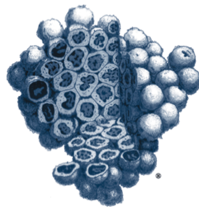

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Evaluation of Cytokines for Expansion of the Megakaryocyte and Granulocyte Lineages

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Key Words. *Megakaryocytes · Granulocytes · Progenitors · Serum-free medium · Liquid culture · CD34⁺ cells · Peripheral blood*

ABSTRACT

The goal of our study was to identify cytokine combinations that would result in simultaneous ex vivo expansion of both the megakaryocyte (Mk) and granulocyte lineages, since these cell types have the potential to reduce the periods of thrombocytopenia and neutropenia following chemotherapy. We investigated the effects of cytokine combinations on expansion of the Mk (CD41a$^+$ cells and colony forming unit [CFU]-Mk) and granulocyte (CD15$^+$ cells and CFU-granulocyte/monocyte [GM]) lineages. Peripheral blood CD34$^+$ cells were cultured in serum-free medium with interleukin 3 (IL-3), stem cell factor (SCF), and various combinations of thrombopoietin (TPO), IL-6, GM-CSF, and/or G-CSF. The Mk lineage was primarily influenced by TPO in our cultures, although Mk and CFU-Mk numbers were increased when TPO was combined with IL-6. The primary stimulator of the granulocyte lineage was G-CSF, although many synergistic and additive effects were observed with addition of other factors. Expansion of CFU-GM increased upon addition of more cytokines. The cytokine combination of IL-3, SCF, TPO, IL-6, GM-CSF and G-CSF produced the greatest number of granulocytes and CFU-GM. The minimum cytokines necessary for expansion of both the Mk and granulocyte lineages included TPO and G-CSF, since no other factors examined could increase Mk and granulocyte numbers to the same extent. The number of hematopoietic progenitors produced in our culture system should be sufficient for successful engraftment following myelosuppressive therapy if produced on a scale of about one liter. *Stem Cells* *1997;15:198-206*

INTRODUCTION

The major side effects associated with transplantation of bone marrow (BM) or peripheral blood (PB) progenitors are periods of chemotherapy-induced neutropenia and thrombocytopenia. For PB progenitors, neutrophil counts take approximately eight to nine days and platelet counts take approximately 12 days to recover [1]. Conceptually, these periods of cytopenia could be largely eliminated by administering mature cells and late neutrophil and megakaryocyte (Mk) progenitors as a supplement to uncultured cells for myeloablative therapy or alone for myelosuppressive therapy. Ex vivo expansion promises to improve the current methodology for blood cell progenitor transplantation following chemotherapy by increasing both the number and maturity of cells available for transplantation.

Several clinical studies are evaluating transplantation of ex vivo expanded hematopoietic cells. In these clinical trials, either bone marrow mononuclear cells (MNC) or PB CD34$^+$ cells have been expanded for 9 to 14 days with various cytokine combinations including interleukin 3 (IL-3), GM-CSF, erythropoietin (EPO), and stem cell factor (SCF) [2]; PIXY321 and EPO [3]; PIXY321 [4]; and IL-3, IL-6, IL-1, EPO, and SCF [5, 6]. Although results of these initial Phase I clinical ex vivo expansion studies look promising, platelet and neutrophil recovery times were not greatly improved. Further studies to examine cytokine combinations for expansion of the Mk and neutrophil lineages may solve some of the problems associated with platelet and neutrophil recovery. Since platelet recovery times are often longer and more variable than neutrophil recovery times, thrombopoietin (TPO), which promotes both proliferation and maturation of megakaryocytes, will be an invaluable addition to cytokine mixtures. Addition of TPO to cultures of human PB CD34$^+$ cells has resulted in cultures with

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20%-100% Mks [7]. In colony assays, TPO has been found to synergize with SCF and to have an additive effect when combined with IL-3 [7]. When TPO is present, Mk ploidy is increased, and Mks can produce functional platelets [8].

Our initial study focused on maximizing expansion of the Mk lineage without depleting the granulocyte lineage. All cultures in our study were supplemented with IL-3 and SCF, since IL-3 has been shown to increase the number and size of Mk colonies [9], and a further increase in colony number was found upon addition of SCF to IL-3 [10, 11]. We evaluated TPO in combination with IL-6 and GM-CSF because these latter factors have been found to affect various stages of megakaryocytopoiesis [11-20]. IL-6 and GM-CSF have also been found to promote expansion of the granulocyte lineage. Addition of G-CSF to the cytokine combinations mentioned above was also examined to determine if we could further increase granulocyte expansion in serum-free medium and thereby produce clinically relevant numbers of Mks, granulocytes, and colony-forming cells (CFC).

