Role of Cytokines in Sustaining Long-Term Human Megakaryocytopoiesis In Vitro

By Robert A. Briddell, John E. Brandt, Thomas B. Leemhuis, and Ronald Hoffman

An in vitro liquid suspension culture system was used to determine the role of cytokines in sustaining long-term human megakaryocytopoiesis. Bone marrow cells expressing CD34 but not HLA-DR (CD34^DR^) were used as the inoculum of cells to initiate long-term bone marrow cultures (LTBMC). CD34^DR^ cells (5 x 10^5/) mL initially contained 0.0 ± 0.0 assayable colony-forming unit-megakaryocytes (CFU-MK), 6.2 ± 0.4 assayable burst-forming unit-megakaryocytes (BFU-MK), and 0.0 ± 0.0 megakaryocytes (MK). LTBMCs were recharged every 48 hours with granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1α (IL-1α), IL-3, and/or IL-6, alone or in combination. LTBMCs were demidepopulated weekly or biweekly, the number of cells and MK enumerated, and then assayed for BFU-MK and MK. LTBMCs receiving no cytokine(s) contained no assayable CFU-MK or BFU-MK and no observ-

able MK. LTBMCs receiving GM-CSF, IL-1α, and/or IL-3 contained assayable CFU-MK and MK but no BFU-MK for 10 weeks of culture. The effects of GM-CSF and IL-3, IL-1α and IL-3, but not GM-CSF and IL-1α were additive with regards to their ability to augment the numbers of assayable CFU-MK during LTBMC. LTBMCs supplemented with IL-6 contained modest numbers of assayable CFU-MK for only 4 weeks; this effect was not additive to that of GM-CSF, IL-1α, or IL-3. The addition of GM-CSF, IL-1α, and IL-3 alone or in combination each led to the appearance of significant numbers of MKs during LTBMC. By contrast, IL-6 supplemented cultures contained relatively few MK. These studies suggest that CD34^DR^ cells are capable of initiating long-term megakaryocytopoiesis in vitro and that a hierarchy of cytokines exists capable of sustaining this process.

Several laboratories, including our own, have shown that the cells present in normal human bone marrow (BM) responsible for initiating and sustaining long-term hematopoiesis in vitro express CD34 but not the major histocompatibility class II locus HLA-DR.1-4 This same cell population (CD34^DR^) contains a variety of other primitive hematopoietic progenitor cells, including the colony-forming unit-blast (CFU-B), the high proliferative potential–colony-forming cell (HPP-CFC), and the burst-forming unit-megakaryocyte (BFU-MK).1-3,5,6

A large number of purified and recombinant cytokines are now known to affect human megakaryocytopoiesis.6-17 Our laboratory has recently concentrated its efforts on defining those cytokines that regulate the most primitive megakaryocyte progenitor cell, the BFU-MK.5,7 We have shown that granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) are each capable of promoting BFU-MK–derived colony formation by CD34^DR^ BM cells, and that the action of these cytokines is additive.5,7 In addition, IL-1α, but not IL-6, while incapable of promoting BFU-MK–derived colony formation by itself, augments the ability of IL-3 to promote the formation of BFU-MK–derived colonies.5,7

Previous attempts to establish long-term culture systems in which megakaryocytopoiesis was maintained for several weeks have met with limited success.16,26 To establish such a culture system, we have used CD34^DR^ cells as the initial cellular inoculum and sustained the proliferation of megakaryocyte (MK) progenitor cells with cytokines previously recognized to affect CFU-MK– and BFU-MK–derived colony formation in vitro.6,18 Based on these studies, we conclude that CD34^DR^ cells are capable of initiating long-term megakaryocytopoiesis and that GM-CSF, IL-1α, and IL-3 each appear to play a role in sustaining this process.

MATERIALS AND METHODS

BM 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. BM aspirates were immediately diluted 1:1 with Iscove’s modified Dulbecco’s media (IMDM; GIBCO, Grand Island, NY) containing 20 U sodium heparin/mL. Low-density mononuclear cells (LDMC) were obtained.19 LDMC were further separated by counterflow centrifugal elutriation to obtain those cells eluting at flow rates between 12 and 14 mL/min (FR 12-14).3 To further purify FR 12-14 cells for primitive progenitor cells, we used immunofluorescent flow cytometric cell sorting on a Coulter Epics 753 dual laser flow cytometer (Coulter, Hialeah, FL).1 Cell populations containing high densities of CD34 without HLA-DR expression were used for our experiments. The purity of CD34^DR^ cells was determined by post sort analysis of an aliquot of the isolated fraction of cells. The CD34^DR^ cell fraction consisted of greater than 90% CD34-positive cells and less than 10% HLA-DR–positive cells.

