

Intracerebral Peripheral Blood Stem Cell (CD34⁺) Implantation Induces Neuroplasticity by Enhancing β 1 Integrin-Mediated Angiogenesis in Chronic Stroke Rats

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Although stem cell-based treatments for stroke and other neurodegenerative diseases have advanced rapidly, there are still few clinical treatments available. In this study, rats receiving intracerebral peripheral blood hematopoietic stem cell (CD34⁺) (PBSC) transplantation showed much more improvement in neurological function after chronic cerebral ischemia in comparison with vehicle-treated control rats. Using laser-scanning confocal microscopy, implanted PBSCs were seen to differentiate into glial cells [GFAP⁺ (glial fibrillary acidic protein-positive)], neurons [Nestin⁺, MAP-2⁺ (microtubule-associated protein 2-positive), Neu-N⁺ (neuronal nuclear antigen-positive)], and vascular endothelial cells [vWF⁺ (von Willebrand factor-positive)], thereby enhancing neuroplastic effects in the ischemic brain. Cortical neuronal activity, as evaluated by ¹H-MRS (proton magnetic resonance spectroscopy), also increased considerably in PBSC-treated rats compared with a vehicle-treated control group. In addition, PBSC implantation promoted the formation of new vessels, thereby increasing the local cortical blood flow in the ischemic hemisphere. These observations may be explained by the involvement of stem cell-derived macrophage/microglial cells, and β 1 integrin expression, which might enhance this angiogenic architecture over the ischemic brain. Furthermore, quantitative reverse transcription-PCR analysis showed significantly increased modulation of neurotrophic factor expression in the ischemic hemisphere of the PBSC-transplanted rats compared with vehicle-treated control rats. Thus, intracerebral PBSC transplantation might have potential as a therapeutic strategy for treating cerebrovascular diseases.

Key words: peripheral blood hematopoietic stem cells (PBSCs); CD34⁺; chronic stroke animal model; neuroplasticity; angiogenesis; β 1 integrin

Introduction

Because neuronal death is the major pathogenetic mechanism in neurodegenerative diseases such as cerebrovascular disease, stem cell therapy has been proposed as an effective “regenerative” approach (Lindvall et al., 2004; Taguchi et al., 2004). However, the transplantation of embryonic grafts, as used in previous clinical trials (Boer, 1994; Barinaga, 2000), is hampered by logistical and ethical considerations, and so it is important to find alternative sources of stem cells. Stem cells can be isolated from various tissues (Prockop, 1997; Erices et al., 2000). For example, adult bone marrow contains mesenchymal stem cells (MSCs) or stromal cells, which can potentially differentiate into a variety of cell types such as adipocytes, chondrocytes, and myoblasts (Prockop, 1997; Dennis and Charbord, 2002). Human umbilical cord blood

(hUCB) (Prockop, 1997; Erices et al., 2000) has also been shown to contain large numbers of hematopoietic colony-forming cells (Nakahata and Ogawa, 1982). The mononuclear cells derived from hUCB cells, can be induced to express class III β -tubulin, glial fibrillary acidic protein (GFAP), GalC (an oligodendrocyte marker) (Galvin-Parton, 2003), and microtubule-associated protein 2 (MAP-2) (Ha et al., 2001). However, the complicated culturing conditions for *in vitro* expansion of MSCs, together with the required HLA (human histocompatibility leukocyte antigen) matching for hUCB transplantation between donor and recipient, may limit the clinical application of these cells. Therefore, the use of autologous stem cells without *in vitro* expansion may be an appropriate strategy for clinical applications.

Peripheral blood hematopoietic stem cells (PBSCs) have been increasingly used as a source of hematopoietic stem cells for transplantation. In comparison with bone marrow transplants, intracerebral transplants with PBSCs have been shown to lead to faster hematological recovery with less supportive care requirement (To et al., 1992), although the concentration of PBSCs under steady-state conditions with no cytokine induction is very low (Elfenbein and Sackstein, 2004). In a recent report, granulocyte colony-stimulating factor (G-CSF) was shown to mobilize hematopoietic stem cells (HSCs) into the peripheral blood from

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bone marrow (Demetri and Griffin, 1991), thus amplifying the concentration of PBSCs. Furthermore, PBSCs have already been used in transplantation for the regeneration of nonhematopoietic tissues, such as skeletal muscle, heart (Orlic et al., 2001), and neurons (Sigurdsson et al., 2005).

In this study, we investigate the administration of intracerebral PBSC transplantation as a therapeutic strategy to determine its effect on chronic stroke in rats. In addition, we examine whether these strategies could be of practical use in clinical medicine.

Materials and Methods

Animal brain ischemia/reperfusion model. Adult male Sprague Dawley rats (weighing 250–300 g) were used in this study. Experimental animals were subjected to three-vessel ligation. All surgical procedures were performed using sterile/aseptic techniques in accordance with university institutional guidelines. The rats were anesthetized with chloral hydrate (0.4 g/kg, i.p.). Ligation of the right middle cerebral artery (MCA) and bilateral common carotids [common carotid arteries (CCAs)] were performed by modified methods described previously (Chen et al., 1986). Bilateral CCAs were clamped with nontraumatic arterial clips. Using a surgical microscope, a 2×2 mm craniotomy was drilled where the zygoma fuses to the squamosal bone. The right MCA was ligated with a 10-0 nylon suture. Cortical blood flow was measured continuously with a laser Doppler flowmeter (LDF) (PF-5010; Periflux system; Perimed, Stockholm, Sweden) in anesthetized animals. A burr hole (1 mm diameter) was made in the right frontoparietal region to allow placement of photodetectors. A probe (0.45 mm in diameter) was stereotactically placed in the cortex (1.3 mm posterior, 2.8 mm lateral to the bregma, and 1.0 mm below the dura). After 90 min of ligation, the suture on the MCA and arterial clips on CCAs were removed to allow reperfusion. Core body temperature was monitored with a thermistor probe and maintained at 37°C with a heating pad during anesthesia. After recovery from anesthesia, body temperature was maintained at 37°C with a heat lamp.

Purification and selection of CD34⁺ PBSCs. To purify the PBSCs, rats were injected with G-CSF (50 µg/kg) subcutaneously for 5 d. Immediately, peripheral blood was collected in sterile tubes containing citrate-dextrose solution as an anticoagulant. Mononuclear cells (MNCs) were isolated from peripheral blood using the Ficoll-Histopaque (Sigma, St. Louis, MO) centrifugation method (Asahara et al., 1997). The MNC layer was collected and washed twice with 1 mM EDTA in PBS. CD34⁺ MNCs were separated from 2×10^8 MNCs by a magnetic bead separation method [magnetic-activated cell sorting (MACS)] (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's instructions. In brief, MNCs were suspended in 300 µl of PBS and 5 mM EDTA. These cells were labeled with a hapten-conjugated monoclonal antibody against CD34 (PharMingen, San Diego, CA), followed by an anti-hapten antibody coupled with microbeads, and were incubated with beads at ratios of 100 µl of beads per 10^8 cells for 15 min at 4°C. The bead-positive cells (CD34⁺ MNCs) were enriched on positive-selection columns set in a magnetic field. FACS analysis using anti-CD34 antibodies (PharMingen) labeled with phycoerythrin (Becton Dickinson, Mountain View, CA) of MACS-sorted cells showed that $96 \pm 3\%$ of the selected cells were positive for CD34 (Fig. 1B). Then, cells labeled with 1 µg/ml bisbenzimidazole (Hoechst 33342; Sigma) were cultured in RPMI (Invitrogen, Grand Island, NY) plus 10% FBS (HyClone, Logan, UT) at 37°C in a humidified atmosphere of 5% CO₂/95% air and antibiotics for 1 h, and prepared for transplantation.

