Bone Marrow as a Source of Endothelial Cells and NeuN-Expressing Cells After Stroke

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Background and Purpose—After an ischemic event, bone marrow–derived cells may be involved in reparative processes. There is increasing evidence that bone marrow–derived stem cells may be a source of endothelial cells and organ-specific cells. Our objectives were to determine whether bone marrow–derived cells were a source of endothelial cells and neurons after cerebral ischemia.

Methods—We transplanted bone marrow from male C57 BL/6-TgN (ACTbEGFP)1Osb mice, which express green fluorescent protein (GFP), into female C57 BL/6J mice. The recipient mice then underwent suture occlusion of the middle cerebral artery (MCA), and bone marrow–derived cells were tracked by GFP epifluorescence and Y chromosome probe.

Results—Within 3 days and at 7 and 14 days after MCA occlusion, bone marrow–derived cells incorporated into the vasculature in the ischemic zone and expressed an endothelial cell phenotype. Few bone marrow–derived cells incorporated into the vasculature 24 hours after MCA occlusion. Some bone marrow–derived cells also expressed the neuronal marker NeuN at 7 and 14 days after ischemia.

Conclusions—Postnatal vasculogenesis occurs in the brain in the setting of a cerebral infarction. Bone marrow–derived cells are a source of endothelial cells and NeuN-expressing cells after cerebral infarction. This plasticity may be exploited in the future to enhance recovery after stroke. (Stroke. 2002;33:1362-1368.)

Key Words: bone marrow ■ cerebral ischemia ■ endothelial ■ regeneration

After a cerebral infarction, there is an acute inflammatory response with entry of neutrophils, macrophages, and other blood elements into the ischemic zone. A large body of evidence suggests that this inflammatory response is harmful and contributes to tissue injury.1,2 However, inflammation may be a double-edged sword, and inflammatory cells and blood elements may also be involved in reparative and restorative processes.3 The precise role of inflammatory cells in regeneration and in tissue remodeling remains to be defined.

After a cerebral infarction, there is neovascularization with growth of new vessels in the area of ischemia.3-5 The classic paradigm for neovascularization after ischemia is “angiogenesis,” a process by which new vessels and new endothelial cells are derived from sprouting from preexisting differentiated endothelial cells.6 However, recent studies in the peripheral and coronary circulation demonstrate that some of these new vessels and “new” endothelial cells in the setting of ischemia are derived from circulating endothelial progenitor cells (EPCs) of bone marrow origin, a process known as “postnatal vasculogenesis.”7 Vasculogenesis is the paradigm for development of the primordial vascular network in the embryo. It is not known whether this postnatal vasculogenesis occurs in the setting of a cerebral infarction.

Bone marrow–derived stem cells may also differentiate into other cell types after cerebral ischemia. Bone marrow–derived precursor cells differentiate into microglia and astrocytes in normal brain.8 More surprisingly, bone marrow–derived cells differentiate into neurons in the early postnatal brain and in the olfactory lobe in adult brain.9,10 When bone marrow–derived cells are transplanted into rat brain, they have the ability to migrate along known migratory pathways for neurons and astrocytes.11 Moreover, intrastriatal transplantation of bone marrow stromal cells in rats gives rise to cells that express neuronal markers and improve functional outcome in stroke.12

Although there is now evidence that bone marrow–derived cells can differentiate into cells that express neuronal markers in the normal brain, this transdifferentiation has been limited to the early postnatal period,9 to older animals only in the olfactory bulb,10 or to exogenously transplanted bone marrow stromal cells.12 We hypothesize that brain injury, specifically cerebral ischemia, enhances bone marrow plasticity and provides an environment that supports the differentiation of
Bone marrow–derived cells into endothelial cells and neurons. To test this hypothesis, we used a radiation chimera model in which bone marrow from green fluorescent protein (GFP)–expressing male mice was transplanted into recipient female mice and bone marrow–derived cells were tracked after middle cerebral artery (MCA) occlusion.

