From stem cell to platelet
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Chapter 1

Clinical approaches involving thrombopoietin, to shorten the period of thrombocytopenia after high-dose chemotherapy

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Abstract

High-dose chemotherapy followed by a peripheral blood stem cell transplant is successfully used for a wide variety of malignancies. A major drawback, however, is the delay in platelet recovery. Several clinical strategies using thrombopoietin (Tpo) have been developed in an attempt to speed up platelet repopulation. In contrast to its success in immune thrombocytopenia and in low-dose toxic chemotherapeutic regimes, Tpo appears less effective in the case of high-dose chemotherapy and peripheral blood stem cell transplant. To develop a successful therapeutic approach, more knowledge is needed on several aspects of the megakaryocyte (progenitor) biology, such as homing to the bone marrow, endomitosis, and platelet formation. Interactions of the megakaryocytes with the marrow vasculature and the microvascular microenvironment are other key factors for optimal thrombocytopoiesis. The present report reviews the background of the inefficiency of Tpo after intensive chemotherapy and describes possible strategies that might lead to successful therapies to treat chemotherapy-induced thrombocytopenia.

1. General introduction

Platelets are small anucleated circulating particles whose main function is the formation of a hemostatic plug in response to vascular injury. Platelets are produced upon fragmentation of their precursor cell in the bone marrow (BM), the megakaryocyte (MK). megakaryocytes are formed from the hematopoietic stem cell (HSC) in a critically timed and spatially organized process. In short, proliferation and maturation of the MK require the cell to travel from the endosteal stem cell niche to the marrow vasculature. All the processes involved in MK development are regulated by maturation-modulated signaling. This signaling is induced by several cytokines and interactions with the BM microenvironment, which consists of stromal and vascular cells and their matrix proteins. At the end, every MK releases thousands of platelets into the circulation. Although other growth factors have some megakaryocytopoietic potency, thrombopoietin (Tpo) is the key growth factor in megakaryocytopoiesis. Moreover, the level of unbound Tpo in the plasma, regulated by the platelet and MK mass, determines its efficiency to induce (new) megakaryocytopoiesis. This realization led to Tpo-mediated therapeutic approaches to treat thrombocytopenia. Thrombopoietin is effective in increasing platelet numbers in normal volunteers, in patients with idiopathic thrombocytopenia, and in patients treated with low-dose chemotherapy. In contrast, the period of severe thrombocytopenia after intensive chemotherapy, as potentially the most interesting therapeutic target, is not shortened by Tpo treatment. Different reasons might explain this disappointing finding. The first is that endogenous Tpo levels in these conditions may be so high that exogenous Tpo does not improve megakaryocytopoiesis. In addition, Tpo appears not to be involved in the terminal platelet forming process. Finally, the intensive chemotherapy reduces the first target cells of Tpo, namely, the HSCs and MK progenitors. These targets cells need to be administered, that is, with a peripheral blood stem cell transplant (PBSCT), and subsequently home to a microenvironment that effectively supports Tpo-mediated MK formation, expansion, and platelet formation. It might also be that Tpo is not able to accelerate MK formation from HSCs, like granulocyte-colony stimulating factor (G-CSF) can for granulocytopoiesis.
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For efficient treatment of chemotherapy-induced thrombocytopenia, more knowledge on the mechanism of Tpo action is needed. This includes the need for understanding the role of additional, usually redundant, factors involved in megakaryocytopoiesis and subsequent platelet formation. This review focuses on different available and possible future strategies for using Tpo to shorten chemotherapy-induced thrombocytopenic periods.

