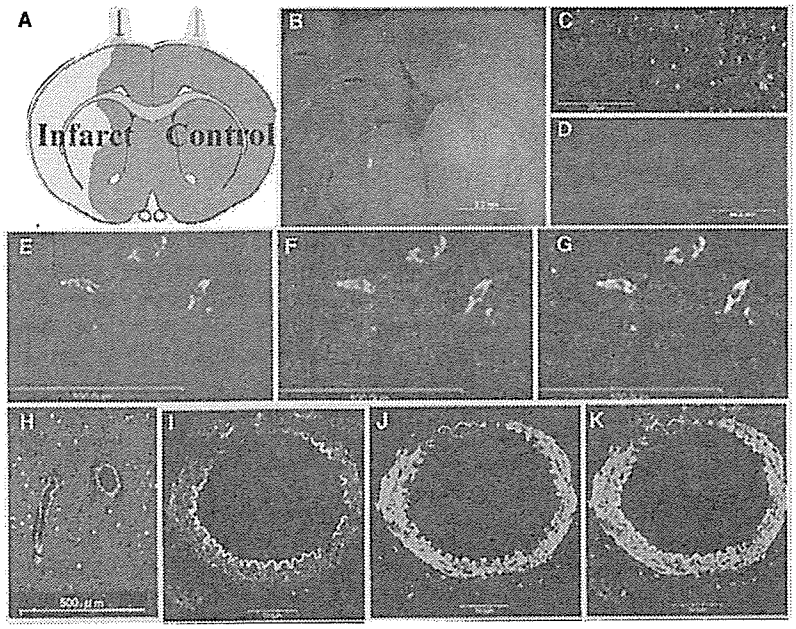

FIGURE 3. *Distribution of administrated EPCs. rCoBF was measured at 2 mm lateral and 1 mm caudal to the bregma. A, immunohistochemical evaluation was performed in the symmetrical regions of the lateral cortex, including the infarct and noninfarct regions, at 24 hours. B, EPCs expressing GFP were mainly distributed in the ischemic hemisphere. Although few cells expressing GFP were observed in the contralateral hemisphere (B, lower magnification; D, higher magnification), many of them were widely observed in the brain parenchyma of the ischemic side (B, lower magnification; C, higher magnification) with expression of vWF at 24 hours (F and G, merged). H, EPCs were also distributed in the inner layers of many arteries in the ischemic lesion. They were located medial to the smooth muscle layer (I), which was demonstrated by staining with α-smooth muscle actin (J and K are merged).* Scale bars = *2 mm* (B), *200 μm* (C and D), *100 μm* (E–G), *500 μm* (H), *50 μm* (I—K).

rectal temperature was kept between 36.5 and 37.0°C. Its change was expressed as the ratio to the preoperative value.

Infiltration of Myeloperoxidase-immunoreactive Cells

Rats were perfused with PBS (-) and 4% PBPA under deep anesthesia at 24 hours. A 10-μm thick frozen coronal section from the center of the ischemic lesion at the level of the anterior commissure was stained with polyclonal rabbit anti-human myeloperoxidase (MPO) antibody (DAKO) to count the number of inflammatory neutrophils. The density of MPO-immunoreactive cells throughout the whole infarct region was expressed by the number counted at ×400 magnification per infarct area (cells/mm^2).

Statistical Analysis

Data were expressed as mean and standard deviations for the given number of animals and statistically evaluated using the unpaired Student's *t* test. Differences were accepted as being significant at a *P* value less than 0.05.

RESULTS

Molecular Characteristics of BM-derived EPCs

BM-derived mononuclear cells showed a cobblestone appearance similar to ECs (*Fig. 1A*) with expression of vWF (*Fig. 1B*). Immunoblotting revealed expression of Tie-2, Flk-1, and vWF, which are specifically expressed in ECs. However, they did not express ICAM-1 (*Fig. 1C*). They endocyted 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acLDL (*Fig. 1D*) and were bound to fluorescein isothiocyanate-labeled isolectin B4, which is a murine-specific surface marker of ECs (*Fig. 1, E and F*) (19).

