Thrombopoiesis

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The production of platelets is a complex process that involves hematopoietic stem cells (HSCs), their differentiated progeny, the marrow microenvironment and hematopoietic cytokines. Much has been learned in the 110 years since James Homer Wright postulated that marrow megakaryocytes were responsible for blood platelet production, at a time when platelets were termed the "dust of the blood". In the 1980s a number of in vitro culture systems were developed that could produce megakaryocytes, followed by the identification of several cytokines that could stimulate the process in vitro. However, none of these cytokines produced a substantial thrombocytosis when injected into animals or people, nor were blood levels inversely related to platelet count, the sine qua non of a physiological regulator. A major milestone in our understanding of thrombopoiesis occurred in 1994 when thrombopoietin, the primary regulator of platelet production was cloned and initially characterized. Since that time many of the molecular mechanisms of thrombopoiesis have been identified, including the effects of thrombopoietin on the survival, proliferation, and differentiation of hematopoietic stem and progenitor cells, the development of polyploidy and proplatelet formation, the final fragmentation of megakaryocyte cytoplasm to yield blood platelets, and the regulation of this process. While much progress has been made, several outstanding questions remain, such as the nature of the signals for final platelet formation, the molecular nature of the regulation of marrow stromal thrombopoietin production, and the role of these physiological processes in malignant hematopoiesis.
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THE HISTORY OF THROMBOPOIESIS

Carnot, Wright, and others in the early 20th century defined the critical role of platelets in blood coagulation, and their origin from the marrow megakaryocyte based on elegant *camera lucida* images. Based on an evolving understanding of erythropoiesis, particularly the identification of erythropoietin as the humoral regulator of erythrocyte production, Keleman coined the term "thrombopoietin" in 1958 to describe the humoral substance responsible for platelet production.[1]

In the mid-1960s and 1970s, several groups attempted to purify thrombopoietin from the plasma of thrombocytopenic animals; these early efforts were severely handicapped by inconvenient and insensitive assays for the hormone, the in vivo incorporation of radiolabeled methionine into newly formed platelets, and the attempts failed to produce unequivocal proof of the existence of thrombopoietin. In the 1980s a number of in vitro megakaryocyte differentiation assays were developed, facilitating additional purification attempts; however, while some claims were made of its biological activities, attempts to produce a cDNA for thrombopoietin failed.

Occasionally in science, a finding from one field, although in itself important, can have a catalytic and profound effect on a *seemingly* unrelated area of research. The discovery and characterization of the murine myeloproliferative leukemia virus (MPLV) had such an impact on the search for thrombopoietin. MPLV causes an acute myeloproliferative neoplasm in infected mice[2]; in 1990, the responsible oncogene (*v-mpl*) was cloned, and the proto-oncogene (*c-Mpl*) obtained 2 years later.[3,4] Based on the predicted structure of the encoded protein it was immediately evident that *c-Mpl* encodes a member of the hematopoietic cytokine receptor family, which includes the receptors for erythropoietin, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, growth hormone, prolactin, and several interleukins (ILs). However, when "c-Mpl receptor" was cloned, the corresponding "c-Mpl ligand" was unknown. Based on the cell from which the receptor was cloned, the bipotent erythroid/megakaryocytic cell line HEL, we, and others, postulated that the c-Mpl ligand might be thrombopoietin.

THE CLONING AND CHARACTERIZATION OF THROMBOPOIETIN

Three distinct approaches yielded cDNA for thrombopoietin. Using an in vitro megakaryocyte-based assay, and

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the plasma from 1,100 thrombocytopenic rats, scientists at Kirin Pharmaceuticals employed a 12-step conventional purification scheme to obtain sufficient purified thrombopoietin to obtain amino acid sequence, and then cloned cDNA for rat and then multiple species of thrombopoietin, including the human hormone.[5] Using the *c-Mpl* proto-oncogene product coupled to an affinity matrix, scientists at Genentech obtained sufficient purified porcine Mpl ligand to allow amino acid sequencing and cDNA cloning[6] of ovine and human hormones. In contrast to the biochemical purifications utilized by these groups, an expression cloning strategy using a chemically mutated c-Mpl-bearing cell line was used by Lok and Kaushansky to obtain cDNA for murine and then human thrombopoietin.[7,8] Initial in vitro experiments using the corresponding recombinant proteins demonstrated the effect of thrombopoietin on megakaryocyte maturation, and injections into normal mice resulted in impressive increases in marrow megakaryocytes and peripheral blood platelet counts.[8]

