Bone Marrow–Derived Endothelial Progenitor Cells Participate in Cerebral Neovascularization After Focal Cerebral Ischemia in the Adult Mouse

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Abstract—We investigated whether circulating endothelial progenitor cells contribute to neovascularization after stroke. Donor bone marrow cells obtained from transgenic mice constitutively expressing β-galactosidase transcriptionally regulated by an endothelial-specific promoter, Tie2, were injected into adult mice. Focal cerebral ischemia was induced by embolic middle cerebral artery (MCA) occlusion and changes of cerebral blood flow (CBF) were measured by perfusion-weighted magnetic resonance imaging (MRI). Laser scanning confocal microscopy (LSCM), immunohistochemistry and X-gal staining were performed. Perfusion-weighted MRI demonstrated increases in CBF around the boundary of an infarct area 1 month after ischemia. Morphological and 3-dimensional image analyses revealed enlarged and thin-walled blood vessels with sprouting or intussusception at the boundary of the ischemic lesion, which closely corresponded to elevated CBF areas detected on perfusion-weighted MRI, indicating the presence of neovascularization. X-gal and double immunostaining demonstrated that Tie2-lacZ–positive cells incorporated into sites of neovascularization at the border of the infarct, and these cells exhibited an endothelial antigenic marker (von Willebrand factor). In addition, bone marrow recipient mice without ischemia showed incorporation of Tie2-lacZ–expressing cells into vessels of the choroid plexus. These data suggest that formation of new blood vessels in the adult brain after stroke is not restricted to angiogenesis but also involves vasculogenesis and that circulating endothelial progenitor cells from bone marrow contribute to the vascular substructure of the choroid plexus. (Circ Res. 2002;90:284-288.)

Key Words: bone marrow ■ endothelial progenitor cells ■ neovascularization ■ cerebral ischemia ■ Tie2

In the early embryogenesis, the vascular system develops from vasculogenesis in which angioblasts differentiate into endothelial cells to form a primitive capillary network, whereas angiogenesis, the sprouting of capillaries from preexisting blood vessels, is involved in the late stage of embryogenesis and in the adult.1 Angioblast-like circulating endothelial progenitor cells are present in the peripheral blood and have been isolated from adult animals.2,3 Injection of circulating endothelial progenitor cells into animals with hindlimb or myocardial ischemia results in incorporation of circulating endothelial progenitor cells into neovasculature at the site of ischemia, suggesting that circulating endothelial progenitor cells contribute to formation of new blood vessels in the adult tissue.4

The endothelial cells of cerebral capillaries differ functionally and morphologically from those of noncerebral capillaries.5,6 During embryonic development, the cerebral vascular system originates from the perineural plexus when vascular sprouts invade the proliferating neuroectoderm, indicating that the cerebral vascular system is primarily developed by angiogenesis and not by vasculogenesis.7 The cerebral endothelial cells are linked by complex tight junctions that form the blood brain barrier (BBB).5,6 In the adult brain, proliferation of the cerebral endothelial cells ceases, and the turnover rate of endothelial cells is approximately 3 years.8,9 Studies from human and experimental stroke indicate that neovascularization is present in the adult brain after ischemia.10,11 However, development of new blood vessels in ischemic adult brain is incompletely understood, and it remains unknown whether newly formed vessels are induced by proliferation of preexisting vascular endothelial cells or by recruitment of circulating endothelial progenitor cells to the brain after stroke. In the present study, in ischemic adult mouse brain, we measured bone marrow cells obtained from transgenic mice constitutively expressing β-galactosidase (lacZ) transcriptionally regulated by an endothelial-specific promoter, Tie2.12

Materials and Methods
All experimental procedures have been approved by the Care of Experimental Animals Committee of Henry Ford Hospital.
Animal Model
Focal embolic cerebral ischemia was induced in male FVB mice (26 to 30 g, n=24) as previously reported with some modifications. Briefly, the right middle cerebral artery (MCA) was occluded by placing a single 8-mm length intact, fibrin-rich, 24-hours-old, homologous clot at the origin of the MCA via an 8-mm length of modified PE-50 catheter. The rationale for selecting a relatively short clot is that a long (10 mm) clot induces the ipsilateral cortical and subcortical lesions supplied by the right MCA, and mice with this type of large ischemic lesion usually do not survive for a week, whereas a short clot induces primarily a subcortical lesion and animals can survive for months. This model of embolic stroke is the most relevant one to human stroke.

