Interacting Cytokines Regulate In Vitro Human Megakaryocytopoiesis

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The effects of hematopoietic growth factors on in vitro human megakaryocytopoiesis were studied using a serum-depleted culture system. Both recombinant interleukin-3 (rIL-3) and recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) increased megakaryocyte (MK) colony formation (P < .01) above that observed in baseline cultures. Recombinant interleukin-4 (rIL-4) and interleukin 1α (rIL-1α) failed either to promote MK colony formation alone or to increase rIL-3 or rGM-CSF promoted colony formation. Recombinant erythropoietin (rEpo) and purified thrombocytopoiesis-stimulating factor (TSF) did not increase (P > .05) MK colony formation when added alone but synergized with rIL-1α, leading to a twofold increase in MK colony formation. Such a synergistic relationship was not observed between rIL-4 and rEpo. In addition, TSF enhanced the ability of rIL-3 but not rGM-CSF to promote MK colony formation. Addition of rEpo to optimal or suboptimal concentrations of rGM-CSF or suboptimal concentrations of rIL-3 resulted in a significant increase (P < .05) in the number of MK-containing colonies, due to the appearance of multilineage colonies containing MKs. The addition of rEpo to optimal concentrations of rIL-3 resulted in increased numbers of multilineage colonies containing MKs; however, the number of total MK-containing colonies was not significantly increased when compared to assays containing rIL-3 alone. By contrast, transforming growth factor-β (TGF-β) inhibited both rIL-3, and rGM-CSF promoted MK colony formation, with optimal inhibition resulting in a 35%-45% reduction of MK colony formation. These data suggest that a number of growth factors can regulate in vitro human megakaryocytopoiesis by either promoting or inhibiting MK colony formation.
polycythemic mouse assay and a radioimmunoassay using anti-Epo antibody) was purchased from AmGen Biologicals, Thousand Oaks, CA. Recombinant human interleukin-3 (rIL-3, specific activity 10^8 CFU/mg protein based on its ability to promote the formation of mixed colonies composed of granulocyte, macrophage, megakaryocyte, and eosinophil elements from human bone marrow cells), recombinant human granulocyte-macrophage colony-stimulating factor (rGM-CSF, specific activity greater than 5 × 10^3 CFU/mg protein based on the generation of CFU-GM derived colonies from 7.5 × 10^6 human bone marrow cells in soft agarose after 14 days of incubation) and recombinant human interleukin-4 (rIL-4, specific activity 10^5 proliferation U/mg protein based on a coproliferation assay using human tonsillar B cells and antihuman IgG) were purchased from the Genzyme Corp, Boston. Recombinant human interleukin-1α (IL-1α) was a gift of Dr Peter LoMedico, Hoffman LaRoche, Nutley, NJ, and had a specific activity of 10^6 U/mg protein, as determined by proliferative effects on D-10 cells. Transforming growth factor-β (TGF-β, assayd for its stimulation of the growth of NRK-1 cells in soft agar in the concentration range of 0.1 to 5.0 ng/mL) was purchased from Biomedical Technologies Inc, Stoughton, MA. Step III thrombocytopoiesis stimulatory factor (1SF, purified from human embryonic kidney cell-conditioned media (HEKM), was kindly provided by Dr Ted McDonald. Step III 1SF is a highly purified preparation of 1SF, the characteristics of which have been previously described by McDonald et al. 1SF samples were dissolved in IMDM to form a stock solution of 30 µg/mL and stored at −20°C until use in culture.

