely. From ongoing experiments in rhesus monkeys we know that starting TPO treatment concurrent with injection of the bone marrow graft does not improve the results. (Hartong and Wagemaker, unpublished observations)

Analysis of the regeneration pattern of 5 Gy irradiated TPO treated monkeys reveals, at least for reticulocyte reconstitution, a biphasic pattern. Two phases of thrombocyte regeneration were also clearly present in 5 Gy irradiated, TPO/G-CSF treated monkeys and could be obscured in animals treated with TPO alone by the maximal thrombocyte production. Presumably regeneration enhancement in the first phase results from maturational effects of TPO on committed progenitors, whereas the second phase of hematopoietic reconstitution results from more ancestral cells. It occurs later in time and is adequately regulated by endogenously produced cytokines as can be deduced from the reconstitution of placebo treated animals. Therefore the absence of a TPO effect in the second week after high dose TBI and BMT might be explained by differences in number or quality of committed progenitors. In the 5 Gy myelosuppression model, the residual cells are less than 2 orders of magnitude depleted in stem cells and progenitor cells, and remain in their normal stromal environment. 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 although the graft is sufficient for sustained reconstitution, and regeneration in placebo animals is similar to that seen in myelosuppressed monkeys, it is deficient in committed progenitors and therefore the first phase cannot be stimulated by TPO. However, this still does not explain the lack of stimulation of cells responsible for sustained engraftment. These cells are present, as can be deduced from reconstitution in placebo treated monkeys, and can be stimulated by TPO, as can be deduced from results in TPO treated myelosuppressed monkeys.

The differential effects of TPO in normal and myelosuppressed animals indicate that (part of) the TPO response is dependent on endogenously produced co-factors. Stimulation of immature bone marrow progenitor cells by TPO *in vitro* also requires other cytokines, such as SCF and IL-3. (22,27-32) Differences in the extent of stromal damage resulting in different endogenous cytokine production profiles or other micro-environmental variations might therefore provide additional hypotheses to be explored.

So, although it is possible to accelerate hematopoietic reconstitution after high dose TBI by giving a stem cell transplant, exogenous growth factors seem to add little to reconstitution.

7.5 EFFECTS OF TPO ADMINISTRATION IN PATIENTS

The clinical data on TPO so far are scattered. In several phase I trials, administration of TPO to cancer patients prior to chemotherapy resulted in dose dependent thrombocytosis and increased numbers of bone marrow megakaryocytes, comparable to the effects described in experimental animals. (79-81) Adverse effects attributable to drug administration or the high platelet counts were not seen. The platelets produced were morphologically normal and showed unchanged aggregation and ATP release responses in *in vitro* assays. (82) These data are promising with respect to the tolerability of pharmacological doses of TPO in humans and the reproducibility of the results obtained in animals, but not in itself predictive for the effects of TPO in myelosuppressed patients.
Trials to assess the usefulness of TPO in accelerating platelet reconstitution should document an actual reduction in the number of thrombocyte transfusions needed, through an elevation of thrombocyte nadir and/or a decrease in the duration of thrombocytopenia. This means that elevation of the thrombocyte nadir with TPO treatment after a chemotherapy regimen in which placebo treated patients do not become transfusion dependent (platelet nadir 111 $x10^9$/L) is clinically not relevant. (83) Others did not observe obvious differences in the thrombocyte nadir or duration of thrombocytopenia (79), while only preliminary data on TPO treatment in de-novo AML patients exist, in which a slightly decreased the period of thrombocytopenia is accompanied by a reduction in thrombocyte transfusions requirements. (84) The report documenting these findings has not yet been published. However, preliminary unpublished evidence reveals more promising results if TPO is administered prior to chemotherapy at a dose of 2.4 µg/kg followed by a short course at the same dose thereafter, results that could in our opinion be improved if the dose and/or schedule was adjusted to achieve a plasma level of 20-60 ng/mL throughout the chemotherapy course. (Chapter 6)
The combination of MGDF and G-CSF has also been used, in patients with advanced solid tumors. (85) Differences were not found in platelet nadir or transfusion requirements (20 vs 29%) between placebo treated patients or any of the dosing groups, but recovery to baseline values was accelerated by MGDF. All patients were given G-CSF and co-administration of TPO did not alter the neutrophil reconstitution. The frequency of chemotherapy dose reductions, one of the endpoints in the trial, was the same in all groups. (85) Numbers of peripheral blood progenitor cells were also assessed as an indicator of the efficacy of TPO to mobilize hematopoietic stem cells into the peripheral blood. Patients treated with the combination of TPO and G-CSF had more circulating BFU-E, GM-CFC and Meg-CFC, compared to patients treated with G-CSF alone (85). This most likely represents a spill-over into the peripheral blood from increased numbers of bone marrow progenitor cells, rather than growth factor induced selective mobilization. (86) In two other trials TPO was administered to high dose chemotherapy patients and in both trials the transfusion requirement was not altered compared to

