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Cilostazol Enhances Neovascularization in the Mouse Hippocampus After Transient Forebrain Ischemia

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Cilostazol is known to be a specific type III phosphodiesterase inhibitor, which promotes increased intracellular cAMP levels. We assessed the effect of cilostazol on production of angioneurins and chemokines and recruitment of new endothelial cells for vasculogenesis in a mouse model of transient forebrain ischemia. Pyramidal cell loss was prominently evident 3–28 days postischemia, which was markedly ameliorated by cilostazol treatment. Expression of angioneurins, including endothelial nitric oxide synthase, vascular endothelial growth factor, and brain-derived neurotrophic factor, was up-regulated by cilostazol treatment in the postischemic hippocampus. Cilostazol also increased Sca-1/vascular endothelial growth factor receptor-2 positive cells in the bone marrow and circulating peripheral blood and the number of stromal cell-derived factor-1α-positive cells in the molecular layer of the hippocampus, which colocalized with CD31. CXCR4 chemokine receptors were up-regulated by cilostazol in mouse bone marrow-derived endothelial progenitor cells, suggesting that cilostazol may be important in targeting or homing in of bone marrow-derived stem cells to areas of injured tissues. CD31-positive cells were colocalized with almost all bromodeoxyuridine-positive cells in the molecular layer, indicating stimulation of endothelial cell proliferation by cilostazol. These data suggest that cilostazol markedly enhances neovascularization in the hippocampus CA1 area in a mouse model of transient forebrain ischemia, providing a beneficial interface in which both bone marrow-derived endothelial progenitor cells and angioneurins influence neurogenesis in injured tissue.

Key words: CD31; cilostazol; endothelial progenitor cells; transient forebrain ischemia; vasculogenesis

Under physiological conditions, new neurons are continuously generated in limited regions such as the subventricular zone, which lines the lateral ventricles and produces neuronal progenitor cells that migrate to the olfactory bulb, and the subgranular zone (SGZ) of the dentate gyrus, from which neuronal progenitors develop granular cell morphology and neuronal markers and migrate to the CA3 region of the hippocampus (Eriksson et al., 1998; Markakis and Gage, 1999). These observations indicate the potential ability of the brain to replace injured neuronal cells. Under the influence of a variety of modulators, including neurotransmitters, hormones, aging, and stress, neural progenitor cells in the SGZ of the dentate gyrus proliferate and differentiate, and the newly formed cells migrate to the granule cell layer (GCL; Kuhn et al., 1996; Eriksson et al., 1998). Hippocampal CA1 neurons are particularly sensitive to global ischemia (Kirino, 1982).

Neurogenic regions require unique and highly specialized microenvironments (vascular niches), such as

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production of angiogenic factors that, along with neovascularization, tightly regulates the neuronal development of adult neural stem cells. A growing body of evidence indicates that endothelial progenitor cells (EPCs) participate in endothelial repair and neovascularization of ischemic organs (Urbich and Dimmeler, 2004). Consistent with this suggestion, up-regulation of the chemokine stromal cell-derived factor-1α (SDF-1α)/CXCL12 and its receptor, CXC chemokine receptor 4 (CXCR4), mediates EPC recruitment along hypoxic gradients, thereby contributing to neovascularization of ischemic tissue (Ceradini and Gurtner, 2005; Schober et al., 2006).

On the other hand, there are many molecules that affect both neurogenesis and angiogenesis, which are called *angioneurins* (Zacchigna et al., 2008). Neuronal expression of brain-derived neurotrophic factor (BDNF) is up-regulated by cyclic AMP (cAMP) in rat cortex and hippocampus (Morinobu et al., 1999; Nakahashi et al., 2000), and cAMP responsive element-binding protein (CREB) is implicated in the increase of BDNF expression (Finkbeiner et al., 1997). Vascular endothelial growth factor (VEGF), which is up-regulated by hypoxia, has been proposed to regulate EPC proliferation and to enhance neovascularization and neurogenesis (Sun et al., 2003; Schanzer et al., 2004; Hristov and Weber, 2004). Furthermore, dibutyryl cAMP treatment increases mRNA expression of the angiogenic cytokines VEGF and SDF-1α in vivo in wound tissue and in cultured fibroblasts and macrophages in vitro (Asai et al., 2006).

Cilostazol (6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)butoxy]-3,4-dihydro-2-(1*H*)-quinolinone) increases intracellular cAMP levels by inhibiting type III phosphodiesterase (Kimura et al., 1985). Most recently, Lee et al. (2009) showed that, in an ischemic rat subjected to two-vessel occlusion with hypotension (37–42 mm Hg during occlusion for 10 min), cilostazol exhibited dual beneficial effects by preserving the CA1 hippocampal region and promoting the generation of immature migratory neuroblasts in the dentate gyrus by up-regulation of CREB phosphorylation after transient forebrain ischemia. Additionally, cilostazol increases the phosphorylation of endothelial nitric oxide synthase (eNOS) at Ser1177 and of Akt at Ser473 in association with increased cAMP levels and enhances endothelial tube formation in human aortic endothelial cells (Hashimoto et al., 2006).

