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Preclinical Evaluation of Thrombopoietin and Flt-3 ligand Treatment

Preklinische evaluatie van de behandeling met thrombopoietine en flt-3 ligand

PROEFSCHRIFT

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Aan mijn ouders
Aan Felix

Contents

CHAPTER 1

General introduction

1.1 Hematopoiesis

The hematopoietic system is a cell renewal system in which mature peripheral blood cells with a limited life span are continuously replenished by proliferation and differentiation of lineage-specific progenitor cells derived from scarce pluripotent hematopoietic stem cells [1, 2, 3]. The process of blood cell formation, hematopoiesis, takes approximately 20-30 cell divisions and takes mainly place in the bone marrow (BM) in adult mammals. In fetal hematopoiesis, liver and spleen also play an important role, whereas there is still residual hematopoiesis in the spleen of adult mice. The process of hematopoiesis is tightly controlled by hormone like proteins the hematopoietic growth factors (HGFs), by other cytokines, by complex interactions between hematopoietic cells and stromal cells in the BM, via extracellular matrix molecules and cell-cell interactions through specialized adhesion molecules [4]. Different models have been developed in which stem cell commitment and differentiation are described as either a stochastic/random process or in which stem cell fate is regulated by external stimuli [5, 6, 7].

1.2 Hematopoietic stem cells

Most of the small pool of pluripotent hematopoietic stem cells remain in a quiescent state. A few proliferating HSC are responsible for the formation of daughter cells which after multiple cell divisions gradually loose their multipotent differentiation potential and acquire the characteristic phenotypical and functional properties of the individual blood cell lineages. Studies in mice showed that the number of stem cells is genetically determined and age dependent [8, 9, 10, 11]. Mice with a longer lifespan had a larger stem cell pool, and less proliferative activity. Furthermore, older mice had higher numbers of primitive stem cell subsets [10].

HSC are characterized by the expression of the CD34 antigen, which is present on committed and uncommitted hematopoietic progenitor cells and is used as a crude parameter to assess stem cell content of bone marrow and peripheral blood [12]. The CD34 molecule is a transmembrane glycoprotein of the mucin membrane molecule family, which is expressed on hematopoietic progenitor cells in normal bone marrow (0.5%-2%), peripheral blood (PB) (0.01%-0.15%), umbilical cord blood cells (UCB) (0.1%-0.4%) and first trimester fetal blood (5±1%) [13]. The CD34 antigen is further expressed on vascular endothelium, high endothelial venules and some fibroblasts [14, 15]. The binding epitopes of different monoclonal antibodies against CD34 are differentially sensitive to neuraminidase (NA), chymopapain (CP) and Pasteurella hemolytica-derived glycoprotease (GP) [16]. On this basis, CD34 monoclonal antibodies have been divided into three classes: class I antibodies are directed against epitopes that are sensitive to NA, CP, and GP. Class II antibodies detect epitopes sensitive to CP and GP and class III antibodies detect epitopes that are insensitive to these enzymes. The CD34 epitope is involved in stem-cell and progenitor-cell localization / adhesion in the bone marrow [17, 18, 19], and is determined by the CD34 epitope class expression. Class III expressing

hematopoietic progenitor cells (HPCs) are enriched for CFU-GM and BFU-E and cells coexpressing CD13, CD33, c-kit and CD71 compared to class II expressing CD34+ cells. CD34+ cells exclusively expressing class III epitopes uniformly display CD13 and CD33; they have a high clonogenic capacity and may include cells upto the maturity level of, and morphological characteristics of promyelocytes and myelocytes [16]. The distribution of the CD34 antigen makes it a useful marker to select for immature cells, transplantation studies with CD34 positive cells show that these cells include progenitor cells as well as immature HSC with long term repopulating capacity [20, 21, 22, 23].

1.3 Hematopoietic Growth Factors

Hematopoietic Growth Factors (HGFs) regulate proliferation, differentiation and maturation of hematopoietic cells and modulate the functions of mature blood cells [4] (Figure 1). Many HGFs have become known in the last 25 years and new cytokines, cytokine receptors and intracellular pathways are still being identified. HGFs can be divided in several classes; early acting cytokines, lineage restricted growth factors that act relatively late in the development of their mature end cell, and cytokines which influence more than one lineage or act at other stages in the development of blood cells as well (Figure 1) [24]. HGFs may act both directly on the hematopoietic cells and indirectly through accessory cells releasing other cytokines [3]. Most HGFs have multiple functions and are a least involved in both the regulation of blood cell production as well as modulation of end cell functions.

The range of activity of the growth factors is determined by the distribution of their specific receptors. Two HGF families can be distinguished on the basis of their receptor type: the tyrosine kinase receptor family [25] and the hematopoietin or cytokine receptor superfamily [26]. The tyrosine kinase receptors have a large extracellular ligand binding domain and a cytoplasmic domain with an intrinsic tyrosine kinase activity. Binding of ligands to the extracellular domain induces receptor dimerization, which is important for activation of the receptor tyrosine kinases, resulting in intracellular signal transduction. Mutations that activate receptor tyrosine kinases abrogate requirements for ligands and constitutively transmit signals. Therefore, genes for tyrosine kinase receptors are also known as proto-oncogenes [25, 27, 28]. The surface receptors of the tyrosine kinase receptor family are classified in three subclasses on the basis of their structure. HGFs which bind to the tyrosine kinase receptors include epidermal growth factor (EGF)(subclass I), insulin (subclass II), stem cell factor (SCF), Flt3-Ligand (FL) and platelet-derived growth factor (PDGF) (all subclass III). The hemopoietin receptors are type I membrane glycoproteins with a single hydrophobic transmembrane domain. The extracellular domain contains two major regions of homology with an amino-terminal half and a carboxy-terminal half. IL-2, IL-3, IL-5, IL-6, IL-7, IL-9, G-CSF, GM-CSF, EPO and TPO belong to the cytokine receptor superfamily. Hematopoietic growth factors can also be clustered according to their phylogenetic background. EPO and TPO for instance, both have cysteins at similar positions [29, 30] and their genes are structurally related. SCF,

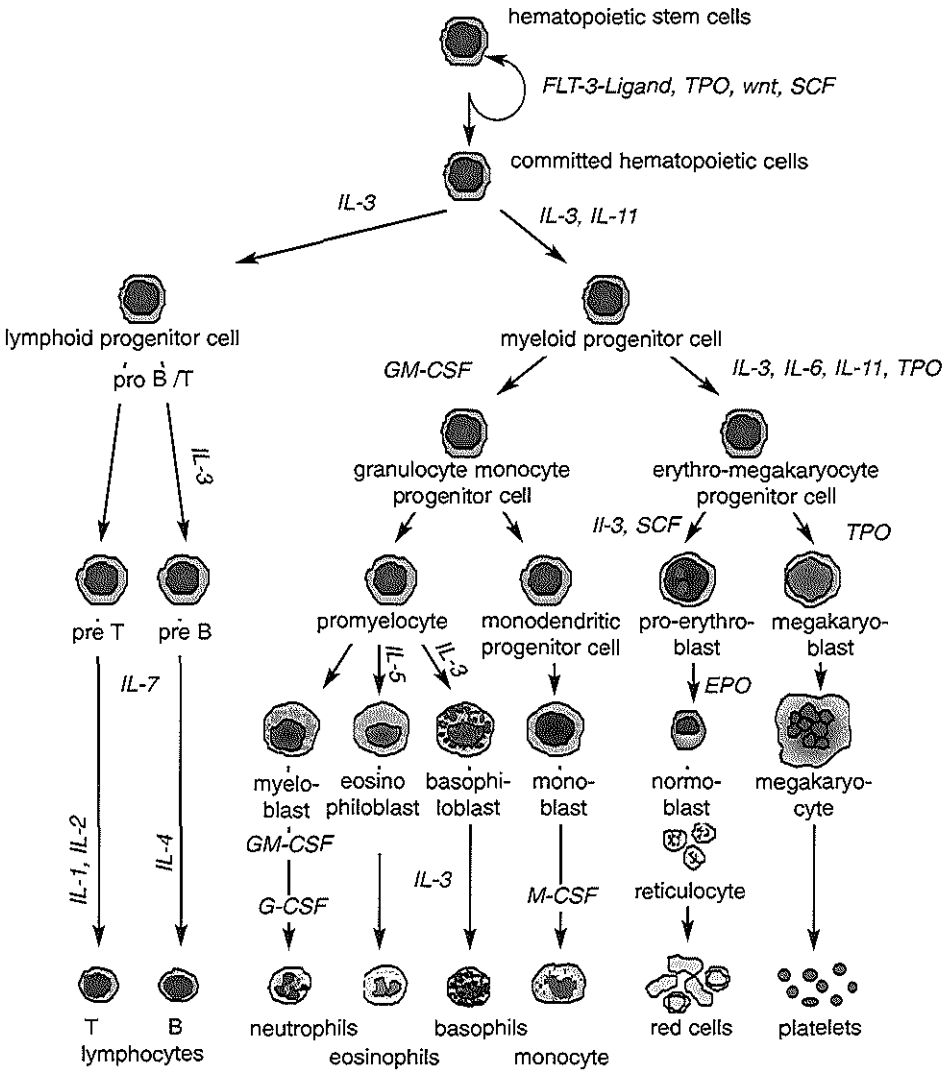


Figure 1. Schematic representation of hematopoietic stem cell differentiation.
Mature blood cells originate from pluripotent stem cells. This process is regulated by various hematopoietic growth factors. IL: interleukin, SCF: stem cell factor, G-CSF: granulocyte colony-stimulating factor, FLT-3 ligand: fetal-liver tyrosine kinase-3 ligand, GM-CSF: granulocyte macrophage colony-stimulating factor. EPO: erythropoietin, TPO: thrombopoietin M-CSF: macrophage colony-stimulating factor.

M-CSF and FL may have been derived from a common ancestral gene because of their similar intron/extron structures [31, 32]. Similarly, IL-3, IL-5 and GM-CSF may have arisen from gene duplications in view of their proximity in the genome and structural similarity of their genes [33, 34, 35]. The actions of individual HGFs on stem cell maintenance, proliferation, commitment and differentiation have become gradually better understood, in particular by the use of transgenic or KO mice in addition to selective in vitro assays and in vivo introduction of pharmacological concentrations of growth factors. Here, TPO, GM-CSF and FL, which are used in the studies described in this thesis, are discussed in more detail.

1.3.1 Thrombopoietin

Discovery

In the fifties of the former century, the existence of a factor with thrombopoietic capacity has been suggested by analogy with EPO. Plasma, serum and urine of thrombocytopenic animals possessed thrombopoietic activity [36, 37]. Via the transforming oncogene *v-mpl*, (of a myeloproliferative leukemia virus (MPLV)), and its human homologue *c-mpl*, mpl-ligand (thrombopoietin (TPO) or megakaryocyte growth and development factor (MDGF)) was cloned in 1994 [29, 38, 39, 40, 41, 42, 43]. *C-mpl* was expressed in purified CD34+ cells, megakaryocytes and thrombocytes [44]. The antisense oligodeoxynucleotide of *c-mpl* inhibited *in vitro* megakaryocyte colony formation, whereas erythroid and granulocyte-macrophage colony formation was unaffected [44]. This suggested that the ligand for the mpl receptor was a cytokine that specifically regulates megakaryocytopoiesis. This was confirmed in studies; 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 [29, 40, 41, 45, 46, 47, 48].

Structure of TPO

The TPO-gene is located on chromosome 3q27-28 [49]. The coding region consists of 6 exons and encodes a protein of 353 amino acids. Two domains within TPO can be distinguished: the amino-terminal and the carboxy-terminal domain. The amino-terminal domain bears 23% sequence homology with erythropoietin (EPO) [49]. This domain of human TPO bears more than 80% homology with porcine and murine TPO and has two receptor-binding sites. Functional truncation studies showed that this amino-terminal domain is responsible for the hormonal activity of TPO [29, 41]. The carboxy-terminal domain is more unique; it has no similarity with other known proteins and is less conserved between the species. The C-terminal domain, in particular the N-glycosylation sites, was shown to be involved in the secretion and stabilization of TPO with enhancement of the circulating half-life of TPO [50]. Isoforms of a protein can be created through alternative splicing or proteolysis. Alternatively spliced isoforms of TPO (TPO2 and TPO3) are found in the kidney (TPO2), fetal liver and mouse bone marrow [30, 51,

52]. The exact function of these isoforms is unknown. TPO2 was found to be poorly secreted and did not influence proliferation of Ba/F3-*mpl* cells [30]. Analysis of different human carcinoma cell lines revealed the existence of three other isoforms, designated TPO4, TPO5 and TPO6 [53]..

TPO production sites and regulation of TPO levels

The main production sites of TPO in both humans and animals, as determined by the number of TPO transcripts, are the liver, and to a lesser extent, the kidney. In addition, TPO mRNA transcripts have been detected in various other organs and tissues, such as bone marrow, spleen, brain and placenta [29, 40, 41, 49, 54]. According to in situ hybridization studies in the liver; both hepatic parenchymal and sinusoidal endothelial cells are responsible for TPO production [55, 56]. Renal TPO production is confined to the cells of the proximal convoluted tubuli [55]. Patients with severe liver diseases often suffer from thrombocytopenia; which also demonstrates the dominant role of the liver in TPO production; orthotopic liver transplantation reconstitutes TPO production and subsequently thrombocyte levels are restored [57, 58].

Several studies demonstrated that thrombocytes bind and metabolize TPO. TPO levels are inversely correlated with the number of thrombocytes as well as the amount of megakaryocytes. This has been demonstrated by a decrease of TPO levels after administration of thrombocyte transfusions and elevated TPO levels in presence of thrombocytopenia after chemotherapy. Also in cyclic thrombocytopenia, TPO levels show an inverse correlation with thrombocyte levels [59, 60, 61, 62, 63, 64, 65]. Thus, the total mass of megakaryocytes and thrombocytes, which both express Mpl, determines the levels of circulating TPO [66, 67] (Figure 2). It was previously shown 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 [60]. The initial binding and uptake of TPO to *c-mpl* is concentration dependent and becomes saturated at higher doses [68]. 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. Additional mechanisms in the regulation of TPO levels include upregulation of TPO production at the transcriptional level by bone marrow stroma cells [55], release of TPO by activated platelets [69] and proteolytic cleavage of TPO by thrombin [70].

In vitro and in vivo activity of TPO

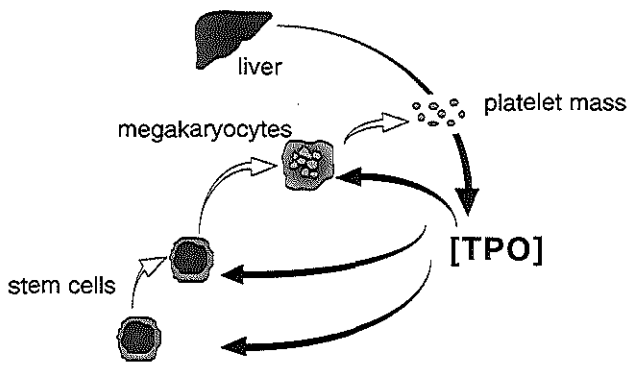
In vitro studies in which the effect of recombinant TPO on megakaryocyte formation and development was assessed have shown that TPO is involved in the proliferation, differentiation and maturation of megakaryocytic progenitor cells up to the induction of proplatelet formation [29, 41, 71, 72, 73]. The importance of mpl-ligand (TPO) in megakaryocytopoiesis *in vivo* is shown by TPO and *c-mpl* knockout mice, which have strongly reduced thrombocyte counts, approximately 10-20% of wild type mice. In addition, the number of megakaryocytes in the bone marrow is decreased, whereas the

numbers of cells of all other lineages are normal [74, 75, 76]. Furthermore, an effect of TPO on stem cells is demonstrated by hematopoietic stem cell deficiencies in c-mpl knockout mice [76, 77] whereas *in vitro* studies with TPO show a synergistic effect with early-acting cytokines in expanding long-term repopulating hematopoietic stem cells [78, 79, 80, 81]. Another important effect of TPO is the ability to inhibit p53-dependent apoptosis and TPO may play a role in the process of self-renewal of hematopoietic progenitor cells *in vivo* [82, 83, 84]. In clinical trials, TPO has been shown to accelerate thrombocyte recovery and improve the thrombocyte nadir in patients treated with chemotherapy or irradiation. TPO administration was well tolerated and induced a dose-dependent increase in functionally normal thrombocytes [85, 86, 87]. This resulted in a reduced requirement of thrombocyte transfusions [88]. Recombinant TPO administration in combination with high-dose chemotherapy with peripheral blood progenitor support was not effective in enhancing platelet recovery or in reducing the number of thrombocyte transfusions [89, 90]. Furthermore, a feasibility study on reinfusion of ex vivo expanded autologous megakaryocyte progenitor cells in combination with unmanipulated cells yielded promising results [91].

In vitro, TPO has been shown to be involved in thrombocyte activation. TPO can directly induce thrombocyte activation and aggregation at high concentrations (1000-fold above normal levels) [92, 93]. However, even under pathological conditions, TPO levels are not more than 100-fold increased. At this concentration, TPO has a priming effect on responses to agonists such as ADP or thrombin [94, 95]. In vivo, no increased incidence of thrombotic events has been reported after administration of TPO to both healthy individuals and thrombocytopenic patients [96].

In all studies, rHuTPO and PEG-rHuMGDF proved to be safe and well tolerated. However, in 10% of the healthy volunteers and in a few cancer patients who received PEG-rHuMGDF, neutralizing antibodies were formed that cross-reacted with endogenous TPO, resulting in thrombocytopenia [97, 98, 99]. This resulted in withdrawal of further clinical studies with PEG-rHuMGDF. A possible solution for this problem may be found in thrombopoietin mimetic peptides, which are functionally similar, but bear no sequence homology with TPO [100, 101], or synthetic non protein agonists .

Figure 2.



1.3.2 Flt3 Ligand

Discovery

The flt3/flk2 gene was cloned in 1991 simultaneously in two laboratories, by using two different strategies: the first consisted of low-stringency hybridization of cDNA libraries with a c-fms probe encompassing the kinase domain of the molecule [102], whereas the second used PCR amplification of tyrosine kinase cDNA segments from stem cell-enriched fetal liver cells, with degenerated oligonucleotides [103]. This receptor was identified in testicular cDNAs and termed flt3 [104]. It shows significant sequence and structural homology to the class III receptor tyrosine kinases, which include the colony-stimulating factor 1 (CSF-1) receptor (c-fms), the stem cell factor (SCF) receptor (c-kit), and the a and b platelet derived growth factor receptors [105]. The flt3 gene encodes a glycosylated protein of 993 amino acids in man and 1000 amino acids in mouse. The mature form of the protein is expressed at the cell surface [106].

Structure

The *flt3* receptor gene is located on human chromosome 13q12 and on mouse chromosome 5, close to *flt1*, another tyrosine kinase [102]. The genomic organization of the flt3 gene (also called STK-1) has been characterized in the human: it encompasses at least 96 kb and comprises 24 exons [107]. The gene of the fms-like tyrosine kinase 3 ligand (FL) has been mapped to chromosome 19q13, which is syntenic with mouse chromosome 7 [108]. The flt3 receptor has an extracellular domain consisting of 5 immunoglobulin-like subdomains of which 3 are involved in ligand binding, and 2 are involved in dimerization of the receptor. The cytoplasmic carboxy-terminal domain contains the catalytic region [32].

FL expression and regulation of FL levels

FL is expressed as a membrane-bound protein on the surface of cells and can be proteolytically cleaved to generate a soluble protein. Both the membrane-bound and soluble forms are biologically active [109]. In humans, flt-3 receptor expression was detected by PCR in liver, spleen, thymus, bone marrow, and weakly in the placenta [110]. The flt3 receptor (clustered as the CD135 antigen) is expressed in a fraction of quiescent hematopoietic stem cells. During differentiation, flt3 expression is down-regulated until extinction and its decline is more rapid than that of c-kit expression [111]. Targeted disruption of the flt3 gene leads to normally developed, healthy and fertile adult animals with normal mature hematopoietic populations. Only B cell progenitors are somehow deficient, as well as natural killer and dendritic cells [112, 113]. In hematopoietic malignancies, flt3 is expressed in a large majority of acute myeloid leukemias (AML), as well as acute B cell lymphoid leukemias [110]. Flt3 internal tandem duplication mutations are present in about 18-27% of all AML cases and are therefore one of the most frequent somatic gene mutations in AML. The presence of the mutation is associated with increased relapse risk, shorter event-free survival and lower overall survival [114, 115,

116, 117, 118]. The ligand of the flt3 receptor exists in membrane-bound and soluble forms. FL mRNA is expressed in stromal cells, endothelial cells, fibroblasts from hematopoietic tissues and T-cells throughout the body of both mouse and man [119, 120, 121]. The normal level of FL in human serum is 54 ± 35 pg/mL and an inverse relationship exists between the number of bone marrow progenitors and the level of FL [122]. This inverse relationship is also demonstrated in patients with Fanconi anemia and acquired aplastic anemia who have levels of about 1300 and 450 pg/mL respectively [123]. In myelodysplastic patients, endogenous FL levels are associated with disease stage [124], whereas FL levels after chemotherapy are related to bone marrow damage and recovery [125, 126, 127].

In vitro and in vivo activity of FL

The range of activities of FL on hematopoietic stem cells and progenitors has been extensively studied in murine as well as in human cells in vitro. When used alone, FL is generally described as widely inefficient for cell proliferation [128, 129, 130]. However, FL is able to stimulate in vitro expansion of very immature cells in long-term cultures (LTCIC) and FL is also relevant for the maintenance of CFU-GM and primitive progenitors in HPP-CFCs (high proliferative potential-colony forming cells) [130, 131, 132]. This effect is especially of importance for ex vivo retrovirus mediated transduction experiments [133] in which FL promotes preservation of the generative capacity of primitive human hematopoietic cell in suspension culture. FL acts in synergy with TPO and other hematopoietic growth factors to stimulate multilineage growth of progenitors and progenitor cell expansion [79, 129, 134, 135, 136, 137]. FL is especially of importance for primitive HSCs, myelomonocytic cells, B lymphoid cells and dendritic cells. Furthermore, FL has shown activity on megakaryocytopoiesis where it augments effects of other growth factors [128, 138], whereas the effect on erythroid progenitors is controversial and effects on mast cell differentiation are absent (in contrast to SCF) [119, 129, 133, 134, 135].

In vivo experiments with FL are directed at stem cell preservation during myelosuppression by chemotherapy or irradiation. FL administration in mice before irradiation proved to be radioprotective [139] whereas FL improved survival in irradiated mice with metastatic lung cancer by enhancing tumor antigen presentation [140]. FL has been shown to induce expansion of primitive hematopoietic progenitor cells in SCID-hu mice transplanted with human fetal bone fragments [141]. FL has also been tested in mobilization models together with GM-CSF or G-CSF. This combination of growth factors resulted in synergistic mobilization of hematopoietic progenitors [142, 143, 144, 145], which led to multilineage outgrowth after transplantation. FL as a single agent administered in normal mice mobilizes hematopoietic progenitors [146], and leads to dramatic numerical increases in dendritic cells (DC) throughout the body [147]. These functionally competent DCs may be of value to stimulate T-cell immunity by their use as vectors for immunotherapy vaccines [147, 148], directed against tumors and infectious diseases.

1.4.3 GM-CSF

Structure

Several colony-stimulating factors influence the survival, proliferation and differentiation of hematopoietic progenitors: macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF). The prefix GM of GM-CSF indicates that this factor stimulates the formation of granulocyte and/or macrophage colonies, which appeared to be only a part of its action on murine hematopoietic cells [149]. The gene that encodes GM-CSF is located at mouse chromosome 11 or its human analog chromosome 5 and is located in close proximity to the IL-3 gene [150]. These genes likely originate from a gene duplication, since they are structurally similar, and share overlapping functions. Human GM-CSF has a relative molecular mass of 20-23 kD and consists of 144 amino acids, 17 of which are removed from the precursor protein. GM-CSF contains 2 potential N-linked glycosylation sites [151] and is highly glycosylated. *In vitro*, deglycosylated human GM-CSF showed increased affinity for its receptor and increased biological activity [152]. Human and murine GM-CSF are species-specific. The receptor for GM-CSF belongs to the hematopoietin or cytokine receptor superfamily [153]. GM-CSF is produced by stroma cells, lymphocytes, monocytes, granulocytes, endothelial cells, and lung cells [149, 154].

In vitro and in vivo activity of GM-CSF

In vitro studies show that GM-CSF promotes the survival, proliferation, and maturation of myeloid cells. It also enhances the function of mature neutrophils and monocytes [155, 156, 157]. Together with erythropoietin, GM-CSF induced the proliferation and differentiation of early erythroid (BFU-E) and multipotential progenitors (CFU-GEM) from simian bone marrow mononuclear cells [158]. GM-CSF proved to have megakaryocyte colony stimulating activity and augments the megakaryocyte colony formation in the presence of IL-3 [159].

In vivo studies with GM-CSF in normal dogs [157] showed a significant in crease in circulating blood neutrophils and eosinophils. During GM-CSF administration, a dose-dependent thrombocytopenia developed, but reversed after cessation of treatment. In non-human primate studies, recombinant human GM-CSF (rhGM-CSF) resulted in an increased leukocyte count and hypercellular bone marrow (without adverse effects) [158, 160], megakaryocyte maturation stage development was stimulated but did not result in a consistent thrombocytopoietic response [161]. Furthermore, rhGM-CSF enhanced bone marrow reconstitution (including thrombocyte recovery) both after severe myelosuppression and after bone marrow transplantation [162, 163, 164, 165]. After high-dose chemotherapy and autologous BMT in patients with Non-Hodgkin's lymphoma, rhGM-CSF reduced neutropenia and hospitalization stay, but there was no difference in relapse rate or number of deaths after one year [166].

1.4 Introduction to the experimental work

Cytotoxic agents are commonly used in the treatment of patients with either hematologic or non-hematologic malignant diseases. Myelosuppression is the major dose-limiting toxicity of systemic anti-cancer chemotherapy, rendering many patients at risk for anemia, life-threatening infections due to neutropenia and major bleeding due to thrombocytopenia [167]. Acute damage and recovery of hematopoietic stem and precursor cells following exposure to whole-body irradiation has been studied extensively in standardized rhesus monkey models for myelosuppression and bone marrow transplantation. In the course of these studies and facilitated by the development of a supportive care system, i.e., prevention of infections by reverse barrier isolation and gastrointestinal decontamination, as well as deliberate transfusion of -mainly- platelets, extensive baseline data have become available for hematopoietic reconstitution following total body irradiation (TBI). In the dose range of 4-9 Gy (X-rays), data on the efficacy and side effects of hematopoietic growth factors have been obtained, in particular GM-CSF, G-CSF, TPO and combinations of these. Placebo controlled studies on TPO [168, 169, 170] have revealed it to be highly effective agent in accelerating thrombocyte reconstitution following 5 Gy TBI as a model for myelosuppression resulting in 3 weeks of pancytopenia. Briefly, TPO accelerated the reconstitution of thrombocytes and strongly promoted the initial reconstitution of immature bone marrow CD34+ cells to two log higher levels two weeks after TBI compared to placebo monkeys. TPO also potentiated the action of growth factors such as G-CSF and GM-CSF in stimulating neutrophilic reconstitution. TPO was not effective following transplantation of as few as 3 x 10^4/kg CD34, RhLA-Drdull cells in 8 Gy irradiated rhesus monkeys, which results in 3-4 weeks of thrombocytopenia. Similarly, TPO was not effective in a pilot study with limited numbers of unfractionated autologous bone marrow cells.

TPO was further evaluated for efficacy with concurrent administration of either GM-CSF or G-CSF [chapter 2]. Although these HGFs belong to the same hematopoietin receptor superfamily, GM-CSF and G-CSF have another phylogenetic background than TPO and may therefore act via different receptor pathways, which is hypothesized to result in increased efficacy. Single IV dose treatment with TPO 1 day after 5 Gy TBI effectively counteracted the need for thrombocyte transfusions and accelerated thrombocyte reconstitution to reach normal levels two weeks earlier than placebo controls. TPO/GM-CSF was more effective than single dose TPO alone in stimulating thrombocyte regeneration, with a less profound nadir and a further accelerated recovery to normal thrombocyte counts. Also reticulocyte production was stimulated by TPO and further augmented in monkeys treated with TPO/GM-CSF. TPO alone did not stimulate neutrophil regeneration whereas GM-CSF shortened the period of neutrophils < 0.5 x 10^9/L approximately one week; TPO/GM-CSF treatment elevated the neutrophil nadir but did not further accelerate recovery to normal values. Based on these results, the combination TPO/GM-CSF has also been examined after autologous bone marrow transplantation (Chapter 3).

In chapter 4, the effect of growth factors on immature CD34+ cells is further detailed relative to the establishment of normal values for bone marrow and peripheral blood CD34+ cells. The CD34+ cells in bone marrow and peripheral blood and hematologic parameters measured after myelosuppression and bone marrow transplantation during bone marrow reconstitution were correlated in more than 80 animals used in standardized protocols.

The effect of Flt-3 Ligand (FL) playing a role in stem cell stimulation as well as B-cell and dendritic cell stimulation, was studied together with rhesus TPO after myelosuppression (Chapter 5). The receptor of FL belongs, in contrast to that of TPO, to the tyrosine kinase receptor family and acts via a different signal transduction pathway, which excludes competition with respect to growth factor receptors. In vitro studies show synergistic effects of TPO and FL on stem cells [80, 171], and it was hypothesized that TPO and FL might also stimulate stem cell reconstitution in vivo. Furthermore, endogenous FL levels with regard to reconstitution by measuring FL levels after growth factor treated myelosuppressed and transplanted animals were related to recovery parameters (Chapter 6).

Using the stem cell transplantation model in rhesus monkeys, the safety and efficacy of thrombocyte transfusions from TPO stimulated donors was studied. Such an approach would potentially allow for repetitive use of a single (MHC matched, family) donor, which would reduce the present use of multiple donors, contribute to prevention of alloimmunization, and lower the risk of infections (Chapter 7).

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CHAPTER 2

The efficacy of single dose administration of thrombopoietin with co-administration of either GM-CSF or G-CSF in myelosuppressed rhesus monkeys

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Abstract

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 three 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 iv dose treatment with TPO 1 day after TBI effectively counteracted the need for thrombocyte transfusions (provided whenever thrombocyte levels were $< 40x10^9$/L) and accelerated platelet reconstitution to reach normal levels two weeks earlier than placebo controls. TPO/GM-CSF was more effective than single dose TPO alone in stimulating thrombocyte regeneration, with a less profound nadir and a further accelerated recovery to normal thrombocyte counts, as well as a slight overshoot to supranormal levels of thrombocytes. Monkeys treated with TPO/GM-CSF uniformly did not require thrombocyte transfusions, whereas those treated with GM-CSF alone needed 2-3 transfusions, similar to the placebo treated monkeys, which required, on average, 3 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 $0.5x10^9$/L by approximately one week; TPO/GM-CSF treatment elevated the neutrophil nadir, but did not further accelerate recovery to normal values. TPO also augmented the neutrophil response to G-CSF, resulting in similar patterns of reconstitution following TPO/G-CSF and TPO/GM-CSF treatment. TPO/GM-CSF treatment resulted in significantly increased reconstitution of CD34 positive 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 (i) a single iv administration of TPO is sufficient to prevent severe thrombocytopenia following myelosuppression, (ii) 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 (iii) TPO significantly improves the performance of the CSFs in alleviating severe neutropenia.

Introduction

Identification of thrombopoietin (TPO) [1-4] as the major regulator of thrombocyte production [5,6] has resulted in novel insights in the regulation of immature hemopoietic cell differentiation [7-9], and has potentially provided a therapeutic approach 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 than detailed experimental animal in vivo studies. We have presently focused on growth factors that are likely to be used clinically with TPO, i.e., GM-CSF and G-CSF.

In previous studies in myelosuppressed mice and rhesus monkeys, a supraoptimal dose of human TPO and concurrent administration of G-CSF was found to prevent thrombocytopenia, accelerate platelet and red blood cell reconstitution, alleviate neutropenia, and promote the recovery of immature bone marrow cells [13-18]. 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 anti-cancer 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 20-25 days. It is therefore of considerable importance to select combinations of growth factors that provide optimal costimulatory efficacy.

The study was undertaken (i) 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 (ii) 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 TBI, which results in a profound pancytopenia for three weeks, and the 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 as well as the monitoring of adverse effects.

Materials and methods

Animals: Purpose-bred male rhesus monkeys (*Macaca Mulatta*), weighing 2.5-4.0 kilograms and aged 2 to 3 years, were used. The monkeys were housed in groups of 4 to 6 monkeys in stainless steel cages in rooms equipped with 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, fresh fruits, and received acidified drinking water. All animals were free of intestinal parasites and 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.

Total body irradiation: Monkeys were irradiated with a single dose of 5 Gy total body irradiation (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 0.20-0.22 Gy/min. During TBI, the animals were placed in a cylindrical polycarbonate cage which 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 the gastrointestinal tract was selectively decontaminated by administering oral Ciprofloxacin (Bayer, Mijdrecht, the Netherlands), Nystatin (Sanofi BV, Maassluis, The Netherlands) and Polymyxin B (Pfizer, New York, NY, USA). 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, the 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 g irradiation) platelet transfusions whenever thrombocyte counts were less than 40 x 10^9/L, packed red cells whenever hematocrits were lower than 20% and, occasionally, whole blood transfusions in case coincidence of both transfusion criteria. The criterion of transfusion of thrombocytes at counts < 40 x 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 Chines Hamster Ovary (CHO) cells was supplied by Genentech Inc. (South San Francisco, CA). The dose used was 0.5, 5 or 50 µg/kg given 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 prior to administration. Placebo treated monkeys were only given the same volume of diluent. Recombinant human G-CSF (Neupogen, Amgen Inc., Thousand Oaks, CA) was administered in a dose

of 10 µg/kg/day, sc, once daily during days 1 to 14 after TBI. Recombinant human GM-CSF (Leukine, Immunex Corporation, Seattle, WA) was given in a dose of 25 µg/kg/day, 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 intravenously at 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/day sc from day 1-14 (n=4), TPO and GM-CSF (TPO/GM-CSF)(n=4), G-CSF at a dose of 10 µg/kg/day 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 which also received GM-CSF or G-CSF sc for 14 consecutive days.

Bone marrow aspirates: Bone marrow was aspirated under neurolept anesthesia using Ketalar (Apharmo, Arnhem, the Netherlands) and Vetranquil (Sanofi, Maassluis, the Netherlands). Small bone marrow aspirates for analytical purposes were taken 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 a Ficoll (density 1.077)(Nycomed Pharma AS, Oslo, Norway) separation.

