Effect of c-kit Ligand on In Vitro Human Megakaryocytopoiesis

By Robert A. Briddell, Edward Bruno, Ryan J. Cooper, John E. Brandt, and Ronald Hoffman

An evaluation of the effects of a recombinant, soluble form of the c-kit ligand and in combination with either granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) on the regulation of human megakaryocytopoiesis was performed using a serum-depleted clonal assay system and a long-term bone marrow culture system. The effects of the c-kit ligand on the primitive megakaryocyte (MK) progenitor cell, the burst-forming unit-megakaryocyte (BFU-MK), and the more differentiated colony-forming unit-megakaryocyte (CFU-MK) were determined. The c-kit ligand alone had no megakaryocyte colony-stimulating activity (MK-CSA) but was capable of augmenting the MK-CSA of both GM-CSF and IL-3. The range of synergistic interactions of c-kit ligand varied with the class of MK progenitor cell assayed. In the case of the BFU-MK, the c-kit ligand synergistically augmented the numbers of colonies formed in the presence of IL-3, but not GM-CSF, but increased the size of BFU-MK-derived colonies cloned in the presence of both of these cytokines. However, at the level of the CFU-MK, c-kit ligand synergized with both GM-CSF and IL-3 by increasing both colony numbers and size. Although the c-kit ligand alone exhibited limited potential in sustaining long-term megakaryocytopoiesis in vitro, it synergistically augmented the ability of IL-3, but not GM-CSF, to promote long-term megakaryocytopoiesis. These data indicate that multiple cytokines are necessary to optimally stimulate the proliferation of both classes of MK progenitor cells and that the c-kit ligand plays a significant role in this process by amplifying the MK-CSA of both GM-CSF and IL-3.

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REGULATION of human megakaryocytopoiesis in vitro is controlled by a complex network of interacting cytokines. Recombinant granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-1α (IL-1α), IL-3, and IL-6 each have been shown to have effects, either alone or in various combinations, on the proliferation and differentiation of human megakaryocyte (MK) progenitor cells. GM-CSF and IL-3, in particular, have been noted to have potent synergistic action in conjunction with other CFSs resulting in increased colony number and size. The c-kit ligand has been variously referred to as mast cell growth factor, stem cell factor, and steel locus activity, not solely to c-kit ligand activity, but to its role in the regulation of megakaryocytopoiesis. These data indicate that multiple cytokines are necessary to optimally stimulate the proliferation of both classes of MK progenitor cells and that the c-kit ligand plays a significant role in this process by amplifying the MK-CSA of both GM-CSF and IL-3.

We have investigated the proliferative effects of a recombinant murine form of the c-kit ligand, MGF, alone and in combination with either GM-CSF or IL-3, in both CD34+ DR+ and CD34+ DR- human bone marrow subpopulations to determine if this cytokine affects MK progenitor cells and/or long-term in vitro megakaryocytopoiesis. While the CD34+ DR- population is enriched for the burst-forming unit-megakaryocyte (BFU-MK), and the cell responsible for initiating long-term hematopoiesis in vitro, the CD34+ DR+ subpopulation contains the more differentiated MK progenitor cell, the colony-forming unit-megakaryocyte (CFU-MK). Although MGF alone had no significant influence on either CFU-MK or BFU-MK proliferation, it synergistically augmented the MK-CSA of both GM-CSF and IL-3 and the ability of IL-3 to sustain long-term megakaryocytopoiesis in vitro. These data indicate that the c-kit ligand affects multiple stages of MK development by enhancing the effects of several cytokines on this process. These data and the observation that abnormal hematopoiesis, and likely megakaryocytopoiesis, in the SI mutant mouse is due to a deficiency of this cytokine, suggest that the c-kit ligand plays an important role in mammalian megakaryocytopoiesis.

MATERIALS AND METHODS

Bone marrow aspirates were obtained under local anesthesia from the posterior iliac crests of hematologically normal volunteers. Informed consent was obtained from the donors according to guidelines previously established by the Human Investigations Committee of the Indiana University School of Medicine, which adheres to the principles of the Declaration of Helsinki.

