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REVIEW ARTICLE

Thrombopoietin: The Primary Regulator of Platelet Production

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ALTHOUGH THE CONCEPT was advanced some 37 years ago, evidence for the existence of a humoral regulator of platelet production, first termed thrombopoietin (Tpo) by Kelemen et al., accumulated very slowly. Initial studies in the 1960s and 1970s indicated that thrombocytopenia evokes characteristic marrow changes including increases in the number, size, ploidy, and maturation rate of megakaryocytes. Platelet transfusion-induced thrombocytosis was associated with the opposite effects. These findings were later confirmed with the availability of improved methods of assessing cellular DNA content and megakaryocyte maturation. Odell et al. are generally acknowledged as providing the first description of a humoral substance in the sera of thrombocytopenic rats which induced thrombocytosis in recipient animals. Subsequent investigators have reported that thrombocytopenic serum also induces many of the marrow effects associated with thrombocytopenia.

However, significant progress in the field awaited the development of reliable, albeit cumbersome, assays of Tpo activity. Attempts to biochemically purify Tpo from plasma, serum, urine, and conditioned culture medium from various cell lines occupied much effort during the 1980s. Unfortunately, because of the complex nature of the starting material and difficulties associated with the use of an in vivo assay, these attempts failed to yield homogeneous protein adequate for protein sequencing and cDNA cloning, the official rite of passage for a hematopoietic growth factor. Furthermore, a number of cytokines were described in the late 1980s and early 1990s that possessed activity in various assays of megakaryocyte development. During the past several years, many investigators suggested that a distinct, lineage-specific thrombopoietin may not exist.

THE SEARCH FOR TPO

Four groups have published extensively on the purification of substances that either augment platelet production or stimulate megakaryocyte development. Levin's group has reported on the biologic effects of Tpo partially purified from thrombocytopenic rabbit plasma. These workers used ammonium sulfate fractionation, two types of lectin affinity chromatography, and gel filtration on high-pressure liquid chromatography (HPLC) columns to obtain material that reproducibly increased platelet production in recipient animals. Its reported molecular weight (M) was 40 to 47 kD. Additional biologic activities ascribed to partially purified Tpo include augmentation of megakaryocyte ploidy, both in vitro and in vivo; maturation of single, cultured megakaryocytes; and increased mean platelet volume and 35S methionine incorporation into the platelets of animals receiving Tpo. Further purification of this material has not been reported.

McDonald et al. have attempted to purify a platelet-promoting cytokine from the conditioned medium of human embryonic kidney (HEK) cells. This material was termed thrombocytopoiesis-stimulating factor (TSF) and was fractionated using differential precipitation, ion-exchange and size-exclusion columns, and affinity chromatography on immobilized lectins. The M, of TSF was estimated to be 32 kD, and its reported biologic activities are similar to that of the Tpo partially purified from thrombocytopenic plasma. When administered to mice, TSF increased platelet volume and level, stimulated megakaryocyte size and number, and increased the proportion of cells thought to be marrow megakaryocyte precursors, the small acetylcholinesterase-positive (SAChE+) cell. Furthermore, TSF induced the maturation of SAChE+ cells into large megakaryocytes in vitro. Thus, other than the apparent difference in M, the physical and functional properties of plasma Tpo and TSF were reported to be quite similar.

Rosenberg et al. have used both thrombocytopenic plasma and HEK cell conditioned medium to purify a factor they initially termed megakaryocyte stimulatory factor (MSF), based on its ability to enhance production of platelet factor 4 in a rat megakaryocyte cell line. These investigators used separation methods very similar to those of Levin and McDonald, including differential ammonium sulfate precipitation, lectin affinity chromatography, and two gel filtration steps. Using these techniques, the investigators enriched a 15-kD protein nearly 1 million-fold over that present in the starting material. Purified MSF was reported to stimulate increased uptake of 14C into platelet factor 4 and 35S-sulfate into proteoglycans of purified rat marrow megakaryocytes. It was reported not to support the proliferation of any type of hematopoietic progenitor cell. Unfortunately, subsequent studies of this material failed to yield a distinct protein se-

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quence (R. Rosenberg, personal communication, August 1994); it thus bears no relationship to the thrombopoietin subsequently identified by these and other investigators 7 years later (vida infra).

