Endothelial Precursor Cells As a Model of Tumor Endothelium: Characterization and Comparison with Mature Endothelial Cells

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ABSTRACT

Human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMVEC) have been the standards for cell-based assays in the field of angiogenesis research and in antiangiogenic drug discovery. These normal mature endothelial cells may not be the most representative of human tumor endothelial cells. Human AC133+/CD34+ bone marrow progenitor cells were established in cell culture media containing vascular endothelial growth factor, basic fibroblast growth factor (bFGF), and heparin to drive differentiation toward the endothelial phenotype. The resulting cells designated endothelial precursor cells (EPC) have many of the same functional properties as mature endothelial cells represented by HUVEC and HMVEC. By SAGE analysis, the genes expressed by EPC are more similar to the genes expressed by endothelial cells isolated from fresh surgical specimens of human tumors than are the genes expressed by HUVEC and HMVEC. Analysis of several cell surface markers by flow cytometry showed that EPC, HUVEC, and HMVEC have similar expression of P1H12, vascular endothelial growth factor 2, and endoglin but that EPC have much lower expression of ICAM1, ICAM2, VCAM1, and thrombomodulin than do HUVEC and HMVEC. The EPC generated can form tubes/networks on Matrigel, migrate through porous membranes, and invade through thin layers of Matrigel similarly to HUVEC and HMVEC. However, in a coculture assay using human SKOV3 ovarian cancer cell clusters in collagen as a stimulus for invasion through Matrigel, EPC were able to invade into the malignant cell cluster, whereas HMVEC were not able to invade the malignant cell cluster. In vivo, a Matrigel plug assay where human EPC were suspended in the Matrigel allowed tube/network formation by human EPC to be carried out in a murine host. EPC may be a better model of human tumor endothelial cells than HUVEC and HMVEC and, thus, may provide an improved cell-based model for second generation antineoplastic angiogenic drug discovery.

INTRODUCTION

The importance of normal cells and tissues to support the growth of tumors has been recognized for centuries. The observations of Van der Kolk (1), Jones (2), and Paget (3) documented this knowledge in the clinical science literature. Algire and Chalkey (4) reported that host vascular reactions could be elicited by growing tumors and described in detail the extent and tumor-specific nature of the induction of host capillaries by transplanted tumors. The central hypothesis of Algire and Chalkey was that vascular induction by solid tumors may be the major distinguishing factor leading to tumor growth beyond normal tissue control levels. By the late 1960s, Folkman et al. (5–7) had begun the search for a tumor angiogenesis factor, and in 1971, Folkman proposed “angiogenesi” as a means of holding tumors in a nonvascularized dormant state (8).

Investigators working in embryogenesis distinguished between angiogenesis, vessels arising from sprouts on existing vessels, and vasculogenesis, vessels arising from endothelial progenitor cells (angioblasts; Refs. 9 and 10). The abnormality of tumor vasculature and value of working with fresh, noncultured live endothelial cells isolated from solid tumors were recognized by cancer researchers (11), and the role of endothelial precursor cells from bone marrow was recognized by researchers studying mammalian development (12, 13). Asahara et al. (14–16) isolated putative endothelial precursor cells or angioblasts from human peripheral blood by magnetic bead selection and described a role for these cells in postnatal vasculogenesis and pathological neovascularization. It was later shown that the recruitment of the progenitor cells from the bone marrow requires that activity of matrix metalloproteinase-9 mediation of the release of Kit-ligand (17). Studies in allogeneic bone marrow transplant recipients confirmed that circulating endothelial precursor cells or angioblasts in peripheral blood originated from the bone marrow (18). Gehling et al. (19) identified CD34+/AC133+ progenitor cells from bone marrow as a subpopulation of cells that in vitro could differentiate into endothelial cells.

