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REVIEW

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Biologic effects of thrombopoietin, the Mpl ligand, and its therapeutic potential

Abstract In vitro experiments and data from studies in Mpl-null and thrombopoietin (TPO)-null mice have shown that TPO is the major regulator of megakaryocytopoiesis. In addition to its effect on megakaryocyte production and maturation, TPO has a proliferative effect on other progenitors, particularly erythroid progenitors, when used in combination with other cytokines (erythropoietin, interleukin 3, and kit ligand). The precise effect of TPO on platelet release is uncertain and warrants further study. In myeloablated animal models (mice and primates), TPO shortened the period of critical neutrocytopenia and accelerated the recovery of platelet counts, thereby showing great promise for use in patients postchemotherapy and posttransplantation.

Key words Mpl ligand · Thrombopoietin · Megakaryocytopoiesis · Platelets

Introduction

Although the existence of a specific factor stimulating thrombocytopoiesis was inferred more than 30 years ago [17], such a factor has only recently been cloned and expressed independently by four laboratories using different approaches [2, 9, 20, 21]. This factor has been identified as the ligand of the cMpl receptor and the purified protein has been named thrombopoietin (TPO), megakaryocytic growth and development factor (MGDF), or megapoietin. All these proteins appear to have the same sequence and a novel two-domain structure: the amino-terminal domain shows significant homology with erythropoietin (Epo-like domain), whereas the carboxy-terminal domain contains several potential N-linked glycosylation sites (carbohydrate domain).

Ample experimental in vitro and in vivo evidence suggests that the Mpl ligand is the primary physiologic regulator in megakaryocytopoietic development and in platelet homeostasis [1, 3–6, 13, 15, 22, 30]. In vitro experiments show that TPO has the properties both of a megakaryocytic colony-stimulating factor (Meg-CSF), inducing megakaryocytic colony formation (CFU-Mk) in purified populations of murine and human progenitors, and of TPO, inducing terminal maturation of megakaryocytes (increasing their ploidy, their cytoplasmic maturation and size, and the formation of specific megakaryocytic proteins). In vivo, TPO increases numbers of megakaryocytic precursors and platelet production in normal animals and enhances recovery of platelets in myeloablated hosts [11, 15]. The central role of TPO in thrombopoiesis is also supported by data obtained using cMpl-null mice, which show a dramatic decrease in the number of circulating platelets and of megakaryocytes in their bone marrow and increased levels of circulating thrombopoietin [12]. Recent data on TPO-null mice lend additional support to this view [10].

In vitro proliferative effects

The biologic effects of TPO in vitro have been investigated by several laboratories ([13] and references therein). Using recombinant murine TPO and suspension cultures of murine mononuclear bone-marrow cells, it was first shown that TPO dramatically increases the number of megakaryocytes in culture [14]. Not only are the numbers of megakaryocytes increased, but their size and their ploidy are also enhanced as indicated by several specific studies [4, 22]. In addition to the effects of TPO as a single cytokine, several studies ([13 and references therein) have demonstrated that TPO cooperates with early-acting cytokines, such as interleukin 3 (IL-3) and kit ligand (KL), and later-acting

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cytokines, such as IL-6, IL-11, and erythropoietin (EPO). The data from these studies collectively show that there is a significant enhancement in colony formation in vitro (both the numbers and the size of CFU-Mk-derived colonies increase) when IL-3 is added to TPO-containing cultures. As background colonies always appear in IL-3-containing cultures, it is thought that the effects of IL-3 and TPO are at least additive [4], whereas those of KL and TPO or of EPO and TPO are synergistic, as the number of background colonies appearing in the presence of KL alone or EPO alone is very low.

