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Thrombopoietin, the Mpl ligand, is essential for full megakaryocyte development

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ABSTRACT **The development of megakaryocytes (MKs) from their marrow precursors is one of the least understood aspects of hematopoiesis. Current models suggest that early-acting MK colony-stimulating factors, such as interleukin (IL) 3 or c-*kit* ligand, are required for expansion of hematopoietic progenitors into cells capable of responding to late-acting MK potentiators, including IL-6 and IL-11. Recently, the Mpl ligand, or thrombopoietin (Tpo), has been shown to display both MK colony-stimulating factor and potentiator activities, at potencies far greater than that of other cytokines. In light of these findings, we tested the hypothesis that Tpo is absolutely necessary for MK development. In this report we demonstrate that neutralizing the biological activity of Tpo eliminates MK formation in response to c-*kit* ligand, IL-6, and IL-11, alone and in combination, but that these reagents only partially reduce MK formation in the presence of combinations of cytokines including IL-3. However, despite the capacity of IL-3 to support the proliferation and initial stages of MK differentiation, elimination of Tpo prevents the full maturation of IL-3-induced MK. These data indicate that two populations of MK progenitors can be identified: one that is responsive to IL-3 but can fully develop only in the presence of Tpo and a second that is dependent on Tpo for both proliferation and differentiation. Thus, our results strongly suggest that Tpo is the primary regulator of MK development and platelet production.**

The generation of megakaryocytes (MKs) is a complex process dependent on the interaction of hematopoietic progenitor cells, cytokines, and stromal elements (1–3). Committed MK progenitor cells must undergo a series of mitotic divisions, shift to endomitotic replication, express specific membrane glycoproteins, and undergo cytoplasmic maturation in preparation for platelet shedding. Models of erythropoiesis and granulopoiesis have been established that stress the importance of both early-acting and late-acting cytokines or hormones for completion of the erythroid and granulocytic developmental programs (4–6). A number of investigators have shown that interleukin (IL) 3 and c-*kit* ligand (KL) can support the production of MK colonies from their progenitors [MK colony-forming units (CFU-MK)] in semisolid medium and of individual MKs in suspension culture (7–10). Moreover, although IL-6, IL-11, and leukemia inhibitory factor are not reported to support MK formation alone, these cytokines augment the MK response to IL-3 or KL (11–14). Recently, we (15) and other groups (16, 17) have cloned and expressed the ligand for the c-Mpl receptor. Based on its capacity to induce increases in the size, ploidy, and maturation of MKs, its inverse relationship with platelet levels, and its ability to increase platelet production manyfold (18, 19), we proposed that the Mpl ligand is identical to thrombopoietin (Tpo) (20, 21).

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Like IL-3 and KL, Tpo can support the growth of MKs in both semisolid and suspension culture systems. However, interpretation of results using either of these techniques has limitations: agar cultures usually contain serum, which could introduce known or unknown growth factors, and suspension cultures usually contain unfractionated marrow cells at high density, which could facilitate endogenous production of cytokines or direct cellular interactions. To determine whether IL-3, KL, or Tpo acts directly to induce MK differentiation, we tested these cytokines, alone and in combination with IL-6 and IL-11, for their capacity to promote MK growth in the presence of reagents that neutralize Tpo activity. In the present work we provide evidence for the existence of two populations of MK progenitor cells: the first absolutely dependent on the presence of Tpo and the second able to initially develop in response to IL-3 but requiring Tpo for full maturation. Thus, our results strongly suggest that full MK development and platelet production is dependent on the presence of Tpo.

