Bone Marrow Origin of Endothelial Progenitor Cells Responsible for Postnatal Vasculogenesis in Physiological and Pathological Neovascularization

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Abstract—Circulating endothelial progenitor cells (EPCs) have been isolated in peripheral blood of adult species. To determine the origin and role of EPCs contributing to postnatal vasculogenesis, transgenic mice constitutively expressing β-galactosidase under the transcriptional regulation of an endothelial cell–specific promoter (Flk-1/LZ or Tie-2/LZ) were used as transplant donors. Localization of EPCs, indicated by flk-1 or tie-2/lacZ fusion transcripts, were identified in corpus luteal and endometrial neovascularature after inductive ovulation. Mouse syngeneic colon cancer cells (MCA38) were implanted subcutaneously into Flk-1/LZ/BMT (bone marrow transplantation) and Tie-2/LZ/BMT mice; tumor samples harvested at 1 week disclosed abundant flk-1/lacZ and tie-2/lacZ fusion transcripts, and sections stained with X-gal demonstrated that the neovasculature of the developing tumor frequently comprised Flk-1– or Tie-2–expressing EPCs. Cutaneous wounds examined at 4 days and 7 days after skin removal by punch biopsy disclosed EPCs incorporated into foci of neovascularization at high frequency. One week after the onset of hindlimb ischemia, lacZ-positive EPCs were identified incorporated into capillaries among skeletal myocytes. After permanent ligation of the left anterior descending coronary artery, histological samples from sites of myocardial infarction demonstrated incorporation of EPCs into foci of neovascularization at the border of the infarct. These findings indicate that postnatal neovascularization does not rely exclusively on sprouting from preexisting blood vessels (angiogenesis); instead, EPCs circulate from bone marrow to incorporate into and thus contribute to postnatal physiological and pathological neovascularization, which is consistent with postnatal vasculogenesis. (Circ Res. 1999;85:221-228.)

Key Words: vasculogenesis ■ endothelial progenitor cell ■ bone marrow transplantation ■ Flk-1 ■ Tie-2

In the embryonic yolk sac, vasculogenesis involves growth and fusion of multiple blood islands that ultimately give rise to the yolk sac capillary network; after the onset of blood circulation, this network differentiates into an arteriovenous vascular system. The integral relationship between the elements that circulate in the vascular system (the blood cells) and the cells that are principally responsible for the vessels themselves (endothelial cells [ECs]) is implied by the composition of the embryonic blood islands. The cells destined to generate hematopoietic cells are situated in the center of the blood island and are termed hematopoietic stem cells. Endothelial progenitor cells (EPCs), or angioblasts, are located at the periphery of the blood islands. In addition to this spatial association, hematopoietic stem cells and EPCs share certain antigenic determinants, including Flk-1, Tie-2, Sca-1, and CD34. These progenitor cells have consequently been considered to derive from a common precursor, putatively termed a hemangioblast. Circulating CD34 antigen–positive EPCs were recently isolated from adult species; once adherent, these cells were shown to differentiate in vitro. Heterologous, homologous, or autologous EPCs administered systemically to animals with operatively induced hindlimb ischemia were found to incorporate into foci of neovascularization in ischemic muscles of the affected hindlimb. These findings, together with other recent studies, are consistent with the notion of postnatal “vasculogenesis.”

To determine the origin and role of EPCs contributing to postnatal vasculogenesis, we established 2 murine models of bone marrow transplantation (BMT). In each case, immunodeficient mice underwent BMT from transgenic mice constitutively expressing β-galactosidase (lacZ) transcriptionally regulated by an EC-specific promoter, Flk-1 or Tie-2. Reconstitution of the transplanted BM yielded Flk-1/LZ/BMT or Tie-2/LZ/BMT mice, in which expression of lacZ is restricted to BM cells expressing Flk-1 or Tie-2; lacZ expression is not
observed in other somatic cells. We then sought evidence of lacZ expression and the promoter/lacZ transcripts, flk-1/lacZ and tie-2/lacZ, in both pathological and physiological neovascular foci as a means of assessing the contribution of BM-derived EPCs to postnatal neovascularization.

