Porcine Brain Microvascular Endothelial Cells Support the In Vitro Expansion of Human Primitive Hematopoietic Bone Marrow Progenitor Cells With a High Replating Potential: Requirement for Cell-to-Cell Interactions and Colony-Stimulating Factors

By Thomas A. Davis, Douglas H. Robinson, Kelvin P. Lee*, and Steven W. Kessler*

Primary autologous as well as allogeneic and xenogeneic stroma will support human stem cell proliferation and differentiation for several months. In the present study, we investigated the capacity of porcine microvascular endothelial cells (PMVECs) together with combinations of cytokines (granulocyte-macrophage colony-stimulating factor [GM-CSF] + stem cell factor [SCF], interleukin-3 [IL-3] + SCF + IL-6, and GM-CSF + IL-3 + SCF + IL-6) to support the expansion and development of purified human CD34+ bone marrow cells. In short-term cultures (7 days), the greatest expansion of nonadherent hematopoietic cells and clonogenic progenitors was seen with CD34+ cells in direct contact with PMVEC monolayers (PMVEC contact), followed by PMVEC noncontact and liquid suspension cultures, respectively. Maximal expansion of nonadherent cells (42-fold) and total CD34+ cells (12.6-fold) occurred in PMVEC contact cultures treated with GM-CSF + IL-3 + SCF + IL-6, with similar increases in the number of granulocyte-macrophage colony-forming units (CFU-GM), CFU-mix, erythroid burst-forming units (BFU-E), CFU-blast and CFU-megakaryocyte (CFU-Mk) progenitor cells. Moreover, the number of CD34+ CD38- and CD34+ CD38+ cells increased 148.1-fold and 8.0-fold, respectively. Replating studies show that cells from day 7 dispersed blast cell colonies generated on cytokine-treated PMVEC monolayers have a high replating potential for multilineage progenitor cells. In long-term PMVEC contact cultures, CD34+ cells seeded onto PMVEC monolayers with GM-CSF + IL-3 + 6CF + IL-6 showed a total calculated expansion of over 5,000,000-fold of nonadherent cells over 35 days in culture. Maximal clonogenic cell production was observed at day 28, with 6,353-fold for total CFC and comparable increases for CFU-GM, CFU-mix, CFU-blast, BFU-E, and CFU-Mk. The total number of CD34+ cells increased 2,584-fold at day 28. Furthermore, the extended growth kinetics of these cultures indicates that these phenotypically primitive progenitor cells are also functionally expanded on PMVEC monolayers. These results support the hypothesis that direct contact with a PMVEC monolayer supports the initial expansion of hematopoietic progenitor cells with a high replating potential and, possibly, a more primitive phenotype (CD34+, CD34+/CD38-).

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of primitive (CD34+) BM progenitor cells in this culture system.

MATERIALS AND METHODS

**CD34+ BM cells.** Human vertebral body BM was procured from cadavers as part of an existing tissue procurement protocol in the Immune Cell Biology Program Center at the Naval Medical Research Institute, Bethesda, MD. The procedure used to procure BM is described in The Procuring and Processing of Human Cadaveric Bone Marrow. Briefly, BM was obtained from the bone matrix by sterile technique and placed in sterile culture support media. Low density mononuclear cells were separated over Ficoll-Hypaque (specific gravity, 1.077 g/mL; Pharmacia Fine Chemicals, Piscataway, NJ) density gradients at 400 g for 30 minutes at 22°C. Low density cells at the interfaces were procured, washed twice by centrifugation (400 g for 10 minutes) and resuspended in immunoselection/wash medium consisting of Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT) 100 µg/mL L-glutamine (GIBCO, Grand Island, NY), 1,000 U/mL DNAse (400 U/mL), and 10% heat-inactivated FCS, prewarmed (37°C) complete culture medium consisting of IMDM supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT) 100 µg/mL L-glutamine (GIBCO, Grand Island, NY), 1,000 U/mL DNAse (400 U/mL), and 10% heat-inactivated FCS, prewarmed (37°C) complete culture medium. The thawed CD34+ BM cells were washed twice by centrifugation (400 g for 10 minutes) and resuspended in immunoselection/wash medium consisting of Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT) 100 µg/mL L-glutamine (GIBCO, Grand Island, NY), 1,000 U/mL DNAse (400 U/mL), and 10% heat-inactivated FCS, prewarmed (37°C) complete culture medium consisting of IMDM supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT) 100 µg/mL L-glutamine (GIBCO, Grand Island, NY), 1,000 U/mL DNAse (400 U/mL), and 10% heat-inactivated FCS, prewarmed (37°C) complete culture medium.

**CD34+ BM progenitor cells.** Further purified by positive immunomagnetic selection as previously described. Briefly, low density bone marrow mononuclear cells were rosetted using a biotinylated monoclonal antibody (MoAb) specific for the CD34 antigen (K6.1) linked to magnetic Dynabeads (Dynal Incorporated, Great Neck, NY). After three to four cycles of magnetic attraction, the beads were dissociated from the cells with an excess of biotin (GIBCO) and separated from the cells magnetically. Cells by this procedure showed greater than 99% positive reactivity with a second noncross-blocking CD34-specific MoAb (HPA-2 fluorescein isothiocyanate [FITC], Becton Dickinson Immunocytochemistry Systems, San Jose, CA), by flow cytometric analysis. The majority of the CD34+ cells coexpressed CD38 (96.8% ± 2.4%). Isolated CD34+ BM cells were cryopreserved (1 to 5 x 10^6 cells/vial) and stored under liquid nitrogen before experimentation.