MATERIALS AND METHODS

Reagents. Murine Tpo was produced in BHK cells under serum-free conditions as described (15). By definition, 50 units is that amount which supports half-maximal proliferation in a BaF3/mpl MTT assay. Soluble murine c-Mpl containing a 5-residue cAMP-dependent kinase domain (RRASV; ref. 22) and a six-residue polyhistidine tail at the carboxyl terminus of the extracytoplasmic domain was prepared by site-directed mutagenesis. The cDNA was expressed in BHK cells, purified by metal chelation affinity chromatography, and eluted with imidazole buffer (23). For use in culture, purified soluble receptor was twice dialyzed against pyrogen-free phosphate-buffered saline and filter-sterilized. Protein concentrations were estimated by comparing intensity of Coomassie blue staining with albumin controls. Murine (m) IL-3, mKL, human IL-6, and human IL-11 were purchased from Genzyme. Nutridoma-SP culture supplement was purchased from Boehringer Mannheim and horse serum was from GIBCO/BRL. The neutralizing anti-mIL-3 monoclonal antibody (mAb) 19B3, provided by John Abrams (DNAX), was used at 5 μg/ml and completely eliminated MK formation in suspension culture and BaF3/mpl cell proliferation in response to IL-3 at 20 ng/ml (15). The neutralizing anti-murine c-*kit* mAb ACK-2, provided by Shin-Ichi Nishikawa, was used as hybridoma supernatant at 5% (vol/vol) and completely eliminated acetylcholinesterase (AChE) activity in suspension cultures in the presence of KL at 20 ng/ml (14). The neutralizing rabbit anti-murine c-Mpl antiserum (raised against soluble Mpl receptor by Zymogenetics) was purified by protein A-Sepharose chromatography (Pharmacia) and used at 5 μg/ml. At this

Abbreviations: MK, megakaryocyte; IL, interleukin; KL, c-*kit* ligand; CSF, colony-stimulating factor; Tpo, thrombopoietin; CFU, colony-forming unit(s); mAb, monoclonal antibody; AChE, acetylcholinesterase; m, murine; GM, granulocyte–macrophage; E, erythroid.

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concentration, it completely blocked the proliferation of BaF3/mpl cells grown in Tpo at 250 units/ml.

Marrow Cell Culture Conditions. Marrow cells from BDF_1 mice were obtained by flushing femurs with Iscove's modified Dulbecco's medium (IMDM) with 10% (vol/vol) fetal calf serum. Cells were aspirated through 20- and 25-gauge needles and filtered through Nytex mesh to break up clumps and to ensure that mature MKs were destroyed. For CFU-MK colony assays, cells were plated at 1 to 4×10^5 cells per ml in IMDM supplemented with 15% (vol/vol) preselected horse serum and antibiotics and were made semisolid with 0.275% agar. Only at the highest cell concentrations were MK colonies detected in IL-6- or IL-11-containing cultures. MK colonies contained greater than three polyploid AChE-positive cells. For suspension cultures, the cells were washed free of fetal calf serum and resuspended at 1×10^6 cells per ml in IMDM supplemented with 1% Nutridoma-SP as described (11). All cultures were incubated for 5 days at 37°C in a 5% CO_2/95% air fully humidified atmosphere. MK mass was evaluated by determining AChE activity levels in a fluorescence assay (11). MK size was measured by calibrated microscopy after AChE staining, and ploidy was determined as reported (18). Cultures for erythroid [erythroid CFU (CFU-E)] and myeloid [granulocyte–macrophage CFU (CFU-GM)] progenitor cell-derived colonies were grown in plasma clot and methylcellulose assays, respectively, as described (24, 25).

Detection of Tpo. The biological activity of Tpo was detected by using a specific BaF3/mpl proliferation assay (15) in which any mIL-3 present was neutralized using mAb 19B3. These cells fail to respond to IL-5, IL-6, IL-11, KL, granulocyte–macrophage colony-stimulating factor (GM-CSF), or erythropoietin. To detect Tpo-specific mRNA, marrow cells similar to those used to initiate cultures were lysed and RNA was prepared. First-strand cDNA was prepared using Superscript reverse transcriptase (GIBCO/BRL) and 30 cycles of PCR was carried out with sense and antisense primers located 412 and 1087 nt from the initiation codon (15). Glyceraldehyde 3-phosphate dehydrogenase primers were included as a measure of cDNA integrity. PCR products were size-fractionated on 0.8% agarose gels and visualized with ethidium bromide staining.