Experimental animals treated with PBSC transplantation and vehicle control. The treatment protocol for experimental rats was divided into two groups (Fig. 1A): PBSC transplantation and a control group. Cerebral ischemia was induced in every experimental rat on day 0. Experimental rats in the allograft PBSC transplantation group were injected stereotactically with $\sim 2 \times 10^5$ cells of PBSCs (CD34⁺) labeled with bisbenzimidazole in a 3–5 µl PBS suspension through a 26-gauge Hamilton syringe into three cortical areas, 3.0–5.0 mm below the dura. The approximate coordinates for these sites were 1.0–2.0 mm anterior to the bregma and 3.5–4.0 mm lateral to the midline, 0.5–1.5 mm posterior to

the bregma and 4.0–4.5 mm lateral to the midline, and 3.0–4.0 mm posterior to the bregma and 4.5–5.0 mm lateral to the midline. The needle was retained in place for 5 min after each injection, and a piece of bone wax was applied to the skull defects to prevent leakage of the injected solution. Experimental rats in the vehicle control group were treated with saline stereotactically.

Neurological behavioral measurement. Behavioral assessments were performed 3 d before cerebral ischemia, and 1, 7, 14, 21, and 28 d after treatment. The tests measured (1) body asymmetry, (2) locomotor activity, and (3) grip strength measurement. Pretreatment scores were recorded to normalize those taken after cerebral ischemia. (1) Body asymmetry: The elevated body swing test was used to assess body asymmetry after MCA ligation and evaluated quantitatively, as described previously (Borlongan et al., 1998). Initially, animals were examined for lateral movement, with their bodies suspended by their tails. The frequency of initial head swing contralateral to the ischemic side was counted in 20 continuous tests and normalized, as described previously (Chang et al., 2003). (2) Locomotor activity: Rats were subjected to VersaMax Animal Activity monitoring (Accuscan Instruments, Columbus, OH) for ~ 2 h for behavioral recording of each of the experimental rats. The VersaMax Animal Activity monitor contained 16 horizontal and 8 vertical infrared sensors spaced 87 cm apart. The vertical sensors were situated 10 cm from the floor of the chamber. Locomotor activity was counted as the number of beams broken by a rat's movement in the chamber. Three vertical parameters defined in the manufacturer's menu option were calculated over 2 h at night: vertical activity, vertical time, and number of vertical movements. (3) Grip strength was analyzed using a Grip Strength Meter (TSE Systems, Bad Homburg, Germany) as described previously, with modification (Dunnett et al., 1998). In brief, the grip strength ratio of each forelimb was measured separately and was calculated as the ratio between the mean strength out of 20 pulls of the side contralateral to the ischemia and that of ipsilateral side. In addition, the ratio of grip strength after treatment and before treatment was also calculated, and changes were presented as a percentage of the pretreatment value. Overall, the investigators were blinded when measuring behavioral changes in the PBSC-treated and control group.

Magnetic resonance imaging examinations. To evaluate the infarcted area, experimental animals were imaged 3, 7, 14, and 28 d after treatments in each of the PBSC-treated and control groups. Magnetic resonance imaging (MRI) was obtained for the animals on a 3.0 Tesla whole-body Signa EchoSpeed MR scanner (General Electric, Milwaukee, WI). The animals were anesthetized with chloral hydrate (0.4 g/kg, i.p.), supported on a wooden cradle, and their heads were placed in a home-made birdcage coil with a 5 cm outer diameter. After the acquisition of scout images, six to eight coronal plane images were taken from between 3 mm behind the olfactory bulb and the caudal portion of the cerebellum. Each slice was 2 mm thick without any gaps, with a matrix size of 320×160 and an 8×4 cm field of view. T2-weighted fast-spin echo sequences were optimized to detect the lesion size. The acquisition parameters were as follows: echo time (TE)/repetition time (TR), 105/4000 ms; echo train length, 53; number of excitations, 8. Each image was determined by a consensus of two observers blinded to the PBSC-treated and control group.

Proton magnetic resonance spectroscopy assessment. To assess the therapeutic potential of each of the PBSC-treated and control rats, animals were imaged 3, 7, 14, and 28 d after the treatment. Proton magnetic resonance spectroscopy (¹H-MRS) was performed using the same MRI scanner using a multivoxel technique. T2-weighted transverse, coronal, or sagittal images were used to localize the volume of interest (VOI). The VOI ($0.5 \times 0.5 \times 0.5$ cm³) was precisely localized centrally to the infarcted brain using two or three images (transverse and sagittal/coronal). The spectroscopic acquisition parameters were as follows: TR, 3.0 s; TE, 30 ms; and number of signal, 36 average with PROBE (proton brain exam) (GE Medical Systems, Fairfield, CT). CHESS (chemical shift selective) sequence was used for suppression of the H₂O signal. All raw data were transferred to a Sun Sparc-10 workstation (SUN Computer, Sunnyvale, CA), and processed using SA/GE (spectral analysis/General Electric) software (GE Medical Systems) incorporating low-frequency filtering of residual water signals, apodization by 0.5 Hz of exponential line broad-

ening, zero-filling of 8k, Fourier transformation, and Lorentzian-to-Gaussian transformation according to the scheme described previously (Kreis et al., 1992). Metabolic peaks were fitted by the Lorentzian line shape at known frequencies of *N*-acetylaspartate (NAA) at 2.02 ppm, creatine (Cr) at 3.03 ppm, and choline and choline-containing compounds (Cho) at 3.22 ppm. The values of the [NAA/Cr] and [NAA/Cho] ratios were calculated. The result of the metabolic ratio is presented as mean \pm SE.

Bromodeoxyuridine labeling. Bromodeoxyuridine (BrdU), a thymidine analog that is incorporated into the DNA of dividing cells during S-phase, was used for mitotic labeling (Sigma). The labeling protocol has been described previously (Zhang et al., 2001). The BrdU immunostaining procedure with specific antibody against BrdU (1:400; Sigma) and quantification of BrdU-immunoreactive cells have been described previously (Zhang et al., 2001).

Immunohistochemistry of brain tissue. Experimental rats were anesthetized with chloral hydrate (0.4 g/kg, i.p.), and their brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde, before being removed and embedded in 30% sucrose. A series of adjacent 20- μ m-thick sections were cut from each brain in the coronal plane, stained with hematoxylin and eosin, and observed by light microscopy (E600; Nikon, Tokyo, Japan). For BrdU immunostaining, DNA was first denatured by incubating each section in 50% formamide of 2 \times standard saline citrate at 65°C for 2 h, and then in 2N HCl at 37°C for 30 min, and finally rinsed in 0.1 M boric acid with pH 8.5. Sections were then rinsed with Tris buffer and treated with 1% H₂O₂ to block endogenous peroxidase. The immunostaining procedure was performed using the labeled streptavidin–biotin (LSAB) method (LSAB-2 kit; peroxidase; DakoCytomation, Carpinteria, CA). Brain tissue, on a silane-coated slide, was placed in boiling citrate buffer, pH 6 (ChemMate; DakoCytomation), twice for 5 min in a microwave oven at 750 W, after deparaffinization and rehydration. Tissues were then incubated with the appropriate diluted primary antibodies to BrdU (for nuclear identification; dilution, 1:400; Sigma), at room temperature for 1 h. After washing with TBS-T (Tris-buffered saline containing 0.1% Tween 20), the specimens were sequentially incubated for 10–30 min with biotinylated anti-rabbit and anti-mouse (1:200; R&D Systems, Minneapolis, MN) Igs and peroxidase-labeled streptavidin. Staining was performed after a 10 min incubation with a freshly prepared substrate–chromogen solution, containing 3% 3-amino-9-ethylcarbazole and hydrogen peroxide. Finally, the slides were lightly counterstained with hematoxylin, washed with water, and mounted. Negative control sections were stained with identical preparations of brain tissue specimen, except that primary antibodies were omitted. Quantification of BrdU-immunoreactive cells was counted digitally using a 60 \times objective lens (LSM510; Carl Zeiss, Oberkochen, Germany) via a computer imaging analysis system (Imaging Research, St. Catharines, Ontario, Canada).