Evidence garnered from in vitro studies of megakaryopoiesis support the hypothesis that two types of factors are involved in platelet production: early acting, megakaryocyte colony-stimulating factors (Meg-CSFs) and late-acting, megakaryocyte potentiators.53-56 This hypothesis is quite similar to that advanced for the generation of other blood cells, such as the "early" and "late" roles played by c-kit ligand (KL) and erythropoietin (Epo) in erythropoiesis,57-59 granulocyte-macrophage-CSF (GM-CSF) and macrophage-CSF (M-CSF) for monocytes,60-62 interleukin-3 (IL-3) and IL-5 in eosinophil production,61 and IL-3, GM-CSF, or KL and G-CSF in the generation of neutrophils.63-65 Because neither partially purified Tpo, TSF, nor MSF enhanced megakaryocyte colony formation66,67 but did promote different aspects of megakaryocyte differentiation,68-70 they were thought to represent late-acting factors. In contrast, a number of well-characterized, recombinant cytokines act as Meg-CSFs. IL-3, GM-CSF, and KL all support the formation of megakaryocyte colonies in serum- or plasma-containing assays.71-73 However, because all of these cytokines have multilineage effects and have only minor effects on platelet production in vivo, the possibility remained that a lineage-specific Meg-CSF may exist.

Murphy et al have attempted to purify such a Meg-CSF from the urine of thrombocytopenic patients.19,64 Based on its capacity to stimulate the formation of murine megakaryocyte colonies in serum-containing assays, Erickson-Miller has reported the partial purification of Meg-CSF using ultrafiltration, cation-exchange and gel-filtration chromatography, preparative gel electrophoresis, chromatofocusing, and ion-exchange HPLC. This material, functionally and immunologically distinct from M-, G-, or GM-CSF or IL-1, -3, -6, -9, or -11, stimulated the formation of megakaryocyte colonies in serum-free culture in the absence of accessory cells. Its reported Mr was 15 kD, further emphasizing an apparent distinction between Meg-CSF and Tpo or TSF.

THE WOULD-BE TPOS

In recent years, three well-characterized cytokines (IL-6, IL-11, and leukemia-inhibitory factor [LIF]) have been proposed, on the basis of various effects on megakaryocyte development, to function as thrombopoietins. IL-6 displays a myriad of biologic activities including effects on hematopoiesis. For example, IL-6 acts synergistically with IL-3 and KL to augment the expansion of very primitive hematopoietic progenitor cells in blast-cell colony assays.74 Moreover, although not able to stimulate megakaryocyte progenitor cells alone, IL-6 acts together with IL-3 to promote CFU-Mk–derived colony formation and supports the development of mature megakaryocytes in suspension culture.29,37,38 These studies led to preclinical and clinical trials of the agent for thrombocytopenia. Unfortunately, results have been disappointing. In normal mice, even toxic doses of IL-6 increase platelet counts by only 70%.75 Responses in humans have also been unsatisfactory. Furthermore, IL-6 levels do not vary inversely with platelet levels,65,76 the sine qua non for a physiologic regulator of platelet production. However, recent results suggest that IL-6 may play a role in reactive or neoplastic thrombocytosis. Several groups have reported statistically significant correlations between IL-6 serum or ascitic fluid levels and thrombocytosis in patients with inflammatory and neoplastic conditions.67,68 Moreover, administration of an anti–IL-6 monoclonal antibody to patients with renal cell carcinoma, elevated serum IL-6 levels, and thrombocytosis returned platelet counts to normal.77 Together, the available data strongly suggest that IL-6 is distinct from Tpo but that it may account for many cases of reactive thrombocytosis.

IL-11 is a distinct polypeptide initially cloned from a primary marrow stromal cell line that could support the proliferation of IL-6–dependent cells.69 Like IL-6, IL-11 has pleiotropic actions, including effects on hematopoiesis. IL-11 can synergize with IL-3 to enhance the production of megakaryocytic colonies from marrow precursors and shorten the $G_0$ period of very early hematopoietic progenitors.40,41 However, IL-11 does not appear to be the factor in serum (Tpo) that can synergize with Epo to produce megakaryocyte colonies.11 Furthermore, as with IL-6, administration of very high doses of IL-11 resulted in a modest 30% to 50% increase in circulating platelets, even in splenectomized animals.72,73 Clinical trials of the agent are underway.

LIF is another cytokine that shares several features with IL-6, including a common component of its receptor, pleiotropic actions, and effects on hematopoietic cells. As with IL-6 and IL-11, LIF works with IL-3 to promote expansion of CFU-Mk29,42 and, when administered to animals, promotes a modest increase in platelet count.43 Of note, all three of these cytokines are pleiotropic in effect, at odds with the commonly held assumption that Tpo is tissue specific.