RESULTS

Soluble Murine c-Mpl Neutralizes the Biological Activity of Tpo. Dose–response curves using Tpo and soluble receptor (1.5 μg/ml) were constructed by using murine marrow MK colony-forming assays. As shown in Fig. 1, concentrations of Tpo at or below 100 units/ml were completely neutralized by soluble c-Mpl at 1.5 μg/ml, higher concentrations of Tpo (3000 units/ml) were able to overcome some of this effect. However,

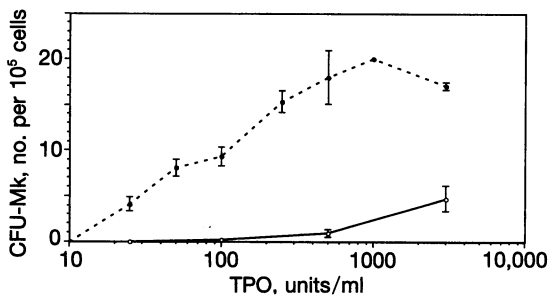

FIG. 1. Soluble Mpl inhibits Tpo activity. MK colony formation was assessed in response to increasing concentrations of Tpo in the absence (dashed line) or presence (solid line) of soluble Mpl receptor at 1.5 μg/ml. The data represent the mean (±SEM) number of colonies detected in triplicate plates. The experiment has been repeated two more times, with different Tpo and soluble receptor preparations, with essentially identical results.

to be certain that the results using the soluble receptor were specific to megakaryopoiesis and not due to nonspecific toxicity, we tested for effects on CFU-E- and CFU-GM-derived colony formation and on IL-3-induced proliferation of BaF3 cells. There were no significant differences in the number of either erythroid colonies (CFU-E = 199 ± 2 vs. 191 ± 9.5 in response to 0.1–0.5 unit of erythopoietin, $n = 2$) or myeloid colonies (CFU-GM = 113 ± 4.3 vs. 115 ± 4.7 in response to IL-3 at 20 ng/ml, $n = 3$) formed in absence or presence of soluble c-Mpl at 1.5 μg/ml, respectively. BaF3 cells grown in IL-3 at 30 units/ml were unaffected by the soluble receptor.

Soluble c-Mpl Inhibits MK Formation in Response to IL-3, KL, IL-6, and IL-11. Numerous groups (7–14) have described the effects of cytokines on MK colony growth, most notably for IL-3, KL, IL-6, and IL-11, alone and in combination. However, all of the reported results utilize culture systems containing a large number of potential accessory cells and/or serum that might produce or contain Tpo. To test which cytokines are ultimately responsible for megakaryopoiesis, murine marrow cells were cultured with MK-promoting proteins in the presence and absence of soluble c-Mpl. In the presence of soluble receptor, KL-, IL-6-, and IL-11-induced colony formation was essentially eliminated, and IL-3-induced colonies were reduced to 39% of control cultures (Fig. 2*A*). MK colony formation in response to combinations of these cytokines was also affected by soluble c-Mpl, although the effects on combinations including IL-3 were much less pronounced. Similar