Infarct Volume

Ninety-minute occlusion of MCA induced a large cerebral infarct, including the whole caudoputamen and part of the lateral cortex. The infarct volume of the PBS group (*Fig. 1H*) and the EPC group (*Fig. 1I*) was 41.3 ± 10.5% (n = 9) and 28.7 ± 13.8% (n = 8), respectively, at 24 hours. The volume of each group at 48 hours was 51.4 ± 12.2% (n = 9) and 35.1 ± 16.7% (n = 8), respectively. The infarct volumes both at 24 and 48 hours were significantly lower in the EPC group than in the control ($P < 0.05$) (*Fig. 2A*). The infarct volume at 24 hours of the A10 transplanted and delayed EPC groups was 52.2 ± 11.6% (n = 9) and 46.5 ± 13.3% (n = 3), respectively. Only the early EPC group showed a significant reduction of infarct volume ($P < 0.05$) (*Fig. 3B*).

Neurological Deficits

Motor function scores of the PBS and EPC groups were 4.57 ± 2.84 (n = 7) and 7.91 ± 2.45 rpm (n = 9), respectively, at 24 hours and 2.61 ± 1.15 (n = 8) and 6.79 ± 3.46 rpm (n = 8), respectively, at 48 hours. The score of the EPC group was significantly higher than that of the control at both 24 and 48 hours ($P < 0.05$ at 24 hr, $P < 0.01$ at 48 hr) (*Fig. 3C*). The motor function scores of the A10 and delayed EPC groups at 24 hours were 4.0 ± 1.91 (n = 9) and 6.6 ± 0.36 rpm (n = 3), respectively. Delayed EPC group showed significant improvement ($P < 0.05$) (*Fig. 3D*).

rCoBF

The value of rCoBF of the control (n = 5) was decreased to 54.2 ± 10.2% of the preoperative value at 24 hours and remained 50.6 ± 13.7% at 48 hours. The value of rCoBF of the EPC group (n = 5) was decreased to 68.9 ± 15.3% at 24 hours, but recovered to 84.4 ± 12.0% at 48 hours. The value of rCoBF in the EPC group at 48 hours was significantly higher than that of the control ($P < 0.05$) (*Fig. 3E*).

Distribution of Administrated EPCs

Distribution of EPCs was estimated in the similar lateral cortex (*Fig. 3A*). Although few cells expressing GFP were observed in the contralateral hemisphere (*Fig. 3, B* and *D*), many were observed widely in the ischemic brain parenchyma (*Fig. 3, B* and *C*) with expression of vWF at 24 hours (*Fig. 3, E–G*). EPCs seemed to replace the endothelial layer entirely in some arteries (*Fig. 3H*). They were located medial to the smooth muscle layer (*Fig. 3, I–K*). These observations were also confirmed at 48 hours. In the rats in which EPCs were administered without ischemic insult, few cells expressing GFP were observed in the brain (data not shown).

Density of MPO-immunoreactive Cells

Administration of EPCs significantly reduced the infiltration of MPO-immunoreactive cells in the ischemic hemisphere at 24 hours ($P < 0.05$) (*Fig. 4A*). The density of the MPO-immunoreactive cells in the ischemic hemisphere at 24 hours was 37.4 ± 14.0 cells/mm^2 (control, n = 4, *Fig. 4B*) and 15.3 ± 3.95 cells/mm^2 (EPCs, n = 4, *Fig. 4C*). ICAM-1 positive cells were distributed throughout the ischemic hemisphere, but its expression was not colocalized with the transplanted EPCs (*Fig. 4, D–F*).

Expression of eNOS, VEGF, and IGF-1 in Administered EPCs

Administered EPCs expressing GFP were distributed in the brain parenchyma and around the endothelial layer of pial arteries in the ischemic lesions at 24 hours (*Fig. 5, A, D,* and *G*). Most of the cells also expressed eNOS (*Fig. 5, B* and *C*). Expression of VEGF (*Fig. 5, E* and *F*) and IGF-1 (*Fig. 5, H* and *I*) was mainly observed in the cells other than EPCs in the ischemic lesion.

DISCUSSION

EPCs can be isolated from peripheral blood, BM, and umbilical cord blood (3, 5, 19, 26). In this study, we used EPCs obtained from autologous BM by culture with VEGF on fibronectin-coated dishes by removing the nonadherent cells because this method seemed to provide more purified EPCs than culture of freshly isolated CD34-positive cells (26). Molecular characteristics of EPCs isolated and expanded from BM were similar to that of vascular ECs, such as uptake of acLDL, binding to lectin, and expression of vWF, Flk-1, and Tie-2, except for the expression of ICAM-1.