Given that neovasculogenesis improves the tissue microenvironment around the hippocampal CA1 ischemic area as a vascular niche and favors the proliferation and differentiation of neuronal precursor cells, it is predictable that enhancement of neovascularization could provide a beneficial therapeutic option for the improvement of neurogenesis and neurologic functional recovery after stroke. However, the importance of the neovascularization in the hippocampus area after transient forebrain ischemia remains to be clarified. Several lines of evidence suggest that transient forebrain cerebral ischemia induces selective and delayed neuronal death in the hippocampal CA1 region (Nitatori et al., 1995). Here, we present data showing that cilostazol stimulates hippocampal neovascularization after transient forebrain ischemia, potentially a powerful mechanism to improve functional recovery.

MATERIALS AND METHODS

Transient Forebrain Ischemia

All procedures were conducted in accordance with the animal care guidelines of the Animal Experimental Committee of Pusan National University. Male C57BL/6 mice (Samtako BioKorea) weighing 20–25 g were anesthetized with chloral hydrate (450 mg/kg, intraperitoneally) and were allowed spontaneous respiration throughout the surgical procedures. Both common carotid arteries were exposed through a midline cervical incision. The exposed arteries were carefully separated from the vagus nerve and occluded for 20 min with microclips. For reperfusion, the clips were removed and the patency of the arteries was confirmed by inspection. The rectal temperature was monitored and maintained at 37°C ± 0.5°C with a feedback-controlled heating pad (Homeothermic Blanket System; Harvard Apparatus, Holliston, MA). The animals received cilostazol (10 mg/kg/day, intraperitoneally; Otsuka Pharmaceutical, Tokushima, Japan) or vehicle (100% DMSO, 50 μl) 1 hr after the operation and afterward once daily for 3, 7, 14, or 28 days. Weight measurements were taken just before the operation and then twice per week.

Western Blotting

To assess the implication of endogenous angiogenic factors, we determined expression of eNOS, VEGF, and BDNF in the hippocampal area obtained from normal mice after cilostazol treatment. The entire hippocampal region obtained at 3, 7, and 28 days after cilostazol treatment was homogenized in lysis buffer (20 mmol/liter Tris-HCl, pH 7.4, 150 mmol/liter NaCl, 1 mmol/liter EGTA, 1 mmol/liter EDTA, 10% glycerol, 1% Triton-X, 1 mmol/liter Na_3VO_4). After centrifugation at 12,000 rpm, 30 μg of total proteins from each sample was loaded into 10% SDS-polyacrylamide gel, and the separated proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The membrane was blocked with 5% skim milk, followed by incubation with a 1:1,000 dilution of anti-eNOS (BD Biosciences, Franklin Lakes, NJ), anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-BDNF (Santa Cruz Biotechnology) in 5% bovine serum albumin in PBS. After the blots had been incubated with IgG horseradish peroxidase-conjugated secondary antibody, the intensity of chemiluminescence was measured by a LAS-3000 System (Fuji Photo Film, Tokyo, Japan). The membrane was reprobed with an anti-β-actin antibody (MP Biomedicals, Aurora, OH) as an internal control.

Tissue Preparations and Histological Assessment

Three, seven, fourteen, and twenty-eight days after transient forebrain ischemia, mice were deeply anesthetized with thiopental sodium and perfused transcardially with PBS. The isolated brains were frozen in an optical cutting temperature

medium for frozen tissue specimens (Sakura Finetek, Torrance, CA) by immersion in liquid nitrogen-equilibrated isopentane. Frozen specimens were stored at −70°C. Serial 10-μm-thick sections were cut on a cryostat and stained with cresyl violet to assess CA1 hippocampal neuronal cell damage. The quantity of damaged pyramidal cells in CA1 areas was measured in Metamorph software (Molecular Devices, Sunnyvale, CA) and expressed as a percentage of damaged pyramidal cells of the entire CA1 region of the untreated group. Viable and damaged pyramidal cells were evaluated in a blind fashion.

BrdU Administration and Immunofluorescence

5-Bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% PBS containing 10 mmol/liter NaOH. Mice were injected with BrdU (50 mg/kg, intraperitoneally) twice daily for a total dosage of 100 mg/kg/day for 1–3 days after transient forebrain ischemia. For BrdU immunolabeling, DNA was denatured with 2 N HCl at 37°C for 30 min, and sections were neutralized with 0.1 mol/liter borate buffer at room temperature for 10 min and washed twice for 5 min in PBS. Sections were incubated with blocking solution (CAS Block, Zymed Laboratories, South San Francisco, CA) for 10 min and then overnight with a 1:100 dilution of mouse anti-BrdU antibody (Sigma-Aldrich). In double-labeling experiments, the incubating solution contained a 1:100 dilution of anti-CD31 antibody (BD Biosciences) with the same dilution of BrdU and anti-SDF1α antibodies (R&D Systems, Minneapolis, MN) or antiglial fibrillary acidic protein (GFAP; Dako, Carpinteria, CA). After washing, the sections were incubated with a 1:100 dilution of secondary antibody solution containing Cy3-conjugated anti-mouse IgG (Vector Laboratories, Burlingame, CA) and fluorescein anti-rat IgG (Vector Laboratories) or fluorescein anti-rabbit IgG (Vector Laboratories) for 1 hr at room temperature. Each sample was washed, counterstained with 0.1 μg/ml Hoechst 33258 for 15 min, and mounted with Vectorshield mounting medium (Vector Laboratories). Fluorescence was detected using an Axiovert 200 fluorescence microscope (Carl Zeiss, Oberkochen, Germany) and quantified in Metamorph software (Molecular Devices). Quantitative analyses are expressed as a percentage of the sham-control group.

