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Thrombopoietin, the Ligand for the Mpl Receptor, Synergizes With Steel Factor and Other Early Acting Cytokines in Supporting Proliferation of Primitive Hematopoietic Progenitors of Mice

By Hsun Ku, Yuji Yonemura, Kenneth Kaushansky, and Makio Ogawa

Recently, the ligand for the Mpl receptor (ML) was identified to be thrombopoietin, the principal regulator of megakaryocytopoiesis and thrombopoiesis. We examined the effects of ML, as a single factor or in combinations with early acting factors such as steel factor (SF), interleukin (IL)-3, IL-11, IL-6, and granulocyte colony-stimulating factor (G-CSF), on colony formation from primitive progenitors of mice. Cells enriched for cell cycle dormant primitive progenitors were isolated from bone marrow cells of 5-fluorouracil (5-FU)–treated mice by a combination of Nycodenz density gradient separation, immunomagnetic selection for lineage-negative cells, and fluorescence-activated cell sorter (FACS) sorting for Ly-6A/E$^+$Kit$^+$ cells. ML, in the presence of erythropoietin, could support the formation of only a few megakaryocyte colonies. However, ML acted synergistically with SF or IL-3 to support the formation of multiple types of hematopoietic colonies including multilineage colonies. Effects of the combination of ML and SF on multipotential progenitors were not mediated through other cells, as demonstrated by micromanipulation of individual progenitors. In suspension culture, the combination of ML and SF increased the number of multipotential progenitors. ML also acted synergistically with IL-11, IL-6, or G-CSF to support colony formation in serum-containing, but not in serum-free, cultures. However, the multilineage colony formation seen in serum-containing culture was completely abrogated by addition of ACK2, a neutralizing antibody to Kit protein. Serial observation (mapping studies) of colony development from multipotential progenitors suggested that ML triggers the cell division of dormant progenitors. Based on these observations, we propose that ML can function as an early acting cytokine and stimulate the proliferation of cell cycle dormant progenitors by shortening their G_0 period.

FOR HEMATOPOIETIC STEM cells to proliferate and mature in multiple lineages, coordinated support by several cytokines is required. Using clonal cell culture assays, we have characterized cytokine interactions regulating the proliferation of primitive progenitors (see review[1]). Based on their functional similarities, these cytokines may be classified into three groups. Interleukin-3 (IL-3),[2] IL-4[3] and granulocyte-macrophage colony-stimulating factor (GM-CSF)[4,5] appear to form one group since, as single agents, these cytokines can support the proliferation of multipotential progenitors after they exit from the cell-cycle dormancy state (G_0 period). IL-6,[6] IL-11,[7] granulocyte-CSF (G-CSF),[8] leukemia inhibitory factor (LIF)[9,10] and IL-12[11,12] form the second group. These cytokines, which are related to each other through similarities in their structure or in the structure of their receptors, appear to be involved in triggering of the cell cycle-dormant multipotential progenitors by synergizing with IL-3 or steel factor (SF; the ligand for Kit). SF[11,13,14] and the ligand for Flt3/Flk2 (FL)[15] form the third group. These ligands can interact synergistically with the cytokines in the first and second groups, with minor exceptions.[13-15] Combinations of two factors within a group, however, do not support colony formation from cells in G_0.

Recently, the ligand for the Mpl receptor (ML) was cloned and identified to be thrombopoietin (TPO), the primary regulator of megakaryocytopoiesis and platelet production.[16-21] Injection of recombinant ML into normal mice caused an increase in megakaryocyte colony-forming units (CFU) in bone marrow (BM) and spleen,[18] as well as an increase in the number of circulating platelets.[17] ML has also been shown to increase the size and DNA content of megakaryocytes.[22] Although it has been proposed that ML acts directly on committed megakaryocyte progenitors, it is also possible that the increase in megakaryocyte progenitors induced by ML reflects activation of multipotential progenitors. To test the hypothesis that ML acts on primitive multipotential progenitors, we performed clonal cell culture studies using a population that is highly enriched for dormant multipotential progenitors. We tested the effects of ML, alone and in combinations with multiple hematopoietic cytokines, on colony formation from highly enriched marrow cells isolated from 5-fluorouracil (5-FU)–treated mice. We report here that ML synergizes with SF or IL-3 in support of multilineage colony formation. ML also acted synergistically with IL-11, IL-6, or G-CSF to support colony formation in serum-containing, but not in serum-free, cultures. ML, like IL-6, IL-11, G-CSF, and IL-12, appears to belong to the group of cytokines that trigger the dormant hematopoietic progenitors into cell cycle.

