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RAPID COMMUNICATION

In Vitro Megakaryocytopoietic and Thrombopoietic Activity of c-mpl Ligand (TPO) on Purified Murine Hematopoietic Stem Cells

By Francis C. Zeigler, Frederic de Sauvage, H. Ramon Widmer, Gilbert A. Keller, Christopher Donahue, Robert D. Schreiber, Beth Malloy, Phillip Hass, Dan Eaton, and William Matthews

Recently, the ligand for c-mpl has been identified and cloned. Initial studies of this molecule indicate that it is the platelet regulatory factor, thrombopoietin (TPO). Previous work has indicated that c-mpl is expressed in very immature hematopoietic precursors and thus raised the possibility that TPO may act directly on the hematopoietic stem cell. Therefore, in these studies, we investigate the effects of TPO on hematopoietic stem cell populations isolated from the murine fetal liver and bone marrow. Cocultivation of stem cells with fetal liver stroma gives rise to multilineage expansion of the stem cells but with little or no megakaryocytopoiesis.

PLATELET HOMEOSTASIS is a vital facet of hematopoietic regulation, particularly in patients undergoing chemotherapy or bone marrow transplantation. Consequently, the identification of a thrombopoietin molecule (TPO) has been a major quest in the field of hematology and oncology. Very recently, the cognate ligand for the cytokine receptor c-mpl has been cloned and initial biologic studies of this molecule have shown both a megakaryocytopoietic and thrombopoietic activity.¹⁻⁴ These results strongly infer that the c-mpl ligand is the long-awaited TPO.

Previously, c-mpl expression has been shown by polymerase chain reaction (PCR) analysis in human CD34⁺ and CD34⁺ CD38⁻ hematopoietic cells.⁵ Furthermore, in colony assays, antisense oligonucleotides against c-mpl inhibited megakaryocyte production from CD34⁺ cells.⁵ These data suggest that the initial megakaryocytopoietic effects of TPO are elicited upon an early progenitor cell and perhaps upon the hematopoietic stem cell itself. Additionally, the presence of c-mpl in early progenitor populations also raises the possibility that TPO may be involved in the proliferation of these early progenitor cells as well as in megakaryocytopoiesis.

To further elucidate the biologic effects of TPO, we have investigated its actions on murine stem cell populations that have been previously shown to result in long-term engraftment of lethally irradiated hosts.⁶ These studies show that TPO can induce proliferation of stem cell populations, with the most marked effects being the enhancement of megakaryocytopoiesis and thrombocytopoiesis.

MATERIALS AND METHODS

Isolation of hematopoietic stem cell populations. The hematopoietic stem cell populations AA4⁺ Sca⁺, AA4⁺ CD34⁺ kit⁺, and Lin^{lo} Sca⁺ were isolated as previously described.⁶

Antibody production. The antimurine c-mpl monoclonal antibody was raised in Syrian hamsters using a murine c-mpl-Fc chimeric protein¹ as immunogen. The hybridoma 2F5 was derived from hamster splenocytes fused with the murine myeloma P3X63Ag8U.1. For fluorescence-activated cell sorter (FACS) analysis, 2F5 was purified by protein A affinity and conjugated to biotin. The specificity of this reagent was tested using 293 cells stably transfected with murine c-mpl by calcium phosphate transfection.⁷ The non-transfected parental 293 line was used as control.

Stromal cell/stem cell coculture assays. Coculture assays were

Addition of TPO to these cocultures gives significant megakaryocyte production. This production is enhanced in combination with Kit ligand or interleukin-3. The addition of TPO to stem cell suspension cultures produces a dynamic thrombopoietic system in which stem cells undergo differentiation to produce megakaryocytes and proplatelets. These experiments show that the megakaryocytopoietic and thrombopoietic activities of TPO are initiated at the level of an early progenitor cell or upon the hematopoietic stem cell.

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performed on the stromal cell line 7-4 as previously described.⁶ Growth factors were used at the following concentrations: Kit ligand at 50 ng/mL; interleukin-3 (IL-3) at 1 ng/mL (Genzyme, Cambridge, MA); granulocyte-macrophage colony-stimulating factor (GM-CSF) at 2 ng/mL (R&D Systems, Minneapolis, MN); and IC2-310 at 40 µg/mL.

Stem cell suspension cultures. Stem cells were isolated as described.⁶ Ten thousand of these cells were plated into individual wells in a 12-well format in DMEM/f12 media supplemented with 10% fetal calf serum (FCS), 10⁻⁷ mol/L Selenium, 5 µg/mL Apotransferin, 5 µg/mL insulin, and 10⁻⁸ mol/L progesterone. Additional growth factors were added at the same concentrations indicated above.