Serum-free medium was used in our study because serum inhibits megakaryocytopoiesis [8, 21-23], contains both inhibitory and stimulatory factors that complicate analysis of cytokine interactions [24], and results in an undefined and variable growth medium. Cultures were grown under 20% oxygen to increase production of Mks [25]. PB CD34$^+$ cells were used instead of MNC to provide a more uniform starting population depleted of mature cell types. A low inoculum density was used to maximize expansion and differentiation [26, 27], while reducing endogenous release of cytokines [12].

Materials and Methods

Cells and Cell Separation Procedures

Patient samples (Response Oncology; Memphis, TN) were collected after informed consent under protocols approved by the respective Institutional Review Boards. Apheresis products were collected from cancer patients following stem cell mobilization regimens consisting of treatment with G-CSF with or without chemotherapy. Positive selection of CD34$^+$ cells was performed using one of two methods. The MiniMacs column was used according to the directions of the manufacturer (Miltenyi Biotec; Auburn, CA). The other method has been previously described [28], and involved incubation of apheresis products with mouse anti-CD34 antibody (9069, Baxter Immunotherapy Division; Irvine, CA), capture of CD34$^+$ cells using sheep antimouse IgG-Fc-coated paramagnetic beads (Dynal; Oslo, Norway), and release of CD34$^+$ cells from the beads using chymopapain (Chymodiactin, Bootes Pharmaceutical; Lincolnshire, IL). The average purity of the CD34$^+$ cell populations was approximately 90%.

Culture and Harvest of Cells

All cultures were performed with XVIVO-20 medium (BioWhittaker; Walkersville, MD) supplemented with 1.5 ng/ml IL-3 (R&D Systems; Minneapolis, MN) and 50 ng/ml SCF (R&D Systems). The following cytokines were added to the base combination as indicated: 10 ng/ml IL-6 (R&D Systems), 4 ng/ml GM-CSF (Immunex; Seattle, WA), 1.5 ng/ml G-CSF (Amgen; Thousand Oaks, CA), and 25 ng/ml TPO (Genentech; S. San Francisco, CA). CD34$^+$ cells were plated at 2×10^4 cells/ml in 1 ml volumes in the center eight wells of 24-well plates. The outer wells were filled with water to minimize evaporation from the inner wells. Cells were cultured for 10 days at 37°C under fully humidified conditions in an atmosphere of 5% CO_2 in air. Cultures were fed at day 6 by doubling the culture volume with fresh growth factor-containing medium. Cultures were harvested at day 10 by removing the cell suspension from the culture plate, rinsing the plate with cell dissociation solution (Sigma Chemical; St. Louis, MO; C-5789) to remove any adherent cells, and then washing the plate with phosphate-buffered saline (PBS). To determine the total number of cells in each culture, a small volume of cell suspension was added to 10 ml of cetrimide solution, and the released nuclei were counted on a Coulter counter (Coulter Electronics; Hialeah, FL). All reagents were obtained from Sigma Chemical unless otherwise stated.

Colony Assays

Approximately 1-2.5 $\times 10^3$ cells/ml were inoculated into Iscove's modified Dulbecco's medium containing 1.1% methylcellulose (Dow; Midland, MI), 30% fetal bovine serum (Hyclone; Logan, UT), 2% bovine serum albumin (Intergen; Purchase, NY), 50 mg/ml gentamicin sulfate (GIBCO; Grand Island, NY), 10^{-4} M 2-mercaptoethanol, 1.5 ng/ml IL-3, 4 ng/ml GM-CSF, 1.5 ng/ml G-CSF, 10 ng/ml IL-6, 3 U/ml EPO (Amgen), and 50 ng/ml SCF. One ml aliquots were plated in duplicate into 35 mm suspension culture dishes (Nunc; Naperville, IL) for 14 days at 37°C under fully humidified conditions in an atmosphere of 5% CO_2 and 5% O_2 in N_2. Colonies of 50 or more white cells were scored as colony forming unit-granulocyte/monocyte (CFU-GM) using a dark-field stereomicroscope.

CFU-Mk assays were performed using the method previously described by *Hunt et al*. [7]. Cells were plated at 2×10^4 cells/ml in XVIVO-20 supplemented with 1.5 ng/ml IL-3, 50 ng/ml SCF, and 25 ng/ml TPO. Aliquots of 300 µl were plated in triplicate into 96-well plates for 12 days at 37°C under fully humidified conditions in an atmosphere of 5% CO_2 in air. The outer wells of the plates were filled with water to minimize evaporation from the inner wells. After 12 days, cells were fixed in the wells with glutaraldehyde and the Mks were stained using anti-CD41a antibody as described below.

