THROMBOPOIETIN PHYSIOLOGY AND PRECLINICAL BIOLOGY OF THE PRIMARY REGULATOR OF MEGAKARYOCYTE AND PLATELET PRODUCTION

Kenneth Kaushansky

A humoral mediator of platelet production was first suggested to exist in the late 1950s but remained conceptual until thrombopoietin (TPO) was cloned two years ago.[1-6] Based on the work of Levin, McDonald, Murphy, Rosenberg and others using various in vitro and in vivo systems, many physiologic properties of the protein were identified.[7,8] With the availability of the recombinant protein, investigators have verified some of these physiologic concepts and have advanced others. This paper will review the in vitro physiology of thrombopoietin and correlate this information with what is known of the properties of the agent when administered to normal animals and in preclinical models of myelosuppression.

Thrombopoietin Stimulates Multiple Aspects of In Vitro Megakaryocyte Production

Most investigators reported that partially purified thrombopoietin was primarily if not exclusively a megakaryocyte differentiation factor.[7,8] Initial studies with the recombinant protein have indicated that the hormone is a potent stimulus of megakaryocyte differentiation. Compared to other cytokines, thrombopoietin increases the size of megakaryocytes by 50–70%, increases the geometric mean ploidy severalfold, and results in cultures predominated by gpIIb/IIIa- and gpIb-expressing cells.[9,10] When viewed in the electron microscope, megakaryocytes cultured in TPO contain abundant demarcation membranes, platelet-specific granules and "platelet fields."(10,11) Moreover, if a substratum matrix is also present, the cells form "proplatelet processes,"[12] thought to be the in vitro equivalent of the cytoplasmic extensions that occur between marrow sinusoidal endothelial cells[13] and from which megakaryocyte cytoplasm or whole cells move into the circulation. From such cultures numerous morphologically normal platelets can be obtained, cells which appear to function normally (e.g., they exteriorize P-selectin upon exposure to classic platelet agonists).[14]

As noted above, thrombopoietin was not initially thought to support the proliferation of megakaryocytic progenitor cells. However, work in multiple laboratories has indicated that thrombopoietin is a potent stimulus of megakaryocyte colony growth in vitro. By itself, the recombinant protein supports the proliferation of as many as 75% of all megakaryocyte colony-forming cells in normal marrow.[9] Most of the derived colonies contain low to intermediate numbers of very large cells, in contrast to colonies obtained in the presence of IL-3 or c-kit ligand (KL), in which up to one-third of the colonies contain 20–200 megakaryocytes.[15] Nonetheless, thrombopoietin acts additively with IL-3 and synergistically with KL, IL-11 and erythropoietin (EPO) to enhance the proliferation of CFU-MK.[16]

Thrombopoietin Stimulates Platelet Production in Both Mouse and Man

Given this growing body of data on the effects of thrombopoietin on megakaryopoiesis in vitro, it was not surprising to find that the administration of the protein to normal mice, dogs, baboons and humans has been associated with profound increases in platelet levels.[2,3,9,17,18] Within three days of initiating treatment of mice, and within seven days in primates, platelet counts begin to increase and are maximal at four to ten times normal levels just at or beyond termination of its administration. Elevated platelet levels are maintained for as long as administration continues and begin to wane five to seven days after its discontinuation. The reason for the thrombocytosis is increased platelet production; marrow (and, in mice, splenic) megakaryocyte numbers and size are increased up to tenfold within a week of initiation of treatment,[9,17] effects that subside within one to two weeks of ceasing therapy. Platelet morphology is essentially normal, except for an increase in the number of "reticulated platelet" cells, which bear rough endoplasmic reticulum and stain readily for RNA. Such platelets resemble those found in states of rapid platelet turnover, such as autoimmune thrombocytopenia. And platelet function in vivo appears entirely normal, even when tested in a prothrombotic vascular graft model.[19]

Is Thrombopoietin Essential for Megakaryocyte and Platelet Production?

Several lines of evidence indicate that, although thrombopoietin is critical for the normal level of platelet production, it is not absolutely essential. We have recently explored this issue with in vitro studies. Over the past several years, a number of cytokines have been shown to support the proliferation and differentiation of megakaryocytes in vitro; several of these agents have also been tested for their capacity to stimulate platelet production in thrombocytopenic patients. If thrombopoietin were absolutely essential for megakaryocyte and platelet development, these other cytokines would have to act through endogenous thrombopoietin in the various culture systems used in previous studies. As most reports using IL-3, IL-6, IL-11 and KL have either utilized serum or plasma, which contain the hormone, or culture systems that contain modest to large numbers of marrow stromal cells, which have been shown to produce thrombopoietin,[20] we investigated whether eliminating thrombopoietin from cytokine-stimulated marrow cell cultures would eliminate megakaryocyte production. Using a soluble form of the Mpl receptor, we recently showed that megakaryocytic colony formation in the presence of combinations of KL, IL-6 and IL-11 is obliterated in the presence of the soluble receptor. [15] In contrast, although megakaryocytic numbers were reduced by the soluble receptor in colony forming assays and suspension cultures, they were never eliminated in the presence of combinations of cytokines including IL-3. In addition, the number of megakaryocyte colonies formed using optimal levels of thrombopoietin were always increased if IL-3 was also present in the culture system, and the number of colonies containing large numbers of megakaryocytes was far greater when IL-3 was present. These results indicate that IL-3 stimulates a megakaryocytic progenitor cell of greater developmental potential than that supported by thrombopoietin. However, none of these results indicate the maturity of the megakaryocytes that form in the absence of thrombopoietin. Using suspension cultures, we also evaluated the level of cellular