Further studies in which soluble Mpl has been used to inhibit the effect of TPO, either added exogenously or endogenously produced, have shown that colony formation is abrogated in all non-IL-3-containing cytokine combinations [16]. By contrast, IL-3-containing cultures show only partial inhibition. The implication of this finding is that IL-3 is responsible for the proliferative expansion of a class of megakaryocytic progenitors that does not appear to be TPO-dependent. Additional evidence supports this hypothesis. When TPO addition is delayed, the output of megakaryocytic precursors ($CD41^+$ cells) in suspension culture is significantly curtailed in the presence of all cytokine combinations (i.e., KL or EPO) except those containing IL-3 ([26] and Papayannopoulou, unpublished data). Furthermore, Mpl-null mice [11] show residual megakaryocytic cell production (approximately 15%), and one may speculate that this may be due to the presence of IL-3 or a combination of IL-3 and other factors (i.e., IL-11) influencing megakaryocytopoiesis. The cooperation of TPO and EPO in the output of both megakaryocytic and erythroid cells in culture is also of special interest [15, 25, 26]. The bidirectional effect of TPO on both megakaryocytic and erythroid cells is largely mediated by a direct proliferative action of TPO on early and late erythroid progenitors expressing c-Mpl [15, 18, 26]. Enhancement of erythropoiesis by TPO administration has also been seen in vivo, particularly in myeloablated animals; both platelet and reticulocyte counts recovered faster in such animals [11, 15].

Maturational activity and ultrastructure studies

When EPO alone is present in suspension cultures of bone-marrow $CD34^+$ cells, there is an initial decline in total nucleated cell numbers but a subsequent proliferation of mostly megakaryocytic cells as indicated by their GpIIb/IIIa positivity (CD41). A high proportion of these cells are of typical megakaryocytic appearance, displaying polyploid nuclei and cytoplasmic features characteristic of megakaryocytes. However, it should be noted that not all of these cells, even after 2 weeks in culture, achieve the maturational features of in vivo megakaryocytes. Ultrastructure studies have indicated the presence of α-granules and demarcation membranes distributed throughout the cytoplasm of some megakaryocytes, resembling megakaryocytes in vivo. However, when a significant number of culture-derived megakaryocytes were analyzed several maturational defects were apparent (Papayannopoulou and Thorning, unpublished data). A disparity or asynchrony between α-granule and demarcation membrane formation was noted in the cytoplasm of many megakaryocytes. For example, cells with a plethora of α-granules were frequently observed; however, well-developed and normally distributed demarcation membranes were scarce in such cells. In addition, some megakaryocytes showed demarcation membrane aggregation only in some areas of their cytoplasm and very few α-granules.

The reason for these in vitro abnormalities is unclear. As maturational abnormalities in other lineages in vitro, i.e., the erythroid lineage or granulocytic lineage, are not uncommon, it is possible that they are attributable to the abnormal in vitro conditions. However, it is noteworthy that normal megakaryocytic maturation has never been observed when TPO is omitted from culture media, even when a combination of several cytokines, such as IL-3, IL-6, and KL, which generates a large number of $CD41^+$ cells in these cultures, is used. It is also noteworthy that the addition of a combination of cytokines to TPO-containing cultures does not appear to enhance further their ploidy or maturation status as observed in TPO-only-containing cultures. Given the presence of proper substrata, i.e., extracellular matrix [5] or stromal underlayers [25] (endothelium), mature megakaryocytes form many cytoplasmic processes (proplatelets), which may give rise to platelet-sized fragments in the media. However, the precise requirements for proplatelet or platelet formation in culture are poorly understood. The presence of TPO does not appear to be necessary for in vitro process (proplatelet) formation by mature megakaryocytes.

Target progenitor cells of TPO

Attempts to purify megakaryocytic progenitors that are targets for TPO action have made use of bone-marrow $CD34^+$ human cells and antibodies to megakaryocyte-specific proteins, such as anti-GpIIb-IIIa, or antibodies to the cMpl receptor [6–8]. It has been found that $CD41^+/CD34^+$ cells contain most of the CFU-Mk colonies grown in culture, and the number of these colonies does not appear to increase beyond that observed with optimal levels of TPO when combinations of other cytokines are used, although some increase in size is observed [8]. By contrast, CD34 cells negative for CD41 yield fewer CFU-Mk colonies; however, a higher proportion of these consist of large numbers of cells (>50 cells/colony) [8, 26]. The implication of these findings is that late CFU-Mk are present in large numbers in the $CD41^+/CD34^+$ subsets but that earlier progenitors with higher proliferative potential are present in the $CD41^-$ subsets [10, 26].