Colony assays: Cells were plated in 35-mm dishes (Becton Dickinson, Leiden, The Netherlands) in 1 mL a-DMEM (GIBCO, Gaithersburg, MD) containing 0.8% methylcellulose, 5% FCS, and additives as described previously [36-38]. For burst-forming units-erythroid (BFU-E), cultures were supplemented with hemin ($2x10^{-4}$ 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 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 in B. licheniformis and purified as described previously [39,40], and KL. Low density cells were plated at $5x10^4$ cells per dish in duplicate. Colony counts were calculated per mL of bone marrow aspirated using the recovery of cells over the Ficoll density gradient. Colony numbers represent the mean ± standard deviation 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., LTD., 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 was diluted in 1 mL PBS/EDTA/azide and one 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, Leiden, The Netherlands) and analyzed using the Reticount software.

Measurements of surface antigens: Once weekly, a FACScan analysis was made on peripheral blood (PB) and bone marrow (BM) 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 monoclonal antibody MO1-FITC (Coulter Immunology, Hialeah, FL) was used and for CD34 a monoclonal antibody (MoAb) against human CD34 (MoAb 566) that had been fluoresceinated with fluorescein isothiocyanate (FITC; Sigma, St Louis, USA) 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 L) 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 100µL 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 the Lysis II software (Becton Dickinson).

Statistics: Standard deviations were calculated and are presented in the text and in the 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 of 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 4 monkeys treated in this way is shown in figure 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/day. The data for the four concurrent control monkeys have been included in figure 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 2 thrombocyte transfusions (one in each of two monkeys), as opposed to the four placebo controls which needed a total of 13 transfusions (mean 3, range 1-6), since their thrombocytes had decreased to less than 40 x 10^9/L. This is statistically significant ($P < .04$; table 1). In addition, the TPO treated monkeys displayed a clearly accelerated thrombocyte reconstitution and reached normal thrombocyte levels two weeks before the placebo treated controls ($P < 0.03$).

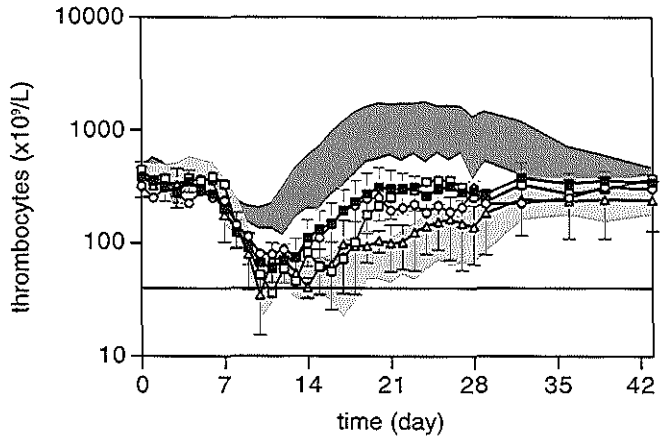

Figure 1. Thrombocyte counts after 5 Gy TBI (day 0) for monkeys treated with rhesus TPO 5 µg/kg (■, n = 4), TPO 50 µg/kg (□), TPO 0.5 µg/kg (O, n = 2), and the concurrent placebo treatment (Δ, n = 4). The lower shaded area represents the mean ± SD of 12 control monkeys; the upper shaded area is the mean ± SD of 4 monkeys treated with human TPO 10µg/kg 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 x 10^9/L) below which thrombocyte transfusions are given.

Table 1. Transfusion requirements and blood cell regeneration after 5 Gy TBI and Growth Factor treatment.

Treatment	No. of monkeys	No. of transfusions	Thrombocytes > 40x 10^9/L (d)	Reticulocytes > 1% (d)	Neutrophils > 0.5 x10^9/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 x 10^9/L not reached in the majority of the monkeys.* statistically significantly different from placebo-treated monkeys ($P < .05$)

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 figure 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 x 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 one day after TBI. The monkeys treated with 0.5 µg/kg both had nadirs lower than 40 x 10^9/L, and needed one and two thrombocyte transfusions, respectively, providing a further validation of the dose of 5 µg/kg as close to the minimum required to get 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 and TPO and 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 cells counts in monkeys treated with TPO/GM-CSF and TPO/G-CSF:
In contrast to the monkeys treated with TPO alone, of which two needed a single thrombocyte transfusion, the monkeys treated with TPO/GM-CSF remained completely transfusion-free and showed an even more elevated nadir, with two of the four monkeys never reaching thrombocyte levels less than 100 x 10^9/L. Recovery to normal values was accelerated by 23 days compared to placebo-treated monkeys ($p<0.003$) and an

overshoot to supranormal values was apparent, which returned to normal values after cessation of GM-CSF treatment (Figure 2). Monkeys treated with TPO/G-CSF (Figure 2) 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 1-3 transfusions. Although there was no significant difference with the placebo treated controls in 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 (Figure 2).

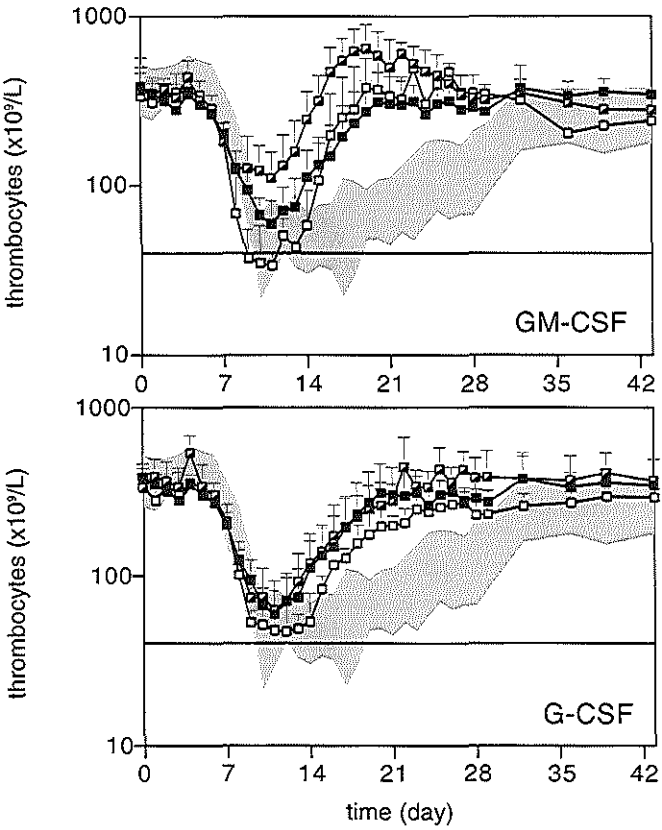

Figure 2. Thrombocyte counts after 5 Gy TBI (day 0) for monkeys treated with rhesus TPO (■, n = 4),TPO/GM-CSF (◪, n = 4), GM-CSF (□, n = 4), in the upper panel and TPO/G-CSF (◪, n = 4) or G-CSF (□, n = 3) in the lower panel. The shaded area represents the mean ± SD of 12 control monkeys. Data represent the arithmetic mean ± SD of the various treatment groups. The horizontal line defines the level of thrombocytopenia (40 x 10^9/L) below which thrombocyte transfusions are given.

Reticulocyte regeneration to values more than 1% occurred approximately one week earlier in TPO treated monkeys than in placebo treated controls ($p<0.03$), and showed a biphasic response (Figure 3). Again, TPO/G-CSF was very 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 four days ($p<0.04$) and G-CSF alone by three days (Figure 3). This pattern was also reflected in recovery of red blood cell counts after the initial decline, which is largely due to diagnostic bleeding, the TPO/GM-CSF group of monkeys being the fastest to recover (data not shown).

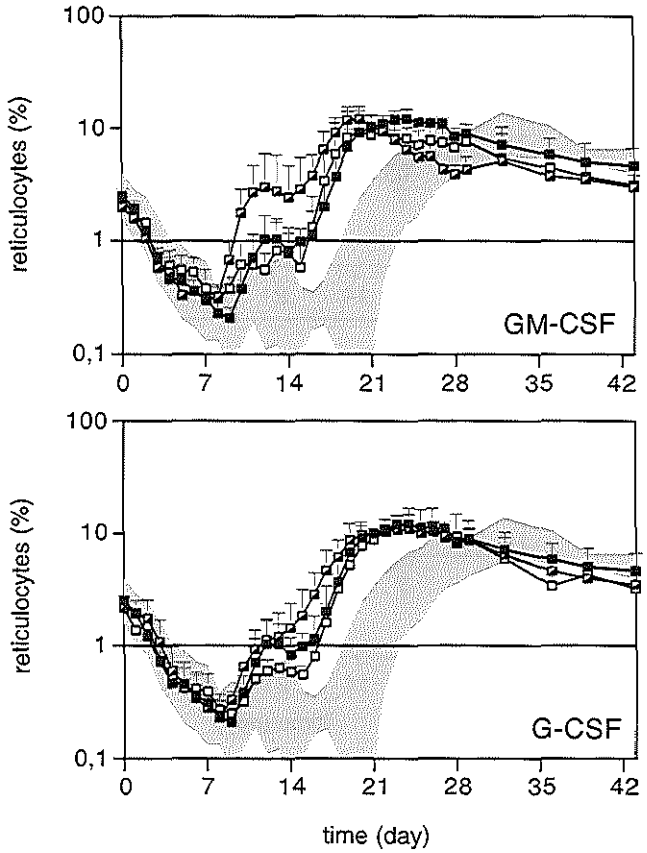

Figure 3. Reticulocyte regeneration after 5 Gy TBI (day 0) for monkeys treated with rhesus TPO (■, n = 4),TPO/GM-CSF (◪, n = 4), GM-CSF (□, n = 4), in the upper panel and TPO/G-CSF (◪, n = 4) or G-CSF (□, n = 3) in the lower panel., The shaded area represents the mean ± SD of 12 control monkeys. Data represent the arithmetic mean ± SD of the various treatment groups. The horizontal line defines the level of 1%, a regeneration marker.

Neutrophil reconstitution in TPO treated monkeys was within the range of that for the placebo treated monkeys with recovery to levels more than 0.5 10^9/L occurring around day 21 (Figure 4). Both TPO/GM-CSF and TPO/G-CSF treatment elevated the neutrophil nadir, reflecting an accelerated recovery to reach levels > 0.5 10^9/L one 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 with GM-CSF alone had very similar neutrophil recovery patterns to reach levels more than 0.5x10^9/L 5 days before placebo treated monkeys ($p<0.03$) (Figure 4). The treatment with the CSFs resulted in a slightly earlier, but not in a detectably less profound neutrophil nadir.

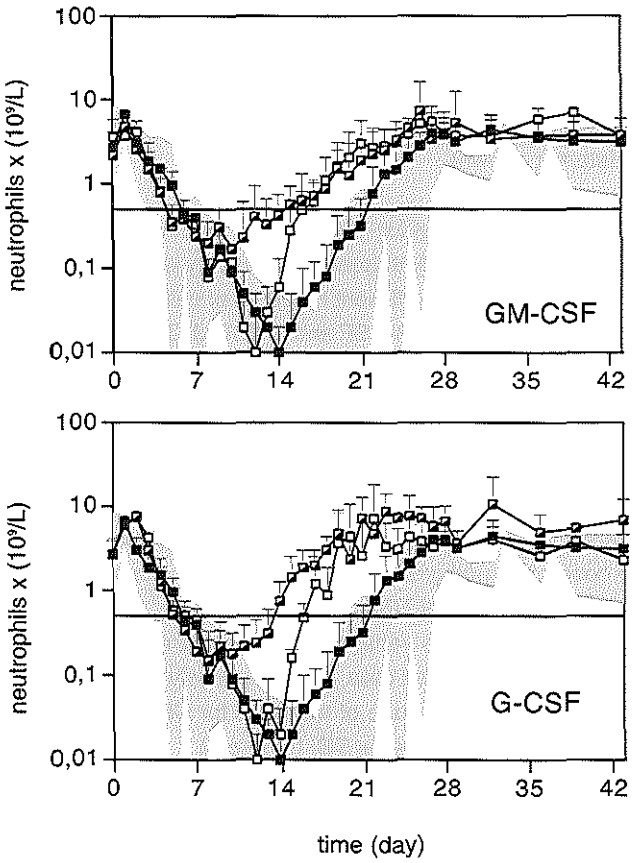

Figure 4. Neutrophil regeneration after 5 Gy TBI (day 0). Symbols are as in Figure 3. The horizontal line defines the level of 0.5 x 10^9/L.

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 positive 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 those treated with TPO/GM-CSF 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 (Figure 4) regeneration.
CD8 positive lymphocytes recovered to normal levels within the observation period of 6 weeks in all treatment groups without significant differences. All animals had subnormal levels CD4 positive T lymphocytes and CD20 positive 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 treated 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 the fourth week after TBI. The stimulating effect of single administration of 5 µg/kg TPO on bone marrow cellularity, CD34 positive 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/day 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, i.e., those obtained in the TPO/GM-CSF group of monkeys for CD34 positive cells at day 15 and for GM-CFU and BFU-E at day 22 after TBI (Figure 5).

At day 15 after TBI, CD34 positive cell numbers significantly exceeded those of placebo controls in the TPO/GM-CSF ($p<0.03$) (Fig. 5) and the G-CSF ($p<0.01$) (not shown) treatment groups. For GM-CFU, only the TPO/GM-CSF group exceeded significantly ($p<0.01$) the placebo controls by approximately one log at day 22 after TBI (Figure 5). The TPO/GM-CSF group was also richer in GM-CFU than animals treated with GM-CSF alone ($P < 0.04$), 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 ($p<0.02$) better than the placebo controls (Figure 5).

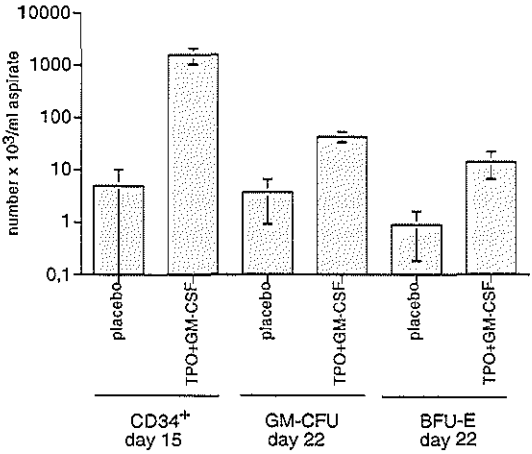

Figure 5. Bone marrow CD34+ cells , GM-CFU and BFU-E, 2,3 and 3 weeks after 5 Gy TBI, respectively (arithmetic mean ± SE). Since the variance of this type of data is inevitably large due to variations of bone marrow content at the puncture site and admixture with peripheral blood cells, in addition to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

Other on study parameters and febrile episodes

No abnormalities were observed for most of the serum clinical chemistry. Albumin concentrations were stable at around 40 g/L, except for the GM-CSF treated monkeys which developed edema (vide infra). Electrolyte disturbances were minor due to gastrointestinal radiation damage 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 the TPO/GM-CSF and the 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 with the other 26 consecutive monkeys described in this report, that did not receive GM-CSF, the phenomenon proved statistically significant (p=0.02, Fisher's exact test). However, none of the four TPO/GM-CSF treated monkeys developed edema. 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 one day after a myelosuppressive dose of radiation, that results in three weeks of cytopenia in placebo treated controls, is sufficient to virtually prevent the need for thrombocyte transfusion and to accelerate thrombocyte reconstitution to normal levels by two 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. Co-administration of TPO/GM-CSF augmented thrombocyte, red cell and neutrophil production over either of the individual growth factors alone, whereas in the TPO/G-CSF treatment group only neutrophil reconstitution appeared to benefit from the combination of growth factors.

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 observation is consistent with data in mice [30], 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 a prevention of a physiological death or apoptosis in the presence of TPO similar to that identified for erythropoietin deprived red cell progenitor cells [43,44] or protection of TPO responsive progenitors from radiation induced cell death. Elucidation of such mechanisms not only will provide further insight into the physiological function of TPO, but also will be of considerable importance to achieve the maximum clinical benefit of its therapeutic use. Although the data reveal that a single iv dose of 5 µg/kg of TPO is sufficient to prevent thrombocytopenia at the level of myelosuppression chosen, this finding does not preclude that clinical cytoreductive therapy requires a more intensive TPO treatment regimen.

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 x 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 an 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 x 10^9/L within 2 days after the first transfusion (Figure 1). It can thus be concluded that TPO treatment that prevented the decline of thrombocyte counts to levels less than 40 x 10^9/L early in the second week after irradiation also effectively prevented the propensity to bleeding (Figure 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 treated monkeys may reflect synergism of the two growth factors, since the initial recovery of both thrombocytes (Figure 2) and reticulocytes (Figure 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 [46,48], and, in retrospect, may have been co-dependent on variations in endogenous thrombopoietin 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/day dose (for two weeks) of G-CSF also appeared to stimulate thrombocyte production to a certain extent, in contrast to the 5 µg/kg/day dose (for three 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/day 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 mentioning 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 the TPO/CSF treated animals. G-CSF or GM-CSF treatment alone did not appreciably influence the height of 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 (Figure 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-CS [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 positive cells, and clonogenic progenitor cells such as GM-CFU and BFU-E following supra-optimal 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 very 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 not only be directed at optimal thrombocyte recovery, but also at the most optimal reconstitution of immature bone marrow cells.

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CHAPTER 3

Lack of efficacy of TPO and GM-CSF after total body irradiation and autologous bone marrow transplantation in rhesus monkeys

Published as: Simone C.C. Hartong, Karen J. Neelis, Trudy P. Visser and Gerard Wagemaker Lack of efficacy of TPO and GM-CSF after total body irradiation and autologous bone marrow transplantation in rhesus monkeys Experimental Hematology 28: 753-759; 2000.

Abstract

Objective If administered in a sufficiently high dose to overcome receptor-mediated clearance and in a well-scheduled manner, thrombopoietin (TPO) prominently stimulates hematopoietic reconstitution following myelosuppressive treatment and potentiates the efficacy of both GM-CSF and G-CSF. However, TPO alone is not effective after bone marrow transplantation. Based on results of GM-CSF and TPO treatment after myelosuppression that resulted in augmented thrombocyte, reticulocyte and leukocyte regeneration, we evaluated TPO/GM-CSF treatment after lethal irradiation followed by autologous bone marrow transplantation.

Materials and Methods Young adult Rhesus monkeys were subjected to 8-Gy total body irradiation (TBI) (X-rays) followed by transplantation of 10^7/ kg unfractionated bone marrow cells. Five µg/kg TPO was administered intravenously (iv) at day 0 to obtain rapidly high levels. Animals then were treated with 5 µg/kg rhesus TPO and 25 µg/kg GM-CSF subcutaneously (sc) during days 1-14 after TBI.

Results The grafts shortened the profound pancytopenia induced by 8 Gy TBI from 5-6 weeks to 3 weeks. The combination TPO and GM-CSF did not significantly influence the recovery patterns of thrombocytes ($p= 0.39$), reticulocytes ($p= 0.08$), white blood cells ($p= 0.08$) or bone marrow progenitors compared to TPO alone.

Conclusions The present study demonstrates that, after high-dose total body irradiation and transplantation of a limited number of unfractionated bone marrow cells, simultaneous administration of TPO and GM-CSF after TBI is ineffective in preventing pancytopenia. This result contrasts sharply with the prominent stimulation observed in a 5 Gy TBI myelosuppression model, despite a similar level of pancytopenia in the 8 Gy model of the present study. The discordant results of this growth factor combination in these two models may imply co-dependence of the hematopoietic response to TPO and/or GM-CSF on other factors or cytokines.

Introduction

Thrombopoietin (TPO) is the ligand for the cytokine receptor c-mpl (the myeloproliferative leukemia proto-oncogene [1-3]). TPO plays a major role in stimulating thrombocyte production and counteracts thrombocytopenia after myelosuppression in mice and non-human primates [4-8]. TPO has multilineage hematopoietic effects when administered to myelosuppressed animals [4,9-12], prevents anemia and alleviates neutropenia. In a non-human primate myelosuppression model of 5 Gy total body irradiation (TBI), which results in 3 weeks of pancytopenia, we showed that TPO administration is highly effective in preventing thrombocytopenia, which results in an improved nadir and faster recovery to normal thrombocyte counts [4,5]. A single dose of TPO is fully effective to prevent thrombocytopenia following cytoreductive treatment [5,9,10]. In myelosuppressed mice, it was demonstrated that administration of TPO early after TBI and attainment of a

sufficiently high plasma level in the first hours are critical for an optimal multilineage effect [9,12] attributed to stimulation of immature cells that are capable of multilineage proliferation. In dose-effect survival experiments in mice, TPO prevented mortality after lethal irradiation and shifted the LD50/30d from approximately 9.5 Gy to 10.5 Gy TBI given as a single dose. This effect is related to accelerated neutrophil and thrombocyte recovery and a strong reduction in hemorrhagic complications [10]. However, TPO was ineffective in a bone marrow transplant model in rhesus monkeys (8 Gy TBI) [13,14].

In previous studies, we examined the effect of simultaneous administration of TPO and G-CSF. In the 5 Gy myelosuppression model, this combination resulted in augmented immature precursor cell recruitment, followed by increased neutrophil regeneration [5]. However, in the 8 Gy bone marrow transplant model an effect on neutrophil, thrombocyte or red blood cell regeneration was not observed [13]. This indicates that exogenous G-CSF is not a critical co-factor for TPO in the transplant setting.

Although the pharmacological profiles of G-CSF and GM-CSF are similar, different response patterns in conjunction with TPO were shown in myelosuppressed monkeys [5]. Co-administration of TPO and GM-CSF after 5 Gy TBI in rhesus monkeys augmented thrombocyte, erythrocyte and neutrophil recovery compared to treatment with either of the individual growth factors, whereas the action of G-CSF was restricted to the neutrophil lineage. On this basis, we hypothesized that the synergistic effects of combined administration of TPO and GM-CSF might be extended to the bone marrow transplant model.

Similar to TPO, GM-CSF treatment shows different effects in normal and myelosuppressed subjects. In normal primates, GM-CSF is effective in promoting the maturation stage of megakaryocyte development, although not consistently followed by a thrombocytosis [15], whereas GM-CSF treatment in normal dogs led to thrombocytopenia [16]. In preclinical myelosuppression or transplant studies, GM-CSF was shown to stimulate granulocyte and thrombocyte recovery [17-19], but in clinical chemotherapy induced myelosuppression- or transplantation studies, GM-CSF only shortened the period of neutropenia without effecting thrombocyte counts [20-22].

Presently, mobilized peripheral blood (PB) stem cells are increasingly used. Transplantation of mobilized PB stem cells results in accelerated hematopoiesis compared to transplanted bone marrow stem cells [23-25]. As a consequence, the indication for growth factor treatment following transplantation narrows. Nevertheless, if only a limited number of stem cells is available, e.g. in cord blood transplantation, in gene transfer therapy or in case of prominent allogeneic MHC disparity, growth factor treatment that both accelerates short-term hematopoietic regeneration and promotes sustained reconstitution may be of critical importance. In the present study we examined the simultaneous administration of TPO and GM-CSF to assess their synergistic efficacy and possible stimulatory and/or adverse interactions after sublethal total body irradiation (8 Gy), followed by transplantation of the deliberately limited number of 10^7/kg body weight unfractionated bone marrow cells.

Materials and methods

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

Total body irradiation: After aspiration of bone marrow, monkeys were irradiated with a single dose of 8 Gy total body irradiation (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 0.20-0.22 Gy/min. During TBI, the animals were placed in a cylindrical polycarbonate cage which rotated slowly (3 times per minute) around its vertical axis [26].

Supportive care: Two weeks before TBI, the monkeys were placed in a laminar flow cabinet and the gastrointestinal tract was selectively decontaminated by giving oral Ciprofloxacin (Bayer, Mijdrecht, the Netherlands), Nystatin (Sanofi BV, Maassluis, The Netherlands) and Polymyxin B (Pfizer, New York, NY, USA). This regimen was maintained until leukocyte counts exceeded 10^9/L. Systemic antibiotics were given when leukocyte counts dropped below 10^9/L, in most cases as a combination of ticarcillin (SmithKline Beecham Farma BV, Rijswijk, The Netherlands) and cefuroxim (Eurobase BV, Barneveld, The Netherlands), as guided by fecal bacteriograms. All monkeys received iron supplementation; 0.5 mL Imferon intramuscularly (Fe(III) 50 mg/ml; Fisons Pharmaceuticals, Loughborough, England) for 5 days before irradiation. Two monkeys were treated with Surolan creme(miconazolnitrate/ polymyxin B sulphate/ prednisolonacetate), (Janssen-Cilag, Tilburg, The Netherlands) and Hydrocortiderm creme (neomycinsulphate/ prednisolonacetate) (Vetoquinol, Den Bosch, The Netherlands) for suspected fungal infection of the skin. Dehydration and electrolyte disturbances were treated by appropriate fluid and electrolyte administration subcutaneously. The monkeys received irradiated (15 Gy) platelet transfusions whenever platelet counts were below 40 x 10^9/L, irradiated packed red cells whenever the hematocrit was below 20% and, occasionally, the monkeys received whole blood transfusions in case of simultaneous occurrence of both transfusion criteria.

Test drugs: The two TPO controls received human TPO supplied by Genentech Inc. (South San Francisco, Ca, USA) in one mL vials containing 0.5 mg/mL recombinant human TPO. The 10 µg/kg/day sc dose was given once daily from day 1 to 21 after

irradiation. The daily doses were diluted to a volume of 1 mL with phosphate-buffered saline (PBS)/0.01% Tween 20 before administration. The TPO/GM-CSF animals received 5µ/kg/day rhesus TPO iv at day zero, simultaneous with the transplant, and then for 14 days after transplantation sc. One mL vials containing 0.930 mg/mL recombinant full length rhesus monkey TPO produced by Chinese hamster ovary cells were supplied by Genentech Inc (South San Francisco, CA, USA). The daily doses were diluted to a volume of 1 mL with phosphate-buffered saline (PBS)/0.01% Tween 20 before administration. Recombinant human GM-CSF (Leukine; Immunex Corp, Seattle, WA) was administered in a dose of 25 µg/kg/day, sc, once daily from day 1 to 14 after irradiation The daily doses were diluted to a volume of 1 mL in the solution indicated by the supplier. Placebo treated monkeys were given the same volume of diluent sc from day 1 to 14 after TBI.

Study groups: Studies with Rhesus monkeys are done sequentially using highly codified methods, including radiation and placebo controls at regular intervals. For the present study, assignment to the study groups was at random. Three monkeys were treated with Rhesus TPO and GM-CSF and one placebo control with carrier. The results were compared with TPO treated monkeys [13] and a placebo control.

Bone marrow aspirates: Bone marrow was aspirated 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 Hank's buffered Hepes solution (HHBS) and 2,500 IU sodium heparin (Leo Pharmaceutical Products, Weesp). Low density cells were isolated using a Ficoll (density 1.077) (Nycomed Pharma AS, Oslo, Norway) separation. Erythrocytes in the aspirate were returned to the monkey after washing twice in 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 for analytical purposes were taken from the shafts of the humeri using pediatric spinal needles.

Colony assays: Cells were plated in 35-mm dishes (Becton Dickinson) in 1 mL a-MEM (GIBCO, Gaithersburg, MD) containing 0.8% methylcellulose, 5% FCS, and additives as described earlier [27,28]. For burst-forming units-erythroid (BFU-E), cultures were supplemented with hemin (2 x 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 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 previously described [29,30] and KL. Low density cells were plated at $5x10^4$ cells per dish and sorted cell fractions at 10^3 per dish in duplicate. Colony numbers represent the mean ± standard error of duplicate dishes, assuming that colony counts are Poisson distributed. Progenitor cell assays performed to monitor bone marrow reconstitution were standardized on a per milliliter aspirate basis.

Transplantation: To enrich for mononuclear cells, low density cells were isolated at day zero. The cells were transplanted using a dose of 10^7/ kg body weight and returned to the monkey the same day after TBI.

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

Measurements of surface antigens: Once weekly FACScan analysis was done 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 11aFITC (Becton Dickinson) respectively). For CD11b the monoclonal antibody MO1-FITC (Coulter Immunology, Hialeah, Florida). Red blood cells were removed by incubation of 0.5 mL of whole blood or bone marrow in 10 mL lysing solution (8.26 g ammonium chloride/1.0g potassium bicarbonate and 0.037 g EDTA per 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 non-specific 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 the Cell Quest software (Becton Dickinson).

Clinical chemistry: serum values for sodium, potassium, albumin, total protein, ALAT, LDH1, C reactive protein, creatinine and iron were performed once weekly using an Elan Analyzer (Eppendorf Merck, Hamburg, Germany).

Statistical analysis: Mean, median and range of numeric variables were calculated by the Excel spreadsheet program. Standard deviations were calculated and are given in the text and in the figures on the assumption of a normal distribution. The statistical significance of differences was calculated by the Mann-Whitney test, comparing two unpaired groups each time.

Results

Characterization of transplants:
Following Ficoll isolation of low density cells, colony assays were performed and a FACScan analysis was done for CD34 antigen expression. Calculated as percentages of the aspirated bone marrow cells, CD34++ cells represented 2.9 % ± 1.8 % and CD34+/DR dull cells (from which reconstitution originates [31]), represented 0.94 ± 0.7% of the total cell number. Transplanted numbers of GM-CFU were 22.1 ± 10.1 x 10^3/ kg and of BFU-E 7.4 ±1.4 x10^3 /kg bodyweight.

Peripheral blood cell counts:
The nadir for platelets was not significantly alleviated by TPO nor by the GM-CSF / TPO treatment, and the time needed for normalization of those values was not improved by growth factor administration (Figure 1). The TPO treated monkeys needed both one thrombocyte and one whole blood transfusion and reached a transfusion free platelet level at day 15. The three TPO/GM-CSF treated monkeys required 4, 5 and 6 thrombocyte transfusions respectively and two of the three monkeys needed one whole blood transfusion, reaching transfusion independence at day 16. For the two placebo controls the number of thrombocyte transfusions were 2 (+ 1 whole blood) and 6 (+ 4 whole blood) respectively, reaching transfusion independence at day 18 and 40 (mean: 23 days).
As described previously, the reticulocyte regeneration [32], defined as the day at which the percentage of reticulocytes exceeded 1%, was found to be the least variable parameter to quantify the engraftment potential of BM. This 1% level was on average reached 17,18 and 22 days after TBI for TPO, TPO/GM and placebo treated monkeys respectively. The regeneration curves (Figure 1) suggest that TPO promoted reticulocyte regeneration to a certain extent. The same pattern was seen for white blood cell (WBC) regeneration, with WBC numbers exceeding 1 x 10^9/L for TPO and TPO/GM-CSF treated monkeys at day 16 and 18 and at day 21 after transplantation for placebo treated controls. An additive effect of GM-CSF was not observed (Figure 1). However, the differences did not reach statistical significance.

White blood cell subsets measured by flow cytometry:
A profound nadir was observed for all surface markers tested in peripheral blood. For all subsets, a similar pattern was shown in bone marrow as well as in peripheral blood, differing by approximately 2 log in absolute numbers. Regeneration patterns of CD34+, CD20, CD11, CD 8, CD4 followed a similar pattern in all monkeys. TPO treated monkeys had an improved nadir for CD16 and CD56 positive cells and a tendency to faster recovery to normal values for these subsets than the other monkeys.

Bone marrow cellularity and progenitor cell content
Bone marrow cellularity before irradiation was within the normal range [33]. In all treatment groups cellularity at days 8 and 15 was insufficient to assess colony forming cell numbers

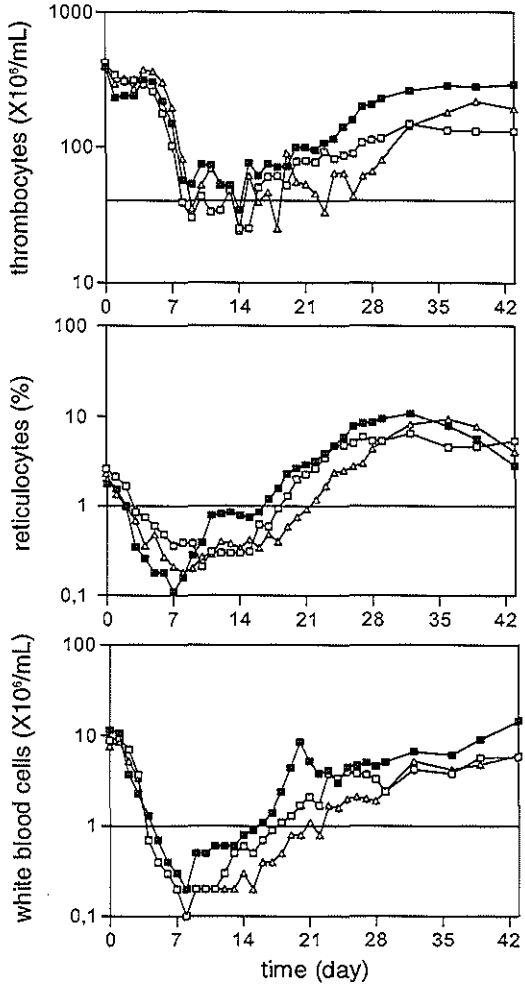

Figure 1. Mean peripheral blood cell counts after 8 Gy TBI and unfractionated bone marrow transplantation. Thrombocytes (upper panel), reticulocytes (middle panel) and white blood cells (lower panel) for TPO (black squares) (n=2, 10µg/kg/d sc 21 days after TBI,) TPO and GM-CSF (open squares)(n=3, 5µg/kg/d rhesus TPO: day 0 iv and days 1-14 sc) and placebo-treated monkeys (open triangles)(n=2). Horizontal lines define degree of cytopenia, 40 x 10^9/L for thrombocytes, 1% for reticulocytes and 1 x 10^9/L for white blood cell numbers.

with the exception of one of the TPO/GM-CSF treated monkeys in which day 15 colony assays were performed. TPO/GM-CSF treated monkeys reached greater marrow cellularity at day 22. At day 29, only placebo controls had regenerated slower, whereas differences in cellularity were not found anymore at day 36. Flow cytometric analysis showed that CD34++/DRdull cells were present at day 8 in the bone marrow of TPO

Table 1. Individual bone marrow cellularity, GM-CFU number, BFU-E number, absolute number CD34+ cells and CD34+/DRdull cells in bone marrow ±SD per mL aspirate after 8-Gy TBI and unfractionated bone marrow transplantation (10^7/kg).