Cytospin analysis. Cytospin analyses of resultant cells were performed as previously described.⁶ Megakaryocytes and other cell lineages were identified by Wright-Giemsa and Megacolor⁸ (Cytocolor Inc, Hinckley, OH). The numbers of megakaryocytes were determined by cell counts following Megacolor staining. We verified the efficiency of Megacolor staining by direct comparison with acetylcholinesterase staining⁹ on control bone marrow cytopsins.

Transmission electron microscopy. Cells were fixed with 1% paraformaldehyde, 1.25% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.2, for 2 hours at 4°C. After washing, the samples were postfixed in 2% osmium in the same buffer, washed in distilled water, en bloc stained in ethanolic uranyl acetate overnight at 4°C, dehydrated through graded ethanol and propylene oxide, and embedded in eponate 12 (Ted Pella, Inc, Redding, CA). Ultrathin sections were cut on a Reichert (Eindhoven, Holland) Ultracut E microtome, counterstained with ethanolic uranyl acetate and lead citrate, examined at 80 kV, and photographed on a Philips (Vienna, Austria) CM12 transmission electron microscope.

Recombinant TPO. Recombinant human TPO was purified by affinity chromatography from conditioned media obtained after

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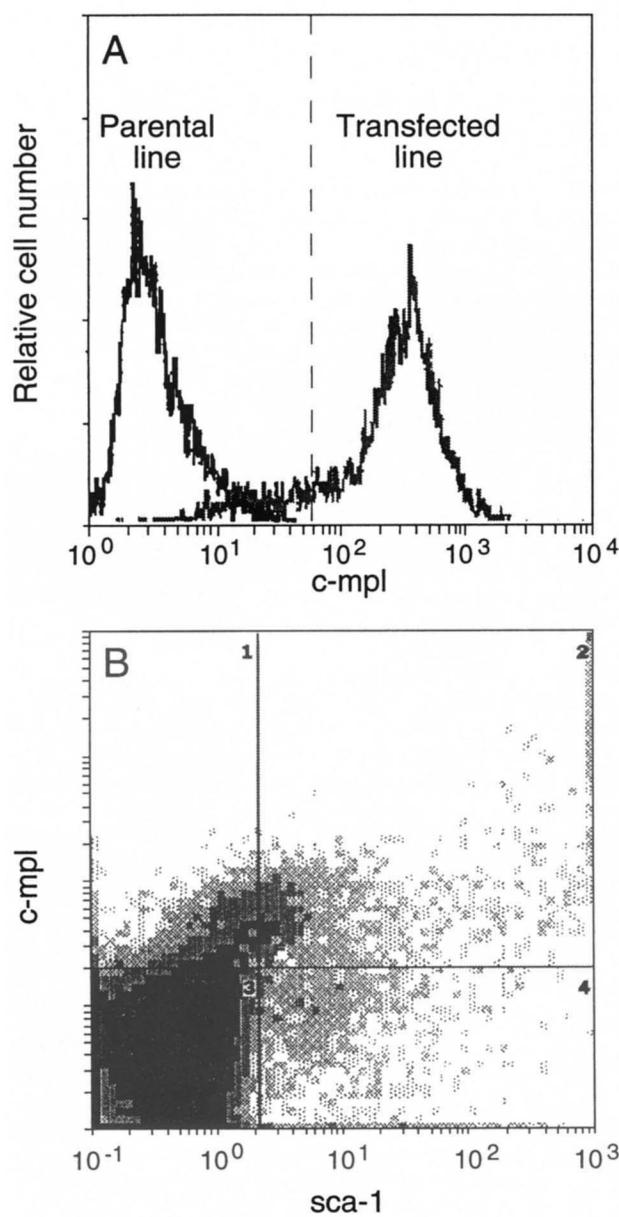


Fig 1. (A) 293 cells were stably transfected with the c-mpl receptor. The parental 293 line and the transfected line were stained using a hamster monoclonal antimurine c-mpl antibody (2F5). (B) AA4⁺ cells were isolated from midgestation fetal liver by immune panning. AA4⁺ cells were subsequently stained using antibodies against Sca-1 and c-mpl (2F5). These experiments were repeated a minimum of three times and gave similar staining profiles on each occasion.

transfection of 293 cells with a recombinant TPO expression vector.¹ Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that the purified material was homogeneous (data not shown). Recombinant human TPO was used in all assays at a concentration of 5,000 U/mL.