Immunofluorescent colocalization analysis by laser-scanning confocal microscopy. To identify cell type-specific markers colocalized with exogenous transplanted PBSCs (bisbenzimidazole-labeled) and endogenous homing stem cells (BrdU immunoreactive), immunofluorescent colocalization analysis by laser-scanning confocal microscopy was performed. To identify cell type-specific markers colocalized with endogenous homing of stem cells (BrdU-labeled, FITC-conjugated; 1:400; Boehringer Mannheim, Mannheim, Germany) and exogenous transplanted mobilized peripheral blood hematopoietic stem cells (MPBSCs) (bisbenzimidazole-labeled; blue fluorescence), immunofluorescent colocalization analysis by laser-scanning confocal microscopy was performed. In immunofluorescent colocalization studies, each coronal section was treated with cell-specific antibodies (Table 1) as follows: GFAP (for astrocyte; 1:400; Sigma), von Willebrand factor (vWF) (for endothelial cell; 1:400; Sigma), neuronal nuclear antigen (Neu-N) (for neuronal nuclei; 1:200; Chemicon, Temecula, CA), Nestin (for neuronal dendrites) (1:200; Chemicon), and MAP-2 (for neuronal dendrites; 1:200; Boehringer Mannheim), with Cy3 (1:500; Jackson ImmunoResearch, West Grove, PA) staining.

Evaluation of PBSC transplantation-induced angiogenesis and vascular remodeling by macrophage/microglial cells. Twenty-eight days after treatment with PBSCs in cerebral ischemic rats, cerebral microcirculation was

Table 1. Antibodies used in the immunohistochemical analysis

Antibody	Dilution	Source
CD34	1:300	PharMingen
BrdU	1:400	Sigma
GFAP	1:400	Sigma
vWF	1:400	Sigma
Neu-N	1:200	Chemicon
Nestin	1:200	Chemicon
MAP-2	1:200	Boehringer Mannheim
CD31	1:100	PharMingen
OX-42	1:400	Serotec
ED-1	1:500	Serotec
β 1 integrin	1:300	Chemicon

analyzed by administering the fluorescent plasma marker (FITC-dextran; Sigma) intravenously to rats and observing them with fluorescent microscopy (Axiovert 200M; Carl Zeiss), as described previously (Morris et al., 1999). In addition, to quantify the cerebral blood vessel density and examine the vascular remodeling by macrophages, experimental rats were anesthetized with chloral hydrate and perfused with 4% paraformaldehyde. Histological sections (6 μ m) were stained with specific antibody to CD-31 (1:100; PharMingen), OX-42 (1:400; Serotec, Indianapolis, IN), and ED-1 (1:500; Serotec), and conjugated with Cy3 (1:500; Jackson ImmunoResearch). The number of blood vessels present was determined as described previously (Taguchi et al., 2004). The total number of positive macrophage/microglial (MA/MI) cells per section was counted as described previously (Pipp et al., 2003).

Western blotting of β 1 integrin expression. To check the expression of β 1 integrin, brain tissue samples (ischemic cortex and striatum) were determined by specific antibody against β 1 integrin (1:300; Chemicon) using Western blotting analysis. Moreover, β 1 integrin activation was blocked by the use of synthetic cyclic RGD peptide (1 μ g/ml; Peptides International, Louisville, KY), which had been added to the PBSCs, and then injected into the ischemic brain. Finally, neurological behavioral measurement and blood vessel density (CD31 immunoreactivity) as described above was examined to evaluate the outcome of these three treatment protocols including PBSCs with synthetic cyclic RGD peptide and control.

Measurement of reactive cerebral blood flow. Experimental rats were positioned in a stereotaxic frame and baseline local cortical blood flow (bCBF) was measured continuously with a LDF (LDF monitor; Moore Instruments, Devon, UK) in an anesthetized state (chloral hydrate) as described previously (Tuettenberg et al., 2001). The reactive cerebral blood flow (rCBF) was examined after intraperitoneal injection of 50 mg/kg acetazolamide (Diamox; Lederle, Pearl River, NY) and defined as percentage change of bCBF.

Quantitative reverse transcription-PCR of growth factor synthesis in vivo. Experimental rats were anesthetized with chloral hydrate (0.4 g/kg, i.p.) 3, 7, 14, and 28 d after initiation of the three treatments. Ischemic cortical and striatal areas were evacuated on ice immediately. Subsequently, brain tissue samples were homogenized by a plastic homogenizer, and total RNA was isolated using the RNeasy (Qiagen, Hilden, Germany). The relative amount of target mRNA was determined by quantitative reverse transcription-PCR (QRT-PCR) using SYBR Green following the manufacturer's instructions (Roche Diagnostics, Alameda, CA), and specific primers were used as summarized in Table 2. The relative expression levels of target mRNA were normalized against a control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The whole procedure of QRT-PCR was the same using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) as described previously with modification (Luo et al., 2004). The conventional RT-PCR was also performed as described previously (Shyu et al., 2004a).

Statistical analysis. All measurements in this study were performed blindly. Results were expressed as mean \pm SEM. The behavioral scores have been evaluated for normality. Student's *t* tests were used to evaluate mean differences between the control and the treated groups.

Table 2. Sequence of PCR primers for neurotrophic factors

Factors	Sequence	PCR fragment (bp)
SDF-1	Sense: TTGCCAGCACAAAGACATCC	243
	Antisense: CTCCTAAGCAACCGAATACAG	
BDNF	Sense: CAGTGGACATGTCGGTGGGACGGTC	533
	Antisense: TTCTTGGCAACGGCAACAAACCACAAC	
GDNF	Sense: CCACACCGTTTAGCGGAATGC	638
	Antisense: CGGGACTCTAAGATGAAGTTATGGG	
NGF	Sense: GTTTTGGCCAGTGGTCGTGCAG	498
	Antisense: CCGCTTGCTCTGTGAGTCCTG	
TGF- β	Sense: CCGCTCTCCCCATGCCGCC	710
	Antisense: CGGGGCGGGGCTTCAGCTGC	
FGF-II	Sense: TCACCTCGCTTCCCGCACTG	252
	Antisense: GCCGTCATCTCTTCATATA	
VEGF	Sense: GCTCTCTGGGTGCACTGGA	431
	Antisense: CACCGCCTTGCTGTGCACA	

Results

Intracerebral PBSC transplantation improves neurological behavior after cerebral ischemia

To evaluate neurological function after cerebral ischemia, a body asymmetry trial was used to assess body swing before and after MCA ligation in PBSC transplantation rats ($n = 10$) and control rats ($n = 10$). The behavioral measurement scores were all normalized by the baseline scores. Because cerebral ischemia causes imbalanced motor activity, all of the experimental rats developed significant body asymmetry, turning contralateral to the side of the ischemic brain on day 1 after cerebral ischemia. PBSCs were separated from peripheral blood of rats after subcutaneous G-CSF injection. The CD34⁺ PBSCs were isolated by a magnetic bead separation method (MACS). The purity of isolated CD34⁺ PBSCs was found to be >96%, as established by fluorescence-activated cell sorter (FACS) analysis (Fig. 1*B*). From 14 to 28 d after treatment, rats treated with intracerebral PBSC transplantation, exhibited significantly reduced body asymmetry in comparison with control rats ($p < 0.05$) (Fig. 1*C*). Locomotor activity was examined before and after cerebral ischemia in all animals. Vertical activity, vertical movement time, and the number of vertical movements significantly increased between 14 and 28 d after cerebral ischemia in rats receiving PBSC transplantation in comparison with control populations ($p < 0.05$) (Fig. 1*D–F*). Furthermore, measurement of grip strength was performed to examine the forelimb strength for all experimental rats before treatment and 28 d after each of the two treatments. The final results revealed that a higher ratio of grip strength was found in the PBSC group (1.63 ± 0.37) in comparison with the control group (0.61 ± 0.35) ($p < 0.05$) (data not shown).