Recent studies have formally tied circulating endothelial precursor cells to the development of tumor vasculature (20–24). Gill et al. (22) found that in patients with vascular insult secondary to burns or coronary artery bypass grafting, there was a ~50-fold increase in circulating endothelial precursor cells within the first 6–12 h after injury and lasting 48–72 h in parallel with the plasma levels of VEGF. A similar pattern of mobilization of endothelial precursor cells from the bone marrow was observed in mice after injection with VEGF. Using the Id1+/-/Id3+/- mutant mouse that has impaired tumor vascular growth, it was shown that transplantation with wild-type bone marrow or with VEGF-mobilized bone marrow stem cells allowed recruitment of endothelial precursor cells sufficient to support tumor growth (23). De Bont et al. (24) found that when NOD/SCID mice bearing human Daudi non-Hodgkin’s lymphoma xenografts were injected i.v. with human CD34+ hematopoietic stem cells or angioblasts that the injected human CD34+ cells homed to the tumor and differentiated along the endothelial lineage. AC133+ multipotent human bone marrow progenitor cells exposed to VEGF in cell culture differentiate into CD34+/VE-cadherin+/VEGFR2+ cells or angioblasts (25). On maintenance in cell culture, these cells will continue to differentiate toward a more mature endothelial phenotype. When human AC133+ progenitor cells were injected i.v. into NOD/SCID mice bearing s.c. murine Lewis lung carcinoma, these cells contributed to the developing tumor vasculature. Additional support for the notion that tumor vasculature arises, in part, through the process of vasculogenesis comes from studies in which murine endothelial precursor cells from bone marrow, peripheral blood, and tumor-infiltrating cells were isolated from mice bearing human breast carcinoma xenografts (26). The number of endothelial precursor cells was elevated in the tumor-bearing mice compared with normal mice. There was a significant number of endothelial precursor cells found in the human breast carcinoma xenografts, and maturation and proliferation of these cells in the tumors were evident. Recently, it was reported that NOD/SCID mice transplanted with human bone marrow and bearing human Namalwa or Granta 519 Burkitt’s lymphoma xenografts had a 7-fold increase in circulating endothelial precursor cells compared with nontumor-bear-

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ing mice (27). Continuous infusion of endostatin into the tumor-bearing mice resulted in inhibition of the mobilization of endothelial precursor cell from the bone marrow.

To date, most research directed toward the development of antian-
giogenic anticancer agents has used cells readily at hand, primarily HUVECs and human microvascular endothelial cells, as the cell-
base models of the tumor endothelium (28). It may be that human endothelial precursor cells are a more representative model of tumor endothelium. The current report compares endothelial precursor cells generated in cell culture to HMVEC and HUVEC grown in culture by the expression of molecular markers and behavior in functional assays.

MATERIALS AND METHODS

Cell Culture. CD34+/AC133+ progenitor cells from human bone marrow cells, HUVECs and HMVECs, were purchased from Cambrex, Inc. (East Rutherford, NJ). The CD34+/AC133+ progenitors (1–2 × 10^6 cells/ml) were grown in IMDM medium (Cambrex, Inc.) supplemented with 15% FBS (Invitrogen Corp., Carlsbad, CA), 50 ng/ml VEGF, (R&D Systems, Minne-
apolis, MN), 50 ng/ml rhbFGF (R&D Systems), and 5 units/ml heparin (Sigma Chemical Co., St. Louis, MO) on fibronectin-coated flasks (BD Biosciences, Franklin Lakes, NJ) at 37°C with humidified 95% air/5% CO_2 to generate EPCs (19, 29–31). Fresh media were added to the cultures every 3–5 days. The adherent cells that were generated from the original population of mixed nonadherent and adherent cells were designated EPC. The EPC were grown to confluency and could be passed up to a dozen times. After the second passage, the EPC were maintained in IMDM media supplemented with 15% 
FBS with additional growth factors. The EPC were divided 2–3–fold at each passage. HUVEC and HMVEC were maintained in endothelial cell growth medium containing 2% FBS (EGM-2; Cambrex, Inc.) at 37°C with humidified 95% air/5% CO_2. Both of the donors for the AC133+/CD34+ progenitor bone marrow cells were normal male healthy volunteers. Both were Caucasian, ages 18 (donor 1) and 23 (donor 2), and both tested negative for HIV and hepatitis B and C infection.

The SKOV-3 human ovarian carcinoma cell line was purchased from American Type Culture Collection (Manassas, VA). SKOV-3 cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS.

Flow Cytometry. EPC, HUVEC, and HUVEC were collected by brief exposure to 0.25% trypsin/1 mM EDTA (Invitrogen Corp.) and washed twice in ice cold phosphate buffered 0.9% saline containing 5 mM EDTA and 5% FBS (FACS buffer). Approximately 2 × 10^5 (5) cells were suspended in a final volume of 100 μl of FACS buffer and incubated for 1 h on ice. The cells were then washed twice with FACS buffer and incubated with secondary antibody, when necessary, for 45 min on ice. The cells were again washed twice with FACS buffer and suspended in a final volume of 500 μl for flow cytometric analysis.