RESULTS

Expression of c-mpl in immature hematopoietic cells. Previously, PCR analysis indicated that c-mpl mRNA is expressed in immature hematopoietic cells. The production of

a hamster monoclonal antibody raised against murine c-mpl allowed further characterization of c-mpl expression on primitive hematopoietic cells. The specificity of this monoclonal antibody (2F5) was shown using 293 cells stably transfected for c-mpl expression (Fig 1A).

To determine the expression of c-mpl in primitive hematopoietic cells, AA4⁺ cells were isolated from the murine fetal liver and stained for Sca-1 and c-mpl expression. Previous experiments have shown that all stem cell activity resides in the AA4⁺ Sca⁺ population¹⁰ (Jordan et al, submitted), with this population being approximately 1,000-fold enriched for the hematopoietic stem cell. All AA4⁺ Sca⁺ cells are CD34⁺ and approximately 90% of this population is c-kit⁺. In both stromal cell coculture and methylcellulose assays, AA4⁺ Sca⁺ cells give rise to both myeloid and lymphoid cells.⁶ The AA4⁺ Sca⁻ population is not capable of long-term reconstitution of lethally irradiated animals. FACS analysis illustrated that approximately 50% of the AA4⁺ Sca⁺ stem cell population was c-mpl⁺. In addition, the AA4⁺ Sca⁻ fraction also contained c-mpl-expressing cells, probably reflecting the presence of more mature megakaryocytic cells in this population. Importantly, these results confirm the previous observations from PCR analysis that c-mpl is expressed in primitive hematopoietic cells.

Cocultivation of stem cells and stroma. We have previously reported that the 7-4 fetal liver stromal cell line gives rise to multilineage expansion of stem cells after 7 days of coculture.⁶ We used this assay to determine the proliferative potential of TPO on two previously identified stem cell populations from the midgestation fetal liver, AA4⁺ Sca⁺ and

Table 1. Cell Proliferation Assays on Fetal Liver Stroma

	Fold Expansion	MEGS per Field	Total MEGs Produced per Well
Media alone	78 ± 7	0	0
	44 ± 3	0	0
TPO	189 ± 14	9 ± 3	680
	174 ± 8	15 ± 3	1,044
IC2-310	121 ± 12	0	0
	94 ± 7	1 ± 1	376
IC2-310/TPO	230 ± 14	15 ± 2	1,380
	112 ± 6	22 ± 7	986
KL	255 ± 14	0	0
	232 ± 12	0	0
KL/TPO	315 ± 14	41 ± 3	5,166
	344 ± 10	17 ± 4	2,339
IL-3	181 ± 44	0	0
	172 ± 30	0	0
IL-3/TPO	227 ± 10	37 ± 3	3,360
	240 ± 14	7 ± 3	672

AA4⁺ CD34⁺ kit⁺ stem cells were isolated from the midgestation fetal liver and cocultivated on the fetal liver stromal cell-line 7-4. After 7 days, the expansion of the original 10,000 cells plated was determined and cells were prepared for cytopsin analysis as described. The presence of megakaryocytes in these cultures was confirmed using Megacolor and Wright-Giemsa staining. Megakaryocyte counts were performed on multiple cytopsins of 25,000 cell aliquots from each well. The total megakaryocyte content of each well was calculated based on overall cell proliferation. Similar results were also obtained using AA4⁺ Sca⁺ stem cells (data not shown). Assays were performed in duplicate and the experiment was repeated twice.

