Pericytes and Endothelial Precursor Cells: Cellular Interactions and Contributions to Malignancy

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Abstract

Tumor vasculature is irregular, abnormal, and essential for tumor growth. Pericytes and endothelial precursor cells (EPC) contribute to the formation of blood vessels under angiogenic conditions. As primary cells in culture, pericytes and EPC share many properties such as tube/network formation and response to kinase inhibitors selective for angiogenic pathways. Expression of cell surface proteins including platelet-derived growth factor receptor, vascular cell adhesion molecule, intercellular adhesion molecule, CD105, desmin, and neural growth proteoglycan 2 was similar between pericytes and EPC, whereas expression of P1H12 and lymphocyte function–associated antigen-1 clearly differentiates the cell types. Further distinction was observed in the molecular profiles for expression of angiogenic genes. Pericytes or EPC enhanced the invasion of MDA-MB-231 breast cancer cells in a coculture assay system. The s.c. coinjection of live pericytes or EPC along with MDA-MB-231 cells resulted in an increased rate of tumor growth compared with coinjection of irradiated pericytes or EPC. Microvessel density analysis indicated there was no difference in MDA-MB-231 tumors with or without EPC or pericytes. However, immunohistochemical staining of vasculature suggested that EPC and pericytes may stabilize or normalize vasculature rather than initiate vasculogenesis. In addition, tumors arising from the coinjection of EPC and cancer cells were more likely to develop lymphatic vessels. These results support the notion that pericytes and EPC contribute to malignancy and that these cell types can be useful as cell-based models for tumor vascular development and selection of agents that may provide therapeutic benefit. (Cancer Res 2005; 65(21): 9741-50)

Introduction

The vasculature of tumors has been described as being more vein like than artery like; vascular structure exists but is abnormal. Folkman hypothesized that the switch of transformed lesions from a dormant state to malignant progression was driven by the development of vasculature; a new focus of drug discovery thus emerged towards attacking endothelial cells responsible for tumor angiogenesis (1). More recently, endothelial precursor cells (EPC) have been identified as contributors to vessel development in both normal physiologic processes, such as wound healing, and in pathologic settings like cancer (2). Pericytes (also known as mural cells, mural cells, or myofibroblasts) wrap around the endothelial cells. Pericytes share the same basal lamina with the endothelial cells and form an incomplete covering around the abluminal surface of endothelial cells (3). Pericytes and endothelial cells communicate with each other through gap junctions, tight junctions, soluble factors, and cell surface adhesion molecules (4, 5).

EPC can be derived from CD34+/CD133+ bone marrow progenitor cells driven by angiogenic stimuli towards an endothelial phenotype (6–8). EPC are characterized by their ability to form tubes and express markers typical of endothelial cells (9). EPC involvement in developing vasculature has been shown in murine tumor models. CD34+ hematopoietic cells delivered to a model of lymphoma doubled tumor growth and were also identified as contributors to vasculature in Lewis lung tumors (9, 10). Vascular endothelial growth factor (VEGF)–mobilized stem cells restored tumor angiogenesis and growth in Id-mutant mice (11). Clinically, a higher population of EPC is associated with inflammatory breast tumors versus a noninflammatory phenotype (12). Additionally, EPC have been detected in the circulation and malignant tissues of patients with multiple myeloma (13).

Pericytes were first described over 100 years ago as adventitial or Rouget cells (14) and were termed pericytes in 1923 (15). Pericytes possess a distinctive morphology comprised of an irregularly shaped plasma membrane with extended cytoplasmic processes. These extensions facilitate interdigitation with endothelial cells (16). Pericytes regulate capillary and venule blood flow through contractile activity and can also control vascular permeability (14, 15). In diabetic retinopathy, a regeneration of retinal capillary pericytes results in microaneurysms (17).

The role of pericytes in normal vasculature is well documented; however, the contribution to developing tumors is just emerging. Pericytes have been identified in tumor vasculature through immunohistochemical staining of sections (18, 19) and could cover 73% to 92% of endothelial sprouts in several murine tumor types. The morphologic shape and association of the pericytes with the endothelial cells in tumor vasculature are abnormal and the cytoplasmic processes extend deep into tumor tissue (18). The recruitment of pericytes to tumors may be attributed in part to platelet-derived growth factor (PDGF) signaling. In a fibrosarcoma model, PDGF-β was identified as an important factor in the recruitment of pericytes to tumor vessels (20). In human gliomas, PDGF-β and PDGF receptor β (PDGF-Rβ) are overexpressed, and in mice, the overexpression of PDGF-β enhanced glioma formation by attracting pericytes (21).