**Serum-depleted assay for megakaryocytic colony formation.** Various hematopoietic growth factors, alone or in combination, were assayed for their ability to promote megakaryocyte colony formation in a serum-depleted fibrin-cot culture system previously described by Bruno et al. Bone marrow cells, aspirated from iliac crests of hematologically normal volunteers, were diluted 1:1 with reconstituted IMDM containing preservative-free sodium heparin (GIBCO) at 20 U/mL and layered over an equal volume of Ficoll-Paque (specific gravity 1.077 g/mL; Pharmacia Fine Chemicals, Piscataway, NJ). Density centrifugation was performed at 500 g for 15 minutes at 4°C in a Beckman model TJ-6R centrifuge (Beckman Instruments, Inc, Fullerton, CA). The interface mononuclear cell layer was collected and washed with IMDM containing no defined growth factors. Whole mononuclear cells were then diluted to 2 × 10^5 cells/mL in IMDM and cultured in a 1-mL volume in 35-mm petri dishes (CoStar, Cambridge, MA). The fibrin clot assay of Dainiak et al was modified to allow for subsequent staining of megakaryocyte colonies. The final culture mixture contained (1) 0.3 ml of a serum substitute mix containing 300 µg/mL iron saturated transferrin and 3 mg/mL BSA; (2) 0.3 ml of backup mix containing 0.1 ml CaCl2 (280 µg/mL), 0.1 ml L asparagine (1 × 10−3 M) and 0.1 ml IMDM; (3) 0.1 ml of various hematopoietic growth factors suspended in IMDM at different concentrations; (4) 0.3 ml of clotting matrix containing 0.1 ml IMDM, 0.1 ml of 2% (wt/vol) purified fibrinogen (Kabi, Stockholm, Sweden) suspended in phosphate buffered saline (PBS), and 0.1 ml of 2.0 U/mL of purified human thombin (95%; Sigma Chemical Co, St Louis) suspended in PBS; and (5) 0.1 ml of marrow cells diluted in IMDM. The purity of the fibrinogen used in the assay system was analyzed by disc gel electrophoresis using a 7.0% polyacrylamide gel. One major band was detected by silver staining with a mob wt of 340,000 daltons as determined by comparison to migration of standard mol wt markers. The purity of the fibrinogen was determined to be >98%. The final culture mixture contained 0.3% (wt/vol) BSA, 0.2% (wt/vol) purified fibrinogen, and 0.2 U/mL of purified human thrombin. The final concentration of fatty acid free BSA in individual culture plates was 300 µg/mL.omp. The concentrations of growth factors added to individual assays was determined by data either reported in this communication or previously published by Bruno et al. To define the effect of interacting cytokines on the ability of rIL-3 and rGM-CSF to promote megakaryocyte colony formation, an optimal and suboptimal dose of either cytokine was chosen. Since rIL-1α, purified TSF, and Epo had been previously shown by our laboratory not to enhance baseline colony formation over a broad range of doses, a single concentration of these cytokines was used. In this report dose-response curves for rIL-4 are reported. Since rIL-4 is shown not to have intrinsic MK-CSA, a single dose was used in those studies testing its interaction with other cytokines. Purified TGF-β was tested at a number of concentrations that approximate those found in human serum.26 Cultures were incubated for ten to 12 days at 37°C in a 100% humidified atmosphere of 5% CO2 in air. Fibrin clots were fixed in situ with methanol:acetone (1:3) for 20 minutes, washed with 0.01 mol/L (PBS), pH 7.2, and double-distilled water, then air dried. Fixed plates were stored frozen at −20°C until immunofluorescent staining was performed.

**Immunofluorescent identification of human megakaryocytic colonies.** Rabbit antisera to human platelet glycoproteins, previously established as an immunologic probe for identifying human megakaryocytes, was diluted in PBS (1:200), layered over fixed fibrin clot cultures, and incubated for 60 minutes at room temperature in 100% humidified air. After washing three times with PBS, the specimens were reincubated for an additional 60 minutes at room temperature with fluorescein-conjugated goat F(ab')2 antirabbit IgG (Tago, Inc, Burlingame, CA) diluted in PBS at a final concentration of 0.76 mg of protein/mL. Specimens were washed in PBS, counterstained with 0.125% Evan's blue for 1.5 minutes, washed with distilled water, and mounted in isotonic barbital buffer, pH 8.6, in glycerol (1:3).