**Processing of CD34+ cells for culture.** Cryopreserved CD34+ cells were thawed rapidly at 37°C, diluted in a 10x volume of prewarmed (37°C) complete culture medium consisting of IMDM supplemented with 10% heat-inactivated FCS, 100 µg/mL L-glutamine (GIBCO) and 100 U/mL penicillin/streptomycin (GIBCO). Unless otherwise noted, this culture medium will be referred to as complete culture medium. The thawed CD34+ BM cells were washed twice in complete culture medium, and resuspended at 1 x 10^5 cells/mL. Cell viability was greater than 99% as determined by trypan blue dye exclusion. At the start of experimentation, a sample of CD34+ cells was cultured to determine the number and type of hematopoietic colony-forming cells (CFCs) using a methylcellulose colony-forming cell assay (below).

**Endothelial cell culture conditions.** PMVEC were isolated and grown as previously described. Briefly, PMVEC (passages 19 through 28) were fed weekly with complete medium. When confluent, PMVEC monolayers were washed with phosphate-buffered saline (PBS), trypsinized (0.25 mg trypsin/ml, 5 mmol/L EDTA, 37°C, 10 minutes; Sigma, St Louis, MO) and subcultured either at a ratio of 1:5 into 75 cm² flasks or at cellular concentration of 1 x 10^5 cells/well in gelatin-coated 6-well tissue culture plates (Costar, Cambridge, MA) containing 3 mL of complete culture medium. After 48 to 72 hours, the adherent PMVEC monolayers (70% to 80% confluent) were washed twice with complete culture medium to remove nonadherent PMVEC and the culture medium was replaced with 5 mL of complete culture medium.

**Proliferation of CD34+ BM cells in culture.** Purified (1 x 10^5) CD34+ cells were added to each well (final volume, 5 mL) of 6-well tissue culture plates (Costar) containing no PMVEC monolayer (liquid cultures) or confluent PMVEC monolayers (contact cultures) and to the upper chamber of a collagen-treated Transwell insert (0.4-µm pore size; Costar) placed on top of PMVEC monolayers (noncontact cultures). Cultures were treated with GM-CSF + stem cell factor (SCF), interleukin-3 (IL-3) + SCF + IL-6, and GM-CSF + IL-3 + SCF + IL-6. All cultures were maintained at 37°C in a humidified atmosphere at 5% CO₂ in air. Recombinant human growth factors (R&D Systems, Minneapolis, MN) used to stimulate colony formation included IL-3 (10 ng/mL), granulocyte-macrophage colony stimulating factor (GM-CSF) (2 ng/mL), IL-6 (10 ng/mL), and stem cell factor (SCF) (100 ng/mL). After various days of culture, nonadherent cells were gently removed from the endothelial cell monolayers, washed twice with complete culture medium, and manual hemacytometer cell counts were performed using trypan blue exclusion dye.

**Long-term marrow cultures.** Purified CD34+ BM cells (5 x 10^5 cells/vial) were inoculated in 75 cm² flasks containing 15 mL of complete culture medium supplemented with GM-CSF + IL-3 + SCF + IL-6 in the presence or absence of PMVEC confluent monolayers. After 7 days, and subsequently at weekly intervals, all nonadherent cells were removed and counted, and 2 x 10^5 of the harvested nonadherent cells were reseeded either onto fresh PMVEC monolayer cultures or into liquid suspension cultures containing fresh medium supplemented with growth factors. Cells that were tightly adherent or embedded (cobblestone foci) in the PMVEC monolayer were not passaged using this method. Nonadherent cells were immunophenotyped for CD34 and CD38 cell surface antigens and assayed in methylcellulose to determine their clonogenic content. Percent nonadherent cell expansion over each 7-day culture interval was calculated as follows: (number of nonadherent cells harvested/number of cells cultured) x 100. Total cell yield over the period in culture was calculated as the product of the interval increases multiplied by 1 x 10^6 cells initially plated.

**In vitro methylcellulose colony-forming assay.** Purified CD34+ bone marrow cells and nonadherent hematopoietic cells from harvested cultures were cultured in 35-mm Lux suspension culture dishes (Miles Laboratories, Naperville, IL) using a modification of the technique as previously described. Briefly, 5 to 50 x 10^5 BM cells were seeded into 1 mL of IMDM, 1% methylcellulose, 30% heat-inactivated FCS, 2 U/mL tissue culture grade erythropoietin (Amgen, Thousand Oaks, CA), 2 ng/mL GM-CSF, 10 ng/mL IL-3 (R&D Systems) and 5% conditioned medium from the bladder carcinoma cell line 5637 (5 x concentrate) as a source of colony-stimulating activity. After 14 days, cultures were evaluated to determine the number of colonies (>50 cells) developed. At day 14, aggregates of hemoglobin-containing cells were recognized as erythroid burst-forming units (BFU-E), granulocyte-macrophage colonies as CFU-GM, and aggregates of hemoglobin cells containing at least granulocytes and/or macrophages and/or megakaryocytes as CFU-Mix. Megakaryocyte colonies (CFU-Mk) were confirmed based upon established morphologic criteria. Morphologic verification of selected colonies was determined using Wright-Giemsa stain. Triplicate assays were set up for each individual data point per experiment.