Bone Marrow Transplantation
Using a syringe with 1 mL phosphate-buffered saline (PBS), fresh bone marrow was harvested aseptically by flushing thibias and femurs from age-matched (3 months) transgenic mice constitutively expressing β-galactosidase under transcriptional regulation of a Tie2 promoter (FVB/N-TgN, Tie2LacZ, The Jackson Laboratory, Bar Harbor, Maine). The Tie2 receptor is expressed in endothelial transplantation, by which time the donor bone marrow cells can be injected into a recipient mouse via a tail vein. At 4 weeks after bone marrow transplantation, by which time the donor bone marrow cells can be detected in the recipient bone marrow cells, recipient mice were subjected to embolic MCA occlusion and euthanized at 2, 30, and 60 days after MCA occlusion.

Bromodeoxyuridine Labeling
Bromodeoxyuridine (BrdU, Sigma Chemical), the thymidine analog that is incorporated into the DNA of dividing cells during S-phase, was used for mitotic labeling. BrdU (50 mg/kg) was intraperitoneally injected daily for 7 consecutive days into ischemic mice starting 7 days after ischemia. These mice (n=4) were euthanized 14 days after ischemia.

Magnetic Resonance Imaging Measurements
To dynamically measure changes of cerebral blood flow (CBF), perfusion-weighted magnetic resonance imaging (MRI) was performed on mice before and 1 month after MCA occlusion using a 7.0 T magnet (Magnex Scientific) equipped with actively shielded gradients and a Surrey Medical Imaging Systems console. MR images were recorded using a birdcage RF coil, as previously described. MR images were recorded using a birdcage RF coil, as previously described. This technique is based on the selective inversion of blood water protons at the level of the carotid arteries prior to H MRI measurement in the brain. Two images were obtained for analysis system (Imaging Research).

Three-Dimensional Image Acquisition
To examine neovascularization in ischemic brain, fluorescein isothiocyanate (FITC) dextran (2×10⁶ molecular weight, Sigma; 0.1 mL of 50 mg/mL) was administered intravenously to the ischemic mice subjected to 30 days of MCA occlusion. FITC-dextran remains dissolved and free in plasma. This dye circulated for 1 minute, after which the anesthetized animals were euthanized by decapitation. The brains were rapidly removed from the severed heads and placed in 4% of paraformaldehyde at 4°C for 48 hours. Coronal sections (100 μm) were cut on a vibratome. The vibratome sections were analyzed with a Bio-Rad MRC 1024 (argon and krypton) laser-scanning confocal imaging system mounted onto a Zeiss microscope (BioRad), as previously described.

LacZ Staining and Immunohistochemistry
Mice were perfused with heparinized saline. Brains were embedded in Tissue-Tek OCT compound (Miles, Inc), frozen in 2-methylbutane (Fisher Scientific), and cooled on dry ice. Coronal brain sections (8 to 40 μm thick) were cut on a cryostat and thaw-mounted onto gelatin coated slides. LacZ was performed using a β-galactosidase reporter gene staining kit according to manufacturer’s protocol (Sigma). Briefly, after postfixation, coronal sections were incubated in X-gal solution for 2 days at 37°C and then counterstained with light eosin. To identify cell types of Tie2-LacZ-expressing cells, double immunofluorescent labeling for von Willebrand factor (vWF), a marker for endothelial cells, or microtubule-associated protein-2 (MAP2), a marker for neurons, or glial fibrillary acidic protein (GFAP), a marker for astrocytes, and β-galactosidase was performed. A monoclonal antibody (mAb) against vWF (DAKO, Carpinteria, Calif), a mAb against MAP2 (clone AP20, Boehringer Mannheim Biochemicals, Indianapolis, Ind), a polyclonal antibody against GFAP (DAKO), and a polyclonal antibody against β-galactosidase (Chemicon) were used at a titer of 1:20, 1:50, 1:200, and 1:200, respectively. Coronal sections were incubated with the antibody against vWF, MAP2, or GFAP for 3 days at 4°C, and sections were then incubated with the anti-mouse or anti-rabbit immunoglobulin antibody conjugated to Cy3 (Vector, Burlingame, Calif). These sections were incubated with the antibody against β-galactosidase for 3 days at 4°C and then with the anti-rabbit immunoglobulin antibody conjugated to FITC. Single immunostaining for vWF was performed for morphological analysis of vessels. For BrdU immunostaining, DNA was first denatured by incubating brain sections (6 μm) in 50% formamide 2X SSC at 65°C for 2 hours and then in 2N HCl at 37°C for 30 minutes. Sections were then rinsed with tris buffer and treated with 1% of H2O2 to block endogenous peroxidase. Sections were incubated with a mouse monoclonal antibody (mAb) against BrdU (1:1000, Boehringer Mannheim) overnight and incubated with biotinylated secondary antibody (1:200, Vector) for 1 hour. Control experiments consisted of staining brain coronal tissue sections as outlined above but omitted the primary antibodies.