To further document the specificity of the antibody-mediated immunofluorescent identification of megakaryocyte colonies, bone marrow aspirate smears from a patient with hyper eosinophilic syndrome were stained with antiplatelet glycoprotein antiserum followed by goat F(ab')2 antirabbit IgG. A matching marrow specimen was stained with Wright’s-Giemsa to document increased numbers of megakaryocytes and eosinophils. Specimens stained with both antiplatelet glycoprotein antiserum and goat antirabbit IgG showed positive fluorescence for both eosinophils and megakaryocytes. The nonspecific immunofluorescent staining of eosinophils was quenched, however, by subsequently counterstaining with 0.125% Evans Blue, allowing for the specific identification of megakaryocytes.

 Cultures were scored in situ to enumerate fluorescein-positive colonies. The 35-mm petri dishes were inverted and the base area completely scanned with a fluorescent microscope at X 100 (Zeiss standard microscope 18 with IV FL vertical fluorescent illuminator; Carl Zeiss, Inc, NY). A pure MK colony was defined as a cluster of three of more fluorescent cells. A mixed colony containing MKs was defined as a cluster of at least 50 cells containing both fluorescent and nonfluorescent cells. Each mixed colony contained at least three fluorescent cells. Each experimental group was cultured in duplicate or quadruplicate.

**Statistical analysis.** The results were expressed as the mean ± SEM of data obtained from three or more experiments performed in duplicate or quadruplicate. Statistical significance was determined using the paired t test.

**RESULTS**

In the serum-depleted assay system, small numbers of megakaryocyte colonies (7.9 ± 1.0/2 × 10^3 cells plated) formed in the absence of the addition of exogenous growth factors. This number represents the results from the assay of bone marrow cells taken from 17 different normal marrow
Additives to Culture (Units/mL)

Fig 1. Effect of the addition of rIL-1α (A and B), rIL-3 (A), and rGM-CSF (B) alone and in combination on human megakaryocyte colony formation by normal human marrow cells. Each point represents the x ± SEM of pooled data from four (A) and three (B) separate experiments. All assays were performed in duplicate or quadruplicate. The capital letters (A through F) represent cytokines added to culture, while the numbers in the parentheses (%) represent units of each cytokine/mL present in the culture mixture. (A) A: No additions; B: rIL-1α(25); C: rIL-1α(50); D: rIL-1α(100); E: rIL-3(12.5); F: rIL-3(50). (B) A': No additions; B': rIL-1α(25); C': rIL-1α(50); D': rIL-1α(100); E': rGM-CSF(1.25); F': rGM-CSF(10).

In the initial group of experiments, the effect of rIL-1α on the ability of rGM-CSF or rIL-3 to promote MK colony formation was tested. In Fig 1 (1A and 1B) it can be seen that both rIL-3 (Fig 1A) and rGM-CSF (Fig 1B) individually promoted the formation of MK colonies to a statistically significant degree over control (P < .01). Varying doses of rIL-1α alone had no MK colony-promoting effect (Fig 1), while the addition of rIL-1α did not influence colony formation promoted by either rIL-3 or rGM-CSF (P > .05).

Similarly, when varying concentrations of rIL-4 were evaluated for their ability to promote MK colony formation (Fig 2A), no effect was detected at the concentrations tested. Furthermore, rIL-4 did not significantly influence rIL-3- or rGM-CSF-promoted MK colony formation (Fig 2B).

In the next series of studies, the effects of rEpo on MK colony formation added alone or in combination with other cytokines were assessed. In Fig 3A, it is shown that, at varying doses, rEpo failed to significantly promote MK colony formation. By contrast, rEpo appeared to have a truly synergistic interaction with rIL-1α, since neither of these cytokines alone was able to promote MK colony formation.

