Human Bone Marrow Microvascular Endothelial Cells Support Long-Term Proliferation and Differentiation of Myeloid and Megakaryocytic Progenitors

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Endothelial cells are a major component of the bone marrow (BM) microenvironment that regulate the trafficking and homing of hematopoietic progenitor and stem cells. In this paper, we provide evidence that BM endothelial cells (BMECs) also support multilineage hematopoiesis by elaboration of soluble cytokines. Hematopoietic progenitor cells incubated in direct contact with BMEC monolayers, or physically separated by microporous membrane, expanded fivefold to sevenfold at 7 days, in the absence of exogenous cytokines. Flow cytometric analysis of proliferating progenitor cells grown in the presence of BMEC monolayers showed that by day 14 of coculture, 70% to 80% of hematopoietic cells were myeloid, expressing CD15 or CD14, and 14% to 19% were megakaryocytic, expressing GPIIb/IIIa or GPIb-CD34+ cells derived from umbilical cord blood, cultured in the upper chamber of transwell culture plates, as well as the cells grown in direct contact with BMEC monolayers, generated progenitors for up to 70 days. Unstimulated BMEC monolayers constitutively produce interleukin-6, Kit-ligand, granulocyte colony-stimulating factor, and granulocyte macrophage colony-stimulating factor. These data suggest that BMEC regulate proliferation of hematopoietic progenitor cells and long-term culture initiating cells by elaboration of lineage-specific cytokines.

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The Bone Marrow (BM) microenvironment is a complex three-dimensional structure where hematopoietic cells proliferate, mature, migrate into the sinusoidal space and enter the circulation in an exquisitely regulated fashion. Stromal cells within the BM microenvironment provide a suitable environment for self-renewal, proliferation, and differentiation of hematopoietic stem cells. BM stromal cells consisting of endothelial cells, fibroblasts, reticular cells, monocytes, osteoclasts, osteoblasts, and adipocytes secrete cytokines and produce extracellular matrix in addition to providing direct cellular contact that regulates hematopoiesis.

Our understanding of stromal regulation of hematopoiesis derives largely from investigations of long-term human BM culture (LTBMC). In LTBMC systems, the irradiated stromal elements, which are comprised of BM fibroblasts, adipocytes, occasional endothelial cells, and macrophages, are seeded with hematopoietic stem and progenitor cells in the presence of standard cell culture medium supplemented with horse serum, fetal calf serum (FCS), and hydrocortisone. BM stroma used in LTBMC system support the proliferation of long-term culture initiating cells (LTCICs), and proliferation of hematopoietic progenitors for several months. BM stromal cells and fibroblast cell lines support the proliferation of predominantly myeloid progenitors. Marine lymphoid progenitors can only be expanded in selected culture conditions originally described by Whitlock et al.

Although stromal cells used in LTBMC occasionally contain a small percentage of contaminating endothelial cells, the relative contribution of this cell type in the regulation of hematopoiesis is not well established. The anatomic location and complex network of BM endothelial cells (BMECs) within the BM microenvironment and its close association with mature hematopoietic elements suggests that it may regulate both early and late proliferation of hematopoietic elements. Much of the study of regulation of hematopoiesis by endothelial cells derives from studies of human umbilical vein endothelial cells (HUVECs). Other than macrophage colony-stimulating factor (M-CSF) and Kit-ligand, resting vascular endothelial cells such as HUVECs have not been reported to constitutively produce significant quantities of hematopoietic cytokines. However, stimulation of HUVEC monolayers with interleukin-1 (IL-1) or tumor necrosis factor results in production of cytokines which include IL-1, IL-4, IL-6, granulocyte-macrophage CSF (GM-CSF), and granulocyte CSF (G-CSF). Phorbol ester–stimulated HUVEC monolayers express messenger RNA for IL-11, but IL-11 protein has not been detected either in resting or phorbol myristate acetate (PMA)-activated HUVECs by IL-11 bioassays or enzyme-linked immunosorbent assay (ELISA). In addition, resting vascular endothelium constitutively produces factors that may regulate hematopoiesis, such as basic fibroblast growth factor (FGF). Transforming growth factor β (TGF-β), and platelet-derived growth factor. Human endothelial cells have recently been reported to express receptors for IL-3, kit-ligand, erythropoietin, thrombopoietin, and basic FGF. Finally, expression of adhesion molecules by resting and activated endothelium may play a critical role in regulating the transit of CD34+ progenitor and mature blood cells in and out of the BM.

We have recently reported the isolation and characterization of BMECs. In this report we provide evidence that resting BMEC monolayers support long-term hematopoiesis, specifically myelopoiesis and megakaryocytogenesis, by the...
elaboration of lineage-specific cytokines. BMEC monolayers maintain their cellular integrity during long-term coculture experiments, and do not require immortalization or growth inhibition with irradiation, allowing the study of the physiologic role of these cells in regulation of hematopoietic cell trafficking, proliferation, and differentiation in vitro. In addition, we provide evidence that direct contact between BMECs and CD34+ progenitor cells may augment megakaryocyte proliferation and enhance hematopoietic progenitor cell expansion.