Fluorescence-Activating Cell Sorting Analysis

The peripheral blood and bone marrow cells from the femur and tibia were obtained from normal mice that had been treated with cilostazol for 3, 7, and 14 days. Monocytes were isolated from the peripheral blood and the bone marrow by density gradient centrifugation with Histopaque-1083 (Sigma). The viable monocyte population was analyzed for the expression of Sca-1-fluorescein isothiocyanate (FITC; e-Bioscience, San Diego, CA) and vascular endothelial growth factor receptor-2-phycoerythrine (VEGFR-2 or Flk-1; e-Bioscience). Isotype-identical antibodies (e-Bioscience) served as control. Single- and two-color flow cytometric analyses were performed by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) quantifying 1×10^4 events using gates to exclude nonvisible cells. Data were evaluated in Cellquest software (ver. 3.3; Becton Dickinson).

Drugs

Cilostazol was donated by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan) and was dissolved in DMSO.

Statistical Analyses

Data are expressed as mean ± SEM. The changes in variable parameters between drug-treated and vehicle groups were analyzed by the unpaired Student's *t*-test. Analysis of each individual comparison (3, 7, 14, and 28 days) was performed by one-way ANOVA followed by Tukey's multiple-comparisons tests. Statistical analysis was performed in Sigmastat software (Systat Software, Point Richmond, CA). A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

There were no significant differences in body weight between vehicle and cilostazol groups over the 28-day observation period after forebrain ischemia. After 20 min of forebrain ischemia, mortality was also similar (n = 2 each) between vehicle (n = 26) and cilostazol (n = 22) groups.

Cilostazol Decreases Neuronal Cell Loss in the Hippocampal CA1 Area

Bilateral common carotid occlusion for 20 min resulted in prominent pyramidal cell loss in the hippocampal CA1 area as assessed by cresyl violet staining (Fig. 1). The numbers of surviving pyramidal cells were significantly higher in the cilostazol-treated group, whereas the pyramidal cell loss was manifested mainly in the hippocampal CA1 region in the vehicle group (day 7, 30.1% ± 13.2%, $P < 0.05$ vs. vehicle group, 55.4% ± 10.1%; day 14, 27.4% ± 7.9%, $P < 0.01$ vs. vehicle group, 57.3% ± 11.7%; day 28, 5.3% ± 2.8%, $P < 0.05$ vs. vehicle group, 20.2% ± 5.7%; Fig. 1A—C).

Cilostazol Enhances Protein Expression of eNOS, VEGF, and BDNF Levels

Hippocampal eNOS and VEGF protein levels were up-regulated by cilostazol at day 7 (eNOS, 152.6% ± 20.4%, $P < 0.01$; VEGF, 150.6% ± 20.6%, $P < 0.05$) and day 28 (eNOS, 143.7% ± 34.3%, $P < 0.01$; VEGF, 156.4% ± 25.2%, $P < 0.05$). Likewise, BDNF expression was increased by cilostazol at day 7 (188.5% ± 36.3%, $P < 0.05$ vs. control mice) and then decreased (86.4% ± 17.5%) at day 28 (Fig. 2).

Cilostazol Stimulates EPC Recruitment in Mice

To test the effect of cilostazol on EPC recruitment, normal C57BL/6 mice were treated with cilostazol (10 mg/kg/day) for 3, 7, or 14 days. EPCs positive for Sca-1 (a mouse stem-cell marker)/VEGFR-2 (an endothelial cell lineage antigen) were quantified by FACS (Fig. 3A). Treatment with cilostazol significantly increased Sca-1/