	Treatment	Before	Week 1	Week 2	Week 3
Cellularity	TPO	32/20	0.3/0.6	1/0.9	5/2
x 10^6/mL	TPO + GM-CSF	17/42/12	0.6/0/0	0.5/3/0.5	21/9/2
	placebo	11/29	0.2/0.2	0.2/0.3	3/1
GM-CFU	TPO	69/85	ND*	ND	0.7/1
x 10^3/mL	TPO + GM-CSF	7/22/4	ND	0.4/0/ND	8/0.2/2
	placebo	4/70	ND	ND	1/ND
BFU-E	TPO	23/18	ND	ND	0.9/0.4
x 10^3/mL	TPO + GM-CSF	4/8/2	ND	0.2/ND/ND	3.6/0.1/0.2
	placebo	1/20	ND	ND	0.1/ND
CD34+	TPO	1490/1920	10/0	40/40/ 0	150/160
x 10^3/mL	TPO + GM-CSF	360/550/110	10/0/0	10/10/40	20/830/40
	placebo	42/1644	1/4	2/9	174/54
CD34+	TPO	449/483	2/7	4/23	28/622
/ DR dul	TPO + GM-CSF	48/55/91	0.4/1/0	2/10/14	11/291/12
x 10^3/mL	placebo	317/25	0/0.4	0.5/0.2	3/120

*ND= not done, due to insufficient cell procurement. Animals were treated with TPO (n=2), TPO and GM-CSF (n=3) or placebo (n=2).

treated monkeys and also in TPO/GM-CSF treated monkeys at day 15, whereas placebo treated controls regenerated much slower, but reached similar numbers after three weeks (Table 1). The progenitor cell content of the bone marrow, expressed as colonies formed per mL aspirate, was subject to the large variation characteristic for exponential growth, and showed no trend in favor of any growth factor regimen.

Clinical chemistry parameters:

For most parameters, abnormalities were not observed. After two weeks, all monkeys had a short period of hypalbuminemia, especially the GM-CSF/TPO treated monkeys showed a more profound decline without signs of edema and/or gain in body weight. ALAT values of placebo treated controls were slightly elevated during the first two weeks, but returned to normal in the other groups within 3 days after a initial rise immediately after TBI. LDH1 values peaked one day after TBI but remained within the normal range in all monkeys during the remainder of the study period. Iron values did not change significantly.

Adverse effects and on study observations:
The TPO and placebo treated monkeys did not develop adverse effects. However, two of the GM-CSF/TPO treated monkeys showed symptoms of a peri-arthritis of elbow, knee and hand 13 days after TBI without apparent *functio laesa* or pain. Symptoms disappeared spontaneously two days after cessation of GM-CSF treatment. The other GM-CSF treated monkey suffered of a fungus-like infection from days 14-24, showing multiple round red lesions with a white squamous center. It healed completely post *aut propter* after therapy with low dose corticosteroid / fungicide combination cream. Cultures and KOH slides prior to the treatment were negative for yeasts or fungi. Another monkey irradiated with 6 Gy and not treated with GM-CSF or rhesus TPO simultaneously developed the same lesions; the monkey recovered similarly.

Discussion

In this study, GM-CSF and TPO were combined to evaluate their effects after transplantation of unfractionated autologous bone marrow into irradiated young adult rhesus monkeys after TBI at a dose of 8 Gy. As published previously [13,14], a significant effect of TPO alone was not expected. It was hypothesized that GM-CSF might act in synergy with TPO. This hypothesis was based on simultaneous administration of TPO and GM-CSF in a myelosuppression model (5 Gy TBI) which resulted in augmented thrombocyte, reticulocyte and leukocyte regeneration compared to the single TPO or GM-CSF treatment [5]. With respect to the previous bone marrow transplant study, dose scheduling of TPO was changed since studies [9] in myelosuppressed mice demonstrated that administration of TPO early after TBI as well as a threshold plasma level to be reached in the first hours are critical for an optimal multilineage effect. Delayed administration causes an increasing decline in efficacy of TPO [9,12]. Therefore it was decided to start TPO administration shortly after TBI. Rhesus monkeys received 5 µg/kg rhesus TPO iv concomitant with the transplant, approximately 2 hours after TBI. Subsequently they received 5µg/kg/day rhesus TPO and 25µg/kg/day GM-CSF sc, days 1-14 after TBI.
The results of the present study differ strikingly from TPO/GM-CSF treatment in the 5 Gy myelosuppression model [4]. TPO/GM-CSF did not prevent thrombocytopenia and did not significantly stimulate regeneration to normal thrombocyte values in the 8 Gy transplant model. The red blood cells and white blood cells of the different groups showed a similar nadir and accelerated regeneration was not observed in the TPO/GM-CSF treated animals compared to TPO treated primates or placebo controls. There was a slight tendency to improved bone marrow regeneration in the TPO/GM-CSF treated monkeys (Table 1). We decided to omit the GM-CSF single treatment controls, since neither TPO, nor G-CSF, nor GM-CSF/TPO or G-CSF/TPO were effective in this model [4,13]
Monkeys exposed to 5 Gy TBI recover to normal thrombocyte counts in the same time as 8 Gy placebo treated monkeys transplanted with 10^7 unfractionated autologous bone

marrow cells (Figure 2). This is no coincidence, since the models were calibrated to achieve this effect based on previous quantitative studies [32]. This implies that the numbers of immature bone marrow cells available for reconstitution after transplantation should be considered similar and sufficient for initial reconstitution in three to four weeks. The absence of a beneficial effect of TPO after bone marrow transplantation could therefore not be attributed to lack of reconstituting cells.

Although capable of stimulating multilineage hematopoietic cells [8,9,34], TPO does not appear to initiate a proliferative stimulus by itself in vitro [35,36]. However, in the presence of other growth factors, in particular SCF and Flt-3 ligand (FL), TPO confers a strong proliferative response [34-41]. We may assume that the concomitant stimulation by other cytokines may also determine the proliferative response to TPO and explain the heterogeneity encountered *in vivo*. In normal non-human primates, TPO results in thrombocytosis and expansion of megakaryocyte formation, without changes in red cell mass or white blood cell counts [42-44]. However, in myelosuppressed animals, TPO elicits a strong multilineage response [4,8,9,45-47], whereas after bone marrow transplantation no response of TPO was obtained [13,14, present study].

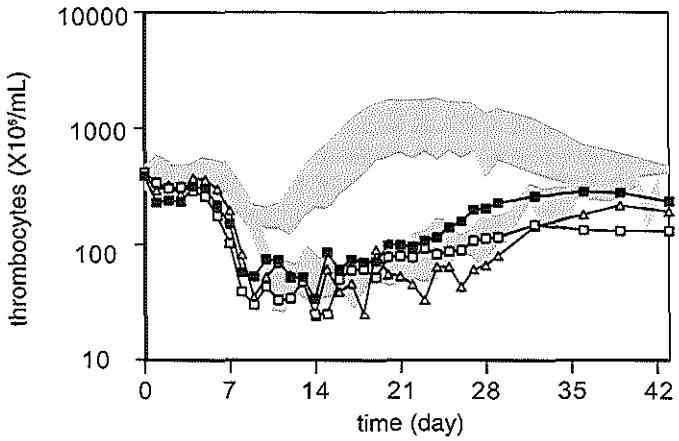

Figure 2. Comparison of the effect of TPO and TPO/GM-CSF on thrombocyte regeneration after transplantation of unfractionated bone marrow cells and after myelosuppression. Mean thrombocyte counts of monkeys after 8 Gy TBI and autologous bone marrow transplantation are represented with symbols and lines; the black squares represent two recombinant human TPO treated monkeys (10µg/kg/d sc days 1-21), open squares represent three TPO/GM-CSF treated monkeys (rhesus TPO 5 µg/kg/d day 0 iv, days 1-14 sc), open triangles represent two placebo controls. The shaded areas represent means ± SDs of thrombocyte counts of 5 Gy irradiated monkeys after TBI; the upper shaded area represents 4 TPO treated monkeys, the lower shaded area 8 placebo controls. The dose and route of TPO in the 5 Gy monkeys were 10µg/kg/day, sc, days 1-21 after TBI (1).

The multilineage effect of TPO is strongly corroborated by the phenotype of c-mpl deficient mice, in which not only thrombocyte counts are dramatically decreased but also total hematopoietic progenitor cell numbers are reduced, including multipotential cells, blast cells and committed progenitors of multiple lineages [48,49]. The difference in effects of administered TPO in normal and myelosuppressed animals might be due to presence or activation of co-factors that are essential for the TPO response and which are not effective after lethal irradiation. Whether these co-factors are other hematopoietic growth factors or regulatory molecules provided by stromal elements, is not clear. Loss or inhibition of an essential co-factor by stromal damage due to lethal irradiation or growth factor disturbances as a result of trauma due to the femoral marrow punctures are possiblilites for further exploration, as are the regulatory molecules involved in homing and subsequent colonization of the bone marrow, such as CXCR4 [50] and integrins [51].

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CHAPTER 4

Immature hematopoietic CD34+ cells: normal levels in bone marrow and peripheral blood and regeneration patterns in myelosuppressed or transplanted, growth factor treated, rhesus monkeys

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Abstract

CD34 positive progenitor cells in bone marrow and peripheral blood of 87 young adult rhesus monkeys were evaluated before and after hematopoietic growth factor treatment in studies involving myelosuppression (TBI; 5 Gy X-rays) and bone marrow transplantation (BMT) after 8 Gy TBI. CD34+ cell numbers in bone marrow (BM) and peripheral blood (PB) were monitored during reconstitution and relations with blood cell parameters such as WBC, reticulocyte percentages and thrombocytes were studied.
BM and PB CD34+ cell numbers (mean values: 1.8 and 0.014 x 10^9/L respectively) appeared to differ two orders of magnitude in absolute numbers and both appeared to vary within a large range with coefficients of variation of 14% and 93%, respectively. The variation appeared to be partly due to fluctuations in CD34+ cell numbers observed in individual monkeys during a three-week observation period. Individual CD34+ cell numbers in BM and PB were not related under steady state conditions.
PB CD34+ cell numbers were significantly related to BM CD34+ cell numbers during regeneration from myelosuppression and also to hematopoietic parameters such as WBC, reticulocytes and thrombocytes (all $p < 0.01$). After bone marrow transplantation, PB CD34+ cell numbers were also related to BM CD34+ cell numbers but relations with hematologic blood cell parameters in PB were were absent. Despite similar hematological recoveries, CD34+ cell reconstitution was delayed in the transplanted 8 Gy animals relative to the regeneration after myelosuppression, associated with refractoriness to growth factor treatment.
It is concluded that enumeration of PB CD34+ cells can be used as a rapid and reliable test of bone marrow CD34+ cell reconstitution.

Introduction

Hematopoietic reconstitution after myelosuppression or bone marrow transplantation originates from a small population of primitive hematopoietic stem cells (HSC) residing in the bone marrow [1, 2]. The CD34 antigen is expressed by committed and uncommitted hematopoietic progenitor cells and is used to assess stem cell content of bone marrow and peripheral blood [3]. Among the three epitope classes of the CD34 antigen, class III epitopes show a broader distribution on normal hematopoietic progenitor cells than the other two classes. The function of CD34 is not fully elucidated but thought to be involved in stem/progenitor-cell localization / adhesion in the bone marrow [4, 5, 6].
Monoclonal antibodies are used to monitor and select CD34+ cells. Several issues are critical in achieving standardized CD34+ enumeration. Firstly, the method of sample processing influences the degree of cell debris and red blood cell concentration. Secondly, the CD34 antibody and the fluorochrome label used, influence the specificity and sensitivity of immunolabeling. Thirdly, the gating strategy, including the denominator for the numbers of CD34+ cells, should be similar for all samples. The level of BM progenitor

cells can only be expressed as a percentage of total cells in the aspirate, but the degree of blood dilution in the aspirate varies significantly according to the technique used and the volume aspirated. Several studies show that increasing volumes of bone marrow aspirates are associated with dilution of peripheral blood [7, 8, 9]. Hence, levels of BM progenitor cells in a BM aspirate which includes PB as well as BM cells may not be a real estimate of the true incidence of progenitor cells in BM. The bone marrow cellularity is also different in distinct parts of the body; sternal aspirates are usually more cellular than iliac spine aspirates [10, 11], and the volume of total body BM remains an estimate [12]. These variables make it difficult to correlate the incidence of progenitor cells in a BM aspirate with the number of progenitor cells in the body [11]. If there would be a relation between the progenitor pools in PB and BM, the level of steady-state PB progenitor cells would be a more accurate indicator of hematopoietic reserve than the incidence of progenitor cells in a BM aspirate [13].

In addition, a study in mice [14] showed that genetic influences determine progenitor cell frequency: stimulation with Granulocyte Colony-Stimulating Factor (G-CSF) resulted in various responses in different mouse strains. The interstrain differences affected the distribution of mature and progenitor cells between peripheral blood, bone marrow and spleen, rather than the total numbers of these cells in the body. Results of an intercross of two strains indicated that regulation of progenitor cell mobilization is a complex genetic trait. The genes involved in the mobilization process are not yet identified. Other studies confirm the genetic basis of stem cell numbers and showed that stem cell numbers fluctuate widely during aging [15, 16].

Although new markers of immature cells have been characterized [17, 18] and some recent studies show that the most immature cells are likely not CD34+ [19, 20, 21, 22], CD34+ cell dose is an established parameter to enumerate as well as select for repopulating cells. Despite the fact that the CD34+/CD38- subpopulation (in rhesus monkeys CD34+/ DRdull) is more immature and responsible for the repopulation capacity [23, 24], the CD34+ cells in total are for practical reasons used in evidence-based medical practice. The number of CD34+ cells in a graft predicts neutrophil engraftment [25, 26], platelet recovery [27, 28] and relapse and survival [29] after hematopoietic stem cell transplantation for hematological malignancies. We evaluated the CD34+ cell numbers in bone marrow and peripheral blood of rhesus monkeys used in hematopoietic growth factor studies under normal circumstances, after 5 Gy total body irradiation (TBI) and after lethal TBI followed by autologous bone marrow derived stem cell transplantation (BMT). CD34+ cell numbers were examined for a relationship with hematology parameters in PB during the regeneration phase. Purpose of the study is to relate bone marrow and peripheral blood CD34+ numbers and to examine the predictive value of PB CD34+ cell numbers for hematopoietic reconstitution.

Materials and methods

Animals: Purpose bred male rhesus monkeys (*Macaca Mulatta)*, weighing 2.5-4.0 kilograms and aged 2 to 3 years, were used. The monkeys were housed in groups of 4 to 6 monkeys in stainless steel cages in rooms equipped with reverse filtered air barrier, normal day light rhythm and conditioned to 20 °C with a relative humidity of 70%. Animals were fed *ad libitum* with commercial primate chow, fresh fruits, and received acidified drinking water. All animals were free of intestinal parasites and seronegative for herpes B, simian T-lymphotropic viruses and simian immunodeficiency virus. Hematology parameters (Hb, Ht, MCV, WBC, RBC, thrombocytes, reticulocytes, WBC differentials) as well as some chemistry parameters (creatinin, electrolytes, iron, glucose, total protein, ALAT, LDH) before the experiment are all within normal values. Housing, experiments and all other conditions were approved by an ethical committee in conformity with legal regulations in the Netherlands. Total number of animals is 87, of which 84 have been irradiated. Three additional animals have been included for the measurement of normal CD34+ numbers in BM and PB.

Total body irradiation: Monkeys were irradiated with a single dose of 5 (n=52), 6 (n=3) 7 (n=3) or 8 (n=26) Gy total body irradiation (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 0.20-0.22 Gy/min. During TBI, the animals were placed in a cylindrical polycarbonate cage which rotated slowly (3 times per minute) around its vertical axis.

Test drugs: Recombinant full length rhesus monkey TPO produced by CHO cells and recombinant human TPO were supplied by Genentech Inc. (South San Francisco, CA). The dose TPO was diluted to a volume of 1 mL with PBS / 0.01% Tween 20 prior to administration. Recombinant human G-CSF (Neupogen, Amgen Inc., Thousand Oaks, CA), recombinant GM-CSF (Leukine; Immunex Corp., Seattle, WA) and recombinant human Flt-3 Ligand (Immunex, Seattle, WA, USA) were given subcutaneously (sc) at several doses and schedules (Table 1). The dose was diluted to a volume of 1 mL with PBS / 0.01% Tween 20 prior to administration. Placebo treated monkeys were only given the same volume of diluent. The daily doses were diluted to a volume of 1 mL in the solution indicated by the supplier.

Study groups: Studies with rhesus monkeys are done sequentially using highly codified and standardized methods, including radiation and placebo controls at regular intervals. Assignment to the treatment groups was at random.

Bone marrow aspirates: Bone marrow was aspirated under neurolept anesthesia using Ketalar (Apharmo, Arnhem, the Netherlands) and Vetranquil (Sanofi, Maassluis, the Netherlands). Small bone marrow aspirates for analytical purposes were taken from the

shafts of the humeri using pediatric spinal needles and collected in bottles containing 2 mL HEPES buffered Hanks' balanced salt solution (HBBS) with 200 IU sodium heparin/mL (Leo Pharmaceutical Products, Weesp, the Netherlands). Cellularity was determined by counting the nucleated cells and calculate this per 10^6/ mL aspirate. Low density cells were isolated using a Ficoll (density 1.077) (Nycomed Pharma AS, Oslo, Norway) separation. In order to determine the amount of blood admixture in the bone marrow aspirate, we adjusted the formula used by Holdrinet et al. [9] who postulated that nearly all hemoglobin in a bone marrow aspirate is in due fact derived from peripheral blood and the number of nucleated cells (NC) derived from PB is proportional to the amount of peripheral hemoglobin present in the sample. Based on this and presumed that bone marrow does not contain T-lymphocytes, we compared CD4 positive cells in paired BM and PB samples. In samples of 63 normal monkeys CD4 percentages were 4.1% ± 1.6% and 20.8% ± 8.1% in BM and PB respectively. To calculate PB admixture we adjusted the formula used by Holdrinet et al.[9] as follows:

$$\frac{[Hb_{BM}]/[Hb_{PB}] \times WBC}{NC_{BM}\text{(nucleated cells BM)}} \rightarrow \frac{(CD4\%_{BM} \times NC_{BM} / CD4\%_{PB} \times WBC) \times WBC}{NC_{BM}}$$

Hematological examinations: Complete blood cell counts were measured daily using a Sysmex F-800 hematology Analyzer (Toa Medical Electronics co., LTD., Kobe, Japan). For reticulocyte measurements, 5 µL EDTA blood was diluted in 1 mL PBS/EDTA/azide and one mL of a thiazole orange dilution was added, using thiazole in a final concentration of 0.5 µg/mL. Measurements were done using a FACScan (Becton Dickinson, Leiden, The Netherlands) and analyzed using the Reticount software.

Measurements of surface antigens: Once weekly, a phenotype analysis was done on peripheral blood (PB) and bone marrow (BM) samples by flowcytometry. A class III 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, St Louis, USA) according to standard procedures, was used to stain CD34+ cells. A 0.5 mL of whole blood or bone marrow was lysed in 10 mL lysing solution (8.26 g ammonium chloride/ 1.0 g potassium bicarbonate and 0.037 g EDTA per L) for 10 minutes at 4 °C. After lysing the cells were washed twice with HBBS containing 2% FCS and 0.05% (wt/vol) sodium azide. The cells were resuspended in 100µL of the latter fluid 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 flowcytometer. Ungated list mode data were collected for 10,000 events and analyzed using the Lysis II software (Becton Dickinson) (Figure 1). All relevant FACS data have retrospectively been reanalyzed, using similar gating strategy for all samples.

Table 1a. Monkeys included in CD34 analysis
Animals received a midlethal dose TBI (without bone marrow transplantation)

Irradiation dose (X-rays) (Gy)	Number of animals	Growth factor treatment			Reference
5	4	Placebo			[34,37,38]
5	4	Rhesus TPO	5	µg/kg iv day 1	[34]
5	1	Rhesus TPO	50	µg/kg iv day 1	[34]
5	2	Rhesus TPO	0.5	µg/kg iv day	[34]
5	2	Rhesus TPO	5	µg/kg iv day 1 and 4	
5	2	Rhesus TPO	5	µg/kg iv day 0, 2 hrs after TBI	
5	1	Rhesus TPO	0.5	µg/kg iv day 0, 2 hrs before TBI	
5	1	Rhesus TPO	1	µg/kg iv day 0, 2 hrs before TBI	
5	6	Rec hum. TPO	10	µg/kg/d sc, days 1-21	[37,38]
5	4	GM-CSF	25	µg/kg/day sc days 1-14	[34]
5	3	G-CSF	10	µg/kg/day sc days 1-14	[34]
5	4	G-CSF	5	µg/kg/day sc days 1-21	[37]
5	2	FL	100	µg/kg/day sc days 1-14	
5	4	Rhesus TPO	5 µg/kg/day iv day 1 and GM-CSF 25 µg/kg days 1-14 sc		[34]
5	4	Rhesus TPO	5 µg/kg/day iv day 1 and G-CSF 10µg/kg days 1-14 sc		[34]
5	4	Rec Hum TPO	10 µg/kg and G-CSF 5 µg/kg sc days 1-21		[37]
5	4	Rhesus TPO	5 µg/kg day 1 iv and FL 100µg/kg day 1-14 sc		

Table 1b. Monkeys included in CD34 analysis.
Animals received a lethal dose TBI followed by bone marrow stem cell transplantation

Irradiation dose (X-rays) (Gy)	Number of animals	Growth factor treatment	Type of bone marrow transplant	Number CD34+ cells x 10^5/kg	Reference
8	3	Radiation controls		2	[35]
8	2	Placebo	CD34++/DRdull sorted	3	[35]
8	4	Rec Hum TPO 10µg/kg sc days 1-21	CD34++/DRdull sorted	3	[35]
8	3	G-CSF 5 µg/kg sc days 1-21	CD34++/DRdull sorted	3	[35]
8	2	Rec Hum TPO 10µg/kg and G-CSF 5 µg/kg sc days 1-21	CD34++/DRdull sorted	3	[35]
8	2	None	CD34++/DRbright sorted	1	
8	1	None	CD34+ beads selected	3.2	
8	1	Rec Hum TPO 10µg/kg sc days 1-21	CD34+ beads selected	1	
8	1	None	CD34++ sorted	1	
8	2	Rec Hum TPO 10µg/kg sc days 1-21	10^7 unfractionated cells/kg	4.7 9.7	[35] [36]
8	3	Rhesus TPO 5µg/kg and GM-CSF 25µg/kg days 1-14 sc	10^7 unfractionated cells/kg	4.6 1.7 1.8	[36]
8	1	G-CSF 10µg/kg days 1-21 sc	10^4 unfractionated cells/kg	0.2	[35]
8	2	Placebo	10^7 unfractionated cells/kg	5.6 0.55	[35], [36]

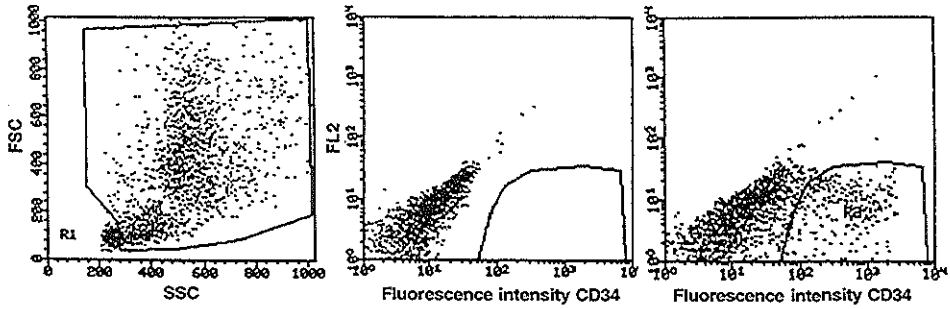

Figure 1. CD34 expression of bone marrow cells. After lysing of erythrocytes, cells were stained with anti CD34 Fitc. Cells in R1*R2 were defined as CD34 positive. On the vertical-axis of the middle and right panel: FL2, in which PE-conjugated antibodies can be shown (not used in this experiment).

Stem cell isolation and transplantation

To enrich for progenitor cells, buffy-coat cells were subjected to a discontinuous bovine serum albumin density gradient [30, 31, 32]. 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 phycoerythtin (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 (100mW). $CD34^{bright}$ /$RhLA\text{-}DR^{dull}$ cells [23] were sorted in the normal C mode and reanalyzed for purity. The sorted cells were transplanted at a dose of 1.4 – 3.0 x 10^4/kg body weight and returned to the monkey on the same day.

Statistical analysis:

Mean, median and range of numeric variables were calculated by the Excel spreadsheet program. Standard deviations were calculated and are given in the text and in the figures on the assumption of a normal distribution. The statistical significance of differences was calculated by the Mann-Whitney test (comparing two unpaired groups each time), the paired t-test for continuous data, and Fisher's exact test for categorical data.

Results

Normal values of CD34+ cells in bone marrow aspirates and peripheral blood

Figure 2 and Table 2 show the absolute numbers of CD34+ cells in BM aspirates and PB in 87 normal young adult rhesus monkeys. Absolute bone marrow CD34+ numbers are approximately 2 orders of magnitude higher than PB CD34+ numbers. Under normal circumstances the individual values are not related ($r = .003$, $p = .979$). CD34+ numbers in bone marrow aspirates are also influenced by blood admixture, hydration state of the monkey and puncture site. Aspiration was standardized by using pediatric fine needles, optimized hydration state of the monkey, taking standardized small samples (0.5 mL per aspiration) with a cumulative volume of 2-5 mL, and by choosing similar puncture sites (alternately the left and right humerus).

Assuming that normal bone marrow does not contain T lymphocytes, percentages of CD4+ T cells in bone marrow and blood were used to calculate blood admixture as detailed in the methods). Peripheral blood admixture in aspirates from normal monkeys is on average 21.3± 6.8 % (median 25.6%) in normal monkeys. Bone marrow cellularity is correlated with peripheral blood admixture ($r = -.654$, $p<.0001$)(Figure 3), as well as with absolute numbers of CD34+ cells in the bone marrow.($r=-.369$, $p=.0032$) in normal rhesus monkeys. When bone marrow CD34+ cell numbers are corrected for PB admixture, the absence of a relationship of PB CD34+ cells and BM CD34+ cells is not influenced, indicating that blood admixture is not a major factor in the way these parameters are associated in normal animals (data not shown).

Because of the observed range in normal CD34 values in different animals, we evaluated the longitudinal variation in both PB and BM CD34+ cell numbers of three healthy rhesus monkeys and of three monkeys at one year after autologous bone marrow transplantation. The animals were daily monitored for 3 weeks to measure CD34+ cells in peripheral blood (PB). After three weeks, a bone marrow aspirate of 1 mL was taken from both humeri of the animals and cellularity of the humeri was compared to examine variability in puncture site. The individual PB CD34+ cells appeared to fluctuate 0.5 log in time within the range previously established for normal monkeys (Figure 4). The values of normal and transplanted monkeys did not differ in number. Bone marrow cellularity showed variation within one animal demonstrating variability due to puncture site, although the difference did not exceed 40%. The percentages CD34+ in the two samples were similar. So, in conclusion, PB34+ and BM34+ cells differ 2 orders of magnitude in absolute counts, are individually determined and both fluctuate in time during steady state in normal rhesus monkeys.

Regeneration of CD34+ cell numbers in BM and PB after 5 Gy TBI (myelosuppression model)

Accumulated data of 5 Gy irradiated animals were used to correlate CD34+ cells in BM and PB and with peripheral blood cell reconstitution. In all treated animals, PB CD34 values follow BM CD34 values, regenerating in a similar pattern (right panels in figure 5).

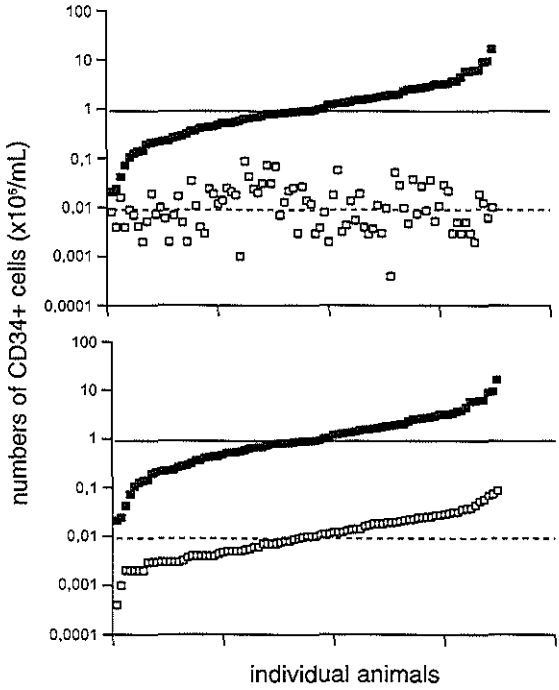

Figure 2. Absolute numbers of CD34+ numbers in bone marrow aspirates (black squares) and peripheral blood (open squares) of 87 young adult normal rhesus monkeys. On the Y-axis the absolute numbers of CD34+ cells x 10^6/mL, and on the X-axis the individual animal numbers (1-87). Upper panel: bone marrow data sorted in ascending order with corresponding peripheral blood data. Lower panel: bone marrow and peripheral blood data are dissociated and both sorted in ascending order. The thin black horizontal line represents the median of CD34+ BM numbers in normal monkeys and the dotted horizontal line represents the median of PB CD34+ numbers in normal monkeys.

Table 2. Distribution parameters of bone marrow aspirate and peripheral blood CD34+ cells of 87 normal rhesus monkeys

	BM CD34+ cells	PB CD34+ cells
Frequency (%)		
Mean ± SD	2.97 ± 2.88	0.17 ± 0.15
Median	2.17	0.13
Range	0.23 – 17.5	0 – 1.09
Absolute number (x 10^9/L)		
Mean ± SD	1.78 ± 2.44	0.014 ± 0.013
Median	0.91	0.009
Range	0.02 – 16.5	0 – 0.087

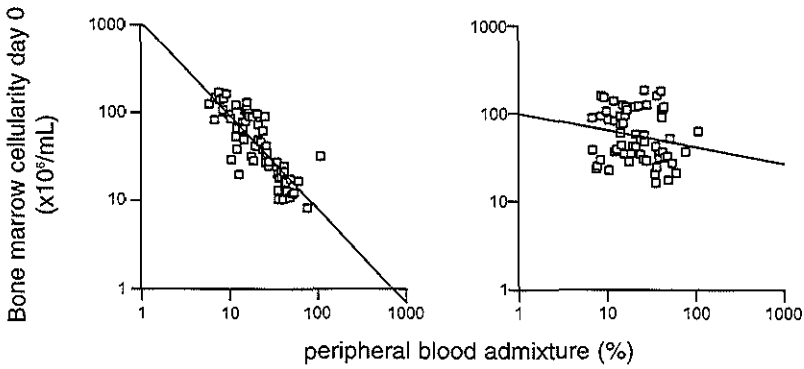

Figure 3. Bone marrow cellularity x10⁶/mL in normal rhesus monkeys on the Y-axis versus peripheral blood admixture (%) as measured by the adjusted formula of Holdrinet (see methods section) on the X-axis. Left panel: measured values of bone marrow cellularity. Right panel: adjusted values of bone marrow cellularity corrected for peripheral blood admixture

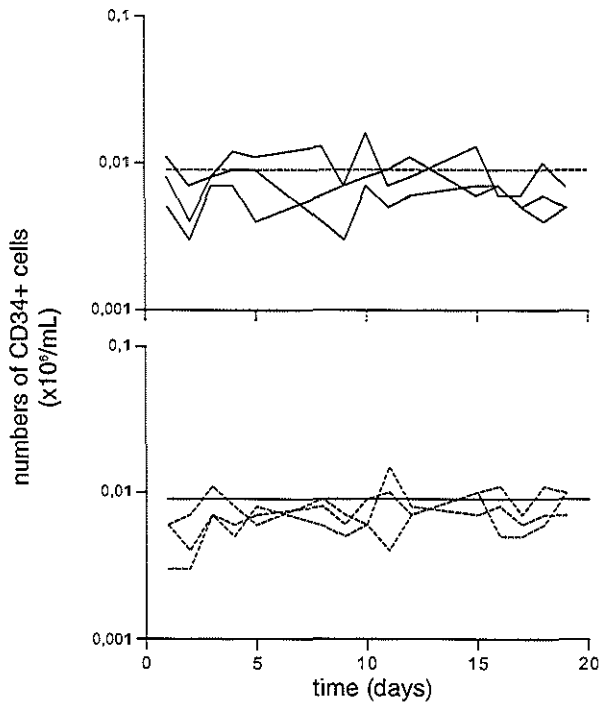

Figure 4 . Absolute numbers of CD34+ cells in PB (Y-axis) of 3 normal rhesus monkeys (black lines) and 3 monkeys at 1 year after autologous BMT (dotted lines) during three weeks (X-axis).

During the regeneration phase, the magnitude of the difference between BM CD34 and PB CD34 numbers decreases from two (in steady state) to one order of magnitude or less immediately after myelosuppression (Figure 6). During myelosuppression, blood admixture shows a wide range up to 90% at day 8 because of lack of cells in the irradiated bone marrow that had been replaced by peripheral blood. PB and BM CD34+ cell numbers are in the same range during that period (Figure 6) which may either be due to lack of bone marrow cells or to selective mobilization CD34+ cells. It is not possible to distinguish BM and PB CD34+ cells in the aspirate, but it can be argued that it is unlikely that a high number of bone marrow derived CD34+ cells is present shortly after irradiation without any circulating stem cells, pointing to the possibility that selective mobilization occurs. Comparing relations of PB CD34+ cells at several time points with peripheral blood and BM parameters, it was concluded that PB CD34+ cell numbers at day 15 show the best relations to the studied parameters. The PB CD34+ cell number is at day 15 already significantly related to the BM CD34+ cell number 8 days after irradiation ($p<.0001$) (Figure 7). The strongest correlation of BM34 and PB34 cells was found after two weeks ($p= .0004$, $n=52$), after which BM and PB values started to vary within normal ranges. Furthermore, the fluctuation in CD34+ cell numbers in PB in steady state (Figure 4) was also observed during recovery from radiation (Figure 8.)

PB CD34+ cells and peripheral blood cell reconstitution after 5 Gy TBI

During regeneration of myelosuppressed bone marrow, reticulocytes are an important and stable parameter to evaluate the recovery of the hematopoiesis [33]. The percentage of reticulocytes was correlated ($p<0.0001$) with the absolute numbers of PB CD 34+ cells and both showed a more rapid increase as a result of growth factor treatment. CD34+ cells in PB on day 15 and thrombocytes or white blood cells at that time point were also correlated (both $p <0.0001$)(Figure 7). PB CD34+ cell numbers at day 15, on which day maximal growth factor effects are shown, are not related to reticulocytes and WBC at day 22 ($r = .186$ and .181 resp., both $p = 0.2$), but do have a significant relation with platelets on day 22 ($r =.517$, $p= .0001$). BM CD34 levels at day 8 were significantly related to PB CD34+ cell numbers, reticulocyte %, WBC and thrombocyte counts at day 15 ($p<.001$), and similarly for BM CD34 and PB CD34 numbers at day 15 ($p = .0004$) (Figure 7).