This observation was extended when $CD41^+$ and $CD41^-$ subsets were placed in suspension cultures and observed for 2 weeks. Suspension cultures of $CD41^+$ subsets gave rise to mature megakaryocytic cells by the end of the 1st week in

culture but showed no expansion of CFU-Mk numbers and only slight, if any, expansion of the total $CD41^+$ population inoculated. By contrast, when $CD41^-$ subsets were placed in suspension culture, there was a significant expansion (6- to 28-fold) in CFU-Mk progenitor numbers and a dramatic increase in the amount of $CD41^+$ cells after 2 weeks in culture [26]. Similar data were obtained using anti-Mpl antibodies [7], although the exact spectrum of TPO target populations may be compromised by the low affinity of the antibody used.

Interestingly, in our own studies we noted that together with the majority of CFU-Mk, late burst-forming units-erythroid (BFU-E) and early BFU-E were copurified with the $CD41^+$ and $CD41^-$ subsets, respectively [23, 24, 26]. More recent studies have also drawn attention to the effect of TPO on the proliferation of other types of progenitor cells, including primitive hemopoietic stem cells [19, 29, 30].

In vivo effects of TPO

When healthy mice were treated with TPO for up to 8 days, platelet counts began to increase by day 4 and reached a peak by days 6–8 [14]. The numbers of megakaryocytes were visibly increased in both the marrow and the spleen of TPO-treated mice, and their progenitors were expanded as shown by clonogenic in vitro assays. However, in addition to expansion of megakaryocytic progenitors, progenitors of other lineages, erythroid or myeloid, also increased in number [15]. Further in vitro studies conducted in our laboratory provided an insight into these early in vivo observations. It was noted that TPO could enhance BFU-E and CFU-E development in vitro in the presence of EPO. This is particularly evident when limiting conditions for the growth of these progenitors are used (i.e., serum-free media or reduced serum, or growth under low oxygen). Although TPO can influence the growth of BFU-E in synergy with IL-3 or stem-cell factor (SCF), enhancement of CFU-E was observed only when TPO was combined with EPO [26].

When, instead of healthy animals, myeloablated animals following irradiation and chemotherapy were treated with TPO, the enhancement in platelet recovery was significant as compared with vehicle-treated control values, and nadir platelet levels were not as low as those seen in control animals [11, 15]. In addition to these effects, it was also noted that reticulocyte recovery and hematocrit levels were higher in TPO-treated animals than in controls. Given the in vitro cooperation between TPO and EPO mentioned above, these in vivo results are not entirely unexpected or surprising. EPO levels are likely to be higher in myeloablated animals than in healthy animals, leading to terminal differentiation/maturation of an expanded pool of early and late erythroid progenitors and an earlier reticulocyte increase. Thus, in states of compromised bone marrow, TPO has the potential to enhance more than one lineage. These results may have both physiologic and therapeutic implications.

TPO treatment prospects

Given the parallels between EPO and TPO in both structure and biologic effects and the success of EPO in appropriate clinical settings, one may speculate about the therapeutic potential of TPO in certain patient populations. Although TPO-deficiency states, unlike that of EPO in kidney failure, are yet to be identifed, other potential therapeutic uses for TPO can be proposed. In postchemotherapy or postbone-marrow transplantation states there is a rapid increase in serum TPO activity that is inversely correlated with platelet levels [22]. However, there is significant variation among individuals in the levels of TPO found. Therefore, several of these cases could potentially be helped by recombinant TPO (rTPO) administration to enhance platelet recovery. TPO use in these states is supported by studies in animals myeloablated by either radiation or chemotherapy [11, 15]. As indicated above, treatment with TPO in these settings leads to significant enhancement of platelet recovery, to higher platelet nadirs, and to earlier erythroid recovery.

Previous investigators have recognized the need to reduce the risks of prolonged thrombocytopenia in the above-mentioned clinical settings and have attempted to do so by employing several cytokines with a promising in vitro activity profile on megakaryocytic cells. These included IL-1, IL-6, IL-11, IL-3, and PIXY-321 (a fusion IL-3–granulocyte-macrophage colony-stimulating factor protein). The results of these studies have previously been reviewed [28]. Although there were responses in some cases, no consistent or long-term effect was noted and many undesirable side effects were observed.