2. The role of Tpo and other growth factors in thrombocytopoiesis

Investigation of megakaryocytopoiesis has been hampered because Tpo was not cloned until 1994 (reviewed in1), even though its existence was already suspected in 1958. Whereas the cytokines involved in lymphocyte, erythrocyte, monocyte and granulocyte production were purified in the 1980s, Tpo was cloned only after the characterization of its receptor, Mpl, in the 1990s. The impetus for this started in an unrelated field with the discovery of the oncogene of the myeloproliferative leukemia virus, v-mpl, which was able to immortalize BM hematopoietic cells from different lineages.3 Its human homologue, c-mpl, was shown to be expressed in CD34+ haematopoietic progenitor cells (HPCs), MKs, and platelets, which encodes a protein highly homologous with members of the hematopoietin receptor superfamily.4 This expression pattern and the observed role of Mpl in MK formation were strong leads towards the fact that its ligand was likely to be identical to Tpo. After the cloning of the Tpo gene, this was confirmed.5

The observation that MK and platelets are not completely absent in Tpo and Mpl knock-out mice demonstrates that although in the normal setting, Tpo is the most important factor responsible for platelet formation, other factors can replace Tpo and stimulate some thrombocytopoiesis. Candidate factors are, for example, the cytokines interleukin 1 (IL-1), IL-3, IL-6, IL-11, erythropoietin (Epo), oncostatin M, and the leukaemia inhibitory factor, which are all able to stimulate megakaryocytopoiesis both in vitro and in vivo.6 However, in the presence of Tpo, the loss of such cytokines by gene targeting did not significantly affect platelet numbers. Furthermore, in double knock-out mice lacking Mpl and IL-3, IL-6, leukaemia inhibitory factor, or IL-11, the number of circulating platelets was not lower than that observed in mice lacking only Mpl.7

In conclusion, other factors or cellular interactions may regulate megakaryocytopoiesis and are responsible for the remaining platelet formation in Mpl or Tpo knock-out mice. In support of this concept, recently, it has been demonstrated that fibroblast growth factor 4 (FGF-4) and stromal derived factor 1 (SDF-1) can restore platelet counts to wild-type levels in Tpo or Mpl knock-out mice.9

3. Megakaryocyte development is a spatially regulated process

The BM consists of specialized regions. These regions contain distinct cellular subtypes of stroma, which provide the appropriate signals to cells of the different hematopoietic lineages in different stages of development. Hematopoietic stem cells reside in the stem cell niche, located near the bone endostial surface. The HSCs are fixed by the surrounding microenvironental cells, which results in a stable potential of self-renewal (reviewed in10). To be able to proliferate and differentiate, HSCs need to
detach and move into the central marrow region.\textsuperscript{11,12,13} During the development of the HPC into an MK, the cell migrates via the stromal cells to the BM endothelial sinusoids. Stromal derived factor 1 can induce migration of MKs but does not influence proliferation or differentiation.\textsuperscript{14} The efficiency of SDF-1 to induce MK migration decreases with MK maturation,\textsuperscript{15} in spite of increased expression of its receptor, CXCR-4, during maturation.\textsuperscript{16} This phenomenon could be explained by concomitant upregulation of a protein that negatively influences signaling from the G protein-coupled CXCR-4. This protein is called regulator of G-protein signaling 16.\textsuperscript{17} Thus, SDF-1 might influence MK positioning in the BM, but this is not certain because the exact in vivo gradients of SDF-1 in the BM are not known.

The MKs interact with the BM cells and extracellular matrix via specific adhesion molecules and by the binding of receptors to (presented) chemokines and growth factors. Such interactions have been studied in in vitro culture. However, these results are hard to extrapolate to the in vivo situation, and the 3-dimensional organization of the BM cannot be totally mimicked by in vitro experiments. Furthermore, as mentioned above, not only the contact with the BM environment but also the cytokines produced and their presentation influence megakaryocytopoiesis. For example, BM microvascular endothelial cells (BMECs) and their matrix support long-term hematopoiesis, specifically myelopoiesis and megakaryocytopoiesis, not only by adhesive interactions, but also by the presence of various cytokines. In this respect, unstimulated BMECs constitutively produce G-CSF, granulocyte-macrophage CSF, IL-6, and stem cell factor (SCF).\textsuperscript{18} Furthermore, the endothelial extracellular matrix is rich in heparan sulphate and other glycosaminoglycans, which enables it to bind to the various cytokines involved in megakaryocytopoiesis, such as b-FGF, SCF, granulocyte-macrophage CSF and transforming growth factor-\(\beta\), of which the latter is a negative regulator (references within\textsuperscript{18}).