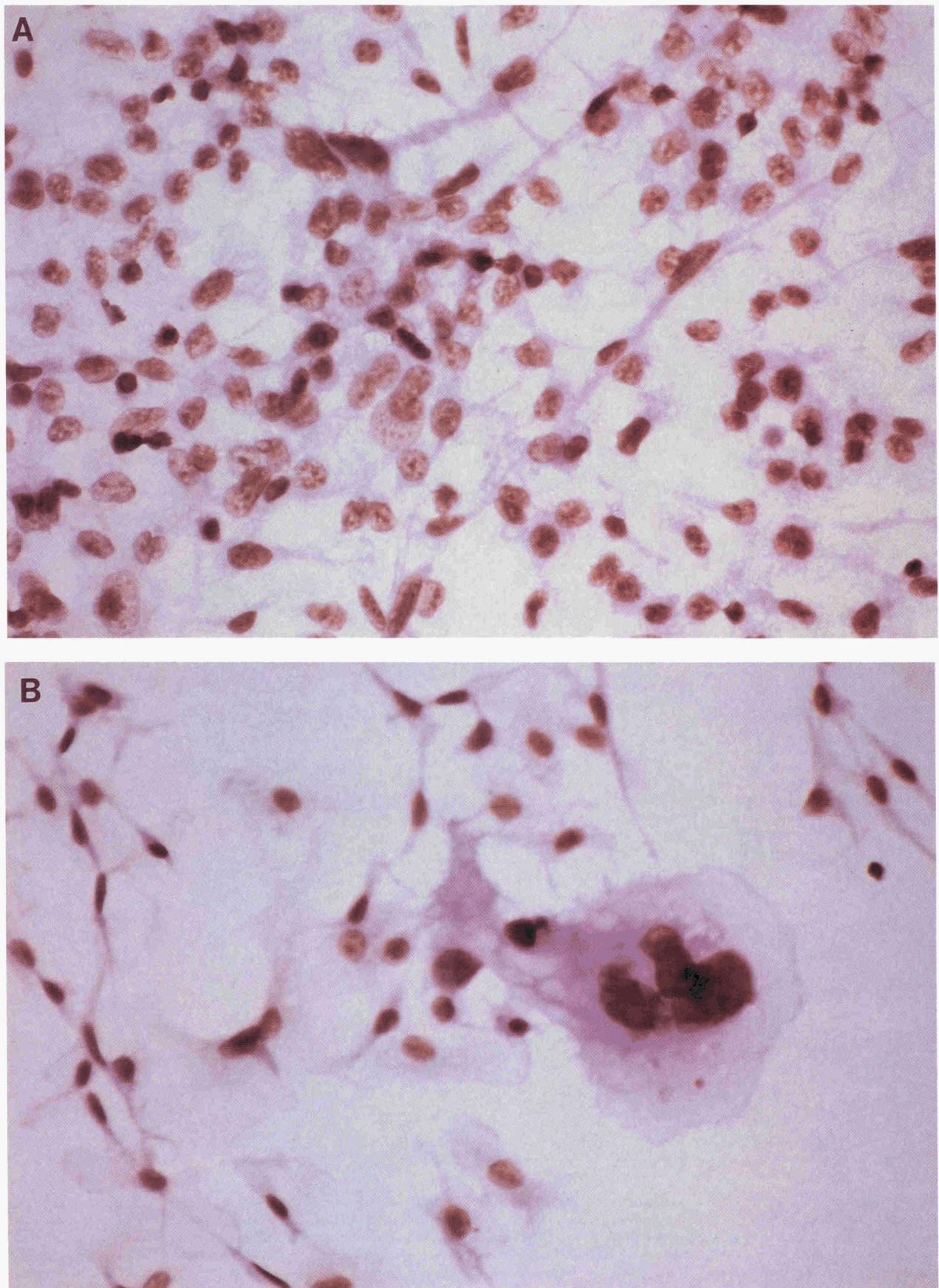


Fig 2. AA4⁺ CD34⁺ kit⁺ stem cells cocultivated on stromal cell line 7-4 for 7 days, fixed *in situ*, and stained with Megacolor (A) on 7-4 stroma alone and (B) 7-4 stroma with the addition of TPO. Original magnification $\times 400$.