An understanding of the role of pericytes has been gained by histologic examination of tissues and studies of the physical interaction between pericytes and normal mature endothelial cells. This report begins characterization of the interaction between pericytes and EPC. The similarities and differences between human pericytes and EPC were investigated by examining expression of cell surface markers and behavior in in vitro assays, cocultures, and potential roles in tumor growth...
using a MDA-MB-231 breast cancer xenograft tumor model. The findings indicate that pericytes and EPC can be useful models for antiangiogenic drug discovery, interact to form primitive vessels, and contribute to malignancy in part through the normalization of tumor vasculature.

**Materials and Methods**

**Cell culture.** CD34+/CD133+ progenitor cells from normal bone marrow cells were supplied as being >90% pure (Camberx, Inc., East Rutherford, NJ). The CD34+/CD133+ cells (1-2 × 10^5 cells/mL) were differentiated in Iscove's modified Dulbecco's medium (IMDM, Cambrex) with 15% fetal bovine serum (FBS, Invitrogen Corp, Carlsbad, CA), 50 ng/mL VEGF165 (R&D Systems, Minneapolis, MN), 25 ng/mL basic fibroblast growth factor (bFGF, R&D Systems), and 4 units/mL heparin (Sigma Chemical Co, St. Louis, MO) on fibronectin-coated flasks (BD Biosciences, Franklin Lakes, NJ) to generate adherent EPC (6, 7). Adherent EPC were maintained in IMDM supplemented with 10% FBS, without additional growth factors. Characterization of EPC has been reported previously (8).

Pericytes from normal fetal brain (ScienCell Research Laboratories, San Diego, CA) were cultured in pericyte medium with 2% FBS and 2 ng/mL each of epidermal growth factor, bFGF, and insulin-like growth factor-1 (IGF-I); 5 μg/mL insulin; 1 μg/mL hydrocortisone; and apo-transferrin (ScienCell Research Laboratories).

Human MDA-MB-231 breast carcinoma cells (American Type Culture Collection, ATCC, Manassas, VA) were maintained in RPMI 1640 with 10% FBS (Invitrogen). Human dermal fibroblasts (HDF, ATCC) were cultured in DMEM high glucose with 10% FBS. Human umbilical vascular endothelial cells (HUVEC) and human microvascular endothelial cells (HMVEC) were cultured in EGM-2 medium (Camberx).**

**Reverse transcription-PCR.** cDNA was isolated from EPC and pericyte cultures by exposure to Trizol (Invitrogen) and chloroform extraction. Samples were applied to RNeasy miniprep spin columns (Qiagen, Valencia, CA) and purified according to instructions. cDNA was generated using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real-time PCR was done with SYBR Green PCR Master Mix with primers on an ABI Prism 7700 Sequence Detection System (Applied Biosystems): actin-1, ANG-2, CD31, CD105, intercellular adhesion molecule-1 (ICAM-1), lymphocyte function-associated antigen (LFA-1), matrix metalloproteinase-1 (MMP-1), MMP-2, MMP-9, IL-8, IGF-1, P1H12, VEGF, transforming growth factor-β3 (TGF-β3), FGF, hepatocyte growth factor (HGF), VEGF2R (FLK-1) (Maxim Biotech, Inc., South San Francisco, CA), PDGF-B, PDGFB-B, vascular cell adhesion molecule-1 (VCAM-1, R&D Systems), and 18S (Applied Biosystems).

**Flow cytometry.** Cells were exposed to 0.25% trypsin/1 mMOL EDTA (Invitrogen) and washed twice in cold 0.9% PBS containing 5% FBS (flow cytometry buffer). Approximately 1 × 10^6 cells were resuspended in 50 μL of flow cytometry buffer and incubated with a primary antibody for 1 hour on ice. Cells were washed twice and incubated with the secondary antibody for 30 minutes on ice. Cells were washed twice and resuspended in a final volume of 500 μL for analysis. For intracellular antigens, the cells were first fixed and permeabilized for 20 minutes with Cytofix/Cytoperm (Invitrogen) and washed twice in cold 0.9% PBS containing 5% FBS (flow cytometry buffer). Approximately 1 × 10^6 cells were resuspended in 50 μL of flow cytometry buffer and incubated with a primary antibody for 1 hour on ice. Cells were washed twice and incubated with the secondary antibody for 30 minutes on ice. Cells were washed twice and resuspended in a final volume of 500 μL for analysis. For intracellular antigens, the cells were first fixed and permeabilized for 20 minutes with Cytofix/Cytoperm buffer (BD Pharmingen, San Diego, CA).