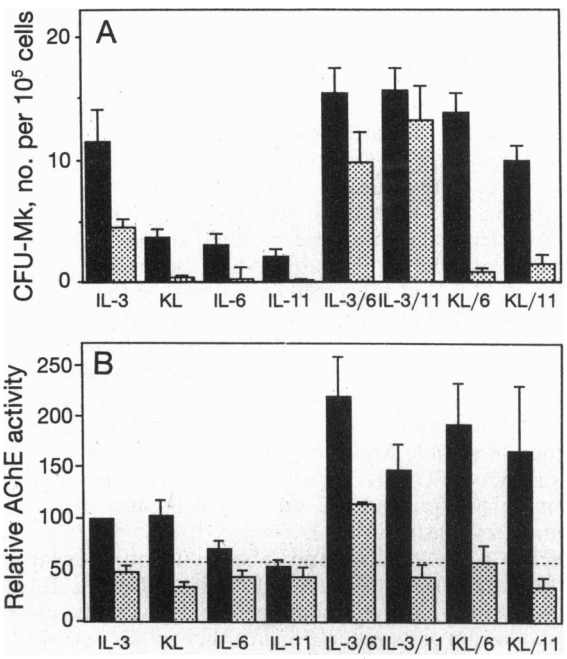

FIG. 2. Tpo mediates cytokine-induced MK formation. (*A*) MK colony formation was assessed in response to IL-3 at 200 ng/ml, KL at 200 ng/ml, IL-6 at 50 ng/ml, or IL-11 at 50 ng/ml, in the absence (solid bars) or presence (stippled bars) of soluble Mpl receptor at 1.5 μg/ml. The results represent the mean (±SEM) of three experiments of triplicate plates. MK colony formation was never detected in the absence of exogenous cytokines in these assays. All pairs of results are statistically significantly different by the Student's *t* test except for the IL-3/6 and IL-3/11 cultures. (*B*) Suspension cultures of murine marrow were incubated with IL-3 at 20 ng/ml, KL at 20 ng/ml, IL-6 at 50 ng/ml, or IL-11 at 50 ng/ml, alone or in combination, either without (solid bars) or with (stippled bars) 1.5 μg of soluble Mpl receptor. The results represent the mean (±SEM) AChE activity compared to that of IL-3 at 20 ng/ml (defined as 100% activity) of from four to eight experiments. The dotted line represents the level of AChE activity present in cultures containing no exogenous cytokines. All results are statistically significantly different except that for IL-6 and IL-11, which are no different than background.

results were obtained using the IgG fraction of an anti-Mpl antiserum that blocks the biological activity of Tpo.

To be certain that the results using Mpl-neutralizing reagents were not due to serum present in the agar cultures, we used a serum-free suspension culture system (14). Alone, IL-3 and KL induced MK formation, as detected by a quantitative fluorescence assay for AChE. Of note, control serum-free cultures containing no exogenous cytokines also produced a moderate number of MKs. This level of megakaryopoiesis was reduced nearly completely in the presence of soluble c-Mpl plus a neutralizing mIL-3 mAb (data not shown). Neither IL-6 nor IL-11 alone significantly induced AChE above control cultures, as reported (12, 14). However, in combination with IL-3 or KL, both IL-6 and IL-11 induced supraadditive levels of AChE activity above that seen in control cultures (Fig. 2*B*). When soluble c-Mpl was added to these cultures, AChE activity was reduced to levels below that produced in control cultures, with the exception of cultures containing IL-3 plus IL-6.

Evidence for Two Populations of MK Progenitors. The results from both colony-forming assays and suspension cultures indicated that a fraction of the IL-3-responsive MK progenitors could, at least partially, develop in the absence of Tpo. As our studies (18) have indicated that Tpo was at least as effective as IL-3 in supporting MK colony formation, we next sought to determine whether the IL-3- and Tpo-responsive CFU-MK represented distinct or overlapping sets of progenitors. At optimal concentrations of each, the effects of IL-3 and Tpo were additive. In seven experiments, IL-3 at 20 ng/ml supported 29.3 ± 3.1% and Tpo at 300 units/ml supported 76.8 ± 8.5% of the maximal number of MK colonies formed in the presence of both cytokines. Moreover, IL-3-induced colonies contained more cells. In two experiments of triplicate plates, the proportion of MK colonies containing >15 cells was 32 ± 3.5%; Tpo failed to induce a single colony of this size. These results suggest a greater proliferative potential for IL-3-responsive progenitors.

Nearly identical results were obtained in suspension culture. In four experiments, the relative AChEase levels were 55.1 ± 13, 93.6 ± 11.5, and 156 ± 8.0 for cultures in the presence of IL-3, Tpo, or IL-3 plus Tpo, respectively. The results represent the mean of triplicate determinations above the background level seen in cultures without exogenous cytokines (12.3 ± 1.0% of maximal levels in these four experiments). Thus, with the partial resistance of IL-3-induced colony formation in the presence of soluble Mpl receptor, these results indicate that two distinct CFU-MK populations can be identified, one responsive to Tpo and the second, of greater proliferative potential, responsive to IL-3.

Marrow Cells Produce Tpo. The reduction or ablation of MK formation in response to IL-3, KL, IL-6, or IL-11 by Tpo-neutralizing reagents could be explained by the presence of Tpo in serum or its generation in either colony or suspension cultures. Tpo would then act with the exogenous cytokine(s) to induce MK formation. It is possible that the spontaneous MK formation seen in control suspension cultures is also dependent on endogenous production of Tpo. Our data indicating that soluble Mpl also reproducibly reduced the AChE activity of control cultures by 40% are consistent with this idea. To test this hypothesis, we assayed spent suspension culture medium for Tpo activity by a specific bioassay. Spent medium did not contain measurable levels of Tpo unless exogenous Tpo was added. As this result could be due to consumption of the Tpo by the proliferating MKs themselves or due to levels of Tpo that were below the level of detection (20 units/ml), we analyzed marrow cells for Tpo-specific mRNA by reverse transcription–PCR. As shown in Fig. 3, Tpo-specific mRNA was readily detected. However, the origin of the specific mRNA (nonhematopoietic cells or the MKs themselves) could not be discerned with our present methods.