THE SUCCESSFUL SEARCH BEGINS

As often happens in science, findings from one arena have provided the critical impetus to success in an unrelated field. The successful cloning of TPO began with the identification of the murine retrovirus myeloproliferative leukemia virus (MPLV). In 1986, Wendling et al26 described a transforming viral complex that induced a myeloproliferative syndrome in recipient mice. The responsible virus was ultimately cloned and the transforming gene, v-mpl, identified in 1990.78 The cellular homologue of the viral oncogene, c-mpl, was cloned from human erythroleukemia cells (HEL) in 1992.79 Primary sequence data from both genes strongly suggested that the normal polypeptide might function as a hematopoietic cytokine receptor.78-81 This conclusion was based on the presence of two copies of the "cytokine receptor motif," a sequence containing approximately 200 amino acids, four characteristically spaced cysteine residues, 14 $\beta$ strands, and a five amino acid sequence (Trp-Ser-Xaa-Trp-Ser, where Xaa encodes any amino acid) near the carboxyl terminus of the unit.78,79 This pattern has been identified in more than 20 distinct polypeptides, all of which form receptors for either interleukins (IL-2, -3, -4, -5, -6, -7, -9, -11, and -13), colony-stimulating factors (G-CSF or GM-CSF), or other hormones or cytokines (LIF, Epo, growth hormone, prolactin, oncostatin-M, and ciliary neutrophic factor). However, the li-
gand for c-Mpl was not known in 1992; c-mpl encoded an orphan cytokine receptor.

Beginning with this sentinel discovery, a number of investigators began to search for the Mpl ligand (ML). Based on the cell that first yielded the c-mpl cDNA (HEL), which may differentiate along either erythroid or megakaryocytic lines,\textsuperscript{7,8} we suspected that ML played an important role in promoting megakaryocyte development. Data generated in the ensuing 18 months reinforced this belief. First, Skoda et al.\textsuperscript{9} using a chimeric IL-4/c-Mpl receptor, showed that the cytoplasmic domain of the latter can transmit a proliferative signal in hematopoietic cells. Vignon et al\textsuperscript{10} also published a similar result using a hybrid G-CSF/c-Mpl receptor. Second, Methia et al\textsuperscript{11} provided evidence that c-mpl expression is limited to normal cell populations that include megakaryocytes, their precursors, or their progeny (fetal liver, marrow, CD34+, megakaryocytes, and platelets) or to cell lines that possess or can be induced to display characteristics of megakaryocytes (UT-7, MO7e, TF-1, HEL, DAMI, and KU-812). In addition, half of the samples examined from patients with acute myelogenous leukemia and one third of those from patients with myelodysplasia expressed c-mpl.\textsuperscript{12} Third, using antisense oligonucleotides, Methia et al\textsuperscript{13} have shown that elimination of c-mpl expression in CD34+ selected marrow cells led to the selective elimination of CFU-Mk-depleted colonies. Erythroid, granulocytic, and monocytic colony formation was unaffected.

Using three different strategies, several groups have now obtained cDNA clones for ML. Scientists at Genentech,\textsuperscript{14} in collaboration with Lawrence Solberg at the Mayo Clinic, have used affinity chromatography based on immobilized recombinant c-Mpl receptor to purify ML from the plasma of irradiated pigs, obtained N-terminal amino acid sequence, and cloned cDNA by conventional strategies. A nearly identical strategy was used by workers at Amgen, except that canine ML was first purified and cloned.\textsuperscript{15} A very different strategy was used by scientists at ZymoGenetics, in collaboration with our group, to clone ML. Chemical mutagenesis was used to generate a cell line producing murine ML; specific cDNA was then obtained by functional expression cloning techniques.\textsuperscript{16} Finally, workers at Kirin Pharmaceuticals and Kuter et al independently used conventional protein fractionation methods to purify a plasma protein from thrombocytopenic rats or sheep, respectively, capable of promoting megakaryocyte differentiation.\textsuperscript{17,18} Amino terminal protein sequence then enabled the isolation of cDNA clones. Except for species-specific differences, all of these groups have reported on the identical molecule.