Fig 2. Effect of the addition of varying concentrations of rIL-4, added either alone (A) or in combination with rIL-3 (B) or rGM-CSF (B), on human megakaryocyte colony formation by normal human marrow cells. Each point represents the x ± SEM of pooled data from three separate experiments. All assays were performed in quadruplicate. The number in the parentheses (%) represents units of each cytokine/mL present in the culture mixture. (B) A: No additions; B: rGM-CSF(2.5); C: rGM-CSF(12.5); D: rGM-CSF(2.5) + rIL-4(30); E: rIL-3(12.5) + rIL-4(30); F: rIL-3(50); G: rIL-3(12.5) + rIL-4(30); H: rIL-3(50) + rIL-4(30).

Fig 3. Effect of the addition of varying concentrations of rEpo added either alone (A, B, C) or in combination with rIL-1α (50 U/mL; A), rIL-4 (30 U/mL; A), a suboptimal concentration of rIL-3 (12.5 U/mL; B) or an optimal concentration of rGM-CSF (2.5 U/mL; C) on human megakaryocyte colony formation by normal human marrow cells. Each point represents the x ± SEM of pooled data from three separate experiments. All assays were performed in duplicate or quadruplicate. (A) — A— rEpo (pure MK colonies); — A— rEpo + rIL-1α (pure MK colonies); — O— rEpo + rIL-3 (total MK containing colonies); — O— rEpo + rGM-CSF (pure MK colonies). (B) — A— rEpo (pure MK colonies); — A— rEpo + rIL-3 (total MK containing colonies); — O— rEpo + rIL-3 (pure MK colonies); — O— rEpo + rGM-CSF (mixing colonies containing MKs). (C) — A— rEpo (pure MK colonies); — A— rEpo + rGM-CSF (total MK containing colonies); — O— rEpo + rGM-CSF (pure MK colonies); — O— rEpo + rGM-CSF (mixed colonies containing MKs).
growth factors alone was able to promote MK colony formation (Fig 1A, 3A), but the combination of rIL-6 (50 U/mL) + rEpo (2 U/mL) was able to promote significantly greater numbers of MK colonies than that observed in control assays (P < .05). The combination of rEpo + rIL-4 resulted in increased MK colony formation, but this increase was not significant using the two-tailed paired t test. The effects of combinations of rEpo and both rIL-3 and rGM-CSF on in vitro megakaryocyte proliferation were also examined. In Fig 3B it can be seen that the combination of a suboptimal concentration of rIL-3 (12.5 U/mL) + rEpo resulted in significantly greater numbers of total MK-containing colonies than that observed with rIL-3 alone (P < .05). When making these assessments the numbers of pure MK colonies and mixed colonies containing MKs were individually enumerated. In the presence of rIL-3, only pure MK colonies and no mixed lineage colonies containing MKs were noted. Therefore the increase in total MK-containing colonies observed in the presence of rIL-3 and rEpo was due to the appearance of mixed colonies that contained MKs. The addition of an optimal concentration of rIL-3 (50 U/mL) + rEpo, although resulting in the appearance of increased numbers of mixed colonies containing MKs, did not result in formation of a significantly greater number of total MK-containing colonies, when compared to an identical dose of rIL-3 alone (data not shown; P = .09). The findings observed when varying concentrations of rEpo were added to an optimal concentration of rGM-CSF are shown in Fig 3C. The addition of rEpo to rGM-CSF resulted in the appearance of a greater number of total MK-containing colonies (P < .05). This increase was due to the appearance of significant numbers of mixed MK-containing colonies. Similar results were attained when varying doses of rEpo were added to a suboptimal concentration of rGM-CSF (data not shown). The number of pure MK colonies in assays containing rEpo + rGM-CSF was similar to that in assays containing the identical dose of rGM-CSF alone.