MATERIALS AND METHODS

Isolation of endothelial cells from BM aspirate. After obtaining consent, BM aspirates were obtained with a standard Jamshidi needle in preservative free heparin (50 U/mL) from posterior or anterior iliac crest of normal volunteer donors undergoing BM harvest at Memorial Sloan-Kettering Cancer Center (New York, NY). BMECs were isolated as described recently. Briefly, the BM aspirate was passed through a 40-μm nylon filter (Falcon, Lincoln Park, NJ), and the retained stromal elements were digested with 5 mL of 0.1% collagenase for 20 to 30 minutes at 37°C. Subsequently, the digested material was passed through a 22-gauge needle, and then refiltered through another 40-μm filter to obtain microvesSEL fragments. The microvesSEL explants were cultured in endothelial cell growth medium (ECGM) containing M199 medium (MA Bioproducts, Walkersville, MD), heparin 90 μg/mL (Sigma Chemical Co, St Louis, MO), 20% FCS, endothelial cell growth factor (ECGF) 20 μg/mL (Organon Teknika Corp, Durham, NC) or 10 ng/mL of vascular collagenase for 20 to 30 minutes at 37°C. Subsequently, the digested endothelial cells were selectively removed by the addition of ECGM containing M199 medium (MA Bioproducts, Walkersville, MD), 20% FCS, endothelial cell growth factor (ECGF) (VEGF) (PeproTech, Rocky Hill, NJ), L-glutamine 2 mmol/L (Sigma) penicillin (80 U/mL), and streptomycin (80 μg/mL), for 5 to 7 days. Subsequently, the endothelial cells were selectively isolated by using Ulex europeus selections as described previously or alternatively, the fibroblasts contaminating the cultures were mechanically waded out. The purity of the BMEC monolayers was assessed by metabolic labeling with Dil-ac-SDL and immunohistochemical staining with monoclonal antibody (MoAb) to factor VIII/von Willebrand factor (vWF). On average, 95% to 98% of BMEC monolayers had characteristic staining with factor VIII/vWF antibody, or were metabolically labeled with Dil-ac-SDL.

The pure BMEC collected from each wash were pooled, centrifuged, then resuspended in ECGM and plated on gelatin-coated six-well Costar tissue culture dishes (Costar, Cambridge, MA). For coculture experiments confluent monolayers of BMEC, HUVEC, or BM, stroma were incubated with CD34+ cells in a modified coculture medium containing Iscove’s modified Dulbecco’s medium (IMDM: MA Bioproducts), 20% FCS, penicillin (80 U/mL), streptomycin (80 μg/mL), with or without 0.5 μg/mL of heparin and 0.1% FCS of ECGM. HUVEC monolayers were cultivated in ECGM, by the technique described by Jaffe et al. BM stroma were obtained from platelet BM aspirate, as previously described. The BM stroma were maintained in IMDM with 10% FCS, 10% horse serum, 2 mmol/L L-glutamine, and 10−4 mol/L hydrocortisone. Less than 2% of the established BM stroma stained with factor VIII/vWF, suggesting that the predominant cell type in the monolayers are BM fibroblasts and reticular cells. All monolayers used in these experiments were between passages 2 and 4.

Immunofluorescent flow cytometry. Hematopoietic cells (106 cells/mL) from the upper chamber of the transwell cultures as well as those proliferating in direct contact with BMECs were obtained by weekly demidepopulation and were placed in Hank’s balanced salt solution (HBSS, Sigma), 0.02% azide, 1% bovine serum albumin (Sigma) containing 1 mmol/L adenine, 2 mmol/L theophylline, and 2 mmol/L EDTA (buffer A) to inhibit megakaryocyte activation or aggregation. Subsequently, the cells were incubated with saturating doses of conjugated and nonconjugated MoAb for 30 minutes at 4°C. The majority of MoAb used in these experiments (Table 1) were conjugated with fluorescein (FITC), phycoerythrin (PE), or rhodamine (RD1). Even though the endothelial cell monolayers retained intact during the experiment period reported here, a few endothelial cells could have detached during demidepopulation of hematopoietic cells grown in direct contact with BMECs or HUVECs. As a result, the demidepopulated cells were initially stained with CD45, which stains all hematopoietic cells. All subsequent staining was gated on CD45+ cells.

Megakaryocytes were quantified by MoAbs to GPIb/IIa and GP Ib. Three different conjugated MoAbs that were specific for different epitopes of GPIb/IIa, were 10E5, PLT-1-FITC (Coulter), and P2-FITC (ImmunoTech). Unconjugated MoAb 10E5 gave similar results to P2 and PLT1. CD61 (GPIIb) was another marker used to confirm the presence of megakaryocytes. Ulex europeus is a lectin that binds to human endothelial cells as well as subtypes of human erythroid precursors and megakaryocytes (unpublished data, November 1994). Because no glycophorin A+ cells were detected in coculture experiments, we used Ulex europeus conjugated with PE or FITC to confirm the presence of megakaryocytes proliferating on the transwell plates.