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Colonies of greater than three CD41a+ cells were scored as CFU-Mk.

Cytospins

Approximately 2×10^4 cells from each sample were centrifuged onto a slide using a Cytospin 2 (Shandon; Pittsburgh, PA). Cells were centrifuged for 5 min at 850 rpm and then immediately fixed with acetone.

Cytochemical Staining

Cytospins of cells were stained for CD41a and CD15 antigens using a Histomark strepavidin-horseradish peroxidase kit (Kirkegard & Perry; Gaithersburg, MD). The staining procedure was performed at 25°C. Slides were soaked in a 15:1 mixture of PBS and 30% H_2O for 10 min to block endogenous peroxidase and then washed with PBS. Slides were then incubated with normal goat serum for 15 min in a humidified environment to block nonspecific staining. Excess serum was subsequently wicked off, and slides were incubated with anti-CD41a antibody (1:500 dilution in PBS) or anti-CD15 antibody (1:100 dilution in PBS) (AMAC; Westbrook, ME) for 1 h. Slides were then incubated with biotinylated goat anti-mouse secondary antibody for 30 min, strepavidin-peroxidase for 30 min, and a chromogen solution (Peroxidase chromogen kit; Biomeda; Foster City, CA) for 2-10 min for color development, with a PBS wash performed prior to each step. Slides were rinsed with Millipore water and then stained with hematoxylin (Biomeda) for 1 min. Slides were rinsed for a final time with tap water and then air-dried, coated with Crystal Mount (Biomeda), and dried overnight before applying a coverslip using Accumount (Scientific Products; McGaw Park, IL). Approximately 400 cells per slide were counted to determine the percentage of CD41a+ or CD15+ cells in the cultures.

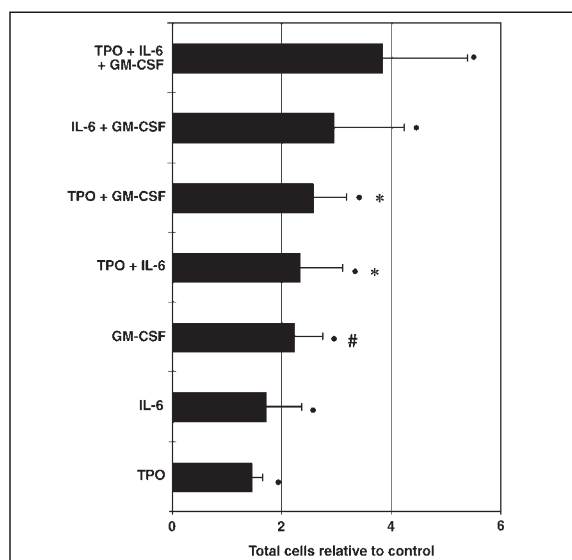

Figure 1. Total cell production from day 10 cultures of PB CD34+ cells using XVIVO-20 supplemented with IL-3, SCF and the indicated cytokines. *The control cultures contained IL-3 and SCF. The means ± SD relative to control cultures of nine experiments are indicated. Control cultures contained 17 ± 7.4 × 10⁴ cells in 2 ml. (•) significant increase (p < 0.05) over control cultures. (*) significant difference (p < 0.05) between two-factor addition and either factor alone. (#) significant increase (p < 0.05) over IL-6 or TPO alone.*

Image Analysis

Images of cultured cells stained with anti-CD41a antibody were captured at 400× magnification as PICT files (240 × 192 pixels) using a Kodak EktaPro video system (Eastman Kodak Company; San Diego, CA) and an Olympus microscope (Model BHTU B061) (Olympus; New York, NY) with a Power Macintosh 7100/80 AV connected to the video out signal. Pixel area measurements were obtained using the program Image v1.59 (NIH) and a slide micrometer was used to convert pixel area measurements to μm^2 area measurements. An average of 80 CD41a+ cells were measured for each culture condition for each experiment.

Statistical Analysis

Except where indicated (Table 1), data (absolute cell numbers) were analyzed using the Wilcoxon signed rank test. Points marked on the graphs indicate differences that were statistically significant, as indicated in the figure legends.

RESULTS

Total Cells

TPO, IL-6, and/or GM-CSF were added to the base combination of IL-3 and SCF to determine if an additive or synergistic increase in either of the megakaryocyte or granulocyte lineages would result by combining these factors. All of the cytokine combinations examined simulated an increase in the total number of cells over the level in control cultures (Fig. 1). The one-factor addition that resulted in the greatest expansion of total cells was GM-CSF. The combinations of TPO plus IL-6 or TPO plus GM-CSF produced a greater number of cells than those produced with TPO, IL-6, or GM-CSF alone.

