From stem cell to platelet
Tijssen, M.R.

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Chapter 9

Summary and general discussion
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The culture of megakaryocytes for the optimization of peripheral blood stem cell transplantation

Profound thrombocytopenia following high dose radio- or chemotherapy is still one of the major complications inherent to hematopoietic stem cell transplantation. Thrombopoietin (Tpo) administration improves thrombocytopenia caused by non-myeloablative treatment, but not that caused by myeloablative treatment. This difference in the effectiveness of Tpo might be due to the low number of megakaryocyte (MK) progenitors in the bone marrow (BM) after myeloablative therapy. Therefore, improving progenitor cell viability after cryopreservation, increasing the number of megakaryocyte progenitors in the transplant by using Tpo as a progenitor cell mobilization factor, or administration of megakaryocytes expanded in vitro could all potentially decrease thrombocytopenia in this setting (Chapter 1).

After harvest from peripheral blood, stem cells are usually cryopreserved before being administered to the patient. The cryopreservation process is detrimental to the stem cells and although in most transplants enough repopulation capacity is maintained to ensure full hematopoietic recovery, optimization of the cryopreservation and thus improvement of the quality of the infused cells, might accelerate recovery. Furthermore, the infusion of DMSO, which is used to protect the cells against damage induced by freezing, can also cause side effects. Current protocols for cryopreservation of peripheral blood stem cell transplants (PBSCTs) have been developed empirically. Freezing methods could be improved by using a fundamental cryobiological approach that addresses the putative causes of cell injury during freezing and thawing. Mathematic modeling of the osmotic events during cryopreservation can be used to predict the optimal cooling rates for specific cell types. The model developed by Woelders and Chaveiro calculates the ‘compromise’ cooling rate for every subzero temperature and its results show that optimal freezing curves are non-linear. In Chapter 2, we used this model to calculate ‘optimal’ freezing curves for PBSCTs with 10% and 5% DMSO using the values for membrane permeability coefficients and other related parameters for cord blood HPCs that were published earlier. Unselected and CD34-selected PBSCs were cryopreserved by means of the standard or the calculated freezing curves and the results were compared. Post-thaw quality was evaluated by cell viability, CFU-GM colony formation and megakaryocyte growth. With 10% DMSO, the use of the calculated freezing curve compared to the standard one resulted in increased viability of CD34+ cells. Lowering the DMSO concentration to 5% improved cell viability and functionality with results similar to the ones obtained with 10% DMSO and the calculated freezing curve. However, there was no further gain from using the calculated freezing curve with the lower concentration of DMSO, suggesting that the cooling rate of the calculated curve for 5% DMSO may have been too high. Indeed preliminary experiments with a slower non-linear freezing rate suggest that further improvement is possible. Thus our results indicate that the current cryopreservation method for PBSCT can be improved by using calculated freezing curves and/or a lower concentration of DMSO, thereby improving cell viability and ultimately the outcome of PBSCT.

During the freezing process, transition from the liquid to the solid phase occurs. Heat of fusion liberated during crystallization just below 0°C results in a sudden increase in temperature. Most current cryopreservation methods use linear freezing curves that provide a progressive (1°C/min) reduction of the temperature of the
material to be frozen and thus compensate for the heat of fusion.\textsuperscript{8} However, the freezing curve obtained with uncontrolled-rate freezing is non-linear and therefore similar to our calculated 'optimal' freezing curve. Several clinical studies have shown that uncontrolled-rate freezing in a -80°C freezer, which does not compensate for the heat of fusion can be safely used for PBSCTs.\textsuperscript{8,9,10,11,12,13} The successful use of uncontrolled-rate freezing in the clinical setting strongly suggests that our calculated freezing curve could safely be applied for cryopreservation of PBSCTs. Reduction of the DMSO concentration has also been shown to be safe for clinical application.\textsuperscript{12,13,14} The major increase in post-thaw viability and \textit{in vitro} functionality we observed in our study with 5% DMSO concurs with these previous reports.

Using 5% DMSO instead of 10%, we observed an increased megakaryocyte growth from 3.9±0.6 MKs per seeded CD34\textsuperscript{+} to 6.3±0.9 (p<0.01). Improving the preservation of early megakaryocyte progenitors could potentially reduce the severity of thrombocytopenia after high-dose chemotherapy followed by a PBSC transplant. The caveat to this is the fact that the percentage of CD41\textsuperscript{+} cells in a PBSC transplant is usually very low. And although it could be argued that the early MK progenitor does not yet express CD41, in our opinion it is the lack of megakaryocyte progenitors and thus the delay for a stem cell to differentiate into platelets that is the critical parameter in the duration of thrombocytopenia. Therefore, an alternative option to decrease the thrombocytopenia after a PBSCT would be to increase the number of MK progenitors in the harvest. This might be accomplished with Tpo as a mobilization agent (Chapter 1).