Cell separation techniques. Bone marrow aspirates were immediately diluted 1:1 with Iscove's Modified Dulbecco's Media (IMDM; GIBCO Laboratories, Life Technologies, Inc, Grand View, CA) immediately diluted 1:1 with Iscove's Modified Dulbecco's Media (IMDM; GIBCO Laboratories, Life Technologies, Inc, Grand View, CA)
Low-density mononuclear cells (LDMC) were obtained by density centrifugation over Ficoll-Paque (Pharmacia LKB Biotechnology, Inc, Piscataway, NJ) at 750g. To obtain a cell population enriched for BFU-MK, we further separated LDMC by countercflow centrifugal elutriation to obtain those cells eluting at flow rates between 12 and 14 mL/min (FR12-14). We further purified FR12-14 cells using monoclonal antibody (MoAb) staining and fluorescence-activated cell sorting (FACS) according to previously described methods. The cell populations containing high densities of CD34 and no detectable density of HLA-DR (CD34+ DR-) were used for experiments concerning the human BFU-MK. The CD34+ DR- cell population consistently contained greater than 90% CD34-positive cells and less than 5% HLA-DR-positive cells. The more differentiated human MK progenitor cell, the CFU-MK, is present in elutriation fractions with a broader range of flow rates; therefore, a nonadherent, low-density, T-cell-depleted mononuclear cell subpopulation (NALDT-1) was isolated from LDMC. We further purified NALDT-1 cells using MoAb labeling and FACS. Cell populations containing high densities of CD34 and detectable densities of HLA-DR (CD34+ DR+) were used for the experiments concerning the human CFU-MK. The CD34+ DR+ cell population consistently contained greater than 90% CD34-positive cells and greater than 95% HLA-DR-positive cells.

Recombinant human and murine cytokines. The following recombinant purified human (h) and murine (m) cytokines were used in these studies: (1) hGM-CSF: specific activity (sp act) = 5.0 x 10^10 U/mg protein determined by granulocyte-macrophage colony formation from human bone marrow cells; Genzyme Corp, Boston, MA. (2) hIL-3: sp act = 1.0 x 10^11 U/mg protein determined by mixed colony formation from human bone marrow cells; Genzyme Corp. (3) m c-kit ligand (MGF): sp act = 1.0 x 10^12 U/mg protein determined by proliferative effects on MC6 cells; Immunix Corp, Seattle, WA.

Long-term suspension cultures. Long-term stromal cell-free bone marrow suspension cultures (LTBMC) were initiated and maintained as previously described. Briefly, polystyrene 35-mm tissue culture dishes containing 1 mL IMDM with 10% fetal bovine serum (Hyclone, Logan, UT) were inoculated with 1.0 x 10^5 CD34+ DR- and CD34+ DR+ cells, respectively, were cultured in 100% humidified 5% CO2 in air. After 24 hours, and every 48 hours thereafter, cultures received no additions, 200.0 pg/mL GM-CSF, 1.0 ng/mL IL-3, 100.0 ng/mL MGF, or combinations of the aforementioned cytokines. Cytokine concentrations used in these studies were the optimal concentrations known to promote in vitro BFU-MK-derived colony formation as previously reported by our laboratory or reported in this communication. At weeks 2, 3, 4, 6, 8, 10, 12, and 14 the cultures were demidepopulated by removal of one half the culture volume, which was replaced with fresh media. Cells in the harvested media were counted and assayed for MK progenitors.

MegaKaryocyte progenitor cell assay system. Sorted cells or cells obtained during LTBMC were assayed for their ability to produce BFU-MK- or CFU-MK-derived colonies. The aforementioned cytokines were used alone or in various combinations as sources of MK-CSA. Cultures initiated were incubated for 14 or 21 days to quantitate CFU-MK- and BFU-MK-derived colonies, respectively, at 37°C in 100% humidified 5% CO2 in air. After incubation, fibrin clots were fixed in situ in methanol-acetone (1:3) for 20 minutes, washed with phosphate-buffered saline, and air-dried.