Materials and Methods

Experimental Design
All procedures followed were within institutional guidelines. Bone marrow from male C57 BL/6-TgN (ACTbEGFP)1Osb mice (Jackson Labs) was transplanted into female C57 BL/6J mice. In this transgenic mouse line, an “enhanced” GFP cDNA under control of a chicken β-actin promoter and cytomegalovirus enhancer makes all tissues, with the exception of erythrocytes and hair, appear green under fluorescent light. Approximately 8 weeks after bone marrow transplantation, the recipient female mice underwent suture occlusion of the left MCA. At various time intervals after suture occlusion, the mice were killed, and brains were examined by histochemical and immunocytochemical techniques to determine the fate of bone marrow–derived cells. Donor marrow cells were “marked” with GFP and the Y chromosome. Mice with bone marrow transplantation but not undergoing suture occlusion of the MCA were examined at similar time points. The overall experimental design is depicted in Figure 1.

Bone Marrow Transplantation
Hematopoietic cell (bone marrow and more differentiated descendant cells) transplantation was accomplished by introduction of cell suspensions intravenously into the retro-orbital sinus of C57 BL/6J mice. Donor stem cells were obtained from the femurs of adult male C57 BL/6-TgN (ACTbEGFP)1Osb mice. Before transplantation, the female recipient mice underwent irradiation with 137Cs with the use of a Nordion Gammacell 40 irradiator. A dose of 9.0 Gy (900 rads) was used to ablate the marrow. Within 24 hours after irradiation, the mice received the bone marrow donation. For introduction of donor cells, animals were restrained by hand, and volumes up to 0.10 mL with up to 1.5×10⁶ cells were introduced into the retro-orbital sinus. Flow cytometry of peripheral blood samples indicates that >75% of the nucleated cells express GFP in this model at 6 weeks after transplantation.

Suture Occlusion MCA Model
Mice were anesthetized with ketamine/xylazine (80 mg/kg+10 mg/kg SC) before surgery. A midline cervical incision was made to expose the left common carotid artery (CCA). The left external carotid artery (ECA) was ligated superior to the bifurcation of the CCA. An additional suture was then tied adjacent to the primary ligation suture, and the ECA was severed between the 2, creating a stump of the ECA. The CCA and internal carotid artery (ICA) were then clamped inferior and superior (respectively) to the bifurcation of the ECA and CCA. A third suture was placed around the stump of the ECA, and a small opening was made in the stump. A 6-0 nylon monofilament suture, with the tip rounded by heating, was introduced into the ICA through the opening in the stump. The suture around the stump was then gently tightened to prevent backflow of blood. The clamp on the ICA was removed, and the nylon suture was advanced until the origin of the MCA was occluded. The suture on the stump was tightened again, and the clamp was removed from the CCA. The incision was then closed with 2-mm suture clips. After 90 minutes of occlusion, the incision was reopened, and the nylon suture was removed to allow reperfusion. Temperature was maintained at 37°C with a recirculating water blanket and was monitored by rectal thermometer.

Tissue Fixation, Embedding, and Sectioning
At the time of death, mice were anesthetized with xylazine (10 mg/kg IM) and ketamine (80 mg/kg IM) and perfused transcardially with saline followed by 3% paraformaldehyde. The brain was removed and sliced into 2-mm coronal sections with the use of a mouse brain matrix. The sections were immersion fixed overnight on a rocker in 3% paraformaldehyde.
The tissue was cleared with 70% ethanol and embedded in Surgiplast formula-R paraffin. The blocks were serially sectioned at 5 or 10 μm, dependent on their intended purpose, and mounted on Superfrost plus slides (Fisher).