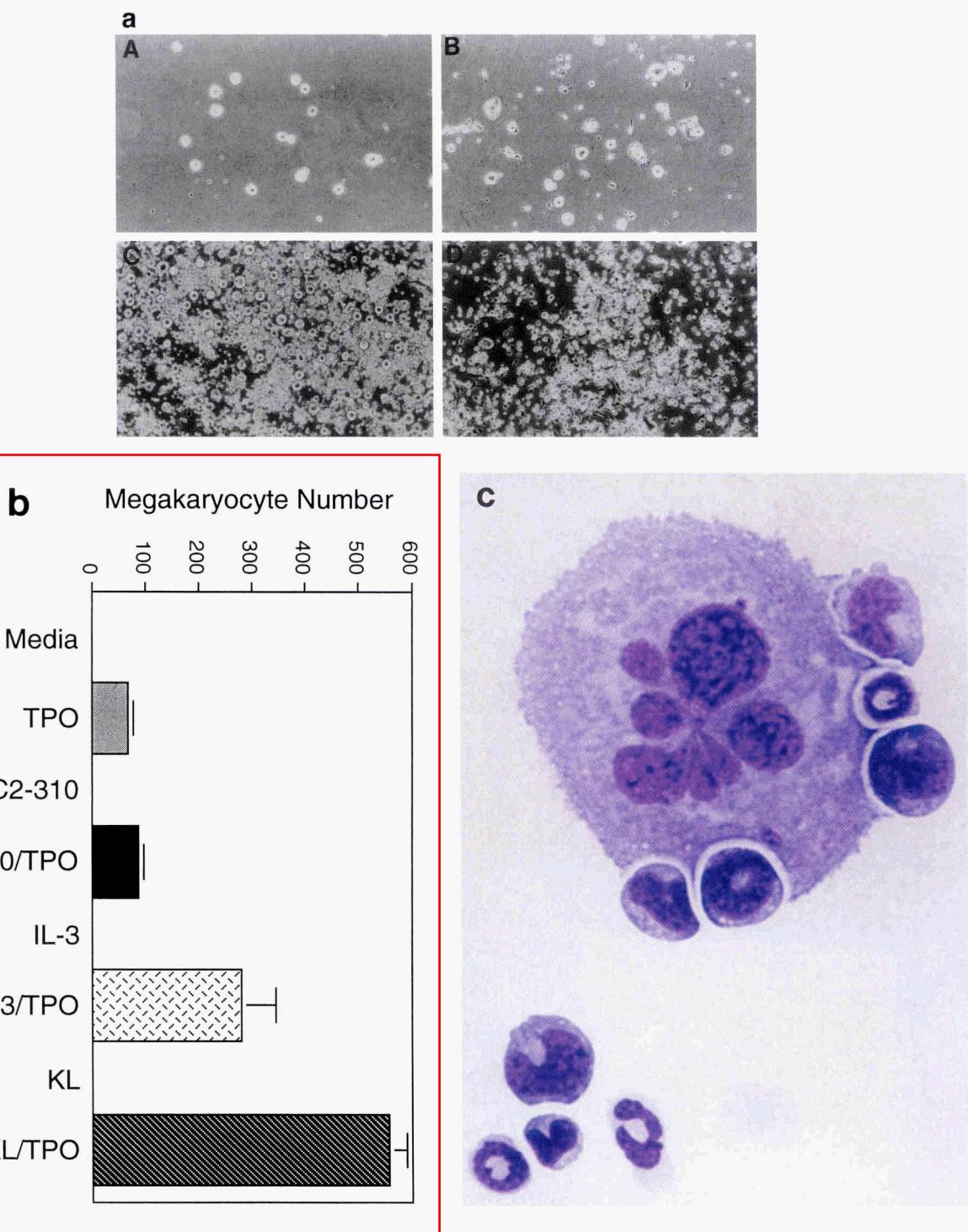


Fig 3. (a) AA4⁺ Sca⁺ stem cells were isolated and cultured in suspension with the addition of the following growth factors: (A) TPO alone; (B) TPO and IC2-310; (C) TPO and KL; and (D) TPO and IL-3. Original magnification $\times 40$. (b) Suspension cultures were performed as described and cells harvested after 6 days. Cytospins of the resultant cells were performed and stained using Wright-Giemsa and Megacolor. Mature megakaryocytes were counted from the Megacolor slides. (c) A representative cytospin from the TPO/KL suspension culture Wright-Giemsa stained to demonstrate the presence of megakaryocytes. Similar results were also obtained using the AA4⁺ CD34⁺ kit⁺ stem cell population.

