

HISTORICAL OVERVIEW OF MEGAKARYOCYTE AND PLATELET PRODUCTION

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Hemopoietic regulators are powerful pharmacologic agents that stimulate the production and growth of blood cells. The formation, maturation and functional activity of most white blood cells (*i.e.*, granulocytes and monocyte-macrophages) are controlled by four of these colony-stimulating factors: GM-CSF, G-CSF, M-CSF and Multi-CSF (IL-3). Each regulator is polyfunctional; while each has some distinctive actions, to a major degree they overlap, and individual cells can simultaneously respond to more than one hemopoietic regulator.

Many hemopoietic regulators have now been purified; complementary DNAs (cDNA) for each isolated and biologically active recombinant factors have been biosynthesized using a variety of expression systems. In addition, cDNAs for each of the unique membrane receptors have also been cloned.

When injected *in vivo*, recombinant hemopoietic regulators are able to induce the increased production of granulocytes and/or macrophages, each one eliciting a distinctive pattern of response. The functional activity of mature granulocytes and monocytes is also enhanced by their administration and, in model systems, the injection of certain hemopoietic regulators can be shown to increase resistance to infections or to reduce mortality from whole-body irradiation or chemotherapy.

Clinical utility to date has been most extensive for G-CSF and GM-CSF, both of which are able to elevate blood and marrow levels of granulocytes and/or monocytes in a range of disease states following cancer chemotherapy or marrow transplantation and the myelodysplasias. The clearest evidence to date of the ability of these regulators to enhance resistance to infections has come from studies on patients with cyclic or congenital neutropenia.

Although thrombopoietic factors or megakaryocyte colony-stimulating activity (MK-CSA) or megakaryocyte colony-stimulating factor (Meg-CSF) in serum, plasma, cell culture medium and urine have long been reported,⁽¹⁻¹⁵⁾ the biochemical isolation of molecularly pure thrombopoietin (TPO) or Meg-CSF resulted in limited success due to extremely low concentrations in natural sources. Our own efforts to purify Meg-CSF from the urine of aplastic anemia patients required almost 20,000 liters of urine from this rare patient population and thirteen years of work.^(6,14)

Recently a protein which is a ligand for the cytokine receptor c-Mpl has been identified, cloned and expressed (c-Mpl ligand). Four research groups have independently isolated this protein from the plasma of myelocompromised animals, and one group exploited chemical mutagenesis to induce autocrine production of the c-Mpl ligand.⁽¹⁷⁻²¹⁾ The endogenous form of this molecule is known as TPO, a name initially coined by Kelemen *et al.* in 1958.⁽²⁾ TPO stimulates the development of megakaryocyte precursors of platelets, leading to an increase in the number of circulating platelets in a manner that is analogous to the erythropoietin (EPO) stimulation of erythroid precursors.

Mpl (the TPO receptor) was discovered as the product of the gene *c-mpl*, the normal homolog of the oncogene *v-mpl*, the transforming gene of a murine myeloproliferative

leukemia virus.^(22,23) Antisense oligonucleotides of c-mpl inhibited colony-forming ability of megakaryocyte progenitors,⁽²²⁾ and “knockout” mice deficient in c-mpl had an 85% reduction in circulating platelets.⁽²⁵⁾ Hence, the Mpl receptor proved to be the key to identification and cloning of TPO.⁽¹⁷⁻²¹⁾

TPO Structure

Human TPO, obtained from the primary translation product by cleavage of a 21-residue signal peptide, is a 332-amino acid (aa) polypeptide.^(19,26,27) It consists of at least two regions. The N-terminal portion (about 154 aa residues) has sequence and general structure homology with EPO. There is a high degree of sequence homology (> 70%) between human, porcine, canine, murine and rat TPOs; homology is higher in the N-terminal (EPO-like) region than the C-terminal region of the molecule. Thus far, no sequence similarity has been discovered between TPO's C-terminal portion and any other known protein.

From TPO's genetic sequence, the mass of TPO is about 35 kDa, but reports vary from 18-70 kDa obtained from TPO in serum or from culture media from recombinant cells, which may be due to its highly glycosylated nature and its consequent susceptibility to proteolytic processing.^(26,27)

TPO Receptor

As mentioned above, the receptor for TPO is the protooncogene c-Mpl, a homolog of an envelope protein of the myeloproliferative leukemia virus.^(22,23) The human and murine forms of the receptor have been cloned and sequenced and display approximately 81% identity at the aa sequence level.^(22,23,28) Both human and murine sources show the presence of multiple forms of the Mpl receptor produced as a result of alternative mRNA splicing, including soluble forms lacking transmembrane and cytoplasmic domains.^(22,23,28) The human and murine Mpl receptors show the conserved sequences and structural organization characteristic of members of the hematopoietin superfamily of cytokine receptors.^(29,30) No consensus sequence for kinase activity is found in the cytoplasmic domain of these receptors.⁽²²⁾ The intracellular domain of the Mpl receptor has been found to consist of two different domains that interact with different signal transduction pathways. One domain is required for induction for Shc phosphorylation and c-fos mRNA synthesis, indicating involvement of the Ras signal transduction pathway.⁽³¹⁾ A separate cytoplasmic domain is involved with activation of the JAK2 kinase and subsequent activation of STAT protein.

TPO Biological Effects

Identification of the types of cells that express the TPO receptor (Mpl) gives a clue to the biological action of TPO. Expression of the c-Mpl gene has been detected by reverse transcription-polymerase chain reaction (RT-PCR) in pluripotent hematopoietic stem cells (*i.e.*, CD34⁺/CD38⁻), but among lineage-committed cell lines or mature cells its

expression is detected only in cells of the megakaryocytic lineage.⁽²⁴⁾
Immunofluorescence studies by flow cytometry confirm this.⁽³²⁾

The dissection of the thrombopoietic activities of TPO has been difficult because of the multiple cytokines known to have general hematopoietic, including thrombopoietic, activities. Kaushansky's recent review⁽³³⁾ identified the major thrombopoietic cytokines as stem cell factor (SCF), IL-3, IL-6 and IL-11. The biological question is, "Where in the sequence from stem cell proliferation to differentiation of committed megakaryocyte precursors and, finally, to the maturation and shedding of functional platelets, does TPO exert its biological effect, and is there synergy with the other thrombopoietic cytokines?" TPO apparently affects the entire thrombopoietic process from the endocytosis of the megakaryocytoblast with concomitant ploidy increases from 32 to 64.⁽³²⁻³⁴⁾ In fact, in vitro complete maturation of megakaryocytes with formation of platelets is observed only in the presence of TPO.⁽²⁶⁾

TPO Summary

As Kaushansky recently summarized, "Thrombopoietin has moved from a theoretical entity to the clinic in two short years. It is perhaps reassuring that most of the characteristics predicted for TPO over the past forty years were confirmed with the availability of recombinant protein. And it is equally gratifying that these same in vitro properties predict, in large measure, its in vivo behavior. As we enter the next phase in its clinical development it seems prudent to keep in mind the lessons learned from its in vitro biology."⁽³⁵⁾ TPO's use alone and in combination with other cytokines provides hope for the clinician and patient alike.

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