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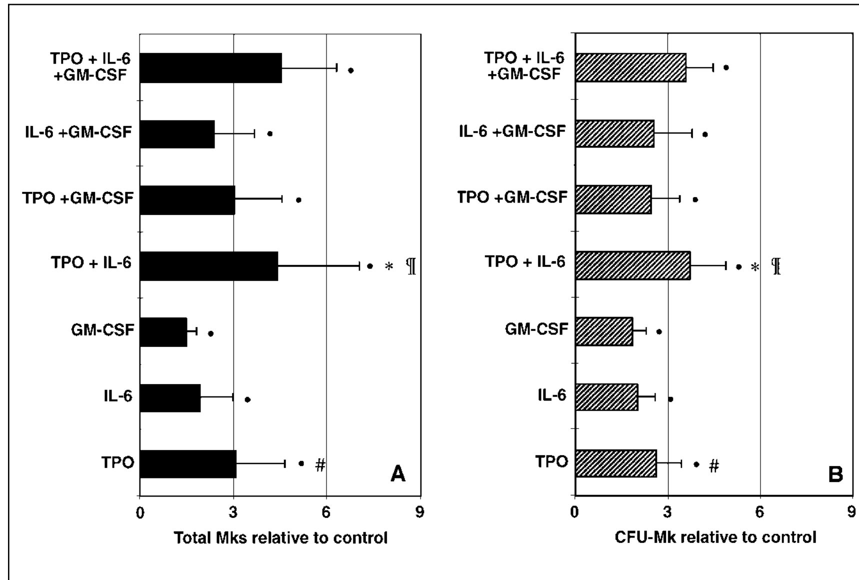

Figure 2. Total Mks (CD41a⁺) (A) and CFU-Mk (B) from day 10 cultures of PB CD34⁺ cells using XVIVO-20 supplemented with IL-3, SCF and the indicated cytokines. *The control cultures contained IL-3 and SCF. The means ± SD relative to control cultures of eight experiments for Mks and six experiments for CFU-Mk are indicated. Control cultures contained 2.7 ± 2.1 × 10⁴ Mks and 2.5 ± 1.0 × 10² CFU-Mk in 2 ml. (•) significant increase (p < 0.05) over control cultures. (*) significant difference (p < 0.05) between two-factor addition and either factor alone. (#) significant increase (p < 0.05) over IL-6 or GM-CSF alone. (¶) significant increase (p < 0.05) over other two-factor additions.*

Megakaryocyte Lineage

The effects of the different cytokine combinations on both Mk and CFU-Mk production are shown in Figure 2. All cytokine additions examined produced a significant increase in the numbers of Mk and CFU-Mk over those produced in control cultures. Cultures supplemented with TPO alone gave the greatest increase in both total Mks and CFU-Mk compared to the other one-factor additions. Treatment of cultures with both TPO and IL-6 produced an almost additive increase (4.4-fold over control) in Mks. Cultures supplemented with both of these cytokines were comprised of 28% Mks on average, while addition of GM-CSF significantly decreased the percent of Mks in the cultures (Fig. 4A). TPO plus IL-6 also increased CFU-Mk formation by 3.7-fold, which was greater than the level produced by either factor alone. Addition of the combination of TPO plus GM-CSF or IL-6 plus GM-CSF resulted in production of the same number of Mks and CFU-Mks as were produced in cultures supplemented with TPO alone. Thus, the two factor addition that resulted in the greatest increase in the total number of Mk and CFU-Mk was TPO plus IL-6. We observed that addition of both IL-6 and TPO did not significantly alter the size of the Mks from that found with TPO alone (201 ± 37 μm^2 for control and 346 ± 20 μm^2 for TPO alone versus 314 ± 75 μm^2 for TPO + IL-6). Further addition of GM-CSF to the combination of IL-6 and TPO did not provide any additional stimulation of the Mk lineage and resulted in a decrease in Mk size (227 ± 51 μm^2 for TPO + IL-6 + GM-CSF).