Human recombinant hematopoietic cytokines. The following human recombinant cytokines were used in these studies: (1) GM-CSF with a specific activity of 5.0 × 10^5 U/mg protein determined by granulocyte-macrophage colony formation from human BM cells (Genzyme, Boston, MA); (2) IL-1α with a specific activity of 1.0 × 10^5 U/mg protein determined by proliferative effects on D-10 cells (Genzyme); (3) IL-3 with a specific activity of 1.0 × 10^5 U/mg protein determined by mixed colony formation from human BM cells (Genzyme); and (4) IL-6 with a specific activity of 1.0 × 10^5 U/mg protein determined by Ig production augmentation to the half maximal level by CESS cells (Genzyme).
SUSTAINED LONG-TERM HUMAN MEGAKARYOCYTOPOIESIS

Long-term suspension cultures. Long-term stromal cell-free suspension cultures were initiated and maintained as previously described. Briefly, polystyrene 35 mm tissue culture dishes containing 1 mL IMDM with 10% vol/vol fetal bovine serum (Hyclone, Logan, UT) were inoculated with $5 \times 10^5$ CD34⁺DR⁻ cells and incubated at 37°C in 100% humidified 5% CO₂ in air. At this time, and every 48 hours thereafter,² cultures received no additions, 200.0 pg/mL GM-CSF, 1.0 ng/mL IL-1α, 1.0 ng/mL IL-3, 30.0 ng/mL IL-6, or combinations of the aforementioned cytokines. Cytokine concentrations chosen to be used in these studies were the optimal concentrations known to promote in vitro BFU-MK colony formation as previously reported by our laboratory.³ At weeks 1, 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; mature MK were identified using the immunofluorescent techniques described below.

MK progenitor cell assay system. Cell samples from each cell fraction were assayed for their ability to produce MK colonies in a single serum-depleted, fibrin clot culture system.⁴ 1.0 ng/mL of IL-3 was used as a source of MK-colony-stimulating activity (MK-CSA). Cultures were incubated for 18 days 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. Fixed plates were stored at 4°C until immunofluorescent staining was performed.

Immunofluorescent identification. A murine monoclonal antibody (MoAb) to the human platelet glycoprotein (GP) Ib-IIIa complex receptor (10E5, provided by Dr Barry S. Coller, Stony Brook, NY) was used as the immunologic probe for identifying human MKs and MK colonies.²² Cultures were scored in situ to enumerate fluorescein-positive colonies. The 35 mm petri dishes were viewed through the objective of a fluorescent microscope at 100×. Mature MK were observed as clusters of 42 or more fluorescent cells distributed in at least two foci of development.²² The results are expressed as the number of previous demidepopulated MK colonies (data not shown). The IL-3–supplemented cultures contained twice the number of assayable MK-MK on weeks 1 to 10 as the cultures receiving GM-CSF (Fig 2). In addition, those cultures receiving the combination of GM-CSF/IL-3 contained significantly greater numbers of assayable MK-MK than those cultures containing either GM-CSF or IL-3 alone (Fig 2, $P < .05$). The effects of GM-CSF and IL-3 appeared, largely, to be additive (Fig 2).

IL-1α alone had a significant ability to sustain long-term megakaryocytopoiesis, leading to a similar cumulative production of CFU-MK over the 10-week period as IL-3 (Fig 3, $P < .05$). The effects of the IL-1α/IL-3 combination on long-term megakaryocytopoiesis were far greater than that observed with either cytokine alone (Fig 3, $P < .05$). Such an additive effect was not observed when IL-1α was added to cultures containing either IL-6 or GM-CSF (data not shown, $P > .05$).