**In vitro clonogenic assay for CFU-blast.** The number of CFU-blast cells were determined using the method described by Gordon et al. Briefly, nonadherent hematopoietic mononuclear cells from harvested cell cultures were added to confluent human stromal cell monolayers cultured in 35-mm Lux tissue culture dishes and incubated for 2 hours to allow for attachment of progenitor cells that give rise to CFU-blast cell colonies. The stromal cell monolayers were washed three times with PBS supplemented with 10% heat-
inactivated FCS and overlaid with 1.0 mL of 0.3% agar in a medium supplemented with 15% FCS and 2 × 10⁻⁴ mol/L methylprednisolone. Cultures were incubated for 7 days and cluster/aggregates (≥ 20 cells) of cells were scored as CFU-blast colonies. Each individual data point per experiment was assayed in triplicate.

**Immunofluorescence staining.** Simultaneous two-color cytometric analysis of cultured nonadherent cells was performed at selected intervals of culture. Briefly, nonadherent cells were harvested, washed twice in complete culture medium, and resuspended in PBS supplemented with 2% heat-inactivated FCS, 2% (wt/vol) bovine serum albumin and 0.1% sodium azide (staining medium). Cells were incubated (separately and in combination) for 30 minutes with saturating concentrations of phycoerythrin (PE)- or FITC-conjugated CD34 MoAb (HPCA-2, IgG1), PE-conjugated CD38 (Leu-17, IgG1), or FITC-conjugated CD15 (Leu-M1, IgM) (Becton Dickinson Immunocytometry Systems) washed twice with staining medium and fixed with 1% paraformaldehyde. Cells stained with the appropriate conjugated isotype matched antibodies were used as controls. Ten thousand events were collected in list mode on a Coulter Elite (Coulter, Hialeah, FL) flow cytometer.

**RESULTS**

**Proliferation of CD34⁺ BM cells under various culture conditions.** We have shown previously that GM-CSF-treated PMVEC monolayers support the growth of peripheral blood hematopoietic progenitor cells.34 After 7 days of culture, PMVECs in the presence of human hematopoietic growth factors (GM-CSF + IL-3 + SCF + IL-6) are capable of supporting the formation of large dispersed macroscopic colonies (Fig 1A) and “cobblestone areas” (Fig 1B) from overlaid CD34⁺ BM cells, typical of cobblestone foci seen after plating bone marrow cells on marrow-derived stromal monolayers.44 The plating efficiency of purified CD34⁺ BM cells on cytokine-treated PMVEC monolayers ranged from 17% to 32% (data not shown). The replating potential of cells derived from day 7 blast cell colonies was determined by replating individual colonies into secondary methylcellulose clonogenic cell cultures (Table 1). Secondary hematopoietic cell cultures contained CFU-GM, BFU-E, and CFU-mix progenitor cells with greater than 92% of the primary day 7 colony giving rise to at least one secondary colony.

To determine the role of direct cell-cell contact and specific cytokines in supporting the growth of CD34⁺ bone marrow cells, purified human CD34⁺ bone marrow cells were distributed over five wells of 6-well tissue culture plates containing no PMVEC monolayers (liquid cultures) or confluent PMVEC monolayers (contact cultures) and to the upper chamber of a collagen-treated Transwell insert placed on top of confluent PMVEC monolayers (noncontact cultures). Cultures were established with combinations of cytokines and incubated for 7 days; the number of nonadherent cells harvested per culture condition are shown in Fig 2 and Table 2. In comparison to CD34⁺ bone marrow cells grown in liquid suspension culture, greater (2.3- to 4.0-fold) nonadherent cell production was detected in both PMVEC noncontact and PMVEC contact culture conditions. Maximal nonadherent cell production with GM-CSF + SCF (15-fold), IL-3 + SCF + IL-6 (26-fold), and GM-CSF + IL-3 + SCF + IL-6 (43-fold) was achieved under PMVEC contact culture conditions. The most effective cytokine combination for nonadherent cell production was GM-CSF + IL-3 + SCF + IL-6, whereas the least effective combination was GM-CSF + SCF. The addition of IL-3 + IL-6 to GM-CSF + SCF-treated cultures increased the number of nonadherent cells 3.1 (liquid), 2.6 (noncontact) and 2.9-fold (contact). None of the cytokine combinations appeared to have any effect on the rate of development or the morphologic organization of the adherent PMVEC monolayers.