A relatively high number of CD34+ cells isolated from umbilical cord blood (CB) or peripheral blood (PB) mononuclear cells by immunoadsorption technique stain, positively with GPIb/IIa antibody. This false-positive result is caused by nonspecific binding of platelets or platelet dust to CD34+ cells. To circumvent this problem, CD34+ cells were washed three times with phosphate-buffered saline (PBS) and 1 mmol/L EDTA and resuspended in PBS for immunohistochemical staining or flow cytometry analysis with antibodies to GPIb/IIa complex. By this method, only 2% of cytoplasms cells were CD34+.

CD2-RD1 (Leu-5b), CD3-RD1, CD4-RD1 (T4), CD8-RD1 (T8), CD19-FITC (B4), and CD20-FITC (B1) were used for the detection of lymphoid series. CD14 (Leu-M3-FITC, and MoRD21 clone) were used for detection of monocytes. CD33-PE (Leu-M9), which binds to myeloid cells in addition to early megakaryocytic precursors, was used as a marker for determination of differentiation state of hematopoietic progenitor cells. CD15-FITC (Leu-M1) was used for detection of early and late myeloid precursors. Although CD13 (Leu-M7) is a suitable marker for detection of myeloid precursors, we could not use this marker in coculture experiments because it is also expressed and released by endothelial cells. HPCA-2-PE, QBEnd10-FITC, and 11.1.6 were three MoAbs used for detection of CD34+ cells during coculture experiments.

Controls were isotype-matched nonimmune IgGs and FITC. PE, or RD1 conjugated antimouse IgG (F(ab)2). Cell-associated immunofluorescence was assayed by quantitative flow cytometry Coulter EPICS, available at Cornell Medical College (New York, NY).

Immunohistochemical techniques. MoAbs to factor VIII/VWF (Dako, Glostrup, Denmark). CD34 (HPCA-2, Becton Dickinson, Mountain View, CA), intercellular adhesion molecule (ICAM) (AMAC), vascular cell adhesion molecule (VCAM) (AMAC), CD15-FITC (Leu-M1), CD14-FITC (Mo2-RD1), GPIb (SZ2, AMAC), GPIb/IIa (10E5, a gift from Dr B. Coller, Mount Sinai Medical Center, New York, NY), or polyclonal antibodies to factor VIII/vWF (Sigma) at different dilutions were incubated with fixed cells for 1 hour. BMEC monolayers with bound hematopoietic cells from different weekly time points of coculture experiments were washed three times in PBS supplemented with 2 mmol/L of calcium and magnesium, fixed in 3% formalin in PBS for 30 minutes, then quenched with 0.1 glycine for 10 minutes, and blocked with 1.5% horse serum. After washing with PBS, biotinylated antiamouse or antirabbit Ig diluted 1:200 in PBS containing 1% BSA was incubated with the cells for 30 minutes at room temperature. After 30 minutes of incubation with avidin-labeled peroxidase or alkaline phosphatase, slides were rinsed and incubated with peroxidase substrate amino-
ethyl carbazole (red stain) or alkaline phosphatase substrate New Fuchsin (Dako) (red stain) for 10 minutes. After a final rinse, the slides were counterstained with 1% hematoxylin.

Samples of BM aspirate or confluent monolayers of BMEC, HUVEC, and BM stroma were grown in chamber slides, air dried, and fixed with acetone/alcohol. Primary mouse MoAbs to factor VIIa, vWF (Dako) were incubated on the slide for 1 hour at room temperature. The cells were washed twice with 1% BSA in PBS, and re-suspended in 1% BSA to a concentration of 1 to 10^6 cells/mL. Photomicrographs were taken with a Nikon fluorescence microscope (Tokyo, Japan) on Kodak Ektachrome 160 ASA color film (Kodak Ltd, Liverpool, UK). FITC or PE was visualized using standard FITC/PE excitation/emission filter combinations.