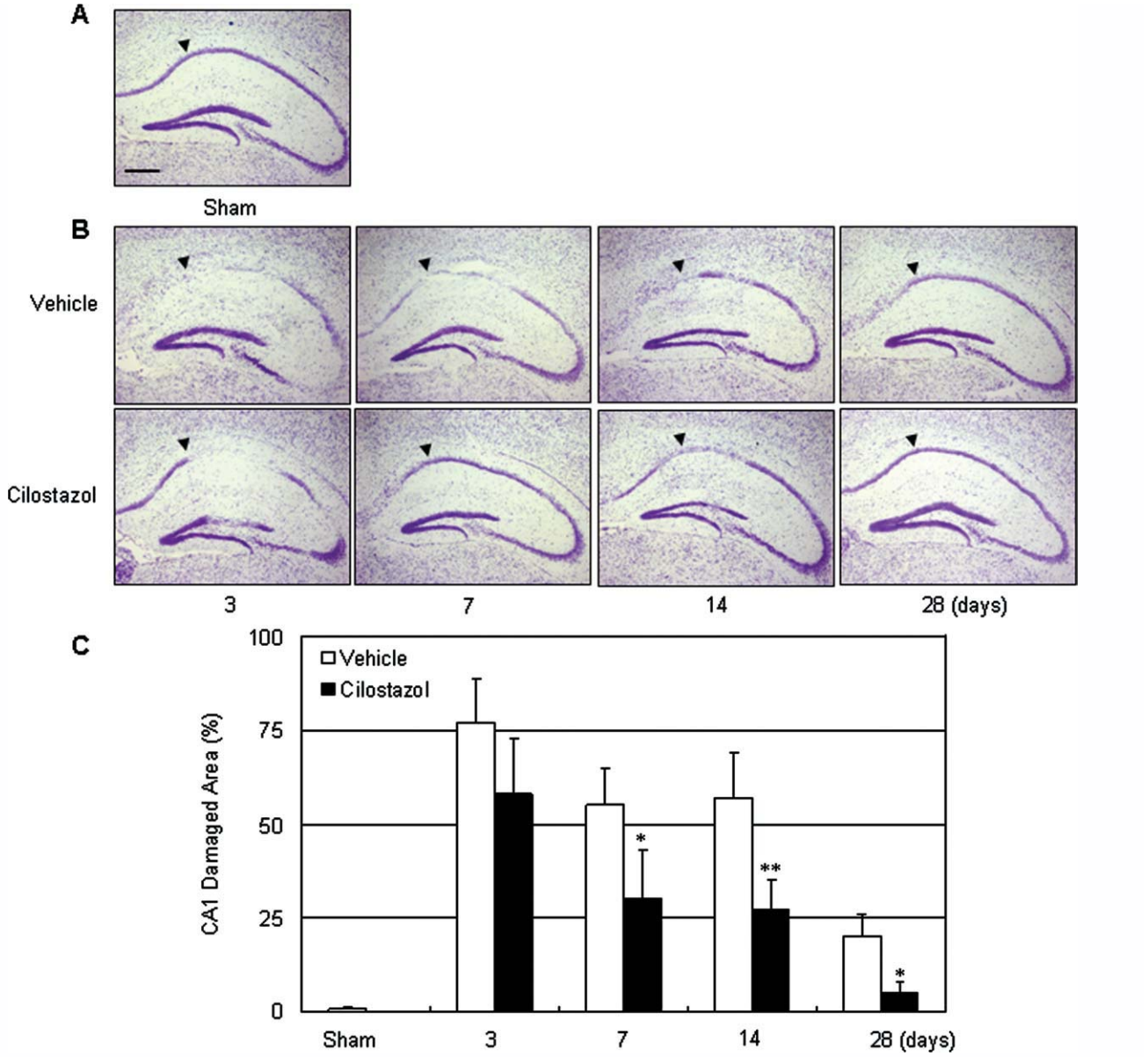

Fig. 1. Histologic evaluation with cresyl violet staining of the hippocampal CA1 sector in ischemic mice. Representative photomicrographs of the cresyl violet-stained mice hippocampal from sham-control mice (**A**) and vehicle- and cilostazol-treated groups (**B**). Mice received vehicle and cilostazol (10 mg/kg/day) for 3, 7, 14, or 28 days. After transient forebrain ischemia with vehicle, pyramidal cell loss was prominent in the hippocampal CA1 region (arrowhead) of the vehicle-treated group, whereas few damaged pyramidal cells were evident in the cilostazol-treated group. **C**: The percentage of CA1 damaged area was quantified and expressed as the mean ± SEM of five mice/group. $*P < 0.05$, $**P < 0.01$ vs. vehicle. Scale bar = 200 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

VEGFR-2-positive EPCs in the bone marrow in a time-dependent manner (day 3, 5.1% ± 1.8%, $P < 0.05$; day 7, 21.4% ± 4.8%, $P < 0.01$; day 14, 35.6% ± 12.9%, $P < 0.01$ vs. vehicle group, 1.2% ± 0.3%; Fig. 3B). A significantly increased number of EPCs was also found in the circulating peripheral blood after 7 days of cilostazol treatment (43.3% ± 8.8%, $P < 0.01$) compared with the vehicle group (5.2% ± 2.2%). These effects remained elevated until day 14 and declined thereafter (Fig. 3C).