Neuronal activity increases in the intracerebral PBSC transplantation group after cerebral ischemia

Representative anatomical pictures (Fig. 2*A,B*) and fluorescent histologies of rat brains (Fig. 2*C,D*) are shown for PBSC-treated and control rats 28 d after cerebral ischemia. MRI was also used to examine the infarcted area of these two groups (Fig. 2*E,F*). To verify the local neuronal metabolism enhanced by the stem cell engraftment, ¹H-MRS was used to assess the neuronal activity of the ischemic rats after each of the two treatments. In normal rats without ischemia, the cerebral cortex as viewed by ¹H-MRS (without ischemia), displayed three important signals: Cho, Cr, and NAA (Lu et al., 1997). The ¹H-MRS of the infarcted brains (using a multivoxel technique) (Fig. 2*G*) showed a sharp decrease in NAA signaling, together with a mild decrease in Cho and Cr

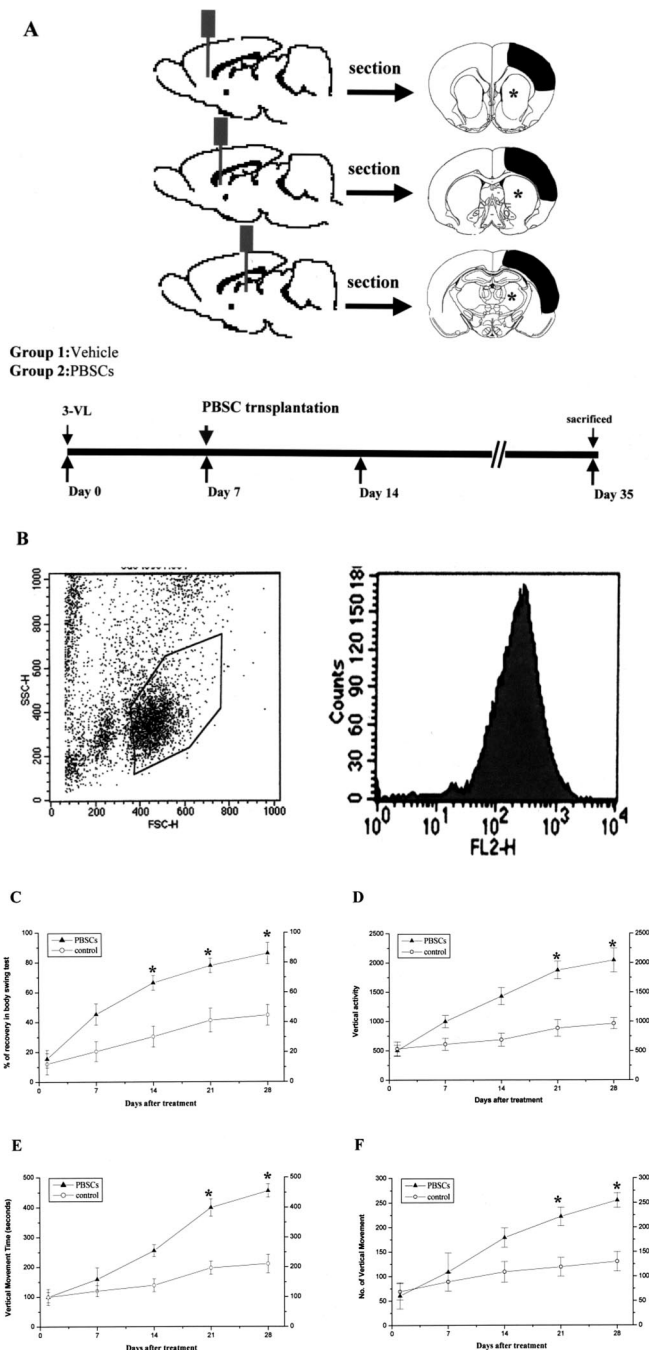


Figure 1. *A*, Schematic representation for the investigation of chronic cerebral ischemia with three therapeutic protocols. Cerebral ischemia was induced by three-vessel ligation (3-VL) in each experimental rat on day 0. In group 1 (vehicle control), rats received saline starting 7 d after cerebral ischemia was induced and were killed on day 28. In group 2 (PBSC transplantation), rats received intracerebral PBSC transplantation on day 7 and were killed on day 28. *B*, Representative FACS analysis of CD34⁺ PBSCs isolated from G-CSF-induced cell mobilization using magnetic immunobeads. More than 96% of the sorted cells expressed the CD34⁺ surface antigen. Thus, PBSC transplantation improves neurological behavior after cerebral ischemia. *C*, A body asymmetry trial was used to assess body swing before and after MCA ligation. Between 14 and 28 d after cerebral ischemia, rats intracerebrally treated with PBSCs exhibited significantly reduced body asymmetry in comparison with controls. *D–F*, Locomotor activity was examined for all experimental rats. Vertical activity, vertical movement time, and the number of vertical movements significantly increased between 14 and 28 d after cerebral ischemia in rats receiving PBSCs in comparison with vehicle control rats. Data are expressed as mean \pm SEM. * $p < 0.05$ versus control.

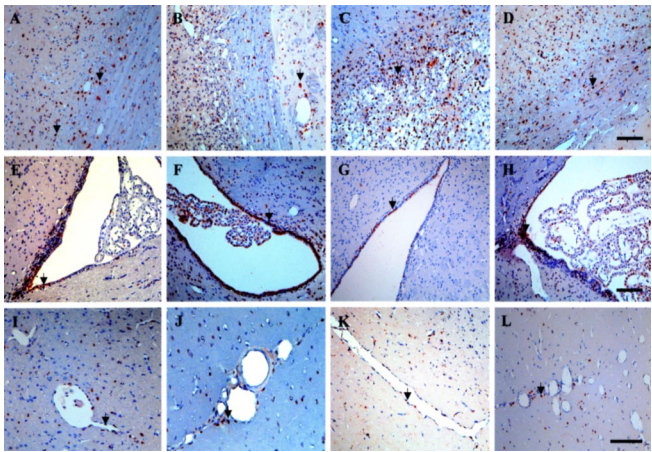
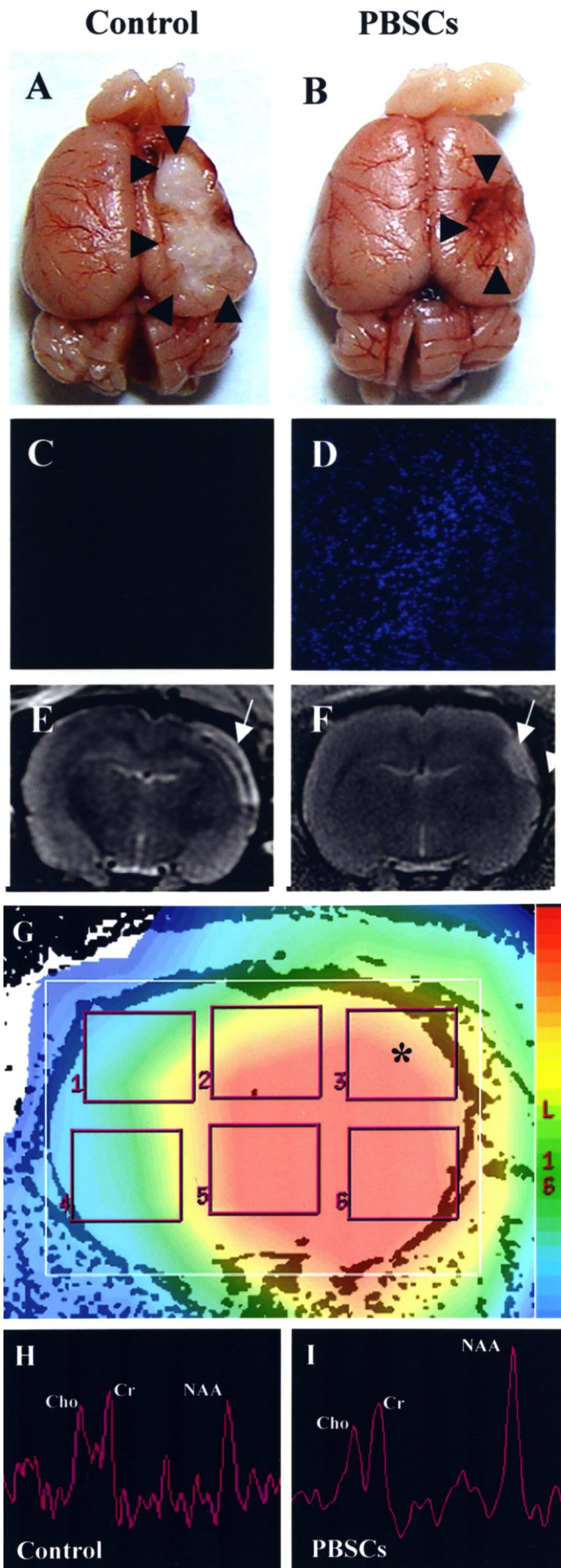