Granulocyte Lineage

The effects of the different cytokine combinations on both CD15⁺ cell and CFU-GM production are shown in Figure 3. All of the cytokine combinations, except TPO alone, produced significantly more granulocytes than control cultures. CFU-GM production was stimulated over control levels by all of the cytokine combinations. When the effects of one-factor additions were examined, we found that IL-6 or GM-CSF gave significantly increased granulocyte numbers compared to those for TPO. In contrast, cultures supplemented with either TPO, IL-6, or GM-CSF all produced a similar increase in CFU-GM formation. For the two-factor combinations examined, we observed a greater number of additive and synergistic effects on the granulocyte lineage than were seen with the Mk lineage. The greatest increase in granulocyte number and percent of granulocytes was found in cultures containing both IL-6 and GM-CSF (Figs. 3A and 4B). Supplementation of cultures with any of the two-factor combinations examined resulted in an additive increase in CFU-GM numbers. Addition of all factors together resulted in a synergistic increase in granulocytes (30-fold over control) and an additive increase in CFU-GM numbers (7.3-fold over control). Although the greatest stimulation in both granulocyte and CFU-GM numbers occurred when cultures were supplemented with TPO, IL-6, and GM-CSF, the percentage of granulocytes found in these cultures was only 7%-13% (Fig. 4B).

Addition of G-CSF to Cultures

In an effort to increase granulocyte numbers and frequency, we examined the effects of adding G-CSF to cultures of CD34⁺ cells. Addition of G-CSF increased the total cell number for all of the cytokine combinations discussed above (Table 1). For all cytokine combinations, addition of G-CSF increased the granulocyte fold-expansion by a factor

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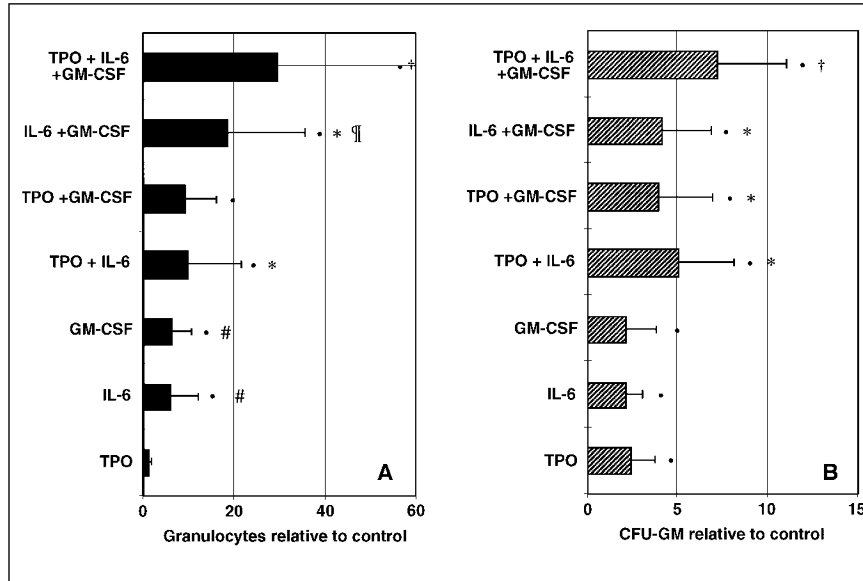

*Figure 3. **Total granulocytes (CD15+) (A) and CFU-GM (B) from day 10 cultures of PB CD34+ cells using XVIVO-20 supplemented with IL-3, SCF and the indicated cytokines.** The control cultures contained IL-3 and SCF. The means ± SD relative to control cultures of seven experiments are indicated. Control cultures contained $5.4 \pm 4.2 \times 10^3$ granulocytes and $1.8 \pm 1.2 \times 10^3$ CFU-GM in 2 ml. (•) significant increase ($p < 0.05$) over control cultures. (*) significant difference ($p < 0.05$) between two-factor addition and either factor alone. (†) significant difference ($p < 0.05$) between three-factor addition and each of the three two-factor additions. (#) significant increase ($p < 0.05$) over TPO alone. (¶) significant increase ($p < 0.05$) over other two-factor additions.*

ranging from 3.3 to 29 (Table 1). In all cases, addition of G-CSF resulted in a much greater increase in granulocyte number than did the addition of GM-CSF. The greatest increase in granulocyte number was found upon addition of TPO, IL-6, GM-CSF and G-CSF. Addition of G-CSF at least doubled the number of CFU-GM for all cytokine combinations (Table 1). As for granulocytes, G-CSF was more potent than GM-CSF in expansion of CFU-GM. CFU-GM production was greatest in cultures containing TPO, GM-CSF, G-CSF and IL-6. While G-CSF greatly influenced both the total number of granulocytes and CFU-GM (Table 1), and the fraction of granulocytes (20%-32% CD15+ cells, Table 2), addition of G-CSF did not significantly change the total number of Mks or CFU-Mk in the cultures (Table 1). As a result, the percentage of Mks in the cultures decreased upon addition of G-CSF (data not shown).