controls. (87,88)
It is too early to conclude whether TPO can reduce the need for platelet support in clinical situations in which prolonged and severe thrombocytopenia occurs. In view of the results obtained with early administration of TPO and the preliminary results in a chemotherapy trial, it might be possible to improve the efficacy of TPO by adjusting the dose schedule, giving TPO concurrent with the chemotherapy in doses sufficient to overcome the initial c-*mpl* mediated clearance.

7.6 FUTURE PROSPECTS

Preclinical evaluation identified TPO as a potential major therapeutic agent to counteract radiation induced pancytopenia, and demonstrated pronounced stimulatory effects on reconstitution of immature hematopoietic cells with multilineage potential. The latter observation explained the potentiation of the hematopoeitic responses to G-CSF and GM-CSF when administered concomitantly. The heterogeneity of the TPO response encountered in the various models used for evaluation points to multiple mechanisms operating on the TPO response and to heterogeneity of its target cells. The finding that a single dose of TPO might be sufficient for a clinically significant response emphasizes its potency and is of practical relevance. Adverse effects of TPO administration to myelosuppressed or stem cell transplanted experimental animals were not observed.
Paradoxically, the initial clinical trials, set up before the experimental animal data were fully completed and processed, failed to show a major benefit of TPO treatment. The mechanistic mouse studies demonstrated that the response of multilineage cells to a single administration of TPO shortly after TBI 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. An evaluation of the presently available data from clinical trials makes clear, that none of these have been designed in keeping with these two principles. It can therefore be predicted, that TPO treatment in, for instance, chemotherapy protocols, can be made effective by maintaining sufficiently high levels of TPO shortly before, during and shortly after chemotherapy treatment. Further elucidation of mechanisms determining efficacy might very well result in a further improvement.
The initially unexpected action of TPO on immature reconstituting stem cells might be useful for both *ex vivo* expansion protocols, especially those that aim for improved platelet reconstitution of stem cell grafts, and in gene transfer protocols. Although TPO by itself did not appear to be a potent mobilizer of stem cells, its capacity to promote $CD34^+$ cell reconstitution in the bone marrow may point to a useful role of TPO in stem cell mobilization protocols in conjunction with other growth factors, and may have the added advantage of accelerated platelet reconstitution. Also patients with thrombocytopenias resulting from HIV infection and those due to large field (>20% of

total bone marrow irradiated) radiotherapy (especially with concurrent chemotherapy) might benefit from well designed TPO treatment protocols. TPO treatment might be tried in thrombocytopenias resulting from primary marrow failure such as MDS and aplastic anemia.

From the basic point of view, there is still much to learn on the mechanisms involved in the action of TPO on very immature cells and its role in stem cell physiology. Especially its role in maintaining stem cell numbers in the bone marrow is as yet insufficiently explored, while the rapid disappearance of multilineage TPO responsive cells following cytoreductive TBI is unexplained and might contain a clue to mechanisms operating on stem cell reconstitution. Further elucidation might be highly relevant for understanding deficiencies in hematopoietic reconstitution and ineffective growth factor treatment, such as has been encountered after transplantation of limited numbers of stem cells.