Although several studies into the role of the BM stroma in MK development have been performed, this role has not been fully elucidated. Some results with BM stroma indicate that fibroblasts or mesencymal cells stimulate proliferation of MKs with a membrane-bound form of SCF,\textsuperscript{19} whereas others indicate that stroma inhibits differentiation into the MK lineage and inhibits MK maturation.\textsuperscript{20,21} Thus, 2 hypotheses on MK development can be deduced. First, detachment of HPCs leads to differentiation toward the MK lineage. The MKs divide during their journey to the endothelium. The second hypothesis implies differentiation of the HSC toward the MK lineage near the sinusoids. This last hypothesis is supported by the description of a second stem cell niche next to the BM sinusoids.\textsuperscript{22} This would enable the HSC compartment to respond directly to differences in the Tpo plasma level.

4. Final stages of MK differentiation and platelet formation

During the late stage of MK differentiation, endomitosis takes place. The initiating molecular signals of this process remain largely unclear. Endomitosis starts as a normal mitosis; the DNA is replicated and the cytoplasmic volume increased. This mitosis, however, is aborted in the anaphase, resulting in a polyploid cell (4-128n). Dysregulation of proteins that normally regulate the nuclear spindle and G1/S transition can be observed during this process. One MK can produce thousands of platelets; therefore, protein production has to be massive. Megakaryocyte
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dpolyploidization results in functional gene amplification to serve this demand.23 This is in accordance with the fact that the number of platelets one MK can produce is related to the size of the MK, which in turn corresponds to the degree of ploidy.24 After polyploidization, the MK starts to form proplatelets, consisting of multiple platelet-sized beads, along their length.25,26 The elongation of proplatelets is guided by microtubules. The transport of platelet-specific organelles and granules to the tip of the proplatelets, where the platelet is assembled and finally shed, also takes place along microtubules (reviewed in27).

Interestingly, proplatelet formation is a form of compartmentalized caspase-dependent cell death.28,29 The core of the MK undergoes apoptosis, whereas the proplatelets are protected. Thrombopoietin plays a stimulating role during MK proliferation and differentiation, but it is likely not needed for the final stage of platelet production and release.30,31 In contrast, Tpo might even inhibit the apoptotic mechanisms required for platelet formation. Moreover, recent studies indicate that Tpo is dispensable for the whole process of platelet production in Tpo and Mpl knock-out mice as long as the MK progenitor interaction with the BM vascular niche is available.32

The role of the vascular niche is very intriguing, because migration of MKs over BMECs is involved in final maturation leading to platelet formation.33 In these in vitro studies, SDF-1 is the trigger for migration, but whether this is also the case in vivo remains unclear. Recent studies indicate that SDF-1 (and FGF-4) also enhances the interaction of the MK progenitors with BMECs, which in the end promotes thrombocytopoiesis.9

The interaction of MKs with endothelial cells can be enhanced by inflammatory cytokines, which results in increased MK differentiation.34 In line with this, activation of BMECs with IL-1ß results in a 3-fold increase in the transendothelial migration of MKs.33 This suggests that the increased interaction is responsible for the thrombocytosis often observed during inflammatory conditions.35

Prudence is in order when the studies using endothelium are interpreted. First of all, in the in vitro studies, the MKs are in contact with the luminal surface of endothelial cells. In contrast, maturing MKs will interact with the subluminal side of BMECs and their deposited matrix in vivo. Second, mostly, interactions between MKs and BMECs and their matrix are studied with respect to platelet fragmentation. However, others have pointed to the possibility of mobilization of mature MKs into the circulation with platelet formation at extramedullary sites, e.g. the pulmonary circulation (reviewed in36).