Optimal Combinations

The cytokine combinations which resulted in the greatest expansion of both the Mk and granulocyte lineages are shown in Table 2. Addition of the lineage-specific factors TPO and G-CSF was necessary for expansion of their respective lineages, especially at the postprogenitor stage. However, in order to achieve maximum expansion of granulocytes, Mks and CFCs, cultures must be supplemented with all six factors. After 10 days in culture with IL-3, SCF, TPO, IL-6, GM-CSF and G-CSF, we obtained 29-fold expansion of total cells and 17-fold expansion of CFU-GM. In addition, these cultures

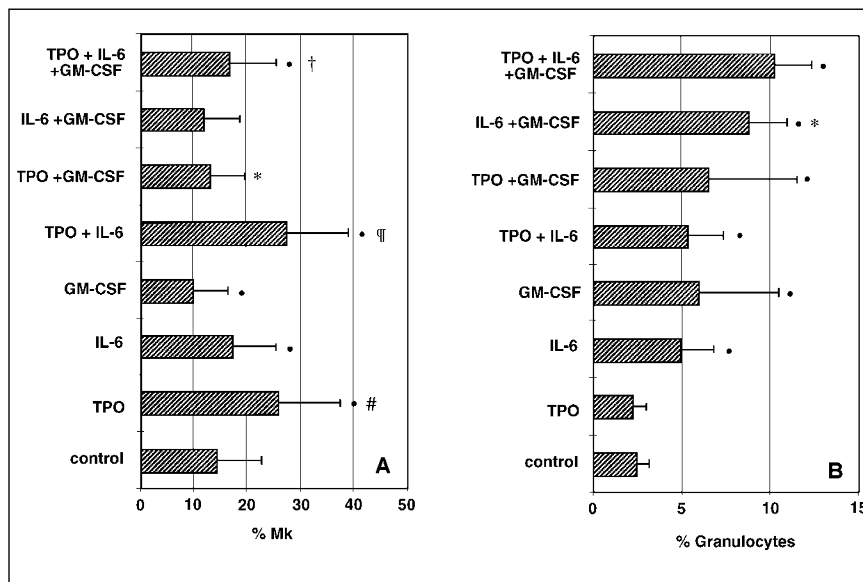

*Figure 4. **Percentage of Mks (A) and granulocytes (B) from day 10 cultures of PB CD34+ cells using XVIVO-20 supplemented with IL-3, SCF and the indicated cytokines.** The control cultures contained IL-3 and SCF. The means ± SD of seven experiments are indicated. (•) significant difference ($p < 0.05$) from control cultures. (*) significant difference ($p < 0.05$) between two-factor addition and either factor alone. (†) significant difference ($p < 0.05$) between three-factor addition and each of the three two-factor additions. (#) significant increase ($p < 0.05$) over IL-6 or GM-CSF alone. (¶) significant increase ($p < 0.05$) over other two-factor additions.*

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Table 1. Effects of G-CSF addition on PB CD34+ cell cultures (relative to control)

	G-CSF	Total cells	CD15+	CFU-GM	CD41a+	CFU-Mk
Control	–	1.0	1.0	1.0	1.0	1.0
	+	1.8 ± 0.8*	29.1 ± 22.2*	6.5 ± 2.7*	1.3 ± 0.4	1.4 ± 0.9
TPO	–	1.4 ± 0.04	1.7 ± 0.3	2.7 ± 1.9	2.4 ± 0.1	2.4 ± 0.6
	+	2.2 ± 1.0*	34.4 ± 17.5*	10.3 ± 6.3*	2.4 ± 0.5	2.5 ± 1.8
IL-6	–	1.4 ± 0.4	5.1 ± 3.4	2.4 ± 1.3	1.4 ± 0.2	1.9 ± 0.9
	+	2.1 ± 0.8*	36.8 ± 24.5*	8.4 ± 2.7*	1.5 ± 0.3	1.9 ± 1.0
GM-CSF	–	2.4 ± 0.9	8.8 ± 9.7	6.4 ± 2.2	1.6 ± 0.4	2.0 ± 0.7
	+	2.9 ± 1.5*	75.7 ± 54.3*	13.7 ± 7.9*	1.6 ± 0.2	2.5 ± 1.6
TPO + IL-6	–	1.9 ± 0.06	11.7 ± 3.4	2.9 ± 3.7	3.2 ± 0.2	3.5 ± 0.3
	+	2.5 ± 0.9*	38.2 ± 20.8*	15.0 ± 6.4*	2.6 ± 0.9	3.0 ± 2.1
TPO + GM-CSF	–	2.6 ± 0.7	12.7 ± 1.8	5.5 ± 3.9	2.2 ± 0.7	2.4 ± 1.8
	+	3.3 ± 1.6*	74.0 ± 52.1*	16.3 ± 4.6*	3.1 ± 1.9	2.6 ± 1.6
IL-6 + GM-CSF	–	2.3 ± 0.9	15.5 ± 6.3	5.2 ± 3.6	1.9 ± 0.7	1.9 ± 0.5
	+	3.2 ± 1.4*	77.8 ± 61.2*	13.7 ± 6.1*	2.0 ± 0.5	2.1 ± 1.2
TPO + IL-6 + GM-CSF	–	3.0 ± 0.8	24.7 ± 15.4	9.7 ± 4.0	3.4 ± 1.1	3.1 ± 1.1
	+	4.1 ± 1.7*	95.7 ± 55.0*	21.6 ± 5.9*	2.7 ± 0.9	2.6 ± 1.8