Three micrograms of the following primary antibodies were added: (a) anti-neural growth proteolytic 2 (NG2, Chemicon International, Temecula, CA), (b) anti-asparagine muscle actin (oSMa, Zymed Laboratories, Inc., South San Francisco, CA), (c) anti-desmin (Chemicon), (d) anti-P1H12 (Chemicon), (e) anti-CD11a/LEA-1 (BD Biosciences, San Diego, CA), (f) anti-CD90/Thy1 (Chemicon), (g) anti-CD54/ICAM (BD Biosciences), (h) anti-CD105/endoglin (BD Biosciences), (i) anti-CD106/VCAM (BD Biosciences), (j) anti-PDGF-Rβ/CD140 (BD Biosciences), and (k) anti-VEGFR2 (clone A-3,Santa Cruz Biotechnology, CA). Matched isotype control antibodies were used (BD Biosciences, Chemicon). Two microliters of PE-labeled secondary antibodies were used for detection (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Positive expression was determined if cells gated at ≥10%.

**In vitro tube formation.** Matrigel (BD Biosciences) was added to a 48-well plate, 150 μL per well. EPC or pericytes (3 × 10^5) were added in 300 μL of medium: IMDM with 2% FBS for EPC and pericyte medium with supplements and 2% FBS for pericytes. Cells were incubated for 24 hours and stained with 8 μg/mL calcein (Molecular Probes, Eugene, OR) for imaging. SU3461 or SU6668 was added at final concentrations ranging from 10 to 50 μmol/L to the cultures at the time that the cells were seeded into the assay plates in triplicate (22). Area of tube formation was quantified as fluorescent pixel area using SCION image software (NIH). Results are expressed as mean ± SD.

**Cocultures.** Matrigel (BD Biosciences) was added to the wells of a 48-well plate as before. EPC were labeled with PKH26 green dye and pericytes were labeled with PKH27 red dye (Sigma, St. Louis, MO). EPC and pericytes were added to Matrigel in a 1:1 ratio (1 × 10^5 cells per well). Cultures were incubated overnight in IMDM with 2% FBS. Cells were visualized by fluorescence using inverted-phase microscope.

**MDA-MB-231 cluster assay.** This model involves cancer clusters cocultured with another cell type in suspension (23). Matrigel (BD Biosciences) was added to the wells of a 24-well plate in a volume of 300 μL. A plug of Matrigel of ~1-mm diameter was removed. The hole was filled with 1 × 10^6 MDA-MB-231 cells, labeled with PKH26 green dye, and suspended in 1% collagen I (Coriolis Technologies, Palo Alto, CA), and allowed to solidify. Other cells (3 × 10^4 cells per well) were labeled with PKH67 green dye for distinction and added in IMDM with 2% FBS in 300 μL final volume. When both EPC and pericytes were included in the same wells, a total of 3 or 6 × 10^4 cells per well were added. Conditioned medium was collected from EPC and pericytes exposed to serum-free RPMI for 48 hours. The cultures were analyzed for up to 5 days. Area of MDA-MB-231 migration was quantified using SCION image software (NIH). Samples were done in triplicate.

**MDA-MB-231 tumor model.** MDA-MB-231 breast carcinoma cells, pericytes, and EPC were grown as described above. Cell suspensions were mixed with Matrigel in a 1:1 (v/v) ratio in volume of 300 μL containing 4 to 9 × 10^6 MDA-MB-231 cells with or without pericytes or EPC in a 2:1 ratio. For some implants, the pericytes or EPC (4 × 10^5 cells/mL) in RPMI were exposed to γ radiation (3 Gy/min) for 1 hour to a total dose of 180 Gy (RS2000 X-ray Irradiator, Rad Source Technologies, Inc., Boca Raton, FL). The cell suspensions in Matrigel were kept cold until implantation by s.c. injection into the rear flank of female beige severe combined immunodeficient mice (C.B-17/IcrScid-scid-bgBR), 7 to 8 weeks of age (Charles River Labs, Wilmington, MA). Tumors were measured twice per week and volume was determined using the formula: (width^2 × length) × 0.52. Data were expressed as mean tumor volume ± SD. Linear regression analysis was used to determine the slopes of the growth curves. A two-way ANOVA analysis determined significance.