**THE GENOMIC STRUCTURE AND REGULATION OF ML**

Several groups have now reported on the genomic structure of the ML locus. Using fluorescence in situ hybridization, the gene maps to the long arm of human chromosome 3, at 3q26 or q27.\textsuperscript{19-24} Of interest, several case reports detailing involvement of this region of chromosome 3 in myeloproliferative diseases have been published,\textsuperscript{25,26} including one in which the serum of a patient with a 3q inversion and thrombocytosis was found to contain elevated levels of Tpo.\textsuperscript{27} The investigators thus predicted that the Tpo gene would be localized at the site of this chromosomal inversion, a prediction supported by current studies of ML.

The gene contains five coding exons, the boundaries of which align precisely with that of the five Epo exons.\textsuperscript{28,29} However, evidence for one or two noncoding upstream exons has come from comparison of ML genomic clones and cDNA or 5' RACE products (Fig 1). One exon resides nearly 1,700 nucleotides upstream of the first coding exon. Although a putative cap site within this exon has been reported,\textsuperscript{30} Chang et al\textsuperscript{31} have presented evidence that transcription initiation occurs an additional 1,200 nucleotides further upstream. These investigators suggest that the second more downstream noncoding exon may undergo alternative splicing. Preliminary analysis of the region upstream of the putative exon 0\textsubscript{b} has shown homologies with SPI and AP-2, but not TATA box promoter motifs.\textsuperscript{32} However, functional correlations of these sequence features are required to confirm their contribution to expression of the gene.

Northern blot and reverse transcription polymerase chain reaction (RT-PCR) analysis of normal human and murine tissues show that ML mRNA is expressed primarily in the liver, kidney, and smooth muscle, with lesser amounts present in the spleen and marrow.\textsuperscript{33-36} These results suggest that ML might be expressed in cell types common to many organs. Using primary cell cultures, several types of endothelial cells and fibroblasts have been shown to express ML-specific mRNA (Ballmaier et al\textsuperscript{37} and McCarty, manuscript in preparation). In addition, embryonic kidney\textsuperscript{38} and primary and cultured hepatocytes\textsuperscript{39,40} express specific transcripts. The implications of this very widespread distribution are not yet understood.

The regulation of ML expression has already become a topic of intense study. Two theories have been proposed. Although it is clear that ML accounts for all of the thrombopoietic activity present in sera of thrombocytopenic animals and that its level varies inversely with the platelet count (or mass),\textsuperscript{41-47} it is unclear whether the rate of gene expression is altered in response to physiologic stimuli. One school theorizes that the rate of ML gene expression is constant; plasma levels are entirely dependent on the rate of platelet Mpl receptor-mediated uptake and destruction.\textsuperscript{48} In this way, when platelet levels are high, ML is rapidly destroyed and circulating levels decrease, thereby limiting megakaryocyte production. A similar mechanism has been invoked to explain regulation of plasma M-CSF levels by its end product, the monocye.\textsuperscript{49} However, discordance between platelet counts and plasma ML levels have been reported. For example, elimination of the erythroid transcription factor NF-E2 in genetically engineered mice led to the unexpected result of disordered megakaryocyte development and severe thrombocytopenia.\textsuperscript{50} Because the ML levels in the sera of these animals are not elevated, additional mechanisms must be responsible for the regulation of ML. Others believe that ML gene expression is a regulated event; feedback is provided by the platelet or megakaryocyte and cellular RNA levels are varied accordingly. These investigators point to the transcriptional regulation of Epo and other cytokines as a model\textsuperscript{106,107} including the findings that, although M-CSF levels may in part be regulated by end-cell destruction, there are other pathways by which its expression is altered.\textsuperscript{108} The two theories are not mutually exclusive.
To begin to explore its regulation, we have used a semi-quantitative RT-PCR approach to measure ML-specific mRNA levels in the organs of mice made thrombocytopenic. Using either antiplatelet serum or radiation and carboplatinum to reduce platelet levels, we have shown that, although hepatic and renal ML transcripts are not altered, ML-specific marrow mRNA levels are inversely related to platelet count. Although liver and kidney contain quantitatively more ML transcripts than marrow, it is tempting to speculate that the marrow is a more physiologically relevant source. Clearly, given the evolving accuracy of semiquantitative PCR and concerns over effects of radiation or immune reactions on ML-producing cells, much additional work is required to fully understand the regulation of ML expression.