MATERIALS AND METHODS

Cytokines. Serum-free conditioned medium (CM) from baby hamster kidney (BHK) cells transfected with the gene encoding murine c-mpl ligand, and control serum-free BHK-CM with no TPO activity, were kindly provided by D. Foster of Zymogenetics (Seattle, WA). Purified human recombinant ML was supplied by the TPO Production Group, Kirin Brewery, Co, Ltd (Maebashi, Japan). Unless specified otherwise, the experiments were performed with CM containing ML. Purified recombinant murine SF was obtained from Immunex (Seattle, WA). Purified recombinant murine IL-3 was purchased from R&D Systems (Minneapolis, MN). Purified recombinant human IL-11 was a gift from P. Schendel of Genetics Institute

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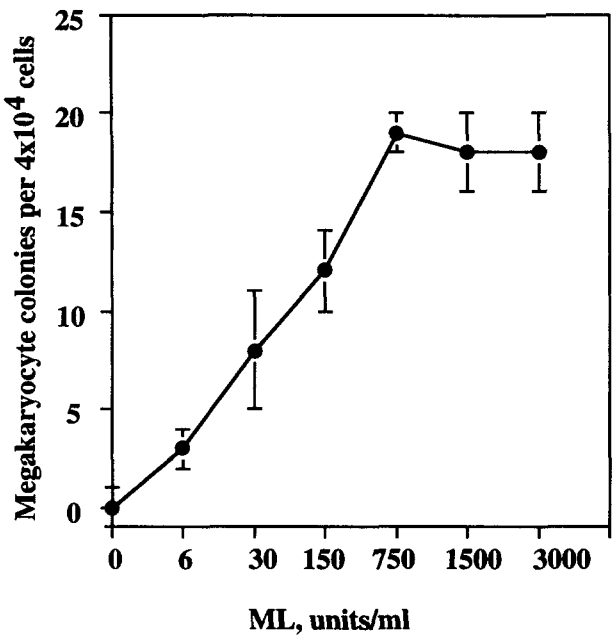

Fig 1. Effects of ML on megakaryocyte colony formation from normal BM cells. A total of 4 × 10^4 cells were plated per dish in serum-containing culture in the presence of 2 U/mL Ep and varying concentrations of ML. Megakaryocyte colonies were examined on day 5 of culture. Data represent the mean and standard deviation of the values obtained from four dishes.

(Cambridge, MA). Purified recombinant human IL-6 was a gift from M. Naruto of Toray Industries (Yokohama, Japan). Purified recombinant human G-CSF was a gift from A. Shimosaka of Kirin Brewery Co, Ltd (Maebashi, Japan). Purified recombinant human erythropoietin (Ep) was provided by the Genetics Institute Clinical Manufacturing Group (Cambridge, MA). Unless otherwise specified, the concentrations of cytokines used were as follows: murine ML (CM), 750 U/mL; human ML (purified), 200 ng/mL; SF, 100 ng/mL; IL-3, 10 ng/mL; IL-6, 100 ng/mL; IL-11, 100 ng/mL; G-CSF, 100 ng/mL; Ep, 2 U/mL.

Monoclonal antibodies (MoAbs). Hybridoma D7 (anti-Ly-6A.2/E.1, rat immunoglobin G [IgG]2a) was a gift from P. Kincade of Oklahoma Medical Research Foundation (Oklahoma City, OK). MoAbs ACK4 and ACK2 (anti-Kit; rat IgG2a and IgG2b, respectively) were provided by S-I. Nishikawa of Kyoto University (Kyoto, Japan). Hybridoma RB6-8C5 (antimouse granulocytes; rat IgG2b) was provided by R.L. Coffman of DNAX (Palo Alto, CA). MoAb TER119 (antimouse erythrocytes; rat IgG2b) was a gift from T. Kina of Kyoto University. Hybridomas 14.8 (anti-B220; rat IgG2b), M1/70.15.11.5 (antimouse macrophages; rat IgG2b), GK1.5 (anti-CD4; rat IgG2b) and 53-6.72 (anti-CD8; rat IgG2a) were purchased from American Type Culture Collection (Rockville, MD).

Cell preparation. Crude marrow cells procured from normal mice were used only in the experiment shown in Fig 1. In all other experiments, BM cells from the femurs and tibias of (C57Bl/6 × DBA/2J) F1 (BDF_1) mice were procured 2 days after intravenous (IV) injection of 150 mg/kg 5-FU (Adria Laboratories, Columbus, OH). Cells were washed twice and then subjected to discontinuous density gradient separation and lineage negative immunomagnetic selection using minor modifications of the protocol we described previously.[23] Cells with densities ranging from 1.063 g/mL to 1.077 g/mL were collected. However, we used Nycodenz (Accurate Chemical and Scientific Corp, Westbury, NY) instead of Metrizamide. Cells reacting to a cocktail of lineage-specific rat MoAbs (RB6-8C5, 14.8, M1/70.15.11.5, GK1.5, TER119 and 53-6.72) were removed twice using immunomagnetic beads (Dynabeads M-450 coupled to sheep antirat IgG, DYNAL, Great Neck, NY). The resulting lineage negative cells (Lin^-) were treated with rat normal IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 20 $\mu g/10^6$ cells to prevent nonspecific binding of MoAbs to Fc receptors. Lin^- cells were then stained with fluorescein isothiocyanate (FITC)-conjugated rat MoAb D7 (anti-Ly-6A/E[24]) and biotin-conjugated rat MoAb ACK4 (anti-Kit[25]). Cells were washed twice before staining with streptavidin-conjugated R-phycoerythrin (PE) (Jackson ImmunoResearch Laboratories). Both FITC-conjugated rat IgG2a and biotin-labeled rat IgG2a (Caltag Laboratories, San Francisco, CA) were used as isotype controls. Ly-6A/E^+Kit^+ cells were collected by sorting on a single laser FACStar^{plus} using Lysis II software (Becton Dickinson Immunocytometry Systems, San Jose, CA). The final population, designated as Lin^-Ly-6A/E^+Kit^+, has a plating efficiency of approximately 50% when cultured with IL-3, SF, IL-6, and Ep in serum-containing culture (unpublished results). About 30% to 40% of the total colonies are multilineage colonies, showing the presence of granulocyte, erythrocyte, macrophage and megakaryocyte lineages.

