Thrombopoiesis and Thrombopoietins

Molecular, Cellular, Preclinical, and Clinical Biology

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Foreword

Shirley Ebbe, MD

The long-standing search for thrombopoietin (TPO) culminated about two years ago when a newly discovered hemopoietic growth factor was identified as TPO. In the subsequent two years the literature about TPO has increased at a meteoric rate. An understanding of cellular and subcellular events associated with megakaryocytopoiesis and its regulator that would have been unimaginable a few years ago is now being achieved. *Thrombopoiesis and Thrombopoietins: Molecular, Cellular, Preclinical, and Clinical Biology* provides a compilation of pertinent historical and recent research and projections about the potential clinical utility of TPO.

Research extending over 40–50 years indicated that there was feedback regulation of megakaryocytopoiesis. Therefore, there was an expectation that TPO existed, but conventional approaches to its purification were unsuccessful. Its identification finally hinged on a seemingly unrelated event when one mouse (of 238) injected with Friend murine leukemia virus developed a peculiar myeloproliferative disorder. The fascinating odyssey from that mouse to the identification of the *c-mpl* proto-oncogene to the realization that *c-mpl* was responsible for producing an orphan hemopoietic cytokine receptor (MPL) is presented by Wendling and Gisselbrecht in Chapter 6. The pivotal studies that identified MPL as a receptor for a growth factor that might specifically control megakaryocytopoiesis are summarized in Chapter 7 by Wendling, Debili, and Vainchenker. As testimony to the importance that was attached to discovering TPO, within a year or so after this work was published several groups had identified the ligand for MPL (Mpl-L) as the elusive TPO.

Since about 1950 there has been a sustained interest in megakaryocytopoiesis and platelet production, with scientific progress sometimes following the development and application of new technology. With the advent of accurate methods to count platelets *(1)* and transfuse them *(2)*, the importance of platelets for hemostasis in irradiated animals was appreciated. Recovery from severe depletion or overtransfusion of platelets in normal animals was characterized by transient overcorrection of the platelet count, and megakaryocyte morphology was sometimes observed to be altered in response to thrombocytopenia *(3,4)*. Therefore, it appeared that platelet production was subject to homeostatic regulation and, by analogy to erythropoietin, the concept of a TPO evolved. The capability to measure platelet survival with radiolabeled tracers permitted classification of thrombocytopenias and allowed for indirect quantification of platelet production. The pattern of labeling of megakaryocyte DNA with tritiated thymidine dispelled the preexisting concept that megakaryocyte cytoplasmic maturation and increasing nuclear DNA content proceeded hand-in-hand *(5)*. From the DNA content of individual megakaryocytes, it became clear that nuclear replication occurred in an orderly fashion

and that it ceased at different ploidy levels *(6)*. Chromosome analysis of bone marrow cells in Philadelphia–chromosome positive leukemia *(7)* and the morphology of splenic colonies in irradiated murine recipients of transplanted stem cells *(8)* demonstrated that megakaryocytes were derived from the same multipotential stem cells as granulocytes and erythroid cells, a finding confirmed by patterns of blood cell enzymes in myeloproliferative disorders.

Jackson, Arnold, Pestina, and Stenberg (Chapter 1) have integrated the results of many early and recent studies to provide a monumental description of the biology of megakaryocytes. They explain a hierarchy of megakaryocyte progenitors, development of polyploidy through mitotic events, kinetics of megakaryocyte development, effects of perturbations of the platelet count and of cytotoxic agents, instructive animal models, and clinical abnormalities of megakaryocytopoiesis. Characteristic features of the megakaryocytic response to thrombocytopenia, such as increases in size and ploidy, are identified, thus establishing some criteria for the action of TPO. The sequential acquisition of biochemical markers, development of ultrastructural organelles, and fragmentation into platelets are analyzed. The plethora of constituents, not all of which have an obvious role in hemostasis, and their meticulous subcompartmentalization are impressive. Levin presents a comparison of mammalian platelets and multifunctional amebocytes of invertebrates in Chapter 3. In addition to their well-known hemostatic potential, platelets also have some of the nonhemostatic capabilities of amebocytes, which he proposes may be evolutionary remnants. However, new insights may demonstrate the importance, or its lack, of the multiple platelet components that seem to have no apparent role in the platelet's primary function of hemostasis.