**Immunohistochemistry.** Tumors were snap-frozen in ornithine carbamyl transferase compound on day 29. Slides were dried for 10 minutes, washed twice in TBS, once in TBST, and fixed in paraformaldehyde buffer for 10 minutes. Slides were rinsed twice, blocked for 10 minutes, rinsed twice, and incubated with antibodies against CD31 (clone MEC13.13, BD Pharmingen), or oSMa (clone 1A4, DAKO, Carpinteria, CA) for 1 hour in a humidified chamber. The oSMa antibody is cross-reactive for mouse and human. Slides were rinsed and incubated with CY2- and CY3-labeled secondary antibodies (Jackson Immunonochemicals, West Grove, PA). For microvessel density quantification, four individual tumors of each group were stained for mCD31 expression; five fields of each tumor were analyzed through a microscope at 20 objective. Data is presented as mean ± SD. For analysis of lymphatic vessels, an antibody against murine LYVE-I was used (R&D Systems).

Endogenous peroxidase was blocked and sections were then exposed to 10% rabbit serum. Slides were incubated with anti-mLYVE-1 at 7.5 μg/mL for 60 minutes at room temperature. A secondary biotinylated antibody was added at 1.5 μg/mL for 30 minutes at room temperature followed by avidin-biotin complex method peroxidase, 3,3′-diaminobenzidine for detection, and counterstained with hematoxylin. Sections were washed between steps. Slides were scanned at ×20 magnification.
Results

The expression of several cell surface markers characteristic of pericytes in tissues has been confirmed by flow cytometry in pericytes maintained as a primary culture (24–28). The expression of characteristic cell surface endothelial markers on EPC has been described previously (8); the EPC are CD45 negative but express CD31, VEGFR2, and VE-cadherin. Several cell surface proteins are expressed similarly by pericytes and EPC, although each cell type is maintained in culture under different conditions (Fig. 1A). The profiles for pericyte and EPC expression of CD54/ICAM, desmin, αSMA, and CD106/VCAM were nearly identical. NG2, VEGFR2, and PDGF-Rβ were strongly expressed on both EPC and pericytes, whereas CD90/Thy-1 was more highly expressed by EPC. VEGFR2 expression has previously been detected in rat brain pericytes and in myofibroblasts associated with human endometrial cancer (29, 30). EPC and pericytes also produce fibronectin that is secreted to generate the basal lamina that is a component of vasculature.

Differences in cell surface protein expression between EPC and pericytes were notable for markers associated with endothelial cells such as CD105/endoglin and P1H12. CD105, a TGF-β receptor, was detectable both on EPC and pericytes but was more strongly expressed by EPC (Fig. 1A). P1H12 and LFA-1, which are strongly expressed by EPC, were weakly expressed by pericytes at the protein level.

Expression of multiple angiogenic genes by EPC and pericytes were determined by reverse transcription-PCR (RT-PCR) and normalized to 18S RNA levels (Fig. 1). Genes expressed by both EPC and pericytes include FGF, IGF-I, MMP-2, PDGF, TGF-β, and VEGF. IL-8 and MMP-1 expression was minor in EPC but significantly up-regulated in pericytes by comparison. Only VEGF was expressed at higher levels in EPC than pericytes, whereas ANG-2 was expressed by pericytes and not EPC. MMP-9 expression was low in both cell types, and neither EPC nor pericytes expressed ANG-1 or HGF. This data indicates that similarities exist between EPC and pericytes in the secretion of angiogenic factors, but differential expression of some genes signifies the distinction between these two primary cell lines. Additional markers commonly associated with vasculature, such as CD31, CD105, VEGFR2, and P1H12, were also confirmed by RT-PCR (Fig. 1C).

Tube formation is an event fundamental to vasculogenesis and growth factors from malignant cells can stimulate the process. In culture, EPC tube formation is dependent upon cell density and serum concentration and is explored here with pericytes. EPC and pericytes were each plated onto Matrigel at optimal cell densities in their respective culture medium. Pericytes actively formed tubes/networks (Fig. 2A). The pericytes formed shorter networks of tubes in a web-like pattern with some symmetry that covered a large area of the well. The tubes formed by EPC seemed longer, more linear, and to have less symmetry. The pericyte tubes remained stable for several days, whereas tubes formed by EPC degenerated after 24 hours (data not shown).

Both pericytes and EPC express the receptors for PDGF and VEGF and therefore may be sensitive to inhibitors of PDGF and VEGF signaling (Fig. 1A and C). SU6668 is a tyrosine kinase inhibitor known to block the function of PDGF-R (19, 22). The effect of SU6668 on the tube formation by EPC and pericytes was evaluated. The model of analysis measuring area was selected as it quantifies the length of the cells as they elongate to form capillary-like networks. By comparison, a single round cell would cover less area than a network of cells that connect with one another. Exposure to SU6668 inhibited tube formation by both EPC and pericytes in a concentration-dependent manner (Fig. 2A). The SU6668 IC50 values for decrease in tube area were ~25 μmol/L for EPC and 2 μmol/L for pericytes suggesting that pericytes are more sensitive to inhibitors of PDGF-R than EPC (Fig. 2B). SU5416, a small-molecule tyrosine kinase inhibitor with selectivity for VEGFR2, also reduced the tube areas formed by EPC and pericytes in a concentration-dependent manner with IC50 values of 35 and 25 μmol/L, respectively (31). These tube formation assay results support the value of incorporating pericytes and EPC into cell-based assays as models of tumor vasculature.