Clonal cell culture. Clonal cell culture was performed in 35-mm Petri dishes (Becton Dickinson Labware, Lincoln Park, NJ). In serum-containing culture, 1 mL of culture mixture contained α-medium (ICN Biomedicals, Aurora, OH), 1.2% 1,500 cp methylcellulose (Shinetsu Chemical, Tokyo, Japan), 1% deionized fraction V bovine serum albumin (BSA; Sigma Chemical, St Louis, MO), 0.1 mmol/L 2-mercaptoethanol (2-ME; Sigma), 30% fetal calf serum (FCS; Intergen, Purchase, NY), 2 U/mL Ep, and designated cytokines. This is our standard culture mixture and is referred to as methylcellulose media. Dishes were incubated at 37°C in a humidified atmosphere with 5% CO_2 in air. In serum-free culture, fraction V BSA and FCS were replaced by a combination of 1% deionized crystallized BSA (Sigma), 600 μg/mL fully iron-saturated human transferrin (>98% pure) (Sigma), 10 μg/mL soybean lecithin (Sigma), 6 μg/mL cholesterol (Sigma) and 1×10^{-7} mol/L sodium selenite (Sigma). Dishes were incubated in a humidified 5% CO_2/5% O_2/90% N_2 atmosphere. Colonies consisting of 50 or more cells were scored on an inverted microscope after 14 days of culture, except that the megakaryocyte colonies shown in Fig 1 were scored on day 5. Megakaryocyte colonies were scored when the colony contained four or more megakaryocytes. Abbreviations for colony types were as follows: GM, granulocyte/macrophage colonies; GEMM, granulocyte/erythrocyte/macrophage/megakaryocyte colonies; GMM, granulocyte/macrophage/megakaryocyte colonies[26]; GEM, granulocyte/erythrocyte/macrophage colonies[26]; Meg, megakaryocyte colonies; and Blast, blast cell colonies.[27]

Single cell micromanipulation. A total of 1,000 Lin^-Ly-6A/E^+Kit^+ cells from the marrow of 5-FU–treated mice were plated in a dish containing α-medium, 1.2% methylcellulose and 30% FCS. The dish was gently swirled so that cells were separated from each other. Cells were then lifted individually from the dish with a fine capillary tube attached to a micromanipulator (Hacker Instruments, Inc, Fairfield, NJ) under direct microscopic visualization[28] and were transferred to secondary dishes containing methylcellulose culture media, SF, ML, and Ep. Colonies were examined after 14 days of culture.

Serial observations of colony formation from progenitors. A total of 150 Lin^-Ly-6A/E^+Kit^+ cells from the marrow of 5-FU–treated mice were plated per dish in the serum-containing culture under four cytokine conditions. In one experimental group, culture was initiated with SF, purified recombinant human ML, and Ep. In another experimental group, culture was initiated in the presence of SF and Ep, and on day 7, ML was carefully layered over the dishes. As controls,

Table 1. Effects of ML, Alone and in Combinations With SF or IL-11, on Colony Formation From Primitive Progenitors

Cytokines	No. of Colonies						
	GM	GEMM	GMM	GEM	Meg	Blast	Total
Serum-containing culture							
None	0	0	0	0	0	0	0
ML	0	0	0	0	5 ± 1		5 ± 1
BHK-CM	0	0	0	0	0	0	0
SF	1 ± 1	0	0	0	1 ± 1	0	1 ± 1
IL-11	0	0 ± 1	0	0	1 ± 1	0	1 ± 1
ML, SF	5 ± 2	7 ± 1	1 ± 1	0 ± 1	18 ± 4	0 ± 1	31 ± 7
BHK-CM, SF	1 ± 1	0	0	0	1 ± 1	0	1 ± 1
ML, IL-11	20 ± 4	7 ± 2	2 ± 2	0 ± 1	6 ± 2	2 ± 2	37 ± 3
BHK-CM, IL-11	0	0 ± 1	0	0	1 ± 1	1 ± 1	1 ± 1
SF, IL-11	42 ± 12	13 ± 3	1 ± 1	0	2 ± 1	0	58 ± 10
Serum-free culture							
None	0	0	0	0	0	0	0
ML	0	0	0	0	3 ± 2	0	3 ± 2
BHK-CM	0	0	0	0	0	0	0
SF	0	0	0	0	0	0	0
IL-11	0	0	0	0	0	0	0
ML, SF	11 ± 2	8 ± 2	2 ± 2	0	11 ± 1	0	32 ± 4
BHK-CM, SF	1 ± 1	0	0	0	0	0	1 ± 1
ML, IL-11	0	0	0	0	6 ± 3	0	6 ± 3
BHK-CM, IL-11	0	0	0	0	0	0	0
SF, IL-11	33 ± 2	12 ± 3	1 ± 1	1 ± 1	2 ± 2	0	48 ± 2