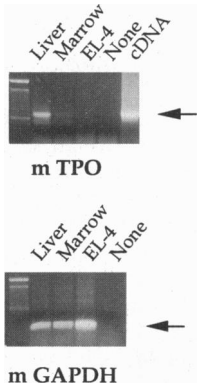

FIG. 3. Reverse transcription–PCR of marrow cell RNA. RNA extracted from normal murine marrow was analyzed by reverse transcription–PCR for the presence of Tpo-specific transcripts. The predicted product of 675 bp is indicated by the arrow. Amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA is shown as a control for reverse transcription and RNA integrity. Liver RNA provided a positive Tpo control (15, 16) and RNA from EL4 lymphocytes provided a negative control.

IL-3-Induced MKs Fail to Mature in the Absence of Tpo. As IL-3 alone supported the formation of colonies of AChE-positive cells, the extent of MK maturation under these conditions was further investigated. Initial studies suggested that IL-3-induced MKs were immature; their mean diameter was only 74% (43 ± 2.0 vs. 58 ± 2.0 μm) and the mean geometric ploidy was only 37% (11.7 vs. 32) that of Tpo-induced cells. Electron microscopic studies confirmed these initial impressions. There was a complete absence of demarcation membranes and a dearth of α granules, while organelles such as mitochondria and rough endoplasmic reticulum remained present in the peripheral zone (Fig. 4*A*). In contrast, MKs obtained from cultures containing Tpo exhibited abundant development of demarcation membranes primarily in the peripheral zone. However, the number and distribution of cytoplasmic organelles were aberrant in showing an excessive number of mitochondria and autophagic vacuoles (Fig. 4*B*). In the presence of IL-3 and Tpo, the formation of demarcation membranes was exuberant, and the range in size of cytoplasmic fragments resembled that seen during normal *in vivo* thrombocytopoiesis (Fig. 4*C*). Thus, IL-3 can support the proliferation and initial stages of MK development; full differentiation, however, to the level of thrombocytopoiesis is dependent on Tpo.

DISCUSSION

Over the past decade a number of distinct cytokines have been shown to influence MK development in various *in vitro* assays, by using marrow cells from both animals and humans. The cytokines that affect megakaryopoiesis have been divided into two groups: MK-CSFs, which affect the proliferation of MK progenitors and include IL-3 (4, 5), KL (9, 10), and GM-CSF (26), and MK potentiators, cytokines that affect the immediate precursors of polyploid MKs including IL-6 (11, 12), IL-11 (13, 14), and leukemia inhibitory factor (14). The recent cloning and biological characterization of the Mpl ligand have provided insights into the biology of MK development. By using recombinant or purified protein, we (18) and others (16, 17, 19) have shown that the Mpl ligand supports both functions; Mpl ligand, alone and in combination with other cytokines, stimulates the production of MK colonies and acts late in MK development to increase MK size, polyploidization, and the expression of platelet-specific cell surface glycoproteins. On the basis of its wide-ranging activities restricted to megakaryo-