**Immunophenotype of cultured BM cells.** The effects of various cytokine combinations and culture conditions on the proliferation and maturation of CD34⁺ hematopoietic bone marrow cells after 7 days of culture was assessed by measuring the expression of CD34, CD38, and CD15 cell surface antigens. Neither unstimulated nor cytokine-treated PMVECs stain positive for antihuman CD34, CD38, or CD15 antigens (data not shown). The results of two-color staining for CD34 and CD38 are shown in Table 2 and Fig 3. After 7 days of culture, the number of CD34⁺ BM cells in PMVEC contact cell cultures increased 4.7 (GM-CSF + SCF), 9.6 (IL-3 + SCF + IL-6), and 12.6-fold (GM-CSF + IL-3 + SCF + IL-6). Moreover, 39% of the harvested CD34⁺ cells from GM-CSF + IL-3 + SCF + IL-6–treated contact cultures were negative for the CD38 antigen. After 7 days of culture, the absolute number of CD34⁺ CD38⁺ and CD34⁺CD38⁻ cells increased 8.0-fold and 148.1-fold, respectively. In contrast, a smaller degree of CD34⁺ stem cell expansion (1.6- to 3.6-fold) was measured in noncontact PMVEC cultures, with greater than 90% of these expressing the CD34⁺CD38⁻ phenotype. In liquid suspension culture, input levels of CD34⁺ bone marrow cells were maintained only with IL-3 + SCF + IL-6 (1.4-fold increase) and GM-CSF + IL-3 + SCF + IL-6 (1.9-fold increase) cytokine treatment. However, greater than 96% of these cells coexpressed the CD38 hematopoietic differentiation antigen.

**Hematopoietic colony formation potential of nonadherent cells harvested from day 7 cultures.** In another set of studies, the growth of hematopoietic progenitor cells from nonadherent cells harvested after 7 days in cytokine-treated liquid suspension cultures, noncontact PMVEC monolayer cultures and contact PMVEC monolayer cultures was investigated. The total number of CFC, CFU-GM, CFU-mix, CFU-blast, CFU-Mk, and BFU-E recovered per culture treatment is shown in Table 3. The total number of CFC generated after 7 days in liquid suspension culture decreased 25% in GM-CSF + SCF–treated cultures, increased 1.5-fold in IL-3 + SCF + IL-6–treated cultures and increased 3.2-fold in the presence of GM-CSF + IL-3 + SCF + IL-6. In contrast, total clonogenic cell content expanded 3.3 (GM-CSF + SCF), 4.4 (IL-3 + SCF + IL-6), and 8.9-fold (GM-CSF + IL-3 + SCF + IL-6) in PMVEC noncontact cultures and 10.5 (GM-CSF + SCF), 20.3 (IL-3 + SCF + IL-6), and 37.4-fold (GM-CSF + IL-3 + SCF + IL-6) in PMVEC contact cultures, respectively. Maximal increases in total nonadherent cells (42-fold), CFU-GM (33.3-fold), CFU-mix (31.0-fold), BFU-E (50.2-fold), BFU-blast (33.6-fold), and CFU-Mk (9.2-fold) were obtained with PMVEC contact cultures in the presence of GM-CSF + IL-3 + SCF + IL-6, emphasizing the importance of direct cell-to-cell contact.
Fig 1. Morphology of a typical colony and cells generated when CD34+ BM cells are cultured on GM-CSF + IL-3 + SCF + IL-6-treated endothelial cell monolayers for 7 days. (A) Dispersed cell colony. (B) Adherent cell foci “cobblestone areas.”

Table 1. Replating Potential of Primary Blast Cell Colonies Generated by CD34+ BM Cells Cocultured on PMVEC Monolayers Treated With GM-CSF + IL-3 + SCF + IL-6

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>No. Primary Cell Colonies Replated</th>
<th>% Replaces With at Least 1 Colony</th>
<th>CFU-GM per Replated Colony</th>
<th>BFU-E per Replated Colony</th>
<th>CFU-mix per Replated Colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>100.0</td>
<td>6.2 ± 1.2</td>
<td>8.5 ± 2.3</td>
<td>3.1 ± 1.3</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>92.0</td>
<td>15.3 ± 2.1</td>
<td>3.9 ± 1.6</td>
<td>2.9 ± 0.9</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
<td>96.0</td>
<td>12.6 ± 3.3</td>
<td>5.4 ± 2.2</td>
<td>4.1 ± 1.7</td>
</tr>
</tbody>
</table>

One hundred purified CD34+ BM cells were cocultured on PMVEC monolayers in the presence of GM-CSF + IL-3 + SCF + IL-6. At day 7, blast cell colonies developed on PMVEC monolayers (17%-32% plating efficiency) were replated in secondary methycellulose cell cultures containing 30% FCS, 10 ng/mL GM-CSF, 100 ng/mL IL-3, 120 ng/mL SCF, 4 U/mL erythropoietin, and 5% 5637-conditioned medium. Secondary colonies were scored at day 14.
Stained cells were analyzed using two-color flow cytometry. The number of each immunophenotype was corrected to reflect the total number stained for phenotypic analysis with FITC-conjugated CD15 (Leu-M1) or FITC-conjugated CD34 (HPCA-2).