maturity when marrow cells are grown in the presence of IL-3 plus the soluble receptor. None of the cells displayed a ploidy greater than 4N, and the cytoplasm was quite immature; there were no demarcation membranes, no platelet specific granules and no platelet fields. The addition of IL-11 to the cultures improved megakaryocyte maturation slightly, but the cells never achieved the developmental potential of cells grown in the presence of thrombopoietin alone.

DeSauvage and colleagues have approached the same question from a different perspective. The availability of the techniques of homologous recombination has provided a unique opportunity to establish the role of a gene in normal and abnormal physiology. Both the thrombopoietin and *c-mpl* receptor genes have now been "knocked out."(21,22) The phenotype of both knock-out animals is similar; such mice display 5–10% of a normal number of marrow megakaryocytes and 10–15% of a normal number of blood platelets. In addition, the megakaryocytes present are somewhat smaller than normal. Thus, consistent with our in vitro studies, these results indicate that thrombopoietin is the primary determinant of platelet production. The knock-out studies also suggest that a low-level, fail-safe mechanism for platelet production exists. Our data would indicate that IL-3 and IL-11 might comprise that mechanism.

Thrombopoietin Is Not a Lineage-Restricted Hormone

Another recent series of in vitro studies have investigated whether thrombopoietin may exert effects on hematopoietic lineages other than megakaryocytes. There are several reasons to suspect this may be the case. First, multiple lines of evidence support the concept of a close relationship between the erythroid and megakaryocytic lineages.(23,24) The two share a number of transcription factors. Most of the available erythroid cell lines display or can be induced to display features of megakaryocytic differentiation. Similar findings have been reported for cell lines initially thought to be exclusively megakaryocytic. Gerald Marguerie and coworkers reported that transgenic mice carrying a suicide gene under the control of a "megakaryocyte specific" promoter (gpIIb) display reduction of both platelet and red cell production upon activation of the gene.(25) And consideration of the pathology of MPLV, the murine myeloproliferative leukemia virus whose identification ultimately lead to the cloning of thrombopoietin, also suggests a more widespread role for thrombopoietin than initially postulated. The virus induces a pan-myeloid expansion of the marrow, leading to transformed cells of erythroid, myeloid and megakaryocytic lineages.(26) This murine retrovirus acquired its oncogenicity by capture and mutation of the gene encoding the thrombopoietin receptor, *c-mpl*.(27) Thus, the pan-myeloid effects of the virus clearly indicate that a constitutively active Mpl receptor *can* stimulate all hematopoietic cell lineages.

Several investigators have found multi-lineage effects of thrombopoietin in vitro. We found that adding thrombopoietin to cultures containing EPO and IL-3 increased the number and size of BFU-E that developed from marrow cells.(28) Moreover, the cytokine was found to augment the generation of CFU-E in a two-stage EPO-containing suspension culture system. Using plucked early BFU-E precursors, Kobayashi found similar results: the presence of thrombopoietin approximately tripled the number of BFU-E that developed in low serum, low oxygen containing cultures.(29) We have found a

similar synergistic interaction between thrombopoietin and KL during granulocyte-macrophage colony development.

More recent studies have indicated that thrombopoietin acts on even more pleuripotent cells. Ogawa has found that in the presence of early-acting hematopoietic growth factors, thrombopoietin affects the primitive CFU-Blast cell population,[(30)] and we have recently shown thrombopoietin to act in synergy with IL-3 or KL to affect the cloning efficiency, rate of entry into the cell cycle, and the number of committed progenitor cells that arise from highly purified hematopoietic stem cells.[(31)] From these *in vitro* studies, in addition to its effects on megakaryocyte biology, thrombopoietin appears to exert a direct effect on primitive stages of hematopoiesis.