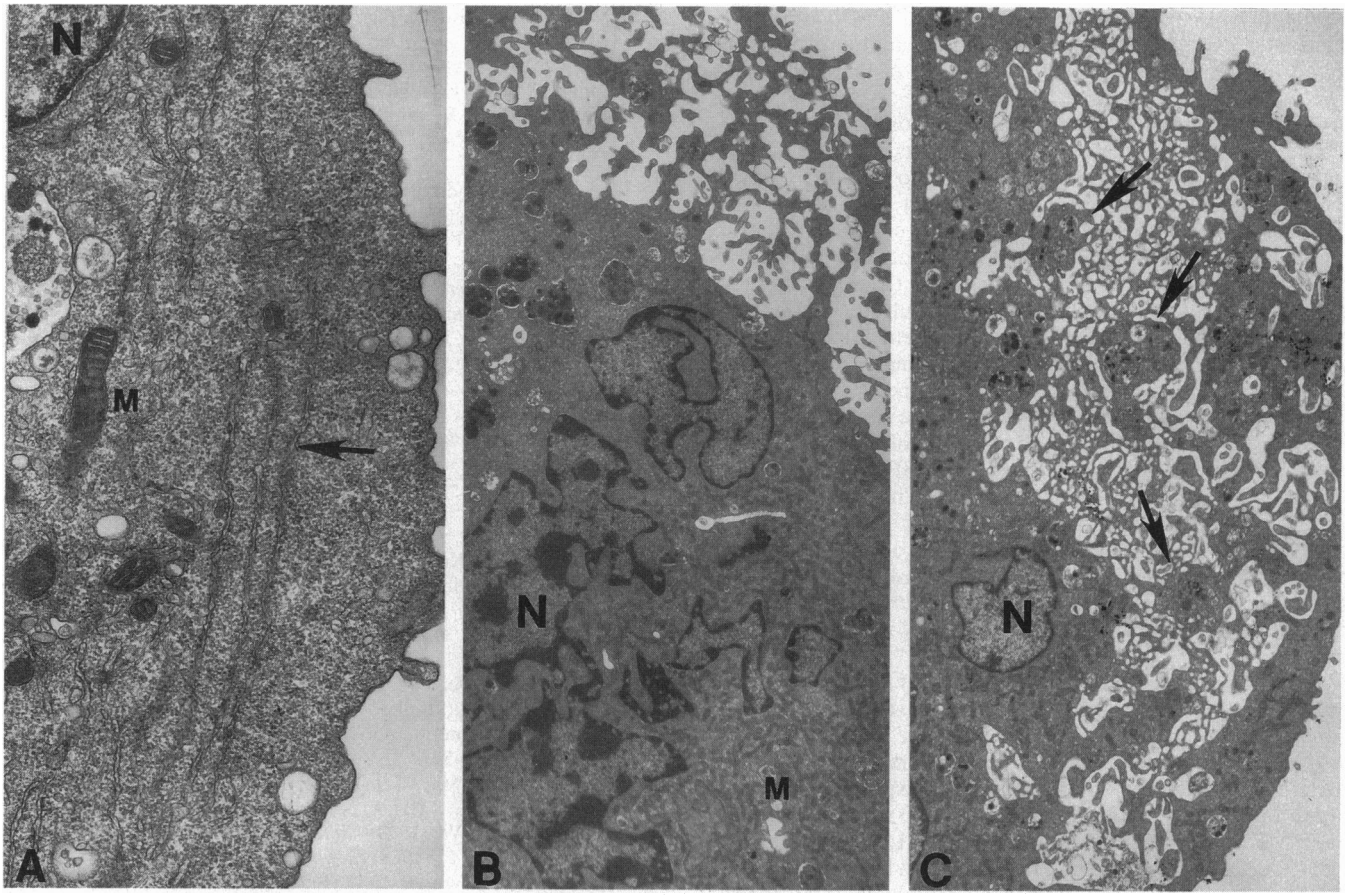

FIG. 4. Morphology of suspension culture MKs. (*A*) Peripheral zone of a MK from a culture treated with IL-3 only. Note absence of demarcation membranes, abundant rough endoplasmic reticulum (arrow), and mitochondria (M). N, nucleus. (×19,000.) (*B*) Detail of MK from cultures treated with Tpo only. Note fragmenting peripheral zone. Granulated appearance of cytoplasm due to excessive number of mitochondria (M). N, nucleus. (×5000.) (*C*) Fragmentation of cytoplasm of MK from cultures treated with IL-3 plus Tpo. Arrows indicate platelet fields with the size of normal platelets. N, nucleus. (×5000.) The consistent absence of MK fragmentation into platelet fields and the persistence of rough endoplasmic reticulum in >50 IL-3-induced MKs examined are remarkable.

poiesis, we proposed that the Mpl ligand be termed thrombopoietin (Tpo) (15, 18).

Given the potency and range of activities of Tpo on megakaryopoiesis, we investigated the possibility that the effects of IL-3, KL, IL-6, and IL-11 were mediated through Tpo. To test this hypothesis, we used reagents that neutralized the biological activities of IL-3, KL, or Tpo, in cultures that support MK development. Our results strongly support this hypothesis. Neutralization of Tpo activity led to the elimination of MK formation in response to KL, IL-6, or IL-11, alone and in combination, to levels below that found in control cultures. In contrast, neutralization of KL had no effect on Tpo-induced megakaryopoiesis.