**Detection of Bone Marrow–Derived Cells**

GFp epifluorescence was detected directly with the use of a fluorescein isothiocyanate (FITC) or GFP filter set. In some cases its detection was enhanced by immunohistochemical amplification. Anti-GFP antibodies were also used (rabbit anti-GFP, Abcam) with detection of the anti-GFP antibody via anti-rabbit IgG FITC-conjugated secondary antibodies (Jackson Immunoresearch). The sections were hybridized overnight at 37°C. When dual labeling was used, the detection of the anti-GFP antibody via anti-rabbit IgG FITC-conjugated secondary antibodies (Jackson Immunoresearch) was usually achieved by using a peroxidase–avidin–biotinylated paint probe was detected with Cy3-conjugated streptavidin (1:100, Jackson Immunoresearch). Biotinylated probes were detected with FITC-conjugated streptavidin (1:100, Jackson Immunoresearch). The antibodies were detected with a peroxidase–antiperoxidase system (Stemmerber) or avidin–biotin complex horse-radish peroxidase system (Vector Laboratories) appropriate to the primary antibody’s species. The antibodies were detected with FITC-conjugated anti–horse-radish peroxidase (1:200, Jackson Immunoresearch) or, if necessary, amplified with biotinylated tyramide, TSA (1:200, NEN SAT-700). Biotinylated probes were detected with FITC-conjugated streptavidin (1:100, Jackson Immunoresearch). Nuclei were counterstained with bis-benzimide (10 mg/mL stock) 1:12 000 in 1× PBS by immersing the sections for 8 minutes, then washing for 2 minutes 3 times in 1× PBS (pH 7.4 to 7.5). Sections were coverslipped with Vectashield (Vector Laboratories) mounting medium. Sections were viewed with a Zeiss Axioplan 2 microscope, and images were captured with a Spot II CCD digital camera.

**Immunohistochemistry**

To identify the cell type derived from the donor bone marrow, double-labeling studies were performed with the use of primary antibodies to astrocytes (rabbit anti–glial fibrillary acidic protein [GFAP], Dako), neurons (NeuN, Chemicon), endothelial cells (rabbit anti–von Willebrand factor, DAKO; CD31, Santa Cruz; fluorescein Griffonia [Bandeiraea] simplicifolia lectin I, isocetin B4, Vector Laboratories), white blood cells (anti-CD45, Pharmingen), and various fluorescent-conjugated secondary antibody detection systems.

**Confocal Imaging**

Confocal imaging was performed with a Zeiss 510 laser scanning confocal microscope with the use of Zeiss software. The objective used was a Zeiss 63x C-Apochromat 1.2 NA. To excite the FITC fluorochrome (green), a 488-nm laser line generated by an argon laser was used, and for the Cy3 fluorochrome (red), a 543-nm laser line from a HeNe laser was used. Filter sets used were a bandpass 500- to 550-nm filter (“green” channel) and a long-pass 585- to 650-nm filter (“red” channel). To confirm whether there were bone marrow–derived neurons in the ischemic brain regions, we identified cells dual labeled for Y chromosome and the neuronal marker NeuN in female mice that had undergone earlier transplantation of bone marrow from male GFP transgenic mice. These cells were then examined by confocal microscopy, and 1-μm step Z series were obtained. Both probes label in the nucleus but with distinctly different patterns. Therefore, dual-label cells will demonstrate the Y chromosome as a spot encased in the surrounding NeuN-positive nucleus. Control slides of male GFP transgenic mouse brain as well as control slides of stroked regions of transplanted animals were used to optimize separate channel parameters for confocal imaging. These included slides in which the dual NeuN/Y chromosome labeling was performed but in which the final Cy3 streptavidin or FITC secondary antibodies were omitted.