At the same time that studies of the kinetics of megakaryocytopoiesis were underway, i.e., from the 1960s through the 1980s, there were efforts to purify TPO from biological sources by concentrating procedures similar to those that were ultimately successful for erythropoietin. Mazur, in Chapter 5, has written an inclusive presentation of searches for a specific regulatory substance. As he describes, a serious problem was the lack of sensitive assays for TPO. A vagary of research is illustrated by the fact that two groups (Chapter 9 by Kuter, Miyazaki, and Kato) finally succeeded in purifying TPO from thrombocytopenic plasma by fractionation techniques at exactly the same time that Mpl-L was produced with the technology of molecular biology. Some of the reasons for their success include innovative bioassays for TPO, removal of inhibitors, and the realization that plasma TPO levels were greater in animals with hypoplastic thrombocytopenia than in those with an intact marrow. Eaton (Chapter 8) describes how Mpl-L was more rapidly characterized by purification from large quantities of plasma from animals with radiation-induced thrombocytopenia, using MPL for receptor–affinity chromatography. Another successful approach depended on the production of Mpl-L by transformation of a cell line engineered to express the receptor. In both cases, the ligand was then used to obtain the DNA encoding it, thus permitting development of recombinant technology to produce and characterize Mpl-L.

When it became possible to demonstrate colonial growth of megakaryocyte progenitor cells in cultures *(9)*, such cultures were applied to the search for specific growth factors, an approach that had been fruitful for growth factors for leukocytes. Specific factors were not identified, but multifunctional factors were found to stimulate megakaryocyte colony formation as well as colonies of other types. Mazur (Chapter 5) and

Hoffman (Chapter 10) provide in-depth discussions of these. Some of these factors affect proliferation of progenitors, whereas others influence maturation, with little apparent overlap, thus supporting a two-level theory of the regulation of megakaryocytopoiesis in which cellular proliferation of early progenitors in vitro is regulated by nonspecific cytokines differing from those, including putative TPO, that regulate such later events as the development of polyploidy and cytoplasmic maturation. Mazur, however, indicates that Mpl-L supports the cellular proliferation phase as well as the later events, thus perhaps questioning the physiological relevance of the two-level model. As described by Debili, Cramer, Wendling, and Vainchenker in Chapter 14, Mpl-L affects proliferation, differentiation, and maturation when applied to progenitor cells in vitro, but the primary target cell for Mpl-L is a relatively mature megakaryocyte progenitor. Though the major effects of Mpl-L are on megakaryocytopoiesis, other lineages are also affected, especially in combination with other cytokines, thus suggesting that Mpl-L may not be totally lineage specific. The potential complexity of regulation is apparent from Hoffman's discussion of inhibitory cytokines, autocrine regulation, the microenvironment, the secondary production of one cytokine by another, and other confounding elements. Therefore, it would probably be premature to conclude that TPO is the only regulator of platelet production in all situations.

Often cited throughout this book are the findings in mice in which the gene for MPL or TPO was disabled or "knocked-out," and these studies are summarized in Chapter 21 by deSauvage and Moore. In both models, thrombocytopenia and megakaryocytopenia with reduced ploidy were seen. The residual platelets were macrocytic, indicating that this feature, known to occur with thrombocytopenia, may not be mediated by the TPO/MPL interaction. The fact that thrombocytopenia and megakaryocytopenia were not absolute is consistent with older observations that platelet hypertransfusion never completely eradicated megakaryocytes in rodents, in contrast to the elimination of erythropoiesis by red cell hypertransfusion. However the reasons for residual platelet production in either case are not apparent. That the TPO/MPL system may affect other hemopoietic cells was again shown by the reduction in knockout mice of colony-forming cells, but not blood cells, of other lineages. Finally, de Sauvage and Moore interpret the findings in these mice to indicate that blood levels of TPO are regulated by direct interaction with platelets rather than by adjustment of TPO production.