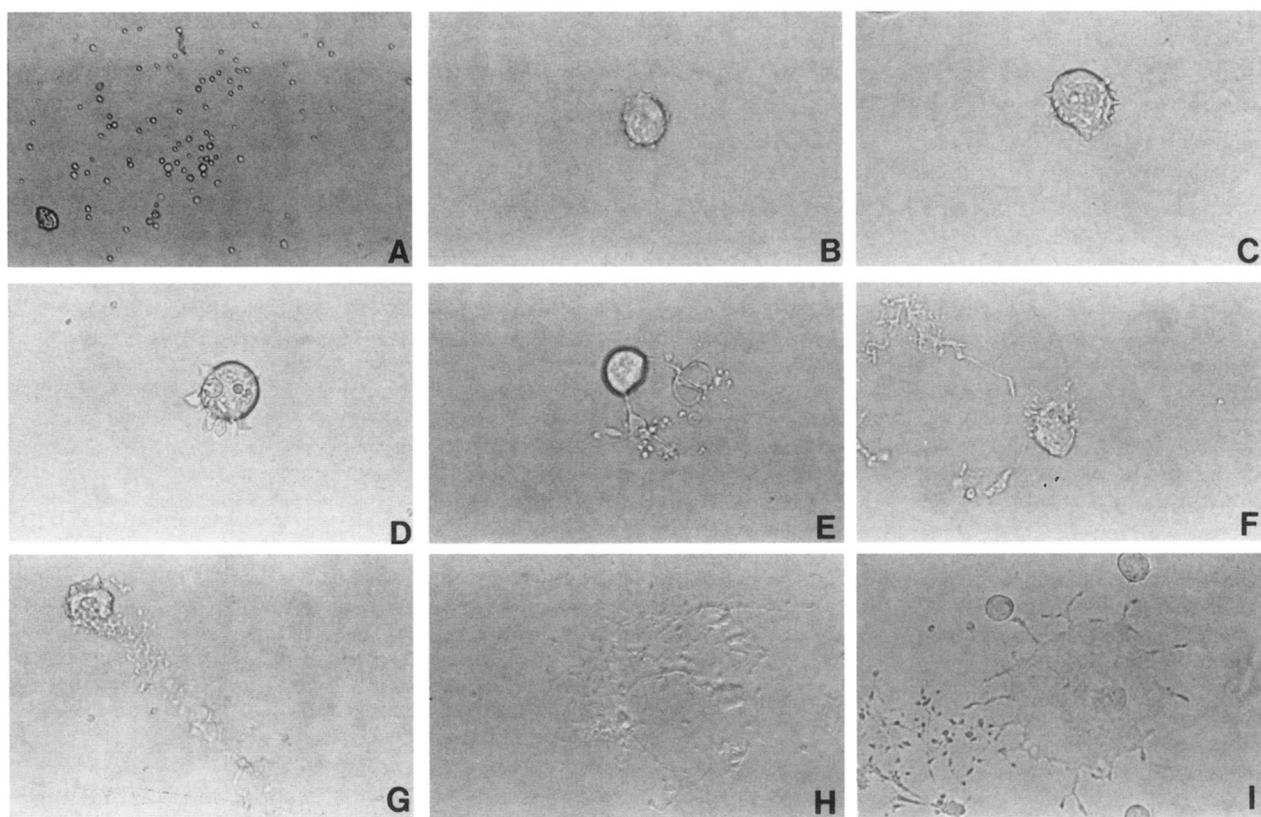


Fig 4. AA4⁺ Sca⁺ cells were isolated and plated in suspension culture or in the presence of matrigel. Under both conditions, the maturation process was essentially equivalent; however, the greater adherence of the megakaryocytes on matrigel made for clearer photography. The matrigel cultures were photographed over a 7-day period. However, the development and maturation of megakaryocytes in this culture system was asynchronous: (A) stem cells after 16 hours in culture; (B) representative megakaryocyte, usually by day 3 of culture; (C) through (G) demonstrate the maturation of megakaryocytes adherent to the matrigel. These cells undergo blebbing of the plasma membrane, followed by the production of pseudopodia. The time course of development was between 3 and 7 days. (H) The resultant pseudopodia detached from the disintegrating megakaryocyte and were shed into the culture media leaving tangles of proplatelets; (I) megakaryocyte remaining adhered to the matrigel after proplatelet release. Original magnification (A) $\times 40$; (B through H) $\times 100$.

AA4⁺ CD34⁺ kit⁺. In these assays, TPO demonstrated an increased proliferation of hematopoietic cells as compared with stroma alone (Table 1). This proliferation could be further enhanced using IC2-310 (an agonist antibody to the flk-2/flt-3 receptor), KL, or IL-3. Cytospin analyses of these cultures illustrated a general increase in myelopoiesis in the presence of TPO; however, megakaryocyte production was only observed when cells were treated with TPO (Table 1 and Fig 2). This production was increased when TPO was used in conjunction with KL or IL-3.

Suspension cultures of hematopoietic stem cells. Stromal cell cocultures clearly demonstrated the proliferative capacity of TPO on hematopoietic stem cell populations. To investigate the effects of TPO on the stem cell in the absence of supporting cells, we used stem cell suspension cultures. AA4⁺ Sca⁺ or AA4⁺ CD34⁺ kit⁺ stem cell populations suspended in serum containing media did not give rise to viable cultures. Strikingly, the addition of TPO to this media gave rise to viable cells of the megakaryocytic lineage. The numbers of megakaryocytes produced in these assays could be enhanced by the use of IC2-310, KL, or IL-3. However, the use of these combinations also led to the production of cells from other myeloid lineages, particularly with KL or IL-3.

(Fig 3). Importantly, if TPO was not added to the cultures, IC2-310, KL, and IL-3 did not give rise to megakaryocytes. The use of AA4⁺ Sca⁻ cells (a population incapable of long-term engraftment of the irradiated host) resulted in the production of megakaryocytes, but in far lower numbers when compared with the stem cell population. These experiments were also repeated using the murine bone marrow stem cell population Lin^{lo} Sca⁺. These cells also gave rise to megakaryocytes when cultured with TPO (82 \pm 8 megakaryocytes per 10,000 cells plated).