In conclusion, platelet production seems highly dependent on the adhesive interactions between the MK and the BM vasculature. Localization of progenitors in the vascular niche promotes MK maturation and platelet production; however, the precise mechanisms, and especially the regulation and localization of platelet formation, still require further characterization.

5. Thrombopoietin in clinical trials

Treatment of hematopoietic malignancies with high-dose (myeloablative) chemotherapy results in the strong depression of blood formation. Even with PBSCT support, reconstitution of the hematopoietic potential takes about 2 weeks. The severe thrombocytopenia in this period, notwithstanding prophylactic platelet
transfusions, can lead to life-threatening bleeding. In addition, formation or boosting of alloantibodies to HLA and/or platelet antigens in some patients can cause a severely decreased survival time of, and sometimes refractoriness to, transfused platelets. Finally, transmission of blood-borne infectious agents is a small but never totally avoided risk of allogeneic transfusions. Similar to the successful use of Epo and G-CSF, to stimulate erythropoiesis and myelopoiesis in anemic and neutropenic periods, respectively, Tpo was likely to have great therapeutic potential in thrombocytopenic periods. Indeed, 2 recombinant forms of Tpo, the pegylated recombinant human MK growth and development factor (PEG-rHuMGDF, pegylation prolongs the half life of MGDF) and the recombinant human Tpo (rhTpo) increased platelet levels and marrow MK mass in healthy volunteers and patients with idiopathic thrombocytopenic purpura (ITP). Recombinant Tpo administration does not lead to increased intravascular coagulation or platelet activation. The only adverse effect of administration of recombinant Tpo seems to be the chance of iatrogenic thrombocytosis, which might lead to a thromboembolic event. However, if present, this risk appears to be low.

Unfortunately, after subcutaneous administration to humans, neutralizing antibodies were detected against the pegylated truncated form of Tpo. The antibodies are able to cross-react with endogenous Tpo, causing clinically significant thrombocytopenia. Subcutaneous administration directly delivers high concentrations of recombinant Tpo in a region containing many dendritic (antigen presenting) cells. This might explain antibody formation with this form of administration. Although, antibody formation has never been observed after intravenous administration, and both PEG-rHuMGDF and rhTpo are still in some clinical use, intravenous administration of these factors is not very practical. Therefore, several Mpl agonists (e.g.) have been developed as alternative for recombinant Tpo. These agonists are now tested in the clinic. The advantage of these agonists is that if antibodies are formed against the exogenous stimulator of megakaryocytopoiesis, these should not be able to cross-react with endogenous Tpo. The second advantage is that these agonists can be administered subcutaneously or orally, which is preferred to intravenous administration.

### 5.1 Tpo treatment of chemotherapy-induced thrombocytopenia

In contrast with its effect on thrombocytopoiesis in healthy donors and patients with ITP, after chemotherapy, the usefulness of Tpo (or Mpl agonists) to attenuate the platelet nadir and to shorten platelet repopulation times after chemotherapy, is not as clear-cut.