TPO is a structurally unique hemopoietic cytokine consisting of two domains, the biologically active domain with partial homology to erythropoietin (EPO) and a long carboxyl domain with many glycosylation sites. Gurney and de Sauvage (Chapter 11) describe the protein structure, forms of mRNA, gene structure, chromosomal location, and similarities of TPO structure in different species. In spite of homology with EPO and resemblance between the receptors for the two factors, there is no cross activation of receptors, thus eliminating the thought that some of the curious interrelationships between erythropoiesis and thrombocytopoiesis might arise from stimulation of one receptor by the cytokine for the other. Foster and Hunt (Chapter 13) expand further on the unique structure of TPO and what it means in terms of biological activity and survival of TPO in the circulation. Full-length TPO and a variety of truncated forms are detectable in human serum. The glycosylated portion appears to be important for synthesis, secretion, and stability in the circulation. It is not clear whether the longer half-life arising from preferential glycosylation or conjugation with poly[ethylene glycol]

over that of truncated forms indicates lower reactivity with platelets, or whether other clearance mechanisms exist.

When cells respond to TPO, a complex of reactions occurs as a result of receptor activation. These are considered by Kaushansky, Broudy, and Drachman (Chapter 16) and Shivdasani (Chapter 12). The organization of the unique MPL receptor and how it may transmit signals for proliferation or differentiation are described. Reasons why lineage-specific patterns of gene expression have not yet been identified are given. However, the importance of nuclear transcription factors in megakaryocytopoiesis is emphasized by the maturation arrest and thrombocytopenia that occur with the elimination of one or the overexpression of another (Chapter 12). Kaushansky et al. also allude to the presence of MPL on leukemic cells, pluripotential hemopoietic cells, and erythroid progenitors. Some of these may account for the nonspecific response to TPO they and others (Farese and MacVittie, Chapter 20) observed in myelosuppressed animals and which was found during the induction of acute thrombocytopenia at the time of exposure to radiation *(10)*.

Megakaryocytes are curious cells. They mature by getting bigger and bigger, and then their cytoplasm fragments into platelets. Levin (Chapter 3) discusses, but remains puzzled by, the possible advantages that this mode of production may have over the cell-by-cell production of amebocytes or nucleated thrombocytes of so-called lower species. The question of how megakaryocyte cytoplasm actually fragments into platelets is surrounded by controversy. In Chapter 17, Choi describes the morphogenesis of megakaryocytes into proplatelets and platelets in vitro and reviews the sometimes confusing and contradictory reports of the roles of various factors on proplatelet formation. She then presents the unexpected finding from her laboratory that TPO inhibits proplatelet formation. It would be interesting to know whether the same inhibition occurs in vivo, i.e., if formation of cytoplasmic projections through the sinusoidal endothelium is affected in TPO-treated animals, since these projections may be the *in situ* counterparts of proplatelets. TPO also seems to inhibit apoptosis in mature megakaryocytes. Does this mean that fragmentation into platelets is actually a part of the process of programmed cell death? This is clearly a work in progress, and the author points out issues that are under investigation. However, it may be relevant to recall the earlier finding that partially hepatectomized rats promptly develop thrombocytopenia owing to deficient platelet production *(11)*. It appeared that the liver produced both TPO, as now thought from the localization of its mRNA, and an unidentified factor that affected release of platelets from the marrow, possibly a factor that might influence proplatelet formation.

Until now, platelet transfusions have been the treatment of choice for thrombocytopenic hemorrhage in patients with marrow aplasia. In Chapter 4, Schiffer explains why they are sometimes ineffective or even hazardous to patients. An exciting prospect is that TPO will prove to be so effective in increasing platelet counts that platelet transfusions can be avoided. Recombinant technology now appears capable of producing seemingly unlimited quantities of active Mpl-Ls, and preclinical analyses of them are promising, so this possibility can be tested. Schiffer identifies categories of patients who will be most likely to benefit from TPO therapy, and he outlines criteria for clinical trials of TPO. Results of clinical trials are not included in *Thrombopoiesis and Thrombopoietins: Molecular, Cellular, Preclinical, and Clinical Biology*, but preclinical data are.