Materials and Methods

Murine BMT Model

All protocols were approved by the St Elizabeth’s Medical Center Institutional Animal Care and Use Committee. In the present study, we established 2 BMT animal models: Flk-1/LZ/BMT mice, which received BM from mice heterozygous for flk-1 null mutation, and Tie-2/LZ/BMT mice, which received BM from transgenic mice constitutively overexpressing β-galactosidase (lacZ) regulated by the Tie-2 promoter. In the flk-1 null mutation, the target gene was replaced with a promoterless lacZ gene from Escherichia coli so that in the heterozygous mouse, expression of lacZ as well as target receptor protein (Flk-1) is subject to regulation by an EC-specific promoter.

BM cells were obtained by flushing the tibias and femurs of age-matched (4 weeks) donor mice heterozygous for Flk-1-null mutation (generous gift from Dr F. Shalaby, Bristol-Myers Squibb Pharmaceutical Research, Princeton, NJ) or Tie-2 transgenic mice (FVB/N-Tg[NITE2lacZ]18Sato, The Jackson Laboratory, Bar Harbor, Maine). BM from C57BL/6j mice, C/BMT, were used as negative controls. BM in lacZ-overexpressing (LZ/BMT) mice (B6.129-TgR[ROSA26]26Sor, The Jackson Laboratory) was also performed to investigate the efficiency of BMT.

Low-density BM mononuclear cells were isolated by density centrifugation over Histopaque-1083 (Sigma). The immunodeficient mice, NIH-III (Tac:NIHS-bg-nu-xidfDF, Taconic Farms, Germantown, NY), were lethally irradiated with 9.0 Gy and received ~2×10^6 donor BM mononuclear cells each intravenously. At 4 weeks post-BMT, by which time the BM of the recipient mice is reconstituted, a variety of surgical experiments were performed. Organs of normal BMT animals and pathological tissues from BMT experimental animal models were harvested for isolation of mRNA and cellular identification of lacZ after intracardial perfusion of phosphate-buffered salt solution to avoid blood cell contamination. In the case of histological examination for identification of lacZ-expressing cells, 1% paraformaldehyde solution was perfused as well.

To determine the transplant efficiency of LZ/BMT, low-density BM mononuclear cells were isolated and cultured for methycellulose semisolid culture assay (MethoCult M3630, StemCell Tech). BM mononuclear cells (1.0×10^5) were cultured in a 35-mm methylcellulose well for 12 days, after which colonies were stained with X-gal solution for 3 hours and then counted by light microscopic examination.

Hormone-Induced Ovulation

To obtain hormone-induced ovulation, 5 IU of pregnant mare serum gonadotropin (Sigma) was injected intraperitoneally 4 weeks after BMT, followed 42 hours later by 5 IU of human chorionic gonadotropin. The mice were killed, and the ovary and uterus were surgically removed at 48 to 72 hours after gonadotropin administration.

Tumor Implantation Model

BMT mice were anesthetized with pentobarbital (160 mg/kg IP). Approximately 2×10^6 mouse syngeneic colon cancer cells (MCA38) were suspended in 100 μL of Matrigel and injected subcutaneously at the flank fold. Large tumors were typically observed 3 to 4 weeks after implantation. Mice were euthanized at 1 week or 3 weeks after macroscopic tumor identification, and sections of the tumor and surrounding tissue were harvested for histological analysis.

Wound Healing Model

The skin was cleansed with 70% alcohol, and a full-thickness wound was made by pinching up a fold of flank skin and using a sterile, disposable 4-mm punch biopsy (Baker Cummings Dermatological) to punch through the 2 layers of skin on one flank. At various intervals after wounding, the mice were euthanized, and wounds were harvested with a perimeter of 1 to 2 mm of normal skin tissue and analyzed for EPC incorporation into wound neovascularization.