While the addition of IL-6 alone to CD34⁺DR⁻ cells resulted in the appearance of assayable MK progenitor cells in LTBMC ($P < .05$), this persisted for only 4 weeks

RESULTS

Each LTBMC was initiated with $5 \times 10^5$ CD34⁺DR⁻ marrow cells to which no cytokines, individual cytokines, or combinations of cytokines were added. Before the initiation of LTBMC, this cell population obtained from four separate normal donors contained no morphologically or immunologically identifiable MKs or assayable CFU-MK, but did contain $6.2 \pm 0.4$ BFU-MK per $5 \times 10^5$ CD34⁺DR⁻ cells. These LTBMCs did not contain a pre-established adherent cell layer, nor did such a layer develop during the period of observation. LTBMCs were demidepopulated, counted, the number of MKs enumerated, and assayed for both BFU-MK and CFU-MK at weeks 1, 2, 3, 4, 6, 8, 10, 12, and 14. In the LTBMCs not receiving any exogenous cytokines, viable cells were not detected after one week (Fig 1). By contrast, each LTBMC receiving individual cytokines or cytokine combinations contained viable cells for 6 to 10 weeks with approximately a 10- to 200-fold cumulative increase in cell numbers (Fig 1). The combination of IL-1α and IL-3 was the most efficient in enhancing cellular proliferation and expansion (Fig 1).

In the LTBMC not receiving exogenous cytokines, assayable CFU-MK and BFU-MK were not detected during the period of observation (Figs 2, 3, and 4). Those LTBMCs that received repeated additions of GM-CSF alone, IL-3 alone, or the combination of GM-CSF/IL-3 contained assayable CFU-MK for not greater than 10 weeks (Fig 2). No assayable BFU-MK were detected during the duration of the LTBMCs (data not shown). The IL-3–supplemented cultures contained nearly twice the number of assayable CFU-MK on weeks 1 to 10 as the cultures receiving GM-CSF (Fig 2). In addition, those cultures receiving the combination of GM-CSF/IL-3 contained significantly greater numbers of assayable MK-MK than those cultures containing either GM-CSF or IL-3 alone (Fig 2, $P < .05$). The effects of GM-CSF and IL-3 appeared, largely, to be additive (Fig 2).

IL-1α alone had a significant ability to sustain long-term megakaryocytopoiesis, leading to a similar cumulative production of CFU-MK over the 10-week period as IL-3 (Fig 3, $P < .05$). The effects of the IL-1α/IL-3 combination on long-term megakaryocytopoiesis were far greater than that observed with either cytokine alone (Fig 3, $P < .05$). Such an additive effect was not observed when IL-1α was added to cultures containing either IL-6 or GM-CSF (data not shown, $P > .05$).

While the addition of IL-6 alone to CD34⁺DR⁻ cells resulted in the appearance of assayable MK progenitor cells in LTBMC ($P < .05$), this persisted for only 4 weeks
Fig 2. Effect of no addition, GM-CSF, IL-3, or GM-CSF/IL-3 on the number of assayable CFU-MK per milliliter during LTBMC. CFU-MK were harvested by demidepopulation from their corresponding LT-BMC and cultured using a serum-depleted, fibrin clot assay system with 1.0 ng IL-3/mL as MK-CSA. Each point represents the corrected cumulative mean of observed CFU-MK-derived colonies determined weekly or biweekly and obtained from four separate experiments.

The numbers of CFU-MK observed in these LTBMCs were significantly less than those receiving GM-CSF, IL-1α, or IL-3 (Fig 4, P < .05). The addition of IL-6 to cultures containing either GM-CSF or IL-3 did not substantially alter the number of assayable CFU-MK during the period of observation when compared with cultures receiving either GM-CSF or IL-3 alone (Fig 4, P > .05).

Mature MKs were not seen in LTBMCs to which no exogenous cytokines were added (data not shown). Repeated additions of IL-6 to LTBMCs led to the appearance of mature MKs for only 4 weeks, while repeated additions of IL-1α or IL-3 resulted in the appearance of mature MKs for 8 and 10 weeks, respectively (Fig 5A). GM-CSF supplementation led to the appearance of MKs for 10 weeks of LTBMC (data not shown). The peak appearance of MKs occurred on week 2 in LTBMCs receiving the combination IL-3/IL-6, while the greatest numbers of MK were observed in cultures receiving IL-1α in combination with IL-3 on weeks 8 to 10 (Fig 5B). The cumulative MK production by the LTBMCs to which the various cytokines were added is shown in Table 1. The greatest overall numbers of MKs appeared in LTBMCs to which GM-CSF, IL-1α, or IL-3 were added, while the IL-6-supplemented LTBMCs contained relatively few MKs (Table 1). The effects of combinations of GM-CSF/IL-3 or IL-1α/IL-3 on the appearance of MKs were additive, resulting in the appearance of far greater numbers of MKs than in LTBMCs supplemented with single cytokines or other cytokine combinations (Table 1). Nonetheless, a somewhat surprising result was the observation that the combination of IL-6 with either GM-CSF or IL-3 led to the appearance of fewer MKs than in the presence of GM-CSF or IL-3 alone (Table 1).

