RAPID COMMUNICATION

Isolation and Characterization of Human Bone Marrow Microvascular Endothelial Cells: Hematopoietic Progenitor Cell Adhesion

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To examine potential mechanisms by which hematopoiesis may be regulated by endothelial cells within the bone marrow (BM) microenvironment, we have devised a technique for the in vitro study of the interaction of human BM microvascular endothelial cells (BMEC) with hematopoietic cells. Microvessels isolated by collagenase digestion of spicules obtained from filtered BM aspirate were plated on gelatin-coated plastic dishes, and colonies of endothelial cells grown from microvessel explants were further purified by Ulex europaeus lectin affinity separation. BMEC monolayers isolated by this technique grew in typical cobblestone fashion, stained positively with antibody to factor VIII/von Willebrand factor, and incorporated acetylated LDL. Immunohistochemical studies showed that BM microvessels and BMEC monolayers express CD34, PECAM, and thrombospondin. In-}

THE BONE MARROW (BM) microenvironment is a complex, three-dimensional structure where hematopoietic elements proliferate, differentiate, mature, and ultimately migrate into the circulation. Stromal cells, which form the backbone of the BM microenvironment, consist of fibroblasts, endothelial cells, adipocytes, osteoclasts, and monocytes. They secrete cytokines, produce extracellular matrix, and mediate direct cellular contact that regulates hematopoiesis. The fibroblasts of the BM adventitia are composed of adventitial reticular cells, perisinusoidal adventitial cells, periarterial adventitial cells, and intersinusoidal reticular cells. Most studies on stromal regulation of hematopoiesis derive from investigations of BM fibroblasts. However, little is known about the role of BM-derived microvascular endothelial cells (BMEC) in the regulation of hematopoiesis. The anatomic location of BM microvascular endothelium suggests that it may serve as a gatekeeper regulating the passage of cells between the marrow and the circulation, but its role in regulating the trafficking and development of hematopoietic cells has not been well studied because of the lack of methodologies to isolate and grow BMEC.

Studies on other endothelial cell types, particularly human umbilical endothelial cells (HUVEC), have shown that the trafficking of immune or inflammatory cells across the endothelium is regulated by the coordinate surface expression of receptors specific for a subset of circulating cells. Several of these surface receptors are induced by inflammatory cytokines such as interleukin-1β (IL-1β). Similarly, the specific expression of unique adhesion molecules on the surface of BM microvascular endothelium may regulate the trafficking of hematopoietic elements, particularly pluripotent stem cells in and out of the BM microenvironment.

Within the BM microenvironment, BMEC reside in close association with other cell types such as fibroblasts, adipocytes, mature megakaryocytes, plasma cells, and hematopoietic cells, and form an interface between the circulation and the hematopoietic compartment. This anatomy suggests that BMEC, through direct cellular contact, may control the final stages of hematopoiesis, regulating the entry of mature cells into the peripheral circulation. The release of cytokines by the BM endothelium, independent of cell contact, may also have a role in the regulation of hematopoiesis. Here we describe a reproducible, and efficient method for isolation, and cultivation of BMEC, and our findings on the potential role of this cell type in the regulation of hematopoiesis by mediating specific adhesion of CD34 progenitor cells.

MATERIALS AND METHODS

Isolation of endothelial cells from BM aspirate. BM aspirates were obtained with a standard Jamshidi needle in preservative-free heparin (50 U/mL) from posterior or anterior iliac crests of normal volunteer donors undergoing BM harvest at Memorial Sloan-Kettering Cancer Center (MSKCC). Informed consent was obtained from each individual before BM harvest. Each 1 mL of BM aspirate obtained from a single BM puncture resulted in removal of approximately 150 to 300 floating spicules with sizes ranging from 250 to 500 μm. Because BM spicules adhere avidly to plastic or glass surfaces, all glassware or plastic pipets used in these experiments were passivated with 1% bovine serum albumin (BSA) to avoid attachment and loss of spicules.
The BM aspirate, consisting of floating fat-laden spicules, was immediately diluted in 1:1 in buffer A (Hanks’ balanced salt solution [HBSS] with 1 mmol/L EDTA), and passed through a 40-μm stainless-steel filter to remove the loosely attached hematopoietic cells. The retained stromal elements, which consisted mostly of fat-laden spicules, were washed with 50 mL of buffer A to remove loosely attached cells and plasma. The filter was then placed in a 50-mL conical tube and the retained spicules were resuspended in 5 mL of buffer A, followed by the addition of 5 mL of 0.2% collagenase (final concentration of 0.1%) for 20 to 30 minutes at 37°C. Occasionally a sample of the material undergoing collagenase digestion was removed and examined by phase-contrast microscopy to assess the adequacy of digestion. The digested material was passed gently through a 20- or 21-gauge needle, and then refiltered through another 40-μm filter to obtain microvessel fragments. The retained microves-}