The following primary antibodies were used at a 1:20 dilution: (a) anti-
CD31-FITC (PharMingen, San Diego, CA); (b) anti-CD34-FITC (PharMingen); (c) anti-CD36-FITC (PharMingen); (d) anti-AC133-PE (Miltenyi Biotech, University Park, PA); (e) anti-CD105 (PharMingen); (f) anti-P1H12 (Chemicon International, Temecula, CA); (g) anti-CD45 (PharMingen); and (h) anti-CD106 (PharMingen). The following unconjugated primary antibodies were used at a 1:10 dilution: (a) anti-VEGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA) and (b) anti-VE-cadherin (Santa Cruz Biotechnology). Un-
conjugated primary antibody against CD36 (PharMingen) was used at a 1:100 dilution, and antibody against CD141 (PharMingen) was used at a 1:500 dilution. The following secondary antibodies were used at a 1:35 dilution: (a) antimouse-FITC (PharMingen or Jackson Immunoresearch, Bar Harbor, ME); (b) anti-rabbit-FITC (Santa Cruz Biotechnology); (c) anti-goat-FITC (Santa Cruz Biotechnology) or at a 1:50 dilution: (d) anti-mouse-PE (Jackson Immunoresearch). EPC and HUVEC were stimulated with 20 ng/ml TNF α (R&D Systems) for 4 h before CD106 staining. Cells were fixed in 4% paraformaldehyde and analyzed within 24 h. Positive expression was determined if cells gated at ≥10%.

Statistical Comparison of SAGE Libraries. SAGE libraries for brain, breast and colon tumor and normal endothelial cells (EC), and SAGE libraries from EPC and HMVEC were constructed as described previously (32). SAGE libraries for brain, breast EC, and EPC were constructed using the long SAGE technology (33). SAGE libraries for colon EC and HMVEC were constructed using microSAGE technology (34). The sample information for all of the libraries constructed is: three brain tumors and two normal brain samples, two primary breast tumors, one breast bone metastasis and one normal breast sample, one colon tumor and one normal colon sample, EPC grown with or without VEGF, and HUVEC in the presence or absence of DMSO. SAGE tags were normalized to 50,000 total library counts except the colon EC libraries, which were normalized to 100,000 total library counts. Long SAGE tags were converted to regular SAGE tags, and tag counts for the same regular SAGE tags and clustered. There were 139,838 unique SAGE tags from the 15 libraries. SAGE tag counts of two or less were filtered out in ≥10 of the 15 libraries to remove erroneous tags. Within tissue comparison of tumor versus normal libraries were also performed through a χ² analysis on the averages of the normal and tumor SAGE tag counts. Confidence interval levels of 90, 95, and 99% were also used for tag filtering and generated 4030 and 762 tags, respectively. Hierarchical clustering and Venn diagrams were performed on filtered libraries using GeneSpring software release 5.0.2 build number 954 (Silicon Genetics, Redwood City, CA). Pearson correlation was for similarity measurement, and the minimum distance was set to 0.001.

Proliferation. The generation times of the EPC, HUVEC, and HMVEC were determined over a 4-day period. Cells were plated in 96-well plate format at 2000 or 3000 cells/well in triplicate. EPC were grown in EGM-2 with 2% FBS or IMDM with 15% FBS and HMVEC and HUVEC were grown in EGM-2 media with 2% FBS. Cells were assayed daily using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) that measures ATP levels. The results are expressed as cells/well +/− SD.

Migration Assay and Invasion Assay. EPC, HUVEC, or HMVEC (5 × 10^5 cells) were placed into the top chamber of a BD Falcon HTS Fluoroblok insert with a PET membrane with eight μm pores (BD Bio-
sciences) in 300 μl of serum-free IMDM for EPC, EGM-2 for HUVEC, or HMVEC in triplicate. For the invasion assay, the FluoroBlok inserts were coated with a thin layer of Matrigel. The inserts were placed into the bottom chamber wells of a 24-well plate containing IMDM or EGM-2 media and FBS (0, 0.5, or 5%) as chemoattractant. For direct comparison of cell lines, 5% FBS was used. At 4, 24, and 48 h, cells that migrated through the pores of the inserts were counted. After 48 h, the inserts were removed using a glass pipette under light vacuum. The hole was filled with SKOV3 cells suspended in 1% collagen I (Cohesion Technologies, Palo Alto, CA) at a concentration of 1 × 10^6 cells in 5 μl and allowed to solidify at 37°C for 30 min. After the Matrigel solidified, EPC, HUVEC, or HMVEC (2–
2.5 × 10^5 cells) were added in 300 μl of media: IMDM with 2% FBS for EPC and AC133+/CD34+ bone marrow progenitor cells, EGM-2 for HMVEC and HUVEC. The cells were incubated at 37°C with humidified 95% air/5% CO_2 for 24 h (35). The tube networks were stained with calcine and quantified by image analysis using Scion image as fluorescent pixel area.