A total of 150 Lin^- Ly-6A/E^+ Kit^+ cells prepared from the marrow of 5-FU–treated mice were plated per dish in the presence of 2 U/mL Ep and designated cytokines. Dishes were examined on day 14 of culture. Data represent the mean and standard deviation of values obtained from quadruplicate dishes.

the same population of cells were cultured in the presence of ML and Ep or SF and Ep. The location, proliferation, and differentiation of emerging blast cell colonies were recorded daily as described previously.[2] Kinetic properties were calculated only on the colonies that later showed GEMM lineages. Doubling times of the individual colonies were calculated by using simple linear regression analysis.

Suspension culture for in vitro expansion of progenitors. A total of 1,500 Lin^-Ly-6A/E^+Kit^+ cells from the marrow of 5-FU–treated mice were plated in 1 mL suspension culture media consisting of α-medium, 1% deionized fraction V BSA, 0.1 mmol/L 2-ME, 30% FCS, Ep, and various cytokines. On day 6, cells were washed and replated in freshly prepared medium, while aliquots were analyzed for cell counts and progenitor content. On day 11, only cell counts and progenitor numbers were determined. CFUs were assessed in clonal cultures containing IL-3, SF, IL-6, and Ep.

Statistical analysis. Student's *t*-test was used to determine statistical significance.

RESULTS

Effects of ML on megakaryocyte colony formation from normal marrow. First, we tested the effects of ML on formation of Meg colonies from normal marrow. As shown in Fig 1, ML supported formation of Meg colonies in a dose-dependent manner. The number of Meg colonies reached a plateau at 750 U/mL ML. Based on this observation, we used 750 U/mL in the subsequent studies. The number of Meg colonies supported by ML was comparable to that reported by Kaushansky et al[18] and Broudy et al.[22]

Synergy between ML and SF and/or IL-11. Colony formation from Lin^-Ly-6A/E^+Kit^+ marrow cells of 5-FU–treated mice is presented in Table 1. ML, SF, and IL-11, in the presence of Ep, individually supported formation of a small number of Meg colonies in serum-containing culture. In serum-free culture, only ML could support formation of Meg colonies. When SF was added to ML, formation of GEMM, GM, and Meg colonies was seen both in serum-containing and serum-free cultures. The combination of ML and IL-11 supported GEMM and GM colony formation in serum-containing culture, but not in serum-free culture. Combination of BHK-CM with SF or IL-11 did not increase the colony number over that supported by SF or IL-11, indicating that the colony-stimulating effects of the ML-CM are dependent on ML.

To eliminate the possibility that the effects of ML and SF on progenitors are indirect and mediated by other cells, single cells were plated by micromanipulation in cultures containing ML, SF, and Ep, and colony formation examined. Ninety-three micromanipulated cells yielded a total of 34 colonies including 5 GEMM, 17 Meg, 6 GM, 3 GMM, and 3 Blast colonies. There were no erythroid burst-forming units (BFU-E) because of the 5-FU treatment of the mice.[29] May-Grunwald-Giemsa staining of the GEMM colonies showed the presence of neutrophils, mast cells, macrophages, erythrocytes, and megakaryocytes. This result showed the effect of ML and SF on multipotential progenitors is direct and does not involve other cells.

The effects of combinations of ML, SF, and IL-11 were tested again in serum-free culture; the results are presented in Table 2. Although the total colony number supported by the three cytokines was significantly ($P < .01$) higher than that supported by the combination of SF and IL-11, the

Table 2. Combined Effects of ML, SF, and IL-11 on Colony Formation in Serum-Free Culture

Cytokines	No. of Colonies					
	GM	GEMM	GMM	GEM	Meg	Total
SF	0	0	0	0	0	0
ML	0	0	0	0	2 ± 1	2 ± 1
IL-11	0	0	0	0	0	0
SF, ML	10 ± 3	7 ± 4	6 ± 3	0	11 ± 2	34 ± 6
SF, IL-11	24 ± 4	17 ± 4	1 ± 1	0	1 ± 1	42 ± 4
SF, ML, IL-11	33 ± 6*	22 ± 2	0 ± 1	0 ± 1	4 ± 2	59 ± 7*

A total of 150 Lin^- Ly-6A/E^+ Kit^+ cells prepared from the marrow of 5-FU–treated mice were plated per dish in the presence of 2 U/mL Ep and designated cytokines. Dishes were examined on day 14 of culture. Data represent the mean and standard deviation of values obtained from quadruplicate dishes.

* Colony numbers are different from that in cultures containing SF and IL-11 at $P < .01$.

number of GEMM colonies between these two groups did not differ significantly.