Although EPC or pericytes when plated at optimal cell density can form tubes/networks, at suboptimal concentrations, these structures do not form. However, when lower cell numbers of EPC and pericytes are cocultured, interactions between the two cell types occur and the formation of tubes/networks proceeds. Structures resembling primitive vessels form involving both cell types, EPC (green) and pericytes (red; Fig. 3A). The structures formed present as multiple lumens that seem to consist of EPC studded with pericytes. Although EPC and pericytes were equal in number, the lumens that developed seemed derived mainly from EPC. The cords and rings comprised of EPC are dotted with pericytes and the connections between cell groups seem mainly pericytes. Because the pericytes frequently seem to connect groups of EPC through extensions, this suggests the possibility that pericytes are activated first and may drive vessel extension (Fig. 3B). Changing the ratio of EPC to pericytes from 1:1 to 1:2 or 2:1 did not generate significantly different results. EPC and pericytes can interact closely to form cords composed of one layer of each cell type (Fig. 3C). EPC seem to migrate along the aligned pericytes to form multicellular vessels. Exposure of EPC and pericyte cocultures to SU6668 resulted in inhibition of the formation of primitive vessels and apparent disruption of interactions between EPC and pericytes, such that the cells cease dissociated and the cultures lack any form of organization (Fig. 3D).

The behavior of EPC and pericytes in the presence of MDA-MB-231 breast cancer cells was investigated. The MDA-MB-231 clusters were prepared as 1- to 2-mm plugs. In the presence of EPC or pericytes, the migration of MDA-MB-231 cells into the Matrigel was enhanced (Fig. 4A). This effect was not observed with conditioned medium collected from EPC or pericytes (data not shown). The pixel area covered by the cancer cells was quantified (Fig. 4B). A comparison was made with HUVEC, HMVEC, and HDF, but these cells did not generate similar results indicating that not all cells possess the ability to enhance the outgrowth and migration of MDA-MB-231 cancer clusters. The area covered by cancer cells migrating into the Matrigel was three times greater in the presence of EPC or pericytes on day 3 versus MDA-MB-231 cells alone. Differences between pericytes and EPC arose when pericytes formed stable tubes for several days, whereas the EPC did not. By comparison, the EPC formed multiple clusters within the wells. Overall, pixel area covered by the migrating cells was similar in MDA-MB-231 cocultures with EPC or pericytes. However, the inclusion of both EPC and pericytes in the same well did not have an additive or synergistic effect.

The tyrosine kinase inhibitor SU6668 resulted in the inhibition of EPC and pericyte tube formation (Fig. 2A and B). To determine if SU6668 could inhibit the migration of MDA-MB-231 clusters in...
Figure 1. A, flow cytometry profiles of EPC and pericytes for expression of cell surface molecular markers characteristic for pericytes or endothelial cells. Solid profiles represent matched IgG isotype controls. Several markers were expressed by both pericytes and EPC including CD105/endoglin, fibronectin, SMA, CD54/ICAM, NG2, desmin, PDGF-R, and Thy-1. Cell surface molecules expressed by EPC but not detected at significant levels in pericytes were P1H12 and LFA-1. B, molecular expression of angiogenic genes by EPC and pericytes as detected by RT-PCR. MMP-2 was highly expressed by both EPC and pericytes, whereas neither cell expressed ANG-1 or HGF. EPC had greater expression of VEGF, whereas pericytes had higher levels of TGFβ3, MMP-1, and IL8. C, expression of markers detected by flow cytometry was confirmed by RT-PCR.
The coculture of EPC or pericytes with MDA-MB-231 breast cancer cells indicated that EPC and pericytes can increase migration of malignant cells in vitro. To determine whether a similar effect could be observed in vivo, MDA-MB-231 breast cancer cells were coinjected s.c. with pericytes or EPC in a 12:1 ratio. The tumors that arose from the implantation of cell mixtures had a greater rate of growth versus those that arose from MDA-MB-231 cells alone. A two-way ANOVA analysis indicated that the differences were significant \( (P < 0.0001) \). Slopes of the growth curves were determined by linear regression analysis. The slope for the tumor growth curve for the EPC/MDA-MB-231 mixture was 2.6-fold greater than for the slope for the MDA-MB-231 cells alone and the slope for the tumor growth curve for the pericyte/MDA-MB-231 cell mixture was 2.5-fold greater than that for the tumor cells alone (Fig. 5A). Thus, the incorporation of EPC or pericytes into the initial implant provided a growth advantage to tumors.