In Vitro Tube Formation. Matrigel (BD Biosciences) was added to the wells of a 48-well plate in a volume of 150 μl and allowed to solidify at 37°C for 30 min. After the Matrigel solidified, EPC, HUVEC, or HMVEC (2–
2.5 × 10^5 cells) were added in 300 μl of media: IMDM with 2% FBS for EPC and AC133+/CD34+ bone marrow progenitor cells, EGM-2 for HMVEC and HUVEC. The cells were incubated at 37°C with humidified 95% air/5% CO_2 for 24 h (35). The tube networks were stained with calcine and quantified by image analysis using Scion image as fluorescent pixel area.

In Vitro Tube Formation. Matrigel (BD Biosciences) was added to the wells of a 24-well plate in a volume of 300 μl and allowed to solidify at 37°C for 30 min (36). A plug of Matrigel of ~1 mm diameter was removed using a glass pipette under light vacuum. The hole was filled with SKOV3 cells suspended in 1% collagen I (Cohesion Technologies, Palo Alto, CA) at a concentration of 1 × 10^6 cells in 5 μl and allowed to solidify at 37°C for 30 min. After Matrigel solidified, EPC or HMVEC were labeled with PKH67 green dye according to the manufacturer’s instructions (Sigma). The cells were incubated in the presence of 2.5 μM dye suspended in diluent for 5 min. The labeling was stopped with 1 ml of PBS for 1 min followed by three washes in serum-containing medium. After the washes, the cells were suspended in IMDM or EGM-2 and counted by hemocytometer. EPC or

2 The abbreviations used are: HUVEC, human microvascular endothelial cells; EPC, endothelial precursor cells; VEGF, vascular endothelial growth factor; ICAM, intercel-
lular adhesion molecule; HUVEC, human umbilical vein endothelial cell; SAGE, serial anal-
ysis of gene expression; FBS, fetal bovine serum; VEGF, vascular endothelial growth factor; rhbFGF, human recombinant basic fibroblast growth factor; FACS, fluo-
rescence activated flow cytometry; bFGF, basic fibroblast growth factor; DAPI, 4, 6-diamidino-2-phenylindole.
HMVEC (3 × 10^5 cells) were added to each well in 300 μl of IMDM or EGM-2 media. The cultures were incubated at 37°C in humidified 95% air/5% CO₂ for 24 or 48 h. The EPC or HMVEC in the wells were visualized using a fluorescein (PKH67) filter on an inverted phase using a fluorescent inverted phase microscope.

In Vivo Matrigel Angiogenesis Assay. EPC were prelabeled with DAPI (Sigma) at 20 μg/ml at 37°C for 20 min, then were washed twice with PBS and used within 24 h. The EPC were collected by exposure to 0.25% trypsin. Approximately 5 × 10⁶ EPC in 100 μl of PBS were mixed with 500 μl of Matrigel (BD Biosciences) containing 40 units/ml heparin and 150 ng/ml rhbFGF. The Matrigel containing EPC mixtures (500 μl) were implanted s.c. into the mid-dorsal region of female nude mice. The Matrigel plugs containing EPC were collected after 7 days in vivo and snap frozen in OCT medium. For detection of DAPI-labeled EPC, 5-μm frozen sections of the Matrigel plugs were rinsed briefly with PBS, fixed in 10% formalin for 10 min, washed twice, and then imaged by fluorescent microscopy after mounting. Other sections were stained with H&E and imaged by bright field microscopy or stained with mouse antihuman CD31 (1 μg/200 μl/slide; clone JCT70A; DAKO, Carpinteria, CA) or rabbit antihuman von Willebrand factor (DAKO) using a Cy3 secondary antibody for immunohistochemistry.