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Summary

Thrombocytopenia, a serious condition that may cause hemorrhage and may in some cases result in mortality, is either the result of increased destruction of platelets or of decreased production. Decreased platelet production due to myelotoxicity of anti-cancer treatment is a well known problem in clinical oncology, and accounts for an increase in morbidity, dose reductions and treatment delays. The possibility to stimulate platelet regeneration would be beneficial for patient care. Counteracting the thrombocytopenia that occurs after bone marrow transplantation would be another application for a potential platelet stimulating factor. With the cloning of the gene for thrombopoietin (TPO) in 1994, enabling production of the recombinant protein, this factor has become available for investigation in (pre)clinical studies.

The aim of this study was to assess the efficacy of TPO in stimulating peripheral blood platelet regeneration after myelosuppression and after bone marrow transplantation in rhesus monkeys. Furthermore, the combination of TPO with the myeloid growth factors G-CSF and GM-CSF was tested to evaluate possible stimulatory and/or adverse interactions. To establish optimal dose and dose schedule and to elucidate the cellular basis of the multilineage effects, a separate set of experiments was carried out in mice.

To gain a rapid insight into the thrombocyte regeneration enhancement potential of TPO, the initial experiments were carried out in a well established model for myelosuppression in rhesus monkeys. Rhesus monkeys were irradiated with 5 Gy TBI, a dose that results in approximately two log stem cell kill and a period of three weeks of pancytopenia. The dose and dose schedule of TPO were based on pilot experiments in normal mice and monkeys and on experience with other hematopoietic cytokines such as GM-CSF and G-CSF. TPO was highly effective in preventing thrombocytopenia, thereby abolishing the need for thrombocyte transfusions, and in addition stimulated erythrocyte regeneration. Combination of TPO and G-CSF resulted in improved neutrophil nadirs and enhanced neutrophil reconstitution. Furthermore, the recovery of immature CD34 positive bone marrow cells was stimulated by TPO administration. (Chapter 2) The prominent erythropoietic stimulation resulted in the depletion of already borderline body iron stores, explaining the development of a microcytic anemia in TPO treated monkeys. (Chapter 3)

The initial dose schedule used for TPO was more intensive than necessary to obtain the main goal of treatment, prevention of the need for thrombocyte transfusions. In an extension of the study, administration of TPO was reduced to a single dose. (Chapter 4) In these experiments combination treatment with G-CSF was compared with the combination TPO/GM-CSF. TPO/GM-CSF was more effective than either the single dose of TPO or TPO/G-CSF in stimulating thrombocyte and reticulocyte regeneration. TPO/G-CSF and TPO/GM-CSF were similarly effective in stimulating neutrophil regeneration. TPO/GM-CSF treatment also significantly increased reconstitution of CD34 positive bone marrow cells and progenitor cells such as GM-CFU and BFU-E.

To evaluate the efficacy of TPO after bone marrow transplantation, TPO was administered to rhesus monkeys that were irradiated with 8 Gy TBI and transplanted

with limited numbers of $CD34^+/HLA\text{-}DR^{dull}$ bone marrow cells. Placebo treated monkeys develop a profound pancytopenia and need four to five trombocyte transfusions. In this setting TPO did not stimulate platelet recovery and neither promoted neutrophil regeneration if coadministered with G-CSF. (Chapter 5) The reason for this lack of efficacy after high dose TBI is as yet not fully elucidated.