Cilostazol Increases SDF-1α/CXCR4 Protein Expression

The number of SDF-1α-positive cells in the molecular layer was significantly increased to 1,633.0% ± 95.9% ($P < 0.05$) in cilostazol-treated mice compared with the vehicle group (897.5% ± 309.0%) at day 14 after ischemia (Fig. 4A,B). CXCR4 protein expression was up-regulated by cilostazol in mouse bone marrow-derived EPCs. Such up-regulation was antagonized by

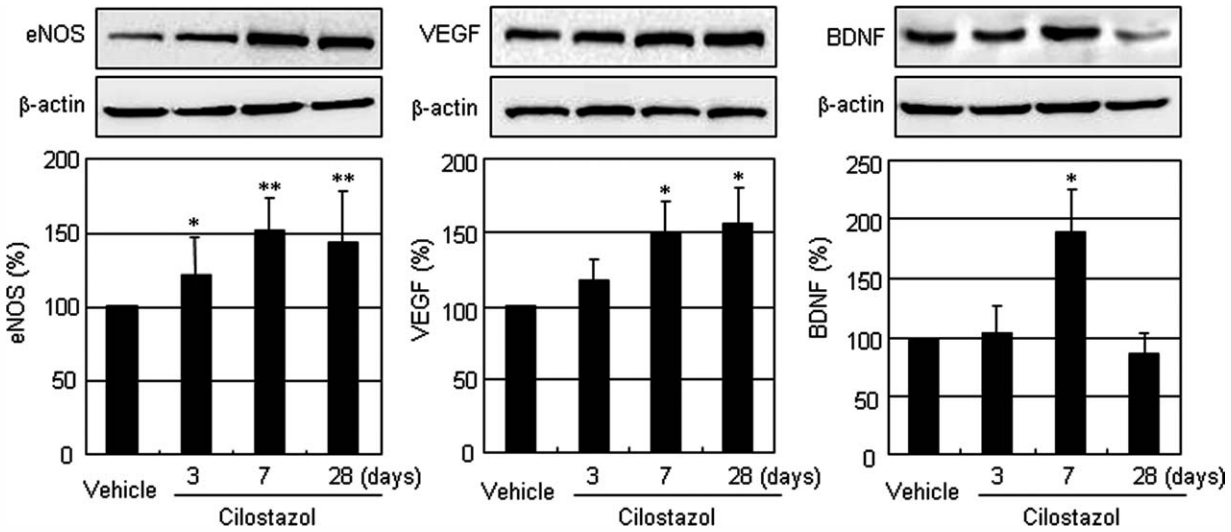

Fig. 2. Effects of cilostazol on eNOS, VEGF, and BDNF protein expression in the hippocampal region. The hippocampal regions harvested at the indicated times were examined by Western blotting. Expression of eNOS, VEGF, and BDNF was significantly increased by cilostazol treatment. Results are expressed as the mean ± SEM of five or six mice/group. $\star P < 0.05$, $\star\star P < 0.01$ vs. vehicle.

AMD3100, a CXCR4 chemokine receptor antagonist (123.3% ± 23.5%, $P < 0.01$) and KT5720, the selective protein kinase A inhibitor (106.4% ± 16.1%, $P < 0.01$) compared with the value of the cilostazol-treated group (10 μmol/liter, 231.7% ± 15.0%; Fig. 4C). Double-immunostaining was performed to determine the relationship between SDF-1α and astrocytes, or SDF-1α and endothelial cells. Almost all SDF-1α-positive cells coexpressed the endothelial cell marker CD31, whereas few SDF-1α-positive cells were also positive for the astrocytic marker GFAP (Fig. 5).

Cilostazol Enhances CD31 Expression In Vivo

Compared with the levels in the sham group (100.2% ± 10.4%), CD31-positive cells increased to 938.2% ± 212.5% ($P < 0.01$) and 860.3% ± 296.0% ($P < 0.05$) in the molecular layer of vehicle group at 7 and 14 days after ischemia, respectively. In the cilostazol-treated group, CD31-positive cells were significantly elevated to 1,324.7% ± 284.8% ($P > 0.05$) and 1,626.9% ± 76.6% ($P < 0.05$ vs. vehicle group), respectively (Fig. 6A,B).

Cilostazol Enhances Endothelial Cell Proliferation In Vivo

BrdU-positive cells in the cilostazol-treated group were significantly increased at day 14 to 1,432.9% ± 283.9% ($P < 0.05$) compared with the vehicle group (433.8% ± 92.9%). The proportion of BrdU-positive cells that was also labeled with CD31-positive cells in the molecular layer was significantly increased at day 14 in the cilostazol-treated group (1,371.6% ± 349.5%) vs. vehicle (568.1% ± 23.7%; $P < 0.05$), suggesting that the increase in these newly generated CD-31-positive cells was triggered by cilostazol. CD31-positive cells running perpendicularly to the pyramidal layer of CA1 area were identified in the inner molecular layer (Fig. 7).

DISCUSSION

The present study was undertaken to assess the effect of cilostazol on the local production of angioneurins and chemokines and recruitment of new endothelial cells for vasculogenesis following 20 min of transient forebrain ischemia in a mouse model. After ischemia (3–28 days), conspicuous pyramidal cell damage was markedly attenuated by cilostazol treatment. In line with these results, the expression of angioneurins such as eNOS, VEGF, and BDNF in hippocampal samples was up-regulated by cilostazol treatment. Furthermore, the number of SDF-1α-positive cells was significantly increased in the molecular layer by cilostazol treatment compared with the vehicle group on postischemic day 14. The SDF-1α-positive cells colabeled with the CD31-positive cells, but less so with GFAP-positive cells. Additionally, CXCR4 chemokine receptors were up-regulated by cilostazol in mouse bone marrow-derived EPCs. Upon treatment with cilostazol, CD31-positive cells were increasingly colabeled as BrdU-positive cells in the molecular layer, suggesting a cilostazol-mediated enhancement of endothelial cell proliferation in the hippocampal area. Previously, Lee et al. (2005) reported elevated cAMP levels in the proximal ascending aorta in mice fed a cilostazol-supplemented high-fat diet. Most recently, Lee et al. (2009) observed that rats subjected to transient forebrain ischemia with arterial hypotension display prominently damaged hippocampal CA1