RESULTS

In the absence of stimulation, AC133+/CD34+ bone marrow cells can be maintained for a short period of time in culture with limited expansion potential. On exposure to the angiogenic factors VEGF165, rhbFGF, and heparin, the AC133+/CD34+ progenitor cells began to proliferate. Within 1–2 weeks, a new phenotype of cells began to emerge and adhere to the flask. After 2 weeks, a confluent, adherent monolayer of elongated cells was obtained. These adherent cells derived by angiogenic growth factor exposure of AC133+/CD34+ progenitor cells in culture were designated EPC. The remaining bone marrow cells in suspension that continued to proliferate and thrive were transferred to a new flask for additional exposure with the angiogenic factors VEGF165, rhbFGF, and heparin and generation of EPC. The EPC maintain significant expansion potential and can be passaged at least 12 times. For the experiments described herein, the EPC were limited to 10 passages. Maintenance of the EPC at a minimum of 50–60% confluence was important to generating high cell numbers with a doubling time of ~3 days. EPC viability remained highest when at near confluence in culture.

The expression of cell surface proteins that are characteristically expressed on bone marrow progenitor cells and mature ECs was assessed on EPC in the presence and absence of EC-associated growth factors and on HUVEC and HMVEC (Table 1). Flow cytometry was used to score relative expression of each marker on the various cell types. AC133+/CD34+ is a M₉, 97,000 five-span transmembrane protein with no known function (37, 38). The expression of the AC133 protein is, in large part, limited to normal bone marrow and some CD34+ leukemias. The expression of progenitor cell marker AC133 on the cell surface EPC was weak. However, AC133 was not detectable on the surface of the mature ECs represented by HUVEC and HMVEC. Sialomucin/CD34 was also a marker in the bone marrow progenitor cell population selected to be the originating cell for EPC development. CD34 is found expressed in vessels in vivo and on ~20% of HUVEC and HMVEC in cell culture (39). EPC, HUVEC, and HMVEC in the current study were weakly positive for CD34 expression.

Several cell surface EC markers were similarly expressed on the EPC, HUVEC, and HMVEC. Among the similarly expressed markers were VEGERF2/Flk-1, endoglin/CD105, and P1H12/CD146. VE-cadherin and pP1B/CD36 were expressed weakly in EPC, HUVEC, and HMVEC. Several markers differentiated EPC from mature ECs represented by HUVEC and HMVEC. ICAM1/CD54, ICAM2/CD102, and thrombomodulin/CD141 were much more strongly expressed on the mature ECs than on the EPC. Finally, PCAM/CD31 was very strongly expressed on the mature ECs represented by HUVEC and HMVEC and was more weakly expressed on the surface of EPC.

In other studies, EPC were tested for uptake of acetylated LDL and binding to UEA-1 lectin, traits that are common for mature ECs, such as HUVEC and HMVEC. The cells were incubated for 4 h at 37°C with 10 μg/ml Dil-Ac-LDL (Biomedical Technologies, Stoughton, MA) or with FITC-labeled UEA-1 lectin (Sigma) for 1 h at 37°C in serum-free media. All cells were washed twice with PBS after incubation. Although HUVEC and HUVEC demonstrated robust uptake of AcLDL and binding of UEA-1 lectin, the EPC did not take up AcLDL and weakly bound UEA-1 lectin (data not shown). The differences between cell surface markers expressed by the EPC and the HUVEC and HMVEC suggest that the EPC population derived in cell culture does not express all of the characteristic markers associated with fully mature ECs. Thus, EPC may be regarded as representing an intermediary cell type between the AC133+/CD34+ progenitor cell and the mature well-differentiated EC.

Gene expression in the EPC was compared with gene expression from human tumor ECs and with gene expression in HMVEC using SAGE analysis (Refs. 33 and 34; Fig. 1). Human tumor ECs were derived from three breast tumors, three brain tumors, and one colon tumor. The seven tumor EC SAGE libraries were compared with the corresponding normal tissue EC SAGE libraries, and the gene expressed at significantly higher levels in the tumor ECs were determined by χ² analysis. The genes expressed in the EPC and HMVEC as determined by SAGE analysis were compared with the genes expressed at three levels of stringency in the pooled tumor EC libraries. At each level of stringency, ≥99%, ≥95%, and ≥90%, there were a larger number of expressed genes in common between the EPC and the tumor ECs than between the HMVEC and the tumor cells. Thus, at the gene expression level, there was greater similarity between EPC and tumor ECs than between HMVEC and tumor ECs.