**Metabolic labeling with Dil-Ac-LDL.** Near confluent monolayers of HUVEC and BMEC or mixed populations of endothelial cells and fibroblasts were incubated with 1 μg/mL of Dil-Ac-LDL (acetylated low-density lipoprotein labeled with diacetylcydine 1,3,3,3-tetramethyl-indocarbocyanine perchlorate), (Biomedical Technologies Inc, Stoughton, MA) was added to the cells at a concentration of 50 pg/mL for 30 minutes at 4°C. The cells were washed twice with 0.1% BSA in PBS, and resuspended to 10^8/mL, and passed through an avidin column. Cells collected in the bead-negative fraction were recovered. For separation using immunomagnetic beads, low-density mononuclear cells were washed twice with 0.1% BSA in PBS and were resuspended to 1 × 10^6 cells/mL. A mouse IgG1 anti-human antibody to CD34 (Biocytosis, Uniondale, NY) was added to the cells at a concentration of 25 pg/mL. Sheep antimouse IgG (Fc) immunomagnetic beads (30 μg/mL), providing a bead-to-cell ratio, were added for 30 minutes at 4°C. The bead-positive fraction was selected with a magnetic separator, resuspended in 20% FCS, and kept overnight at 37°C in 1% porosity.) on the upper chamber with HUVEC, BMEC, or BM stromal layers grown in contact with BMSCs, HUVECs, or stromal monolayers, one half of the coculture medium was removed and replaced with fresh medium. Hematopoietic cells grown on the transwells or PB were plated in triplicate in six-well plates (Costar) in direct contact with HUVEC, BMEC, or BM stromal monolayer. CD34^+ cells (4 × 10^6/mL) isolated from BM were plated in triplicate in six-well plates (Costar) in direct contact with HUVEC, BMEC, or BM stromal monolayer. CD34^+ cells (4 × 10^6/mL) were plated on transwell plates (Costar, 0.4 μm porosity) on the upper chamber with HUVEC, BMEC, or BM stromal layers grown as confluent monolayers separately on the lower chamber. Each week, hematopoietic cells were demidepopulated and replaced with fresh modified coculture medium. For hematopoietic cells grown in contact with HUVECs, HUVECs, or stromal monolayers, one half of the coculture medium was removed and replaced with fresh medium. Hematopoietic cells grown on the transwells were demidepopulated as follows: one half of the volume containing

Table 1. MoAbs Used in This Study

<table>
<thead>
<tr>
<th>Cluster</th>
<th>MoAb</th>
<th>Isotype</th>
<th>Cellular Distribution</th>
<th>Manufacturer</th>
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<tr>
<td>CD2-RD1</td>
<td>T11</td>
<td>IgG1</td>
<td>T cells, thymocytes</td>
<td>C</td>
</tr>
<tr>
<td>CD3-RD1</td>
<td>T3</td>
<td>IgG1</td>
<td>T lymphocytes</td>
<td>C</td>
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<tr>
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<td>T4</td>
<td>IgG1</td>
<td>Helper/inducer cells</td>
<td>C</td>
</tr>
<tr>
<td>CD6-RD1</td>
<td>T8</td>
<td>IgG1</td>
<td>Cytotoxic, suppressor T cells</td>
<td>C</td>
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<td>IgG1</td>
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<td>Monocytes</td>
<td>C</td>
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<td>Dr B. Coller</td>
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<td>Lectin</td>
<td>Endothelial cells, subtype of erythroid cells, Meg</td>
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</table>

Abbreviations: IM, Immunotech; BD, Becton Dickenson; C, Coulter; ND, not defined; Meg, megakaryocyte.
the cells growing on the transwell were demidepopulated each week and replaced with fresh coculture medium. In addition, one half of the medium on the lower chamber of the transwells was removed and replaced with fresh coculture medium. To determine the phenotype of hematopoietic cells adherent to BMECs, HUVECs, or stromal monolayers, each week the monolayers were washed three times with HBSS supplemented with 2 mmol/L magnesium and 2 mmol/L calcium chloride to remove the nonadherent cells, and fixed with formalin or acetone for immunohistochemical staining.

For BMEC activation studies, BMEC monolayers were incubated with 10 U/mL of IL-1β for 18 hours at 37°C. Subsequently, BMEC monolayers were washed five times with ECGM to remove IL-1β and resuspended in modified coculture medium.

**Long-term cultures.** CD34+ cells (4 × 10³/mL) from CB or PB were suspended in modified coculture medium and were placed in either direct contact with BMECs, HUVECs, or stromal monolayers (contact) or separated by a 0.4 μm transwell membrane (noncontact). Each week the wells were demidepopulated, cells counted on a Coulter Counter ZM (Hialeah, FL), and replaced with an equal volume of fresh modified coculture medium. Cultures were continued until no detectable progenitors were generated, though cells were still present.

After complete removal of nonadherent cells from the stroma, the adherent cells were removed with 1 mmol/L EDTA and 0.1% trypsin. Trypsin was deactivated with FCS and the cells were washed three times with PBS. Cells were quantified by hemocytometer and were evaluated for progenitor content using agarose assays and cell surface markers using either flow cytometry or immunocytochemistry as described. All of the trypsinized cells were plated for the agarose assays.

