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## Chapter 4

### **The (patho)physiology of megakaryocytopoiesis: from thrombopoietin in diagnostics and therapy to *ex vivo* generated cellular products**

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## General introduction

The height of the normal level of blood platelets ( $150\text{--}350 \times 10^9/\text{l}$ ) and their short life span (approximately 10 days) explain their production rate of  $150 \times 10^6/\text{min}$ . Thrombopoietin (Tpo) is the key growth factor in this production (reviewed in<sup>1</sup>). Tpo consists of a 153-amino-acid long amino-terminal receptor-binding domain and a 179-amino-acid carboxy-terminal domain, which is involved in its secretion and protection from proteolysis. The Tpo receptor, Mpl, was discovered in 1992<sup>2</sup> and is expressed on haematopoietic stem cells (HSCs;  $\text{CD}34^+$ ), the large polyploid megakaryocyte (MK; platelet progenitor) and platelets themselves.<sup>3</sup> Though genetic elimination of Mpl and Tpo in mice was shown to lead to a strong reduction (85%) of platelet numbers (reviewed in<sup>4</sup>), some platelets are still formed. While synergy with Tpo has been described for SCF,<sup>5</sup> effective platelet formation might also depend on factors like SDF-1 and FGF-4. Upregulation of adhesion molecules by these factors enhances late megakaryocytic development and platelet formation by growth promoting interactions between MK and marrow cells.<sup>6</sup>

## Tpo in diagnostics

After the cloning of the Tpo gene in 1994 (reviewed in<sup>7</sup>), Tpo mRNA was found to be present in many tissues. Its production, however, is dominated by the liver.<sup>8,9,10</sup>

Under normal conditions, Tpo production is constant and its plasma level is regulated by internalization and degradation after binding to Mpl-expressing cells.<sup>11,12</sup> This usually negative correlation between Mpl-expressing cells and Tpo levels can be used to distinguish different forms of thrombocytopenia. For example, patients with normal platelet production but low numbers due to shortened survival time by autoimmune antibodies (AITP), have normal levels of Tpo.<sup>13</sup> In contrast, chemotherapy-induced loss of MKs and platelets, and thus Mpl-expressing cells, leads to high Tpo levels.<sup>14</sup>

Pitfalls in the interpretation of Tpo levels, however, should be discerned. Improperly high Tpo levels are sometimes observed in patients with high platelet counts. For example, in patients with reactive thrombocytosis Tpo levels seem secondary to inflammatory factors like interleukin-6 (IL-6),<sup>15</sup> e.g. after surgery.<sup>16</sup> Furthermore, patients with primary or essential thrombocythaemia (ET) and also other myeloproliferative syndromes have high Tpo levels, probably due to Mpl downregulation or defective Mpl metabolism and thus defective Tpo clearance by the circulating platelets.<sup>17,18</sup>

## Bone marrow and megakaryocytopoietic assays in diagnostics

While bone marrow smears can visualize extreme dysplastic or absent megakaryocytopoiesis, definite conclusions about the effectiveness of platelet production can hardly be made. Moreover, the *in vivo* clearance of radiolabelled blood platelets enables determination of platelet destruction and production rate, but these assays are cumbersome and rarely performed nowadays. Therefore, *in vitro* megakaryocytopoietic assays may be an alternative to distinguish between intrinsic

or exogenous defects in megakaryocytopoiesis. These tests comprise standardized Tpo-driven cultures in which induction of megakaryocytopoiesis from HSCs can be quantified. For example, HSCs of patients with congenital amegakaryocytic thrombocytopenia (CAMT) usually cannot be expanded into MK.<sup>19</sup> This rare disorder is associated with mutations in the Tpo receptor gene, *c-mpl*.<sup>20,21,22</sup> We are presently investigating if these mutations lead to absent or aberrant Mpl expression, and thus reduced Tpo binding or Mpl signalling.

In similar assays, using no or low amounts of Tpo, spontaneous or fast MK formation from HSCs is strongly diagnostic for myeloproliferative diseases like ET or Polycythaemia Vera (PV).<sup>23</sup> Comparison of gene expression in these and normal MKs might reveal genes that play a role in this aberrant megakaryocytopoiesis.

Finally, a European research consortium is presently characterizing the specific gene transcription profiles of cultured MKs (by subtraction of the transcriptome of cultured erythroblasts, which originate from a common progenitor<sup>24</sup>). Moreover, all possible single nucleotide polymorphisms (SNPs) within these genes are determined. The final goal of this work is to correlate the presence or absence of these SNPs with changed platelet functionality in patients, e.g. with cardiovascular disease, thrombosis, or bleeding tendency.