sels were washed with 30 mL of buffer A, and then collected by gentle vortexing or trituration of the filters in a 50-mL conical tube and plated on fibronectin- or gelatin-coated 12- or 6-well cluster plastic dishes. Centrifugation of the microvessels for 2 minutes at 150g accelerated their attachment to the plastic dish.

After 5 to 7 days of growth of microvessel explants in endothelial cell growth medium (ECGM) containing M199 medium (BioWhit-}

taker, Walkersville, MD), heparin 90 μg/mL (Sigma, St Louis, MO), endothelial cell growth factor 20 μg/mL (Organon Teknika Corp, Rockville, MD), L-glutamine 2 mmol/L (Sigma), penicillin (80 U/ mL), and streptomycin (80 μg/mL), a mixed population of endothelial cell colonies and other adventitial cells were present. The endothelial cells originating from the microvessel explants were washed free of hematopoietic cells, but areas of fibroblast growth were occasion-}

ally noted. At this stage, selective metabolic labeling of endothelial cells with Dil-acetylated LDL29 was used to estimate the purity of endothelial cells within each well. Endothelial cells were further purified from wells with smallest amount of fibroblast contamination (endothelial cell to fibroblast ratio: >1:1) by positive selection using Ulex europaeus 1 (UEA1). UEA1 lectin (Sigma) was covalently bound to Tosyl activated Dyna beads M-450 (Dynal, Great Neck, NY) by the method of Jackson et al29 and Holthofer et al.31 The mixed population of cells was treated with 1 mmol/L EDTA and 0.05% collagenase, washed twice in HBSS, and then resuspended in HBSS + 5% fetal calf serum (FCS) at a cell density of 5 × 10⁵ cells/mL. The cells were incubated for 10 minutes at room temperature with Ulex-coated beads (50 beads/endothelial cell). After incubation the BMEC that bound to UEA1-coated beads were washed five times by resuspending them in 10 mL of HBSS + 5% FCS and mixing by end-over-end rotation for 1 minute, followed by separation using a magnetic particle concentrator (MPC) (Dynal). The contaminating cells in the washes were plated for further identification. The endothelial cells were detached from the UEA1 beads by incubation in HBSS + 5% FCS containing 0.01 mol/L sucrose (L-}

isomer) (Sigma) for 10 minutes at 4°C and the beads were removed with MPC. The pure BMEC collected in each wash were pooled, centrifuged, resuspended in ECGM, and plated on gelatin-coated tissue culture dishes. BMEC monolayers isolated in this fashion can be passaged for 8 to 10 times. Early passage cells (1-4) were used for experiments described below.

Because some microvascular endothelial cells may escape filtration during collagenase digestion, the flow through from the digested material can be washed and then treated with Ulex-coated Dyne beads to remove endothelial cells or, alternatively, digested material can be plated on gelatin-coated plastic dishes. Subsequently, after 7 to 10 days of growth, Ulex-coated Dyne beads can be used to isolate endothelial cells from contaminating stromal cells. Substitution of D-valine for L-valine in the culture medium, which reportedly slows the growth of fibroblasts,32 did not increase the yield of BMEC.

**BM mononuclear cell preparation.** BM aspirates were obtained from normal subjects undergoing BM harvest at MSKCC. Ten milli-}

ters of BM aspirate was drawn into syringes containing 50 U/mL of preservative-free heparin, and passed through a 80-μm filter to remove stromal elements. The BM was dilution with buffer A, layered over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) (density 1.077), and centrifuged at 400g for 20 minutes, and the mononuclear cells at the interface were collected, washed in buffer A, and counted. Plastic adherent cells were removed by incubating Ficoll-purified marrow cells (5 × 10⁶ cells/mL) on Costar plastic dishes (Cambridge, MA) at 37°C for 2 hours. The nonadherent cells were passed again through an 80-μm mesh to remove any cell clumps, and resus-}

pended in Iscove’s modified Dulbecco’s medium (IMDM; Sigma), 20% FCS, and monothioglycerol (10 ng/mL) at a density of 5 × 10⁵ cells/mL. For adhesion studies with BMEC, the cells were resus-}

pended in HBSS supplemented with calcium (2 mmol/L) and magne-}

sium (2 mmol/L).