Evidence that thrombopoietin can act as a pleotropic hematopoietic regulator also comes from two in vivo sources. Recently, levels of hematopoietic progenitor cells in the mpl and thrombopoietin knock-out mice have been analyzed. Marrow and splenic numbers of erythroid, myeloid and mixed hematopoietic lineage progenitor cells were all substantially reduced in both settings.[(22)] Results from pre-clinical trials of thrombopoietin have lead to similar conclusions. When administered to normal mice, and to a lesser extent in normal non-human primates, thrombopoietin modestly expands the numbers of marrow and splenic hematopoietic progenitor cells of multiple lineages.[(28)] However, these effects do not result in an increase in red cell or leukocyte production, as the primary regulators of these cell types (EPO and G-CSF) are not increased in normal animals. In contrast, when administered to pancytopenic animals, hematopoietic progenitor recovery of all lineages is greatly accelerated; because of the increased levels of erythroid and myeloid cytokines in these settings, red cell (and, in some studies, leukocyte) recovery is greatly improved.[(28)] Thus, taken together, these data suggest that the hematopoietic response to thrombopoietin will likely be greater than initially anticipated.

Thrombopoietin has evolved from concept to reality in two short years. Its physiological properties have been extensively studied; the results have both supported old concepts and have generated new ones. The most anticipated future results will come from clinical studies, which are currently underway. However, as the cytokine reaches the stage of clinical investigation, it would seem wise to keep the lessons derived from in vitro studies of the cytokine in mind when designing and interpreting the results of clinical trials.

Bibliography

. de Sauvage FJ, Hass PE, Spencer SD, Malloy BE, Gurney AL, Spencer SA, Darbonne WC, Henzel WJ, Wong SC, Kuang W-J, Oles KJ, Hultgren B, Solberg Jr LA, Goeddel DV, Eaton DL: Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. Nature 369:533, 1994

. Lok S, Kaushansky K, Holly RD, Kuijper JL, Lofton-Day CE, Oort PJ, Grant FJ, Helpel MD, Burkhead SK, Kramer JM, Bell LA, Sprecher CA, Blumberg H, Johnson R, Prunkard D, Ching AFT, Mathewes SL, Balley MC, Forstrom JW, Buddle MM, Osborn SG, Evans SJ, Sheppard PO, Presnell SR, O'Hara PJ, Hagen FS, Roth GJ,

Foster DC: Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production *in vivo*. Nature 369:565, 1994

. Bartley TD, Bogenberger J, Hunt P, Li Y-S, Lu H-S, Martin F, Chang M-S, Samal B, Nichol JL, Swift S, Johnson MJ, Hsu R-Y, Parker VP, Suggs S, Skrine JD, Merewether LA, Clogston C, Hsu E, Hokom MM, Hornkohl A, Choi E, Pangelinan M, Sun Y, Mar V, McNinch J, Simonet L, Jacobsen F, Xie C, Shutter J, Chute H, Basu R, Selander L, Trollinger D, Sieu L, Padilla D, Trail G, Elliott G, Izumi R, Covey T, Crouse J, Garcia A, Xu W, Del Castillo J, Biron J, Cole S, Hu MC-T, Pacifici R, Ponting I, Saris C, Wen D, Yung YP, Lin H, Bosselman RA: Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. Cell 77:1117, 1994

. Sohma Y, Akahori H, Seki N, Hori T, Ogami K, Kato T, Shimada Y, Kawamura K, Miyazaki H: Molecular cloning and chromosomal localization of the human thrombopoietin gene. FEBS letters 353:57, 1994

. Kuter DJ, Beeler DL, Rosenberg RD: The purification of megapoietin: a physiological regulator of megakaryocyte growth and platelet production. Proc Natl Acad Sci USA 91:11104-11108, 1994

. Kaushansky K. Thrombopoietin: The primary regulator of platelet production. Blood, 86:419-431, 1995.

. McDonald TP: Thrombopoietin: Its biology, purification, and characterization. Exp Hematol 16:201, 1988

. Hill RJ, Levin J: Regulators of thrombopoiesis: Their biochemistry and physiology. Blood Cells 15:141, 1989

. Kaushansky K, Lok S, Holly RD, Broudy VC, Lin N, Bailey MC, Forstrom JW, Buddle MM, Ort PJ, Hagen FS, Roth GJ, Papayannopoulou T, Foster DC: Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. Nature 369:568, 1994

. Wendling F, Maraskovsky E, Debili N, Florindo C, Teepe M, Titeux M, Methia N, Breton-Gorius J, Cosman D, Vainchenker W: c-Mpl ligand is a humoral regulator of megakaryocytopoiesis. Nature 369:571, 1994

. Zeigler FC, de Sauvage F, Widmer HR, Keller GA, Donahue C, Schreiber RD, Malloy B, Hass P, Eaton D, Matthews W: *In vitro* megakaryocytopoietic and thrombopoietic activity of *c-mpl* ligand (TPO) on purified murine hematopoietic stem cells. Blood 84:4045, 1994