Immunofluorescent identification of megakaryocyte colonies. 10E5 mouse monoclonal IgG antibodies recognizing the human platelet glycoprotein IIb-IIIa complex (provided by Dr Barry S. Collier, State University of New York-Stony Brook) were used as immuno-logic probes for identifying human MKs. 10E5 was subsequently tagged with a fluorescein-labeled, affinity-purified, goat antihuman IgG (H + L) (Kirkegaard and Perry Laboratories, Inc, Gaithersburg, MD). The 35-mm Petri dishes were inverted, and the base area completely scanned at 100X using an inverted microscope with reflected light fluorescent attachment (Olympus Corporation, Lake Success, NY). A CFU-MK-derived colony was defined as a cluster of three or more fluorescent cells. A BFU-MK-derived colony was described by criteria established by Long et al. These colonies appeared in human marrow cultures as clusters ≥ 42 fluorescent cells usually distributed in multiple foci of development, and are identified after 21 days of incubation. Human BFU-MK-derived colonies are distinguished from CFU-MK-derived colonies by duration of incubation required for their appearance in vitro (21 days v 12 days, respectively), colony size (108.6 ± 4.4 cells/colony v 112.2 ± 1.2 cells/colony, respectively), and foci of development (2.3 ± 0.4 foci/colony v 1.2 ± 0.1 foci/colony, respectively).

Statistical analysis. Results are expressed as means ± standard error of the mean obtained from multiple experiments performed in duplicate. Statistical significance was determined using the Student t-test.

RESULTS

Both BFU-MK- and CFU-MK-derived colony formation by CD34+ DR- and CD34+ DR+ cells, respectively, were entirely dependent on the addition of either GM-CSF or IL-3 (Table 1). MGF stimulated neither BFU-MK-derived (Fig 1) nor CFU-MK-derived (Fig 2) colony formation when added alone at concentrations ranging from 12.5 to 100.0 ng/mL. MGF potentiated the ability of both suboptimal (125.0 pg/mL) and optimal (1.0 ng/mL) concentrations of IL-3 to promote BFU-MK-derived colony formation (Fig 1, P < .05). MGF had no effect on the ability of GM-CSF to promote BFU-MK-derived colony formation (data not shown). However, MGF did augment CFU-MK-derived colony formation when added in combination with optimal concentrations of GM-CSF (200.0 pg/mL) (Fig 2, P < .05) and suboptimal concentrations of IL-3 (125.0 pg/mL) (Fig 2, P < .05). MGF had no effect on CFU-MK-derived colony formation when added together with suboptimal concentrations of GM-CSF or optimal concentrations of IL-3 (data not shown). The effect of MGF on the number of cells composing BFU-MK- and CFU-MK-derived colonies is shown in Table 2. MGF increased the cellular

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<td>Cytokines/mL</td>
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</tr>
<tr>
<td>No addition</td>
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<tr>
<td>25.0 pg GM-CSF (suboptimal)</td>
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<td>200.0 pg GM-CSF (optimal)</td>
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*Each point represents the mean ± the standard error of the mean obtained from duplicate experiments.
†Each point represents the number of separate, duplicate experiments for the corresponding data point (n value).

P < .05 when compared with the control culture which received no exogenous cytokine.
composition of both classes of MK colonies cloned in the presence of optimal concentrations of either GM-CSF or IL-3.

LTBMCs were initiated with $5 \times 10^6$ CD34$^+$ DR$^-$ marrow cells to which no cytokines, GM-CSF, IL-3, and MGF were added alone or in combination. Before the initiation of LTBMC, this cell population, obtained from two separate normal donors, contained no morphologically or immunologically identifiable Mk, no assayable CFU-MK, but did contain 6.2 ± 0.4 BFU-MK per $5 \times 10^6$ CD34$^+$ DR$^-$ cells. These LTBMCs did not contain a preestablished adherent cell layer, nor did such a layer develop during the period of observation. LTBMCs were demidepopulated and assayed for both BFU-MK and CFU-MK at weeks 2, 3, 4, 6, 8, 10, 12, and 14. In the LTBMCs not receiving any exogenous cytokines, viable cells were not detected after 1 week; assayable CFU-MK and BFU-MK were never detected during the period of observation (Fig 3). Those LTBMCs that received repeated additions of GM-CSF or IL-3 alone contained assayable CFU-MK for not greater than 10 weeks (Fig 3). No assayable BFU-MK were detected during the duration of the LTBMCs (data not shown). The IL-3-supplemented cultures produced nearly twice the number of assayable CFU-MK over 10 weeks of LTBMC as the cultures receiving GM-CSF (Fig 3). Repeated additions of MGF alone led to the production of small numbers of CFU-MK for approximately 4 weeks (Fig 3). The addition of MGF and GM-CSF in combination did not alter the number of assayable CFU-MK in LTBMC beyond that observed with repeated additions of GM-CSF alone, while the addition of MGF and IL-3 in combination led to a doubling of the cumulative production of CFU-MK during the period of LTBMC over that observed in LTBMC receiving IL-3 alone (Fig 3).
DISCUSSION