The long-term growth of purified CD34+ BM cells (5 \times 10^6) were seeded onto PMVEC monolayers in the presence of GM-CSF + IL-3 + SCF + IL-6 was investigated next. These data represent four separate experiments and at all sampling intervals, the cell viability of harvested nonadherent cells, as determined by trypan blue exclusion, was greater than 95% (data not shown).

In the PMVEC contact cultures, a calculated 5.3 \times 10^6-fold increase in total nonadherent cell numbers occurred over 35 days in culture (Fig 4). Furthermore, increases of 6,353-fold (total CFC), 7,454-fold (CFU-GM), 5,285-fold (CFU-Mix), 5,277-fold (CFU-Blast), 3,483-fold (BFU-E), and 1,944-fold (CFU-Mk) in the number of total clonogenic cells were detected in culture by day 28 (Fig 5, A and B). Although the percentage of cells in culture that expressed the CD34+ surface antigen at days 7, 14, and 21, and 28 decreased to 26.6%, 13.0%, 3.3%, and 1.9%, respectively, the absolute number of CD34+ cells in culture increased 10.0-, 105-, 498-, and 2584-fold over the same time interval (Fig 4). The presence of CD34+ cells was undetectable by fluorescence-activated cell sorting analysis at day 35 (<1% of the harvested nonadherent cell population was CD34+) and this decrease correlated with a significant decrease in the number of assayable CFU-mix, CFU-Mk, CFU-Blast, and BFU-E. However, no significant changes in the number of CFU-GM were observed at day 35 in comparison with day 28 values.

In parallel studies, the kinetics of progenitor-cell production in long-term liquid-suspension cultures (without PMVECs) are shown in Table 4. Nonadherent cells and CFU-GM progenitors expanded 228- and 50-fold, respectively, above input levels after 21 days of culture, but then declined rapidly by day 28. BFU-E numbers expanded 4.0-fold and CFU-mix numbers increased 6.8-fold after 7 days of culture, but then exhibited a steady decrease in cell numbers throughout the culture period. In contrast with PMVEC coculture experiments, CD34+ cell numbers decreased steadily from the beginning of culture.

**CULTURE CONDITION**

Long-term growth potential of BM CD34+ hematopoietic progenitor cells on cytokine-treated PMVEC monolayers. The long-term growth of purified CD34+ seeded onto PMVEC confluent monolayers and in liquid suspension cultures (without PMVECs) in the presence of GM-CSF + IL-3 + SCF + IL-6 was investigated next. These data represent four separate experiments and at all sampling intervals, the cell viability of harvested nonadherent cells, as determined by trypan blue exclusion, was greater than 95% (data not shown).

In the PMVEC contact cultures, a calculated 5.3 \times 10^6-fold increase in total nonadherent cell numbers occurred over 35 days in culture (Fig 4). Furthermore, increases of 6,353-fold (total CFC), 7,454-fold (CFU-GM), 5,285-fold (CFU-Mix), 5,277-fold (CFU-Blast), 3,483-fold (BFU-E), and 1,944-fold (CFU-Mk) in the number of total clonogenic cells were detected in culture by day 28 (Fig 5, A and B). Although the percentage of cells in culture that expressed the CD34+ surface antigen at days 7, 14, and 21, and 28 decreased to 26.6%, 13.0%, 3.3%, and 1.9%, respectively, the absolute number of CD34+ cells in culture increased 10.0-, 105-, 498-, and 2584-fold over the same time interval (Fig 4). The presence of CD34+ cells was undetectable by fluorescence-activated cell sorting analysis at day 35 (<1% of the harvested nonadherent cell population was CD34+) and this decrease correlated with a significant decrease in the number of assayable CFU-mix, CFU-Mk, CFU-Blast, and BFU-E. However, no significant changes in the number of CFU-GM were observed at day 35 in comparison with day 28 values.

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**Table 2. Proliferation of CD34+ Hematopoietic Cell Subsets in Cytokine-Treated PMVEC Cultures**