The cloned human thrombopoietin cDNA encodes a polypeptide of 353 amino acids, including the 21 amino acid secretory leader sequence[7]; the mature protein consists of two domains. The amino-terminal 154 residues are homologous to erythropoietin, which like other members of the hematopoietic cytokine family displays a four helix bundle fold,[9] and binds to the c-Mpl receptor. The carboxyl-terminal domain of thrombopoietin bears no resemblance to any known proteins, and acts to prolong the circulatory half-life of the hormone[10]; it also serves as an intramolecular chaperone, aiding in the proper folding of the polypeptide into the mature hormone.[11]

THE BIOLOGICAL ACTIVITIES OF THROMBOPOIETIN

The availability of the recombinant protein allowed the first detailed studies of the biological properties of thrombopoietin. Previous conjecture was that the hormone was solely a megakaryocyte differentiation factor, driving the maturation of megakaryocytes and platelet formation, but had no effect on immature cells of the lineage or other hematopoietic cell types. Initial studies with recombinant thrombopoietin dispelled many of these incorrect assumptions. Thrombopoietin alone is able to stimulate the proliferation of nearly all marrow megakaryocytic progenitor cells (colony-forming unit, megakaryocyte [CFU-MK]) in vitro, and acts in synergy with other hematopoietic cytokines, such as IL-3, IL-11, and stem cell factor (SCF)[12] to promote the growth of CFU-MK. In vitro, thrombopoietin acts to increase megakaryocyte size and expression of lineage-specific megakaryocyte surface proteins, such as glycoprotein (GP)Ib and GPIIb/IIIa.[8,13] Studies of megakaryocyte ultrastructure show increased demarcation membrane and platelet granule formation following culture with thrombopoietin, indicating that the hormone primes megakaryocytes for platelet production.[14] And culture of marrow megakaryocytes in thrombopoietin leads to pronounced polyploidy.[15] However, the final stages of platelet formation and release appear to be thrombopoietin-independent, as withdrawal of the hormone from late-stage megakaryocyte cultures does not eliminate proplatelet formation; in fact, thrombopoietin withdrawal is reported to stimulate it.[16]

Also at odds with the prevailing conventional wisdom on thrombopoietin, in addition to its effects on megakaryocytic progenitors and mature cells, thrombopoietin affects hematopoietic stem cells (HSC) *in vitro*, especially when used in combination with IL-3 or SCF[17,18]. Numerous studies reported the expression of c-Mpl on the surface of HSCs[19,20], indicating the stem cell effects of thrombopoietin are direct. And based on these results, thrombopoietin has been included in many *ex vivo* cytokine cocktails designed to expand HSCs for therapeutic use.[21,22]

More recently, an intriguing paracrine role for thrombopoietin/c-Mpl in maintaining quiescent $Tie2^{+}$ HSCs at the osteoblastic niche has been identified[23]. Osteoblasts were found to release thrombopoietin, supporting the survival and quiescence of HSCs; inhibition of this interaction reduced the number of HSCs at the osteoblastic niche. Another mechanism by which thrombopoietin affects HSCs is by promoting DNA repair,[24] a finding that could eventually be clinically translated to HSC "protection" from genotypic damage during ionizing radiation or chemotherapy.

In Vivo Thrombopoietic Effects of Hematopoietic Cytokines

Once recombinant thrombopoietin was available purified protein was tested in a range of experimental animals. The initial results were remarkable; within 5 days of daily administration of subnanogram quantities of recombinant murine "c-Mpl ligand", mouse platelet counts were quintupled.[8] These initial experiments made obvious the fact that the c-Mpl ligand obtained by the multiple groups was thrombopoietin, as prior studies of the administration of other megakaryocytic factors (e.g. IL-6, IL-11) would result in, at most, a 50% increase in blood platelet counts. That thrombopoietin is the primary physiological regulator of thrombopoiesis was made clear by genetic studies; the generation of mice engineered to lack either *Thpo* or *c-Mpl* resulted in a 85-90% reduction (although not complete elimination) of platelet counts, and its blood levels were inversely related to platelet count[25,26]. That the *in vitro* HSC effects of thrombopoietin were physiological was then demonstrated when competitive repopulation assays were performed on the *c-Mpl*[27], revealing an approximate 8-fold reduction in HSC activity of marrow cells when compared to normal mouse marrow. Likewise, transplantation of normal HSCs into lethally irradiated normal recipient mice resulted in a 15-20 fold greater increase in post-transplant stem cell expansion compared to transplantation into $Thpo^{-/-}$ recipients.[28] And the final

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