The production and maturation of megakaryocytes in these culture conditions was asynchronous and reflects the presence of the most primitive totipotent hematopoietic cells and multipotent progenitors in the stem cell populations. Megakaryocyte production was rapid in comparison to agar assays and was essentially complete after approximately 3 days in culture, but, as the culture progressed, the megakaryocytes underwent clear maturation processes (Fig 4). Adherence of the megakaryocytes to the tissue culture plastic appeared to be important in this maturation event. In almost all cases, adherence was accompanied by the development of blebbing of the megakaryocyte cell membranes and the production of cytoplasmic processes.

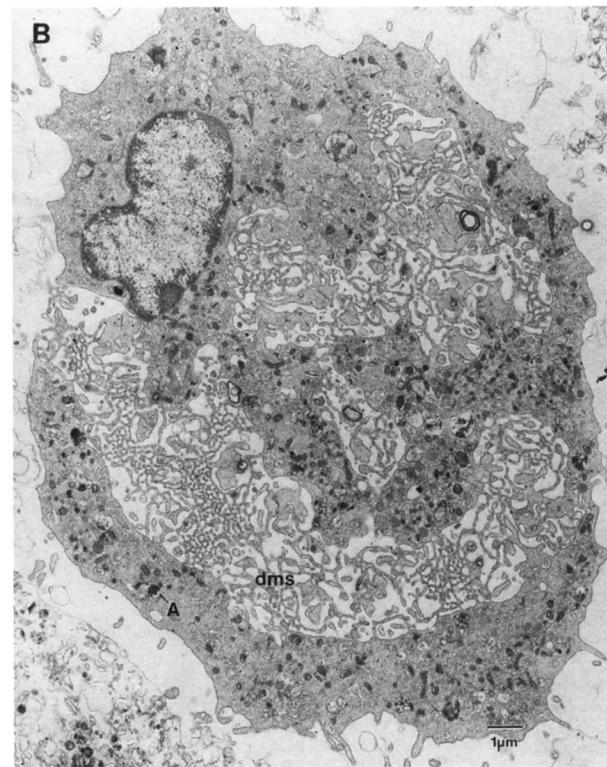
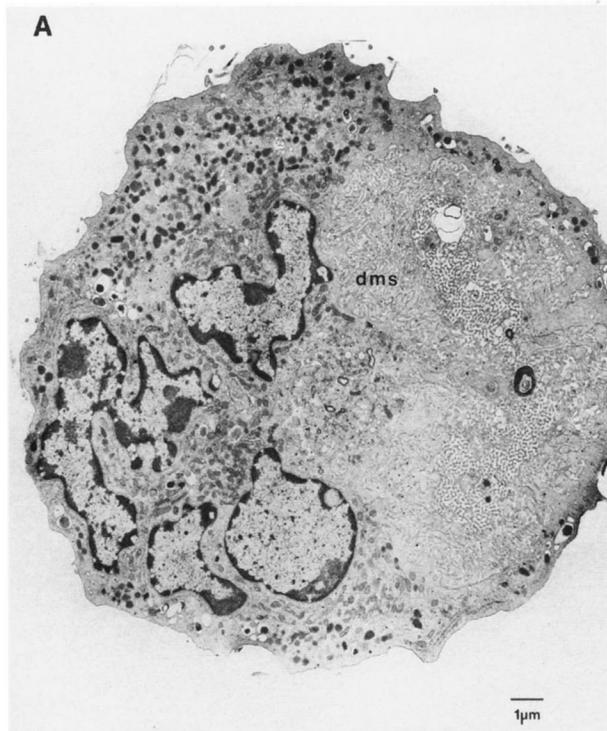
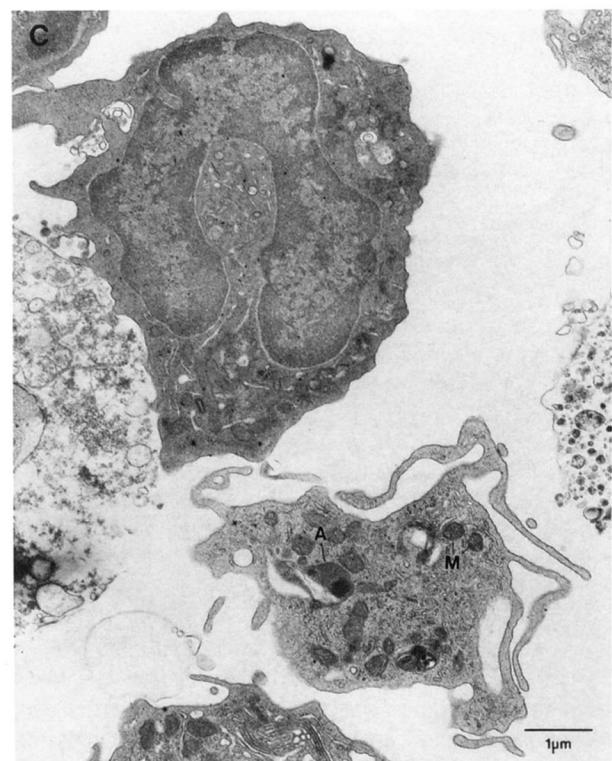