CD34 regeneration after bone marrow transplantation

Monkeys received an autologous bone marrow transplant after a lethal dose of 8 Gy TBI (X-rays). The grafts consisted of either low density cells (1-3 x 10^7/kg), immuno-selected CD34+ cells (1-3 x 10^5/kg), sorted CD34++/DRdull cells (1.4-3 x 10^4/kg) or sorted CD34++/DRbright cells (10^5/kg). Radiation controls only received supportive care (transfusions and fluid replacement when necessary, antibiotics during neutropenia). The purpose of the experiments was to study growth factor (GF) efficacy after transplantation in order to stimulate especially thrombocyte recovery. Radiation controls and animals transplanted with CD34++/DRbright cells (which are depleted of repopulating stem cells) were treated as a distinct group due to delayed blood cell recovery.

During the exponential recovery of the bone marrow in the first three weeks after TBI, PB CD34+cell numbers were related to BM CD34+ cells ($p<.0001$). The difference in CD34+ cell numbers in BM and PB initially diminished to less than one log, but with a large variation (1.5 log after 8 days and 3 logs after 3 weeks) (Figure 6). Correlations of peripheral blood CD34+ cell numbers with either thrombocyte counts, reticulocyte percentages or white blood cell counts were not conclusive because of the large variation. Relations were also absent for BM CD34+ cell numbers and blood cell reconstitution parameters. BM CD34+ levels at day 15 were only predictive for PB CD34+ cell numbers at day 22 ($p = .01$).
In agreement with several clinical stem cell transplantation studies [25,27,28,29], the size (number of CD34+ cells) of the (bone marrow) graft proved to be of importance with respect to regeneration of hematopoiesis. Also the present analysis demonstrated the relationship between reconstitution of WBC ($p = .008$), reticulocytes ($p = .004$) and thrombocytes ($p = .05$) with the CD34+ cell content of the graft (Figure 9).

Effect of growth factors after myelosuppression and bone marrow transplantation on regeneration of CD34+ cells
Several growth factors/growth factor combinations were tested in the myelosuppression model in which the midlethal dose of 5 Gy had been given (Table 1). Early reconstitution of immature BM cells, as measured by BM CD34+ cell numbers, responds well to exogenous TPO stimulation. Recombinant human TPO (10µg/kg/day) given for 21 consecutive days after TBI, accelerates CD34 reconstitution in the bone marrow, resulting in a 2 log augmentation compared with placebo controls at 2 weeks ($p< .05$) (Figure 5). In contrast, recombinant human G-CSF (5µg/kg/day) as a single growth factor was only marginally effective. TPO and G-CSF both given for 21 days did not clearly synergize in accelerating expansion of CD34+ cells in the bone marrow. As a single dose at day 1 intravenously (iv), rhesus TPO is also effective, but to a lesser extent (lower panel Figure 5) due to the much lower total dose. A single dose of rhesus TPO synergizes with G-CSF (10µg/kg, during 14 days sc) resulting in increases in CD34+ cell numbers ($p< .01$) compared to placebo controls at day 15 (lower panel of Figure 5). The single dose rhesus TPO study with GM-CSF showed that GM-CSF, given for 14 days sc at a dose of 25 µg/kg/day accelerates BM CD34+ expansion and results in a significant difference following co-administration of TPO with the placebo controls two weeks after TBI [34]. Co-administration of TPO and FL resulted in an accelerated rise in CD34+ cell numbers in the BM at d 15 (Figure 5) but these cells declined 1 week later [35], contrary to treatment with TPO alone. TPO and GM-CSF were the most effective agents in promoting BM CD34+ cells expansion among those studied.
Based on the results of the 5 Gy experiments, the most effective growth factors were also tested in the 8 Gy bone marrow transplantation model. However, TPO either as single GF or in combination with G-CSF or GM-CSF was ineffective in this setting [36, 37]. After radiation, leukocyte counts dropped and reached a similar nadir, after which various recovery patterns are observed, irrespective of growth factor treatment after

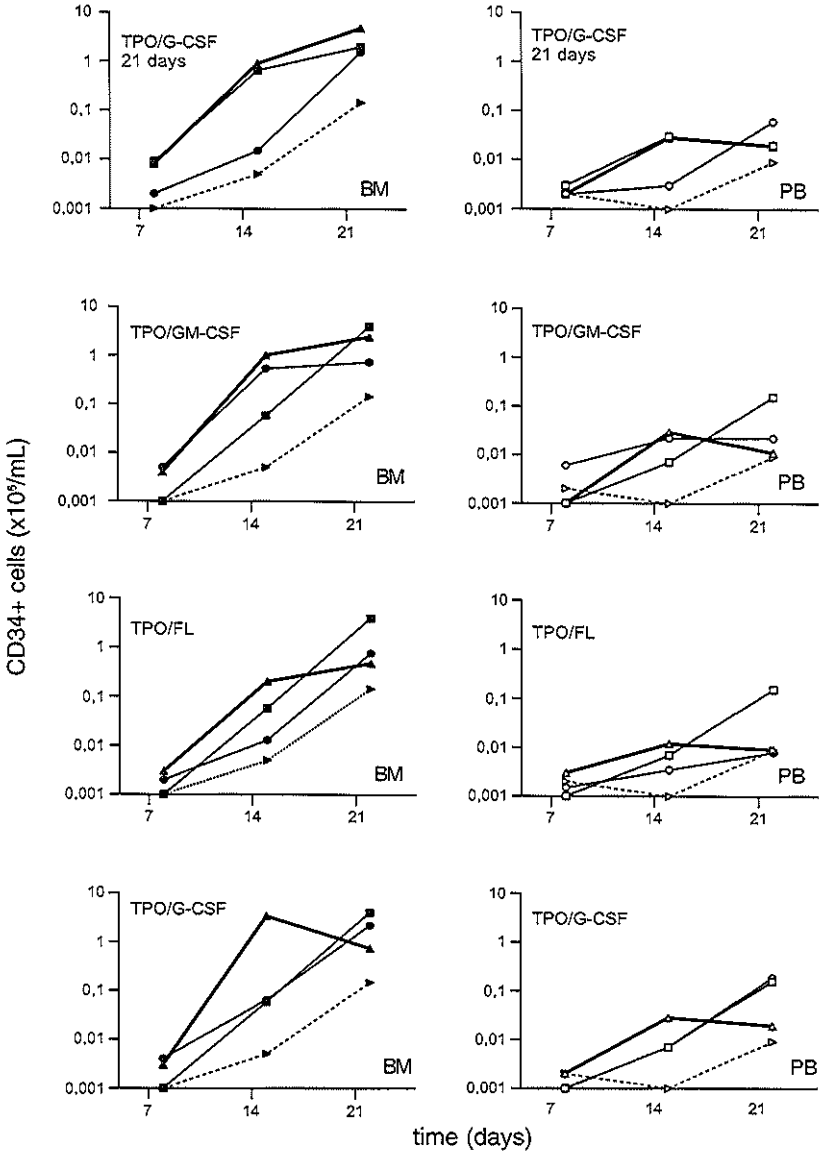

Figure 5. Effect of growth factor treatment after 5 Gy TBI on CD34+ cell regeneration (absolute numbers on log scale on the Y-axis) in BM and PB. On the X-axis: time in days. Black symbols represent BM values (left panels) and open symbols represent peripheral blood values (right panels). Squares represent TPO treated monkeys; 10 µg/kg/sc during 21 days in one study (21 days TPO/G-CSF) and 5µg/kg/iv at day one in the other three studies. Circles represent monkeys treated with G-CSF (5µg/kg/sc for 21 days upper panel, or 10 µg/kg/sc for 14 days, lowest panel), GM-CSF (25µg/kg/sc days 1-14) or FL (100µg/kg/sc days 1-14). Horizontal triangles represent combined growth factor treatment of TPO with one of the other growth factors in doses described above. Right tilted triangles represent placebo controls.

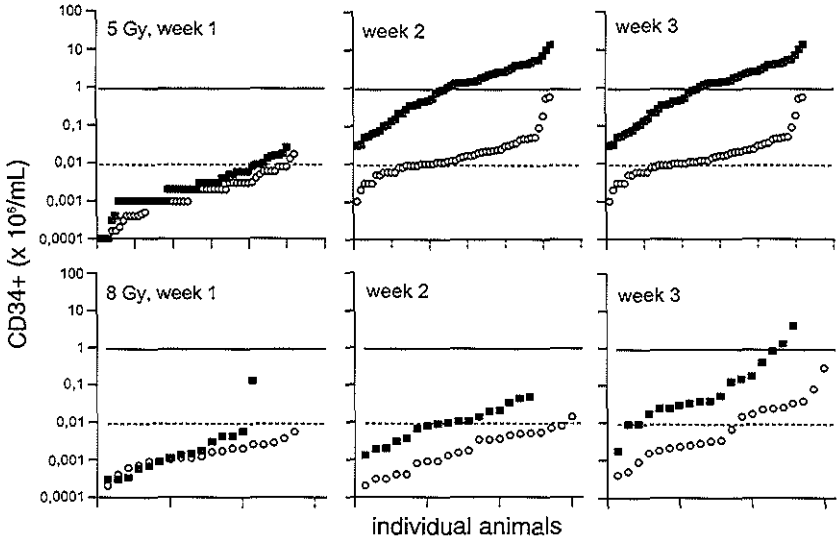

Figure 6. Absolute numbers of CD34+ cells in BM and PB during 3 weeks after 5 Gy (upper three panels, n=52) and 8 Gy TBI (lower three panels, n=22). At some time points, only PB CD34+ were measured in some transplanted animals. Black squares are BM values, open circles are PB values. The thin black horizontal line represents the median of CD34+ BM numbers in normal monkeys and the dotted horizontal line represents the median of PB CD34+ numbers in normal monkeys. BM and PB values are dissociated and both presented in ranked ascending order.

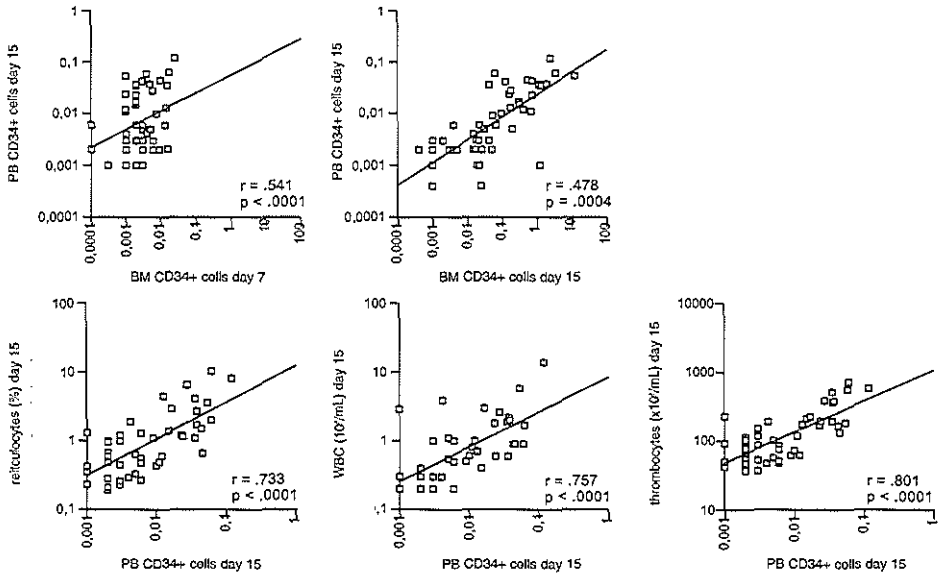

Figure 7. Relations between bone marrow CD34+ numbers, PB CD34+ cell numbers (x10⁶/mL)and hematology parameters at 8 and 15 days after irradiation.

transplantation [36, 37]. Only combined administration of TPO/GM-CSF resulted in limited expansion of CD34+ cells in BM and PB, but this was not reflected by an accelerated hematological response. Signs of stem cell mobilization were not observed in any of the transplanted animals. Despite similar hematological regeneration of transplanted 8 Gy irradiated animals and 5 Gy irradiated control animals [36], it is shown in figure 6 that reconstitution of CD34+ cell numbers in both bone marrow and peripheral blood is delayed in transplanted rhesus monkeys. This lower number of CD34+ cells during regeneration after lethal irradiation and bone marrow transplantation might contribute to the lack of effect of administered hematopoietic growth factors.

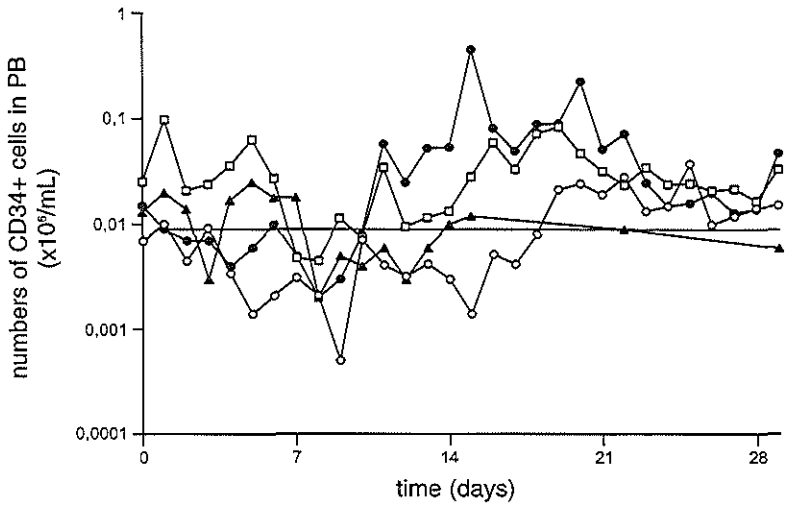

Figure 8. Absolute numbers of PB 34+ cells on a log scale (Y-axis) during the first 4 weeks (X-axis) after TBI. The black line represents the median number of CD34+ cells of 87 normal monkeys, the open circles represent 4 placebo controls, the open squares represent 4 animals treated with human TPO 10µg/kg sc during 21 days, the triangles represent animals treated with 5µg/kg rhesus TPO at day 1 iv plus 100µg/kg FL sc during days 1-14. The black circles represent human TPO and G-CSF (5µg/kg sc) treated animals both during 21 days after TBI.

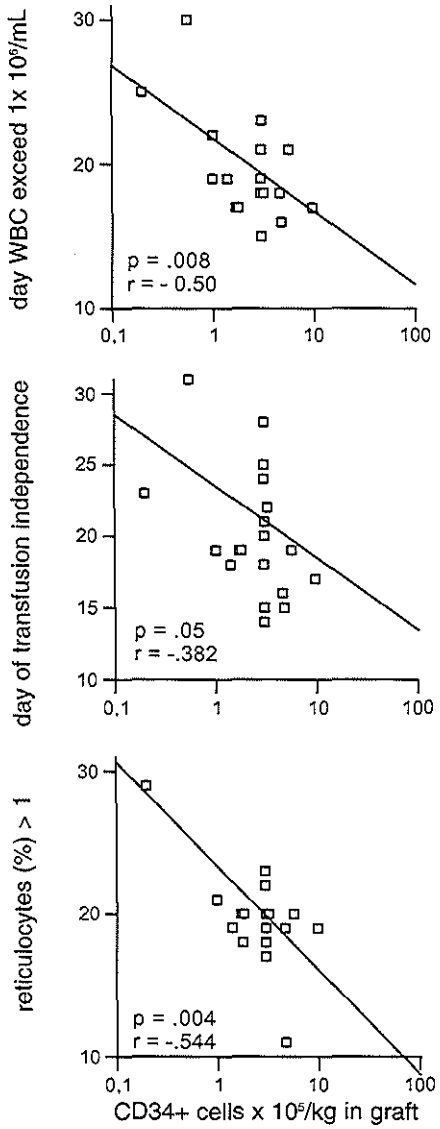

Figure 9 . Relation of the number of CD34+ cells in the graft and hematology parameters. Upper panel: relation with the day at which leukocytes exceed 1 x 10^6/mL ($p = .008$). Middle panel: relation with day after which animals are transfusion independent thrombocytes ($p = .05$). Lower panel: relation of graft with day at which reticulocytes exceed 1% ($p = .004$).

Discussion

CD34+ cell numbers were evaluated in bone marrow and peripheral blood of 87 monkeys used in irradiation, growth factor and bone marrow (stem cell) transplantation studies (Table 1) [34, 35,36, 37, 38, 39]. Three experimental designs were distinguished; 1. normal, untreated rhesus monkeys; 2. monkeys irradiated with 5 Gy TBI (X-rays) which results in a pancytopenic period of three weeks (myelosuppression model); 3. a bone marrow transplant model in which the animals were irradiated with a lethal dose TBI (8 Gy X-rays) resulting in pancytopenia during 6 weeks (with supportive care) which can be shortened by a BM (stem cell) graft to three weeks. Purpose was to identify the relation between PB34+ cells and bone marrow regeneration and with peripheral blood cell reconstitution parameters of white blood cells (WBC), reticulocytes and thrombocytes.
Variability in the measurement of BM CD34+ cells was as much as possible avoided by giving the animals optimal supportive care (hydration state), avoidance of peripheral blood admixture by standardized fine needle and small volume diagnostic punctures, alternately performed in the left and right humerus (large volumes used for the transplant were taken from femurs). To analyze the percentage blood admixture, we compared CD4 positive lymphocytes in BM and PB. Assuming that bone marrow does not contain T-lymphocytes, we calculated that there was on average 21.3% ±6.8% (median 25.6%) blood admixture in aspirates of normal rhesus monkeys by this procedure. It has been shown that normal human bone marrow (obtained by biopsy or operation) contains 6 - 8.9% T-lymphocytes (measured by AET-rosettes) [40, 41]. In rhesus monkeys which were bled under general anesthesia prior to euthanasia the following analysis was performed: all vertebrae, ribs, long bones, scapulae and pelvis were collected and subsequently bone marrow, obtained by extrusion of the cells from the bones in a tissue press, was analyzed. These bone marrow cells contained 1.8% CD4 positive and 6.7% CD2 positive cells versus 17.6% and 50.1% respectively in PB samples of the same day (mean values of 4 animals) [unpublished observation, SCC Hartong, TP Visser, G Wagemaker]. If the number of bone marrow T-cells is distracted from the number of T-cells in the aspirate, this might result in blood admixture in normal rhesus monkeys of 15-20%, demonstrating that the fine needle, small volume procedure successfully limits PB admixture. A crude estimate of blood admixture in the aspirate can also be made by counting the cellularity. Bone marrow cellularity in normal rhesus monkeys is inversely related to the amount of blood admixture ($r=-.654$, $p<.0001$), mean normal cellularity is 38.2 x 10^6/mL (median 54.7 x 10^6/mL, determined in 65 animals) (Figure 3), demonstrating the validity of the approach. In all analyses, CD34+ cell numbers as measured by flow cytometry have been used. In Figure 10, BM CD34+ cell numbers corrected for PB admixture both in normal animals and two weeks after irradiation are shown. The upper panel is identical to Figure 1, whereas in the lower panel both measured and corrected values are shown. Regarding the minimal differences, analyses with measured CD34 numbers were performed.
CD34+ cell numbers in bone marrow and peripheral blood of normal monkeys show a variation which can cover a range of 3 logs between animals and of about 1.5 log

longitudinally within a monkey (Figure 2 and 4). Despite the wide range both intra-individually and inter-individually, there is a clear difference between BM and PB progenitor cell content of approximately 2 orders of magnitude (Table 2, Figure 2) during steady state. Individual BM and PB values did not display a significant correlation. Recently a study in mice [42] showed that CD34 expression by murine hematopoietic stem cells is reversible, this may also play a role in physiological variance in time [15] of CD34 numbers.

Shortly after a myelosuppressive dose of total body irradiation, when the damaged bone marrow lacks functional hematopoietic progenitor and stroma cells, blood admixture calculated with CD4 percentages in the aspirate and in the peripherhal blood, increased up to 90% (mean 89, median 88%). At 8 days after irradiation, the time at which bone marrow cellularity reached its nadir, there is no correlation between the cellularity of the bone marrow and the calculated blood admixture. Also CD34+ cell numbers in BM and PB decline and reach a similar nadir (Figure 6), after which an exponential regeneration pattern is observed in which the difference in absolute numbers of BM and PB gradually increases. These numbers (CD34+ cells in BM and PB) are significantly related during that period of 3 weeks ($p < .001$), also BM CD34+ numbers (BM and PB CD34+cells) at day 8 are predictive for PB CD34+ cell numbers one week later ($p<.001$), and also related well to reticulocyte, WBC and thrombocyte levels at day 15 ($p<.001$). Correlations at several time points within that first period of three weeks show the most strong relationship at 15 days after TBI between BM and PB CD34+ cell numbers ($p = .0004$) (Figure 7), despite the fluctuations in PB CD34 numbers, which also occur during this phase (Figure 8). PB CD34 numbers at day 15 are related as well to WBC and thrombocyte counts at that time point. This confirms close relations within subsets in hematopoiesis. Bone marrow CD34+ cell regeneration can be stimulated by administration of TPO and or GM-CSF/ G-CSF and also in this situation the mutual relationship of BM and PB CD34+ cells and with blood cell reconstitution remains evident. So, after myelosuppression, PB CD34+ cells reflect bone marrow CD34+ regeneration and can thus be used as a parameter of reconstitution.

In the bone marrow transplant model, using a lethal dose of TBI, the effect of growth factors is absent with the exception of a small stimulating effect of combined administration of TPO/GM-CSF on CD34 progenitors in BM and PB [37]. However, this stimulation is not followed by accelerated hematopoiesis. During regeneration in the first three weeks PB34 cells reflect BM34+ cells ($p<.0001$) although relations with blood cell reconstitution parameters were not observed. Figure 6 shows that CD34 regeneration in BM and PB is much slower after bone marrow transplantation (lower three figures) than after a myelosuppressive dose TBI (upper three figures), despite a similar nadir of progenitor cells and blood cells one week after TBI, and despite a similar reconstitution of peripheral blood cells in control (i.e., not treated with GF) animals. This indicates that the delayed regeneration after bone marrow transplantation is not due to a shortage of

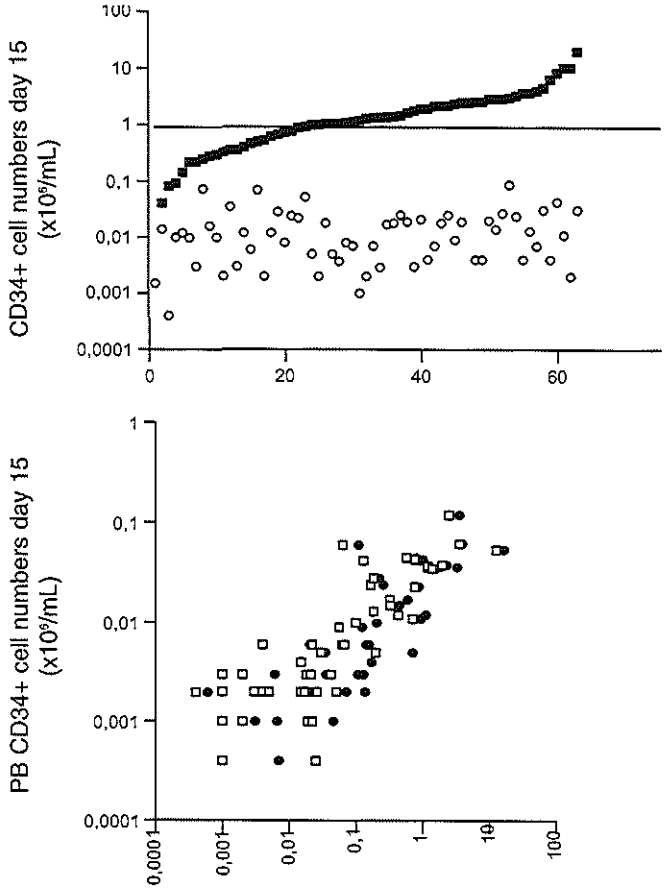

Figure 10. BM CD34+ cell numbers versus PB CD34+ cell numbers (both x 10^6/mL) at day 15 in 5 Gy irradiated rhesus monkeys. Open squares represent measured values. Filled Black circles represent bone marrow CD34+ cell numbers adjusted for PB admixture in the aspirate.

absolute numbers of BM CD34+ cells. Factors such as the disturbed quantities of essential primary and secondary cytokines, changed receptor status, loss of stem cell self-renewal capacity and/or intracellular pathways due to the higher irradiation dose [43, 44] may be relevant. Galotto et al [44] showed that bone marrow transplant (BMT) recipients display prolonged stromal damage after BMT (up to 12 years) with decreased Colony forming units—fibroblasts (CFU-f) and significantly reduced levels of long-term culture-initiating cells (LTC-IC). Analogous to these results, we found that the numbers of CD34+ cells 6 weeks after transplantation in BM and PB are still below normal levels (Figure 11), despite the regenerated normal hematology parameters in these animals and in contrast to the recovered numbers in 5 Gy irradiated animals at that time point. The lower number

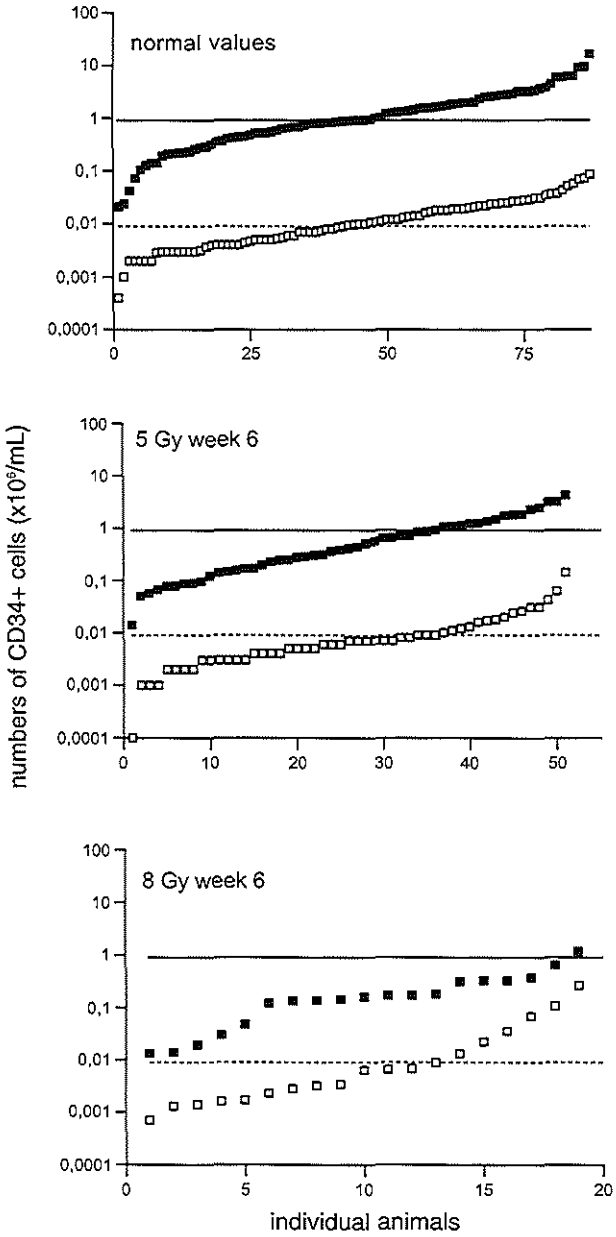

Figure 11. Absolute numbers CD34+ cells in BM and PB in normal rhesus monkeys (upper panel), 6 weeks after 5 Gy (middle panel) and 8 Gy TBI (lower panel). Black squares are BM values, open squares are PB values. The thin black horizontal line represents the median of CD34+ BM numbers in normal monkeys and the dotted horizontal line represents the median of PB CD34+ numbers in normal monkeys. BM and PB values are dissociated and both presented in ranked ascending order.

of CD34+ cells in 8 Gy irradiated and transplanted animals during reconstitution might well be a major cause of the lack of effect of administered hematopoietic growth factors, although the other, above mentioned factors certainly play a role as well. The exact mechanisms of the complex reconstitution after transplantation remains to be elucidated. The characteristics of transplanted cells e.g. bone marrow stem cells versus mobilized peripheral blood stem cells have been proven to be of importance relative to regeneration rate. Furthermore, the range of the CD34+ cell numbers at any time point is influenced by genetic factors, whereas differences in regeneration patterns in animals with apparently similar CD34+ cell numbers can be explained by results of a recent study which shows that phenotypically similar $Sca\text{-}1^{+}c\text{-}kit^{+}Lin^{-}$ cells of two mouse strains differ significantly in proliferative potential after transplantation [45]. This explanation might also be applicable to the bone marrow regeneration of an outbred species such as rhesus monkeys. The above-mentioned factors may interfere with the development from progenitors toward mature functional PB cells. Thus, also after BMT, PB CD34+ cells can be used as a parameter of bone marrow reconstitution, but not of regeneration of peripheral blood parameters such as WBC, thrombocytes or reticulocytes. BM recovery is delayed and not influenced by growth factor administration.

It is concluded that progenitor pools in PB and BM are correlated; under and differ under normal circumstances they differ two log in magnitudeorder of magnitude. Absolute levels fluctuate within time probably on a genetic basis [15, 16, 46]. PB CD34+ cells reflect BM CD34+ cells during regeneration from myelosuppression and bone marrow transplantation, and can thus be used as a rapid reliable test of bone marrow reconstitution.

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Chapter 5

Co-administration of Flt-3 ligand counteracts the actions of Thrombopoietin in myelosuppressed Rhesus Monkeys

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Abstract

This placebo-controlled study evaluated the efficacy of Flt-3 Ligand (FL) combined with TPO in myelosuppressed rhesus monkeys. The monkeys were subjected to 5 Gy total body irradiation (TBI), resulting in three weeks of profound pancytopenia, and received either 5 µg/kg of rhesus TPO IV at day 1 (n=4) and 100 µg/kg/day SC human Flt-3 Ligand (FL) for 14 consecutive days (n=4), or FL alone (n=4), and were compared with results from a concomitant study involving administration of TPO alone (n=4) or placebo (carrier; n=4). The TPO/FL combination was considerably less effective than TPO alone, with a more profound nadir and a slower recovery to thrombocyte counts > 100 x 10^9/L, approaching recovery patterns of placebo controls. Leukocyte regeneration was similar in all animals. Monkeys treated with FL alone displayed a regeneration of reticulocytes and thrombocytes in the lower range of those of the placebo controls. Recovery of bone marrow (BM) cellularity was slightly accelerated in the TPO/FL treated monkeys, but was not reflected by an increase in progenitor cells, in contrast to TPO alone. Monkeys treated with FL alone showed a BM reconstitution similar to placebo-treated controls. FL by itself was not effective as a therapeutic agent in this model for myelosuppression. As FL also suppressed BM CD34+ cell reconstitution, it is concluded that FL competes with TPO at the level of immature cell differentiation.

Introduction

Thrombocytopenia and neutropenia are major dose-limiting complications of radiotherapy and/or chemotherapy, particularly after of multiple cycles of cytotoxic therapy. Thrombopoietin (TPO) has been shown to prevent thrombocytopenia, accelerate thrombocyte regeneration to normal platelet levels and to promote reconstitution of bone marrow cellularity and granulocyte/macrophage and erythroid progenitor cells in radiation-induced myelosuppression in rhesus monkeys (Neelis *et al.*, 1997a). TPO synergizes with G-CSF as well as with GM-CSF in this model by mitigating the nadir of the neutrophils and by accelerating the subsequent recovery of these cells (Neelis *et al.*, 1997c). Promotion of G-CSF and GM-CSF responses by concurrent TPO administration has been attributed to expansion of immature cells along multiple haematopoietic lineages, thus making more G-CSF or GM-CSF target cells available (Neelis *et al.*, 1997c; Sitnicka *et al.*, 1996). Accelerated recovery of bone marrow cellularity, CD34+ cells and progenitor cells following supraoptimal TPO treatment has been reported previously (Neelis *et al.*, 1997a; Farese *et al.*, 1996).

Based on the hypothesis that peripheral reconstitution of blood cells is promoted by accelerated immature bone marrow cell reconstitution, we attempted to further improve the latter by co-administration of TPO and Flt3 (fms like tyrosine kinase 3) Ligand (FL). Flt-3 belongs to the subfamily of tyrosine kinase receptors that includes c-kit, c-fms, and the platelet-derived growth factor (PDGF) A and B receptors (Lyman, 1995). In humans, flt-3 expression was detected by PCR in liver, spleen, thymus, bone marrow, and weakly in

placenta (Rosnet et al., 1993). Among haematopoietic cells, enriched progenitor/stem cell populations selectively express Flt-3, suggesting a role in the regulation of early haematopoiesis (Small *et al.*, 1994; Rasko *et al.*, 1995, Rusten *et al.*, 1996). Targeted disruption of the flt-3 gene leads to a deficiency of early B-cell precursors and multipotential haematopoietic stem cells (Mackarehtschian *et al.*, 1995). FL stimulates the growth of murine and human BM progenitor cells and has been shown to synergize with a number of cytokines such as SCF, GM-CSF, interleukin-3 (IL-3) in stimulating CD34+BM progenitors (Rusten et al., 1996; Hannum et al., 1994). TPO and FL are able to sustain and expand CD34+ umbilical cord blood cells including long-term culture-initiating cells (Piacibello et al., 1997). FL is also a potent inducer of dendritic cells when administered *in vivo* (Maraskovsky et al., 1996).
The present study is based on the stimulating effects of both TPO and FL on immature progenitors, an action that possibly might synergize and overcome the lack of efficacy of TPO in a bone marrow transplant model (Neelis et al., 1997b; Hartong *et al.*, 2000). The study involved rhesus monkeys that had been exposed to 5 Gy total-body-irradiation (TBI), which results in a profound pancytopenia that lasted 3 weeks. The model proved to be highly adequate to study growth factor stimulation and synergism (Neelis *et al.*, 1997a; Neelis *et al.*, 1997c). Rhesus TPO was used at a single dose at the first day intravenously (IV) and FL treatment for the first 14 consecutive days after TBI subcutaneously (SC). This schedule is identical to concomitant studies with TPO combined with G- and GM-CSF, in which a single dose of TPO intravenously was effective and synergized with the CSFs, which were administered for 14 days subcutaneously after irradiation (Neelis *et al.*, 1997c)
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Materials and methods

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

Total body irradiation: Monkeys were irradiated with a single dose of 5 Gy total body irradiation (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 0.20-0.22 Gy/min. During TBI, the animals were placed in a cylindrical polycarbonate cage which rotated slowly (3 times per minute) around its vertical axis. Two monkeys treated with FL alone were irradiated with a single

dose of 6 Gy TBI delivered by a 6 MV linear accelerator (Siemens). The radiation delivered by the linear accelerator has a RBE (relative biological effect) of 0.85 relative to X-rays and, therefore, the dose of 6 Gy is equivalent to 5 Gy X-rays irradiation. During irradiation the monkeys were anaesthetised and placed in a perspex frame. The dose rate was 31 cGy/minute and the focus-skin distance 2 meter. The irradiation is given in two parts, half of the dose in *anterior-posterior* (AP) position, and the other half in PA position. The dose was confirmed by means of a thermoluminescent dosimeter (TLD) fixed to the skin of the monkey.