Finally, to optimize dose and dose schedule and to investigate the origin of the multilineage response to TPO, more basic experiments were carried out in mice. It was demonstrated that a single dose of TPO shortly after 6 Gy TBI prevented the severe thrombocytopenia observed in control mice and in addition stimulated red and white blood cell regeneration. A threshold level of TPO required to overcome initial *c-mpl* mediated clearance was defined. Using short-term transplantation assays, i.e. colony-forming unit-spleen day 13 and the more immature cells with marrow repopulating abilitity it was shown that TPO stimulated CFU-S-13 and transiently depleted MRA. (Chapter 6)

In conclusion, TPO is a hematopoietic cytokine that regulates and stimulates thrombocytopoiesis and, in addition, has important actions on immature, multilineage hematopoietic stem cells, e.g., in suppression of apoptosis. In preclinical animal models it proved to be useful in counteracting radiation induced thrombocytopenia and enhancement of neutrophil regeneration and that of other myeloid lineages. The effects are partly mediated through the actions of TPO on immature bone marrow cells. Further elucidation of the mechanisms involved might lead to improved treatment modalities using TPO in chemotherapy patients as well as in BMT recipients.

Samenvatting

Thrombocytopenie, een tekort aan bloedplaatjes, is een ernstige aandoening omdat het kan leiden tot bloedingen en daardoor soms de dood tot gevolg kan hebben. Een tekort aan bloedplaatjes of thrombocyten kan ontstaan door een verhoogde afbraak of een verminderde productie. Een verminderde thrombocytenproductie als gevolg van de beenmerg toxiciteit van bepaalde chemotherapeutica is een bekend probleem in de oncologie. Het leidt tot verhoogde morbiditeit, dosis aanpassingen en/of uitstel van chemokuren. Als het mogelijk zou zijn het herstel van het aantal bloedplaatjes te stimuleren zou dit de behandeling van de patiënt ten goede komen. Een andere toepassing van een bloedplaatjes-stimulerende factor zou het behandelen van de thrombocytopenie die voorkomt na beenmergtransplantatie kunnen zijn. Doordat in 1994 het gen voor thrombopoietine (TPO) is gecloneerd, waardoor het recombinante eiwit geproduceerd kon worden, is deze factor beschikbaar gekomen voor (pre)klinisch onderzoek.

Het doel van de preklinische studie met rhesusapen was om de effectiviteit van TPO te onderzoeken in het bevorderen van het herstel van het aantal bloedplaatjes na beenmergsuppressie en na beenmergtransplantatie. Verder is de combinatie van TPO met de myeloide groeifactoren G-CSF and GM-CSF onderzocht op eventuele stimulerende en/of negative interacties. Om de dosis en het toedieningsschema te optimaliseren en om de cellulaire basis van het geobserveerde multilineage effect op te helderen werden experimenten met muizen gedaan.

Om snel inzicht te krijgen in het stimulerende effect van TPO op het herstel van bloedplaatjes werden de eerste experimenten uitgevoerd in een bekend model voor beenmergsuppressie in rhesusapen. De apen werden bestraald met een dosis van 5 Gy TBI, een stralingsdosis die resulteert in een reductie van ongeveer twee log van het aantal hematopoietische stamcellen in het beenmerg en een ongeveer drie weken durende pancytopenie. Dosis en toedieningsschema van TPO waren gebaseerd op experimenten in normale muizen en apen en op de ervaring met andere hematopoietische groeifactoren zoals G-CSF en GM-CSF. TPO was in dit model zeer effectief in het voorkomen van thrombocytopenie en daarmee was de noodzaak tot het geven van thrombocyten transfusies verdwenen. Bovendien stimuleerde TPO de regeneratie van het aantal rode bloedcellen. De combinatie van TPO en G-CSF leidde tot een minder ernstige afname van het aantal witte bloedcellen en versnelde het herstel daarvan. Verder werd ook het herstel van het aantal CD34 positieve cellen in het beenmerg versneld door TPO. (Hoofdstuk 2) Het opmerkelijke effect op de rode bloedcel reeks resulteerde in een uitputting van al marginale ijzervoorraden in het lichaam, wat het ontstaan van een microcytaire anemie in TPO behandelde apen verklaart. (Hoofdstuk 3)