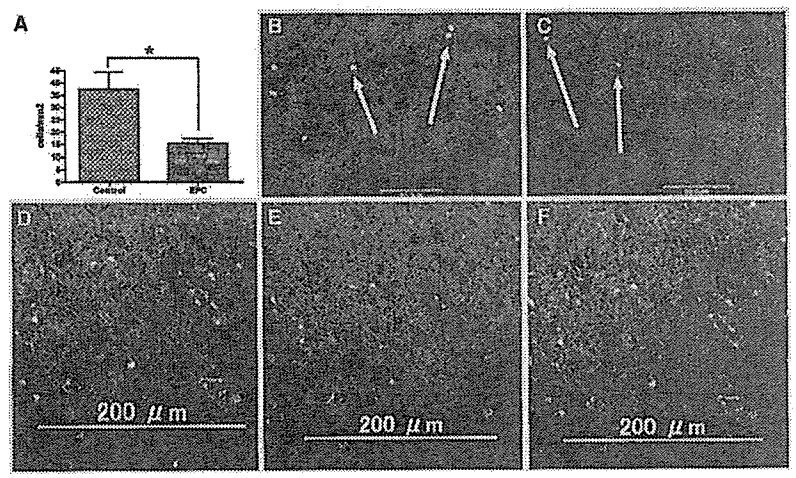

FIGURE 4. *Density of the MPO-immunoreactive cells in the ischemic lesion.* A, *the density of the MPO-immunoreactive cells in the ischemic hemisphere at 24 hours was significantly reduced by administration of EPCs ($P < 0.05$). MPO-immunoreactive cells* (arrows) *in the control* (B) *and EPC group* (C). *ICAM-1 positive cells* (D) *and EPCs* (E) *were distributed throughout the ischemic hemisphere. Their distribution differed* (F, *merged*). Scale bars = *200 μm.*

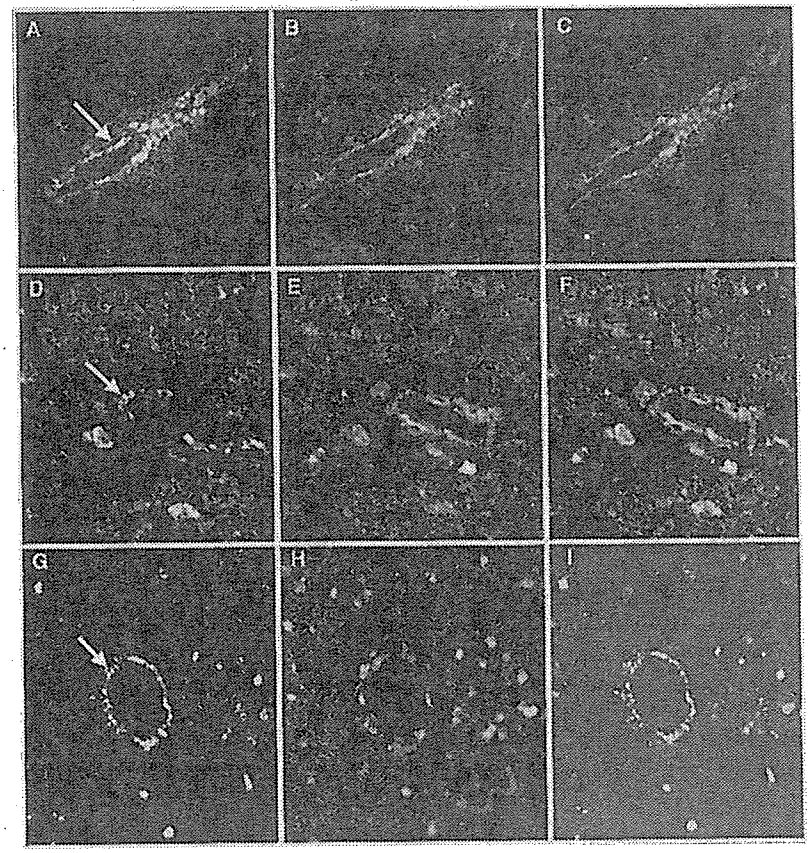

FIGURE 5. *Expression of eNOS, VEGF, and IGF-1 in administered EPCs.* A, D, *and* G, *administered EPCs expressing GFP were distributed in the brain parenchyma and around the endothelial layer of pial arteries in the ischemic lesions at 24 hours. Most of the cells also expressed eNOS* (B *and* C *are merged*). *Expression of VEGF* (E *and* F *are merged*) *and IGF-1* (H *and* I *are merged*) *was observed in the cells other than EPCs in the ischemic lesion.*

Administration of ex vivo-expanded BM-derived EPCs could reduce the cerebral infarct volume and the neurological deficits at 24 and 48 hours after focal cerebral ischemia-reperfusion injury. rCoBF was reduced after ischemia, but significantly recovered by administration of EPCs at 48 hours. Delayed administration also showed partial beneficial effects on the ischemia.