**Progenitor assay (agarose assay).** Starting at day zero, and then weekly thereafter, escalating numbers of cells (1 × 10^3 to 4 × 10^6) collected after demidepopulation were placed in triplicate in 35-mm tissue culture dishes (Corning, Corning, NY), containing 20% FCS in IMDM supplemented with 50 μg/mL gentamicin (GIBCO), monothioglycerol (7.3 × 10^-5 mol/L; Sigma), 0.36% agarose (FMC Bioproducts, Rockland, ME), and a combination of five cytokines: 20 ng/mL human kit-ligand (kindly provided by Immunex, Seattle, WA); 50 ng/mL human IL-3 (Immunex); 20 ng/mL mutein IL-6 (kindly provided by Imclone Systems Inc, New York, NY); 6 IU/mL erythropoietin (Amgen, Thousand Oaks, CA); and 100 ng/mL human G-CSF (Amgen). This combination is abbreviated as K36EG. Cultures were incubated at 37°C in 100% humidified 5% CO2 in air and removed from the incubator at 14 days. Colonies defined as greater than 40 cells were counted using an inverted microscope. Burst-forming unit erythroid (BFU-E) and granulocyte-macrophage-burst-forming unit erythroid (BFU-E) and granulocyte-macrophage (GM)-CFU CFU-GM were observed using this assay. To determine total progenitors generated over time, the area under the curve (AUC) was calculated as the sum of the parallelograms created from the data points (Fig 1, A and B). A simplified formula is: AUC = nA + nB + ... + nX, where nA is the number of progenitors at day zero and nX is the number of progenitors on the last day of culture.

**cDNA-PCR technique.** Hematopoietic cells proliferating in direct contact with BMECs or on the transwell plates separated from BMEC monolayers were removed for determination of GPIbexpression each week. Total RNA was isolated from 2 × 10³ cells proliferating on days 7, 14, and 21 of coculture by RNAzol reagent (Biotecx Lab, Houston, TX). Total RNA was also isolated from intact resting BMEC monolayers. Total RNA was precipitated with ethanol and resuspended in modified coculture medium.

![Fig 1. Long-term culture of CD34+ progenitor cells: CD34+ cells from PB (A) or CB (B) were plated either in contact with BMEC or separated by a 0.4-μm microporous transwell membrane. Each week the nonadherent cells were demidepopulated and progenitors (mean ± SEM, n = 3) were determined in an agarose assay. On days 14, 28, and 42, the adherent hematopoietic cells were removed with enzymatic digestion of the adherent monolayer, and the progenitors were quantified by agarose assay (C). The number of progenitors (mean ± SEM, n = 4) adherent to the BMECs during the coculture experiment comprised a small percentage of overall expanding progenitors. There is no difference between the total number of progenitors (NA + A) that were generated from contact cultures and those from the transwell plates (P > .05 for all time points).](image-url)
0.2 volume of sodium acetate and 2.5 volume of 100% ethanol, dried, and resuspended in DEPC-treated dH2O. RNA was reverse transcribed using the Moloney murine leukemia virus reverse transcriptase, and oligo dt as the primer for 15 minutes at 42°C. DNA amplification was performed in polymerase chain reaction (PCR) buffer using the GPIIb primer pair (5'-CTGCGTCGCTTTGACCTCC-3') and (5'-AACTGGCGCTAGGTTCTCC-3'). For thrombopoietin determination, the primer pair 5'-AGAAATGGCGTCTTGTTCT-3' and the reverse primer 5'-CAGTCTGGCTGAAGGACATGG-3' were used for DNA amplification. Samples were cycled 35 times and the PCR products were analyzed on a 2% NuSieve agarose gel. The expected product at 265 base pair was cut and sequenced to confirm the identity of the product.

Quantification of cytokines elaborated by BMEC and HUVEC monolayers. BMEC or HUVEC-conditioned medium were collected from 3-day-old cultures from equally confluent culture flasks (5.56 x 10^6 cells/flask), filtered, and 200 microliter samples were used in a sandwich ELISA (R & D Systems, Minneapolis, MN) for the quantitative determination of IL-6, G-CSF, GM-CSF, and kit-ligand. Control medium, which contained M199 medium, 20% FCS, was used to sustain endothelial cell monolayers during these experiments.

Statistical analysis. The results are presented as the mean ± SEM of the data obtained from three or more experiments performed in triplicates. Statistical significance was determined using the Student's t-test.

RESULTS

BMEC monolayers induce proliferation of CD34+ cells. CD34+ cells isolated by immunoadsorption from PB or CB were placed in liquid culture in direct cellular contact with BMEC monolayer, or on 0.4-μm microporous transwell plates to separate them from BMEC monolayers. Each week, the nonadherent hematopoietic cells were demidepopulated and placed in agarose assays to evaluate progenitor content. Progenitors grown in direct contact with BMEC expanded fivefold by 7 days and were detectable for up to 56 days in experiments with PB-derived CD34+ cells, and 70 days for CB-derived CD34+ cells (Fig 1, A and B). CB CD34+ cells, grown physically separated from BMEC monolayers in the transwell plates, generated progenitors for up to 63 days, whereas PB CD34+ cells generated progenitors for 49 days. Total progenitors generated from CB CD34+ cells over time as calculated by integrating the AUC was 88,300 progenitors in experiments in which cells were in BMEC contact and 80,200 progenitors in BMEC noncontact experiments. For the first 28 days, a time when the majority of progenitors were generated, AUC for the two conditions was almost identical. However, after 28 days, BMEC contact generated approximately 1 log greater number of progenitors per week. For PB, AUC for BMEC contact was 39,500 progenitors and for BMEC noncontact, 17,500 progenitors. The contact cultures show a slight but consistent advantage in generating progenitors over those of noncontact cultures. However, this difference does not reach statistical significance.