Supportive care: Two weeks before TBI, the monkeys were placed in a laminar flow cabinet and the gastrointestinal tract was selectively decontaminated by giving orally Ciprofloxacin (Bayer, Mijdrecht, the Netherlands), Nystatin (Sanofi BV, Maassluis, The Netherlands) and Polymyxin B (Pfizer, New York, NY, USA). 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 dropped below 1 x 10^9/L. Guided by faecal bacteriograms, the antibiotics were continued until leukocyte counts rose to levels > 1 x 10^9/L. During decontamination, iron supplementation (0.5 mL Imferon (Fe(III) 50 mg/mL; Fisons Pharmaceuticals, Loughborough, England, for 5 consecutive days before irradiation) was administered because of previously demonstrated prominent erythroid stimulation and iron depletion by thrombopoietin (Neelis *et al.*, 1997d). Dehydration and electrolyte disturbances were treated by appropriate fluid and electrolyte administration subcutaneously. The monkeys received irradiated (20 Gy γ-irradiation) platelet transfusions whenever thrombocyte counts reached values below 40 x 10^9/L, packed red cells whenever haematocrits were lower than 20% and, occasionally, whole blood transfusions in cases where transfusion criteria coincided. Transfusions were given when thrombocytes were < 40 x 10^9/L because monkeys are known to develop petechiae and other hemorrhages at this level. Thrombocyte counts in placebo monkeys show a sharp decline after one week (Fig. 1a), which without transfusion results in virtual disappearance of thrombocytes and a life-threatening propensity to bleeding. Indeed, thrombocyte levels < 40 x 10^9/L in monkeys have been associated with mortality at the mid-lethal dose of radiation used (Broerse *et al.*, 1978; Pickering *et al.*, 1955), mostly because at this stage after TBI a value < 40 x 10^9/L results in values < 10 x 10^9/L within 24 hours. Thrombocyte transfusions were collected from adult male donor monkeys kept at the Biomedical Primate Research Centre (Rijswijk, The Netherlands). A volume of 100 mL citrate anticoagulated blood was taken and centrifuged after which thrombocyte-enriched plasma was collected. Transfusions were irradiated with 15 Gy (γ-rays; Gammacell 40; Atomic Energy of Canada, Ottawa, Canada). Thrombocyte transfusions had a mean volume of 31.8 ± 5.4 mL and contained 334 ± 114 x 10^9 thrombocytes per liter. Whole blood transfusions consisted of 43.5 ± 5.1 mL with a haematocrit of 35.4 ± 8.2 %, 235 ± 50 x 10^9 thrombocytes per liter and 4.1 ± 1.4 x 10^9 leukocytes per liter.

Test drugs: Recombinant full-length rhesus monkey TPO produced by CHO cells were supplied by Genentech Inc. (South San Francisco, CA). The dose used was 5 µg/kg given IV on day 1 after TBI. The dose was diluted to a volume of 1 mL with PBS / 0.01% Tween 20 prior to administration. Placebo treated monkeys were only given the same volume of diluent only. Recombinant human Flt-3 Ligand (Immunex, Seattle, WA, USA) was administered in a dose of 100 µg/kg/day, SC, once daily for 14 days after TBI. The daily doses were diluted to a volume of 1 mL in the solution indicated by the suppliers. This dose of FL has a CD34+ cell mobilising effect by itself as well as in conjunction with simultaneously administered G-CSF (Papayannopoulou *et al.*, 1997).

Study groups: Studies with rhesus monkeys are done sequentially using highly codified and standardized methods, including radiation and placebo controls at regular intervals. For the present study, assignment to the treatment groups was at random. Animals received either 5 µg/kg rhesus TPO IV at day 1 after TBI and 100 µg/kg/day Flt-3 Ligand (FL) SC from day 1-14 (n=4) or FL 100µg/kg/day (n=4) from day 1-14). The results were compared with TPO treated monkeys (n=4) and placebo controls (n=4) (Neelis *et al.*, 1997c).

Bone marrow aspirates: Bone marrow was aspirated under neurolept anaesthesia using Ketalar (Apharmo, Arnhem, the Netherlands) and Vetranquil (Sanofi, Maassluis, the Netherlands). Small bone marrow aspirates for analytical purposes were taken from the shafts of the humeri using paediatric spinal needles and collected in bottles containing 2 mL HEPES buffered Hanks' balanced salt solution (HBBS) with 200 IU sodium heparin/mL (Leo Pharmaceutical Products, Weesp, the Netherlands). Low-density cells were isolated using a Ficoll (density 1.077) (Nycomed Pharma AS, Oslo, Norway) separation.

Colony assays: Cells were plated in 35-mm dishes (Becton Dickinson, Leiden, The Netherlands) in 1 mL enriched Dulbecco's medium containing 0.8% methylcellulose, 5% FCS, and additives as described previously (Merchav *et al.*, 1984; Guilbert *et al.*, 1976; Wagemaker *et al.*, 1979). For burst-forming units-erythroid (BFU-E), cultures were supplemented with hemin ($2x10^{-4}$ 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 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 in B. licheniformis and purified as described previously (Van Leen *et al.*, 1991; Burger *et al.*, 1990), and KL. Low-density cells were plated at $5x10^4$ cells per dish in duplicate. Colony counts were calculated per mL of bone marrow aspirated using the recovery of cells over the Ficoll density gradient. Colony numbers represent the mean ± standard deviation of bone marrow samples of individual monkeys.

Haematological examinations: Complete blood cell counts were measured daily using a Sysmex F-800 haematology Analyzer (Toa Medical Electronics co., LTD., Kobe, Japan). For reticulocyte measurements, 5 µL EDTA blood was diluted in 1 mL PBS/EDTA/azide and one mL of a thiazole orange dilution was added, using thiazole in a final concentration of 0.5 µg/mL. Measurements were done using a FACScan (Becton Dickinson, Leiden, The Netherlands) and analysed using the Reticount software.

Measurements of surface antigens: Once weekly, a FACScan analysis was done on peripheral blood (PB) and bone marrow (BM) samples on the following surface antigens: CD8 and CD4 (T-cells), CD20 (B-cell), CD11b (myelomonocytes), CD56 and CD16 (NK cells) and CD34 (immature cells). HLA-DR was also measured on bone marrow cells. Directly labelled 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 11aFITC (Becton Dickinson) respectively). For CD11b the monoclonal antibody MO1-FITC (Coulter Immunology, Hialeah, FL) was used 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, St Louis, USA) according to standard procedures. A phyocoerythrin (PE) –conjugated mAb against human HLA-DR that reacts with rhesus monkey RhLA-DR antigens (Becton Dickinson, San Jose, CA) was used to measure HLA-DR activated CD34+ cells. A 0.5 mL of whole blood or bone marrow was lysed in 10 mL lysing solution (8.26 g ammonium chloride/ 1.0 g potassium bicarbonate and 0.037 g EDTA per L) for 10 minutes at 4 °C. After lysing the cells were washed twice with HBBS containing 2% FCS and 0.05% (wt/vol) sodium azide. The cells were resuspended in 100µL of the latter fluid 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 analysed using the Lysis II software (Becton Dickinson).

Clinical chemistry parameters: Serum concentrations of sodium, potassium, chloride, glucose, albumin, total protein, aspartate-amino transferase, alanine-amino transferase, alkaline phosphatase, lactate dehydrogenase (LDH), gamma-glutamyl transpeptidase, bilirubin, C reactive protein, creatinin, urea and bicarbonate were analysed once a week using an Elan Analyser (Eppendorf Merck, Hamburg, Germany).

Statistical analysis: Mean, median and range of numeric variables were calculated by the Excel spreadsheet program. Standard deviations were calculated and are given in the text and in the figures on the assumption of a normal distribution. The statistical significance of differences was calculated with the Mann-Whitney test, comparing two unpaired groups each time, using Statview 4.1 (Abacus concepts Inc., Berkeley, CA). To analyse the reconstitution patterns obtained in more detail, linear regressions were calculated and regression lines compared using the t distribution to determine the two-tailed probability P

that the differences observed are due to random variation.

Results

Peripheral blood cell counts

Co-administration of TPO and FL in 4 monkeys resulted in transfusion independence in two monkeys, whereas the other two monkeys, respectively, needed one and as many as four thrombocyte transfusions. Figure 1a demonstrates that thrombocyte reconstitution is suppressed relative to monkeys treated with TPO alone. To analyse this observation in more detail, the data of both the rapid initial reconstitutions from the time point of the nadir as well as the slower subsequent reconstitution towards normal values were subjected to a regression analysis (Fig 1b). This demonstrated that the initial reconstitution as well as the platelet levels in the slower phase was significantly suppressed ($p < .001$ and $p < .05$, respectively). FL treated monkeys regenerated similar to, but in the lower range, of the placebo controls and reached transfusion independence after 11,15,19 and 29 (18.5 ± 7.7) days. The FL treated monkeys needed 1,2,3 and 4 thrombocyte transfusions respectively. The latter two animals showed a delayed regeneration pattern to normal levels, reaching levels >100 10^9/L after a protracted period of 32 and 36 days, whereas the other two animals reached this level after 19 and 22 days. Placebo controls needed 1,2,2 and 5 thrombocyte transfusions and three of the four monkeys needed one whole blood transfusion, reaching thrombocyte transfusion independence at days 11,15,17,30 (18.3 ± 8.2) respectively.

Apart from the more pronounced biphasic rise between day 7 and 14 (Fig 2) of reticulocytes in the TPO monkeys, the FL/TPO treated monkeys showed a similar reticulocyte regeneration reaching the 1% level at 15,17,18 and 20 (17.5±2.1) days after irradiation. This level was reached after 17,20,20,23 (20±2.4) and 19,19,20 and 21 (19.7± 1) days in placebo controls and FL treated monkeys, respectively (Fig 2). The latter difference is not statistically significant. FL/TPO treated monkeys did not reach a reticulocyte peak as prominent as the TPO treated monkeys. This might be due to the higher haematocrit values in the FL/TPO monkeys after day 17 and, consequently, lower levels of erythropoietin (not measured). Haematocrit values showed a similar pattern (a slow decline) for all groups except the placebo controls, which reached a lower nadir resulting in the need for a whole blood transfusion in three of the four placebo controls (Fig 2). The reconstitution of the white blood cell count (WBC) to a level of 1 x 10^9/L corresponds with the level of 0.5 10^9 /L of the neutrophilic granulocytes that was used as the reconstitution parameter in previous studies (Neelis *et al*, 1997 a,b,c.) Regeneration of WBC followed the same pattern in all monkeys with values greater than 10^9/L around day 19 (Fig 3).

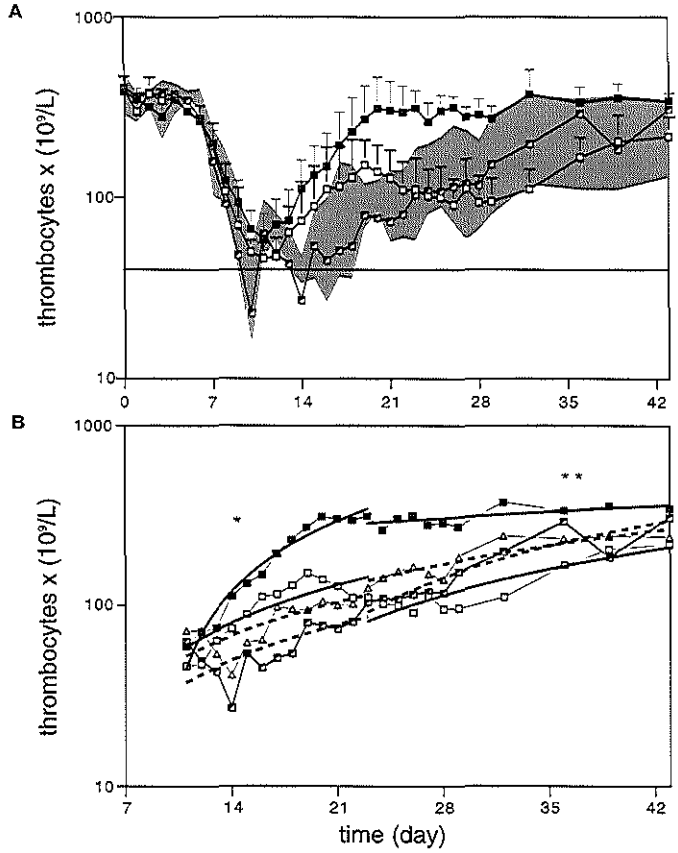

Figure 1 a. (Upper panel) Mean values of thrombocyte regeneration after 5 Gy TBI (day 0). Monkeys were treated with TPO 5 µg/kg/iv day 1 after TBI (filled-squares, n=4), TPO/FL (open squares, n=4), FL 100µg/kg/day/sc day 1-14 after TBI (half-filled squares, n=4). The shaded area represents the arithmetic mean of placebo controls ± SD (n=4). The horizontal line defines the level of thrombocytopenia (40 x 10^9/L) below which thrombocyte transfusions were given.

b. (Lower panel) Mean values of thrombocyte regeneration after 5 Gy TBI (day 0). Symbols as in Figure 1a, with exception of counts of placebo controls which are represented by open triangles (n=4). To analyse the platelet response patterns in more detail, least square linear regression lines were calculated through the rapidly ascending phase of reconstitution from the nadir (days 9-22 after TBI) and the slower second phase approaching normal levels (after day 22). Bold solid lines represent regression lines of TPO and TPO/ FL treated monkeys. Bold dotted lines represent regression lines of FL treated monkeys and placebo controls. The regression lines were evaluated for the statistical significance of differences in slope (reflecting rate of reconstitution): * $p < .001$ TPO vs. TPO/FL, ** $p < .05$ TPO vs. TPO/FL. The difference between the slope of the regression lines of FL and placebo treated animals was not significant. The suppressed initial phase of reconstitution in the FL/TPO treated monkeys relative to the TPO treated monkeys is also reflected in significantly ($p < .001$) lower platelet levels in the second phases of reconstitution

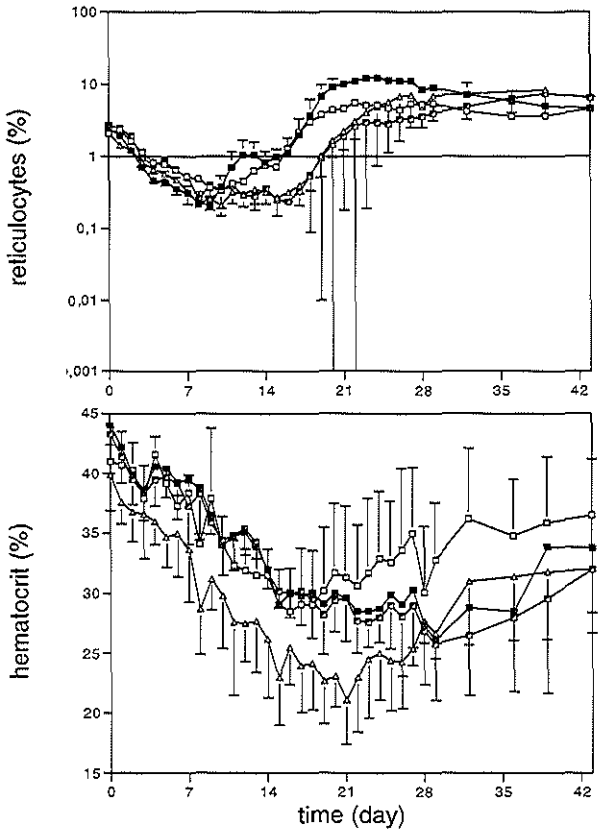

Figure 2. Mean values of reticulocyte percentages after 5 Gy TBI (day 0) in the upper panel and haematocrit regeneration in the lower panel. Monkeys were treated with TPO 5 µg/kg/iv day 1 after TBI (filled-squares, n=4), TPO/FL (open squares, n=4), FL 100 µg/kg/day/sc day 1-14 after TBI (half-filled squares, n=4) or placebo controls (open triangles, n=4). The horizontal line in the reticulocyte figure defines the level of 1%.

White blood cell subsets measured by flow cytometry

The regeneration patterns of CD4, CD8, CD11b, CD20, CD56 and CD16 did not differ between the various treatment groups in bone marrow and peripheral blood. A profound nadir was observed for all cell types in the peripheral blood in all groups. TPO and the combination of TPO and FL initially accelerated bone marrow CD34+ cell reconstitution, resulting in one log augmentation at day 15 compared to placebo controls (Fig.4), but the co-administration of FL subsequently appeared to diminish the CD34+ cell TPO response. This phenomenon was also reflected in the peripheral blood CD34+ levels. A remarkable overshoot of CD11b, CD16 and CD56 was seen after 21 days in monkeys stimulated with a single dose TPO (data not shown). FL alone did not stimulate CD34+ cell or B-lymphocyte recovery after myelosuppression.

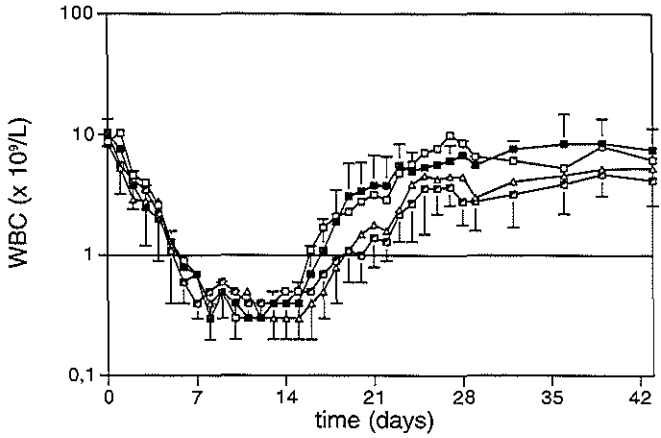

Figure 3. Mean values of WBC numbers after 5 Gy TBI (day 0). Monkeys were treated with TPO 5 µg/kg/iv day 1 after TBI (filled-squares, n=4), TPO/FL (open squares, n=4), FL 100µg/kg/day/sc day 1-14 after TBI (half-filled squares, n=4) or placebo controls (open triangles, n=4). The horizontal line in the figure defines the level of 1x 10^9/L.

Table 1. Mean ± SD of bone marrow cellularities (=total nucleated cells (TNC), GM-CFU numbers, BFU-E numbers and absolute numbers CD34+ cells in bone marrow per mL aspirate after 5-Gy TBI.

	Treatment	Before	Week 1	Week 2	Week 3	Week 4
Cellularity	TPO	71±62	0.3±0.1	0.7±0.7	44±48	55±62
x 10^9/mL	FL	48±67	0.2±0.2	0.3±0.3	7.1±7.5	6.9±7.1
	TPO and FL	42±47	0.5±0.2	2.0±3.0	14±9	12±5
	placebo	43±17	0.6±0.5	0.3±0.2	4±3	13±12
GM-CFU	TPO	58±83	ND	ND	64±98	25±19
x 10^3	FL	66±113	ND	ND	1.6±1.4	1.1±1.3
	TPO and FL	18±15	ND	ND	2±1	2.5±0.1
	placebo	43±31	ND	ND	0/0/3/12	15±24
BFU-E	TPO	31±44	ND	ND	16±28	17±16
x10^3	FL	31±57	ND	ND	0.8±1.2	0.2±0.3
	TPO and FL	17±26	ND	ND	3±3	0.7±0.4
	placebo	21±14	ND	ND	0/0/0.6/3	5±7
CD34+	TPO	1.2±0.95	0.001±0.001	0.058±0.095	4.33±4.33	3.59±5.39
x10^9/mL	FL	1.6±2.9	6.8 x10^{-5}±4.1 x10^{-5}	0.007±0.012	0.46±0.7	0.15±0.18
	TPO and FL	2.7±4.4	0.003±0.002	0.204±0.376	0.48±0.48	0.16±0.19
	placebo	1.1±0.4	0.002±0.002	0.006±0.009	0.16±0.24	0.61±0.65

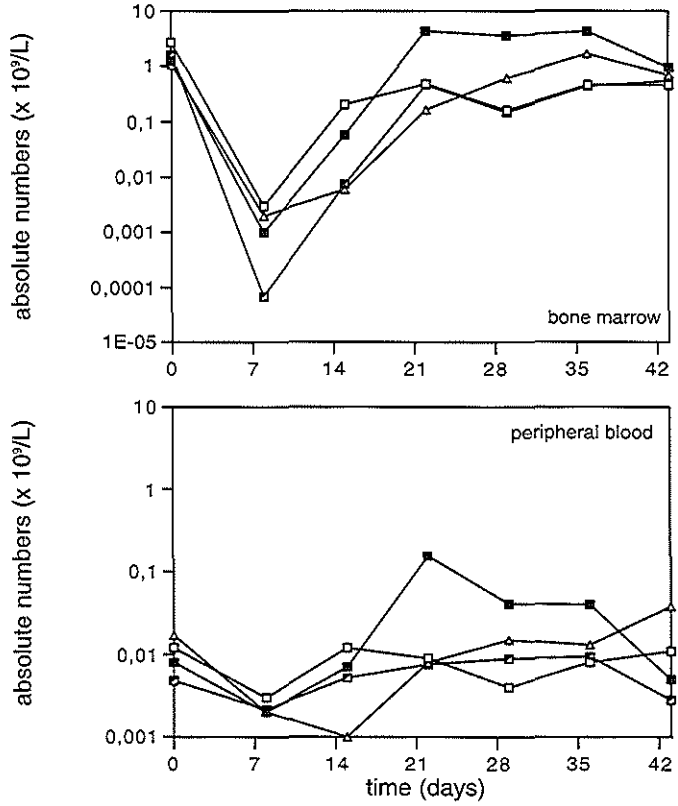

Figure 4. Mean numbers of CD34+ cell levels in bone marrow (upper panel) and peripheral blood (lower panel) after 5 Gy TBI (day 0). Monkeys were treated with TPO 5 µg/kg/iv day 1 after TBI (filled-squares, n=4), TPO/FL (open squares, n=4), FL 100µg/kg/day/sc day 1-14 after TBI (half-filled squares, n=4) or placebo controls (open triangles, n=4)

Bone marrow cellularity and progenitor cell content

The bone marrow cellularity and progenitor cell content before irradiation were within the normal range. Bone marrow aspirations were performed once weekly. Cellularity was very low in the first week after TBI in all monkeys, and did not yield sufficient numbers of cells to perform colony assays. Three weeks after irradiation, bone marrow cellularity was sufficient for colony assays. However, only the TPO treated monkeys displayed clonogenic progenitor cell recovery at this time point (Table 1). Co-administration of TPO and FL resulted in an accelerated rise in CD34+ cell numbers (Table 1) in the bone marrow at day 15 but these cells declined one week later, whereas TPO treatment resulted in an increasing number of CD34+ cells after 3 weeks which persisted until 5 weeks after TBI. The differences between the groups are not prominent, partly due to the small groups, the variability in cell numbers due to puncture site, hydration state, the inevitable admixture of

blood in the bone marrow aspirate, and partly due to the less pronounced effect of TPO if administered as a single dose (Neelis *et al.*, 1997d). Both GM-CFU and BFU-E in the bone marrow were similarly increased as CD34-positive cells in the bone marrow of TPO treated monkeys. FL/TPO treated monkeys reached their pre-irradiation colony counts 5 weeks after TBI and FL treated monkeys one week later (data not shown). Adverse effects and abnormal chemistry parameters were not observed.

Discussion

We have examined the effects of administration of recombinant human FL alone and in combination with rhesus TPO on haematopoietic reconstitution after myelosuppression with 5 Gy TBI in young adult rhesus monkeys. As a single agent, a single dose of 5 µg/kg TPO IV one day after TBI is effective in virtually preventing the need for thrombocyte transfusions and in accelerating thrombocyte reconstitution to normal levels 2 weeks after irradiation (Neelis *et al.*, 1997c). A stimulating effect of TPO on stem cell reconstitution was demonstrated by increased numbers of CD34+ cells two weeks after TBI and by early clonogenic progenitor recovery 15 days after irradiation as compared with placebo controls. Previous studies with G-CSF and GM-CSF in this myelosuppression model showed that co-administration of TPO/GM-CSF augmented thrombocyte, erythrocyte, and neutrophil production, whereas adding G-CSF to TPO predominantly improved neutrophil reconstitution (Neelis *et al.*, 1997c). FL stimulates the proliferation in vitro and the expansion and mobilisation *in vivo* of stem cells and progenitor cells. FL as a single factor has little proliferative activity on stem cells and progenitor cells, but it synergizes with a range of other colony-stimulating factors including TPO to stimulate the proliferation of stem cells in vitro (Piacibello *et al.*, 1997; Papayannopoulou *et al.*, 1997; Kobayashi *et al.*, 1997; Ohmizono *et al.*, 1997; Broxmeyer *et al.*, 1995; Gabbianelli *et al.*, 1995). Therefore, FL was studied in this myelosuppression in conjunction with TPO treatment in a non-human primate model.

This study demonstrated that FL as a single agent did not stimulate CD34+ cell recovery in bone marrow or peripheral blood. In combination with TPO, a minor, probably TPO derived, effect on CD34+ numbers in the bone marrow was observed but this effect was not reflected by an additional increase in progenitors. Mueller *et al.* (2002) reported that FL induced proliferation of mouse bone marrow stem cells, but reduced the engraftment potential of haematopoietic stem cells harvested form the marrow (Mueller *et al.*, 2002). The TPO/FL combination slightly stimulated WBC regeneration similar to TPO (Fig 3) but did not have an additive effect, whereas FL alone followed the reconstitution pattern of the placebo treated controls. The thrombocyte reconstitution of FL treated monkeys was clearly suppressed and similar to the reconstitution pattern of the placebo controls (Fig 1a and 1b, Table 2), partly resulting in a higher need for transfusions. The suppression of thrombocyte reconstitution was even more pronounced in the TPO/FL treated monkeys compared to treatment with TPO alone in the initial rapid regeneration period ($p < .001$,

resulting in a clearly suppressed regeneration pattern ($p < .05$) during the subsequent slow phase toward normal levels. As a consequence, a prolonged period of approximately 3 weeks (similar to placebo controls) was necessary to reach normal (>100x10^9/L) platelet levels. This observation contrasted with the TPO treated monkeys, which reached normal thrombocyte values after 2 weeks (Fig 1, Table 2).

In several studies, a radioprotective effect of FL was observed. One study (Gratwohl *et al.*, 1998) showed a protective effect of FL from otherwise lethal TBI (14Gy) in rabbits; in combination with G-CSF, the nadir of leukocyte and thrombocyte counts was less profound and of shorter duration than that of untreated controls. The rabbits received 500 µg/kg FL, which is 5 times more than in our rhesus monkey model described above. In addition, administration started before TBI, which might be an explain these results because of recruitment of progenitor cells. Also in another study (Hudak *et al.*, 1998) using lethally irradiated mice radioprotective effects of FL, if administered before irradiation, were observed. It should be noted that none of the irradiated controls survived for more than two weeks, which makes evaluation of a stimulatory effect on blood cell reconstitution difficult. The data suggest a stimulating effect of FL on WBC, but the mice experienced a prolonged anaemia and thrombocytopenia similar to the monkeys treated with FL alone in the present study. The antagonism of FL and TPO on thrombocyte stimulation was also encountered in a mobilisation and a transplantation study in mice, and therefore not unique to this monkey model (unpublished observations). The *in vivo* observations contrast in vitro studies, which show synergism of TPO, FL and SCF on stem cell expansion. CD34+ cord blood cells could be expanded in vitro for up to 10 weeks, with a retained repopulating capacity (Kobayashi *et al.*, 1997; Ohmizono *et al.*, 1997; Piacibello *et al.*, 1999). Explanations for this apparent antagonism of TPO and FL *in vivo* might be extrapolated from other in vitro studies, which show that presence of the Flk-2/Flt-3 receptor in primitive haematopoietic cell populations resulted in preferential myeloid differentiation, whereas CFU-Meg colonies were found in the Flk-2/Flt-3 negative fraction (Banu *et al.*, 1999). Flow cytometric studies showed that more than 90% of the CD34+CD38- cells expressed Flt-3. However, during maturation to committed (CD34+CD38+) lineages, Flt-3 expression is lost (Gabbianelli *et al.*, 1995). The present study demonstrates that the suppressive effect of FL on TPO stimulated haematopoiesis is already apparent at the level of progenitor cells, both as detected by CD34 and by colony-formation in vitro. We conclude therefore that competition at an immature cell level between TPO and FL results in suppression of the TPO response in FL treated monkeys.

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CHAPTER 6

Circulating FL levels correlated with hematopoietic reconstitution parameters after myelosuppression and stem cell transplantation are reduced by treatment with GM-CSF

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Abstract

Purpose: To study the relation of Flt-3 Ligand (FL) levels and (growth factor stimulated) regeneration of hematopoiesis after myelosuppression and bone marrow transplantation (BMT).
Methods: FL levels in the peripheral blood (PB) of 56 young adult rhesus monkeys were evaluated before and after hematopoietic growth factor treatment in studies involving myelosuppression by total body irradiation (TBI) at a dose of 5 Gy (X-rays) and BMT after a dose of 8 Gy. FL levels were measured during reconstitution of hematopoiesis and related to hematopoietic reconstitution, as measured by numbers of CD34+ cells in bone marrow (BM) and PB, white blood cell counts (WBC), reticulocyte percentages and thrombocyte counts.
Results: The mean (± SD) normal FL level in 94 samples of normal animals was 180 (± 54) pg/mL, and did not differ between males and females. After myelosuppression, FL levels increased to reach peak values after 7-14 days. Normalization of FL levels occurred after 3 weeks, concurrent with regeneration of hematopoiesis. The area under de curve (AUC) of FL levels after 3 and 6 weeks was significantly related to reconstitution of WBC, reticulocytes and thrombocyte levels, but at earlier time points significance of the relations was absent. FL levels in GM-CSF (± TPO) treated animals were significantly lower than in other groups studied, corresponding to an increase in BM CD34+ cells and a faster regeneration of hematopoiesis. After 8 Gy TBI and BMT, GM-CSF treatment reduced FL levels as well and was similarly accompanied by higher BM CD34+ cell numbers. A significant relation of FL levels and WBC and BM CD34+ cells could be demonstrated whereas relations with other parameters were not observed.
Conclusions: FL levels after myelosuppression as well as after transplantation are inversely related to the regeneration of WBC and BM CD34+ cells, and to the regeneration of reticulocytes and thrombocytes, whereas the latter are not significantly related to FL levels after BMT. Treatment with GM-CSF after both myelosuppression and BMT results in a reduced increase of FL levels, likely related to accelerated CD34+ cell reconstitution.

Introduction

The fms like tyrosine kinase 3 Ligand (Flt-3 ligand, FL) is the ligand for the receptor CD135 which belongs to the subfamily of tyrosine kinase receptors that includes the receptors of c-kit, c-fms, and the platelet-derived growth factor (PDGF) A and B receptors [1]. In humans, flt-3 gene expression was detected by PCR in liver, spleen, thymus, bone marrow, and weakly in placenta [2]. Among hematopoietic cells, enriched progenitor/stem cell populations selectively express Flt-3, suggesting a role in the regulation of early hematopoiesis [3, 4, 5]. Targeted disruption of the flt-3 gene leads to a deficiency of early B-cell precursors and multipotential hematopoietic stem cells [6]. FL stimulates the growth

of murine and human BM progenitor cells and has been shown to synergize with a number of cytokines such as SCF, GM-CSF and interleukin-3 (IL-3) in stimulating CD34+ BM progenitors [5, 7]. Elevated FL serum levels have been demonstrated in patients with aplastic or Fanconi anemia [8] and in patients suffering from bone marrow suppression after chemo- and/or radiotherapy treatment [9, 10]. This is similar to , e.g., G-CSF [11, 12], of which a rise in endogenous levels precedes and correlates with myeloid engraftment. Serum concentrations of FL are inversely correlated to the frequency of colony-forming-cells in the bone marrow, and FL levels are negatively correlated with neutrophil recovery after myelosuppression [9, 10, 13]. Although FL is thought to act at a very early stage prior to the separation of myeloid and megakaryocytic lineages, FL levels provide a more accurate prediction of duration of thrombocytopenia than of neutropenia [9], which can be explained by FL counteracting the effectiveness of TPO [14]. FL levels are not elevated in pure red cell aplasia, Diamond-Blackfan anemia, anemia's of undetermined origin or a thalassemia. This may be due to the phenotypes of these hematopoietic disorders being restricted to the erythroid lineage, and not expressed at the stem cell level [8]. Under physiological steady-state conditions, FL is produced constitutively but retained intracellularly within and close to the Golgi apparatus. In hematopoietic failure, FL is rapidly translocated to the cell surface of T lymphocytes, and the soluble form is probably released by proteolytic cleavage of the transmembrane protein. Only during severe aplasia, an increase in mRNA is observed after 8-10 days in bone marrow and thymus, whereas mRNA in the spleen, kidney, lymph nodes, brain, liver and lung remained unchanged [15,16]. Others have shown that mononuclear cells express the membrane-bound form of FL in aplastic anemia [10], as well as endothelial cells both in larger blood vessels and in bone marrow microvasculature [17]. Bone marrow stromal cells are able to produce Flt3-ligand [18]. As to whether other hematopoietic growth factors influence the complex regulation of FL levels is not known. To investigate to what extent FL levels are a prognostic factor for hematopoietic reconstitution following cytoreductive treatment and/or stem cell transplantion and to study the effects of treatment by other growth factors, we determined circulating FL levels in rhesus monkeys. Data were obtained from several studies involving the effect of thrombopoietin (TPO), Granulocyte Colony Stimulating Factor (G-CSF) and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) after myelosuppression (5 Gy total body irradiation (TBI), X-rays) and bone marrow transplantation (BMT) (8 Gy TBI, X-rays). FL levels have been related with several parameters of hematopoietic reconstitution.