**CD34** progenitor cell isolation.** Low-density BM mononuclear}

cells (less than 1.077 g/mL) were separated over Ficoll-Hypaque. CD34** cells were enriched using a commercially available cell separation system kit from Cell Pro Inc (Botell, WA),27 washed twice with 1% BSA in phosphate-buffered saline (PBS) and resus-}

pended in 1% BSA to a concentration of 2 × 10⁶ cells/mL and incubated for 25 minutes with a biotinylated anti-CD34 IgM monoclonal antibody (MoAb) (12.8) at room temperature. The cells were washed with 1% BSA to remove unbound antibody, then resuspended at 2 × 10⁶ cells/mL in 5% BSA and loaded onto an avidin column. The ad-}

sorbed CD34** cells were released by manually squeezing the gel bed, resuspended in IMDM with 20% FCS, and counted on a Coulter counter (Coulter Electronics, Hialeah, FL).

**Antibodies used.** MoAbs to factor VIII/VWF (Dako), PECAM (Becton Dickinson, San Jose, CA), thrombospondin (11.4; Oncogene Science, Manhasset, NY), ICAM1 (IOL54, AMAC), VCAM (1G11, AMAC), a-actin (Dako), GPIb (SZ2, AMAC). GPIIIa/IIa (10E5, a gift from Dr B. Colter, Story Brook Medical Center, Story Brook, NY), LFA1 (IOT16, AMAC), L-selectin (DREG-56, a gift from E. Butcher, Stanford University, Stanford, CA). CD38 (AMAC), HLA- DR (Coulter), and CD34 (HPCA-1, Becton Dickinson or 11.1.6, Oncogene Science) were used for immunohistochemical and adhesion assays.

**In vitro adhesion assay.** Plastic nonadherent BM mononuclear}

cells (5 × 10⁵ cells/mL) were incubated with HUVEC or BMEC monolayers in HBSS with calcium and magnesium in six-well cluster plates at 37°C for 1 hour with gentle shaking. The nonadherent cells were removed and adherent cells were characterized by light microscopy (Wright/Giemsa staining) and immunohistochemistry using MoAbs against CD34 (HPCA-1, GPIb-S2, AMAC). GPIIIa/IIa (10E5). GPIb- or GPIIIa-positive cells were counted in the entire six-well cluster plates and scored as megakaryocytes, and small round CD34** but factor VIII/von Willebrand factor (vWF) negative cells were scored as CD34** progenitor cells. Erythroid and myeloid progenitors were identified morphologically by standard Wright/Giemsa staining.

CD34** progenitor cells purified by avidin-biotin immunosorptions}

tion column (Cell Pro Hybridoma technology) (10 μL of 10⁶ cells/mL) were added to washed resting or stimulated BMEC monolayers cultured on Tera-}

saki (Nunc, Naperville, IL) or 96 wells. Adhesion assays were performed for 1 hour at 37°C in HBSS supplemented with magnesium (2 mmol/L) and calcium (2 mmol/L), and unbound cells were removed by three washes with HBSS/Ca/Mg. Adherent cells on endothelial surfaces were counted manually using an inverted phase-contrast microscope. For adhesion inhibition studies, MoAbs (10 μg/mL) to CD34 (HPCA-1, Becton Dickinson, or 11.1 Oncogene Science), VCAM (1G11), and LFA1 (IOT16) were incubated with BMEC on ice for 20 minutes before the study. In the case of CD34 inhibition assays, the antibody to CD34 (10 μg/mL) was also added to the fluid phase during the adhesion study.

**Immunohistochemical techniques.** BMEC monolayers and mi-
Bone marrow microvascular endothelial cells

**Fig 1.** Microvessels within BM spicules. (A) Immunofluorescence from an intact BM spicule stained with MoAb to factor VIII/vWF showing selective staining of endothelial cells and megakaryocytes (arrows). Note the complex vascular network, crisscrossing the spicule. Large polyploid megakaryocytes can be seen in association with endothelial cells (original magnification x 100). (B) This is a photograph of partially collagenase (0.1%-digested (10 minutes) BM spicule, retained on a 40-μm mesh, washed three times with buffer A, and transferred to a plastic slide and stained with Wright/Giemsa stain. This photograph shows the complex network of microvasculature within a typical BM spicule. Note that the spicule is denuded of hematopoietic elements and has a predominant central capillary (large arrow) branching into single layered sinusoidal endothelial cells (small arrows) (original magnification x 50).