Total cells, CD41a+ cells, CFU-Mk, CD15+ cells and CFU-GM from day 10 cultures of PB CD34+ cells using XVIVO-20 supplemented with IL-3, SCF and the indicated cytokines. The control cultures contained IL-3 and SCF. The means ± SD relative to control cultures of three experiments are indicated. Control cultures contained $16 \pm 7 \times 10^4$ total cells, $2.2 \pm 0.9 \times 10^3$ CD15+ cells, $1.0 \pm 0.1 \times 10^3$ CFU-GM, $3.5 \pm 2.1 \times 10^4$ CD41a+ cells, and $2.7 \pm 1.2 \times 10^2$ CFU-Mk in 2 ml.

*$p < 0.05$ (according to paired Student's *t*-test) between cultures with and without G-CSF. Due to the small number of samples ($n = 3$), the Wilcoxon signed rank test could not be used to evaluate the statistical significance of the results for this part of the study.

were comprised of 14% Mks and 32% granulocytes. Overall, cultures supplemented with one of the optimal cytokine combinations shown in Table 2 had high expansion of the clinically relevant cell types and were greatly enriched for Mks (14%-25%) and granulocytes (20%-32%).

Discussion

The six-factor combination of IL-3, SCF, TPO, IL-6, GM-CSF, and G-CSF produces cultures highly enriched for Mks, granulocytes, CFU-Mk and CFU-GM. The minimum addition of factors (to the basal combination of SCF plus IL-3) for expansion of the Mk and granulocyte lineages includes TPO and G-CSF. We found that expansion of CFC increased as more factors were added. Most previous studies examining ex vivo expansion of the granulocyte lineage have found that combinations containing five to six factors were most potent for expansion of total cells and CFU-GM [29-32].

The Mk lineage was primarily influenced by TPO in our cultures, although Mk and CFU-Mk numbers were further increased when TPO was combined with IL-6. Addition of IL-6 or GM-CSF alone to the base combination of IL-3 and SCF significantly increased the number of Mks and CFU-Mk, but to a lesser extent than for TPO addition. In contrast, G-CSF addition did not significantly increase the number of Mks or

Table 2. Summary of optimal cytokine combinations

	Number of cells produced (× 10−4)			Cell distribution	
	CFC	CD15+	CD41a+	% CD15+	% CD41a+
TPO + G-CSF	1.7 ± 0.7	6.7 ± 1.3	8.5 ± 6.0	22 ± 4	25 ± 14
TPO + IL-6 + G-CSF	2.3 ± 0.7	7.2 ± 1.2	7.9 ± 4.5	20 ± 2	21 ± 8
TPO + GM-CSF + G-CSF	2.5 ± 0.4	13.6 ± 4.6	8.1 ± 3.1	29 ± 4	17 ± 4
TPO + IL-6 + GM-CSF + G-CSF	3.5 ± 0.5	18.2 ± 4.2	8.5 ± 5.0	32 ± 5	14 ± 6

CFC (CFU-GM + BFU-E + CFU-mix + CFU-Mk), granulocytes (CD15+ cells), and Mks (CD41a+ cells) from 2 ml day 10 cultures of PB CD34+ cells using XVIVO-20. The means ± SD of three experiments are indicated. All of the cultures contained IL-3 and SCF in addition to the cytokines indicated.