Results from several clinical trials published in the last decade on the co-transplantation of megakaryocytes expanded \textit{ex vivo} have shown minimal or no positive effect on platelet recovery after a PBSCT.\textsuperscript{15,16,17,18} To study this in more detail, we investigated platelet formation from \textit{in vitro} cultured MKs in NOD/SCID mice (Chapter 3). Despite the unproven benefit of using a lower concentration of DMSO for the cryopreservation of CD34\textsuperscript{+} cells on thrombocytopenia after transplantation in humans, its effect was clear on the \textit{ex vivo} expansion of autologous megakaryocytes from CD34\textsuperscript{+} cells (Chapter 2). This made the use of 5% DMSO the logical choice for cryopreservation of CD34\textsuperscript{+} cells in this study. When a minimum of 2.25 x 10\textsuperscript{6} stem cells were expanded and differentiated towards megakaryocytes for 7 days and then re-infused in the mice, platelet formation was detected in peripheral blood of the mice as early as three days post-transplant. The human platelets formed in the mice were shown to be activated by TRAP-6, which suggests that they are functional. When stem cells were cultured for a shorter period (overnight or 4 days), platelet formation was delayed. This study demonstrates that CD34\textsuperscript{+} cells differentiated towards MKs for 7 days are able to home to an environment suited for platelet formation, and that these MKs can contribute to early platelet formation. This model can be used to further investigate the fate and properties of cultured MKs.

We did not check whether the transplanted cells ended up in the BM and whether it was there that platelet production occurred. There has been some debate whether the lung or the BM is the site of platelet formation.\textsuperscript{19} A recent study showed that transplantation of cord blood (CB) cells cultured with Tpo in NOD/SCID mice resulted in a 4-fold increase in CFU-MK numbers in the BM 7 days after transplantation.\textsuperscript{20} In that study, 3.8% of the expanded cells were present in the BM 24 hours after injection, but 3.5% of the expanded cells were also detected in the lungs (Y. van Hensbergen personal communication). Therefore it remains unclear whether the lungs, the BM
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or both contribute to the platelet formation. In follow-up experiments, we could consider culturing CD34+ cells by our method and transducing them with GFP to be able to detect the cells in the BM or in the lungs to clarify this issue. Transducing the cultured cells with GFP would also render it possible to make a distinction between platelets formed by cultured MKs from platelets formed by uncultured CD34+ cells.

Recently, an alternative model in which human platelet-rich plasma was injected into NOD/SCID mice has been described. Immediately after injection in the mice, human platelets comprised ~13% of the total circulating platelet population, which decreased to half that number in 24 hours. When the authors transplanted human peripheral blood CD34+ cells in the mice only ~2% of the circulating platelets were human after 14 days. Platelet numbers peaked at ~16% on week 3 after transplantation of 8 x 10^6 CD34+ cells and were hardly detectable after 4 weeks. As the platelets were only detectable in such a narrow window the authors proposed their model as a tool to investigate immune thrombocytopenias, the efficacy of novel antithrombotic, antiplatelet therapeutics and platelet storage. In our NOD/SCID model, the percentage of human platelets peaked at day 14 at ~44% (n=5) after transplantation of only 4.5 x 10^6 cells. The percentage declined to ~20% at day 21, and ~3% at day 28 after transplantation. However, as can be seen in chapter 3, during this period human platelets can clearly be detected in the mouse blood. The median number of human platelets per ml mouse blood was 1.7 x 10^7 (n=10) at day 14, 2.7 x 10^7 at day 21 and 4.5 x 10^6 at day 28. After sublethal irradiation the number of mouse platelets per ml of blood decreased from the baseline 1 x 10^9 to below 1 x 10^8 at day 7. Thereafter, the platelet count started to increase. The decrease in percentage of human platelets between day 14 and 28 is thus partly caused by the comparative increase in mouse platelets. Indeed, the number of mouse platelets per ml of blood increased about 4-fold from day 14 to day 28. This shows that in our model more than enough human platelets can be detected for a duration of at least three weeks to do functional studies. The model of Newman et al. is currently the only available model to investigate the effect of storage on platelets. However, our model appears to be more convenient to investigate immune thrombocytopenias and the efficacy of novel antithrombotic, antiplatelet therapeutics, due to the higher percentage of human platelets and the longer window during which functional experiments can be performed.