Het gebruikte toedieningsschema van TPO was intensiever dan nodig om het belangrijkste behandelingsdoel, het voorkomen van thrombocyten transfusies, te bereiken. In een vervolgstudie werd de toediening van TPO teruggebracht tot een eenmalige dosis. (Hoofdstuk 4) In deze studie werd de combinatie TPO/G-CSF vergeleken met TPO/GM-CSF. TPO/GM-CSF was effectiever dan TPO alleen of TPO/G-CSF in het

stimuleren van het herstel van bloedplaatjes en rode bloedcellen. Beide combinaties waren even effectief in het stimuleren van het herstel van witte bloedcellen. Behandeling met TPO/GM-CSF versnelde ook significant het herstel van CD34 positieve beenmergcellen en voorlopercellen zoals de GM-CFU en de BFU-E.
Om de effectiviteit van TPO na beenmergtransplantatie te evalueren werd TPO toegediend aan apen die waren bestraald met een dosis van 8 Gy TBI gevolgd door een beenmergtransplantatie met $CD34^+/HLA\text{-}DR^{dull}$ cellen. Apen die werden behandeld met placebo ontwikkelden een diepe pancytopenie en hadden vier tot vijf thrombocyten transfusies nodig. In deze situatie had TPO geen effect op het herstel van de bloedplaatjes en, in combinatie met G-CSF, ook niet op het herstel van witte bloedcellen. (Hoofdstuk 5) De reden voor dit gebrek aan effect na hoge dosis TBI is tot op heden niet bekend.
Als laatste werden meer basale experimenten uitgevoerd in muizen, om dosis en toedieningsschema te optimaliseren en om de basis van het multilineage effect van TPO te onderzoeken. Een eenmalige dosis TPO, kort na 6 Gy TBI, was voldoende om de ernstige thrombocytopenie die gezien werd in controle muizen te voorkomen en stimuleerde bovendien het herstel van rode en witte bloedcellen. Een drempelwaarde voor TPO, nodig om de initiële clearance door binding aan c-*mpl* op bloedplaatjes te overkomen, werd gedefinieerd. Gebruik makend van korte termijn transplantatie assays, de colony-forming unit-spleen dag 13 (CFU-S-13) en de meer onrijpe cel met beenmerg repopulerend vermogen (MRA), werd aangetoond dat TPO de CFU-S-13 stimuleerde terwijl het de MRA tijdelijk uitputte. (Hoofdstuk 6)
Conclusie: TPO is een hematopoietische groeifactor die de thrombocytopoiese reguleert en stimuleert en bovendien belangrijke effecten heeft op immature multilineage hematopoietische stamcellen, onder meer blijkend uit de onderdrukking van apoptose. In preklinische diermodellen bleek het effectief in het tegengaan van stralingsgeinduceerde thrombocytopenie en het bevorderen van de regeneratie van witte bloedcellen. Deze effecten worden deels gemedieerd door de effecten van TPO op immature beenmerg cellen. Verdere opheldering van de betrokken mechanismen zou kunnen leiden tot een verbetering van de behandeling met TPO in patiënten die chemotherapie krijgen en in patiënten die een beenmergtransplantatie ondergaan.

List of Publications

Articles:

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Curriculum Vitae

Karen J. Neelis was born on July 15, 1968 in Rotterdam. In 1986, after graduating high school at the Gereformeerde Scholengemeenschap Rotterdam, she began medical studies at the Erasmus University Rotterdam. In 1991 she passed her doctoral exam and in 1993 she was registered M.D. (cum laude). In the period from December 1993 till November 1997 she performed the studies presented in this thesis at the Department of Hematology, Erasmus University, Rotterdam, in the research group of dr. G. Wagemaker. In July 1998 she started working at the department of Radiotherapy at the Daniël den Hoed Kliniek Rotterdam. She is married to Aad van der Lugt and mother of Juliët and Suzan.

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