MK progenitor cell development has been shown to be a process closely regulated by a number of interacting cytokines. These studies suggest that cytokines such as GM-CSF, IL-1α, IL-3, and IL-6 can affect human in vitro megakaryocytopoiesis. The effects of the ligand for the c-kit gene product (MGF), on BFU-MK- and CFU-MK-derived colony formation and long-term megakaryocytopoiesis in vitro, was examined to further define the cytokine requirements of human MK progenitor cells. The MK-CSA of the c-kit ligand alone, as well as its effect on the proliferative capacity of MK progenitor cells promoted by recombinant cytokines such as GM-CSF and IL-3, was analyzed.

Zsebo et al reported that the administration of the c-kit ligand in pharmacological doses in vivo to St/St mice not only corrected the severe anemia of these animals but led to leukocytosis and thrombocytosis. Several groups have shown that while the c-kit ligand alone has no CSA in vitro, it potentiates the ability of other cytokines to stimulate hematopoietic colony formation. This stimulatory effect occurred whether unfractionated or CD34-enriched human bone marrow cell fractions were used as target cell populations. The effect of the c-kit ligand on thrombopoiesis in St/St mice and its ability to potentiate the effects of other cytokines thus served as a rationale to determine the effects of the c-kit ligand on human MK progenitor cells.

Although the c-kit ligand alone had no MK-CSA, these studies clearly show that it has an effect on human megakaryocytopoiesis. The influences of the c-kit ligand were observed at the level of both the BFU-MK and CFU-MK. This cytokine potentiated the ability of IL-3, but not GM-CSF, to promote BFU-MK-derived colony formation as well as the number of cells composing BFU-MK-derived colonies cloned in the presence of either GM-CSF or IL-3. The c-kit ligand had a more diverse range of effects on the CFU-MK, because it was able to synergistically interact with both GM-CSF and IL-3 by increasing the number and size of CFU-MK-derived colonies assayed. The ability of c-kit ligand to increase the number of cells comprising individual colonies suggests that this cytokine permits CFU-MKs to undergo additional numbers of divisions before they enter a nonmitotic (endomitotic) phase of MK development.

Recently our laboratory has developed an LTBMCS system for human hematopoiesis that is not dependent on the establishment of an adherent cell layer. In this system, the LTBMCS are initiated with subpopulations of bone marrow cells and are then supplemented with individual cytokines or cytokine combinations every 48 hours for the duration of the culture period. We have previously reported that LTBMCS initiated with CD34+ DR- cells and repeatedly supplemented with GM-CSF, IL-1α, or IL-3 are capable of sustaining long-term hematopoiesis in vitro, and in this report defined the role of c-kit ligand in long-term megakaryocytopoiesis.

MGF alone had limited ability to sustain long-term megakaryocytopoiesis and did not significantly potentiate the action of GM-CSF in this process. By contrast, MGF in combination with IL-3 more than doubled the cumulative production of CFU-MKs during LTBMCS, suggesting that the c-kit ligand likely acts not only on the BFU-MK or CFU-MK but likely on a pre-BFU-MK present in CD34+ DR- marrow cells.

The cytokine relationships reported both here and in other reports in the literature clearly show that human megakaryocytopoiesis is under the regulation of a network of different cytokines including GM-CSF, IL-1α, IL-3, IL-6, and the c-kit gene product. These cytokines appear to act directly on MK progenitor cells by either stimulating colony formation (GM-CSF, IL-3) and/or by augmenting the action of other cytokines (IL-1α, IL-6, c-kit ligand). However, it is important to emphasize that these studies do not negate the possibility that c-kit ligand may also affect human megakaryocytopoiesis by altering accessory cell functions present within the hematopoietic microenvironment. The potentiation of the action of these other cytokines by the c-kit ligand indicates that several cytokines in combination may be required to achieve maximal MK progenitor cell expansion in vitro.

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