The performance of EPC in several cell-based assays in comparison with the mature ECs commonly used in the angiogenesis field, HUVEC and HMVEC, was assessed. EPC were evaluated in proliferation, migration, invasion through Matrigel, and tube/network formation assays. These assays are routinely used to identify and evaluate both proangiogenic and antiangiogenic agents that may be potentially effective in therapeutic clinical settings. Generation times

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<th>Cell type</th>
<th>AC133</th>
<th>Sialomucin</th>
<th>CD34</th>
<th>PIH12</th>
<th>VEGFR2</th>
<th>PCAM</th>
<th>CD105</th>
<th>Endoglin</th>
<th>VE-cadherin</th>
<th>ICAM1</th>
<th>ICAM2</th>
<th>VCA1</th>
<th>ICAM2</th>
<th>pP1B</th>
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Table 1 Comparison of cell surface molecular marker protein expression by EPC, HUVEC, and HMVEC

The cells were immunostained for the markers and analyzed by flow cytometry. Relative expression of % gated or positive cells are shown: 1 = 0–25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100%. EPC grown in the presence or absence of growth factors (GF; VEGF165, rhbFGF, and heparin) were compared.
were determined for HMVEC, HUVEC, and EPC over 96 h (Fig. 2). EPC were grown in either IMDM plus 15% FBS or in complete EGM-2 media that is supplemented with VEGF, bFGF, and 5% FBS. The generation times for EPC in IMDM media with high serum were ~117 h, similar to HMVEC with a generation time of 115 h. When EPCs are grown in EGM-2 media, the generation time decreased to ~36 h, a rate more similar to HUVEC with a doubling time of ~27 h. These results indicate that like mature ECs, EPC respond to growth factors, such as VEGF and bFGF. EPC proliferation rates resemble both HMVEC and HUVEC and comparisons are dependent on the media chosen.

CD34+/VEGFR2+ cells from peripheral blood have been reported to migrate in response to VEGF and bFGF, leading to further differentiation and maturation of a subset of those progenitor cells that were AC133+ (21). The EPC generated in cell culture from AC133+/CD34+ bone marrow progenitor cells exposed to VEGF165 and rh-bFGF was investigated in a migration assay designed to evaluate their chemotactic capacity. The cells in serum-free media were placed in an insert with a membrane containing 8-μm pores. The insert was placed in a well containing media with FBS at increasing concentrations as chemottractant. Fig. 3A depicts the time course of EPC migration at 4, 24, and 48 h in the presence of 0, 0.5, and 5% FBS. EPC began to migrate within 4 h in a FBS concentration-dependent manner and continue to migrate up to 48 h by which time there is likely no longer a serum gradient between the insert and well. Because EPC do not proliferate significantly in a low FBS media, the increase in fluorescence over 48 h indicates a continued migration by the EPC through the pores of the insert membrane. Although the presence of FBS as chemottractant was observed at the earliest time point, by 24 and 48 h, migration of EPC proceeded as well in the absence of FBS as in the presence of FBS. Thus, the EPC can migrate even in the absence of serum (Fig. 3A).

When 0.5% FBS was used as the chemottractant at 24 and 48 h, the number of cells migrating was very similar when EPCs were compared with HUVEC and HMVEC (Fig. 3B). After the EPC population of cells was established by passing twice in the presence of the endothelial growth factors, VEGF165, rhbFGF, and heparin, the EPC were maintained without addition of growth factors to the media.

To determine whether continuous stimulation with VEGF165, rhbFGF, and heparin would affect the EPC behavior in culture, some EPC were maintained in growth factor-rich media for an additional three cell passages. As can be seen in Fig. 3B, there was no difference in the ability to migrate between EPC maintained without growth factors and those maintained in growth factor-rich media.

Invasion through Matrigel is another important property recognized as a characteristic of ECs. The invasion assay used the same insert and well apparatus as the migration assay except that a layer of Matrigel coating the porous membrane through which the cells invade before they can migrate through the pores of the membrane. The invasion by EPC, HUVEC, and HMVEC was examined at 24 and 48 h with 0.5% FBS as the chemottractant (Fig. 4). The EPCs performed as well as the mature ECs, HUVEC and HMVEC, in the cell culture Matrigel invasion assay. In addition, AC133+/CD34+ bone marrow progenitor cells from a second donor were differentiated in EPC. The EPC generated from both individual donors performed equally well in the cell culture Matrigel invasion assay.