Figure 3. Immunohistochemical staining for BrdU. In rats treated with PBSCs and vehicle control after cerebral ischemia, a few BrdU-immunoreactive cells were detected in the ipsilateral cortex close to the following areas: the infarct boundary (**A**, **B**; shown by the arrow), the striatum (**C**, **D**; shown by the arrow), and the subventricular area (**E**–**H**; shown by the arrow). BrdU-immunoreactive cells were also found around blood vessels in the penumbic region of the ipsilateral cortex (**I**–**L**; shown by the arrow). Data are expressed as mean \pm SEM. * $p < 0.05$ versus control. Scale bars, 50 μ m.

signals (Fig. 2*H,I*). Four weeks after the two treatments, significant improvements ($p < 0.05$) in neuronal activity were observed under ^1H -MRS, specifically in regard to NAA/Cho and NAA/Cr (2.77 ± 0.13 and 2.95 ± 0.13 , respectively) ($n = 6$) of the PBSC-treated group, and to NAA/Cho and NAA/Cr (1.69 ± 0.13 and 1.88 ± 0.17 , respectively) ($n = 6$) of the control group.

Intracerebral PBSC transplantation stimulates endogenous stem cell mobilization, homing, and engraftment into the brain after cerebral ischemia

To determine whether intracerebral PBSC transplantation could enhance central (brain parenchymal) and peripheral stem cells homed and engrafted into the brain after ischemia, BrdU labeling was used to follow the growth of these homing stem cells in the brain. BrdU-immunoreactive cells were detected mainly in the penumbra area, striatum, and subventricular area of the lateral ventricle in PBSC transplantation rats. Cumulative labeling of BrdU revealed a few BrdU-immunoreactive cells in the ipsilateral hemisphere near the penumbra area (Fig. 3*A,B*), the striatal region (Fig. 3*C,D*), and the subventricular region (Fig. 3*E–H*) in the PBSC-treated group. BrdU-immunoreactive cells were also found around the lumen of varying calibers of blood vessels (and also in the vessel walls of endothelial cells) (Fig. 3*I–L*) in the penumbic region, suggesting that mobilized stem cells may participate in angiogenesis. In BrdU pulse-labeling experiments, the number of BrdU-immunoreactive cells in the penumbic region of ischemic cortex and striatum significantly increased in the

Figure 2. Intracerebral PBSC treatment increases neuronal activity, as demonstrated by ^1H -MRS. **A–D**, Gross anatomical pictures of infarcted areas (shown by the black arrowhead) (**A**, **B**) and fluorescent histology of rat brains (**C**, **D**) are shown for the PBSC-treated rats, and vehicle control-treated rats 28 d after cerebral ischemia. **E**, **F**, MRI also indicates the infarcted areas (shown by the white arrow) of the PBSC-treated rats and the control-treated rats. **G**, ^1H -MRS was used to assess the neuronal activity of infarcted brain areas using the multivoxel technique (*). **H**, **I**, Four weeks after cell transplantation or vehicle treatment, significant improvement in neuronal activity under ^1H -MRS was observed in the PBSC-treated group compared with the control group. Data are expressed as mean \pm SEM. * $p < 0.05$ versus control.

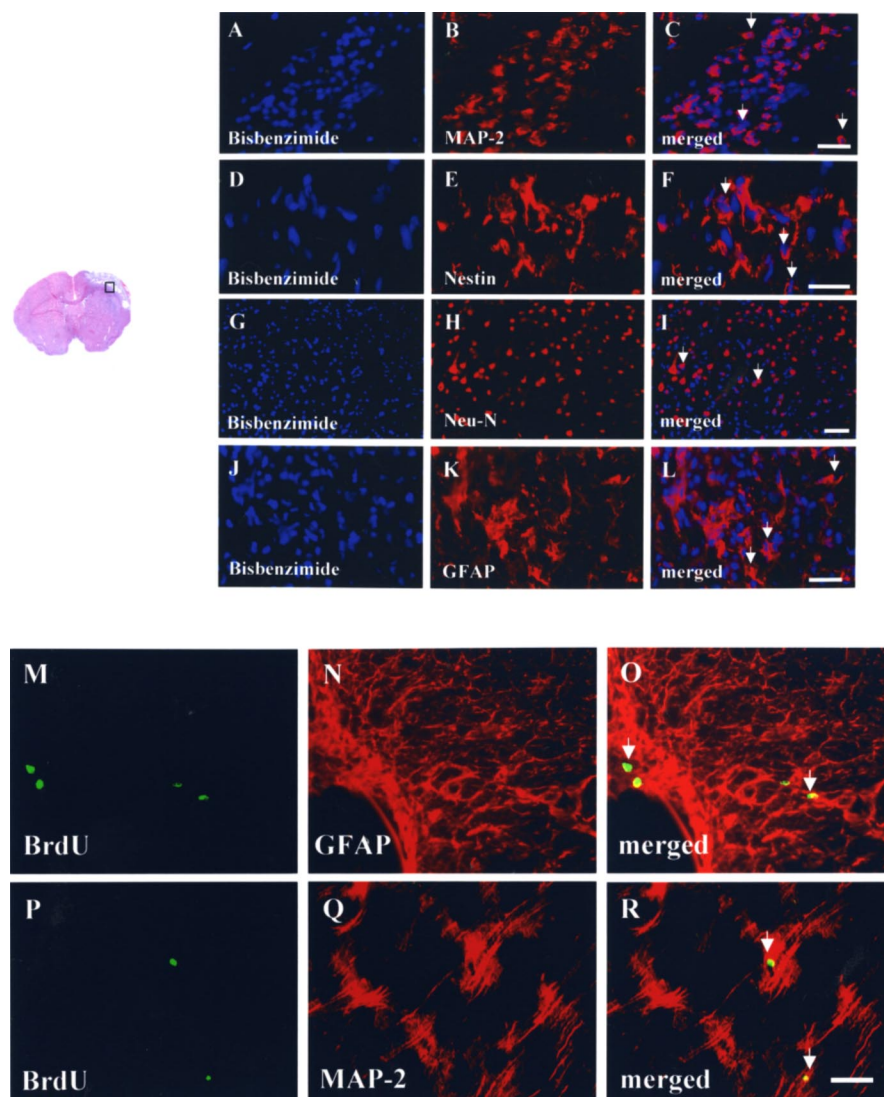


Figure 4. Neuroplastic effects after intracerebral transplantation of PBSCs after cerebral ischemia. **A–C**, In the representative penumbra area (□), the results show many bisbenzimidate-labeled cells (blue for cell nuclei) colocalized with the cellular marker MAP-2 (red for cytoplasm; shown by the white arrow). **D–F**, Several bisbenzimidate-labeled cells also colocalized with the cellular marker Nestin (red for cytoplasm; shown by the white arrow). **G–I**, Some bisbenzimidate-labeled cells also colocalized with the cellular marker Neu-N (red for nuclei; shown by the white arrow). **J–L**, Some bisbenzimidate-labeled cells colocalized with the cellular marker GFAP (red for cytoplasm; shown by the white arrow). **M–R**, In a double immunofluorescent study of endogenous homing HSCs, many BrdU-immunoreactive cells (green for nuclei) colocalized with the cellular markers of GFAP and MAP-2 (red for cytoplasm; shown by the white arrow). Scale bars, 50 μm.