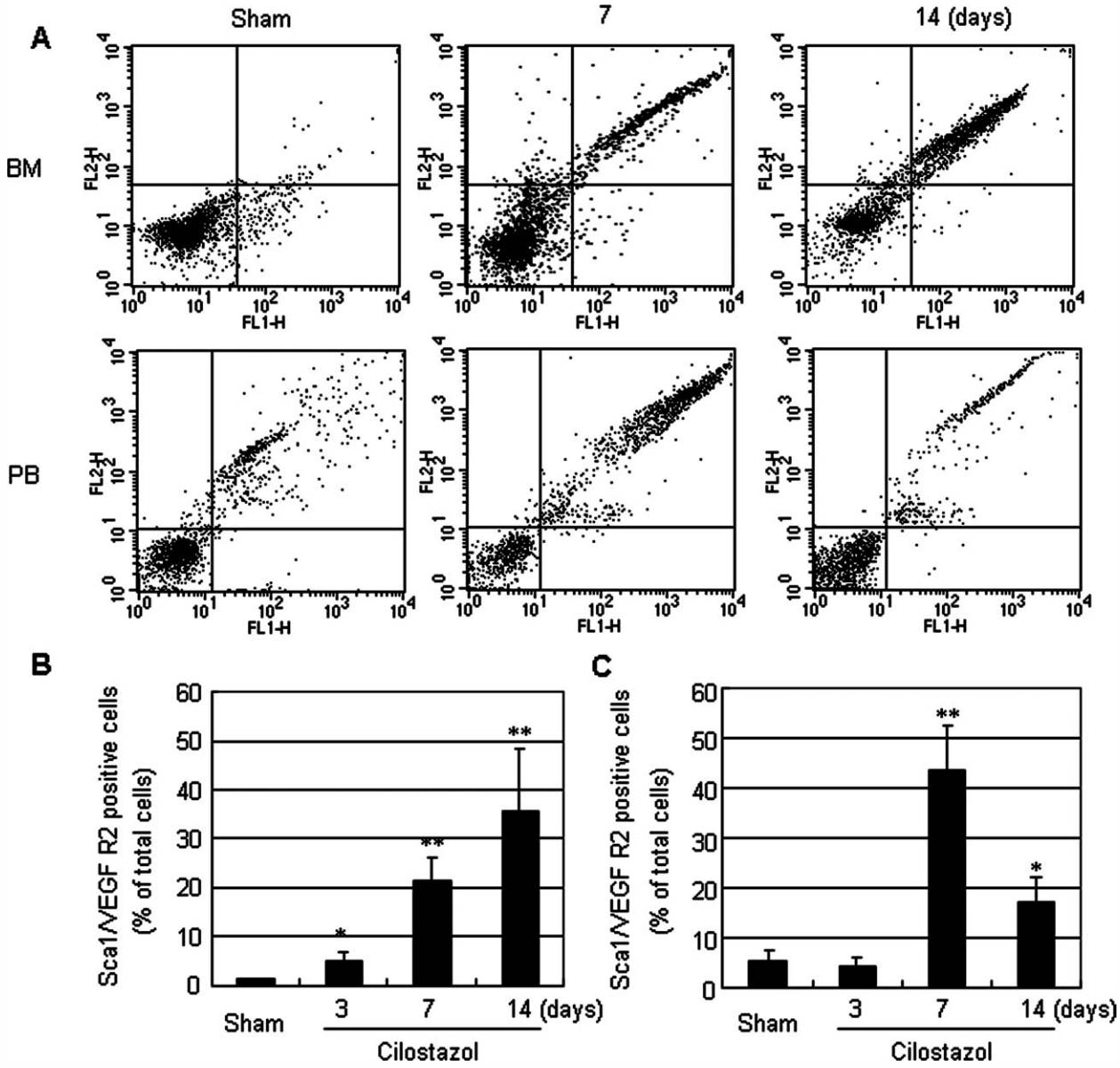

Fig. 3. Cilostazol treatment increases EPC number in the bone marrow and peripheral blood. **A**: Representative four-quadrant analysis of double-positive cells for Sca-1 (a mouse stem cell marker, x axis) and VEGFR2 (an endothelial cell lineage antigen, y axis) in samples of bone marrow (BM, upper) and peripheral blood (PB, lower). Double-positive cells are shown in the right upper quadrant. Quantitative analyses of Sca-1- and VEGFR2-positive cells in the bone marrow (**B**) and peripheral blood (**C**) are expressed as mean ± SEM of four mice/group. $*P < 0.05$ and $**P < 0.01$ vs. sham.

regions; the damage was significantly reduced by cilostazol (60 mg/kg/day, orally) and was associated with increased BrdU-positive/phosphorylated CREB-positive cells in both the hippocampus and the dentate gyrus.