Fig 5. AA4⁺ Sca⁺ stem cell suspension cultures treated with TPO were fixed after 5 days in culture and electron microscopy performed as described in Materials and Methods. (A) A representative micrograph demonstrating the presence of megakaryocytes in these cultures. The cell contains many alpha granules (A) and the demarcation system (dms) is indicated. (B) A megakaryocyte in which the dms has begun to delineate areas of the granule dense areas of the cytoplasm. (C) A megakaryocyte with very little cytoplasm accompanied by a cytoplasmic fragment with large pseudopodia and containing α granules (A) and mitochondria (M).



These processes exuded from the cell giving rise to long pseudopodic formations. These pseudopodia were then released from the megakaryocyte cell body to give rise to many proplatelet structures free floating in the culture medium (Fig 4). It should be noted that the suspension cul-

tures used here did contain serum and we have not been successful in setting up this assay in serum-free conditions. Therefore, we cannot discount the potential importance of any serum component in this proliferation and maturation sequence.

Further confirmation of megakaryocyte production was provided by electron microscopy of fixed cells from the TPO suspension cultures. Analysis of these cells illustrated the presence of megakaryocytic cells of variable morphology (Fig 5). Based on differences in the extent of the megakaryocyte demarcation systems, these morphologic differences appear to represent different stages of megakaryocyte maturation.

DISCUSSION

Megakaryocytopoiesis and thrombopoiesis have previously been considered to be regulated at multiple cellular levels, with different molecules potentially being responsible for proliferation and maturation.¹¹⁻¹⁵ However, the recent cloning of the c-mpl ligand and the investigation of its biologic role indicates that it is indeed the TPO molecule.¹⁻⁴ We have extended the existing work to clearly show that TPO can act on stem cell populations to result in both the proliferation and maturation of megakaryocytes. However, stem cell populations are heterogeneous as to stem cell content and include both stem cells and multipotent precursor cells. Therefore, we assessed the activity of TPO on two different stem cell populations that have been shown to produce long-term reconstitution of lethally irradiated mice. The resultant megakaryocytopoiesis was very similar in each case and suggests that TPO does act directly on the hematopoietic stem cell. This finding is also supported by FACS analysis that demonstrated that 50% of the AA4⁺ Sca⁺ cells were also c-mpl⁺. However, to rigorously demonstrate that c-mpl is expressed on the hematopoietic stem cell will require reconstitution experiments in lethally irradiated hosts. Consequently, we are currently determining the repopulating capacity of the AA4⁺ Sca⁺ mpl⁺ and the AA4 Sca⁺ mpl⁻ subpopulations.

The specificity of action of TPO has not yet been fully investigated. However, none of the known hematopoietic factors has totally lineage-specific effects.¹⁶ The experiments on the 7-4 stromal cell line indicate that TPO has the capacity to synergize with other factors to enhance myelopoiesis in addition to its megakaryocytopoietic activities. However, in the context of stem cell suspension cultures, the predominant effects of TPO are undoubtedly on megakaryocytopoiesis and thrombopoiesis. This finding is in agreement with in vivo studies of TPO activity in which no effects have been evident on other lineages (Eaton et al, manuscript in preparation).

The maturation of megakaryocytes in suspension cultures in the presence of TPO is similar to the events previously reported from other in vitro models of megakaryocytopoiesis.¹⁷⁻²² In these previous studies, a small percentage of megakaryocytes were reported to undergo maturation to produce cytoplasmic processes. Light and electron microscopy of these processes indicated a "proplatelet" structure.^{18,22,23} The addition of TPO to suspension cultures led to the maturation of the majority of megakaryocytes produced therein, with the resultant structures closely resembling the previously described "proplatelets." Currently, the functional potential of these "proplatelets" remains unresolved. From the current studies, it is conceivable that the megakaryocytopoietic and thrombopoietic activity of TPO

may allow for large scale production of these "proplatelets" and thereby allow functional assays to be performed. Clearly, the potential for platelet production *in vitro* may be of considerable therapeutic value in the future.

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