#### ***Use of Tpo/Mpl agonists in therapy***

An overview of clinical studies with recombinant Tpo is given by Kuter *et al.*<sup>25</sup> Tpo was shown to be effective in raising blood platelet counts in both AITP and human immunodeficiency virus (HIV) related idiopathic thrombocytopenic purpura (ITP), but also before surgery and before platelet aphaeresis. This Tpo-increased yield of platelet aphaeresis might become especially interesting if successful cryopreservation of platelets is possible. In this respect, recent tests with autologous freeze-thawed platelets, showed no transfusion reactions and similar increments of platelets as were obtained with fresh allogeneic platelets.<sup>26</sup> Possible Tpo-induced activation and adhesion of platelets,<sup>27</sup> however, are still issues to be looked at. Antibody formation against exogenous Tpo, with the risk of cross reactivity with endogenous Tpo, was initially reported. However, this was never observed after intravenous administration.

Finally, new non-Tpo Mpl agonists should be mentioned. With high Mpl affinity, very low immunogenicity and so far no discernable differences in intracellular signalling and *in vitro* megakaryocytopoietic activity, these agents seem very promising. In accordance, an AMGEN produced Mpl agonist showed clinical effectiveness in AITP patients.<sup>28</sup>

#### ***Ex vivo expanded megakaryocytes for therapy***

Initially, the most important use of Tpo analogous and Mpl agonists was projected for patients with chemotherapy-induced thrombocytopenia and bleeding risk. However, Tpo administration after high-dose chemotherapy did not shorten the period of very low platelet counts or lower the need for platelet transfusions.<sup>29,30</sup> In contrast, Tpo preceding chemotherapy might have a stem cell protecting effect with reduction of the early thrombocytopenia.<sup>31</sup> The lack of sufficient Mpl-expressing cells in this aplastic period appeared to be the limiting factor for Tpo effectiveness. In accordance, an increased presence of megakaryocytic progenitor cells, within stem cell transplants, correlated with reduced platelet recovery periods.<sup>32</sup>

*Ex vivo* predifferentiation and expansion of HSCs towards MKs might thus be a way to shorten post-chemotherapy platelet recovery. Within animal experiments this *ex vivo* culture approach was shown to accelerate platelet count recovery.<sup>33,34</sup> In humans, Bertolini *et al.*<sup>35</sup> were the first to show that *ex vivo* expanded MKs can be administered safely with less need for platelet transfusions compared to historic controls. We are shortly starting a phase 1 trial in which patients with non-Hodgkin disease will receive autologous expanded MKs from purified CD34<sup>+</sup> cells, together with a standard autologous stem cell transplant. Using only megakaryocyte growth and development factor (MGDF)/recombinant human Tpo (rhTpo) and only IL-1, sufficient numbers of megakaryocytic progenitors and MKs for transfusion purposes can be generated.<sup>36</sup>

So far, this treatment modality is not cost-benefit effective in most conditions. It might, however, be an option for Jehovah's Witnesses or patients that are refractory (by HLA, HPA antibodies) for allogeneic platelet transfusions. *Ex vivo* MK expansion might also be a solution for patients with very long blood platelet repopulation times. Drayer *et al.* excluded intrinsic defects in the stem cells of these particular patients by showing normal MK formation in *in vitro* cultures.<sup>37</sup>

An increased efficacy to generate MKs from CD34<sup>+</sup> *in vitro* will allow broader use. Expansion of the HSCs before MK differentiation might be a solution. Tpo itself is known to stimulate self-renewal of CD34<sup>+</sup> HSCs. Very recently, it was demonstrated that Tpo exercises this effect, at least partly, through p38 MAPK activation leading to activation of USF-1, which in turn activates transcription of *HOXB4*.<sup>38</sup> In analogy to recently published approaches,<sup>39,40</sup> we are presently investigating if hyper-expression by addition of pure TAT-HOXB4 or HOXB4 protein increases the MK yield in our cultures.

Finally, the homing of expanded MKs should be optimized. At first we will investigate, using non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse models, which cell type (the CD34<sup>+</sup>CD41<sup>+</sup> MK-progenitor cell or the more mature CD34<sup>+</sup>CD41<sup>+</sup> MK) is responsible for the increased platelet recovery.

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