Myocardial Ischemia Model

The mouse model of myocardial ischemia was based on that previously described by Michael et al.13 Mice were anesthetized, placed in a position rotated 45° to the right of supine, and cannulated with a 22-gauge IV catheter (Johnson & Johnson), and the intubation cannula was connected to the output valve of a mouse ventilator (Harvard Apparatus). The ventilator typically delivered to the mouse a volume of 0.2 to 0.5 mL, 100% O_2, at a rate of 115 strokes per minute. An incision was then made along the left midclavicular line and, using a dissecting microscope, the pectoralis muscle was dissected. A lateral incision was made in the left fourth intercostal space, and the ribs were retracted to open the chest. After removal of pericardium, the left anterior descending (LAD) branch of the left coronary artery was identified under high-power magnification. The LAD was then permanently ligated by passing an 8-0 silk suture under the LAD at the level of the distal margin of the retracted left atrial appendage. The chest wall, muscle layers, and skin were then closed with interrupted 6-0 silk sutures, intubation was discontinued, and the mice were allowed to recover on a heated platform.

Identification of flk-1/lacZ or tie-2/lacZ Transcripts

RNA was extracted from each tissue sample using a total RNA isolation kit (Totally RNA, Ambion Inc). One microgram of total RNA from each sample underwent reverse transcription–polymerase chain reaction (RT-PCR) after DNase treatment. Reverse transcription and PCR were respectively performed using a Superscript Preamplification System kit (GIBCO-BRL) and Gene Amp PCR reagent kit with AmpliTaQ DNA polymerase (Perkin Elmer Corp), according to the supplemented protocol.

Primers for the amplification of flk-1/lacZ or tie-2/lacZ reverse-transcribed products3,4,18 were as follows: 5′-CTG TGT CCC GCA GCC GCA TA-3′ and 1′-AAA GCC CCA TTC GAC ATT CA-3′ (flk-1/lacZ) and 5′-GGG AAG TCG CAA AGT TGT GAG TT-3′ and 5′-GCT GGC CTG ATT CAT TCC-3′ (tie-2/lacZ). Twelve microliters of each PCR mixture was electrophoresed in 2% or 1% agarose gel containing ethidium bromide for flk-1/lacZ and tie-2/lacZ, respectively. RT-PCR products were photographed under a UV transilluminator.

Cellular Identification of lacZ-Expressing Cells

After completing the in vivo experiment, mice were euthanized and the target organs fixed with 4% paraformaldehyde for 3 hours at 4°C and incubated in X-gal solution overnight at 37°C. The tissue samples were then placed in PBS and examined under a dissecting microscope to localize foci of lacZ-expressing cells. Histological sections were counterstained with light hematoxylin and eosin and examined by light microscopy. The control samples from C/BMT mice were examined identically.
Physiological Neovascularization

The efficiency of BMT was assessed by X-gal staining of cell colonies in a methylcellullose colony assay using BM mononuclear cells isolated from LZ/BMT mice. LacZ-expressing donor-derived cells were identified in 92.6±4.2% of BM colonies.

Physiological localization of EPCs to normal (ie, unper- turbed) organs was investigated using RT-PCR to identify flk-1/lacZ and tie-2/lacZ fusion transcripts (Figure 1). Each transcript was indeed detected abundantly in BM, peripheral blood, spleen, and to a lesser extent in lung, liver, intestine, skin, and hindlimb muscle, as well as ovary and uterus. Such constitutive incorporation of BM-derived Flk-1– and Tie-2–expressing cells into these normal organs implies a role for EPCs in physiological organ maintenance. The absence of EPC incorporation into the brain are consistent with findings of Pereira et al15 and may reflect organ-specific differences in vascular and connective tissue structure.

The development and endocrine function of the ovarian corpus luteum have been shown to depend on the development of new vessels.16,17 To show evidence for vasculogenesis in physiological neovascularization of the ovaries and endometrium, ovarian cycling was hormonally induced in mice. LacZ-expressing cells were found in tumor tissues from control BMT animals. Flk-1– and Tie-2–expressing lacZ-positive EPCs were identified as stromal cells adjacent to the neovascularization as well. A linear configuration of BM-derived EPCs was observed frequently. The frequency and distribution of EPCs were consistent within the tumor periphery between 1 week and 3 weeks after macroscopic tumor growth, although at 3 weeks, EPCs were absent from central necrotic foci. No lacZ-stained cells were found in tumor tissues from control BMT animals (C/BMT mice).