In agreement with Tpo treatment in healthy volunteers, thrombocytopoiesis is stimulated by Tpo after nonmyeloablative chemotherapy (reviewed in). In contrast, administration of Tpo, after high-dose chemotherapy in combination with a PBSCT, does not have a clinically significant effect on platelet production (reviewed in). Thrombopoietin might not be effective when there is a shortage of MK progenitors. That the amount of MK progenitors present in a transplant is limiting is shown by the inverse correlation between the time to platelet recovery and the number of CD34+CD41+ MK progenitors in a (G-CSF mobilized) PBSCT. This idea is further supported by the observation that if less than the standard threshold of 2 x 10⁶ CD34+ cells (and likely less CD34+CD41+ cells) per kilogram body weight is infused, delayed platelet recovery may occur. Furthermore, infusion of greater than 5 x 10⁶ CD34+
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cells/kg results in faster platelet recovery. In addition, Tpo might not have a positive effect after intensive chemotherapy, because the handling and cryopreservation of the transplants might harm the mature MK (progenitors) more than the early progenitors. When the mature MK (progenitors) are still present in the transplant and home to the BM, these are likely to form platelets earlier than the more immature progenitors. Finally, endogenous Tpo levels are very high after myeloablative treatment, because the lack of platelets and MKs leads to decreased clearance of the constitutively produced Tpo from the plasma. It has been demonstrated that in relation to the level of thrombocytopenia, Tpo levels increase more with each cycle of chemotherapy. An explanation for this effect is the decrease in MK mass. The additional Tpo is probably not able to accelerate formation of MKs from HSCs. In the nonmyeloablative setting, in contrast, not all HSCs and MKs are gone from the BM, and the endogenous Tpo is increased less. The additional Tpo is then probably able to boost megakaryocytopenesis and to increase the platelet yield from the more substantial numbers of MK (progenitors) still present in the BM.

The above findings seem to suggest that a therapy in which the amount of MK progenitors in the SCT is increased would be most effective. There are 2 Tpo-mediated methods to increase the amount of MK progenitors in the graft, using Tpo as a mobilization factor and ex vivo expansion of MKs.

5.1.1 Thrombopoietin as a stem cell mobilization factor

For many years, administration of filgrastim (G-CSF) has been the criterion standard to mobilize HPCs, but new regimens for mobilizing HPCs are still being sought for several reasons. First of all, G-CSF is usually well tolerated, but sometimes has adverse effects such as bone pain, headache, general fatigue, and occasionally splenic rupture. Moreover, some patients, often heavily treated with chemotherapy or elderly, show poor mobilization with G-CSF. Finally, up to 20% of the normal healthy donors for allogeneic transplants are poor mobilizers. Therefore, the effectiveness of other growth factors for HPC mobilization has been investigated. Several growth factors, such as SCF, macrophage inflammatory protein-1α, IL-1, IL-6, IL-7, IL-8, IL-11, and Flt-3 ligand, have been shown to be able to mobilize HPCs (reviewed in and references within). However, the effect was not as strong as with G-CSF. Nevertheless, some of these growth factors and others, such as IL-3 and Epo, have been shown to synergistically enhance mobilization with G-CSF.

Also, Tpo has been studied with the aim to boost CD34+ yield. It has been shown that administration of PEG-rHuMGDF to humans, despite its “lineage-specific” activity on mature cell development, results in mobilization of HPCs of multiple lineages. Initial studies, with different dose schemes of rhTpo administration, together with G-CSF, showed higher CD34+ yields. The number of apheresis procedures needed to obtain sufficient numbers of CD34+ cells was lower in the groups mobilized with Tpo and G-CSF compared with those mobilized with G-CSF only. Hypothesizing that the administration of Tpo could increase the mobilization of CD34+CD41+ MK progenitors, one could expect to see enhanced platelet recovery after transplantation of cells mobilized with Tpo and G-CSF. However, only one of the studies showed a weak positive effect on platelet repopulation. This study demonstrated that in patients transplanted with cells mobilized with G-CSF and Tpo, granulocyte (P < .0001) and platelet (P = .07) recovery were accelerated and fewer erythrocyte and platelet transfusions were needed compared with G-CSF-mobilized...
patients. Increased percentages of CD41+ cells (within the CD34+ population) were seen after administration of either single or repeated injections at the higher rhTpo doses. However, the goal of this study was to infuse higher number of stem cells, not more MK progenitors, and that might explain why no correlation was sought and reported between the number of CD34+CD41+ cells infused and the speed of platelet recovery. In the other 2 studies CD34+CD41+ levels in the transplant were not reported.