**Fig 2.** Isolated microvessels. (A) Phase contrast micrograph (original magnification x 100) of a typical microvessel isolated from BM aspirate. BM spicules retained on a 40-μm mesh were digested with 0.1% collagenase for 30 minutes at 37°C, and pushed through a 21-gauge needle. The digested material were passed through another 40-μm mesh, and retained microvessel fragments were resuspended in ECGM and transferred to a 24-well cluster plate. Note that microvessel is tightly attached to the gelatin-coated plastic dish. (B) Immunohistochemistry showing factor VIII/vWF staining of a microvessel using monoclonal anti-factor VIII/vWF antibody and immunoperoxidase detection (original magnification x 100). Note the spindle-shaped endothelial cell lining the microvessel (Red stain is peroxidase substrate, aminoethyl carbazole). (C) Colonies of BMEC emerging from attached microvessels after 5 days of incubation in ECGM (original magnification x 50). (D) Monoclonal anti-CD34 (HPCA-1, Becton Dickinson) staining with immunoperoxidase detection of a typical microvessel, showing the strong expression of this antigen throughout the microvessel. Red stain is alkaline phosphatase substrate, fast red (original magnification x 100).

**Fig 3.** Characterization of BMEC. (A) Phase-contrast microscopy of a monolayer of BMEC grown from a microvessel explant and purified after Ulex selection. Note the spindle-like and cobblestone morphology of these endothelial cells (original magnification x 50). (B) Epifluorescence of similar monolayer demonstrating that greater than 98% of cells show fluorescence, characteristic of acetylated-LDL uptake (original magnification x 50). (C) Immunoperoxidase staining with MoAb to factor VIII/vWF of BMEC monolayers (original magnification x 50). (D) Immunoperoxidase staining with MoAb to CD34 antigen (HPCA-1). Only early passage cells express this antigen (original magnification x 120). (E) Immunoperoxidase stain with MoAb to PECAM, showing the expression of this antigen specifically at cellular junctions (original magnification x 50). (F) Negative control. Immunoperoxidase stain with monoclonal mouse IgG. Red stain is peroxidase substrate aminoethyl carbazole (original magnification x 50).
cro vessel explants were fixed in 3% formalin in PBS for 30 minutes then quenched with 0.1 mol/L glycine in PBS pH 7.4 for 10 to 15 minutes with Histochoice (Amresco Solon, OH), and blocked with 1.5% horse serum. MoAbs to factor VIII/vWF, CD34 (HPCA-1), PECAM, thrombospondin, ICAM1, VCAM, α-actin, GPIb, GPIIb/IIIa, or L-selectin, at different dilutions, were incubated with fixed cells for 1 hour. After washing with PBS, biotinylated antimouse or antirabbit Ig diluted 1:200 in PBS containing 1.5% horse serum was incubated with cells for 30 minutes at room temperature. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide in PBS for 30 minutes. Endogenous alkaline phosphatase was quenched with levamisole 1.25 mmol/L for 20 minutes. After 30 minutes of incubation with avidin-labeled peroxidase or alkaline phosphatase, slides were rinsed and incubated with peroxidase substrate, aminoethyl carbazole (red stain), or alkaline phosphatase substrate fast red (red stain) for 10 to 20 minutes. After a final rinse the cells were counterstained with 1% hematoxylin.

Samples of BM aspirate were gently placed on polylysine-coated glass slides, air dried, and fixed with Histochoice or acetone/ alcohol. The samples were stained with a primary mouse MoAb to factor VIII/vWF for 1 hour at room temperature or overnight at 4°C, washed three times with PBS, and counterstained with goat anti-mouse IgG1-fluorescein isothiocyanate (FITC). Photographs were taken with a Nikon fluorescence microscope (Tokyo, Japan) using Kodak Ektachrome 160T ASA color film (Kodak Ltd, Rochester, NY, USA). FITC was visualized using standard FITC excitation/emission filter combinations.