Synergy between ML and IL-3. We also tested for possible synergy between ML and IL-3; the results are presented in Table 3. Because multilineage colonies are very large in the presence of IL-3, only 50 enriched cells were plated per dish to insure clonality of the resulting colonies. In serum-containing culture, the combination of ML and IL-3 marginally ($P = .059$) enhanced total colony numbers over those supported by IL-3. However, in serum-free culture, both total colony numbers ($P = .05$), and the numbers of GEMM colonies ($P = .012$) were significantly enhanced by the combination of IL-3 and ML compared with IL-3.

Serum requirement for synergies between ML and early acting cytokines. As shown in Table 1, colony formation supported by the combination of ML and IL-11 differed between serum-containing and serum-free cultures. This observation suggested that unknown factors in the serum are necessary for synergy between ML and IL-11. To further investigate this finding and to determine whether it applies to other early-acting cytokines,[1] we tested colony formation in serum-free and serum-containing cultures in the presence of ML and either IL-11, IL-6, or G-CSF. As shown in Table 4, in serum-free culture, ML in combination with IL-11, IL-6 or G-CSF supported formation of only a few Meg colonies. In serum-containing cultures, the same combinations of cytokines supported formation of GEMM, GM, and Meg colonies. In contrast to these combinations, the combination of ML and SF supported formation of comparable numbers of GEMM, GM, and Meg colonies both in serum-containing and serum-free cultures.

SF is constitutively expressed by stromal cells.[30] There are detectable levels of SF in the serum of normal adults.[31] We postulated that SF in FCS interacted with ML and IL-11 and thereby accounted for the apparent synergy between

Table 3. Effects of ML and IL-3 on Colony Formation from Primitive Progenitors

Cytokines	No. of Colonies						
	GM	GEMM	GMM	GEM	Meg	Blast	Total
Serum-containing culture							
None	0	0	0	0	0	0	0
ML	0	0	0	0	0 ± 1	0	0 ± 1
BHK-CM	0	0	0	0	0	0	0
IL-3	5 ± 3	4 ± 2	2 ± 1	0 ± 1	0 ± 1	2 ± 0	13 ± 5
SF	0	0 ± 1	0	0	0	0	0 ± 1
ML, IL-3	8 ± 1	6 ± 2	2 ± 1	0	3 ± 2	0 ± 1	19 ± 3
BHK-CM, IL-3	10 ± 5	4 ± 1	1 ± 1	0	1 ± 1	0	15 ± 6
ML, SF	4 ± 3	3 ± 2	1 ± 1	0	3 ± 0	0 ± 1	11 ± 4
ML, SF (150 cells/dish)	9 ± 3	7 ± 3	2 ± 1	1 ± 1	13 ± 1	0 ± 1	32 ± 6
BHK-CM, SF	1 ± 1	0	0	0	0	0	1 ± 1
SF, IL-3	12 ± 3	8 ± 3*	0	0	2 ± 1	0	21 ± 1*
Serum-free culture							
None	0	0	0	0	0	0	0
ML	0	0	0	0	1 ± 1	0	1 ± 1
BHK-CM	0	0	0	0	0	0	0
IL-3	5 ± 3	3 ± 1	2 ± 2	0	2 ± 1	0 ± 1	11 ± 5
SF	0	0	0	0	0	0	0
ML, IL-3	6 ± 3	8 ± 2*	2 ± 2	1 ± 1	4 ± 1	0	21 ± 3*
BHK-CM, IL-3	5 ± 4	3 ± 1	1 ± 1	0	2 ± 1	0	10 ± 4
ML, SF	4 ± 1	4 ± 1	1 ± 1	1 ± 1	3 ± 2	0 ± 1	12 ± 3
ML, SF (150 cells/dish)	13 ± 3	11 ± 4	3 ± 1	1 ± 1	11 ± 3	0 ± 1	38 ± 8
BHK-CM, SF	0 ± 1	0	0	0	0	0	0 ± 1
SF, IL-3	18 ± 4	8 ± 4*	0 ± 1	0	1 ± 0	0	27 ± 4*

Lin^- Ly-6A/E^+ Kit^+ cells were prepared from the marrow of 5-FU–treated mice. Unless specified otherwise, 50 cells were plated per dish in the presence of 2 U/mL Ep and designated cytokines. Dishes were examined on day 14 of culture. Data represent the mean and standard deviation of values obtained from quadruplicate dishes.

* Results are different from IL-3 control at $P < .05$.