We next tested the hypothesis that TSF might also influence MK colony formation in the presence of other cytokines. In Fig 4A it can be seen that purified TSF alone did not promote the formation of pure MK colonies. TSF and rIL-1α, however, had a synergistic relationship, since combinations of these two factors promoted pure MK colony formation while neither factor alone possessed such an ability. In Fig 4B it is shown that the addition of TSF augmented MK colony formation induced by suboptimal amounts of rIL-3 (12.5 U/mL; P < .05) but not by higher concentrations of rIL-3 (50 U/mL). All the colonies observed in assays containing rIL-3 + TSF were pure MK colonies. By contrast (Fig 4C), similar concentrations of TSF when added to assays containing rGM-CSF (10 U/mL) actually inhibited the appearance of pure MK colonies (P < .05) when compared to assays containing the same dose of rGM-CSF alone.

The possibility that there are not only agonists but also inhibitors of MK colony formation was also examined. In Fig 5A it can be seen that TGF-β was capable of inhibiting both rIL-3 and rGM-CSF promoted MK colony formation to a statistically significant degree (P < .05). In Fig 5B the effects of increasing concentrations of TGF-β on MK colony formation formed in the presence of an optimal concentration of rGM-CSF (5 U/mL) is shown. TGF-β did not significantly (P > .05) inhibit baseline colony formation at any dose tested. TGF-β at a concentration of either 0.1 ng/mL or 0.5 ng/mL failed to inhibit rGM-CSF (5 U/mL) stimulated MK colony formation, while inhibition was observed when 1.0 to 5.0 ng/mL of TGF-β was added to such cultures (P < .05).

**DISCUSSION**

The data provided in this report suggest that the regulation of in vitro human CFU-MK proliferation involves a complex network of interacting growth factors. A somewhat surprising observation is the detection of a small number of megakaryocyte colonies cloned in the absence of exogenous growth factors when a serum-depleted assay system was used. Such background colony formation has been universally observed when serum or plasma-containing assay systems have been previously employed. This background colony formation might be due to the presence of small amounts of growth factors in serum or plasma, but this explanation seems unlikely, since these constituents are eliminated from the presently used assay system. Background megakaryocyte colony formation is also not eliminated when highly purified populations of marrow progenitor cells are assayed using this same assay system. This finding would make it unlikely that the background colony formation was due to the elaboration of cytokines by marrow accessory cells. Recently our laboratory has reported the
stimulating factor—L, or IL-4, was first postulated to act as a colony formation. In this communication the findings of et al, our findings do differ from Peschel et al, since in the studies B-cell growth factor but has recently been shown to have a colony formation appears therefore to be totally dependent on the addition of exogenous cytokines. CFU-MK-derived colony formation system, no BFU-MK-derived colony formation occurred in the absence of exogenous growth factors. BFU-MK but not BFU-MK-derived colony formation seems to be applicable to the human system. However, IL-la and to enhance the colony-promoting effects of CSF-IL-la or Epo actually augmented murine megakaryocyte colony formation, it acted together with WEHI-3 cell-conditioned media, a potent source of IL-3, to augment in vitro megakaryocytopoiesis. Combinations of suboptimal concentrations of rIL-3 added to varying concentrations of rEpo lead to the appearance of greater numbers of total colonies containing MKs due to the appearance of mixed colonies containing MKs. In contrast, significantly greater numbers of total MK-containing colonies were not observed when rEpo was added to optimal concentrations of rIL-3. In addition, suboptimal concentrations of rIL-3 and rGM-CSF have been previously shown to be additive in promoting human megakaryocyte colony formation.

Although GM-CSF is also a multi-CSF, its action on in vitro megakaryocyte progenitor cell regulation appears different than rIL-3. GM-CSF has MK-CSA, but its capacity to promote megakaryocyte colony formation is inhibited by the addition of TSF. Its interaction with Epo also differs from that of rIL-3, since rEpo, in the presence of both optimal and suboptimal concentrations of rGM-CSF, promotes the appearance of significantly greater numbers of total colonies containing megakaryocytes when compared to cultures containing identical doses of rGM-CSF alone (P < .05).