The coinjection of irradiated cells with cancer cells has been a technique employed to enhance the establishment of s.c. tumors (32). Therefore, the effects of coinjection of viable versus radiation-killed pericytes or EPC on tumor growth were examined (Fig. 5B and C). The addition of viable pericytes to implantation of MDA-MB-231 cells significantly increased the slope of the tumor growth curve 3-fold \( (P < 0.001) \) versus cancer cells alone, whereas addition of irradiated pericytes to the tumor implantation mixture increased the slope of the tumor growth curve 1.8-fold versus MDA-MB-231 cells alone. Similarly, addition of viable EPC to implantation of MDA-MB-231 cells significantly
increased tumor growth compared with controls or irradiated EPC \( (P < 0.0001) \). The slope of the tumor growth curve for viable EPC increased 2.2-fold versus cancer cells alone, whereas addition of irradiated EPC to MDA-MB-231 cell implantation increased the slope of the tumor growth curve 1.2-fold. Thus, irradiated EPC or pericytes enhance tumor growth slightly, although there were no significant differences when compared with control in contrast to viable EPC or pericytes.

The tumors that arose from MDA-MB-231 cells mixed with EPC or pericytes were further evaluated for vessel morphology and microvessel density (Fig. 6). Endothelium was identified with antibodies against CD31 (red) and perivascular cells with an antibody against \( \alpha \)SMA (green). Although the antibodies cannot specifically identify the human pericytes and EPC, the pattern of staining indicated a change in morphology of the vasculature. In tumors consisting of MDA-MB-231 cells alone (Fig. 6A), there was little association between CD31- and \( \alpha \)SMA-positive cells. In tumors arising from the coinjection with EPC (Fig. 6B), or with pericytes (Fig. 6C), the blood vessels seem stabilized and better organized with distinct lumens and direct contact between endothelium and pericytes. No significant differences in microvessel density were noted between tumors (Fig. 6D) suggesting that EPC or pericytes may enhance tumor growth by stabilizing or normalizing the vasculature, rendering the vessels more capable of supplying oxygen and nutrients to the tumors. The lack of increase in microvessel density suggests that EPC or pericytes are less likely to initiate vasculogenesis, the creation of new blood vessels, and more likely to promote angiogenesis, the extension of existing blood vessels. Further quantitative analysis of the tumors indicated that the inclusion of EPC promoted the development of vessels with a lymphatic component \( (P < 0.05; \text{Fig. 6E}) \). This effect was observed to a lesser degree with pericytes. In humans, expression of the antigen LYVE-1 is associated with a poor prognosis in breast cancer (33). The incorporation of EPC or pericytes into tumors rendered the MDA-MB-231 model more aggressive thus indicating that EPC and pericytes can enhance malignancy, in part, through the normalization of blood vessels.

**Discussion**

The focus of therapeutics against tumor vasculature has been mature endothelial cells. Recent findings expanded that focus to include circulating endothelial cells and EPC mobilized from the bone marrow. Although the existence of pericytes and interactions between pericytes and endothelial cells as functional components of vasculature have been well documented, the importance of pericytes to the function and survival of endothelial cells is only now being appreciated. The distinction between pericytes and other cells types in the vasculature of tissues is often made through immunohistochemical staining for desmin. Other markers expressed by pericytes include NG2, \( \alpha \)SMA, and VCAM (24–28). The expression of some markers can vary depending upon the tissue in which the pericytes reside. For example, \( \alpha \)SMA is detected on pericytes from pre- and post-capillary microvascular segments, but those from midcapillaries do not (34).

Pericytes and EPC share many properties such as the ability to form tubes and express specific molecular markers. CD90 is of particular interest, because its expression is associated with a subset of CD34+ stem cells that are predictive of successful hematopoietic engraftments evaluated in cancer patients undergoing peripheral blood stem cell transplantations (35). The expression of CD90 on EPC and pericytes may be indicative of..
their angiogenic potential and capacity for proliferation. The similarities in cell surface proteins between EPC and pericytes suggest the possibility that these two cell types could arise from a common progenitor cell.