Results
To determine dynamic changes of CBF after ischemia, perfusion-weighted MRI measurements were performed on the bone marrow–transplanted mice 2 hours and 1 month after MCA occlusion. Substantial reduction of CBF in the right subcortex was detected in all mice (n=8) (Figure 1A), indicating the presence of an ischemic lesion. However, an elevated CBF was detected around the boundary of the ischemic lesion 1 month after ischemia (Figure 1B). Quantitative analysis of CBF revealed 74% of CBF reduction in ischemic lesion compared with levels of CBF in the contralateral homologous tissue at 2 hours after MCA occlusion (n=8). In contrast, 86% of CBF increase was detected in the ischemic boundary at 1 month after ischemia (n=8). Laser scanning confocal microscopy (LSCM) images of coronal sections that matched MRI sections from the same animal showed enlarged FITC-dextran–perfused cerebral vessels in the lesion boundary areas (Figure 1C, arrow), which closely
corresponded to the elevated CBF areas detected on perfusion-weighted MRI (Figure 1B, arrow). Morphological analysis of vWF stained vessels revealed enlarged and thin-walled blood vessels at the boundary of the ischemic lesion (Figure 1D) and some of them exhibited sprouting (Figure 1E, arrow) or intussusception (Figure 1F, arrows). Sprouting vessels were also detected in 3-dimensional images (Figure 1G, arrows), indicating neovascularization. Few BrdU immunoreactive cells were detected in vessels in the contralateral hemisphere. In contrast, BrdU immunoreactive cells were present on lumen of small and enlarged vessels (Figure 1H, arrows) around the ischemic lesion 14 days after ischemia. Quantitative measurements of vascular perimeter show that perimeters of blood vessels in the ipsilateral hemisphere (n=6) were significantly (P<0.05) larger than perimeters in the contralateral homologous areas (Figure 1I).

Nonischemic mice with bone marrow transplantation exhibited Tie2-lacZ–expressing cells in the choroid plexus of the both hemispheres (Figure 2A), and higher magnification showed that blue color was localized to the inner layer of choroid plexus (Figure 2B), suggesting these are endothelial cells rather than epithelial cells. Tie2-lacZ–expressing cells were not detected on parenchymal cerebral vessels in these mice (Figure 2A). Nonischemic mice without bone marrow transplantation did not show any Tie2-lacZ–expressing cells as assessed by blue color (Figure 2C). However, ischemic mice receiving bone marrow transplantation exhibited Tie2-lacZ–positive cells at the border of the infarct 1 month after ischemia (Figure 2D), and higher magnification showed that these cells were localized to cerebral microvessels (Figure 2E). LacZ immunostaining revealed lacZ immunoreactive cells in the lumen surface of the vessel (Figure 2F). Double immunofluorescent staining on FITC-dextran–perfused brain tissue showed a vessel perfused with FITC-dextran (Figure 2G and 2J, green) and colocalization of vWF immunoreactivity (Figure 2H and 2J, blue) with lacZ immunoreactivity (Figure 2I and 2J, red, arrows) on luminal surface, suggesting that these cells are endothelial cells. Tie2-lacZ positive cells were not detected outside of ischemic lesion (Figure 2K). Tie2-lacZ–positive cells were also found in recipient bone marrow cells 2 months after transplantation (Figure 2L, A and B).
Discussion

The present study provides evidence that circulating endothelial progenitor cells from bone marrow participate in neovascularization processes in the adult brain of mice after ischemia. In addition, circulating endothelial progenitor cells contribute to the microvascular structure of the choroid plexus.