. Choi ES, Nichol JL, Hokom MM, Hornkohl AC, Hunt P: Platelets generated *in vitro* from proplatelet-displaying human megakaryocytes are functional. Blood 85:402, 1995

. Tavassoli M, Aoki M: Localization of megakaryocytes in the bone marrow. Blood Cells 15:3, 1989

. Debili N, Wendling F, Katz A, Guichard J, Breton-Gorius J, Hunt P, Vainchenker W: The mpl ligand or thrombopoietin or megakaryocyte growth and differentiative factor has both direct proliferative and differentiative activities on human megakaryocyte progenitors. Blood 86:2516, 1995

. Kaushansky K, Broudy VC, Lin N, Jorgensen M, McCarty J, Fox N, Zucker-Franklin D, Lofton-Day C: Thrombopoietin, the Mpl-ligand, is essential for full megakaryocyte development. Proc Natl Acad Sci USA 92:3234, 1995

. Broudy VC, Lin NL, Kaushansky K: Thrombopoietin (c-*mpl* ligand) acts synergistically with erythropoietin, stem cell factor, and IL-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy *in vitro*. Blood 85:1719-1726, 1995

. Farese AM, Hunt P, Boone T, MacVittae TJ: Recombinant human megakaryocyte growth and development factor stimulates thrombocytosis in normal nonhuman primates. Blood 86:54, 1995

. Basser R, Clarke K, Fox R, Green M, Cebo J, Marty J, Menchaca D, Tomita D, Begley G: Randomized, double-blind, placebo-controlled phase I study of pegylated megakaryocyte growth and development factor (PEG-rHuMGDF) administered to patients with advanced cancer before and after chemotherapy. Blood 86: Suppl 1, 257a, 1995

. Harker LA, Hunt P, Marzec UM, Kelly AB, Tomer A, Hanson SR, Stead RB: Regulation of platelet production and function by megakaryocyte growth and development factor in nonhuman primates. Blood 87:1833, 1996

20. McCarty JM, Sprugel KH, Fox NE, Sabath DE, Kaushansky K: Murine thrombopoietin mRNA levels are modulated by platelet count. Blood, 86:3668-3675, 1995.

. Gurney AL, Carver-Moore K, de Sauvage FJ, Moore MW: Thrombocytopenia in *c-mpl*-deficient mice. Science 265:1445, 1994

. de Sauvage FJ, Luoh S-M, Carver-Moore K, Ryan A, Dowd M, Eaton DL, Moore MW: Deficiencies in early and late stages of megakaryocytopoiesis in TPO-KO mice. Blood 84:255a, 1995

. McDonald TP, Sullivan PS: Megakaryocytic and erythrocytic cell lines share a common precursor cell. Exp Hematol 21:1316, 1993

. Hunt P: A bipotential megakaryocyte/erythrocyte progenitor cell: The link between erythropoiesis and megakaryopoiesis becomes stronger. J Lab Clin Med 125:303, 1995

. Tronik-Le-Roux D, Roullot V, Schweitzer A, Berthier R, Marguerie G: Suppression of erythro-megakaryocytopoiesis and the induction of reversible thrombocytopenia in mice transgenic for the thymidine kinase gene targeted by the platelet glycoprotein alpha IIb promoter. J Exp Med 181:2141, 1995

. Wendling F, Varlet P, Charon M, Tambourin P: A retrovirus complex inducing an acute myeloproliferative leukemia disorder in mice. Virology 149:242, 1986

. Vigon I, Mornon J-P, Cocault L, Mitjavila M-T, Tambourin P, Gisselbrecht S, Souyri M: Molecular cloning and characterization of MPL, the human homolog of the v-mpl oncogene: Identification of a member of the hematopoietic growth factor receptor superfamily. Proc Natl Acad Sci USA 89:5640, 1992

. Kaushansky K, Broudy VC, Grossmann A, Humes J, Lin N, Ren H-P, Bailey MC, Papayannopoulou T, Forstrom JW, Sprugel KH: Thrombopoietin expands erythroid progenitors, increases red cell production, and enhances erythroid recovery after myelosuppressive therapy. J Clin Invest 96:1683-1687, 1995.

. Kobayashi M, Laver JH, Kato T, Miyazaki H, Ogawa M: Recombinant human thrombopoietin (Mpl ligand) enhances proliferation of erythroid progenitors. Blood 86:2494, 1995

30. Ku H, Kaushansky K, Ogawa A: Thrombopoietin, the ligand for the Mpl receptor, synergy with steel factor and other early-acting cytokines in supporting proliferation of primitive hematopoietic progenitors of mice. Blood. In press

. Sitnicka E, Lin N, Priestley GV, Fox N, Broudy VC, Wolf NS, Kaushansky K: The effect of thrombopoietin on the proliferation and differentiation of murine hematopoietic stem cells. Blood. In press