The full pluripotency of CD133+ stem cells remains to be characterized, and it is possible that these cells may be driven to a different phenotype under an alternative set of stimulatory conditions. CD105/endoglin expression has also been observed in a small subset of CD34+CD133+ hematopoietic stem cells (36), markers that are present in the progenitor cells used here to derive the EPC. CD34+ is recognized as a hematopoietic stem cell marker, and the detection of a lymphocytic marker, LFA-1, on EPC but not HMVEC or HUVEC (data not shown) maintains a link between EPC and hematopoiesis. LFA-1 is expressed selectively on monocytes and macrophages (37), thereby linking the EPC with cells derived from a hematopoietic precursor. By comparison, pericytes are believed to be of mesenchymal origin, although conclusive evidence is lacking (34).

The multistage nature of differentiation from stem cells to mature endothelial cells results in a spectrum of EPC with varying expression of molecular markers depending upon stage of differentiation. The expression of αSMA on the surface of EPC derived from CD34+ stem cells brings into question the exclusivity of αSMA as a marker of mesenchymal lineage cells or CD34 of as a marker of hematopoietic lineage cells. The detection of αSMA on endothelial cells has been observed previously in endothelial cells isolated from porcine and murine capillaries. The expression of αSMA arose when heparin was removed from cells in culture, thereby driving the endothelial cells toward a less mature phenotype (38). The plasticity of endothelial cells and EPC in culture indicates that these cells respond phenotypically to varying stimuli in the environment.

Most antiangiogenic strategies have been directed toward mature endothelial cells; however, the angiogenesis process driven by EPC may also be a useful target. For example, delivery of endostatin has shown effects toward EPC in models, whereby endostatin inhibited EPC differentiation and mobilization (39, 40). The incorporation of human EPC or pericytes into murine tumors advances the use of traditional models, where previously, the vasculature was solely murine in origin. The development of human-mouse chimeras of vasculature within tumors is a more appropriate setting that better mimics the conditions presented in the clinic and, as such, may be more informative and predictable in the evaluation of novel therapies.

Circulating EPC (CEPC) or endothelial cells may be useful surrogate markers for angiogenesis and for antiangiogenic therapy response. In a human lymphoma model, a strong correlation was found between CEPC and tumor volume. VEGF serum levels paralleled the increase in CEPC (41). In breast cancer and lymphoma patients, CEPC were increased by 5-fold.

Figure 4. A, MDA-MB-231 human breast cancer cell invasion coculture assay images. MDA-MB-231 human breast cancer cells embedded in collagen were inserted into Matrigel in a 24-well plate. EPC or pericytes were added as a single-cell suspension to some of the wells. The sprouting or outgrowth of the cancer cells from their origin was visually greater in the presence of EPC or pericytes on day 5. Top, individual wells; magnification, ×1. Pericytes formed tube networks that were stable on day 5 and extended across the area of cancer cell migration zone. HUVEC did not increase migration of cancer cells. B, Matrigel invasion of MDA-MB-231 cells was quantified using SCION Image Software. Coculture with either EPC and pericytes enhanced the invasion of MDA-MB-231 cells compared with MDA-MB-231 cells alone (control) on day 3. HMVEC, HUVEC, and HDF did not have the same effect. Columns, mean; bars, ±SD.
CEPC numbers were similar between controls and lymphoma patients in remission and in breast cancer patients following surgery (42). In a similar study, peripheral blood was collected from breast cancer patients with infiltrating carcinoma, ductal carcinoma in situ, and from normal controls. mRNA analysis identified CEPC elevation in patients with infiltrating carcinomas (43). Angiogenic profiles were assessed from blood samples of 82 cancer patients presenting a variety of cancers (lymphomas, leukemias, breast and hepatocellular carcinomas, ovarian, neuroendocrine, and lung). Compared with healthy volunteers, mRNA expression indicated that VE-cadherin was increased in patients with tumors (44). Circulating endothelial cells and EPC may be a valuable indicator of patients who may benefit most from antiangiogenic therapy.

EPC and pericytes here were found to contribute to the malignancy of human breast cancer cells using both in vitro and in vivo examples of a tumor microenvironment. However, the degree of contribution by EPC and pericytes to vasculature may vary with tumor type, and the role of EPC, in particular, remains somewhat controversial. In a murine model, orthotopic and s.c. GL261 glioma tumors were evaluated for engraftment of bone marrow–derived endothelial cells. Little engraftment was observed, even in highly vascularized tumors overexpressing VEGF (45). In a transgenic model created to trace the origin of tumor endothelium, results indicated that endothelium of Lewis lung or B6R2 lymphoma tumors did not originate from bone marrow cells (46). By contrast, coinjection of murine endothelial cells with human epidermoid cancer cells in vivo increased tumor size and vascularity (47). Pericyte coverage can vary from tissue to tissue and among tumors. A comparison of human glioblastomas, renal cell, colon, breast, lung, and prostate carcinomas showed that breast and colon tumors having significantly greater pericyte recruitment than gliomas or renal cell carcinomas (48). The expansion of tumor vasculature may be enhanced by interactions between EPC and pericytes as coculture experiments show the ability of these two cell types to form primitive vessels. In addition, molecular analysis revealed the expression of matrix metalloproteinases, particularly by pericytes, which may assist in driving angiogenic reactions by breaking down structural components of the basement membrane thereby facilitating the migration of endothelial cells and pericytes.