Metabolic labeling with Dil-Ac-LDL. Near confluent monolayers of BMEC or mixed populations of endothelial cells and fibroblasts were incubated with 10 μg/mL of Dil-Ac-LDL (acylated low-density lipoprotein labeled with dioctadecyl 3,3,3,3-tetramethylindocarbocyanine perchlorate; Biomedical Technologies Inc, Stoughton, MA) for 4 hours at 37°C.7 The cells were washed with HBSS with calcium and magnesium for 10 minutes, and were examined with a Nikon epifluorescence microscope with phase contrast optics. Dil-Ac-LDL uptake was visualized using standard rhodamine excitation/emission filter combinations.

Immunofluorescent flow cytometry. BMEC adherent and nonadherent CD34+ cells washed twice in PBS and resuspended in HBSS with 1% BSA were incubated with saturating doses of MoAbs to CD34, CD38, HLA-DR, and LFA1 for 30 minutes at 4°C. After washing, cells were stained with saturating amounts of FITC- or rhodamine-conjugated goat antimouse IgG1/F(ab)2 (Coulter). Controls were isotype matched nonimmune IgGs and FITC-conjugated antirabbit IgG F(ab)2 (AMAC). Cell-associated immunofluorescence was assayed by quantitative flow cytometry using a Coulter Profile II.

Electron microscopy. BMEC monolayers were washed three times with HBSS with Ca/Mg, then fixed in 2% paraformaldehyde, 0.5% gluteraldehyde, followed by 1% osmium tetroxide. After dehydration the samples were embedded for electron microscopy. Sixty- to 70-nm (silver-grade) sections were cut using a diamond knife (Diatome, Fort Washington, PA) on a Sorvall MT-5000 microtome (DuPont, Wilmington, DE). Sections were stained with 0.1% lead citrate and examined using a JEOL-100CXII electron microscope (Tokyo, Japan) at an accelerating voltage of 80 kV.

RESULTS

BMEC were obtained from explants of microvessels and were characterized with respect to morphology, expression of factor VIII/vWF, thrombospondin, PECAM, CD34, and acetylated LDL uptake. Figure 1A is a photomicrograph of an intact BM spicule stained with FITC-labeled MoAb to factor VIII/vWF, showing the relative frequency of endothelial cells in the BM and their close association with other hematopoietic elements, particularly mature megakaryocytes. Figure 1B is a photomicrograph of a partially collagenase-digested BM spicule showing the complex vascular network of a typical spicule with a central microvessel branching into single-layered sinusooidal endothelial cells. Figure 2A shows a typical microvessel fragment retained on a 40-μm mesh after 30 minutes of collagenase digestion. Immunohistochemical staining of these microvessel fragments with factor VIII/vWF MoAb (Fig 2B) demonstrated the spindle-shaped endothelial cells lining the microvessel, and relative absence of other adventitial elements such as fibroblasts and adipocytes. Figure 2C shows the proliferation of BMEC colonies from microvessels explants grown for 5 days in culture. Figure 2D shows selective staining of a microvessel within an intact BM spicule with MoAb to CD34. BMEC monolayers isolated from microvessel explants and purified by UEA1 selection (Fig 3) proliferated in a cobblestone fashion (A) and were positive for factor VIII/vWF (C), CD34 (D), PECAM (E), thrombospondin (not shown), and acetylated LDL uptake (B). CD34 was significantly expressed only by the monolayers in the first passage. The degree of CD34 expression was diminished and barely detectable by the second passage. Greater than 98% of the cells in these monolayers demonstrated acetylated LDL incorporation characteristic of endothelial cells. BMEC monolayers did not stain with MoAbs to GPIb, GPIIb/IIIa, α-actin, ICAM, L-selectin, or VCAM (data not shown).

Electron microscopic analysis of BMEC monolayers shows the presence of Weibel-Palade bodies, which is characteristic of vascular endothelium (Fig 4).

To determine if hematopoietic cells selectively interact with resting BMEC monolayers, plastic nonadherent BM mononuclear cells obtained from normal donors were incubated with BMEC and HUVEC monolayers for 1 hour at 37°C. Table 1 shows the results of the characterization and quantification of the BM mononuclear cells that were adherent to endothelial cell monolayers. Although megakaryocytes and CD34+ progenitor cells comprise only 0.05% to 1% of BM mononuclear cells, they represent 22% ± 4%, and 15% ± 2% of the cells adherent to BM monolayers, respectively. This represents a 20- to 30-fold enrichment and suggests adhesion mechanisms specific to these cell types. Megakaryocytes and CD34+ progenitor cells adhered less well to HUVEC monolayers. The remainder of the adherent cells were comprised of plasma cells, mature myeloid precursors, and lymphoid-apparing cells (Table 1).