Of particular interest are studies with IL-6 or IL-11, since these two cytokines attracted clinical interest on the basis of their in vitro enhancement of megakaryocytic maturation. Their in vivo effects have been encouraging, especially when one considers the nonhematologic effects of IL-11, which may be more important than its hematologic effects. For example, regeneration of intestinal mucosa was noted in IL-11-treated hosts, and this could represent a potential clinical benefit.

In cases of bone marrow transplantation, it is well known that platelet recovery is delayed, necessitating multiple platelet transfusions with the attendant immunologic and infectious risks and financial burden. This window of recovery has been narrowed by transplantation of mobilized peripheral blood stem cells ([28] and references therein). Nevertheless, even in these cases, platelet recovery lags behind that of other types of cells. Previous attempts to treat recipients with other cytokines, have shown enhancement of platelet recovery in some patients, but there were undesirable side effects. In view of the current data obtained in myeloablated animals treated with TPO, one can be optimistic that TPO will work more efficiently and more consistently in these settings and, we hope, without untoward effects, in contrast to the experience with the previously used cytokines. Of particular interest is the proposition that beneficial results can be obtained in bone-marrow transplant recipients by treatment of the donors with TPO

[11]. It has recently been shown that pretreatment of donor mice with TPO produces increased enhancement of platelet recovery as compared with treatment of recipients with TPO immediately posttransplantation [11]. Given that granulocyte colony-stimulating factor is widely used for stem-cell mobilization but does not appear to influence platelet recovery, it is possible that TPO could be added to this scheme to obtain beneficial effects in the recipient in terms of platelet recovery.

Other situations in which TPO may be useful are states of bone marrow aplasia or myelodysplasia. As has been observed with EPO, it is possible that TPO levels can vary widely in these patients. It is also likely that patients with inappropriately low TPO levels may exist, and these patients are likely to be helped by TPO administration.

Beyond these obvious clinical settings, it is very difficult to predict with certainty how useful TPO treatment may prove to be. In several congenital and familial thrombocytopenias, in addition to problems with megakaryocytic production, there is speculation that impairment of megakaryocytic maturation could mediate the decrease in platelet count. Some of these patients have normal numbers of megakaryocytes and normal platelet survival, leading to speculation that defects in platelet release may play a role. Another example of hypothetical impairment of maturation of megakaryocytes, in addition to platelet destruction and production problems, is human immunodeficiency virus-related thrombocytopenia. Clearly, time will tell how successful attempts to treat these clinical conditions with TPO will be.

In treating patients with TPO, one needs to assess carefully not only the dose and duration of rTPO treatment but also the effects of rTPO on platelet activation and the clinical implications of this activation [31, 32]. The use of TPO is certain to supplement all current therapeutic strategies for supporting myeloablated patients. Combinations of cytokines, even at lower doses, may provide additional therapeutic benefit, but only prospective studies will define schedules and the appropriate clinical settings for treatment of patients with rTPO.

Future avenues of investigation

Mechanisms of TPO gene regulation and platelet homeostasis in vivo are largely undefined. It is unlikely that platelet levels alone are the sole mechanism that stimulates megakaryocytic production. The role of TPO protein processing in the physiology of thrombopoiesis is also unclear. Different molecular forms of TPO exist, and whether these represent different processing products or are physiologically active forms has not been clarified. The nature of the TPO receptor, c-Mpl, the existence and nature of other potential subunits forming a membrane complex, and the detailed signaling pathways of the engaged receptor remain to be defined. Furthermore, the role of putative new cytokines in the platelet-release process in vivo and in vitro will be an important field for future investigation. The additional effect of TPO on erythroid progenitors and, less so, on GM progenitors implies that progenitors for these lineages coexpress the Mpl receptor. The presence and frequency of bipotent erythroid/megakaryocytic progenitors capable of providing dual progeny (E + Mk) and their placement in the hematopoietic hierarchy remain to be defined [26]. Furthermore, the molecular mechanisms that underlie the proliferative signal that TPO delivers to these progenitors of other lineages remain to be identified.

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