<table>
<thead>
<tr>
<th>Culture Conditions and Factors</th>
<th>Cell Yield \times 10^4</th>
<th>CD34+</th>
<th>CD38+</th>
<th>CD34+CD38-</th>
<th>CD34+CD38+</th>
<th>CD34+CD15+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input</td>
<td>5.0</td>
<td>4.8 ± 0.2</td>
<td>4.2 ± 0.4</td>
<td>0.16 ± 0.1</td>
<td>4.6 ± 0.1</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Liquid culture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF + SCF</td>
<td>18.9 ± 1.1</td>
<td>2.6 ± 0.4</td>
<td>17.3 ± 2.2</td>
<td>0.1 ± 0.1</td>
<td>2.5 ± 0.4</td>
<td>8.8 ± 1.8</td>
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<tr>
<td>IL-3 + SCF + IL-6</td>
<td>44.1 ± 6.7</td>
<td>6.6 ± 1.6</td>
<td>40.6 ± 2.9</td>
<td>0.1 ± 0.1</td>
<td>6.4 ± 1.4</td>
<td>10.7 ± 0.9</td>
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<tr>
<td>GM-CSF + IL-3 + SCF + IL-6</td>
<td>58.1 ± 5.7</td>
<td>9.2 ± 3.1</td>
<td>58.1 ± 4.9</td>
<td>0.1 ± 0.1</td>
<td>9.1 ± 2.2</td>
<td>17.4 ± 1.5</td>
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<tr>
<td>PMVEC noncontact culture</td>
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<tr>
<td>GM-CSF + SCF</td>
<td>45.8 ± 4.7</td>
<td>7.7 ± 2.1</td>
<td>35.1 ± 4.1</td>
<td>0.3 ± 0.2</td>
<td>7.4 ± 1.9</td>
<td>17.9 ± 3.2</td>
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<tr>
<td>IL-3 + SCF + IL-6</td>
<td>69.9 ± 8.0</td>
<td>17.1 ± 2.2</td>
<td>56.1 ± 8.2</td>
<td>0.4 ± 0.2</td>
<td>14.6 ± 3.2</td>
<td>16.8 ± 3.0</td>
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<tr>
<td>GM-CSF + IL-3 + SCF + IL-6</td>
<td>119.0 ± 7.1</td>
<td>17.4 ± 2.8</td>
<td>87.5 ± 14.1</td>
<td>0.6 ± 0.3</td>
<td>16.8 ± 2.3</td>
<td>24.2 ± 5.2</td>
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<td>PMVEC contact culture</td>
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<tr>
<td>GM-CSF + SCF</td>
<td>75.0 ± 5.8</td>
<td>22.7 ± 7.4</td>
<td>62.23 ± 3.3</td>
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<td>21.4 ± 6.5</td>
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<td>IL-3 + SCF + IL-6</td>
<td>131.5 ± 10.9</td>
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<td>GM-CSF + IL-3 + SCF + IL-6</td>
<td>215.8 ± 32.3</td>
<td>60.6 ± 16.4</td>
<td>146.6 ± 30.5</td>
<td>23.7 ± 10.4</td>
<td>36.9 ± 11.6</td>
<td>24.6 ± 1.9</td>
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CD34+ BM cells (5 \times 10^6) were plated per culture treatment. Nonadherent cells were procured on day 7 of culture. Cells of each culture were stained for phenotypic analysis with FITC-conjugated CD15 (Leu-M1) or FITC-conjugated CD34 (HPCA-2) plus PE-conjugated CD38 (Leu-17). Stained cells were analyzed using two-color flow cytometry. The number of each immunophenotype was corrected to reflect the total number of cells procured/culture. Each point represents the mean number of positive cells from three different experiments.
DISCUSSION

We show here that in the presence of a xenogeneic porcine microvascular endothelial cell microenvironment, human CD34+ hematopoietic cell expansion is supported in the short-term and multilineage hematopoietic progenitor cell proliferation is amplified in the long-term. Contact of highly purified CD34+ cells to PMVEC monolayers and exogenous growth factors (GM-CSF + IL-3 + SCF + IL-6) is necessary for maximal expansion.

In liquid suspension cultures, sustained expansion of multipotential progenitor cells has been difficult to attain. Conversely, production of progenitors is more sustained when human hematopoietic progenitors are cultured on primary heterogeneous stroma, murine stroma, and with stroma-derived factors, but the degree of total cell expansion has not been as substantial as in liquid cultures. By comparison, a relatively substantial and sustained level of CD34+ cell expansion and progenitor cell production is achieved when unfractionated CD34+ cells are cocultured with PMVECs and exogenous cytokines. We speculate that CD34+ bone marrow cells possess a capacity for at least limited self-renewal and maintenance of differentiation along multiple cell lineages on cytokine-treated PMVECs.
with the PMVEC monolayer, and some of these cells were observed after 7 to 14 days of culture to have integrated within the PMVEC monolayer to form foci of dispersed round translucent adherent cells or "cobblestone areas," suggesting active sites for cell proliferation and long-term hematopoiesis.\(^4\) Moreover, replating studies show that cells from dispersed macroscopic day 7 blast cell colonies generated on cytokine-treated PMVECs have a high replating potential (>92% of the colonies gave rise to at least one secondary colony) and that they give rise to multilineage and committed progenitor cells when cultured in secondary methylcellulose cultures. The ability of cells generated from primary hematopoietic cell colonies to form second to fourth generation colonies upon replating has been used to probe for "self-renewal" or primitivity.\(^5,6\) Huang and Terstappen,\(^7\) showed that the CD34\(^+\) progenitor population that gives rise to the dispersed growth pattern has myeloid as well as lymphoid potential and has the highest replating efficiency (third and fourth generation progeny) in the presence of hematopoietic growth factors. Short-term (7-day) cultures of CD34\(^+\) BM cells in PMVEC-contact, PMVEC-noncontact, and liquid-suspension cultures showed that the greatest expansion of nonadherent cells (43-fold), total CD34\(^+\) cells (12.6-fold), and CD34\(^+\)CD38\(^-\) (148.1-fold) occurred in PMVEC-contact cultures plus GM-CSF + IL-3 + SCF + IL-6. Comparatively, noncontact PMVEC conditions plus cytokines supported at most a 3.6-fold increase of total CD34\(^+\) cell numbers with a 3.8-fold expansion of the CD34\(^+\)CD38\(^-\) cell population. In contrast with other reports,\(^3,4\) these findings indicate a positive effect of cell-to-cell contact on multipotential progenitor cell expansion. The increase in phenotypically primitive CD34\(^+\) and CD34\(^+\)CD38\(^-\) populations in these cultures is temporally correlated to the expansion of multipotential progenitor cells as detected in clonogenic assays. However, further studies are needed to define more extensively the functionality and immunophenotype of these populations, particularly the CD34\(^+\)CD38\(^-\) subset. The possibility that the low CD38