To examine the mechanism(s) of adhesion in greater detail, BM CD34+ progenitor cells isolated by an avidin-biotin column were incubated with BMEC monolayers at 37°C for 1 hour. Ten percent of the added CD34+ cells were adherent to resting BMEC monolayers. This adhesion was partially blocked by antibodies to CD34 (HPCA-1, 111.1.6), and was blocked with EDTA (1 mmol/L); antibodies to VCAM, ICAM, or LFA1 did not block binding (Fig 5). The phenotype of the adherent CD34+ progenitor cells that were detached from endothelial cells by brief treatment (1 minute) with 0.5 mmol/L EDTA was examined by flow cytometry. This showed a relative enrichment for CD34+CD38- HLA-
Fig 4. Electron microscopic analysis of BMEC. (A) Electron microscopic analysis of BMEC monolayer showing a typical endothelial cell with numerous mitochondria and Weibel-Palade bodies. (B) Higher magnification of the same BMEC showing the presence of cigar-shaped Weibel-Palade bodies (arrows), which is characteristic of vascular endothelium.

DR− cells (Table 2) consistent with a more pluripotent progenitor cell phenotype.35,37 In contrast to the results obtained with resting BMEC monolayers, IL-1β treatment resulted in increased adhesion of CD34+ progenitor cells that was divalent cation independent and not inhibited by antibody to CD34 (Fig 6).

DISCUSSION

During the last few years, microvascular endothelia from brain, retina, adrenal gland, hemangiomas, corpus luteum, lung, adipose tissue, and recently myocardium38-46 have been isolated and characterized and the importance of microvascular endothelial cell proliferation in tumor biology is well established.47,48 There are fundamental differences in the microvasculature of different organs. Microvascular endothelium within each organ express unique types of adhesion molecules, also referred to as organ-specific ECAMs.49,50 Zhu et al51,52 have shown that the murine lung vasculature expresses a specific 90-kD endothelial cell adhesion molecule (Lu-ECAM) that may be responsible for selective metastasis of melanoma cells to the lung. Also, Rettig et al53 have identified a 165-kD vascular endothelial cell antigen called endosialin, which is expressed in tumor microvascula-
BM mononuclear cells obtained from normal donors were isolated on a Ficoll gradient and depleted of monocytes and other plastic adherent cells. The nonadherent hematopoietic cells were incubated with BMEC and HUVEC monolayers for 1 hour at 37°C in PBS supplemented with calcium (2 mmol/L) and magnesium (2 mmol/L). Attached cells were characterized by light microscopy, Wright/Giemsa staining, and immunohistochemistry using MoAbs against CD34 (HPCA-1), GPIb/IIa (10E8), GPIb- or GPIb/IIa-positive cells were counted in two six-well cluster plates and scored as megakaryocytes, and small round CD34+ but factor VIII/vWF-negative cells were scored as CD34+ progenitor cells. Erythroid or myeloid progenitors or plasma cells were identified morphologically by standard Wright/Giemsa staining. Megakaryocytes and CD34+ progenitor cells adhered less well to HUVEC monolayers. The binding of plasma cells, mature myeloid precursors, and lymphoid-appearing cells comprised remainder of the adherent cells.

ture but is not found in blood vessels of normal tissues, or in any of the other normal adult cell types tested in vivo. Given the complex network and close association of BMEC with hematopoietic and other adventitial elements within the BM, we hypothesize that BMEC is a unique type of endothelium that may regulate hematopoiesis by direct cellular contact and/or expression and secretion of specific cytokines.

BMEC are morphologically similar to other types of endothelial cells, such as HUVEC, bovine aortic, and brain microvascular endothelial cells. Although BMEC grow in a cobblestone manner, they are more spindle shaped. They stain positively for factor VIII/vWF, PECAM, CD34 (early passages), and thrombospondin, but do not stain with antibodies to α-actin, GPIIb/IIIa, GPIb, L-selectin, VCAM, or ICAM. BMEC monolayers express higher levels of PECAM at areas of cell-cell contact. PECAM is a 140-kD glycoprotein that is also expressed on monocytes, neutrophils, megakaryocytes, and a subset of CD4 cells, and may function as a transmigratory bridge permitting the exit of mature cells such as neutrophils, lymphocytes, and monocytes out of BM.54,55