Fig 1C shows the number of progenitors from CB that are that adherent to the BMEC monolayers during the coculture period. On day 14 of coculture, approximately 10% of expanding progenitors are adherent to BMEC monolayers. On day 28 and 42 of coculture, a comparable number of progenitors are attached to the underlying stroma. However, the relative number of adherent to nonadherent progenitors are significantly higher because the nonadherent progenitors are decreased. There was no statistical difference between the total number of progenitors (NA + A) generated from contact cultures as compared with those generated from transwell plates.

Phenotype of proliferating hematopoietic cells. Flow cytometric and immunohistochemical staining of the proliferating hematopoietic cells grown in direct contact with BMEC showed that by day 7, 3% ± 2% of hematopoietic cells remained CD34+, 88% ± 2% of cells expressed CD33, 40% ± 5% expressed CD15, and 12% ± 1% were monocytic, expressing CD14 antigen (Table 2). By day 14, the number of mature myeloid precursors CD15 were increased to 47% ± 2%, and monocytic cells expressing CD14 increased to 22% ± 5%. Similar results were obtained in experiments performed in transwell plates (Table 2).

Compared with BM stroma and HUVEC monolayers, BMEC supported greater megakaryocyte proliferation as determined by expression of GPIIb/IIIa and GPIIb in Table 2 and Fig 2. Flow cytometric analysis of cells grown on the transwell plates showed that by day 14, 14% ± 3% of proliferating cells were GPIIb/IIIa+, and CD34+ cells grown in direct contact with BMEC gave rise to 17% ± 5% GPIIb/IIIa+ cells. Because a significant number of megakaryocytes were attached to BMEC and HUVEC monolayers, the number of attached megakaryocytes were quantified each week by direct immunohistochemistry of monolayers with GPIIb/IIIa antibodies. Figure 3 displays the total number of adherent and nonadherent megakaryocytes on day 14 of coculture, which comprised ≈19% of hematopoietic cells grown in contact with BMECs. No adherent GPIIb/IIIa+ cells were detected on the surface of transwell plates. Even though HUVEC and BM stromal monolayers support expansion of myeloid series (85% ± 8% of expanding cells), they support the expansion of only a small percentage of megakaryocytic GPIIb/IIIa+ cells (Table 2), and Fig 2. The presence of megakaryocytes was also confirmed by reverse transcriptase-PCR (RT-PCR) (using oligonucleotide primers specific for GPIIb cDNA) on total RNA that was isolated from the hematopoietic cells proliferating in transwells as well as cells grown in direct contact with BMECs.

In parallel studies, hematopoietic progenitors proliferating in the coculture experiments were also stained with MoAbs to lymphoid and erythroid progenitors. Only a small number of CD2 or CD19 cells were detected. No staining was detected with MoAbs to glycoporphin A, CD3, CD20, CD4, or CD8.

IL-1β stimulation of BMEC monolayers. To study the effect of stimulated endothelium on hematopoiesis, endothelial cells were stimulated with IL-1β (10 U/mL) for 18 hours. Incubation of CD34+ cells on the transwell plates with IL-1β–activated endothelium resulted in 10-fold expansion of progenitor cells over 7 days, and induced a relatively rapid maturation of myeloid cells as evidenced by expression of CD15 (53 ± 3, day 7), and CD14 (25% ± 5%, day 7) (Table 3). By day 14, 70% ± 6% of cells were myeloid, expressing CD33, CD15, or CD14 markers; 6% ± 1% were megakaryocytic, expressing GPIIb/IIIa or GPIb markers.

Morphologic analysis. Figure 2A shows a typical day 1 coculture experiment where CD34+ cells were added to...
confluent monolayers of BMEC. By day 14, large numbers of hematopoietic cell colonies were detected over the underlying intact BMEC monolayer (Fig 2, B and C). Figure 2D shows proliferation of hematopoietic colonies of different size and shape including relatively numerous refractile large cells resembling polyploid megakaryocytes. Wright-Giemsa staining of the cytospins of hematopoietic cells proliferating in coculture experiments showed abundant large polyploid megakaryocytes that stained with GPIIib/IIIa antibody (Fig 4, A and B). Cytospins performed on day 14 of coculture experiments showed abundant large polyploid megakaryocytes that stained with GPIIib/IIIa antibody (Fig 4, C and D). Cytospins performed on day 42 and day 56 showed predominantly mature myeloid cells such as monocytes and neutrophils. Quantification of GPIIib/IIIa* cells detected on the cytospins are consistent with the results obtained from flow cytometry.