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**Table 3. Effect of Various Hematopoietic Factor Combinations on Progenitor Cell Production From CD34\(^+\) BM Cells Cultured for 7 Days in Liquid Suspension Culture, Noncontact PMVEC Culture, and Contact PMVEC Culture**

<table>
<thead>
<tr>
<th>Culture Conditions and Factors</th>
<th>Cell Yield (\times 10^5)</th>
<th>CFU-GM</th>
<th>CFU-GEMM</th>
<th>BFU-E</th>
<th>CFU-Mk</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Input</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Liquid culture</td>
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<tr>
<td>GM-CSF + SCF</td>
<td>20.2 ± 4.7</td>
<td>4.5 ± 3.2</td>
<td>2.4 ± 0.7</td>
<td>0.6 ± 0.4</td>
<td>0.6 ± 0.2</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>IL-3 + SCF + IL-6</td>
<td>32.4 ± 2.1</td>
<td>9.8 ± 4.6</td>
<td>4.3 ± 2.4</td>
<td>1.1 ± 0.4</td>
<td>1.3 ± 1.3</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>GM-CSF + IL-3 + SCF + IL-6</td>
<td>56.1 ± 16.2</td>
<td>19.2 ± 7.9</td>
<td>9.1 ± 2.9</td>
<td>3.1 ± 1.2</td>
<td>3.7 ± 1.9</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td><strong>PMVEC noncontact culture</strong></td>
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<tr>
<td>GM-CSF + SCF</td>
<td>55.2 ± 1.0</td>
<td>5.6 ± 2.7</td>
<td>8.1 ± 1.3</td>
<td>3.4 ± 1.1</td>
<td>5.1 ± 3.1</td>
<td>1.5 ± 0.3</td>
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<tr>
<td>IL-3 + SCF + IL-6</td>
<td>79.3 ± 11.4</td>
<td>25.2 ± 17.9</td>
<td>6.2 ± 5.5</td>
<td>7.4 ± 4.2</td>
<td>6.7 ± 4.9</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td>GM-CSF + IL-3 + SCF + IL-6</td>
<td>87.1 ± 2.7</td>
<td>60.6 ± 39.8</td>
<td>12.3 ± 7.1</td>
<td>8.2 ± 4.7</td>
<td>16.2 ± 11.8</td>
<td>2.6 ± 0.6</td>
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<tr>
<td><strong>PMVEC contact culture</strong></td>
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</tr>
<tr>
<td>GM-CSF + SCF</td>
<td>56.7 ± 11.0</td>
<td>60.1 ± 39.9</td>
<td>22.8 ± 12.1</td>
<td>9.3 ± 5.2</td>
<td>6.5 ± 9.2</td>
<td>3.8 ± 1.8</td>
</tr>
<tr>
<td>IL-3 + SCF + IL-6</td>
<td>94.4 ± 31.1</td>
<td>125.9 ± 107.3</td>
<td>35.6 ± 29.4</td>
<td>22.2 ± 16.2</td>
<td>38.2 ± 25.6</td>
<td>5.2 ± 3.5</td>
</tr>
<tr>
<td>GM-CSF + IL-3 + SCF + IL-6</td>
<td>210.2 ± 120.0</td>
<td>182.9 ± 56.6</td>
<td>86.8 ± 56.6</td>
<td>55.3 ± 30.8</td>
<td>80.6 ± 18.6</td>
<td>20.2 ± 9.1</td>
</tr>
</tbody>
</table>

CD34\(^+\) BM cells (5 \(\times 10^5\)) were plated per culture treatment. Nonadherent cells were procured on day 7 of culture. Nonadherent cells (5-500 \(\times 10^5\)) were cultured in 35-mm tissue culture dishes containing IMDM medium, 1% methylcellulose, 30% FCS, 2 U/mL tissue culture grade erythropoietin, 2 ng/mL GM-CSF, 10 ng/mL IL-3, and 5% conditioned medium from the bladder carcinoma cell line 5637 as a source of colony-stimulating activity. The number of myeloid and erythroid colonies were counted after 14 days of culture, and based on the total number of viable cells per culture, the number of colonies was corrected to reflect the total number of CFC per culture condition. Values represent the number of colonies of triplicate cultures from three different experiments.
expression is a culture artifact is argued against by the observation that CD38 expression on the CD34+/CD38' cell line KG1a does not change when cocultured with either untreated or cytokine treated PMVEC's (T. Davis, unpublished observation, May 1994), suggesting that exogenous enzymatic cleavage, masking of cell-surface CD38, or inducible down-regulation of CD38 by PMVEC contact is not occurring.