**Y Chromosome Detection**

Paraffin-embedded sections were first deparaffinized and hydrated to 70% EtOH, then placed into 0.1% Triton-PBS. The slides were then immersed in 75°C 2× SSC for 10 minutes, rinsed in H2O, serially dehydrated in RT 70%, 85%, 95%, and 100% EtOH, and air dried. The biotinylated Y chromosome paint probe (catalog No. BMP037, Applied Genetics) (in hybridization buffer: 50% formamide/2× SSC/0.1% Tween-20) was heated to 75°C for 10 minutes, then placed in a 37°C water bath for 2 to 3 hours to preanneal. The sections were microwaved in 0.01 mol/L sodium citrate for 10 minutes at a gentle boil, then transferred to ice-cold 70%, 85%, and 100% EtOH and air dried. The slides were warmed to 42°C, then 3 μL of probe in hybridization buffer was applied, and the sections were covered with glass coverslips and sealed with rubber cement. The sections were hybridized overnight at 37°C in a humidified chamber. The next day the slides were placed in 70°C solution of 2× SSC for 10 minutes with gentle agitation, then in 70°C 0.4× SSC with 0.3% Tween-20 for 10 minutes. The slides were immediately transferred to RT 2× SSC with 0.1% Tween-20 for 10 minutes. The biotinylated paint probe was detected with Cy3-conjugated streptavidin (1:1000 in 1× PBS) incubated at 37°C for 30 minutes in a humidified chamber. For dual labeling, the primary antibody (eg, NeuN, anti-CD31; see below) was applied along with the streptavidin. The sections were washed for 2 minutes 3 times in 1× PBS (pH 7.4 to 7.5). When dual labeling was used, the detection of the primary antibody was usually achieved by using a peroxidase–antiperoxidase system (Stemmerber) or avidin–biotin complex horse-radish peroxidase system (Vector Laboratories) appropriate to the primary antibody’s species. The antibodies were detected with FITC-conjugated secondary antibodies (Jackson Immunoresearch) and, if necessary, amplified with biotinylated tyramide, TSA (1:200, NEN SAT-700). Biotinylated probes were detected with FITC-conjugated streptavidin (1:100, Jackson Immunoresearch). Nuclei were counterstained with bis-benzimide (10 mg/mL stock) in 1× PBS by immersing the sections for 8 minutes, then washing for 2 minutes 3 times in 1× PBS (pH 7.4 to 7.5). Sections were coverslipped with Vectashield (Vector Laboratories) mounting medium. Sections were viewed with a Zeiss Axioplan 2 microscope, and images were captured with a Spot II CCD digital camera.

**Figure 3.** A, B, and C, Mice 3, 7, and 14 days after MCA occlusion, respectively. Small vessels from the ischemic side of the brain (basal ganglia) are shown. Column 1 demonstrates GFP epifluorescent (green) cells with the use of an FITC filter set. Bleed-through of the Cy3 (orange/red)-labeled immunohistochemically detected von Willebrand antigen is observable. Column 2 shows the same fields as column 1 with the use of a DAPI filter set and reveals all cell nuclei counterstained with bis-benzimide (blue). Column 3 shows the same fields with the use of a FITC/Cy3/DAPI triple-filter set. The von Willebrand antigen is red, and bis-benzimide–stained nuclei are blue. The primarily nuclear GFP epifluorescence is largely obscured by the brighter bis-benzimide label. Arrows mark GFP-positive, bone marrow–derived cells identified as endothelial cells on the basis of nuclear morphology and von Willebrand colabeling. Bar=20 μm.
Results

Forty mice were transplanted with GFP marrow, and all survived the transplantation. Three mice with infarcts (mice undergoing suture occlusion with infarcts in MCA territory by hematoxylin and eosin staining) were studied at 24 hours and 3, 7, and 14 days after suture occlusion. Fourteen mice died before the time of euthanasia. At 3, 7, and 14 days after occlusion, there were abundant GFP-labeled cells lining the microvasculature of the ischemic zone (Figure 2). At 1 day after occlusion there were few GFP-expressing cells, and these were associated with the vasculature. Only a few scattered GFP-labeled cells were evident outside the ischemic zone or in the noninfarcted hemispheres at all time points. These rare GFP-expressing cells were associated with the vasculature. In mice that did not undergo suture occlusion, only rare GFP-labeled cells were found lining the vasculature. At 3, 7, and 14 days after MCA occlusion in the zone of infarction, many of the GFP-positive cells colocalized with known markers of endothelial cells, ie, von Willebrand factor (Figures 3 and 4), CD31 (Figure 5), and IB4 lectin (not shown), indicating that they were an endothelial phenotype.