Continuous monitoring of BMEC and HUVEC monolayers during the coculture with hematopoietic cells with phase-contrast microscopy showed that endothelial monolayers maintain their morphologic integrity for the first 5 weeks of coculture. However, beyond day 42, the BMEC and HUVEC monolayers grown in direct or indirect contact with hematopoietic cells form characteristic tube-like structures. Endothelial cells maintained in coculture medium in the absence of hematopoietic cells underwent no major morphologic changes. Determination of cytokines produced by BMEC. ELISA assays performed on the conditioned medium obtained from BMEC and HUVEC monolayers are shown in Table 4. Resting BMEC elaborated high levels of kit-ligand, IL-6, GM-CSF, and G-CSF. Resting BMEC monolayers used in these experiments did not express activation-dependent adhesion molecules such as VCAM, ICAM, ELAM, suggesting that constitutive elaboration of cytokines by BMEC was not caused by activation of BMEC, but rather was caused by a physiologic programming of these cells.

**DISCUSSION**

We show that unstimulated human microvascular endothelium derived from BM microenvironment support proliferation and differentiation of CD34+ cells by constitutive production of G-CSF, GM-CSF, IL-6, and Kit-ligand. Constitutive production of cytokines such as G-CSF, GM-CSF, and IL-6 is a unique feature of microvascular endothelium because vascular endothelium derived from lining of aorta,
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Fig 2. Proliferation of CD34⁺ cells. Phase-contrast micrographs of proliferating hematopoietic progenitors in direct contact with resting BMEC monolayers on day 1 (A), day 14 (B and C), and day 21 (D). The small CD34⁺ cells shown in A (arrows), proliferate to give rise to floating colonies (arrows) consistent with CFU seen in agarose assays (B and C). D shows proliferation of numerous large refractile cells resembling megakaryocytes (short arrows). Only a few colonies of hematopoietic cells were attached to the underlying BMEC monolayers. As shown here, the BMEC monolayers maintain their cobblestone appearance during coculture experiments (original magnification × 200).

or umbilical vein can only produce these cytokines upon stimulation with IL-1 or TNF.¹²,¹⁴,¹⁸

Long-term culture studies have shown that BM stroma or fibroblasts support predominantly myelopoiesis.³ BMECs not only support myelopoiesis, but also megakaryocytopoiesis, a feature not seen in BM stroma that were maintained in similar culture conditions. Elaboration of cytokines such as IL-6 and kit-ligand by BMECs induce proliferation of megakaryocytic precursors. CD34⁺ cells that were cocultured in direct contact with BMEC monolayers gave rise to a larger number of megakaryocytes than those that were cocultured on the transwells. This finding suggests that membrane-bound cytokines such as kit-ligand may influence megakaryocyte precursor commitment and proliferation. Previously, it has also been shown that endothelial cell extracellular matrix (ECM), which is rich in heparin sulfate and glycosaminoglycans, functions as a cytokine sink for heparin binding factors. Among these ECM-bound cytokines, b-FGF, kit-ligand, and GM-CSF are positive regulatory factors that promote megakaryocytopoiesis,²⁴,²⁵,²⁶ whereas TGF-β is a potent negative regulatory factor.³⁶ Thus, it is possible that interaction of CD34⁺ cells with these positive and negative regulatory factors released by BMECs have an overall selective advantage for megakaryocyte lineage commitment and proliferation. The critical role of these negative and positive regulatory factors were recently confirmed by Waegell et al.,³⁷ who have shown that neutralization of TGF-β in Dexter-type cultures results in significant proliferation of megakaryocytes. This finding suggests that even though BM stroma produces large amounts of megakaryopoietins such as IL-6,³⁶,³⁹ production of negative regulatory factors such as TGF-β has predominant effect in suppressing megakaryocyte production. Although IL-6 and kit-ligand³⁵ may promote proliferation of megakaryocytic precursors, the presence of polyploid megakaryocytes in our coculture experiments suggests that BMMECs may also elaborate other cytokines such as thrombopoietin.³⁵,⁴⁰ Availability of sensitive ELISA or bioassays may facilitate detection of thrombopoietin in coculture conditioned medium.

We have previously shown that a large number of CD34⁺ cells and megakaryocytes derived from freshly isolated BM mononuclear cells adhere to BMEC monolayers.³⁰ However, during the first 4 weeks of coculture period, only a small fraction of overall proliferating CD34⁺ derived from CB or
PB cells were attached to the BMEC monolayers. The low affinity of both megakaryocytes and myeloid precursors for BMEC monolayers under coculture experiments described in this paper is particularly striking. Continued coculturing of proliferating hematopoietic cells beyond day 56 to 63, results in adhesion of relatively large numbers of monocytes, neutrophils, dendritic as well as polyploid megakaryocytes to BMEC and HUVEC monolayers (data not shown). This enhanced binding probably reflects complex interaction between maturing hematopoietic cells and endothelial cells.