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CFU-Mk. Previous studies have examined the effects of IL-6, GM-CSF and G-CSF on megakaryocytopoiesis. IL-6 in combination with IL-3 has been reported to influence both Mk maturation [12, 13] and colony number [11, 13-17]. GM-CSF was found to have Mk colony-stimulating activity similar to IL-3, although not as potent [18-20]. G-CSF in combination with IL-3 was found to increase the number of murine Mk progenitors in agar assays [33]. The effects of TPO in combination with multiple cytokines on both megakaryocytopoiesis and granulopoiesis have not been examined extensively, although TPO in combination with individual cytokines has been more thoroughly evaluated. Our results are consistent with those of a study examining the effect of TPO on murine BM cultures in which IL-6 and TPO were reported to have an additive effect on CFU-Mk at low TPO concentrations [34]. Our results are also consistent with a preliminary study which found that addition of both IL-6 and TPO to BM CD34$^+$ cells did not alter the ploidy of the Mks compared to that obtained in cultures stimulated by TPO alone [35]. *Broudy et al*. did not observe an increase in CFU-Mk over that found with TPO alone upon addition of both TPO and GM-CSF to murine BM cultures [34], which is consistent with our results.

While the primary stimulator of the granulocyte lineage is G-CSF, many synergistic and additive effects are observed when additional factors are added. The expansion of the granulocyte lineage has been studied more extensively than that of the Mk lineage. Several studies have shown that IL-6, GM-CSF and G-CSF are important for expansion of the granulocyte lineage [36, 37]. Previously, TPO alone was shown to have no effect on CFU-GM formation [38]. However, we found that TPO together with IL-3 and SCF resulted in an increase in CFU-GM over the base combination, which is consistent with the findings of *Papayannopoulou et al*. [39]. The increase in CFU-GM with TPO was comparable to that produced by GM-CSF or IL-6. Although combinations of TPO with IL-6, GM-CSF and/or G-CSF did not stimulate the Mk lineage to a great extent over that with TPO alone, multifactor combinations which included TPO resulted in a significant increase in CFU-GM and granulocyte number.

We have shown that under serum-free conditions a large number of Mks, granulocytes and CFC can be produced with the appropriate growth factors. Only a few studies have evaluated the number of both Mks and granulocytes produced in liquid cultures. Cultures of CD34$^+$ cells performed in serum-containing medium with IL-3, SCF and G-CSF either alone [40], or with IL-6, GM-CSF and IL-1 [30] were comprised of 2%-8% Mks and 39%-40% CD15$^+$ cells after 7-10 days. Another study by *Smith et al*. reported that BM CD34$^+$ cells cultured with serum-containing medium and the cytokine combination of IL-3, SCF, IL-6, G-CSF and GM-CSF were comprised of 52% CD15$^+$ cells at day 10 [28]. Several preliminary reports showed that cultures of murine BM progenitors supplemented with IL-3, SCF and TPO either alone [41], or with IL-6 [42] resulted in expansion of CFU-Mk and CFU-GM. In our studies with the six-factor cytokine combination, cultures were comprised of 32% CD15$^+$ cells and 14% Mks. The 4-factor combination of IL-3, SCF, TPO and G-CSF produced cultures with 22% granulocytes and 25% Mks. The frequency of granulocytes in our experiments was lower than those reported in other studies [40], which is most likely due to our use of serum-free medium. CFU-GM expansions in CD34$^+$ cell cultures have been reported to range from eightfold to 66-fold in serum-containing or plasma-containing media when at least four growth factors were present [28-30, 43-45]. Our 17-fold expansion of CFU-GM in serum-free medium is within this range. Other studies using serum-free medium have reported low expansion of CFU-GM [46-48], which may be due to the use of a smaller number of growth factors in these studies.

The number of hematopoietic cells produced under the conditions described here would be sufficient for successful engraftment following myelosuppressive therapy if produced on a scale of about one liter. Our estimate of the number of hematopoietic cells needed for successful engraftment of ex vivo expanded cells is based on results for transplantation using uncultured hematopoietic cells. Rapid hematopoietic engraftment occurs when 2×10^5 CFU-GM or 2×10^6 CD34$^+$ cells/kg of body weight are infused [49]. However, an 8- to 12-day period of neutropenia and thrombocytopenia still occurs with this transplantation regimen. Addition of mature cells and late progenitors of the neutrophil and megakaryocyte lineage has the potential to decrease this nadir. In our culture system, using the cytokine combination of IL-3, SCF, TPO, IL-6, GM-CSF and G-CSF, we can produce on average 580,000 cells composed of 22,000 CFU-GM, 85,000 Mks and 180,000 granulocytes from 20,000 CD34$^+$ cells in 2 ml of medium. Approximately 13 million CD34$^+$ cells expanded under our culture conditions would provide enough CFU-GM for engraftment, as well as a large number of granulocytes and Mks that have the potential to ameliorate the period of neutropenia and thrombocytopenia.

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