Materials and methods

Animals: A total number of 64 purpose bred young adult rhesus monkeys (*Macaca Mulatta*), weighing 2.5-4.0 kilograms and aged 2 to 3 years, were used. The monkeys were housed in groups of 4 to 6 monkeys in stainless steel cages in rooms equipped with reverse filtered air barrier, normal day light rhythm and conditioned to 20 ºC with a relative

humidity of 70%. Animals were fed *ad libitum* with commercial primate chow, fresh fruits, and received acidified drinking water. All animals were free of intestinal parasites and seronegative for herpes B, simian T-lymphotropic viruses and simian immunodeficiency virus. Hematology parameters (Hb, Ht, MCV, WBC, RBC, thrombocytes, reticulocytes, WBC differentials) as well as some chemistry parameters (creatinin, electrolytes, iron, glucose, total protein, ALAT, LDH) before the experiment are all within normal values. Housing, experiments and all other conditions were approved by an ethical committee in conformity with legal regulations in the Netherlands. Total number of animals used is 64. Eight animals have only been used for measurement of normal levels, while FL levels of 53/ 56 other animals were evaluated before, and FL levels of 56 / 56 animals were evaluated after TBI.

Total body irradiation: Monkeys were irradiated with a single dose of 5 (n=36), or 8 (n=18) Gy total body irradiation (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 0.20-0.22 Gy/min. During TBI, the animals were placed in a cylindrical polycarbonate cage that rotated slowly (3 times per minute) around its vertical axis.
Two additional animals were irradiated with a single dose of 9 Gy TBI delivered by a 6 MV linear accelerator (Siemens). During irradiation, the monkeys were anesthetized and placed in a perspex frame, molded to the body shape of monkeys of similar size. The dose rate was 31 cGy/minute and the focus-skin distance was 2 meter. The radiation was delivered in two parts, half of the dose in anterior-posterior (AP) position, and the other half in PA position. The dose was confirmed by means of TLDs which were fixed on the skin of the monkeys and was adjusted in keeping with a RBE of 0.85 relative to X-rays.

Test drugs: Recombinant full length rhesus monkey TPO produced by CHO cells and recombinant human TPO were supplied by Genentech Inc. (South San Francisco, CA). The dose TPO was diluted to a volume of 1 mL with PBS / 0.01% Tween 20 prior to administration. Recombinant human G-CSF (Neupogen, Amgen Inc., Thousand Oaks, CA), and recombinant GM-CSF (Leukine; Immunex Corp., Seattle, WA) were given subcutaneously (SC) at several doses and schedules [14,24,25,26,27, chapter 3 and unpublished data] (Table 1). Placebo treated monkeys were only given the same volume of diluent. The daily doses were diluted to a volume of 1 mL in the solution indicated by the supplier.

Additional animals:Two animals (97042 and 97102) were stimulated during days -35 until -28 before TBI with rhesus TPO 5 µg/kg sc and FL 100 µg/kg sc. After 7 days of growth factor stimulation, a large bone marrow puncture was taken from the femurs and 35 mL of peripheral blood was collected. A ficoll separation (see below) of both bone marrow aspirate and peripheral blood was performed and the Ficoll separated cells were frozen, erythrocytes were washed several times and returned to the animal the same day. At day

Table 1. Growth factor treatment schemes of animals included in analysis of FL levels

Irradiation dose (X-rays) (Gy)	Number of animals	Growth factor treatment			
5	4	Placebo			
5	2	Rhesus TPO	5 µg/kg iv day 1+4		
5	3	Rhesus TPO	5 µg/kg iv day 1		
5	4	Rec hum. TPO	10 µg/kg/d sc, days 1-21		
5	4	GM-CSF	25 µg/kg/day sc days 1-14		
5	3	G-CSF	10 µg/kg/day sc days 1-14		
5	4	G-CSF	5 µg/kg/day sc days 1-21		
5	4	Rhesus TPO	5 µg/kg/day iv day 1 and GM-CSF 25 µg/kg days 1-14 sc		
5	4	Rhesus TPO	5 µg/kg/day iv day 1 and G-CSF 10 µg/kg days 1-14 sc		
5	4	Rec Hum TPO	10 µg/kg and G-CSF 5 µg/kg sc days 1-21		
Irradiation dose (X-rays) (Gy)	**Number of animals**	**Growth factor treatment**		**Kind of bone marrow transplant**	**Number CD34+ cells x 10^5/kg**
8	2	Irradiation controls			
8	2	Placebo		CD34++/DRdull sorted	0.3
8	3	Rec Hum TPO	10µg/kg sc days 1-21	CD34++/DRdull sorted	0.3
8	3	G-CSF	5 µg/kg sc days 1-21	CD34++/DRdull sorted	0.3
8	1	Rec Hum TPO	10µg/kg and G-CSF 5 µg/kg sc days 1-21	CD34++/DRdull sorted	0.3
8	2	None		CD34++/DRbright sorted	1
8	3	Rhesus TPO	5µg/kg and GM-CSF 25µg/kg days 1-14 sc	10^7 unfractionated cells/kg	4.6 1.7 1.8
8	2	Rhesus TPO	5µg/kg and FL 100 µg/kg sc days -35 until -28	Mobilized CD34+ beads selected BM cells	10
8	2	Placebo		10^7 unfractionated cells/kg	5.6 0.55

1 after TBI, the Ficoll separated cells were thawed and a CD34 beads selection (see: *stem cell separation*) was done. One million CD34-positive cells/kg were infused. Furthermore, animal 97042 received rhesus TPO 5 µg/kg iv at day one with the transplant and the rest of the days sc together with G-CSF at the same dose until the WBC exceeded 1 x 10^9/mL.

Study groups: Studies with rhesus monkeys are done sequentially using highly codified and standardized methods, including radiation and placebo controls at regular intervals. Assignment to the treatment groups was at random.

Bone marrow aspirates: Bone marrow was aspirated under neurolept anesthesia using Ketalar (Apharmo, Arnhem, the Netherlands) and Vetranquil (Sanofi, Maassluis, the Netherlands). Standardized small bone marrow aspirates for analytical purposes were taken from the shafts of the humeri using fine pediatric spinal needles and collected in bottles containing 2 mL HEPES buffered Hanks' balanced salt solution (HBBS) with 200 IU sodium heparin/mL (Leo Pharmaceutical Products, Weesp, the Netherlands). BM Cellularity was determined by counting the nucleated cells and expressed per mL aspirate. Low density cells were isolated using a Ficoll (density 1.077) (Nycomed Pharma AS, Oslo, Norway) separation.

Hematological examinations: Complete blood cell counts were measured daily using a Sysmex F-800 hematology Analyzer (Toa Medical Electronics co., LTD., Kobe, Japan). For reticulocyte measurements, 5 µL EDTA blood was diluted in 1 mL PBS/EDTA/azide and one mL of a thiazole orange dilution was added, using thiazole in a final concentration of 0.5 µg/mL. Measurements were done using a FACScan (Becton Dickinson, Leiden, The Netherlands) and analyzed using the Reticount software.

Measurements of surface antigens: Once weekly, a phenotype analysis was done on peripheral blood (PB) and bone marrow (BM) samples by flow cytometry. A class III 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, St Louis, USA) according to standard procedures, was used to stain CD34+ cells. A 0.5 mL of whole blood or bone marrow was lysed in 10 mL lysing solution (8.26 g ammonium chloride/ 1.0 g potassium bicarbonate and 0.037 g EDTA per L) for 10 minutes at 4 °C. After lysing, the cells were washed twice with HBBS containing 2% FCS and 0.05% (wt/vol) sodium azide. The cells were resuspended in 100µL of the latter fluid 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 by flow cytometry. Ungated list mode data were collected for 10,000 events and analyzed using the Lysis II software (Becton Dickinson).

Stem cell isolation and transplantation

To enrich for progenitor cells, buffy-coat cells were subjected to a discontinuous bovine serum albumin density gradient [18, 19, 20]. 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 phycoerythtin (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 (100mW). CD34bright /RhLA-DRdull cells [22] were sorted in the normal C mode and reanalyzed for purity. The sorted cells were transplanted at a dose of 1.4 – 3.0 x 10^4/kg body weight on the same day.

Statistical analysis:

Mean, median and range of numeric variables were calculated by the Excel spreadsheet program. Standard deviations were calculated and are given in the text and in the figures on the assumption of a normal distribution. The statistical significance of differences was calculated by the Mann-Whitney test (comparing two unpaired groups each time), the paired t-test for continuous data, and Fisher's exact test for categorical data.

Measurement of FL levels

FL levels were determined in serum samples using and ELISA kit for human FL that cross reacts [13] with rhesus FL, according to the manufacturer's recommendations (R&D Systems, London, UK). Serum samples have been taken once or twice weekly during the study period of 6 weeks after TBI. Blood was centrifuged 10 minutes at 2300 rpm, 4 °C, after which the serum was collected and cryopreserved at -20 °C. For the correlation studies, both absolute levels as well as areas under the curve have been used.

Results

Normal FL levels in rhesus monkeys

Normal FL levels have been determined in 94 samples of 61 animals (normal serum samples of three irradiated animals were not available). The mean FL level was 180 pg/mL, the median was 181 pg/mL, the standard deviation 54 pg/mL and the minimal and maximal value were 73 and 339 pg/mL respectively (Table 2). These levels do differ from normal levels in humans which are 14 ± 39 pg/mL [10] or at least below 100 pg/mL [8] but

are consistent with a previous study in non human primates (Macaca fascicularis) [13]. If the mean was taken of the individual samples of each monkey (total of 61 values) the mean of the males (n=49) was 186.4, median 189.8, SD 51 and the mean of the females (n=12) was 182.4, median 181.6, SD 39.2. The difference between male/female samples was not statistically significant (p = 0.8).

Table 2. FL levels in normal rhesus monkeys (pg/mL).The difference between male and female values is not significant (p = .83).

Group	Number (n=)	Mean	SD	Median
all	94 samples of 61 animals*	180	54	181
Male	49	186	51	190
Female	12	182	39	182

*mean ± SD of all single samples was 193 ± 54 (n = 39)
mean ± SD of all double samples was177 ± 39 (n = 14)
mean ± SD of all multiple samples was 164 ± 52 (n = 8)

FL levels in myelosuppressed rhesus monkeys (5 Gy TBI)

FL levels have been measured in serum samples of days 3,7,10,14,17,21,24,28,35 and 43 after irradiation. These samples were available for nearly all animals. FL levels show an increase shortly after irradiation, reach peak values after ± 10-14 days (7-17) and remain elevated until 3 weeks after irradiation after which a decline to normal levels (Figure 1, mean of 4 placebo animals) is observed. Individual peak levels of FL in 5 Gy irradiated animals vary between 1780 and 6565 pg/mL with a mean (±SD) of 3719 (±1066), median of 3660 pg/mL. All animals which have been treated with GM-CSF alone or GM-CSF plus TPO have lower peak levels ($p < .0001$) and earlier normalization of the FL serum concentration. Treatment with TPO, G-CSF of TPO and G-CSF did not influence the maximal level of FL in serum (Figure 2). In general, as shown in figure 3, FL appeared inversely related with WBC, the reticulocyte percentages, the number of CD34+ cells in BM and PB and also with the number of thrombocytes in peripheral blood. Because of the fluctuation of FL levels with time, we decided to use the area under the curve (AUC) for analyses and for correlation with hematology parameters. The AUC was divided in fractions of one week, and was related to parameters per group of animals treated with the same growth factor (combination). Relations were more prominent when calculated over a 6 weeks period than over shorter intervals (Table 3). Significant relations could be demonstrated for all reconstitution parameters, including thrombocyte transfusion independence (i.e., thrombocyte count staying > 40 x 10^9/L), reticulocyte percentages reached > 1% or WBC > 1 x 10^9/L.

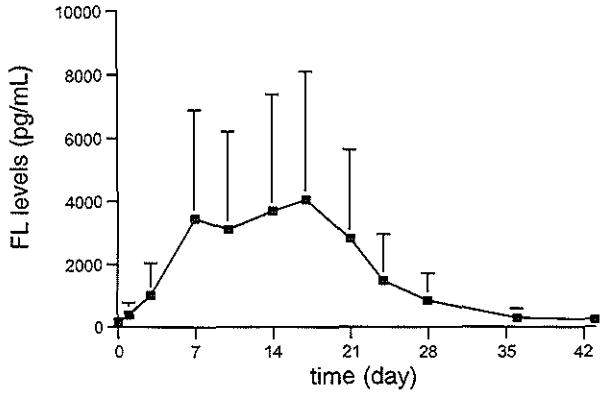

Figure 1. FL levels (vertical axis, pg/mL) as a function of time (days) after 5 Gy TBI in placebo controls (n= 4).

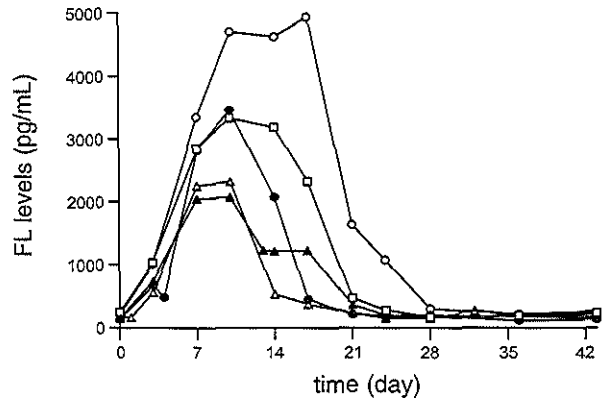

Figure 2. FL levels (vertical axis, pg/mL) after 5 Gy TBI in animals treated with several growth factor regimens. Open squares represent the mean value of 4 animals treated with 5 µg/kg rhesus TPO at day 1 iv, black filled triangles represent GM-CSF (25 µg/kg/day , days 1-14 sc) treated animals, open triangles represent the mean FL levels of 4 monkeys treated with rhesus TPO and GM-CSF in doses described above. Open circles represent the mean of FL levels of 4 monkeys treated with G-CSF 5 µg/kg/day sc, days 1-21 sc and black filled circles the mean FL level of 4 animals treated with 5 µg/kg rhesus TPO at day 1 iv and 10 µg/kg/day G-CSF days 1-14 sc. On the horizontal axis the time in days after TBI.

Furthermore, the values (AUC FL levels) after two weeks significantly correlated with the AUC after 6 weeks ($r = 0.681$, $p = .04$), which indicates a predictive value of the FL levels in the first period of myelosuppression with respect to the regeneration of hematopoiesis. The lower peak levels of FL concentrations in GM-CSF treated animals resulted in a significant difference in the AUC of FL levels two weeks after TBI ($p = .04$) compared with other growth factor regimens. After 1, 3 or 6 weeks, the difference was not significant.

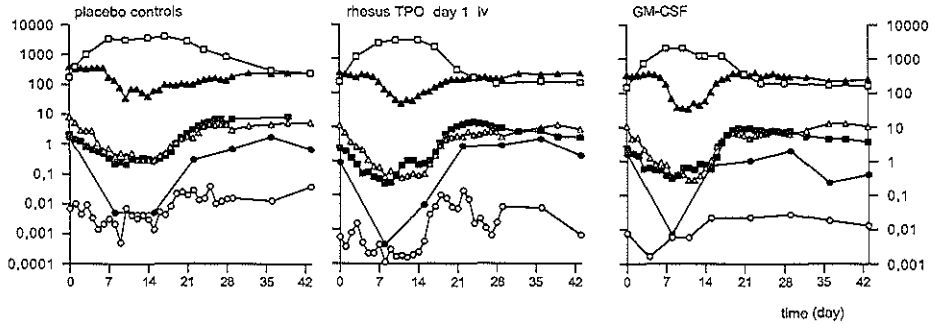

Figure 3. FL levels and reconstitution parameters after 5 Gy TBI in rhesus monkeys. Left panel: placebo controls (n=4), middle panel: rhesus TPO 5 µg/kg iv at day 1 (n=4) , right panel GM-CSF 25 µg/kg/day days 1-14 sc (n=4).
Open squares represent FL levels (pg/mL), open circles represent the absolute number of CD34+ cells in PB (x 10^6/mL), black filled circles represent the absolute number of CD34+ cells in BM aspirates (x 10^6/mL), open triangles represent the WBC (x 10^6/mL), black filled triangles represent the thrombocyte counts (x 10^9/L) and black filled squares represent the percentage reticulocytes in peripheral blood.

Table 3. Relation of the FL levels (values of cumulative areas under the curve (AUTC) per week) with regeneration parameters in myelosuppressed (5Gy TBI) rhesus monkeys. The thrombocyte number of 40 x 10^9/L is the transfusion level. WBC is white blood cell count (x 10^9/L).

FL levels	parameter	Correlation coefficient	P - value
AUTC 6 weeks	Reticulocytes > 1%	0.862	.0015
	Thrombocytes > 40	0.891	.0005
	WBC > 1	0.839	.0028
AUTC 3 weeks	Reticulocytes > 1%	0.741	.02
	Thrombocytes > 40	0.736	.02
	WBC > 1	0.792	.01
AUTC 2 weeks	Reticulocytes > 1%	0.523	n.s. (.15)
	Thrombocytes > 40	0.479	n.s.
	WBC > 1	0.535	n.s. (.14)
AUTC 1 week	Reticulocytes > 1%	0.343	n.s.
	Thrombocytes > 40	0.185	n.s.
	WBC > 1	0.303	n.s.

FL levels in stem cell transplanted rhesus monkeys (8 Gy TBI)

After TBI, individual peak FL levels, reached between 7 and 21 days after TBI, vary between 1934 and 6350 pg/mL. Mean (±SD) peak level is 3561 (± 1085) and the median is 3275 pg/mL. The period after which FL levels decline to normal levels appeared to be more prolonged than in the 5 Gy irradiated animals and was up to 5-6 weeks. In line with the animals in the myelosuppression model, the transplanted animals treated with the combination GM-CSF/ TPO have lower peak levels (mean 2252 ± 402 (n=3)) (p =.01) which are earlier reached after transplantation (all at day 7). However, this difference is not accompanied by earlier reconstitution of hematopoiesis.

In order to examine as to whether elevated FL levels precede reconstitution of hematopoiesis (to study the predictive value) the following analyses were done: absolute FL levels at day 10 and 14 were inversely correlated with WBC at day 14 (p resp. .007 and .004), FL levels at day 17 were correlated with WBC at day 21 ($p = .03$, $r = -.527$). FL levels at other time points were neither related to the number of CD34+ cells in the graft, nor to PB CD34+ cells during regeneration, and only FL levels at day 10 were related to BM CD34+ cells at day 14 ($r = -.552$, $p = .05$). Absolute FL levels were not correlated with regeneration of reticulocytes or thrombocytes. Analyses of the area under the curve of FL levels per week showed only a correlation of the AUC of 6 weeks with the moment after which the WBC are $> 1 \times 10^9$/L ($p = .004$, $r = -.859$) and this relation was not significant anymore three weeks after transplantation ($r = -.545$, $p = .17$). Furthermore, the AUC of 1 and 2 weeks were correlated with the number of CD34+ cells in the bone marrow at day 15 ($r = .829$ and p .02 and $r = .987$, $p < .0001$ respectively). The AUC of FL levels did not show any relation with the other regeneration parameters.

Discussion

Several reports have pointed at the importance of FL levels in cases of radiation accidents [13], chemotherapy [9, 10], Fanconi anemia or acquired aplastic anemia [8]. FL levels are increased during bone marrow aplasia, and FL levels are considered predictive regarding bone marrow reconstitution after myelosuppressive therapy. FL levels are not increased in diseases affecting single blood lineages (for instance in case of only neutropenia or only thrombocytopenia), in contrast to lineage-specific growth factors such as G-CSF, which is elevated in severe neutropenia [23]. In addition to the studies in humans [8, 9, 10], this relation has also been demonstrated in non-human primates [13]. In our previous studies [24, 25, 26,27], several growth factor(s) (combinations) have been tested for efficacy in a rhesus monkey myelosuppression as well as in a bone marrow transplantation model. Studies were performed using highly codified and standardized methods allowing for intercomparison of analyses of several studies. FL levels were measured in serum once or twice weekly during the study period of 6 weeks after TBI and have been correlated with both CD34+ cells in bone marrow and peripheral blood as well as with conventional reconstitution parameters such as WBC, reticulocyte and thrombocyte counts. The inverse relationship of FL levels with WBC regeneration was confirmed in both models. In the myelosuppression model (5 Gy (X-rays) TBI, resulting in a period of three weeks of pancytopenia), also a reciprocal relationship between FL levels and regeneration of reticulocytes and thrombocytes was demonstrated. This relationship could not be demonstrated in transplanted animals, although there is a negative correlation with the number of BM CD34 positive cells. As we have shown before in the 5 Gy model [30, chapter 4], relations exist between CD34+ cells in bone marrow and mature PB cells (thrombocytes, reticulocytes and white blood cells) during reconstitution of hematopoiesis. However, in the rhesus monkey bone marrow transplant model, only a relation between

numbers of BM CD34+ cells en PB CD34+ cells could be demonstrated, but not with mature PB cells.

FL levels were not influenced by administration of TPO and/or G-CSF, whereas treatment with GM-CSF (25 mcg/kg/day sc) resulted in lower FL levels in both models ($p<.0001$ (5 Gy model) and $p = .01$ (8 Gy model). The GM-CSF treated monkeys displayed more prominent reconstitution of both BM and PB CD34+ cells at day 15 ($p= .02$ and $p= .04$ resp.) after myelosuppression and with BM CD34+ cells at days 8 and 22 after transplantation ($p= .01$ and $p= .03$ resp.). This was related to accelerated regeneration of hematopoiesis in the myelosuppression model, especially when co-administered with TPO [24], but without improved reconstitution of hematopoiesis in the BMT model [27].

Soluble and membrane bound FL are produced by various sources throughout the body. High levels of preformed FL are present in T and B lymphocytes and CD34+ hematopoietic precursors and, dependent on the status of the stem cellcompartment, FL is released from intracellular stores [16]. Only during severe aplasia, levels of FL mRNA increase in BM and thymus, suggesting that lymphohematopoietic organs are the major site of FL upregulation in response to BM failure [16]. By analogy to the feed-back mechanism in which an "heme-containing oxygen-sensor" regulates expression of erythropoietin in the kidney [28], a putative "stem cell sensor" responding to bone marrow hypoplasia has been proposed to control the level of FL [10]. This is also suggested by the inverse correlation of FL levels with immature BM cell numbers during reconstitution in both the myelosuppression and the transplant model, and, in addition, may be concluded from figure 4. Whether a similar mechanism is involved in the regulation of TPO levels, i.e., a clearance mechanism mediated by binding to receptors of target cells, plays a role, is an attractive additional hypothesis, but not known. Due to the low number of receptors on CD34+ cells, this mechanism is difficult to examine by flow cytometry. Remarkable are the lower FL levels in GM-CSF treated animals (represented by "standing" open triangles in figure 4). This may be due to an effect of GM-CSF on the FL production by any of these cell types, but, alternatively, may be mediated by the more rapid reconstitution in the GM-CSF treated animals. Although GM-CSF does not affect the most primitive cells, it has been shown to stimulate more mature CFU populations [29], which still may be CD34+. It was not possible to relate FL levels with GM-CFU because of lack of sufficient data immediately after TBI due to shortage of nucleated cells in bone marrow aspirates during the first two weeks after TBI.

Contrary to the myelosuppression model, following BMT a relationship between reconstitution of PB cells and that of BM CD34+ cells was difficult to establish [30]. This may be partly due to the more restricted experimentally induced variation in the transplanted monkeys. It has also been shown that hematopoietic growth factors do stimulate regeneration after myelosuppression, but lack effect after BMT [27]. Hypotheses for this effect are: the disturbed quantities of essential primary and secondary cytokines, changed receptor status, loss of stem cell self-renewal capacity and/or intracellular pathways due to the higher irradiation dose [31,32]. Prolonged stromal damage after bone marrow transplantation (up to 12 years) with decreased Colony forming units—fibroblasts

(CFU-f) and significantly reduced levels of long-term culture-initiating cells (LTC-IC) [32] are also supposed to play a role.

We conclude that FL levels after myelosuppression as well as after transplantation are inversely related to regeneration of bone marrow CD34+ cells and WBC. After myelosuppression, FL levels are also negatively related to the regeneration of reticulocytes and thrombocytes (probably an indirect effect of stem cell regeneration), whereas this latter relation is absent after bone marrow transplantation. FL shows lower maximum levels after treatment with GM-CSF in both the myelosuppression and the bone marrow transplantation model.

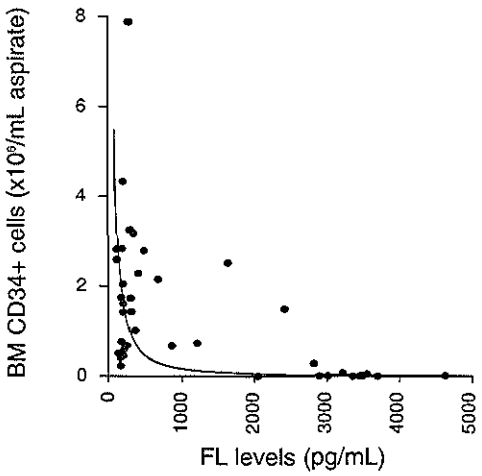

Figure 4. Weekly measured circulating FL levels (pg/mL)(horizontal axis) versus concurrent BM CD34+ cell numbers (10^6/mL aspirate) (vertical axis) in 5 Gy irradiated animals during week 1-6 after TBI. Open squares represent averages of 4 animals treated with rec.human TPO 10 µg/kg SC during 21 days, black squares represent averages of 4 animals treated with G-CSF, 5 µg/kg SC during 21 days. Open circles represent mean values of 4 placebo treated animals, black circles represent means of 5 animals treated with rhesus TPO 5 µg/kg IV on day 1, open triangles the means of 4 GM-CSF treated animals (25 µg/kg, SC, 14 days), black triangles the means of 3 animals treated with 10 µg/kg G-CSF for 14 days SC. Right pointing open triangles represent the means of 4 animals treated with human TPO and G-CSF SC for 21 days in doses described above. Correlation coefficient is -.515, p = .0005.

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CHAPTER 7

Preclinical evaluation of recombinant thrombopoietin administration to platelet donors: highly efficacious in both donor and recipient, but jeopardized by (avoidable) neutralizing antibodies to thrombopoietin in the donors

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Abstract

Due to increasing dose-intensive cancer chemotherapy and the more frequent use of stem cell transplantations, there is an increasing demand for thrombocyte transfusions. Frequent transfusions carry the risk of immune reactions and transmission of bacterial and viral infections. Prevention of these risks includes intensive donor screening (infectious disease marker testing) and leukodepletion of transfusions. A single or a restricted number of HLA matched donors pretreated with a c-mpl agonist (thrombopoietin, TPO) to increase the thrombocyte yield with smaller volumes of donated blood or more efficient apheresis procedures, might be a cost-effective solution. We studied the efficacy of 14 consecutive TPO stimulated thrombocyte transfusions in two rhesus monkey recipients of an autologous stem cell transplant and one radiation control monkey, which all endured a 3-6 week period of profound pancytopenia in the recovery phase after total body irradiation (TBI). The results were compared with 68 consecutive conventional thrombocyte enriched plasma transfusions and 26 consecutive whole blood transfusions in ten concomitant monkeys with a similar duration of pancytopenia,

Donor monkeys displayed a rapid increase in thrombocyte numbers with maximum values of 24 and 33 x 10^{11}/L at 12 and 13 days after initiation of TPO treatment, which is 10 times elevated relative to normal values. The stimulated thrombocyte transfusions had a maximum volume of 15 mL whole blood and were standardized at a thrombocyte number of 10^{10}/transfusion similar to the conventional control transfusions. The small volume TPO stimulated transfusions proved to be at least as effective as conventional whole blood or thrombocyte transfusions resulting in a significantly larger increment of 42 ± 16 thrombocytes x 10^9/L as opposed to 28±16 and 25±14 after conventional thrombocyte and whole blood transfusions, respectively, in concomitant recipients. Neutralizing antibody formation to TPO observed in the donor monkeys could be prevented by restricting the total dose of TPO without loss of efficacy.

It is concluded that TPO stimulation results in highly elevated thrombocyte levels, which can be used safely and effectively for transfusions in irradiated thrombocytopenic recipients. Apart from (avoidable) neutralizing antibody formation to TPO, the procedure did not cause adverse effects in the donors and was well tolerated. Use of a c-mpl agonist in human platelet donors might increase the safety of thrombocyte transfusions, allow the more frequent use of HLA matched family donors, and reduce costs.

Introduction

Thrombopoietin (TPO) is the ligand for the cytokine receptor c-mpl and the physiological regulator of platelet production. *In vitro* studies, in which the effect of recombinant TPO on megakaryocyte formation and development was assessed, have shown that TPO is involved in the proliferation, differentiation and maturation of megakaryocytic progenitor cells up to the induction of proplatelet formation [1, 2, 3, 4, 5, 6, 7]. Thrombocytes formed in culture under the influence of TPO were shown to be of normal morphology and

completely functional [8, 9]. *In vitro*, TPO has been shown to be involved in thrombocyte activation. At high concentrations, i.e. about 1000-fold above normal plasma levels, TPO can directly induce thrombocyte activation and aggregation *in vitro*[10]. Its role as the prime regulator of platelet production has been shown in vivo by generating mice deficient for either TPO or c-mpl [1, 11, 12].These mice have a more than 80-90% reduction in thrombocyte counts, with low numbers of megakaryocytes of low ploidy. In vivo studies which involve TPO administration to normal mice, non-human primates and humans have shown that TPO enhances thrombocytopoiesis; the number of circulating thrombocytes increased in a dose-dependent manner [13, 14, 15, 16, 17, 18, 19].
In cancer patients, thrombocytopenia frequently occurs as a result of radiation or chemotherapy. Administration of TPO in combination with moderate chemotherapy regimens was well tolerated and induced a dose-dependent increase in functionally normal thrombocytes, thrombocyte recovery was enhanced and thrombocyte nadirs were improved [20, 21, 22, 23]. However, TPO administration after bone marrow transplantation or in combination with high-dose chemotherapy with peripheral blood progenitor support was not effective [24, 25, 26]. Therefore, until now, thrombocyte transfusions remain a necessity to prevent and treat bleeding during thrombocytopenia after bone marrow transplantation / myelosuppression. In the past decade, the use of thrombocyte transfusions has increased greatly and is likely to escalate because of the risks of thrombocytopenia in patients receiving dose-intensive cancer chemotherapy, the increased use of hematopoietic progenitor cell transplantation, and the prevalence of human immunodeficiency virus infection. Despite marked advances in procedures for ensuring the safety of thrombocytes, including intensive donor screening, infectious disease marker testing and increased use of expensive leukodepletion techniques, thrombocyte transfusions carry a risk for immune disorders and transmission of bacterial or viral infections [27]. To halt bleeding in patients with severe thrombocytopenia due to bone marrow failure, it is desirable to achieve a post-transfusion blood thrombocyte count of 40 x 10^9/L. Based on calculations of corrected count increments, each 1 x 10^{11} thrombocytes transfused will increase the blood thrombocyte count approximately 10 x 10^9/L per each square meter of patient body surface area [28]. The post-transfusion thrombocyte count likely will be lower in sick patients, e.g., those with sepsis, amphotericin B plus antibiotic therapy, splenomegaly, GvHD, or if thrombocytes are lost due to leukofiltration before transfusion [28]. Thrombocyte transfusions withdrawn from a single donor are safer with regard to immune reactions and infections but require the availability of sufficient numbers of thrombocytes of the donor. We evaluated the feasibility of TPO administration to normal healthy rhesus monkey thrombocyte donors of which repetitively blood was withdrawn in order to treat thrombocytopenia in lethally irradiated and bone marrow transplanted rhesus monkeys.

Materials and methods

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

Total body irradiation: Rhesus monkeys were irradiated with a single dose of 9 Gy TBI delivered by a 6 MV linear accelerator (Siemens). During irradiation the monkeys were anesthetized and placed in a perspex frame. The dose rate was 31 cGy/minute and the focus-skin distance 2 meter. The irradiation is given in two parts, half of the dose in *anterior-posterior* (AP) position, and the other half in PA position. The dose was confirmed by means of TLD fixed on the skin of the monkey. In keeping with a relative biological effectiveness (RBE) of 0.85, the dose was adjusted to 9 Gy to be equivalent to the dose of 8 Gy X-rays used before [24, 29] to enable standardization of results.

Supportive care: Two weeks before TBI, the monkeys were placed in a laminar flow cabinet and the gastrointestinal tract was selectively decontaminated by giving oral Ciprofloxacin (Bayer, Mijdrecht, the Netherlands), Nystatin (Sanofi BV, Maassluis, The Netherlands) and Polymyxin B (Pfizer, New York, NY, USA). This regimen was maintained until leukocyte counts exceeded 10^9/L. Systemic antibiotics were given when leukocyte counts dropped below 10^9/L, in most cases as a combination of ticarcillin (SmithKline Beecham Farma BV, Rijswijk, The Netherlands) and cefuroxim (Eurobase BV, Barneveld, The Netherlands), as guided by fecal bacteriograms. All monkeys received iron supplementation; 0.5 mL Imferon intramuscularly (Fe(III) 50 mg/ml; Fisons Pharmaceuticals, Loughborough, England) for 5 days before irradiation [30]. Dehydration and electrolyte disturbances were treated by appropriate fluid and electrolyte administration subcutaneously.

Regular transfusions

Regular transfusions withdrawn from male rhesus monkeys were provided by the Biomedical Primate Research Center (BPRC) in Rijswijk, The Netherlands. The monkeys in experiment received irradiated (15 Gy) citrate anticoagulated blood derived platelet transfusions whenever platelet counts reached values below 40 x 10^9/L, irradiated (20 Gy) packed red cells whenever the hematocrit was below 20% and the monkeys received whole blood transfusions in case of simultaneous occurrence of both transfusion thresholds. Characteristics of the regular transfusions are shown in Table 1.