Both HUVEC and BMEC monolayers support proliferation of a large number of myeloid cells. Production of G-CSF, GM-CSF, b-FGF, and kit-ligand by resting BMEC account for proliferation and differentiation of myeloid progenitors. Although cellular contact may be critical for megakaryocyte cell proliferation and maturation, direct cellular contact between CD34⁺ progenitor cells and BMEC did not offer any advantage with respect to myeloid cell proliferation. The majority of proliferating myeloid cells, particularly early promyelocytes, were highly granular. This finding is striking because myeloid cells proliferating in the presence of cytokines or BM stroma were significantly less granular than those that were grown in the presence of endothelial cell monolayers.

The number of progenitors generated by BMECs were estimated by calculation of AUC in Fig 1, A and B. AUC reflects the dynamics of proliferation, differentiation, and depletion of hematopoietic stem cells and indicates the net production of progenitors over several weeks in the face of weekly demidepopulation. Figure 1, A and B, shows that in the first few weeks of coculture, BMEC monolayers were generating progenitors, whereas during the remaining period of coculture, there were preservation of progenitors despite weekly demidepopulation. Verfaille et al have shown that direct contact between hematopoietic progenitor cells is not critical for long-term hematopoiesis. Our data show that direct contact cultures consistently show better preservation of progenitors, although this did not reach statistical significance. Adherence of a small subset of proliferating progenitors to BMEC monolayers may preserve or prevent the exhaustion of progenitors during the coculture period and weekly demidepopulation.

IL-1β activation of BMEC results in significant adhesion of CD34⁺ cells to these monolayers. These progenitors remained attached to BMEC monolayers for several days, forming large adherent colonies. Thus, it was difficult to assess the progenitor generated from CD34⁺ cells that were grown in contact with IL-1β-activated BMEC or HUVEC.
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monolayers. However, CD34⁺ cells incubated on transwell plates, physically separated from IL-1β-activated endothelium, show very rapid proliferation. Flow cytometric analysis of the proliferating cells show a predominance of myeloid lineage, suggesting that BMEC activation may result in upregulation of G-CSF and GM-CSF production. This result is consistent with studies that have shown that IL-1β activation of HUVEC cells results in upregulation of G-CSF and GM-CSF, which would favor myeloid cell proliferation and differentiation.12,16-18

The coculture experiments were performed in an IMDM-based medium to supply adequate nutrition for proliferating hematopoietic cells. Very low concentration of heparin and endothelial cell growth supplement were added to IMDM to maintain endothelial cell monolayers. Under these conditions BMEC monolayers remained relatively intact and retained their cobblestone appearance till day 42 to 49 of coculture. Because endothelial cell monolayers, particularly BMEC, are contact inhibited and can maintain their integrity over long periods of time, irradiation or growth inhibition with mitomycin C was not required. Furthermore, addition of low concentrations of ECGF and heparin had no measurable effect on proliferation or phenotype of proliferating hematopoietic cells. However, prolonged coculture beyond day 42 of BMEC and HUVEC monolayers with hematopoietic cells resulted in reproducible shape changes in the endothelial cell monolayers. These changes consisted of reorganization of monolayers into tubular cord-like structures reminiscent of in vivo sinusoidal capillaries and microvessels. The morphologic change in endothelial cells was not only in direct contact cocultures, but also in endothelial cell monolayers that were cocultured with hematopoietic cells on the transwell plates. BMEC monolayers maintained in coculture medium in the absence of hematopoietic cells undergo no major morphologic changes. These findings suggest that soluble factors released by hematopoietic cells may contribute to the changes observed in these experiments.

CD34⁺ cells isolated from BM contain CFU-F cells that may give rise to BM stroma during the coculture conditions described in this study. As a result, in experiments designed in this report only CD34⁺ cells derived from CB and PB were used to avoid the repopulation of the BMEC or HUVEC monolayers with BM stroma, which would influence proliferation and maturation of hematopoietic cells.

In summary, BMEC monolayers support multilineage hematopoiesis by elaboration of soluble cytokines. Because BMEC monolayers can maintain their cellular integrity over long periods of coculture with different types of hematopoietic cells, they provide an ideal feeder layer for LTBM C studies. The reciprocal interaction between BMEC monolayers and proliferating hematopoietic cells may be crucial for self-renewal of pluripotent stem cells, as well as for regulation of differentiation of committed progenitor cells. The coculture model described in this report can be used to study this complex interaction as well as to characterize the cytokines released by BMECs that are responsible for multilineage hematopoiesis and expansion of pluripotent progenitor cells. The study of surface glycoproteins expressed on the luminal and subliminal surface of BMECs in resting and activated state is critical to the characterization of the factors that regulate proliferation of CD34⁺ stem cells as well as the characterization of mature and malignant hematopoietic cells.

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**REFERENCES**


12. Steff CA, Niemeyer CM, Faller DV: The production of hema-
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Human bone marrow microvascular endothelial cells support long-term proliferation and differentiation of myeloid and megakaryocytic progenitors

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