Table 4. Effects of FCS on Colony Formation in Cultures Containing ML and either IL-11, IL-6, or G-CSF

Cytokines	FCS	No. of Colonies					
		GM	GEMM	GMM	Meg	Blast	Total
None	–	0	0	0	0	0	0
None	+	0	0	0	0	0	0
ML, IL-11	–	0	0	0	2 ± 1	0	2 ± 1
ML, IL-11	+	10 ± 3	2 ± 1	2 ± 2	8 ± 1	0 ± 1	22 ± 2
ML, IL-6	–	0	0	0	1 ± 1	0	1 ± 1
ML, IL-6	+	20 ± 3	3 ± 2	2 ± 1	7 ± 2	0	31 ± 3
ML, G-CSF	–	0	0	0	1 ± 1	0	1 ± 1
ML, G-CSF	+	16 ± 2	1 ± 2	1 ± 1	4 ± 2	0	22 ± 2
ML, SF	–	11 ± 2	2 ± 2	2 ± 1	7 ± 3	0	22 ± 4
ML, SF	+	6 ± 3	3 ± 1	0	11 ± 2	0	20 ± 6

A total of 150 Lin^- Ly-6A/E^+ Kit^+ cells were plated per dish in serum-free or serum-containing cultures in the presence of 2 U/mL Ep and designated cytokines. Dishes were examined on day 14 of culture. Data represent the mean and standard deviation of values obtained from quadruplicate dishes.

ML and IL-11 in serum-containing cultures. To test this hypothesis, we used ACK2,[25] a rat MoAb that blocks the effects of SF by binding to its receptor. The results are presented in Table 5. The combination of ML and IL-11 supported formation of various types of colonies in serum-containing culture. Addition of ACK2 significantly decreased the numbers of GEMM, GMM, and GM colonies, suggesting that serum SF is required for the effects of ML and IL-11 on GEMM-, GMM-, and GM-colony formation. However, the number of Meg colonies was not affected by the addition of ACK2. This suggested that the synergy between ML and IL-11 in support of Meg colony formation may not require SF. Rat IgG provided a control for ACK2. Colony formation supported by SF and IL-11 was completely abrogated by ACK2.

To further exclude the possible nonspecific activity of ACK2 in serum-containing culture, we tested the effects of ACK2 on colony formation in the presence of ML, IL-3, or IL-11 and in their combinations. The results shown also in Table 5 clearly demonstrated that ACK2 does not affect colony formation supported by the combination of IL-3 and ML or the combination of IL-3 and IL-11.

Serial observations of colony formation from progenitors. Earlier, we have reported that IL-6,[6] IL-11,[7] G-CSF,[8] and IL-12,[11] individually synergize with SF or IL-3 on colony formation from murine primitive progenitors. Serial observation (mapping studies) of colony formation suggested that these cytokines trigger the dormant multipotential progenitors into cell cycle. The synergy between ML and SF or that between ML and IL-3 described in this report suggested that ML may also shorten the G_0 period of dormant progenitors. To test this hypothesis, we performed serial observation of colony development from primitive progenitors as described in Materials and Methods. In one experimental group, enriched cells were plated in the presence of SF, ML, and Ep. In the other experimental group, the culture was initiated with SF and Ep, and on day 7 of incubation, ML was carefully layered over the dishes. We examined the dishes daily on an inverted microscope for analysis of the growth and differentiation of the emerging Blast colonies and tabulated the kinetic properties of the Blast colonies that later showed GEMM lineages. Neither SF nor ML supported formation of GEMM colonies. The proliferative kinetics of the Blast

Table 5. Effects of MoAb ACK2 on Colony Formation in Serum-Containing Culture

Cytokines and Antibodies	No. of Colonies					
	GM	GEMM	GMM	Meg	Blast	Total
Experiment 1						
ML, IL-11	31 ± 4*	6 ± 3*	5 ± 2*	10 ± 4	2 ± 1	54 ± 2*
ML, IL-11, ACK2	11 ± 1	0	1 ± 1	7 ± 3	0 ± 1	19 ± 4
ML, IL-11, Rat IgG	27 ± 6*	6 ± 2*	3 ± 2	9 ± 1	3 ± 1	47 ± 5*
SF, IL-11	55 ± 4	16 ± 2	0 ± 1	3 ± 2	0	75 ± 1
SF, IL-11, ACK2	0	0	0	0	0	0
SF, IL-11, Rat IgG	61 ± 7	14 ± 1	0	2 ± 1	0	78 ± 7
Experiment 2†						
IL-3	15 ± 2	1 ± 1	1 ± 1	1 ± 1	0	17 ± 3
ML	0	0	0	3 ± 2	0	3 ± 2
IL-11	0 ± 1	0	0	0	0 ± 1	1 ± 1
IL-3, ML	21 ± 4	4 ± 1	1 ± 1	2 ± 1	0	27 ± 4
IL-3, ML, ACK2	17 ± 2	4 ± 1	1 ± 1	2 ± 1	0	24 ± 3
IL-3, ML, Rat IgG	17 ± 6	4 ± 2	1 ± 1	3 ± 1	0 ± 1	25 ± 4
IL-3, IL-11	22 ± 3	3 ± 1	0 ± 1	0 ± 1	0	25 ± 2
IL-3, IL-11, ACK2	22 ± 4	4 ± 2	0 ± 1	1 ± 1	0	27 ± 3
IL-3, IL-11, Rat IgG	25 ± 1	3 ± 3	1 ± 1	1 ± 0	0	30 ± 1

A total of 150 (Experiment 1) or 50 (Experiment 2) Lin^- Ly-6A/E^+ Kit^+ cells prepared from the marrow of 5-FU–treated mice were plated per dish in the presence of 2 U/mL Ep and designated cytokines or antibody. Both ACK2 and rat IgG were added to the culture at the concentration of 50 μg/mL. Dishes were examined on day 14 of culture. Data represent the mean and standard deviation of values obtained from quadruplicate dishes.