A semiquantitative assessment was made of the contribution of bone marrow–derived cells to the endothelium of blood vessels in the region of ischemia. Coronal sections through stroke regions were probed with an antibody to von Willebrand factor. Blood vessels in which GFP epifluorescence was clearly associated with endothelial cells, on the basis of nuclear morphology and colocalization of von Willebrand immunoreactivity, were examined at different time points after MCA occlusion. At least 6 different small blood vessels were counted in 3 animals from each time point. The number of GFP-positive endothelial cells was compared with the total number of endothelial cells in the vessel. At 3 days, 42% of the endothelial cells in the vessels were bone marrow derived; at 7 days, 31% of endothelial cells were bone marrow derived; at 14 days, 26% of endothelial cells were bone marrow derived. The percentage of new bone marrow–derived endothelial cells in these vessels ranged considerably from vessel to vessel. While percentages from the different post–MCA occlusion time points appear to decline with longer survival times, these are not significant differences. The percentage of GFP-positive endothelial cells in vessels from individual animals ranged from 16% to 61%, with an overall average of 34%. Of the 3 animals allowed to survive to 1 day after MCA occlusion, only 3 blood vessels showed single GFP-positive endothelial cells, and few other GFP-positive cells were attached to the lumen or outside of the vessel.

We found that antigen retrieval methods for NeuN staining interfered with methods for detecting GFP epifluorescence. Therefore, we resorted to labeling the Y chromosome to detect bone marrow–derived cells that expressed a neuronal phenotype. At 7 and 14 days after infarction, there were scattered Y chromosome–positive cells expressing NeuN in the ischemic zone (Figure 6). These double-labeled cells were found in the striatum and were rare, with 2 to 4 cells counted per section. Confocal microscopy confirmed colocalization of the Y chromosome probe and NeuN (Figure 7).

No cells were detected that colocalized GFP and GFAP, and no cells clearly colocalized the Y chromosome and...
Discussion

Our studies demonstrate that bone marrow–derived cells differentiate into cerebral endothelial cells after a cerebral infarction. The relatively large number of bone marrow–derived cells lining the vasculature suggests they make a significant contribution to the neovascularization that occurs after a cerebral infarction. However, we did not perform rigorous quantification, and further studies are in progress to more precisely quantify this contribution. These GFP-expressing bone marrow–derived cells had the morphological characteristics of endothelial cells and coexpressed von Willebrand factor, CD31, and IB4 lectin, confirming their identification as endothelial cells. There were only rare bone marrow–derived cells evident in vessels in normal or noninfarcted brain, suggesting that under normal physiological conditions, bone marrow–derived cells rarely differentiate into endothelium or that turnover of endothelial cells is very limited. The rarity of bone marrow–derived endothelial cells at 1 day after MCA occlusion suggests that circulating EPCs have not had sufficient time to be recruited to the damaged vessels. However, by 3 days after MCA occlusion, significant numbers of bone marrow–derived cells were participating in vasculogenic repair of the blood vessels. Our findings demonstrate that the neovascularization that occurs in the setting of brain ischemia does not depend only on sprouting from preexisting vessels but also involves a contribution from bone marrow–derived progenitor cells.

Postnatal vasculogenesis contributes to the neovascularization that occurs during wound healing and ischemia in the peripheral and coronary circulations. Bone marrow transplantation studies in mice indicate that bone marrow–derived

Figure 6. Neurons are identified by anti-NeuN labeling (green). Cells originating from bone marrow are demonstrated by Y chromosome probe labeling (red dots). Putative bone marrow–derived neurons are demonstrated by dual labeling (arrowheads). A, B, and D are from mice 14 days after MCA occlusion; C is from a mouse 7 days after MCA occlusion.

Figure 7. Bone marrow–derived neurons are confirmed by confocal microscopy. Rows A, B, and C are of different NeuN-positive neurons (green). Each row is from 1 plane of a Z series passing through the center of the labeled Y chromosome (red). Column 1 is from the “green” channel, column 2 is from the “red” channel, and column 3 is an overlay of the 2 channels. The neuron in row A is from a mouse 14 days after MCA occlusion, and the neurons in rows B and C are from a mouse 7 days after MCA occlusion. In each case (A, B, and C), the Y chromosome probe is colocalized to the NeuN-labeled nucleus.
cells incorporate into new vessels formed in tumors, wounds, and ischemic myocardium. 13 Endothelial cells of hematopoietic origin constitute 8% to 11% of the endothelial cells in new vessels developing in sponge-induced granulation tissue in mice. 14 In bone marrow transplant studies using donor cells highly enriched for hematopoietic stem cells derived from transgenic mouse that express lac Z, donor cells engrafted into endothelium in ischemic myocardium, primarily in small vessels adjacent to the infarct. 15