In our hands, the number of MKs needed to significantly improve platelet recovery in NOD/SCID mice was high. New studies should address the issue as to how many MKs are needed to improve platelet reconstitution in humans. For most autologous transplant procedures a graft of 4 x10^6 CD34+ cells/kg is sufficient for full hematopoietic recovery, and this can normally be harvested in one leukapheresis procedure. If a further collection of 4 x10^6 CD34+ cells/kg was used for ex vivo expansion of MKs with our 7-day protocol, between 12 x10^6 and 18 x10^6 CD41+ cells/kg could be co-transplanted, rendering this a clinically applicable and potentially beneficial procedure. However, in one of the published clinical studies in which high numbers of CD61+ cells (7-16 x 10^6 CD61+ cells/kg) were transplanted no positive effect was observed. In that particular study, cells were cultured for 10 days in the presence of MGDF and SCF. Perhaps this longer culture period resulted in more mature, larger, and thus more fragile MKs, which could have been damaged through the harvesting and re-injection procedures. The efficient and fast platelet formation from cultured MKs in our animal model implies that a clinical application of cells
cultured according to our protocol could potentially accelerate platelet recovery, but it is possible that not only the number, but also the quality of the CD41+ cells is decisive for the outcome. This should be taken into account when cultures are scaled up, because handling of larger MK transplants might decrease the quality of the cells.

The culture of large amounts of MKs is labor intensive and necessitates large amounts of culture medium and cytokines. The cost of this therapy will quite likely be very high. Thrombocytopenia can be treated with straightforward platelet transfusions, and the number of life-threatening bleedings after PBSCT is currently very small. However, in some patients, platelet transfusions can lead to production of alloantibodies, making further transfusion support more difficult and less effective. It is doubtful whether the use of \textit{ex vivo} expanded MKs will ever be standard clinical practice, but for patients refractory to platelet transfusions or heavily pretreated with chemotherapy, the transplantation of autologous MKs may be justified.

\textbf{The culture of megakaryocytes to investigate megakaryocytopoiesis in a clinical and research setting}

In patients affected with thrombocytopenia of unknown origin, it may be of interest to assess whether bone marrow CD34+ cells are able to form MKs. A simple BM smear can help answer that question, but if no MKs are present in the BM, it is not known whether this is due to an intrinsic defect of the CD34+ cells or a defect of the environment required for these cells to grow and differentiate. As described in Chapter 4, \textit{in vitro} culture of megakaryocytes, by incubating hematopoietic progenitor cells with thrombopoietin, can help to correctly diagnose the pathogenesis of thrombocytopenia in these cases. However, the use of the MK culture for diagnostic purposes is limited. It can only add to the standard tests for the diagnosis of thrombocytopenia in rare cases.

One disease in which the culture can be very helpful is Congenital Amegakaryocytic Thrombocytopenia (CAMT). In this disease, plasma Tpo levels are high but no megakaryocytes are formed when the patient’s BM CD34+ cells are cultured with Tpo. It has been shown that this is caused by defects in the Tpo-receptor, Mpl. The CAMT patients have been divided into two groups. CAMT I patients have a severe thrombocytopenia from birth onwards and early progression into bone marrow failure, whereas the CAMT II patients have a less severe phenotype. CAMT I patients always carry mutations that predict total loss of Mpl protein, and it has been suggested that missense mutations are associated with CAMT II. In Chapter 5 we investigated the functional defects caused by missense mutations identified in CAMT patients and whether this correlated with the course of the disease in these patients. Wild-type Mpl and the different mutants were expressed in the human erythroid leukemia cell line K562. The first mutation, P136H, probably caused mRNA instability, because this mutant could not be detected at the mRNA level. A mutation in the last amino acid of the intracellular signaling domain, P635L, led to lower protein expression, possibly caused by a higher rate of protein degradation. The last two mutants, R102P and R257C, were expressed in the membrane fraction of the K562 cells to a similar extent as wild-type Mpl, although the migration pattern on Western blot differed from that of wild-type possibly due to glycosylation differences. All three mutants that were expressed showed impaired signaling via the MAPK,
JAK/STAT and PI3K pathways after incubation with Tpo. The R102P and R257C mutants induced only minimal phosphorylation of the signaling molecules, while the P635L mutant induced somewhat more phosphorylation, but still less than wild-type Mpl. These results demonstrate that missense mutations can lead to total loss of Mpl function and thus CAMT I. Our results are supported by a recent study in which it was demonstrated that also patients carrying missense mutations can develop early bone marrow failure.\(^{29}\) The only curative treatment available for this disease is hematopoietic stem cell transplantation. It is therefore important to be able to predict which patients are more likely have a severe form of the disease. And because missense mutations can lead to both a CAMT I and a CAMT II type clinical course, it is fundamental to evaluate the effects of these mutations in \textit{in vitro} assays to determine the expression levels and functionality of the mutants.