PBSC-treated rats ($n = 8$) compared with saline control rats ($n = 8$) ($p < 0.05$) (data not shown).

Intracerebral PBSC transplantation enhances neurogenesis *in vivo*

To determine whether exogenous transplanted PBSCs and endogenous homing stem cells could differentiate into neurons, glial cells, or endothelial cells in ischemic brains of the experimental rats, immunofluorescent colocalization studies using a laser-scanning confocal microscope were performed. In a colocalized study of exogenous transplanted PBSCs, the results showed that some bisbenzimidate-labeled cells (blue; cell nuclei fluoresce spontaneously) colocalized with antibodies for MAP-2, Nestin, Neu-N, and GFAP (red; neural cell type-specific markers) (Fig. 4A–L) in the penumbra of PBSC-treated ischemic rat brains. The percentages of bisbenzimidate-labeled cells colocaliz-

ing with the specific markers MAP-2, Nestin, Neu-N, and GFAP were $\approx 3\%$ (6000 cells), $\approx 3\%$ (6000 cells), $\approx 2\%$ (4000 cells), and $\approx 5\%$ (10,000 cells), respectively. Furthermore, in the colocalized study of the endogenous (homing) stem cells, the results showed some BrdU⁺ cells (green for cell nucleus identification) colocalized with markers for MAP-2, GFAP (red for cell type-specific markers) (Fig. 4M–R). These findings indicate that PBSC transplantation might enhance central and peripheral stem cell homing and differentiation, thereby generating new neural tissue for the repair of injured areas of the brain after ischemic injury.

Intracerebral PBSC transplantation induces angiogenesis *in vivo*

To determine whether PBSCs could induce angiogenesis (through endogenous homing HSCs and exogenous transplanted PBSCs differentiated into vascular-endothelial cells at ischemic sites), double immunofluorescent staining, FITC-dextran perfusion studies, and blood vessel density assays were performed on brain slices from PBSC-treated and vehicle control-treated rats. The results indicated that several exogenous transplanted PBSCs (bisbenzimidate-labeled) and endogenous homing HSCs (BrdU-immunoreactive) showed vascular phenotypes (vWF cells) around the perivascular and endothelial regions (Fig. 5A–F) in the penumbra region of ischemic hemispheres of PBSC-treated rats. Visual inspection indicated that treatment with PBSCs ($n = 6$) significantly enhanced cerebral microvascular perfusion with FITC-dextran in comparison with control rats ($n = 6$) ($p < 0.05$) (Fig. 5G,H). Quantitative measurement of blood vessel density examined by immunostaining of CD31 (Fig. 5I,J) showed that ischemic rats treated with PBSCs ($n = 4$) showed significantly more neovascularity in the

penumbra area than control rats ($n = 4$) ($p < 0.05$) (data not shown).

Enhancement of angiogenesis via involvement of stem cell-derived macrophage/microglial cells, and $\beta 1$ integrin expression

To demonstrate the association between angiogenesis and macrophage cells, double immunofluorescent staining and FITC-dextran perfusion studies of the brain specimens of the PBSC transplantation and control groups were examined. The results indicate that several exogenous transplanted PBSCs (bisbenzimidate-labeled) showed MA/Mi phenotypes (OX-42/ED-1 cells) infiltrated around the perivascular regions (FITC-dextran-perfused vessels) (Fig. 5K) of the ischemic hemispheres of PBSC-treated rats. Significantly increased numbers of MA/Mi cells could be observed around the vessels in the PBSC-treated

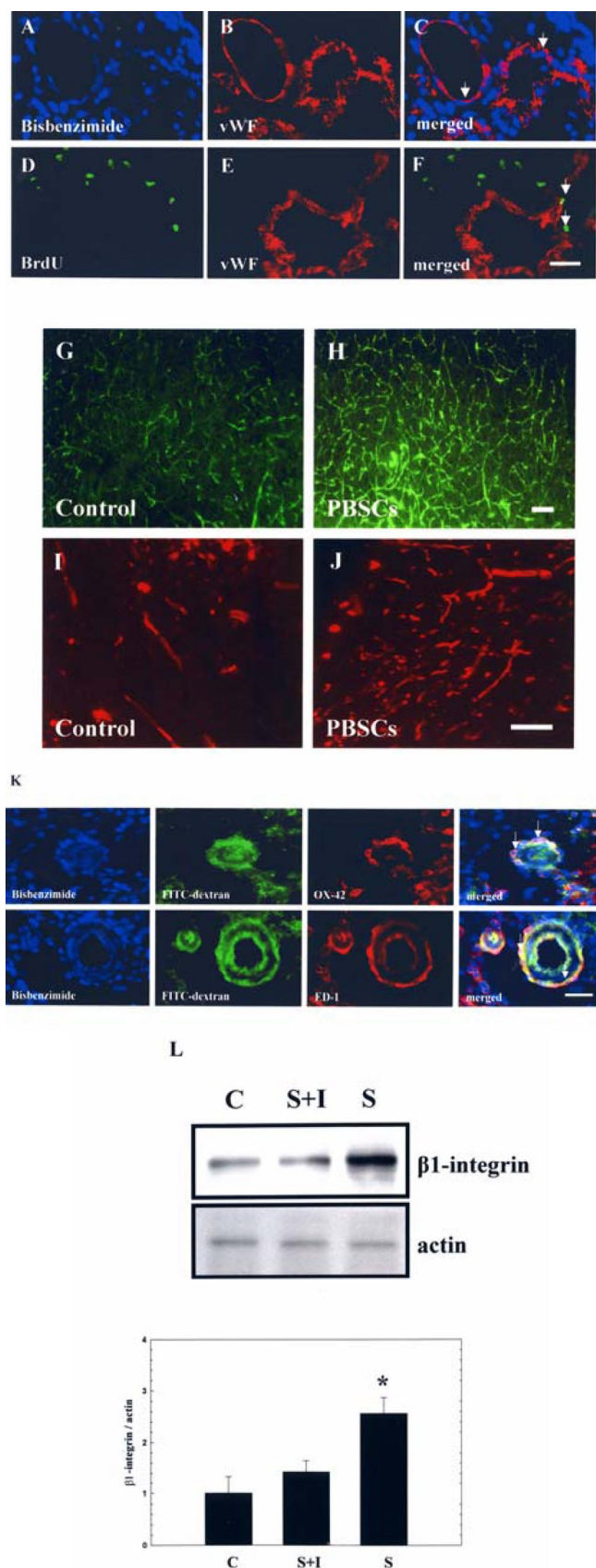


Figure 5. Intracerebral PBSC treatment enhanced the angiogenesis in the ipsilateral hemisphere. **A–F**, In double immunofluorescent studies, some bisbenzimidazole-labeled (exogenous) and BrdU⁺ cells (endogenous) showing vascular phenotypes (vWF⁺ cells) (shown by the white arrow) were found around the perivascular and endothelial regions of the ischemic hemi-

rats compared with control-treated rats ($p < 0.05$) (data not shown). In addition, protein expression of $\beta 1$ integrin showed a significant increase in the PBSC-treated rats ($n = 4$) compared with control rats ($n = 4$) ($p < 0.05$) (Fig. 5L). However, after adding the synthetic cyclic RGD peptide, the expression of $\beta 1$ integrin, the neurological behavior measurement (Fig. 6A), and blood vessel density (CD31 immunoreactive cells) (Fig. 6B) showed no significant difference between these three therapeutic groups ($n = 4$, each group).

Intracerebral PBSC transplantation facilitates rCBF in the ischemic brain

Increased vessel density would enhance neuronal survival, especially associated with an increased cerebral blood flow, which would result in efficient delivery of oxygen and nutrients. Therefore, to examine rCBF in the ischemic brain, experimental rats were injected with Diamox and monitored by LDF under anesthesia after cerebral ischemia. The results showed a significant increase in rCBF in the middle cerebral artery cortex of the ischemic brain in the PBSC-treated rats ($n = 6$) compared with control rats ($n = 6$) ($p < 0.05$) (Fig. 6C).