* These numbers are different from the group of ML, IL-11, and ACK2 at $P < .05$.

† Purified recombinant human ML was used at 200 ng/mL.

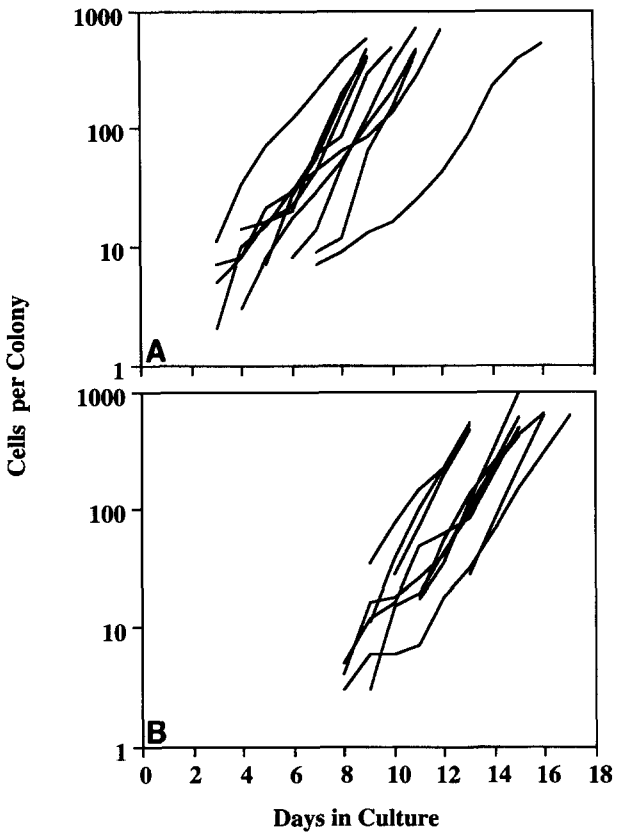

Fig 2. Proliferation of individual Blast cell colonies that later showed GEMM lineages. A total of 150 Lin⁻Ly-6A/E⁺Kit⁺ cells of 5-FU–treated mice were plated per dish and cultured under four cytokine conditions. Cultures supported by ML and Ep or those supported by SF and Ep showed no GEMM colonies. (A) Colony formation from two culture dishes that were initiated with ML, SF, and Ep. (B) Colony formation from four culture dishes that were first prepared with SF and Ep, and on day 7 of culture, received ML. Purified recombinant human ML was used at 200 ng/mL.

colonies later showing GEMM in the two experimental groups are presented in Fig 2. The days for the individual colonies to reach the size of 100 cells significantly differed between the two groups (8.7 ± 2.2 *v* 12.1 ± 1.8 days) at $P < .01$. There were no differences in the doubling times of the individual colonies between the two groups (24.3 ± 7.1 *v* 20.7 ± 3.7 hours). It has been shown that dormant progenitors can survive in culture in the presence of SF alone for several days.[32] These results suggested that ML triggers the cell division of dormant multipotential progenitors.

Expansion of multipotential progenitors by ML and SF. A major clinical application of hematopoietic cytokines is likely to be the expansion of hematopoietic stem cells and progenitors in vitro. The effects of a combination of ML and SF on colony formation from primitive progenitors indicated that these cytokines may be useful for expansion of progenitors. The results of an analysis of progenitor expansion supported by ML and SF are presented in Fig 3. Neither ML nor SF supported cell growth. Only the combination of ML and SF increased the total nucleated cell counts (TNCC) by 135-fold, colony-forming units in culture (CFU-C) by 15-fold, and CFU-GEMM by fourfold after 11 days of culture. These results indicate that the combination of ML and SF stimulates the proliferation of committed and multipotential progenitors in suspension culture and support our conclusion from clonal cell culture that ML enhances the growth of primitive hematopoietic progenitor cells.

DISCUSSION

In this study, we examined the effects of a newly cloned cytokine, the ligand for the Mpl receptor, as a single factor