Bone marrow–derived stem cells may “home” to damaged tissue. In a model of hepatic injury, regenerated hepatic cells were shown to be of bone marrow origin. 16 In a model of muscular dystrophy in mdx mice, bone marrow–derived stem cells preferentially accumulate in dystrophic muscle. 17,18 Chen and colleagues 19 reported that intravenous administration of marrow stromal cells results in accumulation in the ischemic rat brain. A small percentage of these cells (2%) expressed neuronal markers. These findings suggest that the “injured” brain might specifically attract bone marrow–derived cells or that bone marrow–derived cells might “home” to the injured brain.

Regeneration of the brain after cerebral ischemia requires not only the generation of new blood vessels but also the generation of neurons and glial cells. Neural stem cells are concentrated along the subventricular zone, and their migration to the zone of infarction is limited by a long migratory pathway. However, bone marrow cells, being distributed throughout all tissues by the bloodstream, have easy access to the zone of infarction. Our studies indicate that some bone marrow–derived cells express the neuronal marker NeuN. These cells are rare, and it is doubtful that they make a significant contribution to neurological recovery. However, the mere presence of this phenomenon suggests that there may be ways to enhance this transdifferentiation. In in vitro studies, marrow stromal cells differentiate into neurons. 20,21 Moreover, intrastratial and intravenous delivery of marrow stromal cells results in a small percentage of these cells expressing neuronal markers in ischemic brain. 12,20 These findings may not be surprising given evidence that establishment of neural fate may occur by “default.” 22 In our studies it is not clear whether these NeuN-expressing cells function as neurons or integrate into a neural network. Further studies are ongoing to determine the functional characteristics of these NeuN-expressing bone marrow–derived cells.

These studies did not permit us to define the specific bone marrow–derived populations that are involved in neovascularization or in neuronal regeneration. It is highly likely that these may be separate populations of cells. Bone marrow contains at least 2 populations of progenitor cells: hematopoietic and marrow stromal cells. 23,24 Marrow stromal cells have a wide differentiation potential and differentiate into cells that express neuronal markers both in vitro and in vivo. 23 On the other hand, hematopoietic stem cells expressing CD34 are the putative progenitor cells for endothelium. 25 EPCs share antigenic determinants with hematopoietic stem cells and are positive for CD34, Flk-1, and Tie-2. 25

These findings may have clinical import and suggest new therapeutic strategies to enhance regeneration and recovery after stroke. Neovascularization of the peripheral and coro-

nary vasculature can be enhanced by the exogenous delivery of bone marrow–derived EPCs. 26,27 EPCs administered systemically into animals with hind limb ischemia incorporated into neovascularized tissue in ischemic muscles. 26 Administration of human EPCs induced vasculogenesis and angiogenesis and lessened ischemic injury in a rat coronary occlusion model. 27 EPCs are mobilized in response to ischemia and exogenously by cytokine therapy with granulocyte-macrophage colony-stimulating factor (GM-CSF). 26 Cytokines such as GM-CSF or vascular endothelial growth factor (VEGF), by mobilizing EPCs, may enhance postnatal vasculogenesis and angiogenesis. Delayed administration (>48 hours) of VEGF has been shown to enhance angiogenesis and promote functional recovery in murine stroke. 28 It has been observed that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) mobilize EPCs from the marrow, promote their differentiation into endothelial cells, and reduce endothelial apoptosis. 29,30 This suggests a potential role for statins to enhance neovascularization of the brain after an ischemic insult.

Neovascularization and neurogenesis may be tightly linked in the brain. In the adult hippocampus, newly dividing neurons are found in dense clusters associated with the vasculature, suggesting that neurogenesis occurs in an angiogenic niche. 31

The finding that bone marrow–derived progenitor cells can differentiate into endothelial cells and NeuN-expressing cells suggests that strategies to mobilize bone marrow–derived stem cells or enhance their plasticity may be effective treatments to enhance recovery from stroke. Further studies are needed to more accurately quantify this vasculogenesis and “neurogenesis” and examine methods to enhance it. These findings may open up novel therapeutic strategies in the treatment of stroke.

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References


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