The selective homing of transplanted CD34+ progenitor cells to the BM is likely to involve expression of specific adhesion molecules yet to be identified. The movement of mature myeloid and erythroid precursors, and even CD34+ progenitor cells from the BM microenvironment to the peripheral circulation, is a complex phenomenon that is tightly regulated. Regulation of expression of specific adhesion molecules by BMEC or mature hematopoietic elements may allow for selective exit of these mature cells whereas the immature precursors remain bound to adventitial cells within

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**Table 1. Adhesion of BM Mononuclear Cells to Resting Endothelial Cells**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Actual Percentage in BM</th>
<th>Percentage Bound to BMEC</th>
<th>Percentage Bound to HUVEC</th>
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</thead>
<tbody>
<tr>
<td>Megakaryocytes</td>
<td>0.05-0.5</td>
<td>22 ± 4</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>CD34+ cells</td>
<td>0.2-0.5</td>
<td>15 ± 2</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>3-5</td>
<td>15 ± 5</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>Erythroid/myeloid or lymphoid cells</td>
<td>50-80</td>
<td>47 ± 9</td>
<td>67 ± 4</td>
</tr>
</tbody>
</table>

BM mononuclear cells obtained from normal donors were isolated on a Ficoll gradient and depleted of monocytes and other plastic adherent cells. The nonadherent hematopoietic cells were incubated with BMEC and HUVEC monolayers for 1 hour at 37°C in PBS supplemented with calcium (2 mmol/L) and magnesium (2 mmol/L). Attached cells were characterized by light microscopy, Wright/Giemsa staining, and immunohistochemistry using MoAbs against CD34 (HPCA-1), GPIb (S22), and GPIb/IIa (10E8). GPIb- or GPIb/IIa-positive cells were counted in two six-well cluster plates and scored as megakaryocytes, and small round CD34+ but factor VIII/vWF-negative cells were scored as CD34+ progenitor cells. Erythroid or myeloid progenitors or plasma cells were identified morphologically by standard Wright/Giemsa staining. Megakaryocytes and CD34+ progenitor cells adhered less well to HUVEC monolayers. The binding of plasma cells, mature myeloid precursors, and lymphoid-appearing cells comprised remainder of the adherent cells.

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**Table 2. Phenoype of Adherent CD34+ Cells to BMEC Monolayer**

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Adherent</th>
<th>Nonadherent</th>
</tr>
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<tbody>
<tr>
<td>CD34</td>
<td>85 ± 5</td>
<td>87 ± 5</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>52 ± 9</td>
<td>34 ± 10</td>
<td>72 ± 6</td>
</tr>
<tr>
<td>CD38</td>
<td>81 ± 10</td>
<td>39 ± 12</td>
<td>81 ± 5</td>
</tr>
<tr>
<td>LFA1 (CD11A)</td>
<td>15 ± 6</td>
<td>8 ± 7</td>
<td>11 ± 2</td>
</tr>
</tbody>
</table>

CD34+ progenitor cells purified by biotin-avidin immunoadsorption column from BM mononuclear cells were incubated with resting BMEC monolayers at 37°C for 1 hour, and the adherent population of CD34+ cells was detached from BMEC monolayers by brief (1-minute) EDTA (0.5 mmol/L) treatment. The adherent and the nonadherent populations of CD34+ cells were incubated with 10 µg/mL of FITC-labeled MoAbs to CD34 (HPCA-1), HLA-DR, CD38, and LFA1, and the percentage of positive cells was determined by flow cytometry. The figures in the table represent the percent positive cells. As shown in this table the adherent population of CD34+ progenitor cells is enriched for the more pluripotent phenotype CD34+HLA-DR+CD38+.

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**Fig 6. CD34+ progenitor cell adhesion to IL-1β stimulated BMEC monolayers.** BMEC monolayers were incubated with IL-1β (10 ng/mL) for 16 hours, then incubated with BM-derived CD34+ cells isolated by an avidin-biotin column for 1 hour in the presence of antibody to CD34 (HPCA-1 or 11.1.6) or 1 mmol/L EDTA, and adherent cells were quantified by phase-contrast microscopy.