The possibility that inhibitors of CFU-MK proliferation play a role in the control of in vitro megakaryocytopoiesis was initially suggested by the observation that plasma was superior to serum in promoting the formation of megakaryocyte colonies in a variety of clonal assay systems. TGF—B, a relatively abundant constituent of platelet alpha granules, has been suggested by Solberg et al, Ishibashi et al, and Mitjavila et al to be either the primary inhibitor or one of several platelet-derived inhibitors of megakaryocytopoiesis. In this report we clearly demonstrate that TGF—B is capable of partially inhibiting (30% to 40%) human MK colony formation. This phenomenon occurs in our studies at similar concentrations of TGF—B to those previously reported by others.

Our data indicate, furthermore, that TGF—B can inhibit megakaryocyte colony formation promoted by at least two different colony-stimulating factors exhibiting MK-CSA. Such findings suggest that this inhibitory effect occurs at a cellular target common to the action of both rGM-CSF and rIL-3. Therefore one might consider the possibility that

**Fig 5. Effect of the addition of a fixed concentration of TGF—B (5 ng/mL) on background (A), rIL-3 (B), or rGM-CSF (B)—promoted human megakaryocyte colony formation by normal human marrow cells.**

In (B) the effect of varying concentrations of TGF—B on rGM-CSF (8 U/mL)—promoted human megakaryocyte colony formation by normal human marrow cells is shown. Each point represents the mean ± SEM of pooled data from four separate experiments. All assays were performed in quadruplicate. (A) No additions; rIL-3 (25 ng/mL) + TGF—B (5 ng/mL); rGM-CSF (5 ng/mL); rIL-3 (25 U/mL); rGM-CSF (5 ng/mL) + TGF—B (5 ng/mL); (B) —Δ— rGM-CSF + TGF—B; --Δ-- TGF—B.
TGF-β either directly inhibits in vitro CFU-MK proliferation or inhibits elaboration of cytokines by marrow accessory cells. Several groups have demonstrated that the inhibitory effect of TGF-β is not restricted to the CFU-MK, since it can also alter the cloning efficiency of myeloid and erythroid progenitor cells.\(^6,8,9\) In addition, two other platelet constituents, platelet factor-4 and a unique platelet-release glycoprotein recently isolated by Desseypris et al.\(^{31}\) have also been shown to inhibit in vitro human megakaryocyte colony formation.

Our findings indicate that many cytokines may contribute to the regulation of in vitro human megakaryocytopoiesis. Since heterogeneous cell populations were used as a source of target cells in these studies, it is possible that the observed effects were the consequence of cytokines stimulating marrow accessory cells to elaborate factors that then directly influence the CFU-MK. There is some precedence for this hypothesis. IL-1α’s effect on hematopoiesis, for instance, has been in part attributed to its ability to cause either stromal cells, fibroblasts, endothelial cells, or T lymphocytes to elaborate a number of cytokines, including GM-CSF, G-CSF or IL-6, which might then directly influence hematopoietic progenitor cell proliferation.\(^{31,52-60}\)

An alternative explanation for the observed effects of cytokines on in vitro megakaryocytopoiesis, reported in this communication, involves modulation of receptors for various growth factors on committed progenitor cells by other cytokines. This phenomenon has been termed receptor trans-modulation.\(^{59}\) Colony-stimulating factors, while shown not to compete directly for each other’s binding sites, are able to rapidly downmodulate the number of receptors of other CSFs.\(^{59}\) Such receptor downmodulation frequently does not result in loss of responsiveness of these cells to the particular growth factor whose receptor has been downmodulated.\(^{18,19}\) Ligands that downmodulate cytokine receptors generally either synergize with the growth factor with regards to its proliferation-promoting activities or may even mimic the action of the growth factor in its absence.\(^{18}\) Direct analysis of ligand receptor binding on the CFU-MK will be necessary to test whether such a ligand-receptor interaction is operational in the regulation of human megakaryocytopoiesis.

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