Brain microvascular endothelium elaborates BDNF, which contributes significantly to the neurotrophic activity of the vascular endothelium (Leventhal et al., 1999). Recently, neovasculogenesis was demonstrated to play an important role in long-term neurogenesis after stroke, indicating that optimizing vascularization could be an important strategy to promote neurogenesis and repair after stroke (Thored et al., 2007). VEGF is an angiogenic agent that promotes neurogenesis and stem cell/progenitor cell migration (Jin et al., 2002; Sun et al., 2003). BDNF likewise regulates neuronal survival, cell migration, and synaptic function (Aguado et al., 2003; Gorski et al., 2003). Additionally, VEGF up-regulated by hypoxia regulates EPC proliferation and enhances neovascularization and neurogenesis (Schanzer et al., 2004). Accumulating evidence favors the view that

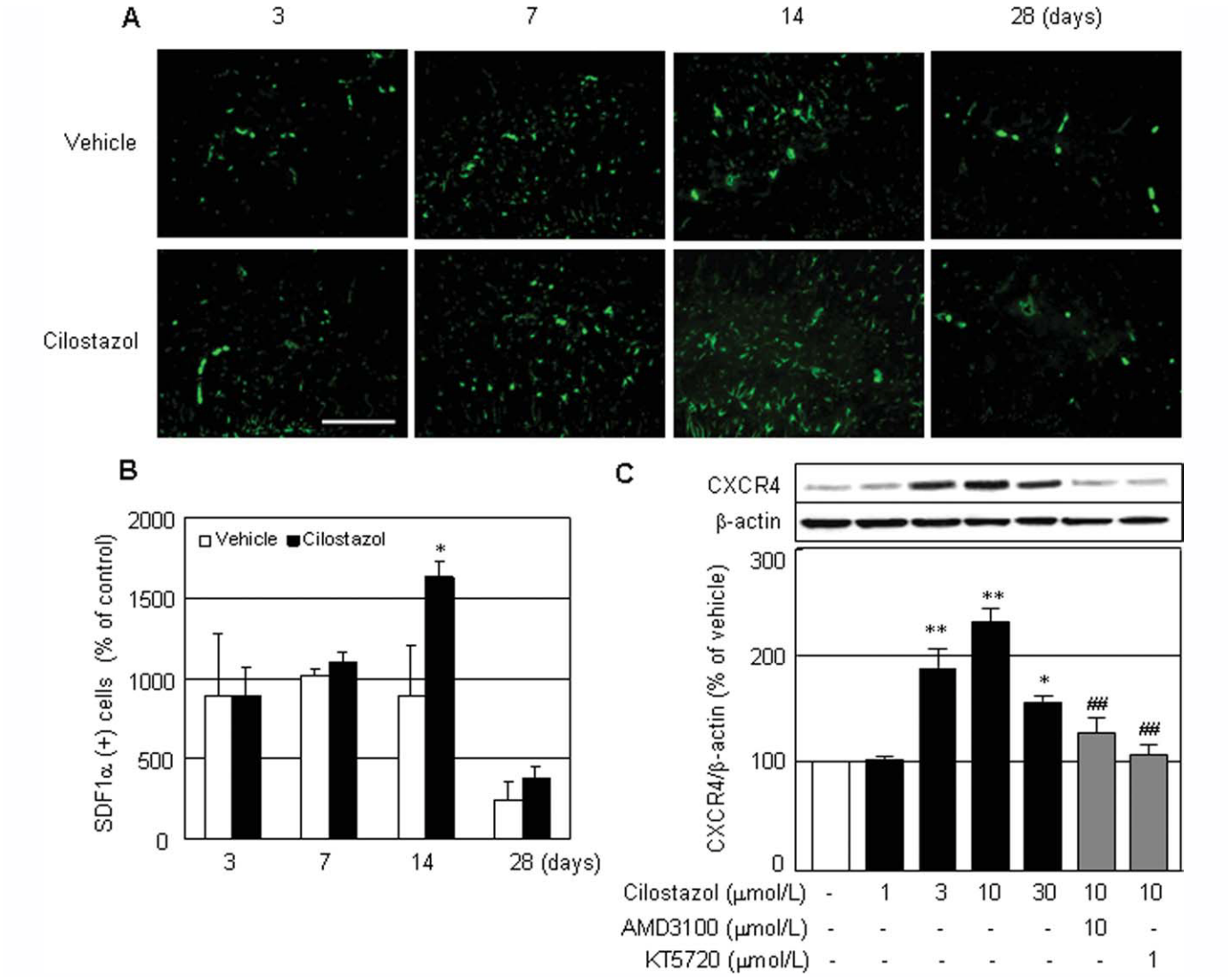

Fig. 4. **A**: Representative photographs showing immunofluorescence staining of SDF-1α in the hippocampus of vehicle- and cilostazol-treated groups at days 3, 7, 14, and 28 after ischemia. In the molecular layer, SDF-1α-positive cells were markedly increased by cilostazol treatment compared with vehicle group at day 14. **B**: Quantitative analyses of the SDF-1α-positive cells expressed as the mean ± SEM from five mice in each group. $\star P < 0.05$ vs. vehicle. **C**: Mouse bone marrow-derived EPCs were incubated with cilostazol (3, 10, and 30 μmol/liter) with or without the CXCR4 chemokine receptor antagonist AMD3100 (10 μmol/liter) and/or the selective protein kinase A inhibitor KT5720 (1 μmol/liter) for 3 hr. Results are expressed as the mean ± SEM of three independent samples/group. $\star P < 0.05$, $\star\star P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. value at 10 μmol/liter cilostazol without antagonists. Scale bar = 200 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