Wound Healing

Healing of cutaneous wounds was examined at 4 days, 7 days, and 4 weeks after skin removal by punch biopsy. At 4 and 7 days, lacZ-positive BN-derived EPCs were incorporated into foci of neovascularization at high frequency (Figure 5). Neovascularation and stromal cells consisting of EPCs were observed mainly in granulation and fat tissues. Flk-1– and Tie-2–expressing EPCs were observed with equal frequency in these tissues. At 4 weeks after wounding, negligible X-gal–stained EPCs could be identified. This was confirmed by down-regulation of promoter/lacZ transcript expression in these tissues (Figure 3).

Limb and Myocardial Ischemia

Evidence that vasculogenesis constitutes a reparatory response to tissue ischemia was observed as well. Unilateral hindlimb ischemia was established in mice by surgical excision of one femoral artery.11 One week after the onset of hindlimb ischemia, Tie-2– or Flk-1–expressing lacZ-positive EPC colonies were observed in tissue stroma at sites of ischemia, and lacZ-positive EPCs were identified incorporated into capillaries among myocytes (Figure 6A and 6B). At 4 weeks after ischemia, EPCs were observed incorporated above-described murine BMT models. Mouse syngeneic colon cancer cells (MCA38) were implanted subcutaneously into Flk-1/LZ/BMT and Tie-2/LZ/BMT mice and harvested at 1 week or 3 weeks after macroscopic tumor identification. The flk-1/lacZ and tie-2/lacZ fusion transcripts were detected in abundance in tumor samples (Figure 3). Tumor tissues stained with X-gal demonstrated that the neovascularity of the developing tumor frequently comprised Flk-1– or Tie-2–expressing EPCs (Figure 4). EPCs were localized focally in highly vascularized lesions, particularly at the tumor periphery; EPCs were incorporated into capillaries and were identified as stromal cells adjacent to the neovascularization as well.

Pathological Neovascularization

Tumor

Pathological neovascularization accompanying tumor growth, wound healing, hindlimb ischemia, and myocardial infarction were evaluated for incorporation of EPCs in the above-described murine BMT models. Mouse syngeneic colon cancer cells (MCA38) were implanted subcutaneously into Flk-1/LZ/BMT and Tie-2/LZ/BMT mice and harvested at 1 week or 3 weeks after macroscopic tumor identification. The flk-1/lacZ and tie-2/lacZ fusion transcripts were detected in abundance in tumor samples (Figure 3). Tumor tissues stained with X-gal demonstrated that the neovascularity of the developing tumor frequently comprised Flk-1– or Tie-2–expressing EPCs (Figure 4). EPCs were localized focally in highly vascularized lesions, particularly at the tumor periphery; EPCs were incorporated into capillaries and were identified as stromal cells adjacent to the neovascularization as well.
into capillaries and interstitial arteries (Figure 6C) at the periphery of the ischemic foci.

After permanent ligation of the LAD coronary artery, 12 histological samples from sites of myocardial infarction were retrieved from BMT mice. Light photomicrographs demonstrated incorporation of EPCs into foci of neovascularization at the border of the infarct, confirmed by immunostaining for CD31 in sections of myocardium harvested at 1 week after infarction (Figure 6D and 6E).

Discussion
This series of BMT experiments establishes proof of the concept that postnatal vasculogenesis contributes to endogenous neovascularization of developing tumors, wound healing, severe hindlimb ischemia, and myocardial ischemia, as well as physiological neovascularization.

BMT recipients received BM from transgenic mice in which constitutive lacZ expression was regulated by an EC-specific promoter, Flk-1 or Tie-2. Vascular endothelial growth factor, the cognate ligand for the EC-specific tyrosine kinase receptor (TKR) Flk-1, has been shown to be essential for EPC (angioblast) differentiation and blood vessel development during embryogenesis13,18 and postnatal neovascularization.19–23 The Tie receptors, Tie-1 and Tie-2, constitute a second family of EC-specific TKRs. Tie-2 receptor has been shown to be expressed in endothelial lineage cells participating in angiogenesis24,25 and, in this regard, essential for blood vessel development and maturation.14,26

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in lung, liver, intestine, skin, and hindlimb muscle, as well as ovary and uterus. Such constitutive incorporation of BM-derived Flk-1– and Tie-2–expressing cells into these normal organs implies a role for EPCs in physiological organ maintenance. The concept of BM-derived progenitor cell reservoirs in normal tissues is consistent with the notion of BM-derived satellite myoblasts and mesenchymal stem cells in muscle or other normal organs. Isolated lacZ-positive EPCs were indeed located at interstitial sites among muscle fibers of healthy hindlimb muscle (Figure 2A). The mouse hindlimb muscle is a highly energy-consuming and physiologically stressed organ. Circulating BM-derived EPCs may home to tissue stroma in certain organs for the purpose of providing maintenance reservoirs of ECs analogous to satellite myoblasts and fibroblasts. The current findings extend this concept to physiological organogenesis in the reproductive system.