The differences we observed in expression level and glycosylation of the Mpl mutants may be an artefact resulting from the use of a cell line. It would be interesting to examine the expression levels of the mutants in the patients’ cells, however due to the scarcity of the material and given the cell numbers necessary to carry out biochemical assays, these experiments cannot be easily performed. Furthermore, attempts at flow cytometric analysis of Mpl expression were unsuccessful. Investigations of mRNA stability of the P136H mutant and protein stability of the P635L mutant would also be of interest. K562 cells do not need cytokine stimulation to grow. Therefore, we could not investigate whether the mutants that gave signaling actually stimulated proliferation. The BaF3 cell line is IL-3 dependent for its growth, and after transduction of Mpl this cell line also grows with Tpo, but transduction of the mutant receptors in this cell line failed.

Currently, patients with CAMT and development of aplastic anaemia can only be treated with life-saving allogeneic bone-marrow transplantation. With the development of peptide and antibody agonists for the Tpo receptor, another treatment possibility arose.\(^{32}\) A recent paper describes so-called minibodies, antibody fragments engineered to more efficiently dimerize Mpl that could to activate the R102P and R257C mutant receptors.\(^{33}\) Our expression model in K562 cells can be used to investigate whether Mpl agonist are able to activate mutant receptors identified in CAMT patients carrying missense mutations. Furthermore, the culture of megakaryocytes from the patient’s BM CD34\(^+\) can be used to examine whether an agonist is able to activate endogenous expressed mutant Mpl.

Megakaryocyte culture was used as a diagnostic tool in one further clinical case. The culture of BM CD34\(^+\) cells taken from a boy with cyclic thrombocytopenia demonstrated the presence of a growth-stimulating factor in his plasma when his platelet numbers were in the ascending phase (\textit{Chapter 6}). We did not detect specific factors influencing the feedback between megakaryocytopenia and platelet mass, but our results would be consistent with cyclic changes in the level of a cytokine that is as yet uncharacterized.

In \textit{Chapter 7} the transcriptomes of megakaryocytes and erythrocytes cultured from cord blood (CB) CD34\(^+\) cells were compared to identify platelet specific genes. Amongst others we identified membrane-bound proteins G6b, G6f, and LRRC32 as platelet-specific genes. SUCNR1 and LRRC32 were also expressed to a higher extent in megakaryocytes than in erythrocytes, but were also found in other blood cell lineages. The expression of these proteins in platelets and on the platelet surface was confirmed by Western blot and by flow cytometry, respectively. Although the precise
biological roles of these proteins are still unclear we demonstrated that succinate, the agonist for SUCE1, potentiates platelet activation by standard agonists at low concentrations. Confirmation of these data by comparing the transcriptome of cultured MKs with MKs isolated directly from bone marrow would be the logical next step. However, MKs are difficult to isolate from bone marrow, as they represent only 0.05-0.1% of the bone marrow cell population and are very fragile. Until now, we have not been able to obtain a population of BM MKs large and pure enough to perform this analysis. However, as these genes were also detected in platelets, both at the mRNA and at the protein level, and as our data significantly overlap with data from earlier studies on the platelet or megakaryocyte transcriptome, it can be assumed that the cultured MKs are comparable to BM MKs with respect to gene expression. To investigate the functional role of the genes identified by this method in human platelets, cultured megakaryocytes can be manipulated to overexpress/downregulate these proteins and then be transplanted into the NOD/SCID mouse, in which platelet function can be studied.

When hematopoietic stem cells start to mature in the presence of Tpo, a bipotent erythroid/megakaryocytic progenitor is formed. In the continuing presence of Tpo this progenitor differentiates into a megakaryoblast that will undergo endomitosis and mature into a multinucleated megakaryocyte. Until now, the process of bifurcation of the erythroid and megakaryocytic lineage is not fully understood. It is known that transcription factors like Fli-1, GATA-1/FOG-1, NF-E2, and BACH1 are involved, but the precise signaling events involved have yet to be elucidated. Insights in this phenomenon are not only of interest to understand basic hematopoiesis, but may also lead to novel clinical strategies to regulate the production of red cells and platelets. The gene lists generated from the comparison of megakaryocytes and erythroblasts in the study described in chapter 7 might include some of the elementary factors involved in this process, and this might open new avenues of investigations.