In anticipation of administering TPO to humans, it is necessary to know whether the function of platelets might be affected. The importance of ultrastructural analysis to evaluate megakaryocytes and platelets produced under the influence of cytokines is underscored by Zucker-Franklin (Chapters 2 and 15). Some cytokines are associated with megakaryocytic or platelet cytoplasmic immaturity or ultrastructural aberrations that are not apparent with other tests. Platelets produced under the influence of cytokines could be hypo-, hyper-, or normally reactive, and ultrastructural analysis is an important tool for predicting which condition may be dominant. Harker, Marzec, and Toombs (Chapter 18) describe the effects of Mpl-L on platelet function when tested in vitro; and in Chapter 19, Harker, Toombs, and Stead disclose its effects in animal models of thrombosis. Mpl-L enhanced the aggregation of platelets by agonists, but there was no increase in thrombus formation in vivo. Thus the enhanced aggregation appeared to be a laboratory phenomenon that they propose may be caused by an increased proportion of young platelets or a sharing of signaling sequences, since both Mpl-L and platelet agonists induce protein tyrosine phosphorylation. It has been demonstrated that platelets and megakaryocytes are heterogeneous for a tyrosine phosphatase and that platelets lacking the enzyme are the more reactive *(12)*. Therefore, it could also be suggested that the ratio of negative to positive platelets might be altered by administration of Mpl-L.

In Chapter 19 the effects of megakaryocyte growth and development factor (MGDF) (a truncated Mpl-L) on platelet production and turnover in normal animals are presented. There was a close correlation between an increase in megakaryocyte mass and the increase in platelet mass turnover. In conjunction with the increasing platelet count, mean platelet volume (MPV) decreased, suggesting again that other factors are responsible for the increased MPV in thrombocytopenic individuals. In these normal animals changes in leukocyte, neutrophil, or red cell counts did not occur. Some human candidates for treatment with TPO will have marrow suppression. Chapter 20 by Farese and MacVittie is a comprehensive summary of the effects of various cytokines, alone and in combinations, in myelosuppressed animal models. Mpl-L was much superior to other cytokines in improving platelet counts, indicating that an excess may be effective even in the presence of presumably high levels of endogenous TPO (Nichol, Chapter 22) and low numbers of progenitors. In contrast to normal animals, Mpl-L also improved leukocyte counts and hematocrits suggesting that marrow cellularity may affect the proliferation and/or differentiation of multilineage or nonmegakaryocytic progenitors. The authors also describe cytokine combinations that may maximize hemopoietic recovery and preview some engineered agonists of cytokine receptors.

The cumbersome TPO bioassays of the past can now be replaced with immunoassays. These and assays for other cytokines that affect thrombocytopoiesis are reviewed by Nichol in Chapter 22, together with blood levels of TPO in different clinical conditions. Patients with hypo- or aplastic marrows show an inverse correlation between platelet count and TPO level in contrast to idiopathic thrombocytopenic purpura (ITP) in which TPO levels are normal or only slightly elevated. She speculates that the difference may be caused by increased platelet production in ITP with absorption of TPO by newly produced, but short-lived, platelets or to an effect of the megakaryocyte mass on TPO levels. Others have attributed the low levels of TPO in thrombocytolytic thrombocytopenias to the megakaryocyte mass *(13)*, a conclusion that may be favored

by the knowledge that platelet production is not always increased in ITP *(14)*. That TPO levels could be affected by megakaryocyte mass might have relevance to an understanding of some compensated hypomegakaryocytic states that have been observed in animals (Chapter 1) and may have an analogy to the observation that EPO levels are influenced by the amount of erythroid marrow *(15)*. It would be instructive to know comparative survivals of TPO in the circulation of normal animals and in those with antibody-mediated or aplastic thrombocytopenia. Other interesting, and as yet unresolved, issues raised by Nichol are the elevated TPO levels in patients with primary thrombocytoses and the exceedingly high levels in some patients with chronic liver disease and persistent thrombocytopenia.