The hormonally induced ovulation cycles were associated with upregulated ovarian expression of flk-1/lacZ and tie-2/lacZ. Histological examination of the harvested reproduction

Figure 4. Histological detection of EPCs in mouse syngeneic colon cancer after BMT. A, Macroscopic tumor sample stained with X-gal after tissue fixation. Blue staining, denoting EPC-rich region, principally involves periphery of tumor site. Histology of tumor specimen demonstrates vasculature comprising Flk-1–expressing EPCs (B and C) in Flk-1/BMT mice. Double staining of X-gal (blue) and BS-1 lectin (brown) (C) demonstrates colonizing ECs derived from EPCs expressing Flk-1. D and E, Tumor specimen from Tie-2/BMT reveals frequent Tie-2–expressing EPCs in vasculature (arrow) and stroma (arrowhead). Double staining for X-gal (blue) and CD31 (red) identifies incorporation of EPCs within blood vessels as well as isolated CD31-positive stromal cells. Bars = 25 μm.

Figure 5. Light microscopic detection of EPCs in wound-healing site created in skin of Flk-1/LZ/BMT and Tie-2/LZ/BMT mice. In tissue specimen harvested from wound healing site, neovasculature and stromal lacZ-positive cells are observed principally in granulation tissue and adipose tissue. Flk-1–expressing EPCs in granulation tissue (A and B) of Flk-1/LZ/BMT and Tie-2–expressing EPCs in the adipose tissue (C) and blood vessels (D) of Tie-2/LZ/BMT were observed 4 days after wounding. Brown hue in panels C and D identifies cells stained with BS-1 lectin, indicative of endothelial lineage. EPCs are identified by arrows in vasculature and arrowheads in stromal tissue. Bar = 25 μm.
systems disclosed BM-derived Flk-1– and Tie-2– expressing cells in corpus lutea and in the uterus endometrium and stroma. The findings indicate that EPCs contribute to physiological neovascularization associated with postnatal regenerative processes. Detection of flk-1/lacZ and tie-2/lacZ transcripts indicates natural homing of EPCs to organs devoid of pathological alterations and, in the case of the ovary and endometrium, characterized by cyclic, physiological neovascularization. In these tissues, cyclic neovascularization is associated with abundant expression of angiogenic growth factors, including vascular endothelial growth factor, basic fibroblast growth factor, and angiopoietins. These cytokines, together with as-yet-unidentified cell adhesion molecules and supportive stromal cells, likely contribute to enhanced homing of circulating EPCs to corpus lutea and endometrial tissue, in which they may further differentiate and/or incorporate into foci of neovascularization.

EPCs expressing Flk-1 or Tie-2 were found incorporated into the nascent vasculature of these respective pathological lesions. Indeed, not only were EPCs identified incorporated into blood vessels, but lacZ-positive colonies or isolated lacZ-positive cells were frequently observed in stroma of tumors, ischemic hindlimbs, and sites of wound healing. This finding is consistent with the recent finding of BM-derived mesenchymal stem cells. BMT experiments performed by Pereira et al suggest that mesenchymal stem cells in BM serve as a continuing source for a variety of mesenchymal cells, such as fibroblasts, osteoblasts, chondroblasts, adipocytes, and myoblasts. More recently, Ferrari et al showed evidence of muscle regeneration from BM-derived myogenic progenitors using BMT experiments similar to those described here. Their data, together with ours, suggest that vascular endothelium as well as mesenchymal cells may be derived from BM and contribute to organ regeneration and maintenance.