The effect of combining Tpo with G-CSF has also been investigated in the mouse model. In mice, much higher doses of Tpo can be administered to investigate the maximal effects on platelet levels and progenitor mobilization. Administration of PEG-rHuMGDF to mice has similar effects as in humans; a rise in platelet, but not in erythrocyte and white blood cell numbers, and mobilization of multipotent and more primitive (spleen colony-forming unit [CFU-S]) progenitors. There is a difference in the kinetics of the mobilization and the rise in platelet level, suggesting that the enhanced HPC mobilization is not related to the enhanced megakaryocytopoiesis. In initial dose-finding experiments, mobilization of HPCs was dose dependent up to Tpo doses of 300 μg/kg a day, whereas platelet counts were already maximal at 30 μg/kg a day.56 Concurrent administration of optimal doses of PEG-rHuMGDF and recombinant human (rhG-CSF) only increased the number of MK progenitors (colony-forming unit-megakaryocyte [CFU-MK]) in the peripheral blood (PB) and not of the other progenitors. At suboptimal doses of both growth factors, significantly higher numbers of progenitors of all lineages are mobilized into the PB, compared with mice treated with either factor alone.

Treatment with PEG-rHuMGDF resulted in an expansion of the number of progenitors in the BM before the maximal increase in circulating PB progenitor cells was observed, suggesting the migration of the expanded BM cells into the PB.56 Furthermore, treatment with rhG-CSF caused a decrease in the number of progenitors in the BM, whereas PEG-rHuMGDF did not. These different effects of the 2 growth factors on HPCs in the BM suggest different mechanisms of PB stem cell (PBSC) mobilization by the 2 factors. This hypothesis is supported by the difference in the kinetics of mobilization by the 2 growth factors. Addition of PEG-rHuMGDF for 10 days gave optimal mobilization, whereas rhG-CSF only had to be added for 5 days. In addition, mainly relatively committed progenitor cells were mobilized by PEG-rHuMGDF, whereas rhG-CSF mobilized mainly immature progenitor cells. Although cells mobilized with Tpo are able to induce long-term repopulation in mice, this mobilization of relatively mature progenitors by Tpo should be taken in consideration when Tpo would be used in the clinic. Dosing schemes involving Tpo, which mobilize enough long-term repopulating cells for proper BM reconstitution, should be established.

Curiously, although different mechanisms seem to result in mobilization by these 2 growth factors, they do influence each other. As mentioned above, the combination of suboptimal doses of Tpo and G-CSF in mice results in synergistic mobilization.56 Furthermore, treatment with G-CSF is less efficient for PBSC mobilization in Mpl knock-out mice than in control mice, suggesting that Tpo (signaling) takes part in PBSC mobilization induced by G-CSF.57

Besides using Tpo as a mobilizing agent, it could also be used as an HSC and MK progenitor cell-protecting agent. In this regard, Tpo has been demonstrated to be involved in HSC self-renewal and proliferation58,59 and also prevents apoptosis of
MKs (progenitors). Indeed, pretreatment of patients with Tpo seems to reduce the severity of chemotherapy-induced thrombocytopenia.

Thus, from the available clinical studies, it is so far unclear whether addition of Tpo to the mobilization scheme increases MK progenitor mobilization and content of the PBSCT. But data from mice studies seem to point to the merits of this approach. The most optimal dose and timing of Tpo, on its own or in combination with G-CSF, remains to be determined. At least Tpo shows slower responses and thus should be administered before G-CSF treatment is started.