THE STRUCTURE OF ML

Complementary DNA for murine, rat, canine, porcine, and human ML have been obtained and share from 71% (murine v human) to 88% (murine v rat) sequence identity. The predicted mature polypeptides range from 305 (rat) to 335 (murine) amino acids, considerably longer than all of the other ligands that use receptors of the hematopoietic cytokine receptor family. Based on homology to other proteins, in particular Epo, the ML polypeptide can be divided into two domains (Fig 1). The amino terminal 154 residues of human ML share 23% sequence identity to human Epo and lesser homology to the interferons. This domain is also most highly conserved between the homologues of ML. For example, murine and rat ML are 96% homologous in this domain but only 81% identical in the remainder of the protein. The carboxyl terminal domain bears no homology with other known proteins, but is rich in serine, threonine, and proline residues, a characteristic of proteins that bear significant levels of O-linked carbohydrate. This region also contains several potential sites of N-linked carbohydrate modification. Characterization of recombinant murine ML confirms these predictions; nearly half of the 70-kD molecular mass is carbohydrate.

Several lines of evidence suggest that ML may undergo proteolytic processing. Although the majority of recombinant murine ML purified from serum-free tissue culture medium migrates with an apparent M₅ of 70 kD, the material purified from irradiated rat, pig, or canine plasma was reported to be 18 to 32 kD. Of some interest in this regard, immediately following the Epo-like domain of ML, an Arg-Arg dipeptide is present at positions 153/154, a sequence commonly found within the recognition sequence of a number of processing proteases. Moreover, recombinant forms of ML, artificially truncated 20 residues downstream of this or following the first of two arginine residues, are biologically active. Recombinant and natural Epo also undergo proteolytic processing near their carboxyl termini. Although only a single residue is removed, this finding indicates that a processing signal might be shared between the two hormones. Therefore, it is possible that proteolytic processing plays an important role in the normal physiology of ML. However, a better understanding of whether this form circulates or if its presence after purifica-
Fig 2. Comparative effects of IL-3 or KL in combination with IL-6, IL-11, or ML. Normal murine marrow cells were plated at $1 \times 10^6$/mL in serum-free medium supplemented only with 1% Nutridoma medium, in the presence of combinations of early acting (either control medium, IL-3, or KL) and late-acting cytokines (either IL-6 [III], IL-11 [III], or ML [III]). Cytokine concentrations in nanograms per milliliter are shown below the data, which represent the mean (±SEM) percentage of maximal acetylcholinesterase (AChE) activity, a measure of total megakaryocyte mass, of three to nine experiments. All results were indexed to the AChE activity of cultures containing 20 ng/mL IL-3 plus 50 ng/mL IL-6.

The in vitro activities of ML

Much of the initial biologic characterization of recombinant ML was conducted using the murine homologue. As originally defined, Tpo is the primary regulator of megakaryocyte differentiation. To test ML for such properties, serum-free suspension culture systems have been employed. Direct examination of acetylcholinesterase (AChE)-stained megakaryocytes, a fluorescence assay of whole culture AChE activity and fluorescence-activated cell sorting (FACS) were used to assess the effects of murine ML, alone and together with other cytokines, on megakaryocyte morphology, size, ploidy, and expression of platelet-specific membrane glycoproteins. Initial studies were focused on comparing the effects of cytokine combinations on megakaryocyte formation. In one series of serum-free suspension culture experiments, the effects of 750 pg/mL murine ML were superior to that of 5 ng/mL IL-6 or IL-11, either alone or in the presence of IL-3 or KL, in promoting AChE activity. The use of 50 ng/mL of either IL-6 or IL-11 was required to approach the activity induced by the low dose of ML (Fig 2). To determine whether this was the result of increased megakaryocyte numbers or size, we assessed individual AChE-stained cells. Both the number and size of the resultant megakaryocytes were greater in the presence of ML than in cultures containing IL-6 or IL-11.110 Previous work by Levin et al indicated that plasma Tpo induces endomitosis, resulting in enhanced megakaryocyte ploidy. Using murine marrow cells, a megakaryocyte-specific monoclonal antibody, propidium iodide staining, and FACS, individual megakaryocyte ploidy in response to various cytokines was determined. Compared with IL-11, ML induced a higher geometric mean ploidy, with modal ploidy values of 32N or 64N in different experiments. This finding was also readily apparent from morphologic assessment of the megakaryocytes grown in the presence of ML; most cells contained a very large nuclear mass. Wendling et al102 presented similar results; the ML present in aplastic porcine serum stimulated a shift in megakaryocyte ploidy. Several groups of investigators have now determined the effects of ML on the expression of platelet-specific membrane proteins. We have shown that murine ML engages the human Mpl receptor and can stimulate the expression of Gp Ib on the surface of the human megakaryocytic cell line CMK. Similar results for the HU-3 cell line were more recently reported by Morgan et al. Bartley et al87 and Papayannopoulou et al112 have detected Gp Ib and Gp IIb/IIIa on the surface of human CD34+ selected cells grown in the presence of recombinant human IL and human plasma. de Sauvage et al86 have shown that both truncated and full-length recombinant ML stimulate the formation of Gp IIb/IIIa-positive cells from peripheral blood progenitors.