The effects of neutralizing Tpo in cultures containing cytokine combinations including IL-3 were more complex. At 20 ng/ml, IL-3-induced MK formation, alone or in combination with low concentrations of IL-6 (5 ng/ml) or IL-11 (5 ng/ml), was eliminated by soluble Mpl receptor (data not shown). However, the presence of 10-fold higher concentrations of IL-3, especially in combination with IL-6 at 50 ng/ml or IL-11 at 50 ng/ml, could partially overcome this effect. These results suggest that IL-3 acts through both Tpo-dependent and Tpo-independent mechanisms. But despite the capacity of the cytokine to support the initial stages of development, IL-3-induced MKs fail to fully mature. This conclusion is based on the size, ploidy, and electron microscopic morphology of IL-3-induced MKs. Thus, IL-3-induced MK progenitors likely require Tpo for the formation and release of platelets.

The hypothesis that megakaryopoiesis is regulated at multiple levels was advanced by Williams *et al.* (27) and others (36, 37). On the basis of existing data relating to the effects of IL-3 on single MKs and their progenitors, these investigators proposed that both IL-3-dependent and IL-3-independent (Tpo) pathways could give rise to marrow MKs. Although our data conflict with some of the details of these earlier models (e.g., it is clear that Tpo influences not only the differentiation of MKs but also the proliferation of MK progenitor cells), our results are consistent with the basic principles of a dual system of megakaryopoietic regulation, mediated by two populations of progenitors and two distinct cytokines. However, because of the complex cellular nature of our culture systems, absolute verification of this hypothesis awaits the development of a single-cell serum-free culture system for purified MK progenitors.

Results from recent gene knock-out studies are also consistent with a dual system of MK development. By using homologous recombination at the murine c-*mpl* locus to eliminate receptor expression, Gurney *et al.* (28) have shown that MK numbers and platelet counts are substantially reduced but not eliminated. Unfortunately, characteristics of the residual MKs and platelets were not provided in this report. These workers hypothesized that another cytokine system is responsible for the residual thrombopoiesis or that elevated Tpo levels in the −/− mice operate through an alternate receptor. Our results cannot distinguish between these possibilities. Although MKs fail to fully mature in the absence of Tpo, IL-3-induced MKs may be able to sustain residual platelet

production *in vivo*. However, such pathways to platelet production are very unlikely to provide the rapid and profound expansion of platelet production in states of increased need. The crossing of Mpl −/− and IL-3 −/− strains may thus provide important insights into thrombopoiesis.

The simplest explanation for our results is that accessory cells in our culture systems produce the Tpo necessary for MK formation. The absence of Tpo activity in a biological assay suggests that Tpo produced in these culture systems might be rapidly consumed or be present at levels too low to detect. A similar conclusion was drawn regarding the production of other CSFs in long-term bone marrow cultures (LTBMCs). Numerous biological assays failed to detect macrophage-, granulocyte-, or GM-CSF in the spent medium from such cultures (29). The apparent absence of specific protein was initially taken as evidence that CSFs are not produced in LTBMCs. Only after the withdrawal of proliferating hematopoietic cells (which actively consume CSFs) or the use of sensitive immunological assays was CSF protein detectable (30, 31). Despite our inability to detect Tpo by bioassay, our results using reverse transcription–PCR demonstrating the presence of Tpo-specific mRNA in marrow strongly support our contention that Tpo is produced *in vitro* and that development of the majority of MKs is directly attributable to its presence.

Our conclusions about the dependence of megakaryopoiesis on Tpo are quite similar to results from studies of erythropoiesis. A number of cytokines enhance the growth and development of early erythroid progenitors (erythroid blast-forming units). The cytokines that display such burst-promoting activity are remarkably similar to those that support early MK colony formation and include IL-3, KL, and GM-CSF (26, 32–34). And like the role of Tpo in MK development, the presence of erythropoietin is absolutely essential for the production of mature erythrocytes (for review, see ref. 35). Thus, it would appear that cytokine control of megakaryopoiesis is organized much like that of erythropoiesis.

Finally, in addition to providing a better understanding of MK physiology, our results have important implications for the clinical application of cytokines in states of decreased platelet production. As the development and maturation of MKs appears to be absolutely dependent on the presence of Tpo, it is very likely that this molecule, rather than other cytokines that act indirectly or in synergy with it, will have the most profound effect on platelet production in clinical settings of natural or therapy-induced thrombocytopenia.

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