Like platelets, endothelial cells are a source of PDGFβ (49). EPC, as precursors to mature endothelial cells, secrete PDGFβ (Fig. 1B) contributing to the structural network of EPC and pericytes in early stages of vascular development. PDGFβ is essential for normal vascular development; thus, genetic ablation of PDGFβ in mice resulted in impaired recruitment of pericytes to blood vessels (50). Pericytes were validated as a therapeutic target using kinase inhibitors selective for PDGF-Rβ. Pancreatic tumors in Rip1Tag2 mice regressed in part by destabilization of interactions between pericytes and endothelial cells following treatment with Gleevec or SU6668 (19, 22). Inhibiting VEGF/PDGF signaling in glial cells produced tumor regression by interfering with pericycle-mediated endothelial cell survival mechanisms (51). The pericytes and EPC presented here were sensitive to kinase inhibitors selective for PDGF-Rβ. Molecular analysis revealed the expression of matrix metalloproteinases, particularly by pericytes, which may assist in driving angiogenic reactions by breaking down structural components of the basement membrane thereby facilitating the migration of endothelial cells and pericytes.

Development of the first generation of antiangiogenic drugs used mature, fully differentiated endothelial cells, such as HMVEC and HUVEC, in the discovery process; yet, these agents met limited clinical success. The incorporation of pericytes and EPC as models of tumor vasculature into drug development schemes may generate more fruitful results. Previous serial analysis of gene expression analysis of EPC, HMVEC, and endothelial cells isolated from several tumor types indicated that EPC expressed more of the

Figure 5. A, MDA-MB-231 human breast cancer cells were implanted s.c. into the flank of female beige severe combined immunodeficient mice either alone or with human EPC or pericytes. MDA-MB-231 cells (9 × 10^6) were mixed with EPC or pericytes (7.5 × 10^5) in Matrigel/RPMI 1640 (1:1) before injection. The presence EPC or pericytes increased the initial rate of tumor growth. Points, mean (n = 5); bars, ± SD. Two-way ANOVA analysis indicated differences were significant (P < 0.0001). B-C, MDA-MB-231 tumor growth after s.c. coinjection of MDA-MB-231 cancer cells (4 × 10^6) alone or with live or irradiated pericytes or EPC (3.3 × 10^6). The rate of tumor growth was greater following coinjection with live pericytes or EPC than with killed pericytes or EPC versus tumor growth rate with tumor cells alone. Points, mean (n = 5); bars, ± SD. Two-way ANOVA analysis indicated differences were significant when EPC or pericytes were viable compared with control or irradiated cells (P < 0.0001).
Pericytes and Endothelial Precursor Cells

Figure 6. Vasculature of tumors was analyzed by immunohistochemistry with antibodies against CD31 (red) and αSMA (green). A, blood vessels in control tumors are disorganized with little association between endothelium and perivascular cells. B, blood vessels of a tumor arising from the coinjection of MDA-MB-231 cells and EPC; direct contact is evident between CD31+ and αSMA+ cells. C, coinjection of cancer cells and pericytes generated vasculature that is organized with stable lumen formation. D, microvessel density analysis of MDA-MB-231 tumors with or without EPC or pericytes. Tumors were stained for mCD31 expression and vessel density was determined over five fields per sample (n = 4). There was no difference in microvessel density when EPC or pericytes were coinjected with cancer cells. E, lymphatic vessels were identified with an antibody against murine LYVE-1. The degree of lymphatic vessels that developed was increased by the incorporation of EPC (P < 0.05, Student’s t test). The effect of pericytes on lymphatic development was not as significant. Inset, immunohistochemical staining for LYVE-1 in a tumor generated from the coinjection of EPC and MDA-MB-231 cancer cells. Bar, 100 μm.

Genes selectively up-regulated by tumor endothelium than HMVEC
(11). Pericytes and EPC are important participants among the many cell types that give rise to progressing malignant disease. Targeting pericytes and EPC may lead to more effective therapies for cancer and increase our understanding of tumor development.

References
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