Intracerebral PBSC transplantation modulates neurotrophic factor expression in the ischemic hemisphere

To study the improvement of neurological function after cerebral ischemia in PBSC-treated rats, we examined the expression of mRNA species coding for trophic factors known to neuroprotect the ischemic cortical area. This was done at four time points (3, 7, 14, and 28 d) after PBSC transplantation. We evaluated neurotrophic factors, including stromal cell-derived factor 1 (SDF-1), BDNF, glial cell line-derived neurotrophic factor (GDNF), NGF, TGF- β , FGF-II, and vascular endothelial growth factor (VEGF) in the PBSC group ($n = 5$) and the control group ($n = 5$). In conventional RT-PCR, the results revealed significantly increased expression of SDF-1, VEGF, and BDNF in ischemic rats treated with PBSCs in comparison with the vehicle control group (Fig. 7A). In QRT-PCR analysis, the ratio of expression level of SDF-1, VEGF, and BDNF to GAPDH peaked at about a twofold increase in the PBSC-treated group, in comparison with the vehicle control group ($p < 0.05$) (Fig. 7B).

Discussion

In this study, rats receiving intracerebral PBSC transplantation showed significantly improved neurological function after chronic cerebral ischemia in comparison with vehicle-treated control rats. In the PBSC group, exogenous transplanted PBSCs and endogenous homing stem cells were seen to migrate toward the cerebral infarcted zone and differentiate into glial cells (GFAP⁺), neurons (Nestin⁺, MAP-2⁺, and Neu-N⁺) and vascular endothelial cells (vWF⁺), thereby enhancing neuroplasticity in the ischemic brain. In addition, cortical neuronal activity

spheres of PBSC-treated rats. **G, H**, To assess penumbra cerebral microvascular perfusion, FITC-dextran was administered intravenously to evaluate the microvascular pattern of PBSC-treated and control rats. **I, J**, Calculating the cerebral blood vessel density stained by CD31 immunoreactivity. **K**, In colocalized immunofluorescent studies, some bisbenzimidazole-labeled cells showing macrophage/microglial phenotypes (OX-42⁺/ED-1 cells) (shown by the white arrow) were found around the perivascular (FITC-dextran-perfused vessels) regions of the ischemic hemispheres of PBSC-treated rats. **L**, Results of Western blotting analysis of expression of $\beta 1$ integrin with or without adding synthetic cyclic RGD peptide in different treatment groups: PBSCs (S), control (C), and PBSCs adding RGD (S+I). Data are expressed as mean \pm SEM. * $p < 0.05$ versus control. Scale bars, 50 μ m.

as evaluated by ^1H -MRS increased considerably in the PBSC transplantation group of rats in comparison with the control group. The PBSC transplantation group promoted the formation of new vessels, to increase the local cortical blood flow in the ischemic hemisphere. Cellular and molecular mechanisms of vascular remodeling in local neovascularization of the ischemic brain have been demonstrated by perivascular infiltration of stem cell-derived macrophage/microglial cells and increased expression of $\beta 1$ integrin. Finally, the PBSC transplantation group also exhibited a significant increase in modulation of neurotrophic factor expression in the ischemic hemisphere.

In our study, transplanted PBSCs and endogenous homing HSCs included major populations of $\text{CD}34^+$ cells, which have been proven to enhance neovascularization in the penumbra region of the ischemic brain and subsequently promote prominent neurogenesis (Taguchi et al., 2004). A relationship between angiogenesis and neurogenesis induced by $\text{CD}34^+$ cells would be consistent with the regeneration of parenchymal cells after injury seen in other organs (Toda et al., 1999; Ross et al., 2001). Thus, the major mechanism underlying this observation of coexistence of neurogenesis and angiogenesis might include enhanced production of trophic factors such as BDNF (Leventhal et al., 1999; Borlongan et al., 2004) and PDGF (Johe et al., 1996) by $\text{CD}34^+$ cell-derived neovasculatures, and directly result in differentiation of these $\text{CD}34^+$ stem cells into neuron/glia cells (Hao et al., 2003). Furthermore, $\text{CD}34^+$ cell-derived neovasculatures contributing to maintain newly formed neuron/glia cells have been shown to integrate into networks in adult animals (Louissaint et al., 2002), and to provide an environment conducive to neurogenesis (Valable et al., 2003). In conclusion, we believe that the combination of endogenous homing stem cell targeting plus the allograft exogenous PBSC transplantation reported here represents an attractive treatment for cerebrovascular diseases.

As demonstrated previously, new blood vessel growth could be exclusively dependent on the growth of endothelial cells (Schaper and Ito, 1996). However, the exact mechanism that leads to angiogenesis or collateral circulation of arteries after stem cell transplantation is not yet known. A recent study indicated that not only endothelial cells but also MA/MI cells may be essential (Heil et al., 2002). In this study, immunofluorescent colocalization analysis in FITC-dextran-perfused blood vessels was used to count the number of recruited MA/MI cells, demonstrating that the activated MA/MI cells are involved in remodeling of new vascular growth. We found that MA/MI cell numbers are significantly increased over the penumbral area in chronic stroke rats treated with the PBSCs compared with control-treated rats. From double immunofluorescent analysis, stem cell-derived MA/MI cells (bisbenzimidazole-labeled) were seen to adhere to the perivascular region of the ischemic brain in PBSC-treated rats. In addition, increased expression of $\beta 1$ integrin and improvement in neurological behavior were both blocked by the addition of synthetic RGD peptide ($\beta 1$ integrin inhibitor). Finally, in this study, angiogenic growth factors such as SDF-1 and VEGF were significantly upregulated in PBSC-treated rats compared with control-treated rats. Therefore, angiogenic growth factor and some adhesion molecules produced by an interaction between implanted stem cells and host brain parenchymal cells might initiate MA/MI adherence to sites of occluded vessels (Buschmann et al., 2003; Pipp et al., 2003; Shastri and Tyagi, 2004; Li et al., 2005; Tucker et al., 2005). Subsequently, MA/MI began to release some proteinases to guide vascular remodeling in the ischemic brain (Fujiiyama et al., 2003; Milner and Campbell, 2003; Pipp et al., 2003). In the PBSC-treated group, profound angiogenesis would have to