Angiogenesis, a critical step in tumor development, is hypothesized to occur through the secretion of angiogenic growth factors and cytokines by malignant cells and perhaps other normal cells in the vicinity of the emerging tumor. The gradient of angiogenic factors attracts EPC in a process resembling vasculogenesis and activates sprouting from existing endothelium causing neovascularization. The coculture assay including human SKOV3 ovarian cancer cells suspended in a collagen plug surrounded by Matrigel allows assessment...
of the invasion potential of cells under the influence of factors secreted by the malignant SKOV3 tumor cells (Fig. 5). EPC and HMVEC were evaluated for their ability to respond to human SKOV3 ovarian cancer cells in the three-dimensional coculture assay. The SKOV3 cancer cells (1 x 10^6 cells) were clustered within a 1 mm^3 collagen plug surrounded by Matrigel in a 24-well plate. EPC or HMVEC (3 x 10^5 cells) fluorescently labeled with PKH67 were added to the well, and their mobility was monitored for 48 h. As shown in Fig. 5, EPC but not HMVEC invaded into the SKOV3 ovarian cancer cell clusters and concentrated into the center of the clusters. Thus, EPC can be differentiated from mature ECs represented by HMVEC by their ability to invade through Matrigel and collagen under the influence of factors secreted by human SKOV3 ovarian carcinoma cells.

The ability to form tubes or networks in Matrigel is a hallmark of EC behavior that models the formation of new vessels or vasculature in vivo. For the tube/network formation assay, AC133+/CD34+ bone marrow progenitor cells, EPC, HUVEC, and HMVEC, were plated onto a layer of Matrigel and allowed to incubate for 24 h (Fig. 6). The more undifferentiated AC133+/CD34+ bone marrow progenitor cells did not form tubes or networks on the Matrigel. However, the EPC formed tubes/networks that appear quite similar to those formed by HUVEC and HMVEC. Thus, differentiation of the CD34+/AC133+ bone marrow progenitor cells toward the EC phenotype as represented by EPC allows the cells to form tubes/networks on Matrigel indicating that on exposure to proangiogenic factors, cells derived from bone marrow can develop several properties similar to mature ECs, like HUVEC and HMVEC.

To develop a convenient in vivo model for testing potential antiangiogenic agents against human vascular targets expressed on EPC, EPC (5 x 10^5 cells) labeled with a tracer amount of the fluorescent nuclear-stain DAPI were mixed into Matrigel (500 μl) and injected s.c. into nude mice. After 7 days, the cell-laden Matrigel plugs were collected and snap frozen. Sections from the plugs were evaluated for tube/network formation and retention of the EPC (Fig. 7, A–E). The tubes/network formed throughout the plugs, and apparent degradation of the Matrigel support was visualized by staining with H&E (Fig. 7, A and B). Fluorescence microscopy allowed visualization of the nuclei of DAPI-labeled EPC in the tubes/network (Fig. 7C). Fig. 7D shows staining of the EPC for CD31, and Fig. 7E shows staining for von Willebrand’s factor by fluorescent immunohistochemistry.

DISCUSSION
Research has shown that progenitor cells derived from adult human bone marrow or from umbilical cord blood and can be recruited into circulation and give rise to well-differentiated cell types. VEGF and bFGF in particular have been implicated in the differentiation of these circulating progenitor cells into ECs (29). VEGF has been shown to modulate postnatal EPC kinetics in normal mice by increasing migration and chemotaxis (15, 16). Stromal cell derived factor-1 and other cytokines up-regulate MMP-9, which is required in the recruitment of hematopoietic stem cells and EPC from bone marrow (17). Insulin-like growth factor-1, granulocyte colony-stimulating factor, and stem cell growth factor also can drive progenitor cells toward an endothe-
lial phenotype. Thus, EPC maturation can occur under a multitude of conditions supporting the notion that the multifaceted potential these progenitor cells possess enables them to function and respond to different pathological settings.