A unified concept about the regulation of platelet production is presented by Kuter in Chapter 23, based on work in his and other laboratories. He envisions that the only physiologically relevant regulator of platelet production is TPO that is constitutively produced by the liver. The amount of TPO available to influence megakaryocytopoiesis would be that amount that is not bound and removed from the circulation by platelets. The amount bound would be determined by the total body platelet mass and the ability of platelets to metabolize TPO. Elevated TPO levels in myeloproliferative disorders might be caused by dysfunctional metabolism of TPO. With the capability now to measure several facets of TPO metabolism, such as the ability of platelets to bind TPO, the number of Mpl receptors on platelets, and mRNA for TPO in different tissues, some of these postulates can now be tested experimentally.

This book contains a wealth of information about megakaryocytopoiesis and platelet production, their regulation, and areas for ongoing or future research. All authors are recognized authorities on their subjects. I have previewed the chapters, and the emphases and commentaries reflect my personal perspective. The new knowledge about TPO and its receptor opens the door to the possibility for addition of a valuable therapeutic agent to the physician's armamentarium, and it is furthering the understanding of megakaryocyte and platelet physiology. This book will serve as a valuable reference for the uninitiated as well as veteran megakaryocyte researchers, but we must continue to watch the latest publications for information in this rapidly expanding field of research.

Finally I would like to remember two men whose works contributed mightily to the scientific progress summarized in this book and who died recently: Dr. Olav Behnke and Dr. N. Raphael Shulman. They will be missed.

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Preface

Our principal aims in bringing together a book on thrombopoiesis at this point after the discovery of its long-sought physiologic regulator, the Mpl ligand, are to educate investigators and to stimulate further research. We hope that we have succeeded on both counts and would like to thank the many outstanding scientists who have contributed directly and indirectly to this volume.

Since different perspectives often help one to arrive at a closer approximation to the truth, we have not attempted to remove areas of either controversy or overlap between the various chapters.

We would like to especially thank Paul Dolgert and Fran Lipton of Humana Press for their persistence and responsiveness; Lawrence Transue for technical typing; Dr. MaryAnn Foote for her tireless and patient attention to detail; and Dr. Shirley Ebbe for reading the book in its entirety and preparing the Foreword.

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1

INTRODUCTION

1

Megakaryocyte Biology

Carl W. Jackson, Julie T. Arnold, Tamara I. Pestina, and Paula E. Stenberg

1. Introduction

Megakaryocytes are giant, polyploid cells of the hemopoietic tissues, whose final differentiation step culminates in the subdivision and release of their cytoplasm into the circulation as platelets. The earliest recognizable megakaryocyte in Romanovsky-stained marrow smears is a large basophilic cell with a high nuclear-to-cytoplasmic ratio and plasma membrane blebbing. As the cells mature, the nuclear-to-cytoplasmic ratio decreases as the amount of cytoplasm dramatically increases and becomes acidophilic, with abundant cytoplasmic granules, while the nucleus becomes lobulated and the chromatin condenses. Megakaryocytes are readily distinguished from osteoclasts, the other large cells in the marrow, by their nuclear morphology; megakaryocytes usually have only one large, lobulated nucleus, whereas osteoclasts contain several small nuclei. Megakaryocytes differentiate from a committed progenitor, which, by definition, has restricted differentiation capabilities. This committed progenitor is derived from a pluripotential hemopoietic precursor. A bipotential progenitor intermediate between the pluripotential and committed precursor with capacity to differentiate along either the megakaryocyte or erythroid pathways is suggested by some studies *(1,2)*. A scheme for megakaryocyte differentiation is presented in Fig. 1.

2. Unique and Identifying Features of Megakaryocytes and Megakaryocyte Development

Megakaryocytes have several unique and identifying features, including polyploid nuclei; subdivision of their cytoplasm into membrane-bound packages, the platelets; formation of cytoplasmic dense granules containing adenine nucleotides, neuropeptides, and divalent cations; α-granules containing plasma proteins and endogenously synthesized proteins; and proteins, such as membrane glycoprotein IIb, uniquely expressed by megakaryocytes and platelets.