To investigate the mechanism of the beneficial effects of EPCs at 24 hours, we focused on the cellular interactions between EPCs and inflammatory neutrophils. Endothelial dysfunction has been reported to be significantly involved in neural injury after focal cerebral ischemia-reperfusion injury (6), and MPO-immunoreactive cells are regarded as a general marker of acute endothelial damage. Infiltration of MPO-positive cells is initiated within a few hours after insult because of the interaction with ECs of enhanced expression of ICAM-1 (6, 21, 25). Administration of EPCs actually suppressed the infiltration of MPO-immunoreactive cells in the infarct region. Although EPCs were assembled widely in the parenchyma of the ischemic hemisphere, and especially in the inner layer of several pial arteries at 24 hours, distribution of EPCs was different from that of ICAM-1 positive cells. We think that lack of expression of ICAM-1 on the surface of the EPCs might be related to the inhibitory effects.

rCoBF in the ischemic penumbra was reduced at 24 hours after ischemia, but significantly recovered by administration of EPCs at 48 hours. Attenuation of infarct volume and neurological deficit at 48 hours might be related to the recovery of rCoBF.

To investigate the molecular mechanisms in attenuation of acute ischemic injury by administration of EPCs, we carried out an immunohistochemical study of expression of eNOS, VEGF, and IGF-1 in the EPCs. EPCs were reported to produce several vasodilatory or growth factors such as nitric oxide, VEGF, and IGF-1 (15). Most of the EPCs distributed in the ischemic lesion expressed eNOS, but few expressed VEGF and IGF-1. These observations suggest that nitric oxide produced by EPCs might be related to the attenuation of endothelial dysfunction or ischemic injury (9, 16, 36).

Zhang et al. (37) reported focal brain ischemia mobilized EPCs from BM to ischemic lesions at 30 days after insult. They were differentiated into ECs in the ischemic region participating in the neovascularization with recovery of cerebral blood flow. Our study revealed that exogenously administered EPCs assembled widely in the parenchyma of the ischemic hemisphere and in the inner layer of several pial arteries with endothelial differentiation, even in the acute ischemic stage. Endothelial differentiation in the ischemic region might occur earlier in the exogenous EPCs than endogenous ones from the BM. This is clinically important in that exogenously administrated EPCs might have therapeutic potential for acute brain ischemia.

Although Taguchi et al. (29) reported beneficial effects of CD34-positive cells derived from human umbilical cord on mouse focal brain ischemia, their method needs immunosuppression for clinical application. Iihoshi et al. (17) reported beneficial effects of administration of stromal cells from autologous BM for focal ischemia, but the target of cells or molecules in their method remains somewhat ambiguous because stromal cells have multipotency. Our method also has some limitations to be solved for clinical application. Delayed administration of EPCs had partial effects on ischemia in our study. In addition, our method takes several days for differentiation of the cells before administration. However, relief of endothelial dysfunction by administration of expanded autologous EPCs will be another possible therapeutic option in the treatment of acute brain ischemia.

CONCLUSION

In summary, administration of ex vivo-expanded autologous BM-derived EPCs diminished the early neural injury after focal cerebral ischemia-reperfusion injury. This might be caused, in part, by attenuation of acute inflammatory endothelial dysfunction.

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COMMENTS

The authors demonstrate neuroprotection and improved functional outcome in the acute phase of reperfused stroke after an intra-arterial injection of ex vivo expanded bone marrow endothelial progenitor cells. They propose that this neuroprotection derives from an attenuation of endothelial dysfunction, reflected by a reduction in the density of myeloperoxidase-positive cells in the area of the infarction and an increase in regional blood flow, assessed by laser Doppler flowmetry from 24 to 48 hours in the treated animals. Although previous work has demonstrated that endothelial progenitor cells (EPCs) ameliorate functional outcome in the chronic phase of stroke, this is the first study to assess functional benefit in the acute phase. As a further advantage of this experimental paradigm, the authors derive these cells from autologous bone marrow, avoiding the need for potentially confounding immunosuppression.