DISCUSSION

LTBMC systems have been used extensively to study in vitro mammalian hematopoiesis. Dexter et al initially developed a murine system from which hematopoietic stem cells and granulocytic progenitor cells could be assayed for several months, while erythroid and MK progenitor cells appeared for far shorter periods of time.23 The maintenance of these cultures was dependent on the formation of an adherent stromal layer composed of endothelial cells, adipocytes, reticular cells, and macrophages.23 Similar methods have also been adapted for the study of human hematopoiesis.24-26 In each of these reports, granulopoiesis predominated for long periods of time, while erythropoiesis was sustained for far shorter periods of time. Long-term megakaryocytopoiesis has not frequently been quantitated in such publications and has been the subject of only a handful of reports.26-28

Recently, our laboratory has developed a LTBMC system for human hematopoiesis that is not dependent on the establishment of an adherent cell layer.7 In this system, the
SUSTAINED LONG-TERM HUMAN MEGAKARYOCYTOPOIESIS

LTBMCs are initiated with subpopulations of BM cells and are then supplemented with individual cytokines or cytokine combinations every 48 hours for the duration of the culture period. These LTBMCs were initiated with CD34+DR- cells and were repeatedly supplemented with several cytokines known to promote MK colony formation in vitro. GM-CSF, IL-1α, IL-3, and IL-6 were each evaluated as cytokines potentially capable of sustaining long-term megakaryocytopoiesis in vitro.

The CD34+DR- marrow subpopulation used to initiate these LTBMCs is known not only to contain the BFU-MK, but also the CFU-BI, and the LTBMC-initiating cells (LTB-MIC), in addition to the HPP-CFC. The BFU-MK is a lineage-restricted progenitor cell, whereas the CFU-BI and the LTBMC are known to be capable of differentiation into multiple hematopoietic lineages.

It is at present unknown whether all or just some of these primitive hematopoietic elements contribute to the long-term production of MK progenitor cells observed in these studies. Because the initial cellular inoculum used to initiate the LTBMC contained limited numbers of BFU-MK and no CFU-MK, the cumulative production of over 400 CFU-MK-derived colonies over the 10 weeks of LTBMC suggests extensive commitment to and expansion of the megakaryocytic lineage occurring during the culture period.

Both GM-CSF and IL-3 have been shown individually by a number of laboratories to promote BFU-MK- and CFU-MK-derived colony formation in vitro. The activities of these two cytokines have also been shown to be additive. GM-CSF and IL-3, in the present studies, were each also shown to be capable of sustaining long-term megakaryocytopoiesis; these actions also were additive. These data again emphasize the pivotal role that these cytokines play in MK development.

IL-1α alone is incapable of stimulating either CFU-MK- or BFU-MK-derived colony formation. However, IL-1α was shown by Briddell and Hoffman to be capable of enhancing the ability of IL-3 to promote BFU-MK-derived colony formation. IL-1α was also found, in the present studies, to be able to promote long-term megakaryocytopoiesis. This effect on long-term megakaryocytopoiesis was additive with that of IL-3 but not GM-CSF. Because IL-1α has no MK-CSA alone, it is possible that its effect on long-term megakaryocytopoiesis is due to an ability to promote commitment to this particular lineage. At present, it is impossible to determine the exact cellular target of IL-1α in producing its effect on long-term megakaryocytopoiesis. Our group has previously presented data to suggest that IL-1α's effect on IL-3-promoted BFU-MK-derived colony formation was due to its direct action on the BFU-MK and not due to the secondary release of cytokines

Table 1. Effect of Various Cytokines Added Alone or in Combination on Cumulative MK Production During LTEMC

<table>
<thead>
<tr>
<th>Cytokine(s)</th>
<th>Cumulative MK Production*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>0†</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>946</td>
</tr>
<tr>
<td>IL-1α</td>
<td>575</td>
</tr>
<tr>
<td>IL-3</td>
<td>1,360</td>
</tr>
<tr>
<td>IL-6</td>
<td>156</td>
</tr>
<tr>
<td>GM-CSF/IL-1α</td>
<td>1,137</td>
</tr>
<tr>
<td>GM-CSF/IL-3</td>
<td>3,100†</td>
</tr>
<tr>
<td>GM-CSF/IL-6</td>
<td>510†</td>
</tr>
<tr>
<td>IL-1α/IL-3</td>
<td>3,380†</td>
</tr>
<tr>
<td>GM-CSF/IL-1α</td>
<td>425</td>
</tr>
<tr>
<td>IL-3/IL-6</td>
<td>394†</td>
</tr>
</tbody>
</table>

Concentrations of cytokines used: GM-CSF, 250.0 pg/mL; IL-1α, 1.0 ng/mL; IL-3, 1.0 ng/mL; IL-6, 30.0 ng/mL.