3. Developmental Sequence of Megakaryocytes

3.1. Pluripotent Hemopoietic Stem Cells

The cell from which megakaryocytes and the other hemopoietic cell lineages are derived is a pluripotential hemopoietic progenitor that by definition, can provide long-term hemopoietic repopulation of the marrow of a lethally irradiated animal *(3)*.

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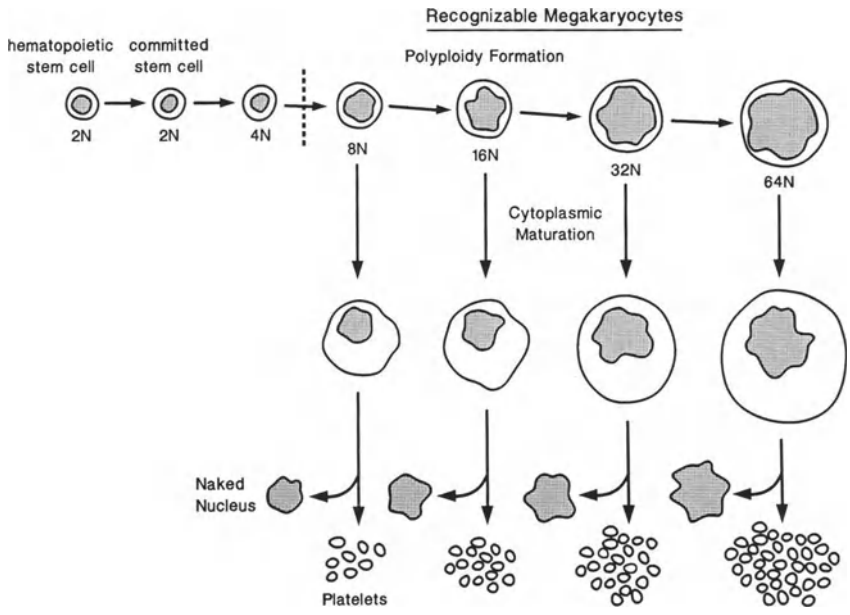

Fig. 1. Scheme depicting stages of megakaryocyte development: (1) commitment of hemopoietic precursors to the megakaryocyte lineage; (2) differentiation of megakaryocyte progenitors to recognizable megakaryocytes; (3) polyploid formation; (4) cytoplasmic maturation; and (5) platelet shedding. The scheme illustrates that polyploidization precedes and is completed before the rapid expansion and maturation of megakaryocyte cytoplasm; megakaryocytes can cease polyploidization, undergo cytoplasmic maturation, and platelet shedding at any level of polyploidy; and megakaryocyte size is related both to the degree of polyploidization and stage of maturation.

3.2. Committed Precursors: MK-BFC and MK-CFC

The megakaryocyte colony-forming cells (MK-CFC) were the first population of committed megakaryocyte progenitor cells to be identified, and the in vitro assays used for their recognition were developed in the 1970s *(4–6)*. The MK-CFC represent a heterogeneous population of cells that vary in their proliferation potential and buoyant density *(7–10)*. Murine MK-CFC undergo 1–8 cell divisions (average colony size 16–32 cells), and are more than 5 days removed from platelet formation *(4,11)*. Maximal megakaryocyte colony formation in vitro is dependent on two factors: a colony-stimulating factor (MK-CSF) required for proliferation of clonable progenitor cells and a maturation factor, megakaryocyte potentiator (MK-Pot), which enhances the DNA content and cell size of individual megakaryocytes within a colony *(11–14)*. While interleukin (IL)-3 *(15,16)* and Mpl ligand *(17,18)* independently have both MK-CSF and MK-Pot activity, maximal numbers of megakaryocyte colonies are produced when both IL-3 and thrombopoietin (TPO) are added to the culture media *(19)*.