Table 1. Characteristics of regular thrombocyte and whole blood transfusions and TPO stimulated transfusions

	Regular trombocyte transfusions (n = 68)	Regular whole blood transfusions (n = 26)	TPO stimulated transfusions (n= 14)
Number of thrombocytes ($x10^9$) per transfusion	9.2 ± 3.2	11 ± 3.1	9.1 ± 4.9 (citrate) 13.4 ± 5.1 (EDTA)
Increment in thrombocyte count (min. – max.)	28 ± 16 (-7 - 81)	25.4 ±13.8 (-3 - 64)	42 ±16 (13 - 68)
Volume of the transfusion (mL)	31.7 ± 6.7	42.5 ±5.2	9.3 ±2.7
Number of RBC ($x 10^{12}$) per transfusion	0.002 ±0.001	0.184 ± .046	0.052 ± 0.017
Increment in Ht (%)	-1.5 ± 3.7	6.5 ± 2.3	-0.3 ± 1.9

TPO stimulated transfusions

The threshold for transfusion was identical to that of the regular transfusions. Citrate anticoagulated blood was withdrawn by means of syringes and needles flushed in advance with Hank's buffered Hepes solution (HHBS) containing 2% Fetal Calf Serum (FCS)(GIBCO BRL NV Life Technologies SA, Merelbeke, Belgium) to coat the material with protein in order to prevent thrombocyte adherence to the material. The quantity of citrate anticoagulated blood procured, with a maximum of 15 mL per transfusion, was made dependent on the thrombocyte count in order to achieve a standardized transfusion of 10 x 10^9 thrombocytes. The blood was given as a 'mini' whole blood transfusion after irradiation with a dose of 20 Gy g rays delivered by a 137Cs source (Gammacell, Atomic Energy of Canada, Ottawa, Canada).

Growth factor stimulation

One mL vials containing 0.930 mg/mL recombinant full length rhesus monkey TPO produced by Chinese hamster ovary cells were supplied by Genentech Inc (South San Francisco, CA, USA). The dose; 5 µg/kg, was diluted to a volume of 1 mL with phosphate-buffered saline (PBS)/0.01% Tween 20 before administration. TPO was subcutaneously administered in schedules indicated under Results and depicted in the Figures displaying the results. Because of previous demonstrated iron deficiency after TPO administration [30], all monkeys received iron supplementation; 0.5 mL Imferon intramuscularly (Fe(III) 50 mg/ml; Fisons Pharmaceuticals, Loughborough, England) alternately in the left or right thigh, simultaneously with the growth factor administration.

Study groups: Studies with rhesus monkeys are done sequentially using highly codified and standardized methods, including radiation and placebo controls at regular intervals.

Assignment of monkeys to experimental protocols is at random. Among the three recipients of TPO stimulated blood transfusion, two monkeys were included in a follow-up study directed at antagonism of FL and TPO [31] and recipients of *in vivo* FL and TPO stimulated, isolated CD34+ cells as previously described [29], and one monkey was a 9 Gy radiation control, all three expected to have protracted periods of thrombocytopenia. The ten control monkey recipients of conventional whole blood and platelet enriched plasma transfusions were included in either the same study or in concomitant studies and also recipients of CD34+ cells with a similar duration of thrombocytopenia.

Hematological examinations: Complete blood cell counts (which also includes recovery of thrombocyte transfusions) were measured once daily in EDTA anticoagulated blood between 8 and 9 AM using a Sysmex F-800 hematology analyzer (Toa Medical Electronics co., LTD., Kobe, Japan). For reticulocyte measurements 5 µL EDTA blood was diluted in 1 mL PBS/EDTA (0.5M)/azide (0.05% wt/vol) and one mL of a thiazole orange dilution was added in a final concentration of 0.5 µg/mL. Measurements were done on a FACScan (Becton Dickinson) and analyzed using the Reticount software (Becton Dickinson). Serum of donors and monkeys in experiment was weekly collected and frozen at - 20 ºC.

Measurement of aggregation and adenosine triphosphate release
Five hundred mL dextran T 500, 6 % solution in 0.9% NaCL (Pharmacia, Woerden, The Netherlands) was added to 5 mL citrate anticoagulated blood (B&D vacutainer, 0.105M, Plymouth, UK) and was kept under an angle of 45 degrees at room temperature, until platelet rich plasma (prp) had developed. PRP was removed and autologous platelet poor plasma was added to reach a final concentration of 200 x 10^9 thrombocytes/mL PRP suspension. Platelet poor plasma of the same animal was prepared by centrifugation at 2300 rpm during 10 minutes at 4°C . Trombocyte aggregation and secretion using Chrono-Lume reagent was recorded with the Chrono-Log lumiaggregometer (Chronolog via Kordia supplies, Leiden, The Netherlands). Thrombocytes (450 µL PRP plus 45 µL Chrono-Lume reagent) were stimulated by addition of 5µL collagen (Type 1, from bovine achilles tendon, Sigma-aldrich chemie, Zwijndrecht, The Netherlands) 0.75, 1.25, 2.5, 5 or 10 µg/mL final concentration or thrombin resp. 0.25, 0.5, 1.0 or 2.0 U/mL final concentration.

Detection of TPO antibodies
The concentration of anti-TPO antibodies was measured by using a sensitive enzyme-linked immunosorbent assay (ELISA) with a detection limit of 10 pg/mL. Microtiter Covalink 96 wells plates (Nunc, Rockslide, Denmark) were activated by treatment with a dithiobis (succinimidyl proprionate) (Pierce, Rockford, Illinois, USA) solution in methanol, during 30 minutes at room temperature. Afterwards, wells were incubated with 50 µL (1 µg/mL) recombinant full-length rhesus monkey TPO produced by Chinese hamster ovary cells (Genentech, South San Fransisco, CA) in PBS (pH 7.4) during 2 hours at room temperature (RT). Non-specific binding sites were blocked with a PBS-albumin solution

(1% BSA, Sigma A7030) , 250 µL per well and plates were stored overnight at 4 °C. Before use, plates were washed 6 times with 200 µL PBS/Tween-20 (0,05%) solution at room temperature. Subsequently, 50 µL serum 1/100 diluted in PBS-Tween-1% BSA was added to the wells and the plate incubated for one hour at RT. After a 6 times wash, Rabbit-anti-rhesus-Ig-PO (A2054, Sigma, Zwijndrecht, The Netherlands) diluted 1/35,000 according to manufacturer's prescriptions was added, followed by an incubation of 1 hour at RT. After incubation wells were washed 6 times with 200 µL PBS/Tween solution and 100 µL TMB substrate (Kirkegaard and Perry Laboratories, Maryland) was added. All incubations were performed while shaking the plates (75 rpm). The colorimetric reaction was stopped by addition of 100 µL 1M Ortho-Phosphoric acid (Merck, Darmstadt, Germany) and the absorbance at 450 nm was measured in a microplate reader (Bio-Rad Laboratories Inc. Hercules, CA, USA). For comparison in each experiment a dose response curve was incorporated by coating a set of wells with rHuman TPO and one with rRhesus TPO, incubating with dilutions of mouse monoclonal anti-Human-TPO (R&D systems, Minneapolis, MN, USA) and detection with a 1/10,000 dilution of GAM-PO (A8924, Sigma, Zwijndrecht, The Netherlands). Samples were measured in quadriplicate. The results are expressed as optical density (OD) .

Statistical analysis: Mean, median and range of numeric variables were calculated by the Excell spreadsheet program. Standard deviations were calculated and are given in the text and the figures on the assumption of a normal distribution. Fisher's Exact Test was used for categorical variables. P values of <0.05 are considered statistically significant

Results

Peripheral blood analysis of donor animals

Approximately 12-13 days after the start of the TPO administration, thrombocyte counts reached a maximum ten-fold increase, which was stable as long as TPO administration was continued every other day (Figure 1). Administration of TPO three times a week (Monday-Wednesday-Friday) resulted in a smaller increase in absolute counts, while administration of TPO twice a week was less effective, resulting in thrombocyte counts between 600 and 1000 x 10^9/L. Also a reticulocytosis (Figure 1) occurred, which can be explained from the supplementation of iron, TPO administration [30], and from the withdrawal of blood. Leukocytes did not vary abnormally in number (Figure 1, lower panel) and neither did the number of CD34-positive cells in the peripheral blood (data not shown). To prevent anemia and hypovolemia of the donors, a maximum of 15 mL of blood was withdrawn periodically. From these two donors a cumulative volume of respectively 97 and 31 mL was withdrawn within a month, which did not result in a decrease of the hematocrit of the donors (Figure 2). A significant decline in hematocrit to a lower limit of 25% was set as criterion to discontinue blood withdrawal. Approximately 40 and 50 days after the start of the TPO stimulation, donor monkeys developed a thrombocytopenia

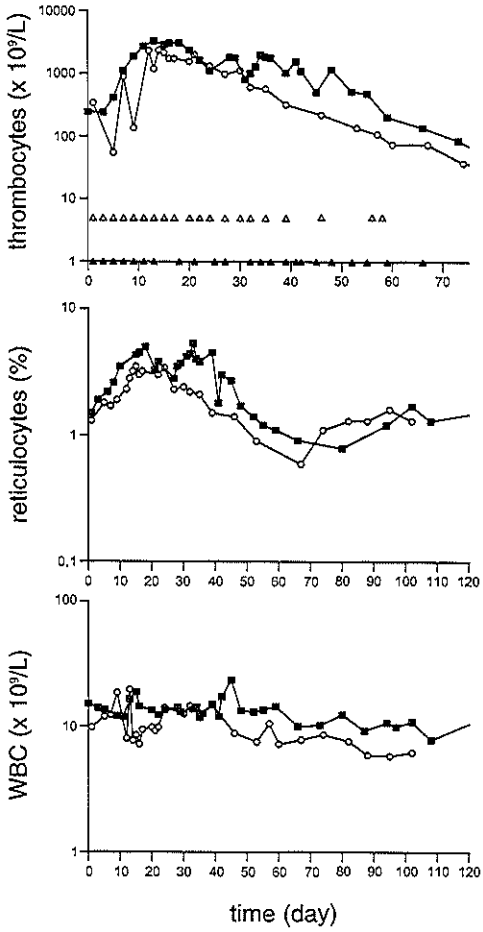

Figure 1
Upper panel: Thrombocyte levels (x 10^9/L) in peripheral blood of the two donor monkeys (y-axis). On the X-axis the time in days. Animal 97117 is represented by black filled figures; squares represent thrombocyte counts whereas triangles represent the days at which TPO was administered. Animal 97361 is represented by open figures; circles represent thrombocyte counts whereas triangles represent TPO administration.

Middle panel: On the Y-axis the percentage reticulocytes in the two donors during TPO stimulation and blood donation. Animal 97117 is represented by black filled symbols whereas animal 97361 is represented by open symbols. On the X-axis the time in days.

Lower panel: On the Y-axis the white blood cell counts (WBC, x 10^9/L) in peripheral blood of donor monkeys during TPO stimulation. Animal 97117 is represented by black filled symbols whereas animal 97361 is represented by open symbols. On the X-axis the time in days.

reaching a nadir of 24 x 10^9/L and 21 x 10^9/L after 95 and 97 days respectively (Figure 4). From earlier experience with rhesus TPO in normal monkeys, it was suspected that this thrombocytopenia was due to neutralizing antibody formation and eventually transient. One of the two donor monkeys reached thrombocyte levels > 100 x 10^9/L at 122 days, after which systematic monitoring was discontinued, the other displayed a more gradual rise in platelet numbers and reached 60 x 10^9/L after 262 days. A third animal, which was treated with TPO in a pilot study directed at the feasibility of of TPO stimulated thrombocyte transfusions, developed a nadir of 29 x 10^9/L at day 127 and reached a platelet count of 141 x 10^9/L after 259 days. Systematic monitoring was discontinued after half a year due to transfer of the monkeys to a more animal friendly group housing; eventually all three monkeys reached pre-treatment thrombocyte levels as concluded from periodic routine examinations. In these monkeys, the thrombocytopenia did not cause bleedings, demonstrated by the absence of purpurae. Cross-reacting antibodies against rhesus TPO were the presumed cause and the presence of antibodies against TPO in serum samples of the animals was subsequently examined (vide infra).

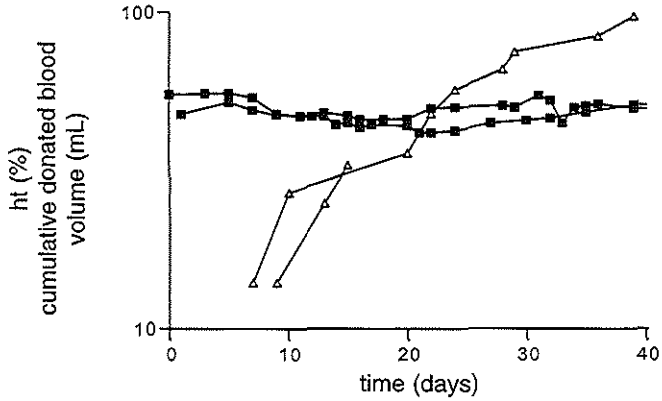

Figure 2. On the Y-axis the hematocrit (%) (squares) and the cumulative blood volume (mL) (triangles) which was withdrawn during the 6 weeks (time period on the X-axis in days) of the experiment. Data of two animals are separately represented.

Efficacy of TPO stimulated thrombocyte transfusions versus regular transfusions

To study the efficacy of TPO stimulated thrombocyte transfusions, a comparison in thrombocyte increment was performed between the TPO stimulated transfusions and conventional thrombocyte and blood transfusions. The regular transfusions included either thrombocyte-enriched plasma suspensions or whole blood transfusions, which had a volume of 31.7 ± 6.7 and 42.5 ± 5.2 mL respectively. The experimental 'mini' whole blood transfusions had an average volume of 9.3 ± 2.7 mL (range 5-14mL) (Table 1). The number of thrombocytes as measured in the citrate anticoagulated 'mini' transfusions was similar to the number of both types of regular transfusions. Although the number of RBC was approximately 25-fold higher than that of the thrombocyte-enriched plasma suspensions, the hematocrit increments did not differ significantly after transfusion. The thrombocyte increment was more pronounced after the TPO stimulated 'mini' transfusions (42 ± 16x 10^9/L) compared to the conventional transfusions (28 ± 16 and 25 ±14, respectively) ($p < .01$). The transfusion volume was based on the platelet counts in citrated anticoagulated blood, which was withdrawn by means of protein-coated syringes. Despite the coating, a discrepancy was noted with the thrombocyte counts of simultaneously sampled EDTA blood withdrawn without interference of a syringe. This likely results from greater adherence of TPO activated platelets to the syringe system, but does not affect our comparison, since all values for conventional transfusions are also obtained from citrated blood.

Table 2 shows the thrombocyte increments after the different transfusions in the first five weeks after TBI. The increment was stratified per week because of the dependence of the thrombocyte increment on host factors such as the rapid decline in thrombocyte numbers in the second week after irradiation, followed by a slow onset reconstitution of thrombocyte numbers in the weeks to follow, in addition to administration of antibiotics, and thrombocyte consuming conditions such as septicaemia and/or fever which are more frequent shortly after irradiation than after three weeks. Although the difference is

moderate, transfusions given five weeks after TBI resulted in more prominent increments than transfusions given at two weeks after TBI (p = .05). This effect is not significant after the 'mini' transfusions, likely due to greater efficacy in weeks 2 and 3 relative to the conventional transfusions. Also in the stratified analysis, the TPO stimulated transfusions resulted in greater increments than the conventional transfusions.

Table 2. Platelet increment (x 10^9/L) after transfusions per week after TBI

	Week 2	Week 3	Week 4	Week 5
Regular trombocyte transfusions	25 ± 15 (n=20)	27 ± 13 (n=22)	29 ±22 (n=15)	35 ±14 (n=11)
Regular whole blood transfusions	- (n=8)	19 ± 10 (n=9)	22 ± 19 (n=9)	26 ± 11
TPO stimulated transfusions	41 ± 11 (n=4)	38 ± 16 (n=3) (56,25,34)	38 ± 23 (n=3) (46,16,56)	48 ± 19 (n=4)

Measurement of aggregation

Thrombocyte aggregation and ATP-release in response to a specific agonist such as collagen of in vivo stimulated thrombocytes versus thrombocytes of normal rhesus monkeys showed that in vivo stimulation with rhesus TPO results in a significant increased aggregation and secretion of ATP (Figure 3). In contrast, aggregation and secretion was not significantly different for thrombin (data not shown). TPO stimulated thrombocytes do not aggregate spontaneously, but agonist induced aggregation of TPO stimulated thrombocytes was more pronounced than that of normal platelets.

Adverse effects

Subcutaneous TPO administration to the donors was without local adverse effects at the injection sites and not associated with thrombosis or any other adverse effect. However, similar to PEG-rHuMGDF treated human donors [32], monkey donors developed thrombocytopenia with thrombocyte nadirs around 10% of normal values. The low thrombocyte counts were not accompanied by an increased bleeding risk, demonstrated by the absence of petechiae, purpurae, or gingival bleedings. Figure 4 shows the relation between the number of thrombocytes and the level of antibodies against TPO in five monkeys, including two, which had not served as thrombocyte donors. In three of these monkeys, antibody formation resulted in profound thrombocytopenia. In the other two, which were on a more restricted dose scheduling, antibody formation, if any, did not result in decreased platelet counts. The presence of the antibodies was temporary, and thrombocyte counts gradually became normal inversely related to the antibody titers and, thus, it is concluded that the antibodies generated by exogenous TPO administration cross-reacted with endogenous TPO. Endogenous TPO levels apparently were insufficient, or endogenous TPO was insufficiently antigenic, to sustain antibody formation.

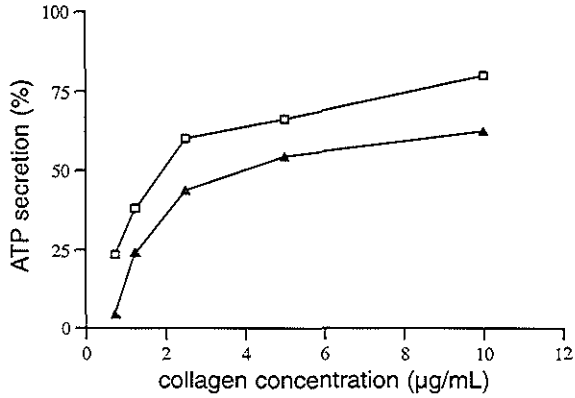

Figure 3. ATP secretion (Y-axis) of in vivo stimulated thrombocytes (triangles) and thrombocytes of normal rhesus monkeys (squares) after stimulation with collagen in several concentrations (X-axis) (mcg/mL).

Avoidance of antibody formation

It was subsequently investigated as to whether further restriction of the dose schedule could effectively prevent antibody formation, as was suggested by the results displayed in Figure 4. To this end, we took advantage of mathematical model simulations demonstrating that the total dose of TPO could be reduced by its administration on 4 consecutive days without loss of efficacy [33] Two monkeys received such a dose schedule repeatedly with 40 day intervals. Figure 5 demonstrates that during the first two dose schedules, antibody titers were not detectable, while the efficacy of TPO was only slightly diminished compared to the more intensely treated monkeys detailed above. Only when this dose schedule was applied for the third time, (very low) antibody titers were detectable which did not jeopardize TPO's efficacy and did not result in decreased platelet counts. Lack of neutralizing antibodies following restricted dose schedules (data of Figures 4 and 5) is statistically significant ($p<0.03$).

Discussion

In this study, we compared the efficacy of TPO stimulated thrombocyte transfusions and regular thrombocyte containing transfusions. Transfusions were standardized to contain 10^{10} thrombocytes per transfusion, which is equivalent to (a much larger volume of) conventional transfusions. Two normal donor rhesus monkeys were stimulated with 5 µg/kg rhesus TPO subcutaneously every other day, which resulted in a rapid increase in thrombocyte numbers with maximum values of 24×10^{11} and 33×10^{11} at days 12 and 13 after initiation of TPO treatment. These maximum values are approximately 10 times elevated relative to normal values. As has been shown before, TPO facilitates thrombocyte activation by specific agonists, but does not induce thrombocyte aggregation

by itself [20, 34, 35]. This was confirmed by the lack of symptomatic thrombotic events. Although the donor monkeys were repeatedly bled during the observation period, the hematocrits ranged from 42-52 and from 45-57, respectively, which is within the normal to slightly elevated range for monkeys. Leukocyte counts remained essentially normal

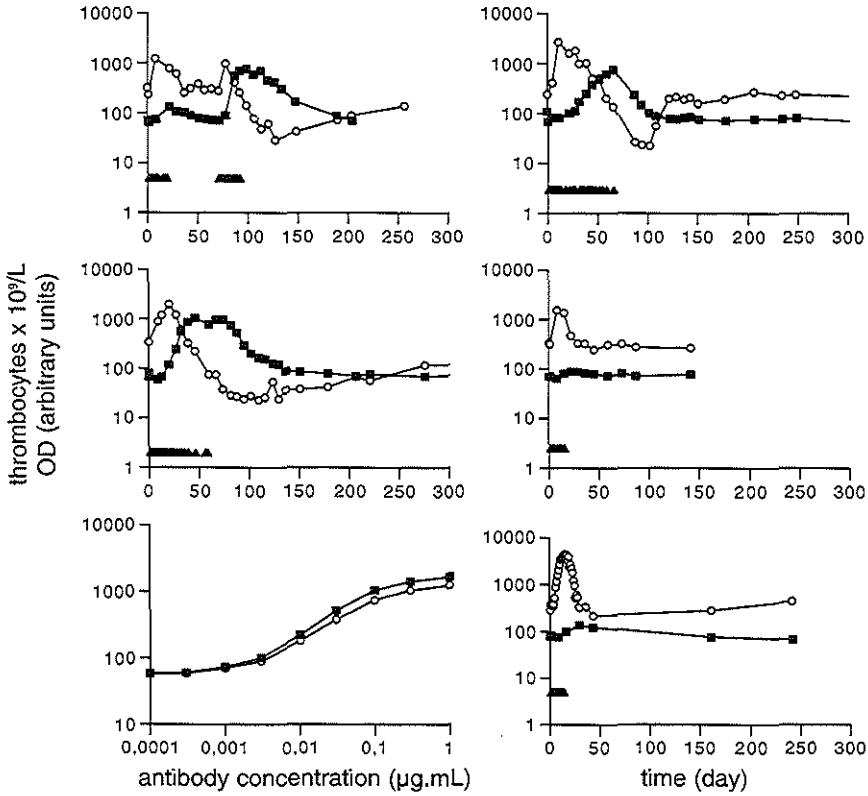

Figure 4 Antibody levels (filled squares) in 5 normal rhesus monkeys stimulated with rhesus TPO 5 µg/kg s.c. at alternate days during several periods of time (filled triangles; each triangle is a dose). Thrombocyte counts are shown by open circles. Five figures represent five normal animals, three out of five show neutralizing antibody formation, whereas from the other two, which both were on a limited dose schedule, very low antibody levels were detected but without resulting thrombocytopenia. The upper right panel and the left middle panel show results of monkeys used as the thrombocyte donors in this study. The upper left panel shows the result of the first animal used in the pilot study for the thrombocyte transfusions The right middle panel shows the results of an animal which was stimulated several times in a dose-scheme developed to avoid antibody development and in the lower right panel results are shown of an animal which had been stimulated with TPO with CD34+ cell mobilization as an end point. The lower left figure shows a standard curve of Mouse-anti-human-TPO (black circles) and Rabbit-anti-rhesus-Ig-PO (open circles) against a titration of rhesus TPO.

without signs of infection and a normal body temperature. Signs of hypovolemia were not observed in the donor monkeys, both TPO administration and repeated blood sampling were well tolerated. The increment in the thrombocyte counts of the recipients resulting from the TPO stimulated 'mini' whole blood transfusions was greater than that of the conventional controls. Based on the standardized citrate anti-coagulated blood counts, the TPO stimulated thrombocytes were therefore more efficacious. Although statistically significant, it will require a prospective, randomized study using the same recipients of alternate conventional and TPO stimulated transfusions to evaluate this difference further. We therefore conclude that the TPO stimulated transfusion were at least as effective as the conventional ones. We further note that the well documented facilitated thrombocyte activation by TPO [10, 36, 37, 38, 39] may have caused variations in standardization of thrombocyte numbers due to variation in adherence and may also be relevant when the procedure is extended to preparing thrombocyte-enriched plasma. The TPO induced facilitated aggregation by specific agonists might be of advantage in thrombocytopenic patients with increased bleeding risks.

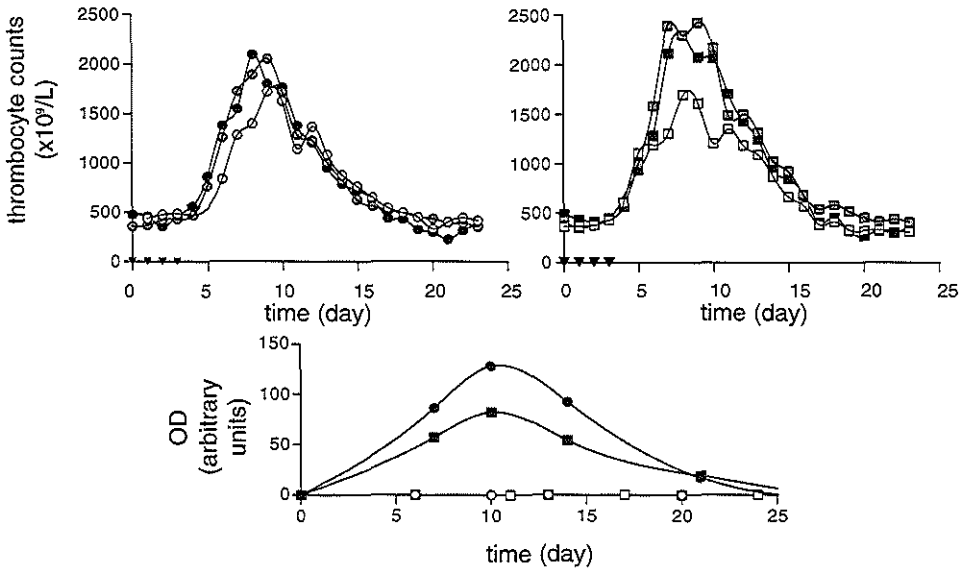

Figure 5. Efficacy (upper panels) and antibody titers (lower panel) of of a total of 6 cycles of 5 µg/kg sc rhesus monkey TPO administered on 4 consecutive days in 2 monkeys receiving 3 cycles each. The interval between the first and the second cycle was 4 months, that between the second and the third 3 months. Symbols: upper panel: triangles represent administration of TPO. Data of the two monkeys are shown, the first monkey by circles, the second by squares. The open symbols represent the first stimulation with TPO, the grey symbols the second, and the black ones represent results after the third cycle; Lower panel: resulting antibody titers, corresponding symbols

In this animal model, which has been demonstrated to show similar thrombocyte kinetics as in humans [40], 'mini' whole blood transfusions are feasible to counteract thrombocytopenia and this might also be feasible for HLA-matched family donors. For other donors however, leukocyte filtration and radiation remain necessary to prevent alloimmunization. This problem of filtration/ irradiation might be avoided by collection of autologous thrombocytes. This approach has been tested by Bentley et al. [41], who show that autologous recombinant human TPO stimulated platelets, harvested by apheresis and cryopreserved, can be safely used in patients treated with high-dose chemotherapy and peripheral blood progenitor cell transplantation. In this study, the adverse effect of antibody formation was not observed, likely due to TPO administration within an immunosuppressive chemotherapy scheme. It has recently been shown in healthy apheresis donors that a single injection with PEG-rHuMGDF resulted in greater platelet yields and shorter apheresis procedures [42]. These stimulated thrombocytes were transfused in thrombocytopenic patients and resulted in greater platelet count increments and prolonged transfusion free intervals compared to conventional platelet transfusions [43]. This clinical study, that, however, did not standardize platelet numbers transfused, corroborates the results of our preclinical evaluation.

In the donor rhesus monkeys, a thrombocytopenia was observed due to anti-TPO antibodies caused by repetitive administration of rhesus TPO. The antibody issue remains a potential risk in normal human donors, although antibodies were not observed in the study performed by Goodnough and Kuter [42, 43], using single dose administration of PEG-rh-MGDF . In the present study, we demonstrate directly that adjustment of the dose schedule successfully prevents antibody formation without loss of efficacy. Despite this observation, the risk of antibody formation warrants the development of alternative c-mpl agonists [44, 45, 46, 47].

It is concluded that thrombopoietin administration results in highly elevated thrombocyte levels. These large amounts of thrombocytes can be used safely and effectively for transfusions in thrombocytopenic recipients and also effectively reduce the blood volume required per transfusion. The comparison of conventional and TPO stimulatedthrombocyte transfusions showed that the TPO stimulated transfusions to the pancytopenic recipients were at least as effective as standard whole blood or thrombocyte transfusions, which required 4- to 10-fold, respectively, larger volume of blood than the TPO stimulated transfusions. Use of a c-mpl agonist in human donors might increase the safety of platelet transfusions, allow the frequent use of HLA matched family donors and reduce costs.

Acknowledgments

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Chapter 8

GENERAL DISCUSSION

8.1 Introduction

Myelosuppression is the major dose-limiting toxicity of systemic anti-cancer chemotherapy, rendering many patients at risk for bleeding due to thrombocytopenia, life-threatening infections due to neutropenia and anemia. Administration of the growth factor granulocyte colony-stimulating factor (G-CSF) incorporated in chemotherapy-schedules is well established in clinical practice. G-CSF administration is followed by a less profound nadir and accelerated recovery of granulocytes, although not necessarily leading to less febrile episodes, infections, or a shorter stay in the hospital [1, 2]. Intensive chemotherapy and stem cell transplantation may still be accompanied with prolonged thrombocytopenia. Growth factor treatment resulting in accelerated thrombocyte recovery could decrease the number of erythrocyte and thrombocyte transfusions, and thereby reduce the occurrence of allosensitization to thrombocytes, diminish the risk of transmission of infectious diseases and reduce costs. In preclinical experiments in rhesus monkeys, the efficacy of thrombopoietin (TPO) alone or in conjunction with granulocyte/macrophage colony-stimulating factor (GM-CSF) or Flt3-ligand (fms-like tyrosine kinase-3 ligand; FL) after myelosuppression was investigated as well as the effect of TPO and GM-CSF after bone marrow transplantation. Furthermore, the transfusion efficacy of *in vivo* stimulated thrombocytes was compared to transfusion of non-stimulated thrombocytes.

8.2 Role of TPO in hematopoietic reconstitution

In vivo effects of TPO

The identification of TPO as the major regulator of thrombocyte production [3, 4, 5] has resulted in novel insights in the regulation of immature hematopoietic cell differentiation [6, 7, 8, 9] and has potentially provided a therapeutic approach for counteracting thrombocytopenic states, in particular those associated with intensive chemotherapy schedules in the treatment of malignancies. Preclinical evaluation of (rhesus) TPO in a nonhuman primate model demonstrated that a single IV dose of TPO, administered one day after a myelosuppressive dose of radiation, is sufficient to virtually prevent the need for thrombocyte transfusions and to accelerate thrombocyte reconstitution to normal levels by two weeks. In contrast, placebo controls needed 2-6 thrombocyte transfusions during the three weeks period of pancytopenia [10]. The effect of a single injection of TPO in this model was highly dose-dependent and showed that TPO is clearly more effective in stimulating thrombocyte reconstitution than other growth factors known to stimulate thrombocyte production, such as the gp 130 agonist IL-6 [12, 13, 14], IL-11 [15, 16], IL-3 [11, 13, 14], and IL-1 [17].

Furthermore, in contrast to effects of TPO administration under normal circumstances, multilineage effects were invariably observed in myelosuppression models [18, 19, 20], with a predominance for the erythroid lineage [21, 22, 23]. This effect occurs concomitantly to the thrombopoietic effect. The effect of TPO on hematopoietic progenitor

cells of other lineages is consistent with the observation that its receptor (c-mpl) is present on immature progenitors and stem cells [22, 24, 25]. These multilineage effects are also confirmed by studies with TPO or c-mpl knock-out mice, which do not only have the expected reduced thrombocyte numbers [9], but also reduced progenitor cell numbers of other lineages [26, 27]. These phenomena, as well as the expression of c-mpl by CD34+ cells [24], demonstrate a physiological role of TPO in regulating early stages of hematopoiesis. Except the thrombocytes, mature cell numbers of other lineages are normal in the TPO or TPO-receptor deficient mice, indicating that compensatory mechanisms operate for the TPO deficiency effects on early cells and that TPO is not essential for outgrowth of other lineages *in vivo*.

Effects of TPO combined with other growth factors

Both in vitro as well as *in vivo*, TPO acts in synergy with other growth factors on the expansion of immature stem cells or reconstitution of multilineage hematopoiesis [10, 25, 28, 29, 30, 31, 32, 33]. Co-administration of TPO with G-CSF, GM-CSF or FL in the rhesus monkey myelosuppression model displayed distinct response patterns. The combination of TPO/GM-CSF augmented thrombocyte, erythrocyte and neutrophil reconstitution relative to either of the growth factors alone, whereas the combination of TPO/G-CSF augmented solely the neutrophil recovery [10], emphasizing the specificity of G-CSF. The combination of FL and TPO did not improve hematopoietic reconstitution, on the contrary, a diminished thrombocyte response was seen after FL/TPO, similar to the pattern of placebo controls [34], and effects on neutrophils were absent. FL alone displayed similar reconstitution patterns as placebo controls and is therefore not useful as a therapeutic agent in myelosuppression. The combination of TPO/GM-CSF was superior to the other growth factor combinations tested. The increased thrombocyte reconstitution of the TPO/GM-CSF-treated monkeys may reflect synergism of the two growth factors, since the initial recovery of both thrombocytes and reticulocytes in the TPO/GM-CSF group exceeded the sum of the responses in the monkeys treated with either of the growth factors alone [10]. GM-CSF alone is known to have a stimulating effect on megakaryocytes [35, 36], although the results of *in vivo* treatment with GM-CSF on megakaryocytes have been shown to be heterogeneous [35, 36, 37, 38], and are presumably codependent on variations in endogenous TPO levels.

TPO-treatment after stem cell transplantation

In a transplant model in rhesus monkeys, involving a high dose of radiation followed by infusion of autologous unfractionated bone marrow, administration of TPO and GM-CSF was ineffective [39]. The combination of TPO/FL has not been tested in the transplant model because of the counteracting effects of the two growth factors in the myelosuppression model.

PEG-rHuMGDF (polyetylene glycol-conjugated recombinant human megakaryocyte growth and development factor), is a PEG-conjugated truncated non-glycosylated form of TPO that is PEG-conjugated to prolong its circulating half-life. PEG-rHuMGDF enhanced

thrombocyte recovery in mice after BMT and following peripheral blood progenitor cell transplantation (PBPCT) [40, 41]. These results have as yet not been confirmed in a clinical setting [42, 43, 44].
The absence of the stimulating effect of TPO in a bone marrow transplant setting has not been fully elucidated. After myelotoxic damage, hematopoietic reconstitution relies on remaining or infused stem cells and progenitor cells. Without growth factor treatment, reconstitution of hematopoiesis is controlled by endogenous mechanisms including cytokines, resulting in the pattern observed in placebo controls [10, 32, 39]. Placebo treated monkeys transplanted with autologous bone marrow cells and control monkeys in the myelosuppression model display a similar reconstitution pattern after a pancytopenic period of three weeks. This suggests the availability of adequate numbers of stem cells and their direct progeny for hematopoietic reconstitution in both models and argues against the hypothesis that lack of sufficient numbers of reconstituting cells after transplantation causes of a lack of response to TPO administration. It is as yet not clear why these cells are susceptible to growth factor stimulation in the myelosuppression model and apparently refractory to or not available for growth factor stimulation in the high radiation dose/stem cell transplant model. Factors such as the disturbed quantities of essential cytokines, changed receptor status and expression of endothelial adhesion molecules or altered intercellular pathways due to the higher radiation dose [45, 46, 47] may be of importance. The radiation-induced stromal damage has been suggested to last up to at least 12 years, and thereby results in reduced levels of long-term culture-initiating cells (LTC-IC; an in vitro assay to analyse the production of committed progenitor cells from immature cells related to long-term repopulating cells) [46]. Each of these factors may contribute to the failure to provide an optimal condition in which TPO-target cells may mature and expand the way they do in the 5 Gy myelosuppression model.