**Effect of thrombopoietin on endothelium**

Mature megakaryocytes form platelets by a process that is still largely unknown, but recently some major advances have been made in our understanding of this process. Megakaryocytes protrude long cytoplasmic extensions, called pro-platelets, through the endothelium into the blood stream. At the tips of these extensions platelets are shed into the blood stream. However, the environmental cues that trigger the formation of proplatelets and induce platelet shedding are not known. We have tried extensively to produce platelets from megakaryocytes in liquid cultures, but despite several studies claiming the generation of a large amount of platelets in vitro, we were not able to reproduce these results. It is likely that some key elements of the BM niche - as yet unknown - are important for platelet formation. For the actual platelet shedding, it may for example be necessary that megakaryocytes migrate over endothelium. Several studies have indeed shown an increase in platelet formation when MKs are allowed to migrate over endothelial cells. We are currently testing this hypothesis, but preliminary experiments indicate that migration of MKs over bone marrow endothelial cells does not induce significant platelet formation. As seen in our attempts to produce platelets in liquid culture, only platelet particles carrying platelet activation markers were detected with flow cytometry. It is possible that proplatelets need to be exposed to the shear of the blood flow before platelets
can be shed. In support of this notion, proplatelet-like structures can be detected in blood, and therefore it is likely that the proplatelets mature into platelets in the bloodstream.\textsuperscript{38}

In Chapter 8, we demonstrated that Tpo, via its receptor Mpl, increased the electrical resistance of human umbilical vein endothelial cell (HUVEC) and human bone marrow endothelial cells (HBMEC) cell monolayers. This increase in resistance was accompanied by a decrease in phosphotyrosine staining and co-localization with VE-Cadherin. We also showed that Tpo can activate Rap1 in endothelial cells. Together, these results suggest that Tpo plays a role in endothelial cell-cell junction formation. Tpo is released from platelets recruited to a site of vascular injury, and may thus play a role in the repair of the endothelial cell layer of the vessel wall.

Besides its role in vascular repair, the observed effect of Tpo on the endothelium might also be important for platelet formation. To our knowledge, we are the first to demonstrate the presence of Mpl protein in human bone marrow endothelial cells (HBMECs). In the bone marrow, the interaction between the microvascular endothelium and megakaryocytes is critical for both thrombocytopoiesis and marrow revascularization after myelosuppression.\textsuperscript{41} During megakaryocytopoiesis the cell migrates from the endosteal region to the sinusoids. At the sinusoids, in close contact with the endothelium, the final stages of megakaryocyte differentiation take place. As mentioned above, the external signals that induce the protrusion of proplatelets through the endothelium and the shedding of the platelets are currently not known.

The bone marrow microvasculature is a discontinuous endothelium, to allow mobilization and homing of hematopoietic cells. This implies that the protrusion of proplatelets is not hampered by close cell-cell contacts. As we have shown, Tpo increases cell-cell contacts in endothelial cells, and this could potentially impair this process. Furthermore, it has been demonstrated that Tpo is not necessary for the final stages of megakaryocytopoiesis.\textsuperscript{42,43} These results suggest that localized variations in Tpo concentration may exist in the bone marrow. If we were to set up an assay in which MKs would be allowed to migrate over endothelium in the presence of flow, and if this would result in platelet shedding, it would be interesting to investigate whether the presence of Tpo inhibits the formation of platelets.

In conclusion, the data presented in this thesis show that the hematopoietic recovery and in particular platelet recovery after stem cell transplantation can potentially be improved. Using the growth of megakaryocytes from peripheral blood stem cells as a quality marker, we were able to demonstrate that cryopreservation of PBSC transplants can be optimized. Furthermore, cultured megakaryocytes were shown to efficiently form platelets in an 	extit{in vivo} murine model, demonstrating the potential clinical application of this method. The culture of megakaryocytes was also shown to be useful to investigate rare cases of thrombocytopenia and to better understand the fundamental process of megakaryocytopoiesis. Many research groups are currently investigating the final stages of megakaryocytopoiesis, including interactions with the endothelium. It may be possible in the near future that culture \textit{in vitro} \textit{“from stem cell to platelet”} would graduate from mere hypothesis to exciting and promising reality.
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