The growth of tumors to a clinically relevant size is dependent on an adequate blood supply. This is achieved by the process of tumor stroma generation in which the formation of new capillaries is a central event. Several molecular and cellular mechanisms have been identified by which tumor parenchyma may exert angiogenic effects on ECs. As a result of this paracrine influence, tumor-associated ECs have been considered to acquire an “immature” phenotype manifested by rapid proliferation, migration, release of proteases, and expression of EC-specific TKRs (eg, Flk-1, Tie-2, or Tie-1). This is not only the case with tumors. Wound healing as well as severely ischemic muscles and myocardium evolve abundant stroma and neovascularature after paracrine and autocrine expression of angiogenic cytokines.

BM-derived EPCs were observed within tissue stroma at sites of pathological neovascularization. On the basis of the expression patterns of EPCs during embryogenesis, EPCs in postnatal stromal tissues would be anticipated to proliferate, differentiate, and migrate in situ in the process of neovascularization. In stromal tissues, after the inflammatory response of extravasated blood cells such as platelets and monocyte/macrophage-delivering cytokines, EC-like or fibroblast-like cells follow closely behind for neovascularization. As Hatva et al have shown, a subpopulation of cells in hemangioblastoma stroma express Flk-1, Flt-1, and Tie-1 receptors. These observations are consistent with the notion that EPCs home to

Figure 6. Histological detection of EPCs in tissues harvested from ischemic hindlimb and myocardium. A and B, One week after onset of ischemia, Tie-2–expressing EPCs are observed migrating in stroma or within capillaries among myocytes. Histological sections were double stained with BS-1 lectin (brown) (A) and CD31 (red) antibody (B). At 4 weeks after ischemia, lacZ-positive EPCs were observed incorporated into capillaries and interstitial arteries (C) at the periphery of the ischemic foci. Immunostaining for CD31 (red) indicates incorporation of EPCs into blood vessels at 1 week after myocardial infarction in Tie-2/LZ/BMT (D and E). Bar=25 μm.

and differentiate in the stromal environment of tumors, ischemic muscles, and sites of wound healing, presumably for the purpose of promoting neovascularization.

The temporal sequence associated with pathological vasculogenesis in these BMT models varied according to the pathological state. In the case of a neoplasm, a period of 1 to 3 weeks after initiation of the so-called angiogenic switch is characterized by persistence of the initial stage of neovascularization, including cellular differentiation and development of neovascular sprouts rather than vessel maturation or regression: such persistent vasculogenesis is reminiscent of Dvorak's characterization of tumors as "wounds that do not heal." In contrast, neovascularization of the ischemic hindlimb at 1 week includes lacZ-positive EPC colonization and sprouting, but by 4 weeks demonstrates a marked increase in capillary density and arteries. This suggests that ischemia initiates sequential stages from initial vasculogenesis to maturation of blood vessels required for persistent collateral circulation. In the wound healing model, after an initial stage of potent neovascularization, most of the neovascularization composed of EPCs undergoes regression leading to scar tissue.

The expression of lacZ regulated by Flk-1 and Tie-2 promoters could potentially identify both BM-derived differentiated ECs and EPCs. The lack of an epitope of which the expression is restricted exclusively to either fully differentiated ECs or EPCs makes it difficult to resolve this issue simply on the basis of cell sorting. There are at least 3 lines of evidence, however, that suggest that EPCs constitute the preponderance of such circulating BM-derived endothelial lineage cells. First, previous work has shown that freshly isolated CD34-positive cells display a paucity of EC-specific markers, in contrast to plated cells cultured for 7 days. Second, recent work from our own laboratory has shown that in contrast to ECs, heterologously transplanted differentiated ECs rarely incorporate into foci of neovascularization. Third, previous work indicates that the population of circulating EPCs far exceeds the number of differentiated ECs in peripheral blood.

These findings underscore the notion that postnatal neovascularization is not synonymous with angiogenesis, at least as the latter has been classically defined, to consist of sprouts that originate as the result of proliferation and migration of differentiated ECs from parent vessels. The current findings indicate that BM-derived EPCs home to and incorporate into sites of neovascularization where differentiation into ECs is completed, which is consistent with postnatal vasculogenesis. As a corollary, augmented or retarded neovascularization, whether endogenous or iatrogenic, likely includes enhancement or impairment of vasculogenesis.

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