Mechanisms of TPO effects
In myelosuppressed mice, TPO has multilineage effects and stimulates colony-forming-unit-spleen (CFU-S) (a measure for relatively immature repopulating stem cells, associated with the initial, short-term wave of hematopoietic reconstitution, which lasts for several months [48, 49]), whereas TPO administration on the other hand resulted in a transient depletion of the more immature cells with (sustained) marrow repopulating ability (MRA) [50, 51]. The increase of CFU-S day 13 and the concomitant decrease of cells with MRA most likely indicate recruitment of multilineage short-term repopulating cells from a more immature ancestral population. These data show that TPO promotes short-term multilineage repopulating cells at the relative expense of more immature ancestral cells, thereby preventing pancytopenia. The mechanism by which TPO makes multilineage cells available for accelerated hematopoietic reconstitution remains to be further elucidated.
The lack of efficacy of TPO in the rhesus monkey bone marrow transplant model may be caused by absence of the necessary "target" cells available in the 5 Gy model. In analogy with the mouse model, where short term repopulating cells home to the spleen, the TPO target cells may not engraft with sufficient efficiency after BMT. Alternatively, accessory

cells that are necessary for TPO action may be depleted as a result of the myeloablative treatment. Thus, further identification of TPO target cells and their homing pattern after transplantation may help to explain and be useful to improve the effect of TPO after BMT. The actions of TPO suggest that the TPO receptor, i.e., c-mpl, is present on very immature progenitors as well as on erythroid progenitors. Immunophenotyping studies with Mpl antibodies and with biotinylated TPO indicate that cells with detectable Mpl expression are mainly located in the population of CD34-dull cells that express the megakaryocyte marker CD41, on megakaryocytes and on mature platelets [52]. However, it has been shown that cells, which could not be detected by flow cytometry, do proliferate in vitro after stimulation with a single growth factor of which the receptor expression was examined [53, 54]. This is due to the rather high detection level limit of flow cytometry, which is about 50 receptor molecules/cell [53, 54, 55]. Despite this limitation of the analysis, the CD34dull/CD41a+ subset was shown to contain differentiating megakaryocyte progenitors and, possibly, erythroid progenitors as well [56]. CD34+/CD45RA- and CD34+/CD38- subfractions in cord blood, which are considered to contain primitive and multi-potent progenitor cells, were both enriched for megakaryocyte progenitors as determined in a semisolid megakaryocyte-colony forming unit (CFU-Meg) assay, whereas they also expanded (19-50 fold) with TPO as single growth factor in a suspension culture [57]. However, in mobilized peripheral blood, virtually no megakaryocytic progenitor cells have been found in the CD34+/CD38- fraction [58]. Thus, in contrast to mobilized peripheral blood, cord blood contains a relatively large number of primitive megakaryocytic progenitor cells, which do not express CD41 and are responsive to TPO as single factor [57]. Also in BM a considerable number of megakaryocytic progenitors with high proliferative capacity were derived from CD34+/ CD41- subfraction [59].

Thus, both the source and the phenotype of immature progenitors determine the ability to respond to TPO. Together with other growth factors, such as SCF and FL, TPO stimulates clonal growth of CD34+/CD38- cells [60] and also megakaryocytopoiesis, myelopoiesis and expansion of CD34+ cells from single CD34+/Thy-1+/Lin- primitive progenitor cells [61]. In rhesus monkeys, immature bone marrow cells are characterized by the phenotype CD34+/RhLA-DRdull [53, 62] and it is hypothesized that these cells too are Mpl+.

Hematopoietic stem cells in non-human primates have been shown to be relatively quiescent and to require prolonged periods of time to cycle [63]. TPO alone is sufficient to promote the viability of Lin-Sca-1+ mouse progenitor cells that had not multiplied after 40 hours in vitro. Without other growth factors, clonal growth could not be initiated by TPO alone, but apoptosis could be prevented [64]. Among the other growth factors, SCF, which also strongly synergizes with TPO to produce a proliferative response, has been implicated as the most potent viability factor for primitive hematopoietic progenitor cells [65, 66].

Timing of TPO administration
A single IV dose of TPO, shortly after intensive cytoreductive treatment and sufficient to saturate the c-mpl mediated clearance mechanism in nonhuman primates, effectively alleviates the course of thrombocytopenia to transfusion independence. The finding is consistent with data in mice [23], with the kinetics of TPO-stimulated thrombocyte reconstitution [25, 32], and with the decline in response when TPO administration is delayed [67]. These studies all point to a critical time frame early after TBI during which TPO has to be administered to achieve an optimal response. In clinical studies, the effect of TPO/ PEG-rHuMGDF could therefore have been disappointing due to suboptimal timing and dosing [69, 70, 71]. In general, TPO / PEG-rHuMGDF administration is well tolerated, but the further pharmaceutical development of TPO has been hampered by the development of antibodies [72, 73, 74, 75] which cross-react with endogenous TPO and neutralize its biological activity, resulting in thrombocytopenia. Alternative molecules which share no homology with TPO, but which do compete with TPO for binding to its receptor, could be used to avoid the formation of TPO-neutralizing antibodies [76].

8.3 CD34-positive cells during hematopoietic reconstitution

CD34 positive cells are generally recognized as hematopoietic progenitor cells, which mainly reside in the bone marrow [62, 77, 78]. A small part of the peripheral blood cells is also CD34+ (0.10% - 0.15%), and, in normal circumstances, fluctuates approximately half a log in time within the normal range (chapter 4). Absolute bone marrow CD34+ numbers are approximately 2 orders of magnitude numerous than PB CD34+ numbers, although under normal circumstances the individual values are not related ($r = .003$, $p = .979$) [79]. The absolute number of the CD34+ cells in blood is further determined by a complex genetic trait and fluctuates widely during aging [80, 81]. During the regeneration phase after myelosuppression or bone marrow transplantation, absolute numbers fluctuate in a similar way as occurs under normal circumstances. Due to this physiologic fluctuation, the absolute number of PB CD34+ cells in healthy subjects is difficult to relate to other parameters of hematopoiesis.
Despite the variation in absolute numbers, there is a strict correlation between PB CD34+ cells and BM CD34+ cells during regeneration after myelosuppression (chapter 4). The recovery of both BM and PB CD34+ cells is exponential, starting in the same range, shortly after irradiation. Afterwards, a similar reconstitution pattern of BM and PB CD34+ cells is observed. This is in contrast with mobilization under "normal" circumstances, where immature cells are expulsed from the bone marrow. In the myelosuppression model, TPO stimulates stem cells in bone marrow, followed by accelerated hematopoiesis, reflected by increasing numbers of several types of immature and mature peripheral blood cells. TPO does interfere at several levels of hematopoiesis: stimulating stem cells and inducing megakaryocyte proliferation and maturation, resulting in prolonged thrombocyte production. Due to its stimulation of immature, multilineage progenitor cells, TPO also augments the response to other exogenous growth factors,

such as G- and GM-CSF.
Not only PB CD34+ cells reflect CD34+ bone marrow regeneration, the latter is also reflected (and to a certain extent "predicted") by levels of circulating FL in peripheral blood shortly after irradiation or in chemotherapy-induced myelosuppression [82, 83, 84, chapter 6]. Similar to the inverse relation of G-CSF with myeloid engraftment [85, 86, 87] or TPO with thrombocyte and megakaryocyte numbers [18, 88, 89, 90], FL levels are inversely correlated with the number of bone marrow immature CD34+ cells. This relationship is especially clear for the area under the curve of FL levels, composed of values measured twice a week, for a 3 or 6 weeks period. For time intervals shorter than 3 weeks, the relation of FL levels and stem cell numbers or parameters of peripheral blood reconstitution was not significant. Remarkably, FL levels in GM-CSF treated monkeys appeared to be less pronounced. The mechanism of this phenomenon has not yet been elucidated. Peak levels of FL shortly after irradiation are of little importance but could aid as a prognostic factor combined with other parameters measured for myelosuppression. An example of such an additional, be it apparently not independent parameter could be the number of PB CD34+ cells (chapter 4). It is known that the capacity of the stem cells to form cobblestone areas (CAFC assay) [113] is related to their capacity to engraft and to cause successful thrombocyte and neutrophil recovery after transplantation [91]. However, this assay takes 6 weeks before the results can be interpreted, and makes it thus not useful to predict recovery after myelosuppression and stem cell transplantation. Both (CD34+) peripheral blood cells and FL levels measured in the first period after myelosuppression may serve as prognostic factors for bone marrow recovery, but not as rapid as would be desirable from the perspective of early therapeutic intervention.

8.4 TPO stimulated transfusions

Thrombocyte transfusions are essential to prevent morbidity and mortality in patients who are severely thrombocytopenic. Thrombocytes for transfusions are currently obtained either by fractionation of whole blood or by platelet apheresis [92, 93]. The risk of immunization and transmission of infections [94] increases with the number of donors required for the transfusions. Therefore, it would be preferable to obtain the transfusions from one single (HLA-matched) donor, whenever possible. However, platelet yield of apheresis depends on many factors, especially the donor's preapheresis count, total blood volume and the duration of the procedure are major determinants [95]. Donor comfort and convenience limit the last parameter.
in vivo studies involving TPO administration to normal mice, non-human primates or humans, have shown that TPO enhances thrombocytopoiesis; the number of circulating thrombocytes increased in a dose-dependent manner [96, 97, 98, 99]. In a rhesus monkey model, we tested the efficacy of TPO stimulated thrombocytes by using those in 'mini' whole blood transfusions in thrombocytopenic animals (chapter 7). Thrombopoietin administration resulted in highly elevated thrombocyte levels, which could be used safely and effectively for transfusions in thrombocytopenic recipients and also effectively

reduced the amount of blood volume required per transfusion. The comparison of conventional and TPO-stimulated thrombocyte transfusions showed that the TPO-stimulated transfusions to the pancytopenic recipients were at least as effective as standard whole blood or thrombocyte transfusions, while the blood volume needed was 4- to 10-fold less, respectively. Due to the higher and prolonged recovery, the number of transfusions needed was smaller.

It is known that TPO facilitates thrombocyte activation by specific agonists, but TPO does not activate thrombocyte aggregation by itself [100, 101, 102]. The facilitated thrombocyte activation by TPO [96, 103, 104, 105, 106] may be a drawback in collecting thrombocyte-enriched plasma, but except for a small variation in measured thrombocyte numbers, such an effect did not appear to be of much relevance. Thrombotic events associated with TPO administration have not been observed. The TPO induced facilitated aggregation by specific agonists might be of advantage in thrombocytopenic patients with increased bleeding risks. Another benefit of activated platelets is the ability to internalize bacteria and HIV and therefore contribute to the immunity of the pancytopenic patient [107]. The presence of a higher number of thrombocytes during a longer time after transfusion, might be a result from the younger age and the slightly prolonged lifespan of the stimulated thrombocytes [102]. However, PEG-rHuMGDF stimulated thrombocytes appeared to have a half-life equivalent to thrombocytes released under normal physiological conditions [100]. This discrepancy might be explained by the increase in reticulated platelets, which are expulsed from the bone marrow [100].
TPO administration in healthy donors appeared to be complicated by one single major adverse effect: the development of cross-reacting antibodies, which neutralize the effect of endogenous TPO, and thereby cause thrombocytopenia. Antibody induction, relatively common following administration of recombinant proteins, remains a potential risk in normal human donors and patients without immunosuppressive treatment, although antibodies were not observed in the study of Goodnough and Kuter [109, 110] using single dose administration of TPO. The dosing scheme proves therefore to be of significance. We found a balance between optimal efficacy of thrombocyte stimulation and avoidance of antibody formation, by using a scheme in which TPO was administered on 4 consecutive days (Chapter 7). An alternative for dose adjustment, which reduces, but does not entirely eliminate the risk of antibody formation, could be the development of thrombopoietin mimetic peptides, which bind to and activate the Mpl receptor. Several groups [76, 111, 112] have generated peptides which compete with TPO for the Mpl receptor and which are equipotent in stimulating megakaryocyte proliferation. If the side-effects of such peptide molecules or alternative non-protein c-mpl agonists are minimal and antibody formation is irrelevant, such molecules will offer several options for the further pharmaceutical development of therapeutic molecules based on the discovery of TPO and the characterization of its pharmacological profile.

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SUMMARY

Many patients with malignant diseases are treated with systemic chemotherapy. Among its side-effects, myelosuppression may place patients at risk for life-threatening infections, and major bleedings and anemia. Severe myelosuppression is therefore dose-limiting to cancer therapy. To achieve optimal therapeutic efficacy, co-administration of specific hematopoietic growth factors has been proposed to counteract myelosuppression. The work described in this thesis focuses on the therapeutic efficacy of the hematopoietic growth factors thrombopoietin and flt-3 ligand on stem cell and peripheral blood reconstitution following cytoreductive total body irradiation (TBI). In the experiments, well-documented preclinical rhesus monkey myelosuppression and bone marrow transplantation models were used.

Chapter 1 provides a general introduction. It opens with a short description of the process of hematopoiesis, stem cells and hematopoietic growth factors. As the growth factors thrombopoietin, flt-3 ligand and GM-CSF play a central role in this thesis, the functions of these growth factors have been described in more detail.

Chapter 2 describes the results of a study on a single dose administration of recombinant rhesus TPO sc one day after TBI, which effectively counteracted the need for thrombocyte transfusions and resulted in accelerated thrombocyte reconstitution to normal levels. The combination of TPO and GM-CSF, day 1-14 sc, 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. Treatment with GM-CSF alone resulted in an equal need for transfusions as placebo treated controls. 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 with neutrophil counts less than 0.5 x 10^9/L by approximately one week. TPO/GM-CSF treatment elevated the neutrophil nadir, but did not further accelerate recovery to normal values. TPO/GM-CSF resulted in significantly increased reconstitution of CD34+ bone marrow cells and progenitor cells such as GM-CFU and BFU-E. The synergism of TPO and GM-CSF is explained from TPO induced accelerated recovery of immature progenitor cells, including those that respond to GM-CSF.

In **Chapter 3** a study with TPO/GM-CSF treatment in a bone marrow transplantation model has been described. Based on the effects of this highly effective GF combination in the myelosuppression model, TPO and GM-CSF were both administered for 14 days after autologous BMT. However, despite a similar level of pancytopenia, simultaneous administration of TPO and GM-CSF was ineffective in prevention and treatment of pancytopenia after transplantion of a limited number of unfractionated bone marrow cells. The discordant results of this growth factor combination in these two models may imply codependence of the response to TPO and / or GM-CSF on other factors or cytokines, or more simply, lack of sufficient numbers of available target cells to elicit a clinically relevant response.

In **Chapter 4** CD34 positive progenitor cells in bone marrow and peripheral blood of 87 young adult rhesus monkeys were evaluated before and after hematopoietic growth factor treatment in studies involving myelosuppression and bone marrow transplantation in conjunction with growth factor treatment. CD34+ cell numbers in bone marrow (BM) and peripheral blood (PB) were monitored and correlated with hematologic parameters such as WBC, reticulocyte percentages and thrombocytes. BM and PB CD34+ cell numbers appeared to differ two log in absolute numbers and both vary within a large range of approximately 1.5 log. PB CD34+ cell numbers are significantly related with BM CD34+ cell numbers during regeneration from myelosuppression and also with peripheral blood parameters such as WBC, reticulocytes and thrombocytes. After bone marrow transplantation, PB CD34+ cell numbers are also related with BM CD34+ cell numbers but relations with parameters in PB are absent. Despite similar hematological recoveries, CD34+ cell reconstitution was delayed for the 8 Gy animals relative to regeneration after myelosuppression, the delay being associated with refractoriness to growth factor treatment. It is concluded that enumeration of PB CD34+ cells can be used as a rapid reliable test of bone marrow CD34+ cells in the reconstitution phase and that PB CD34+ cells are also related to blood cell reconstitution parameters after myelosuppression.
Based on the actions of FL on stem cells and the synergizing effect with other growth factors, it was hypothesized that FL in combination with TPO might augment stem cell reconstitution and accelerate hematopoietic recovery. The results of this study are described in **Chapter 5.** However, the combination of TPO with FL was less effective than TPO alone in stimulating thrombocyte regeneration with a more profound nadir and a slower recovery to thrombocyte counts > 100 x 10^9/L, approaching recovery patterns of the placebo controls. Leukocyte regeneration was similar in all animals. Monkeys treated with FL alone showed an impaired regeneration of reticulocytes and thrombocytes similar to placebo controls. Recovery of bone marrow cellularity was slightly accelerated in the TPO/FL treated monkeys, but not reflected in an increase in progenitor cells, in contrast to the accelerated bone marrow reconstitution of TPO treated monkeys. Monkeys treated with FL alone showed bone marrow reconstitution similar to placebo treated controls. It was concluded that FL was not effective as a therapeutic agent to counteract myelosuppression and that FL competed with TPO at an immature cell level.
Chapter 6 shows the results of a study on the relation between Flt-3 Ligand (FL) levels and (growth factor stimulated) regeneration of hematopoiesis after myelosuppression and bone marrow transplantation (BMT) in 56 young adult rhesus monkeys. FL levels were measured during reconstitution of hematopoiesis and related to hematopoietic reconstitution. After myelosuppression, FL levels increased and reached peak values after 7-14 days. Normalization of FL levels occurred after 3 weeks, concurrent with regeneration of hematopoiesis. The area under the curve (AUC) of FL levels after 3 and 6 weeks was significantly related to regeneration of WBC, reticulocyte percentages and thrombocyte counts, but at earlier time point's significance of the relations was absent. FL levels in GM-CSF (± TPO) treated animals were significantly lower than in other groups studied, corresponding to an increase in BM CD34+ cells and a faster regeneration of hematopoiesis. After 8 Gy TBI and BMT, the reducing effect of GM-CSF treatment on FL

levels was observed as well and similarly accompanied by higher BM CD34+ cell numbers. A significant relation of FL levels and WBC and BM CD34+ cells could be demonstrated whereas relations with other parameters were not observed.
Thus, FL levels after myelosuppression as well as after transplantation are inversely related to regeneration of WBC and BM CD34+ cells. FL levels are inversely related to the regeneration of reticulocytes and thrombocytes, whereas such a relationship is absent after bone marrow transplantation. Treatment with GM-CSF after both myelosuppression and BMT resulted in a reduced increase of FL levels
In **Chapter 7** we examined the feasibility of thrombocyte transfusions donated by single donors stimulated with TPO, which resulted in an increase of the thrombocyte count allowing much smaller volumes of donated blood. The efficacy of 14 consecutive TPO stimulated thrombocyte transfusions was studied in two rhesus monkey recipients of an autologous stem cell transplant and one radiation control monkey, which endure a 3-6 week period of profound pancytopenia. This was compared to 68 conventional thrombocyte and 26 whole blood transfusions in parallel studies. Donor monkeys displayed a rapid increase in thrombocyte numbers with maximum values of 24 and 33 x 10^{11}/L at 12 and 13 days after initiation of TPO treatment, which is 10 times elevated relative to normal values. The thrombocyte transfusions were standardized at a thrombocyte number of 10^{10}/transfusion, enabling direct comparison of TPO stimulated and conventional transfusions. The TPO stimulated transfusions proved to be at least as effective as conventional whole blood or thrombocyte transfusions resulting in an increment of 42 ± 16 thrombocytes x 10^9/L (as opposed to 28±16 and 25±14 after conventional thrombocyte and whole blood transfusions, respectively). Restricting the total dose of TPO, without essential loss of efficacy, could prevent neutralizing antibody formation to TPO observed in these monkeys. Use of a c-mpl agonist in human platelet donors might increase the safety of thrombocyte transfusions, allow the more frequent use of HLA matched family donors, and reduce costs.
Chapter 8 is a general discussion of the results described in Chapters 2-7. It is concluded that the studies described in Chapters 2-7 further define and elucidate the therapeutic potential of TPO and FL in the reconstitution of hematopoiesis following intensive cytoreductive therapy. The efficacy of TPO to counteract myelosuppression and its potential role in improving thrombocyte transfusion technology warrants the further development of non-immunogenic administration schedules and/or alternative, non-immunogenic c-mpl agonists.

Samenvatting

Veel patiënten met maligniteiten worden behandeld met systemische chemotherapie. Eén van de bijwerkingen is myelosuppressie. De pancytopenie die hiervan het gevolg is, kan leiden tot levensbedreigende infecties en /of een sterk verhoogde bloedingsneiging en anemie. Om een optimaal therapeutisch effect te krijgen, is een hoge dosis chemotherapie soms onvermijdelijk. Gelijktijdige toediening van hematopoietische groeifactoren kan een mogelijke oplossing bieden om de myelosuppressie te verminderen en/of te voorkomen. Het onderzoek beschreven in dit proefschrift richt zich op de therapeutische effectiviteit van de groeifactoren thrombopoietine en flt-3 ligand op het herstel van de aantallen stamcellen en perifere bloedcellen. In de experimenten is gebruik gemaakt van goed gedocumenteerde preklinische modellen met de resusaap als proefdier voor beenmergonderdrukking en voor transplantatie van stamcellen uit het beenmerg.

Hoofdstuk 1 is een algemene inleiding. Het beschrijft kort het proces van hematopoiese (bloedvorming), stamcellen en hematopoietische groeifactoren. De groeifactoren thrombopoietine (TPO), flt-3 ligand (FL) en GM-CSF, die een centrale rol spelen in de studies beschreven in dit proefschrift, worden nader toegelicht.

Hoofdstuk 2 beschrijft de resultaten van een studie waarin een éénmalige intraveneuze (iv) toediening van TPO één dag na bestraling leidt tot een drastische afname van het aantal benodigde thrombocytentransfusies en een versneld herstel tot normale thrombocyten aantallen. De combinatie van die éénmalige gift TPO met toediening van GM-CSF, 14 dagen subcutaan (sc) was nog effectiever dan TPO alleen en leidde tot een hogere thrombocyten nadir en nog sneller herstel tot normale thrombocyten aantallen. Thrombocyten stegen zelfs tot licht verhoogde waarden. Placebo behandeling en behandeling met GM-CSF alleen leidde tot de zelfde behoefte aan thrombocyten transfusies. De productie van reticulocyten werd eveneens gestimuleerd door TPO en versterkt door combinatie met GM-CSF. TPO had geen effect op het herstel van neutrofiele granulocyten, terwijl GM-CSF de duur van de neutropenie met een week verkortte. De combinatie van TPO en GM-CSF leidde tot een iets hogere nadir van neutrofiele granulocyten maar versnelde het herstel naar normale waarden niet verder dan GM-CSF alleen. De combinatie van TPO/GM-CSF resulteerde in een significant stimulerend effect op de regeneratie van CD34-positieve beenmerg cellen en voorlopercellen zoals GM-CFU en BFU-E. De synergie van TPO en GM-CSF wordt verklaard uit het versnelde herstel van voorlopercellen onder invloed van TPO, waaronder ook voorlopercellen die reageren op de toediening van GM-CSF.

In **Hoofdstuk 3** werd de combinatie van TPO/GM-CSF bestudeerd in een beenmerg transplantatie (BMT) model. Gebaseerd op de resultaten van deze groeifactorcombinatie in het myelosuppressiemodel, werden beide groeifactoren 14 dagen sc toegediend na bestraling en autologe BMT. Ondanks een vergelijkbare duur van de pancytopenie als waargenomen in het myelosuppressie model, was de combinatie van TPO en GM-CSF niet effectief in het voorkomen en het behandelen van het tekort aan leukocyten, erythrocyten en thrombocyten. De tegenstrijdige resultaten van de combinatie TPO/GM-

CSF in beide modellen kan toegeschreven worden aan afhankelijkheid van andere benodigde groeifactoren, of aan gebrek aan voldoende gevoelige cellen (voor deze groeifactoren) om een klinisch relevante respons te verkrijgen.
In **Hoofdstuk 4** wordt een studie beschreven waarin het aantal CD34+ voorloper cellen in het beenmerg (BM) en perifere bloed (PB) van 87 resusapen is geëvalueerd in het myelosuppressie en het beenmergtransplantatie model, in samenhang met de toediening van groeifactoren om het herstel te bevorderen. De aantallen CD34+ cellen werden gecorreleerd met hematologische parameters zoals het aantal leukocyten (WBC), het reticulocytenpercentage en het aantal thrombocyten. Onder normale omstandigheden verschilt het aantal CD34+ cellen in BM en PB twee log in absolute aantallen, waarbij ze ieder nog variëren binnen een ruime range van 1.5 log. Het aantal PB CD34+ cellen is significant gecorreleerd met het aantal BM CD34+ cellen gedurende regeneratie na myelosuppressie en eveneens met het herstel van de perifere bloedcellen. Na BMT is de relatie tussen aantallen CD34+ cellen in BM en PB eveneens aanwezig, maar kon geen relatie worden vastgesteld met het herstel van bloedcellen. Ondanks vergelijkbaar perifeer herstel in het myelosuppressie en in het BMT model, is het herstel van het aantal CD34+ cellen vertraagd in het BMT model, en dit vertraagde herstel is geassocieerd met de afwezigheid van een klinische relevante respons op hematopoietische groeifactoren. Geconcludeerd kan worden dat het aantal PB CD34+ cellen een maat is voor het aantal BM34+ cellen gedurende de herstelfase na myelosuppressie, en het aantal PB CD34+ cellen tevens gerelateerd is aan het herstel van perifere bloedcellen.
Hoofdstuk 5 beschrijft de resultaten van de combinatie van TPO en FL in het myelosuppressie model. Gebaseerd op het stimulerende effect van FL op stamcellen in vitro en de waargenomen synergie met andere groeifactoren, werd verondersteld dat FL en TPO het herstel van stamcellen en de bloedvorming zouden versnellen. De resultaten lieten echter zien dat de combinatie van TPO/FL minder effectief was dan TPO alleen in het stimuleren van de thrombocyten regeneratie, met een diepere nadir en een trager herstel naar normale waarden, bijna vergelijkbaar met het herstelpatroon van placebocontroles. Behandeling met FL alleen liet een regeneratie van thrombocyten en reticulocyten zien, die vergelijkbaar was met het herstel in placebo's. Het herstel van leukocyten aantallen werd evenmin gestimuleerd. De beenmerg cellulariteit nam aanvankelijk snel toe in de FL/TPO behandelde apen, maar dit effect werd niet weerspiegeld in een toename van voorlopercellen, in contrast tot het snelle herstel van beenmergfunctie in TPO behandelde apen. Herstel van beenmerg- en voorlopercellen verschilde niet in placebo's en FL behandelde dieren. Behandeling met FL is dus niet zinvol om effecten van myelosuppressie te voorkomen. FL werkt TPO zelfs tegen op het niveau van voorloper cellen.
In **Hoofdstuk 6** is de relatie bestudeerd tussen spiegels van FL in het bloed van 56 bestraalde en/of getransplanteerde resusapen en het herstel van de bloedvorming gedurende de eerste 6 weken na bestraling. Na bestraling namen de FL spiegels toe en bereikten ze een maximale waarde na 7-14 dagen. De spiegels normaliseerden na ongeveer 3 weken, gelijktijdig met het herstel van de bloedvorming. In het myelosuppressie model was de 'area under the curve' (AUC) van FL spiegels na 3 en 6

weken significant gerelateerd aan het herstel van WBC, reticulocyten percentages en thrombocyten aantallen. Op tijdstippen eerder dan 3 weken was deze relatie afwezig. Het was opvallend dat in GM-CSF behandelde apen (met of zonder TPO) FL spiegels significant lager waren dan in andere dieren. Dit correspondeerde met een toename van BM CD34+ cellen en een versneld herstel van de bloedcelproductie. Na 8 Gy bestraling en BMT was dit effect van GM-CSF op de FL spiegels ook aanwezig en correleerde het ook met de aantallen CD34+ cellen in het BM, en met het herstel van WBC maar niet met de overige perifere bloedcellen. FL spiegels zijn dus zowel na myelosuppressie als na transplantatie omgekeerd gerelateerd aan regeneratie van BM CD34+ cellen als WBC, en de spiegels van FL zijn significant lager in GM-CSF behandelde dieren.

Hoofdstuk 7 beschrijft de resultaten van een onderzoek waarin TPO toegediend is aan normale resusapen met als doel het verkrijgen van thrombocyten transfusies van één enkele donor. Door de stijging van het aantal thrombocyten zijn maar kleine volumes nodig voor het afnemen van een voldoende aantal thrombocyten. Het effect van 14 TPO gestimuleerde thrombocyten transfusies werd bestudeerd in twee resusapen die een autoloog stamceltransplantaat hebben gekregen en in één stralingscontrole. Deze dieren zijn gedurende 3-6 weken pancytopeen. Het resultaat werd vergeleken met 68 conventionele thrombocyten transfusies en 26 volbloed transfusies in parallelle studies. Aan twee donor resusapen werd TPO om de dag toegediend, wat leidde tot een snelle stijging tot 10 maal de normale waarde van het thrombocytengetal tot 24 en 33 x 10^{11}/L na 12-13 dagen. Alle thrombocytentransfusies waren gestandaardiseerd op een gemiddeld aantal van 10^{10} thrombocyten per transfusie, zodat rechtstreekse vergelijking mogelijk was. De TPO gestimuleerde transfusies waren minstens zo effectief als de conventionele thrombocyten- en volbloed transfusies, resulterend in een gemiddelde stijging van 42 ± 16 thrombocyten x 10^9/L, vergeleken met 18±16 en 25±14 na conventionele thrombocyten- en volbloed transfusies, respectievelijk. Het ontstaan van (passagère) neutraliserende antilichamen tegen TPO in de donoren kon voorkomen worden door de totale dosis TPO te beperken, zonder de effectiviteit essentieel te verminderen. Het gebruik van een c-mpl agonist in humane thrombocyten donoren zou de veiligheid van de thrombocyten transfusies (nu doorgaans van 5 donoren) verhogen, het gebruik van HLA compatibele (familie) donoren vergemakkelijken en kosten-effectief zijn.

Hoofdstuk 8 is een algemene discussie van de resultaten beschreven in hoofdstuk 2-7. Geconcludeerd wordt dat de studies beschreven in het proefschrift het therapeutisch potentieel van TPO en FL m.b.t. het herstel van de bloedcelvorming na intensieve cytoreductieve therapie nader definiëren en ophelderen. De effectiviteit van TPO voor het tegengaan van myelosuppressie en de potentiële rol in het verbeteren van de thrombocytentransfusie technologie rechtvaardigt de verdere ontwikkeling van niet-immunogene toediening-schema's en/of alternatieve, niet-immunogene c-mpl agonisten.

List of Abbreviations

BFU-E	burst-forming unit-erythroid
BM	bone marrow
BMT	bone marrow transplantation
BSA	bovine serum albumin
CAFC	cobblestone area forming cell
CD	cluster of differentiation
CFU-E	colony-forming unit-erythrocyte
CFU-GM	colony-forming unit-granulocyte/macrophage
Cy-5	cyanin-5
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked-immuno-absorbent assay
EPO	erythropoietin
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FL	flt-3 ligand
Flt-3	fms like tyrosine kinase 3
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte/macrophage colony stimulating factor
Hb	hemoglobin
HBN	HHBS containing BSA and sodium azide
HGF	hematopoietic growth factor
HHBS	Hanks' buffered Hepes solution
HSC	hematopoietic stem cells
IL	interleukine
iv/IV	intravenous
LDH	lactate dehydrogenase
Moab/mAb	monoclonal antibody
MGDF	megakaryocyte growth and development factor
Mpl	myeloproliferative leukemia
ND	not done (not performed)
PB	peripheral blood
PBS	phosphate buffered saline
PBSC	peripheral blood stem cells
PE	phycoerythrin
PI	propidium iodide
RhLA	Rhesus leukocyte antigen
sc	subcutaneous
SCF	stem cell factor
SD	standard deviation
SIV	simian immunodeficiency virus
STLV	simian T-lymphotrophic virus
TBI	total body irradiation
TNC	total nucleated cells
TPO	thrombopoietin
UAN	unique animal number
WBC	white blood cells

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 11 juni 1969 te Bussum. In 1987 behaalde zij het VWO diploma aan het Christelijk College "Stad en Lande" te Huizen. Zij startte in hetzelfde jaar met de studie Geneeskunde aan de Vrije Universiteit te Amsterdam, waar in 1994 het artsexamen werd verkregen. Van 1 juli 1994 tot 1 januari 1996 werkte zij als AGNIO (assistent geneeskundige niet in opleiding) interne geneeskunde in het Havenziekenhuis. Per 1 januari 1996 begon zij met het in dit proefschrift beschreven onderzoek op de afdeling hematologie van de Erasmus Universiteit Rotterdam (begeleider: Dr. G. Wagemaker) in het kader van een door NWO gesteunde AGIKO (assistent geneeskundige in opleiding tot klinisch onderzoeker) constructie. Van 1 januari 1998 tot en met 31 december 1999 volgde zij de eerste twee jaar van de opleiding tot internist in het Havenziekenhuis (opleider: Dr. A.G.C. Bauer), waarna zij in het jaar 2000 haar onderzoek vervolgde op de afdeling Hematologie van de Erasmus Universiteit. Sinds 1 januari 2001 is zij werkzaam op de afdeling interne geneeskunde van het Erasmus MC (voorheen Academisch Ziekenhuis Rotterdam, locatie Dijkzigt) (opleider Prof.dr. H.A.P. Pols) om de opleiding tot internist te voltooien. Vanaf 1 september 2003 volgt zij daar het aandachtsgebied vasculaire geneeskunde (opleider Dr. A.H. van den Meiracker).

Publications

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Simone C.C. Hartong, Trudi P. Visser, Huub van Vliet and Gerard Wagemaker. Preclinical evalutation of recombinant thrombopoietin administration to platelet donors : highly efficacious in both donor and recipient, but jeopardized by (avoidable) neutralizing antibodies to thrombopoietin in the donors. Submitted

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