Enlarged thin-walled vessels are termed “mother” vessels and have been found in the pathological angiogenesis. Mother vessels can develop into small vessels by sprouting, by invaginating, or by forming transmural endothelial bridges to structure smaller caliber daughter vessels and glomeruloid bodies. In parallel, we observed that enlarged and thin-walled blood vessels localized to the ischemic boundary and that some of these vessels sprout or show intussusception at the boundary of the ischemic lesion. These data indicate neovascularization occurs in the adult ischemic brain. Furthermore, colocalization of increased CBF with neovascularization implies that these newly formed vessels function. This is consistent with previous reports that functional imaging of stroke patients shows increased cerebral blood flow and metabolism in tissue surrounding focal brain infarcts. Our observation of a near absence of BrdU immunoreactive cells in vessels of the contralateral hemisphere is consistent with that proliferation of the cerebral endothelial cells ceases, whereas increases in proliferated endothelial cells in the ischemic vessels may reflect angiogenesis. Formation of new vessels may arise from the proliferation and migration of endothelial cells from the adjacent tissue and from circulating endothelial progenitor cells.

In the present study, using bone marrow transplantation from transgenic mice in which constitutive lacZ expression was regulated by an endothelial-specific promoter, Tie2, we found that bone marrow recipient mice incorporated Tie2-lacZ–expressing cells into the site of neovascularization around infarct areas after ischemia. Double immunostaining analysis shows that Tie2-lacZ immunoreactive cells had an endothelial antigenic marker, indicating that marrow-derived cells may differentiate into endothelial cells and contribute to neovascularization. These data suggest that formation of new blood vessels in the adult brain after stroke is not restricted to angiogenesis but also involves vasculogenesis. Our findings are consistent with the concept that vasculogenesis contributes to neovascularization in the adult muscle and heart.

Interestingly, nonischemic mice with bone marrow transplantation showed incorporation of Tie2-lacZ–expressing cells into the choroid plexus. The choroid plexus is composed of a single layer of epithelial cells surrounding a central core containing capillaries. It is believed that endothelial cells in the choroid plexus do not proliferate. Our observations of presence of Tie2-lacZ–expressing cells in the inner side of the choroid plexus indicate that these cells are endothelial cells on capillaries. Using male bone marrow cells, others have reported the presence of male bone marrow–derived cells in the choroid plexus of female mice. Taken together, these data suggest that circulating endothelial progenitor cells contribute to the microvascular structure of the choroid plexus. Recent studies suggest that active vascular recruitment and endothelial cells provide cues mediating neurogenesis in the brain. Neurogenesis takes place in the subventricular zone and the dentate gyrus throughout of the rodent life. Observation of presence of circulating endothelial progenitor cells in the choroid plexus suggest that these cells may support neurogenesis in the subventricular zone and the dentate gyrus by secreting growth factors associated with neurogenesis, such as vascular endothelial growth factor or brain-derived neurotrophic factor.

Cells derived from bone marrow can migrate into the brains of adult mice and differentiate into astrocytes, microglia, and neurons, indicating that bone marrow–derived progenitor cells are reservoirs of normal tissues. An absence of neuronal and glial markers for Tie2-lacZ–expressing cells in the brain of any transplant recipients suggests that a subclass of bone marrow–derived progenitor cells may contribute to differentiation of endothelial cells. In fact, circulating endothelial progenitor cells have been isolated. Exogenous angioblasts might be used to augment compromised vascularization in ischemic brain. Our observations of incorporation of circulating endothelial progenitor cells into sites of neovascularization suggest that recruitment of circulating endothelial progenitor cells induced by focal cerebral ischemia may be regulated by cytokines, soluble receptors, and adhesive molecules released from the ischemic lesion. Maximizing the contribution of circulating endothelial progenitor cells to new vessel formation by exogenous angioblasts or by augmentation of endogenous endothelial progenitor cells may provide another avenue for treatment of stroke.

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