Development of reliable assays for human MK-CFC *(20–23)* initially proved difficult because of the lack of a suitable marker that allowed detection of megakaryocytes. Identification of human MK-CFC is now routinely achieved by immunoperoxidase labeling of platelet-specific glycoproteins (GP), such as GPIIb/IIIa *(24,25)*. The growth requirements of human MK-CFC are similar to those of the murine system, being dependent on factors that promote proliferation and maturation *(26,27)*, but again it is

apparent that the addition of Mpl ligand alone to cultures of human $CD34^+$ cells can promote growth of MK-CFC *(28,29)*.

A population of megakaryocyte progenitors more primitive than MK-CFC was first described in the mouse *(30,31)*. Originally termed a megakaryocyte burst-forming cell, MK-BFC, with high proliferative potential, this population of cells also has been denoted as Meg-HPP-CFC *(32)* or HPP-CFC-Mk *(33,34)*. In addition, a mixed high-proliferative-potential megakaryocyte (HPP-Meg-Mix) cell, a trilineage murine hemapoietic progenitor *(35)*, which is presumably even more primitive than the unilineage Meg-HPP-CFC, has been reported. Detection of murine MK-BFC in culture requires phorbol ester stimulation *(30)* or multiple early acting cytokines *(33,34)*, and incubation for 12–14 days, as opposed to 7 days for MK-CFC. Whether TPO is active on the primitive MK-BFC progenitor remains to be determined, although it is clear that TPO is able to synergize in culture with other early acting growth factors to promote megakaryocyte development from murine hemopoietic stem cells *(36,37)*.

The MK-BFC have a high proliferative capacity giving rise to large colonies of megakaryocytes (40–500 cells/colony) comprised of single *(33)* or multiple foci *(30)*. The morphologic criteria defining murine MK-BFC also apply to human MK-BFC *(38)*. Both human MK-BFC and MK-CFC express the CD34 antigen. However, only MK-CFC express detectable quantities of the HLA-DR antigen *(38,39)*. The MK-BFC and MK-CFC progenitor cell populations also differ in their elutriation and velocity sedimentation profiles and the growth-factor requirements needed for in vitro detection *(38,40–42)*. Human MK-BFC have been detected in fetal cord blood *(43,44)*, and increased numbers are reported in peripheral blood of patients after chemotherapy *(45)* and growth factor-induced mobilization *(46,47)*.

In vivo assays for identification of megakaryocyte progenitors cells have been developed only in the murine system *(48,49)*. The progenitor cells, termed megakaryocyte colony-forming cell-spleen (MK-CFC-S), were detected within the marrow of mice receiving high doses of 5-fluorouracil (5-FU) *(48)*. When the marrow from 5-FU-treated mice was transplanted into lethally irradiated mice, the MK-CFC-S cells were capable of forming large megakaryocyte colonies in the spleens of the recipients. The exact relationship of this cell to either the MK-BFC or MK-CFC is unclear. However, the MK-CFC-S most likely corresponds to the MK-BFC population based on its relative insensitivity to 5-FU treatment and its ability to generate a colony of megakaryocytes composed of several hundred cells *(49)*.

3.3. Detection and Characteristics of Immediate Megakaryocyte Precursors

Cells of the megakaryocyte lineage smaller than recognizable megakaryocytes were first detected by histochemical reactions. Zajicek found that in blood and hemopoietic tissues, the enzyme acetylcholinesterase (AChE), although primarily restricted to the erythroid lineage in humans, was found predominantly in megakaryocytes and platelets in rodents and the cat *(50)*. He observed that in addition to morphologically recognizable megakaryocytes, some smaller marrow cells expressed AChE activity. Unfortunately, the reaction product was diffusible, so that further characterization of these cells could not be done at that time. Breton-Gorius and Guichard then developed an ultrastructural histochemical technique for the demonstration of platelet peroxidase