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**Fig 5. CD34+ progenitor cell adhesion to BMEC.** BM-derived CD34+ cells isolated by immunoadsorption technique were incubated with BMEC monolayers at 37°C, in the presence or absence of blocking antibodies for 1 hour and adherent cells were quantified by phase-contrast microscopy.
the marrow. CD34 antigen is a 110-kD glycosylated protein, which is expressed on pluripotent hematopoietic progenitor cells, as well as on other vascular endothelial cells such as HUVEC, capillaries of different tissues,60,67 and neoplastic tissues, such as angiosarcomas, Kaposi's sarcomas, and hepatic hemangioendotheliomas.68,69 However, endothelium from large veins, arteries, placental, and lymphatics are CD34 negative.68 Our findings, along with recent observations that CD34 is concentrated on interdigitating endothelial membrane processes,66,68 suggest that CD34 may function as an adhesion molecule that mediates the transit of peripheral progenitor cells to the BM. Our immunohistochemical studies indicate that only first-passage BMEC monolayers are positive for CD34 but do not express this glycoprotein with subsequent passages. Adhesion studies show that CD34+ progenitor cells display affinity for resting BMEC (passages 2 through 4) monolayers that is divalent cation dependent and is partially inhibited by anti-CD34 antibody. Previous reports have shown that CD34+ progenitor cell binding to fibroblasts is enhanced with cytokine stimulation and expression of VCAM and ICAM.15,16 adhesion molecules. However, antibodies to VCAM and ICAM do not inhibit binding, suggesting that selective binding of BM-derived CD34+ progenitor cells to BMEC may be mediated by other adhesion molecules. Recently, vascular CD34 has been identified as a counter receptor for leukocyte L-selectin,60 raising the possibility that a similar interaction might mediate progenitor cell adhesion to L-selectin expressed by endothelium. However, our data show that BMEC (resting or stimulated with IL-1β) do not express L-selectin. These data suggest CD34+ progenitor cell binding to BMEC may be mediated by CD34+ cell interaction with a ligand expressed specifically by resting BMEC. Several studies have shown that homing of murine progenitor cells to BM is regulated by a calcium-dependent C-peptide.11,12 Our adhesion studies indicate that CD34+ cell binding to unstimulated BMEC monolayers can be completely abrogated with EDTA, supporting the notion that a specific calcium-dependent adhesion molecule may be responsible for the homing of CD34+ progenitor cells to the BM.

The CD34+ cells that are adherent to BMEC monolayers are enriched for the CD34+ HLA-DR+ CD38+ phenotype, which is reported to be a more pluripotent cell type within the CD34+ progenitor cell population.65,67 Furthermore, adherent CD34+ progenitor cells proliferated in coculture with BMEC (unpublished data, 1993), suggesting that regulation of hematopoiesis may be affected by direct contact or by cytokines derived from this endothelium.

The role of inflammatory mediators such as IL-1β in regulating hematopoiesis is incompletely understood. IL-1β is known to regulate adhesion mechanisms that govern the transit of inflammatory cells from the circulation by increasing the expression of ICAM and VCAM on the luminal surface of the endothelium, resulting in upregulation of monocyte and polymorphonuclear cell adhesion.73 Our studies show a similar increase in CD34+ progenitor cell adhesion to IL-1β-treated BMEC monolayers. In contrast to the adhesion observed to resting (untreated) monolayers, adhesion of CD34+ progenitor cells to IL-1β-treated BMEC monolayers was divalent cation independent and not inhibited by anti-CD34, VCAM, or ICAM. The regulation of BMEC adhesion of progenitor cells by IL-1β raises the possibility that increased transit mediated by increased adherence of these circulating hematopoietic cells is one mechanism by which the BM can respond to inflammatory states. That independent mechanisms for cell adhesion are exhibited by resting and IL-1β-stimulated BMEC fits well with experimental data showing that CD34 mRNA expression by HUVEC monolayers is rapidly downregulated by IL-1β, γ-interferon and tumor necrosis factor.81

The specific enrichment of megakaryocytes, together with their previously described close association with the submucosal surface of the sinusoidal endothelium,62,63 raises the possibility that the final stages of megakaryocyte maturation and platelet formation may be regulated by BM endothelium. The adhesion of a large number of megakaryocytes to BMEC monolayers relative to HUVEC monolayers was particularly striking. Furthermore, immunofluorescence studies of multiple, intact spicules, as shown in Fig 1A, show that mature, large polyploid megakaryocytes reside at the submucosal surface of BMEC. Whether the interaction of BMEC with megakaryocytes are critical for megakaryocytopenesis or thrombopoiesis remains to be determined.

ACKNOWLEDGMENT

We are grateful to Dr Jim Young and Janice Godwin, MT, for providing samples and support of this project.

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