Few studies have explored the role of endothelial precursor cells in cerebral ischemia/reperfusion, and this is the first to investigate the neuroprotective effects in the acute phase of such therapy. It is likely that the protection offered by these cells derives from additional protective mechanisms (such as the production of growth factors) that merit further investigation. These effects, as well as the potential of these cells to induce neovascularization, provide the opportunity for neurorestoration at later time points than examined in this article (1). Significantly less cumbersome efforts to attenuate endothelial dysfunction in the acute phase of stroke have been previously investigated (adhesion molecule blockade) and have been shown to ultimately fail in the clinical arena.

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1. Zhang ZG, Zhang L, Jiang Q, Chopp M: Bone marrow-derived endothelial progenitor cells participate in cerebral neovascularization after focal cerebral ischemia in the adult mouse. Circ Res 90:284–288, 2002.

The authors tested the effects of bone marrow-derived EPCs on a rat stroke model. When given immediately after vessel occlusion, behavioral deficits and infarct volumes were reduced. They theorize that these effects were through reduction in endothelial dysfunction, perhaps mediated through nitric oxide. It was interesting to note that the majority of cells were found in the infarct region rather than in other brain locations, a finding noted by others after cell delivery via vascular injection. There are numerous limitations in a study such as this, and these are appropriately addressed in the report. The work is novel and should be pursued further.

Cell delivery two hours after vessel occlusion had reduced effects. Obviously, the temporal nature of any effects needs to be clarified. In the clinical setting, a window of several hours would be imperative. It is interesting to note how few patients actually receive TPA for their stroke, given the time limits on its utility.

Much of the prior work on cellular repair after rodent cerebral infarction has focused on neuronal implantation or striatal tissue grafts. Through an evaluation of endothelial cell effects, this group has chosen a different path that may prove to be an important new avenue for research. Given the number of studies showing measurable behavioral improvement using cellular repair concepts, neurosurgeons should be excited about the future of both basic and clinical research in this area.

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In this article, the authors present experimental evidence that intra-arterial administration of autologous ex vivo-expanded marrow-derived EPCs is beneficial after focal cerebral ischemi rat model. They demonstrate that application of this treatmen egy reduces the volume of infarct and neurological deficits as determined by standard testing in the experimental animals compared with controls after transient occlusion of the middle cerebral artery. In addition, the number of inflammatory cells in the ischemic lesion was also reduced in the treatment group at 24 hours, along with an increase in regional cortical blood flow at 48 hours. As the authors point out, while the beneficial effects of EPC administration may be due, in part, to the attenuation of endothelial dysfunction, other previously identified effects of cellular transplantation may also contribute to the beneficial effect. The use of autologous EPCs is interesting in that it may obviate the need for immunosuppression. It is disappointing that the group receiving the delayed treatment 2 hours after reperfusion did not benefit as much, with obvious implications for ultimate clinical relevance. In addition, the strategy of autologous transplantation requires a prolonged delay between graft harvest, preparation, and transplantation, making it perhaps more appropriate for chronic degenerative disorders than acute ischemia. Nevertheless, the results presented here are interesting and identify endothelial dysfunction as a therapeutic target for treatment of transient focal brain ischemia.

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Ohta et al. characterized EPCs from rat bone marrow for intra-arterial administration after stroke. Behavioral changes were noted and rats were sacrificed for assessment of infarct volume in treated and control groups. Modest improvement in Rota rod (Muromachi, Tokyo, Japan) performance was noted in early-treated rats and a diminution of infarct size was observed. However, there was no statistical difference in mortality in the two groups. They demonstrate the luminal location of injected EPCs and speculate on the mechanism of neuroprotection.

A few additions to the study may help address the feasibility of such cell therapy as a viable treatment option. A less direct method of administration could be tried as opposed to carotid injection. Administration of EPCs could be delayed to better simulate timing of administered therapies, and the period of observation after treatment should be extended to enhance the understanding of the longer-term effects on performance scores and mortality. Additionally, it may be interesting to address the effect of EPC administration on the extent of edema after cerebral ischemia in this model, hypothesizing that improving vascular integrity by limiting endothelial cell dysfunction may reduce cerebral edema, which so often complicates the treatment of patients with large infarcts.

To our knowledge, this is the first report of transplanted bone marrow-derived endothelial cell precursors used as a novel treatment attempt after stroke, and the authors are commended for adding to our existing knowledge of the potential for restorative cell therapy after central nervous system injury. An additional requisite next step is an enhanced understanding into the mechanism of action of transplanted EPCs in this model.

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