5.1.2 The use of MKs ex vivo expanded with Tpo

Several clinical studies have been performed to investigate whether the chemotherapy-induced thrombocytopenic period could be shortened by the addition of ex vivo-generated autologous MKs to standard PBSCTs. Comparison of these studies is difficult, because patients with various malignancies were included, different culture protocols for the production of MKs were used, and different numbers of cells were transplanted. For successful platelet repopulation, the MKs need to meet specific requirements. First of all, short-term platelet repopulation probably needs more mature, and thus more fragile, MKs. However, it is likely that the harvesting and transplantation of in vitro cultured MKs is more damaging for mature than for immature MKs. Second, platelet repopulation from MKs and megakaryoblasts in the transplant can only occur if these cells are still able to home to a site that supports proplatelet formation and fragmentation into platelets. In view of the above, the homing efficiency to these sites might be different for the mature and immature MK progenitors within the transplant. For example, as mentioned earlier, the maturation influences the sensitivity of the MK for the chemokine SDF-1.

Two studies with expanded MKs suggested a positive effect on platelet reconstitution. In the first described clinical trial with ex vivo-expanded MKs, 2 patients that received the highest doses of MKs (9.8 and 21.3 x 10^5 CD41+ cells/kg body weight) did not need a platelet transfusion, whereas all patients of a retrospective control group required allogeneic platelet transfusion support. In support of this concept, in a recent study in which patients served as their own controls in separate cycles of chemotherapy, there was faster neutrophil and platelet recovery when patients received additional ex vivo-expanded cells (average of 10.1 x 10^6 CD41+ cells/kg). This study, however, also included G-CSF in the ex vivo expansion cytokine cocktail. This resulted in a faster neutrophil recovery, which, by reduction of the risk of febrile neutropenia, may be partially responsible for the acceleration of platelet recovery and the diminished need for platelet transfusions. However, reports on in vivo administration of G-CSF do not make this a likely scenario.

In 2 other clinical studies, similar amounts of cultured CD41+ cells were transplanted, but no effect on the platelet nadir or recovery, or the number of required platelet transfusions, was observed. In most expansion protocols, cells are cultured for at least 7 days. Ratajczak et al. demonstrated that short priming (36 hours) of murine progenitor cells with a cocktail including Tpo significantly increased platelet recovery after transplantation. This priming was most efficient when it was done before cryopreservation. However, whether priming is able to hasten platelet recovery in humans has yet to be investigated.

The conflicting clinical results indicate that more research needs to be done before
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clinical application of ex vivo-expanded autologous PB CD34+ cells will successfully shorten the thrombocytopenic period after intensive chemotherapy and a subsequent PBSC. In this respect, knowledge of MK homing and subsequent platelet formation is very important. Currently, little is known of the homing capacity of (cultured) MKs. Two studies in mice show enhanced human platelet formation with MKs expanded from cord blood (CB) CD34+ cells. Bruno et al. detected human platelets in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice 3 days after transplantation. When uncultured CD34+ cells were injected, human platelets were first identified after 3 weeks, indicating that the platelets formed after 3 days are derived from MK-committed cells. This indicates that cultured MKs are able to home to an environment in which they can form platelets. Whether this occurs in the BM or the lung is still a matter of debate. In contrast to the 2 studies described above, transplantation into mice of ex vivo cytokine-stimulated human PB HPCs provided delayed and transient platelet production in mice compared with that seen with unstimulated PB progenitor cells. The source of the CD34+ cells used for MK expansion might in this respect be important. Megakaryocytes generated from CB cells are less mature (lower level of CD41 expression and ploidy), and therefore, less fragile than PB-derived MKs. Clinical trials with expanded cord blood cells might thus be a more practical target, especially while unmanipulated cord blood transplants are known for their slow platelet recovery potential. However, in an ongoing experiment where ex vivo-expanded PB CD34+ cells were transplanted into NOD/SCID mice, alone or in combination with unexpanded CD34+ cells, we observed enhanced human platelet formation by ex vivo-expanded PB cells. These cells formed platelets after 3 days and platelet numbers increased till day 10 and thereafter decreased [unpublished observations]. This indicates that the ex vivo-expanded PB cells can indeed be used to overcome the 2-week period of thrombocytopenia seen after high-dose chemotherapy. Perhaps, our culture protocol is more applicable for the production of the MK cells needed for fast platelet repopulation. But the conflicting results might also be explained by the fact that we transplanted higher cell numbers. It should be mentioned that until today, it is not known whether the number of transplanted immature CD34+CD41+ megakaryoblasts is more important than the number of transplanted mature CD41+ MKs. More mature cells are able to form platelets faster, but might also be more prone to damage by transplant handling.