To determine whether ML can complete the program of megakaryocyte differentiation and generate mature platelets, we performed electron microscopic studies of megakaryocytes in collaboration with Dr Dorothea Zucker-Franklin. As shown in Fig 3, ML induced the formation of a mature peripheral zone devoid of organelles, a well-developed demarcation membrane system, and, in many cells, fragmentation into platelets. Neither IL-3,113 IL-6, nor IL-11 (D. Zucker-Franklin, personal communication, December 1994) induced megakaryocytes were fully mature. These data indicate that ML can induce the full developmental program required to produce mature platelets.

Although not part of the classical definition of a thrombopoietin, several groups have now tested both recombinant ML and that present in aplastic serum for the capacity to support megakaryocyte colony formation. We found...
Fig 3. Ultrastructural study of megakaryopoiesis. Murine marrow was cultured under serum-free conditions in the presence of 1 ng/mL murine Tpo and fixed for electron microscopy. A peripheral zone devoid of organelles, platelet-specific granules, a well-developed demarcation membrane system, and fragmentation of platelet fields, illustrated in this figure, were commonly seen. These features stand in contrast to the underdeveloped megakaryocytes growth in the presence of IL-3, IL-6, or IL-11, in which granule formation is deficient and demarcation membrane development arrested. Original magnification × 4,500.

that in serum-containing cultures purified recombinant murine ML (approximately 150 pg/mL) -induced megakaryocyte colonies were small, suggesting that they had developed from relatively late, committed progenitors. Colony formation was augmented in the presence of either IL-3 or KL: for IL-3, the effects on colony number were additive; for KL, true synergy could be demonstrated. Delayed addition of ML (up to day 3 of the 6-day culture period) to IL-3- or KL-initiated cultures failed to alter colony number or size. Together with the small size of ML-induced megakaryocyte colonies, these data suggest that ML works late in megakaryocyte progenitor development. At higher concentrations of ML (approximately 1 ng/mL), megakaryocyte colony formation was enhanced but was not optimal. The addition of IL-3 consistently increased both the number of megakaryocyte-containing colonies and the number of megakaryocytes per colony. Wendling et al have also presented data suggesting that ML is a Meg-CSF. These investigators showed that aplastic porcine serum could support the formation of megakaryocytic colonies and that this activity was eliminated in the presence of an Mpl-Ig fusion protein. The suggestion that ML is a potent Meg-CSF has more recently been supported by several investigators using enriched progenitor cell populations. Although none of these reports use serum- or plasma-free conditions, the overwhelming weight of evidence thus far supports the conclusion that ML is a Meg-CSF.

Recent data have been generated that extend our understanding of the complexity of megakaryopoiesis. Several groups have previously shown that Epo exerts a positive influence on megakaryocyte formation in response to the thrombopoietic activity of aplastic plasma or serum. Using recombinant ML and Epo, we have recently shown clear synergy in the induction of megakaryocytic colony formation. Although results from in vivo studies have been less clear, these in vitro data suggest that Epo acts in
synergy with Tpo to promote platelet formation. These data also force a careful re-examination of the effects of Epo and ML.

The effects of ML on the final steps of platelet production, megakaryocyte proplatelet formation and fragmentation, have recently been studied using suspension culture systems. Miyazaki, Zeigler, and Choi and their colleagues have all noted the development of megakaryocyte proplatelet formation several days after the addition of ML to suspension cultures of purified progenitor cells.115,126,127 These cytoplasmic extensions are thought to be the antecedent structures that extrude through marrow sinusoidal endothelial cells and, in response to shear stress, yield mature platelets.128 Taken together, the accumulating data strongly supports the contention that ML is identical to plasma Tpo.