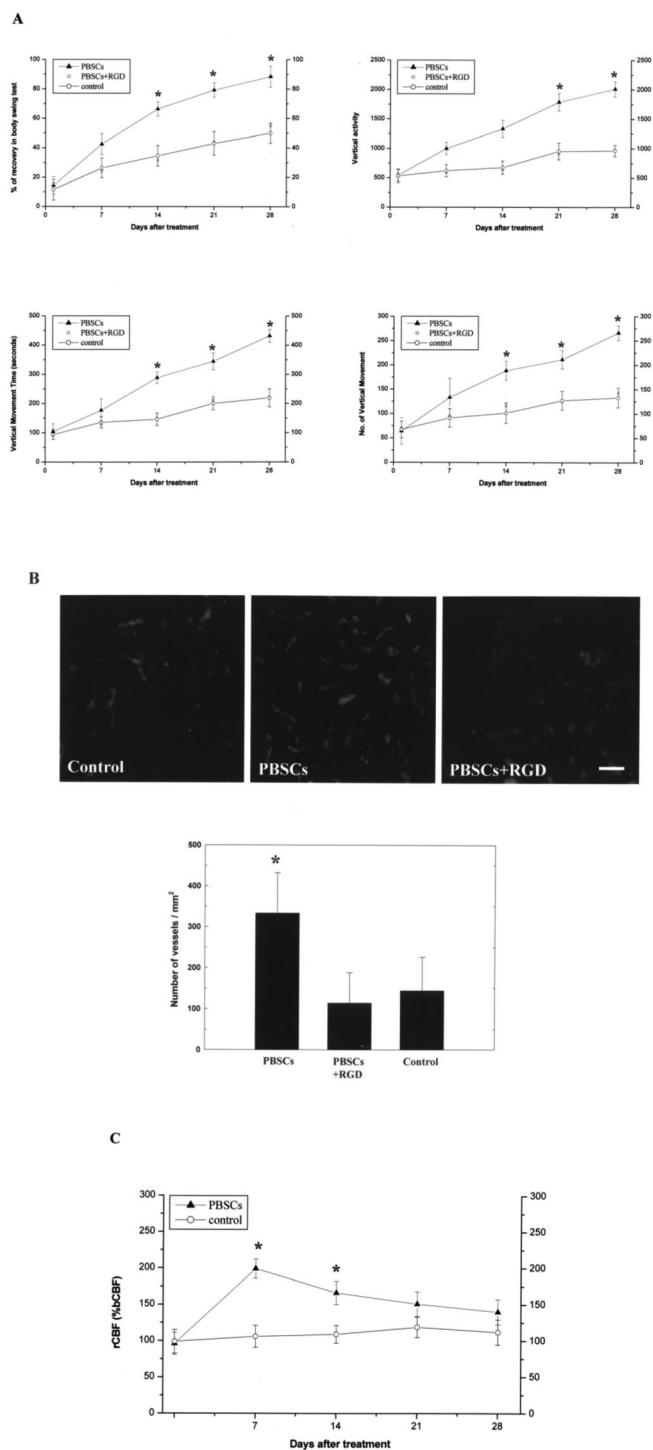


Figure 6. PBSC-derived angiogenesis could enhance cerebral blood flow in the ischemic hemisphere. **A**, Data for neurological behavioral measurements in the three treatment groups: S+I, S, and C. **B**, The results for numbers of blood vessel density in the three treatment groups. **C**, The results from analysis of rCBF using LDF monitoring with Diamox injection showed a significant increase in rCBF in the MCA cortex of the ischemic brain in the PBSC-treated rats compared with control rats. Data are expressed as mean \pm SEM. * $p < 0.05$ versus control. Scale bar, 50 μm .

occur to recover the cerebral blood flow over the ischemic brain. It might be the case that enhanced neuroplastic effect via functional angiogenesis (Taguchi et al., 2004) provides better neurological outcomes.

A recent report indicated that SDF-1/CXC chemokine recep-

tor 4 (CXCR4) is a strong chemoattractant for CD34⁺ cells and also plays an important role in HSC trafficking between peripheral circulation and bone marrow (Petit et al., 2002). Recently, another study demonstrated that focal cerebral ischemia causes an increase in SDF-1/CXCR4 expression in regions adjacent to the infarcted area (Hill et al., 2004). This lesion-induced upregulation of endothelial SDF-1 (Stumm et al., 2002) as well as the appearance of increased CXCR4 expression in the ischemic hemisphere 4 h after ischemia, as explained in our recent report (Shyu et al., 2004b), together indicate that cerebroendothelial SDF-1 could be a chemoattractant for peripheral blood cells. However, upregulation of SDF-1 in the penumbra area has been shown to diminish significantly 7 d after the induction of cerebral ischemia (Hill et al., 2004). In our “chronic” stroke model, the expression of SDF-1 may have decreased progressively in comparison with the “acute” ischemic brain. However, in this study, interaction of transplanted PBSCs with injured brain tissue may lead PBSCs and/or parenchymal cells to produce trophic factors (Lachmund et al., 1994) including SDF-1 that may contribute to the recovery of neural functions lost as a result of tissue injury (Williams et al., 1986). Therefore, it could be that decreased expression of SDF-1 1 week after cerebral ischemia is compensated by the transplanted PBSCs and may cause enough stem cells to home into the ischemic brain; this may lead to the recovery of postischemic neurological performance after PBSC treatment seen in this study.

In this study, we have developed a novel therapeutic strategy to treat chronic stroke rats using intracerebral PBSC transplantation. Neurological dysfunction after chronic stroke showed significant improvement in a PBSC-treated rat population compared with vehicle control-treated rats. Exogenous PBSC transplantation was found to increase the number of endogenously central and peripheral stem cells homing to the lesioned brain, resulting in a significant improvement in neurological function after chronic stroke. Furthermore, exogenous PBSCs transplanted into the ischemic hemisphere resulted in significant increases in neurotrophic factors including SDF-1 and BDNF in PBSC-treated rats compared with control-treated rats. Therefore, we could speculate that these trophic factors not only increased the survival rates of the ischemic neurons in the penumbra region, but also induced endogenous HSCs to migrate and home into the ischemic brain to repair it. In addition, one of these factors, SDF-1, might be the key substance that induced endogenous stem cell targeting to the ischemic hemisphere. Recently, it was demonstrated that focal cerebral ischemia causes an increase in SDF-1 expression in regions adjacent to the infarcted area (Stumm et al., 2002). SDF-1 is a CXC chemokine constitutively produced by bone marrow stromal cells and is a potent chemoattractant for stem cells. By attracting endogenous stem cells to the ischemic region, an SDF-1/CXCR4 interaction may be directly involved in vascular remodeling, angiogenesis (De Falco et al., 2004), and neurogenesis (Stumm et al., 2002), thereby alleviating

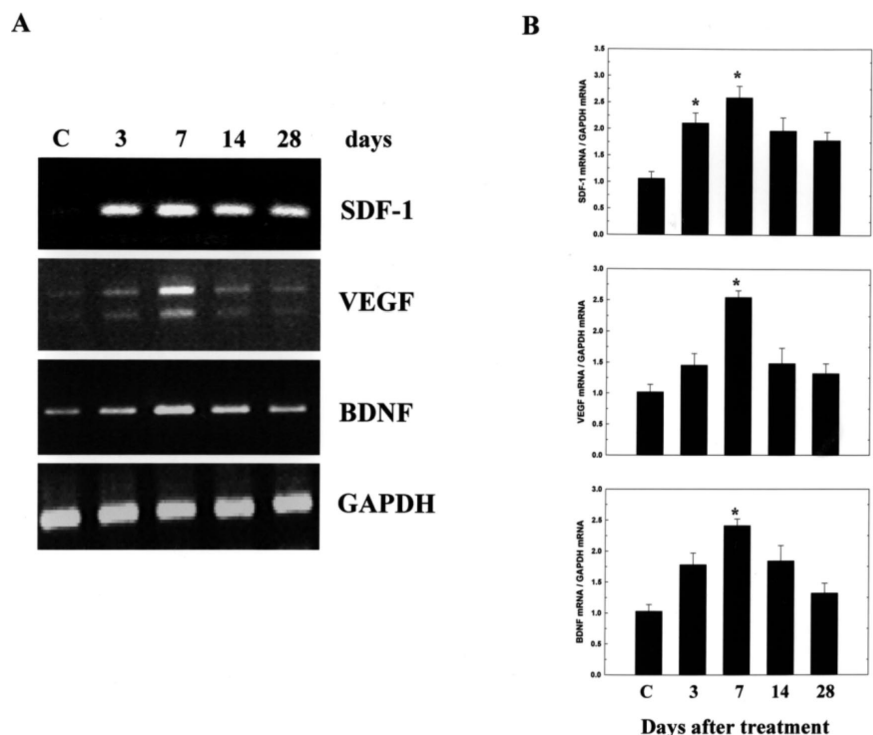


Figure 7. PBSC transplantation modulates neurotrophic factor expression in ischemic rats. **A**, Results of conventional RT-PCR analysis. Using gene-specific primers, RT-PCR was performed for SDF-1, VEGF, and BDNF in brain samples from the cortex and striatum of PBSC-treated rats at different time points (3, 7, 14, and 28 d) after the initiation of treatment protocol. GAPDH was used as an internal control. Brain samples of vehicle control rats are indicated by “C.” **B**, In the results of QRT-PCR analysis, the ratio of expression level of SDF-1, VEGF, and BDNF mRNA was relative to GAPDH mRNA. The mean \pm SEM is shown. * $p < 0.05$ versus control.

stroke symptoms. As a consequence of this autocrine regulatory pathway, endothelial and neuronal progenitor cells may mobilize and fuse with each other, a step required for subsequent formation of a structured network of branching vessels and neurons (Chen et al., 2003).

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