ECs can arise from a subset of common hematopoietic stem cell precursors identified by the markers AC133 and CD34. In cell culture on exposure to VEGF165, rhbFGF, and heparin, the loss of AC133 expression occurs early as the progenitor cells differentiate through stages to a cell with a phenotype resembling ECs, herein described as EPC. The EPC generated express several molecular markers associated with ECs, such as P1H12, VEGFR2, PECAM, and endoglin, and demonstrate migration properties very similar to HUVEC and HMVEC (30, 31, 39). However, other EC markers, such as thrombomodulin, ICAM1, ICAM2, and VCAM1, were not found on EPC. The adhesion molecules ICAM and VCAM mediate the interaction between ECs and T and NK cells, as well as between ECs and stromal tissue or cancer cells (40). Thrombomodulin is a cell surface anticoagulant that modulates the activity of the hemostatic protease thrombin and blocks thrombin’s procoagulant effects and enhances thrombin-dependent activation of anticoagulant protein C (41, 42).

The EPC were obtained from bone marrow cells expressing CD34 and AC133; however, the full potential of this subpopulation of progenitor cells remains to be elucidated. Although expression of AC133 protein appears to be limited to bone marrow and some leukemias from immunohistochemical staining, the message for AC133 is present in other tissues, including kidney and pancreas (37). It is possible that under specific stimulatory conditions that AC133+ progenitor cells can differentiate into various cell types. A second isoform of AC133 expressed in human stem cells other than hematopoietic tissue has been identified (38).

The EPC examined in these studies are likely intermediary between early progenitor cells and fully mature ECs. Like HUVEC and HMVEC, EPC have the capacity to migrate, invade through Matrigel, and form tubes/networks on a Matrigel-coated substrate. The in vivo environment cannot be wholly mimicked in culture, and all of the components that contribute to the maturation and maintenance of ECs...
have yet to be fully characterized. However, there was a clear difference in the behavior of EPC and HMVEC in the coculture assay where human SKOV3 ovarian cancer cells provided the stimulus for vasculogenesis/neoangiogenesis. In that assay, the EPC were able to invade into the tight cluster of malignant cells, whereas the HMVEC did not have the capacity for invasion. SAGE analysis for gene expression allowed us to compare EPC and HMVEC to gene expression in tumor ECs isolated from clinical surgical samples of breast, colon, and brain cancer. The data show that EPC are more similar in expressed genes to tumor EC than are HMVEC. Loading human EPC into Matrigel and the injection of the Matrigel as a s.c. plug into murine hosts resulted in formation of a network/vasculature that was likely a mosaic of human and mouse cells after 7 days. On visualization of the DAPI-labeled EPC, it was evident from the presence of unlabeled cells that some regions of the vasculature were not comprised of EPC but rather consisted of murine ECs. Another possibility is that host macrophages could enter the Matrigel and engulf the human EPC; however, the number of pyknotic cells in the Matrigel plugs was very low (0.1%), and macrophage-like cells were seen. The anastomoses of the EPC and host ECs have generated a basic model of human vasculature in a murine host. Preclinical models comprised at least in part of ECs of human origin are valuable in evaluating the efficacy of potential antiangiogenic therapeutics. Continued characterization of EPC, effects of cytokines and growth factors on EPC differentiation, and identification of the capabilities of EPC in preclinical and clinical settings will continue is important.

Like other areas of drug discovery, the field of antineoplastic antiangiogenic drug discovery has been hampered by the use of nonideal models for human tumor vasculature and endothelium. The...
cell-based models that have been the standard for the field, HUVEC and HMVEC, are mature, well-differentiated, normal human ECs. Through the study of genes expressed in human tumor ECs isolated from fresh surgical specimens of human tumors and corresponding normal tissues as determined by SAGE analysis, it is clear that human tumor endothelium is not well represented by HUVEC and HMVEC. The search for better cell-based models for human tumor ECs has yielded the EPC which in cell culture were developed from AC133+/CD34+ bone marrow progenitor cells. The EPC retain the basic functions of migration and tube formation and have greater proliferative capacity and greater invasive capacity than HUVEC and HMVEC. Recently, endothastin has been shown to inhibit the mobilization of murine EPC in tumor-bearing mice into circulation and reduce the effectiveness of xenotransplantation of human bone marrow cells into SCID mice (27). Utilization of EPC rather than HUVEC or HMVEC in drug discovery for evaluating potential antiangiogenic therapies in the preclinical setting may result in the selection of targets and agents that will prove to be more effective in the clinic.

REFERENCES

Endothelial Precursor Cells As a Model of Tumor Endothelium: Characterization and Comparison with Mature Endothelial Cells

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