Although it is clear that Tpo supports the proliferation, differentiation, and maturation of megakaryocytes and their precursors and results in their terminal fragmentation into platelets, it is less clear whether the production of megakaryocytes and platelets is absolutely dependent on Tpo. Although previous investigators have shown that IL-3, KL, and GM-CSF support the development of megakaryocytic colonies,29,30 it is possible that accessory cells or serum in such cultures may provide the Tpo necessary for the process. To test this hypothesis, we used a soluble form of the Mpl receptor to eliminate all Tpo from semisolid and suspension cultures of megakaryocytic progenitor cells. In the presence of KL, IL-6, or IL-11, alone or in combination, all megakaryocyte formation was eliminated.13 Neutralizing antibodies to KL had no effect on Tpo-induced megakaryocyte colony formation. However, in contrast to the experiments with KL, megakaryocyte formation in response to combinations of cytokines including IL-3 was only partially reduced in the presence of the blocking reagent. However, the resultant megakaryocytes were underdeveloped. The geometric mean ploidy of megakaryocytes was threefold less than that seen in the presence of Tpo. Less than one third of the megakaryocytes contained 16N or greater. In contrast, nearly 80% of the Tpo-induced cells contained greater than 16N ploidy. In addition, cytoplasmic development, as assessed by the formation of platelet-specific granules and demarcation membranes, was quite immature. These data suggest that, although IL-3 can, in the absence of Tpo, induce early stages of megakaryocyte development, the latter is required for full megakaryocyte maturation. Results from Mpl-deficient mice are consistent with this conclusion. Using homologous recombination, workers at Genetech generated mice in which Mpl receptor expression was eliminated.150 Megakaryocyte and platelet levels were reduced to 15% of litter mate controls. These results establish Tpo as the primary regulator of megakaryocyte development. However, the failure of the Mpl receptor knock-out to absolutely eliminate marrow megakaryocytes or circulating platelets argues that alternative routes to platelet production exist. Our data suggest that this may be mediated by IL-3 plus IL-6 or, more likely, IL-3 plus IL-11.

Taken together, these findings suggest a model of the regulation of thrombopoiesis. Because Tpo does not appear to influence hematopoietic stem cells (E. Sitnika, personal communication, June 1994), the development of megakaryocyte-specific progenitors, like most hematopoietic lineages, is dependent on the early acting cytokines IL-3 or KL. Our data indicate that IL-3 can lead to early megakaryocyte development independent of Tpo, but that KL cannot. The former would therefore appear to be more “committed” to the megakaryocytic lineage. However, KL deficiency has a more pronounced effect on marrow megakaryocytes than does the absence of IL-3.130,131 Both cytokines may fill this role in vivo. Once formed, the latter stages of committed progenitor cell and megakaryocyte development become dependent on additional cytokines. Although IL-6 or IL-11 can induce cytoplasmic changes beyond that seen in response to IL-3 alone, megakaryocyte development is incomplete compared with that seen in the presence of Tpo. The development of highly polyploid fully mature megakaryocytes and their fragmentation into platelets is thus primarily controlled by Tpo. These concepts are illustrated in Fig 4.

In many respects, this model of thrombopoiesis is very similar to that proposed for erythropoiesis. In drawing such comparisons, 2N megakaryocytes (the small AChE-positive cell) should be considered functionally equivalent to CFU-E, both the primary proliferative targets of their respective cytokines. This parallel is easily justified. Both cell types undergo approximately 6 to 8 divisions before the end cell(s) is fully mature. In the case of the megakaryocyte, endomitotic division takes the place of mitosis. Stages of development just before the CFU-E or 2N megakaryocyte, such as the late erythroid burst or the CFU-MK able to form small colonies (capable of only 2 to 4 cell divisions), are also supported by these “late-acting” hormones. However, erythroid or megakaryocytic progenitors capable of greater proliferative potential require additional cytokines, ie, KL in the case of erythropoiesis and IL-3 (or possibly KL) for megakaryopoiesis. It is likely that these parallels also extend to erythroid (eg, globin induction) and megakaryocytic (eg, specific surface membrane glycoprotein expression) maturation. Studies of the direct effect of Tpo on genes responsible for the megakaryocytic phenotype will be required to establish this aspect of the model. However, the development of functional platelets from Tpo containing serum-free cultures of enriched hematopoietic progenitor cells (vida infra) strongly supports the hypothesis.