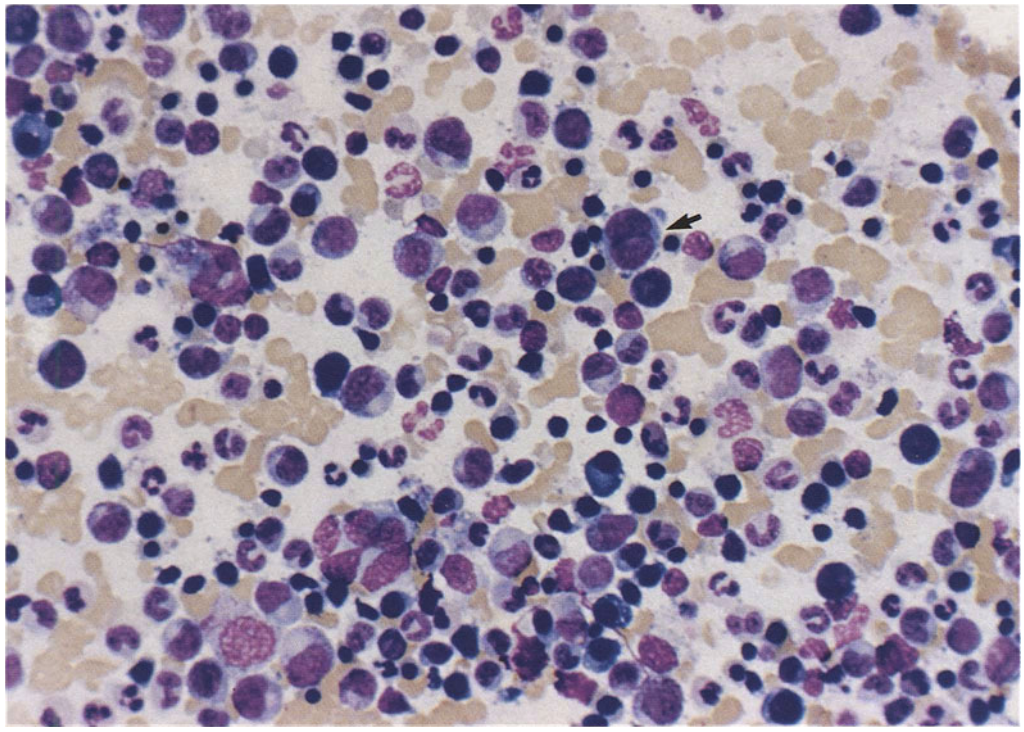

Fig. 2. Photomicrograph of a small, immature human megakaryocyte showing a high nuclear-to-cytoplasmic ratio, an even distribution of nuclear chromatin, basophilic staining of the cytoplasm, and characteristic blebbing of the plasma membrane.

and detected cells smaller than megakaryocytes that contained this enzyme activity *(51)*. However, they later found that this enzyme activity was not strictly megakaryocyte-lineage-specific, since it also was present in a population of early erythroid precursors *(51a)*. Subsequently, Karnovsky and Roots *(52)* developed an improved histochemical technique for demonstration of AChE activity that Jackson applied to analysis of the megakaryocyte lineage in rats *(53)*. Jackson confirmed the presence of $AChE^+$ cells smaller than megakaryocytes and demonstrated that the proportion of $AChE^+$ cells increased early after induction of acute thrombocytopenia. Long and Henry then reported that these cells decreased when platelet number was elevated by platelet hypertransfusion *(54)*. These data strongly suggested that the small $AChE^+$ cells were early cells of the megakaryocyte lineage. At the ultrastructural level, these cells resembled small lymphocytes with a large nucleus usually containing one nucleolus, an occasional profile of rough endoplasmic reticulum (RER), and abundant free ribsomes, all features of undifferentiated cells *(55)*. Small $AChE^+$ cells in the late stages of mitosis were occasionally observed, indicating that at least a portion of these cells are capable of cell division *(55,56)*. Jackson subsequently showed that these small cells also were labeled by platelet-specific antibody, providing more conclusive evidence that they were megakaryocyte precursors *(57)*. This led to the use of platelet antibodies directed against platelet-specific membrane proteins to detect megakaryocyte-lineage cells in human marrow *(24)*.