* Cumulative MK production = the mean of the cumulative production of MK from two separate LTBMCs each initiated with 5 x 10⁶ CD34+DR- marrow cells, and to which the above listed cytokine(s) were added every 48 hours. One-half milliliter samples from each LTBMC were obtained weekly or biweekly and the numbers of MKs enumerated using immunofluorescent antibody labeling.

† Each point represents the mean of cumulative MK production taken from two separate experiments.

< .05 when compared with either of the two individual cytokines added alone to culture.
by marrow auxiliary cells.\textsuperscript{7} Kimura et al have recently suggested that IL-1\textbeta can dramatically alter thrombopoiesis by causing the secondary release of IL-6, or a combination of other cytokines, which then accelerates thrombocytopoiesis.\textsuperscript{25} It seems unlikely that the ability of IL-1\textalpha to promote long-term megakaryocytopoiesis is due to secondary elaboration of IL-6, because IL-6 by itself is unable either to promote long-term megakaryocytopoiesis or to enhance the effect of IL-3.\textsuperscript{26,29}

Mature MKs were easily identifiable within the cytokine-supplemented LTBMCS. The differing kinetics of appearance of these mature MKs in the LTBMCS supplemented with various cytokines are consistent with the known hierarchical effects of cytokines affecting the various stages of human megakaryocytopoiesis.\textsuperscript{28} The cumulative production of MKs in the various LTBMCS is shown in Table 1. The cultures supplemented with IL-6 produced few MKs, but those that did appear were present by 2 weeks of culture. By contrast, the largest numbers of MKs appeared in cultures receiving GM-CSF, IL-1\textalpha, and IL-3, alone or in combination, with the greatest production occurring after 6 to 10 weeks of LTBM. Over the period of LTBM, the $5 \times 10^3$ CD34\textsuperscript*DR$^-$ cells that received repeated additions of IL-1\textalpha/IL-3 and GM-CSF/IL-3 combinations produced over 3,000 MKs, while the IL-6-supplemented cultures produced a meager 150 (Table 1). Although IL-6 is known to act as a maturation factor on immature and mature MK elements, it has little effect on either the CFU-MK or BFU-MK.\textsuperscript{33,34,35} The relative lack of efficacy of IL-6 in producing the appearance of mature MKs in LTBMCS can be accounted for by the absence of MKs in the initial CD34\textsuperscript*DR$^-$ cell population and its lack of ability to generate those cells from the cellular inoculum. The cause of the apparent blunting of the effect of GM-CSF or IL-3 by IL-6 on the production of MKs remains unknown but might be due to an adverse effect of IL-6 on the commitment process to the MK lineage. By contrast, the appearance of greater numbers of MKs in cultures receiving GM-CSF, IL-1\textalpha, or IL-3 is consistent with the primary action of these cytokines being on the CFU-MK and/or the BFU-MK generated from the CD34\textsuperscript*DR$^-$ cells.\textsuperscript{5,7,14} The additional known MK maturation effects of both GM-CSF and IL-3 likely contribute to the terminal maturation of MKs observed in these cultures.\textsuperscript{36}

The suspension culture system used in the present studies provides a means of defining the biologic behavior and cytokine requirements of primitive hematopoietic progenitor cells capable of sustaining megakaryocytopoiesis over a prolonged period of time. Based on these studies, at least three cytokines, GM-CSF, IL-1\textalpha, and IL-3, each appear to play an important role in the regulation of these cellular events. IL-6 appears incapable of providing such a proliferative stimulus to cellular elements capable of sustaining long-term megakaryocytopoiesis in vitro, but does act as a potent maturation factor acting on more differentiated megakaryocytic elements.\textsuperscript{5,35} These findings suggest that a hierarchy of cytokines exists that is capable of affecting a number of cellular stages occurring during megakaryocyte development.

ACKNOWLEDGMENT

We thank Stephanie A. McGillem and Deborah A. Navarro for their secretarial assistance in the preparation of this manuscript.

REFERENCES


Role of cytokines in sustaining long-term human megakaryocytopoiesis in vitro

RA Briddell, JE Brandt, TB Leemhuis and R Hoffman