6. Summary and discussion

Despite numerous attempts with different approaches, severe thrombocytopenia after high-dose chemotherapy, cannot be treated other than with platelet transfusions. Thrombopoietin administration during or after high-dose chemotherapy was not successful in reducing the platelet nadir and repopulation time. Considering the efficacy of Tpo in the treatment of ITP and low-dose chemotherapy induced-thrombocytopenia, the findings after high-dose chemotherapy need reflection. As mentioned, the inefficacy of Tpo administration to shorten platelet recovery under these conditions might be due to several factors, including an inability of Tpo to accelerate the HSC development into MKs and platelet production (figure 1).

Other approaches that might overcome this problem are the ex vivo expansion of MKs (with a growth factor cocktail containing Tpo), or using Tpo as mobilization factor to increase the MK progenitor content of the SCT. Animal studies are promising, but
so far, only a few studies in humans have been reported, showing only limited benefit. No easy applicable therapy has yet evolved from these approaches.

A different approach to overcome chemotherapy-induced thrombocytopenia might be a better preservation of the MK population in the graft. The necessary cryopreservation of autologous SCTs is likely to damage the more fragile and larger MK progenitors. The transplant is usually frozen in 10% dimethyl sulfoxide (DMSO) in a rate-controlled freezer to cool at fixed temperatures from -160 °C to -180°C. Current protocols are based on those developed for BM mononuclear cells. The optimal freezing curve for the CD34+CD41+ MK progenitors is probably different. Adjusting the freezing program such that MK progenitor cell viability after thawing is increased will likely result in faster platelet repopulation after high-dose chemotherapy and PBSCT. A second way to improve cell viability after cryopreservation may be to decrease the percentage of DMSO used. Abrahamsen et al. have shown in several recent articles that when cells are frozen according to the standard freezing curve, 5% DMSO preserves HPCs better than 10% DMSO. Lowering the DMSO concentration also has
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the advantage that patients will suffer less from DMSO-related adverse effects when
cryopreserved cells are reinfused.

In addition to all the above approaches, we should realize that fast platelet
recovery after a PBSCT is not only determined by the number of MK progenitors in
the graft. It will depend foremost on the efficacy of homing of the MK progenitors
to a designated environment, either the BM or possibly the lung. Furthermore, the
capacity to terminally differentiate and form platelets in this environment is also
a critical parameter. Considering the use of Tpo, there is currently no evidence for
involvement of Tpo in either of these mechanisms.

A totally different approach, without the requirement of manipulating in vivo
platelet production, might be to treat patients with autologous platelets, isolated and
cryopreserved after Tpo-mobilization in the period after intensive chemotherapy.75

In conclusion, if we want to improve platelet repopulation after high-dose
chemotherapy, we might use mobilization with Tpo or ex vivo MK expansion to increase
the number of MKs (or MK progenitors) in the transplant. However, the homing
capacity of the manipulated MKs and the integrity of the BM microenvironment
should be sufficient for platelet formation and if possible improved. Bearing in mind
the importance of the vascular niche for MK homing as well as for platelet formation,
high-dose chemotherapy is known to particularly injure the BM vasculature. Another
strategy might thus be to protect BM integrity during, and improve regeneration
after, intensive chemotherapy. Proangiogenic factors, such as angiopoietin 1, might
mediate this.76

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Clinical use of Tpo