In long-term growth studies, PMVEC contact plus GM-CSF + IL-3 + SCF + IL-6 supports continuous myeloid, erythroid, and megakaryocyte proliferation and differentiation. Sustained production of nonadherent cells, total CFC, CFU-blast, CFU-GM, CFU-Mk, and BFU-E progenitor cells over 35 days of culture is consistent with the observed expansion of phenotypically primitive CD34+ cells and hematopoietic cells with a high repopulating potential. Particularly, the detection of assayable CFU-blast from day 0 to 28 correlates with the presence of a significant number of CD34+ cells in culture. Although long-term data is only reported out to 5 weeks in this study, we have found that the PMVEC culture system is capable of sustained CFU-GM production out to 11 weeks in a bioreactor configuration (T. Davis, manuscript in preparation). In comparison, cytokine-treated liquid suspension cell cultures in our hands and others typically result in a comparatively less substantial and sustained increase in total and progenitor cells numbers. These findings support the hypothesis that the PMVEC culture system can support and expand at least a trilineage multipotential progenitor cell population.

Previous reports have also shown that human myeloid progenitor cells can be maintained on xenogeneic stromal monolayers. The mechanisms by which PMVECs support the expansion of multipotential progenitor cells is not clear. However, studies of fetal hematopoiesis may be instructive. These studies show that hematopoiesis is initially extraembryonic and occurs in foci within the yolk sac, known as blood islands. Blood islands consist of peripheral cells that elongate to form capillary endothelium (angioblasts) and central hematopoietic cells (hemoblasts). Thus,
PROGENITOR CELL EXPANSION ON PMVEC MONOLAYERS

during early embryonic hematopoiesis, proliferation and expansion of primitive hematopoietic stem cells occurs in association with an endothelial cell microenvironment. Hematopoietic cell production on PMVEC monolayers suggests that this culture system recapitulates the microenvironmental conditions found during fetal hematopoiesis. A recent report by Yoder et al.\(^1\) showing that transformed murine endodermal and mesodermal cell lines can support murine hematopoiesis is consistent with this hypothesis.

Our findings are consistent with previous reports indicating that primitive hematopoietic progenitor cells require a combination of hematopoietic growth factors for maximal proliferation\(^1^9,2^0\) and that these cells can be maintained, but not significantly expanded, in long-term bone marrow stromal cell cultures without direct cell-to-cell contact.\(^4^5,5^2-5^4\)

Consistent with reports of Verfaillie,\(^1^1,4^5\) soluble-stromal factors in the PMVEC noncontact cultures appeared to support CD34\(^+\) cell expansion. In contrast, we find that optimal CD34\(^+\) expansion requires hematopoietic cell-endothelial cell contact. This finding may reflect differences in the stromal cells studied or may reflect differences in a homogeneous stromal cell microenvironment (PMVEC) versus a heterogeneous microenvironment.

Several mechanisms and interactions may be operative in the PMVEC hematopoietic microenvironment. The increase in total and CD34\(^+\) cell numbers in the PMVEC-noncontact versus liquid culture suggests that soluble PMVEC derived factors may be involved.\(^5^5,5^6\) We have previously reported that GM-CSF may be playing an important role by inducing the PMVEC monolayers to be hematopoietically permissive.\(^5^9\) In addition, PMVEC-derived extracellular matrix proteins may bind exogenous growth factors and present these molecules in active form to the hematopoietic stem cells.\(^5^2,5^4\) Other reports have shown that cytokines are concentrated by glycosaminoglycans\(^2^5\) present in the stromal extracellular matrix and are not found at significant levels in unconditioned conditioned medium.\(^5^2,5^9\) The requirement of cell-to-cell contact for maximal expansion suggests that critical components are membrane associated or may possibly be more active than their soluble counterparts.\(^3^7,5^1-5^9\) Others have reported that endothelial cells express membrane hematopoietic growth factors.\(^5^9\) The involvement of novel cytokines and adhesive interactions between PMVECs and stem or progenitor cells and their effects on the regulation of hematopoiesis in vitro remains to be elucidated.

In summary, coculturing of human CD34\(^+\) bone marrow cells in contact with porcine microvascular endothelial cell monolayers (plus exogenous growth factors) results in a robust and sustained expansion of multipotential progenitor cells. Concurrently, there is an increase in cell numbers phenotypically characterized by CD34 surface expression. Functionally, this is reflected in the expansion of cells with a high replating capacity (which also have a dispersed cell/cobblestone foci colony pattern), increases in CFU-blast numbers, and a sustained output of committed CFCs over 5 weeks of culture. The PMVEC culture system has the potential to be a useful scientific tool for the study of important cell-to-cell interactions that may be involved in stem cell self-renewal and expansion. Additionally, the ability of the PMVEC culture system to expand bone marrow progenitor cells ex vivo may have therapeutic use in autologous bone marrow transplantation.

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Porcine brain microvascular endothelial cells support the in vitro expansion of human primitive hematopoietic bone marrow progenitor cells with a high replating potential: requirement for cell-to-cell interactions and colony-stimulating factors

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