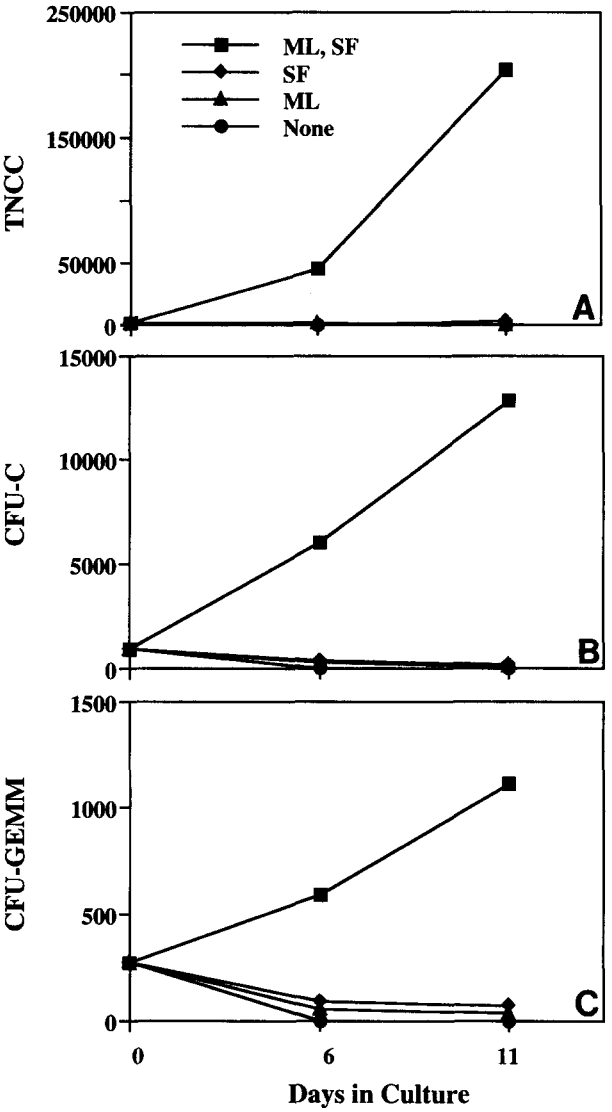

Fig 3. Expansion of cells and progenitors in suspension culture with ML and SF; (A) total nucleated cell counts (TNCC), (B) CFU-C, and (C) CFU-GEMM. Lin⁻Ly-6A/E⁺Kit⁺ cells were prepared from the marrow of 5-FU–treated mice. A total of 1,500 cells were plated in 1 mL of suspension medium containing 30% FCS, Ep, and designated cytokines. On day 6, cells were replated in freshly prepared medium, whereas aliquots were analyzed for TNCC and progenitor content in clonal cultures containing IL-3, SF, IL-6, and Ep. On day 11, only cell counts and progenitor numbers were determined.

or in combinations with SF, IL-3, IL-11, IL-6, or G-CSF on primitive hematopoietic progenitor cells. The progenitors were procured from the marrow of 5-FU–treated mice, enriched by density gradient centrifugation, depleted of mature, lineage-specific cells and FACS-sorted for Ly-6A/E$^+$Kit$^+$ cells. This purification protocol resulted in a cell population that was highly enriched for dormant hematopoietic progenitors including stem cells with long-term repopulating ability. Injection of 100 such cells into lethally irradiated mice resulted in a high percentage of donor-cell reconstitution 4 to 6 months after cell transplantation.[33]

The present studies clearly showed that a combination of ML and SF can support proliferation of primitive progenitors to form multilineage colonies in clonal cell culture and to produce a number of committed and multipotential progenitors in suspension culture. In contrast, neither IL-11, IL-6, nor G-CSF acted in synergy with ML on colony formation from primitive progenitors in serum-free culture. The synergy between these cytokines in serum-containing cultures was apparently due to SF introduced by the serum. Addition of a neutralizing antibody to the murine SF receptor abrogated all of the GEMM- and some of the GMM- and GM-colony formation supported by a combination of ML and IL-11.

We also found that ML and IL-3 synergize in support of multilineage colony formation. Earlier, our laboratory has shown that IL-6,[6] IL-11,[7] G-CSF,[8] or SF[13] individually are able to synergize with IL-3 in support of proliferation of primitive hematopoietic progenitors in part by shortening the G_0 period of the progenitors. In this study, we used mapping of blast cell colony development to show that the addition of ML to cultures triggers the cell division of dormant multipotential progenitors. These results suggest that ML, like IL-11, IL-6, and G-CSF, shortens the G_0 period of cell-cycle dormant hematopoietic progenitors.

Other studies have also suggested that targets of ML may include early hematopoietic progenitors. Methia et al[34] showed that the c-mpl transcript is present in human CD34$^+$CD38$^{-/low}$ cells, which are highly enriched for primitive progenitors. Berardi et al[35] proposed that single dormant human hematopoietic stem cells express c-mpl mRNA. After ML became available, Zeigler et al[36] reported that a combination of ML and SF is able to expand the population of megakaryocytes, suggesting that ML can act on progenitors at earlier stages. Recently, Kobayashi et al[37] and Kaushansky et al[38] in our laboratories observed that ML possesses erythroid burst-promoting activity. The clonal cell culture and micromanipulation studies with purified murine primitive progenitors described in this report are in agreement with these studies and provide the direct evidence for the ability of ML to regulate primitive multipotential progenitors.

In previous studies, we presented evidence that G-CSF, another apparently late-acting cytokine, can stimulate cell cycling of primitive progenitors in G_0.[8] We have now shown that ML also synergizes with SF or IL-3 to stimulate proliferation of primitive progenitors. During states of natural or iatrogenic marrow failure, blood levels of platelets and neutrophils are critical determinants of survival. It is perhaps logical to speculate that under such circumstances, ML and G-CSF not only support the proliferation and maturation of committed megakaryocyte and neutrophil progenitors, but also stimulate proliferation of primitive cell cycle-dormant progenitors to assure a continued supply of cells committed to the megakaryocytic and neutrophilic lineages.

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