Recently, the effects of ML on platelet function has begun to be evaluated. Initial studies suggest that platelets produced in vitro in the presence of Tpo can be stimulated to display the activated form of GpIIb/IIIa when exposed to the physiologic agonist ADP.127 Furthermore, when incubated with human Tpo ex vivo, although not directly induced to aggregate, the dose-response curve to ADP-induced aggregation is shifted to more sensitive values.132 This “priming” of the platelet response in the presence of the primary regulator of their production is not surprising; both GM-CSF and G-CSF prime neutrophils to undergo an oxidative burst in response to physiologic stimuli.133-135 Previous in vitro studies have suggested that both G-CSF and KL might modulate platelet function.136,137 Subsequent preclinical and clinical trials have failed to show such a role. Whether the in vitro priming of Tpo-treated platelets translates into physiologically important effects has yet to be demonstrated. Obviously, in light of the envisioned widespread therapeutic use of this
agent, a thorough understanding of the hemostatic capacity of ML-induced platelets is critical. Additional careful studies are now required to investigate these important issues.

**THE IN VIVO ACTIVITIES OF TPO**

When administered to normal Balb/c mice for 6 days, Tpo induced a profound increase in marrow and splenic CFU-Mk and megakaryocytes and a fourfold increase in platelet counts. These effects were relatively specific for the megakaryocytic lineage; although no effects were noted on the peripheral white or red blood cell counts in normal animals, reproducible effects were noted on erythroid and granulocyte/macrophage progenitors. Splenic CFU-E, marrow and splenic BFU-E, and marrow and splenic CFU-GM were reproducibly increased up to threefold. This latter effect was also noted by Levin et al after the induction of acute thrombocytopenia using antiplatelet serum and is quite similar to the pan-myeloid progenitor effects noted after the administration of Epo. Precise data relating to the relative specific activities of the two homologues is not yet available; it is likely that the discrepant results between these three reports is due to species-specific differences between the murine and human proteins. More recent data strongly support this contention; compared with our results using ~100 ng/mouse/day, the administration of approximately 30-fold more human Tpo resulted in an equivalent increase in platelet levels.

**THE ROLE OF TPO IN STATES OF MARROW FAILURE**

Although only now just beginning, preclinical trials of Tpo have provided very encouraging results. When administered to animals first rendered pancytopenic by the administration of radiation and carboplatinum, recombinant murine Tpo reduced the platelet nadir experienced by vehicle-treated animals and shortened the platelet recovery period substantially. The number of days below normal platelet levels was reduced by 50% (11 v 22) and that below 50% of normal by 70% (5 v 16). The use of human Tpo in a murine model of
radiation- or chemotherapy-induced thrombocytopenia was clearly superior to vehicle treatment alone. More recently, the results of preclinical trials in subhuman primates have been reported. After sublethal irradiation, the administration of human Tpo was associated with a significantly improved nadir platelet count and shortened duration of thrombocytopenia. Although in its earliest stages of testing, it is clear that Tpo will play an important clinical role to enhance the recovery of platelet production in both natural and iatrogenic states of marrow failure.

SUMMARY AND FUTURE DIRECTIONS

Although only very recently cloned and expressed, initial studies indicate that Tpo stimulates both the proliferation and differentiation of megakaryocytic progenitor cells, is essential for the full maturation of megakaryocytes, and acts to enhance platelet production and to speed recovery after cytoreductive therapies. Together with the finding that all of the thrombopoietic activity of thrombocytopenic plasma can be removed with immobilized Mpl receptor and that in the presence of Tpo nearly all assayable CFU-Mk can be induced to develop into megakaryocytic colonies, it has become clear that (1) ML is identical with the long sought after hormone, Tpo, and that (2) Tpo is the primary physiologic regulator of platelet production. Despite premature conclusions to the contrary, to paraphrase Descartes, ‘it has been cloned, therefore, it exists.’

Despite these early gains, much work remains. The mechanisms of regulation of Tpo expression are unclear. Whether end cell destruction modulates Tpo blood levels needs verification; if operative, it is unlikely to be the sole mechanism of regulation. Other systems need to be uncovered and investigated. The role of protein processing in the physiology of Tpo is unclear. Whether the smaller forms obtained from plasma are truncated secondary to proteolysis introduced during purification or represent the physiologically active forms that circulate needs careful study. If physiologic, what is the structural basis and the reason for processing? Such mechanisms now in place? The answer to these two questions will require the demonstration of platelet formation in a single-cell, serum-free CFU-Mk assay system containing only defined, purified cytokine combinations. We are presently at the threshold of a molecular understanding of megakaryopoiesis; we now have the questions and important new tools with which to answer them. It is hoped that a thorough understanding of these and of other aspects of thrombopoiesis will derive from productive studies in the ensuing years.

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