BDNF expression is up-regulated by cAMP in rat hippocampal neurons (Morinobu et al., 1999; Nakahashi et al., 2000) and implicates the CREB transcription factor in the increased expression of BDNF (Finkbeiner et al., 1997). Hashimoto et al. (2006) recently showed that cilostazol increases phosphorylation of eNOS in association with increased cAMP levels and enhances endothelial tube formation in human aortic endothelial cells. Consistently with these reports, our study showed a significant cilostazol-mediated up-regulation of eNOS, VEGF, and BDNF expressions in the mouse hippocampus.

One of the best characterized functions of EPC is to promote neoangiogenesis (Asahara et al., 1997). Circulating cells derived from the bone marrow that exhibit the phenotypic features of endothelial cells are capable of homing in to the sites of endothelial disruption and incorporating into nascent endothelium (Asahara et al., 1997; Shi et al., 1998). cAMP plays specific roles in endothelial cell induction and differentiation from VEGFR2-positive vascular progenitors (Yurugi-Kobayashi et al., 2006). In the present study, treatment of C57BL/6 mice with cilostazol produced a significant increase of EPCs positive for Sca-1/VEGFR-2 in the

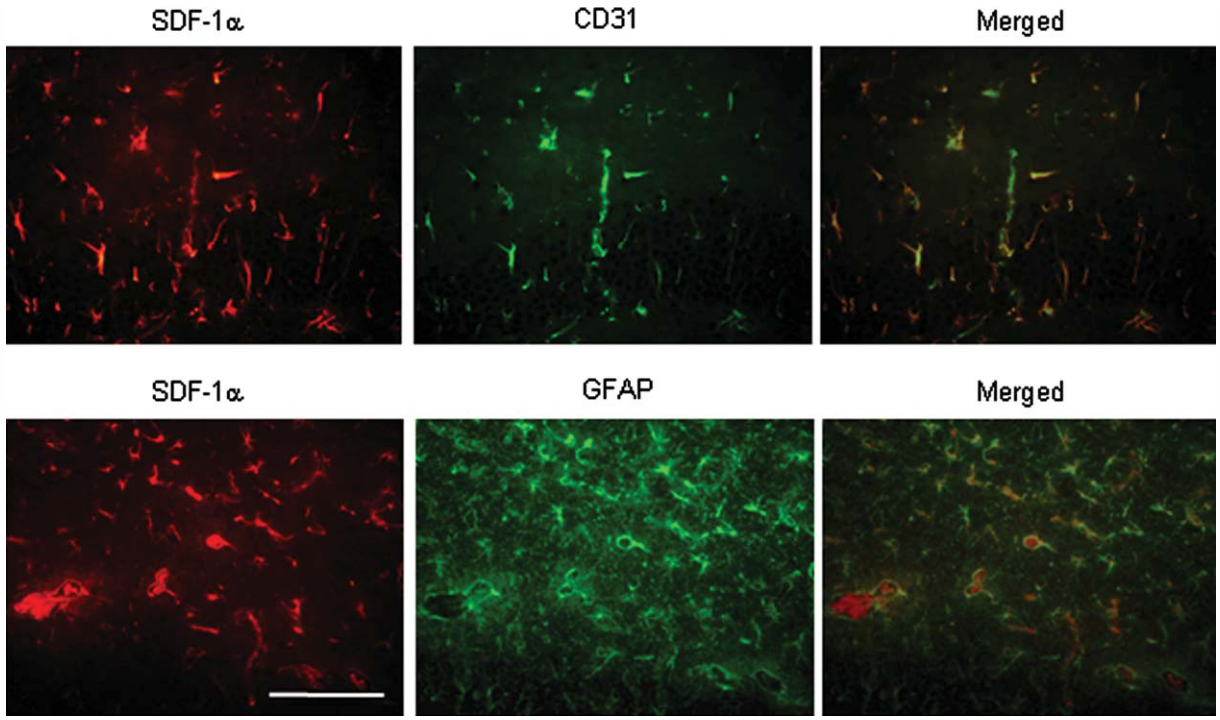

Fig. 5. Representative photographs showing double-immunostaining of SDF1α-positive cells (red) and CD31-positive cells (green) or GFAP-positive cells (green) in the hippocampus of cilostazol-treated mice at day 7 after ischemia. Almost all SDF-1α-positive cells coexpress the endothelial cell marker CD31, whereas fewer SDF-1α-positive cells were also GFAP positive. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

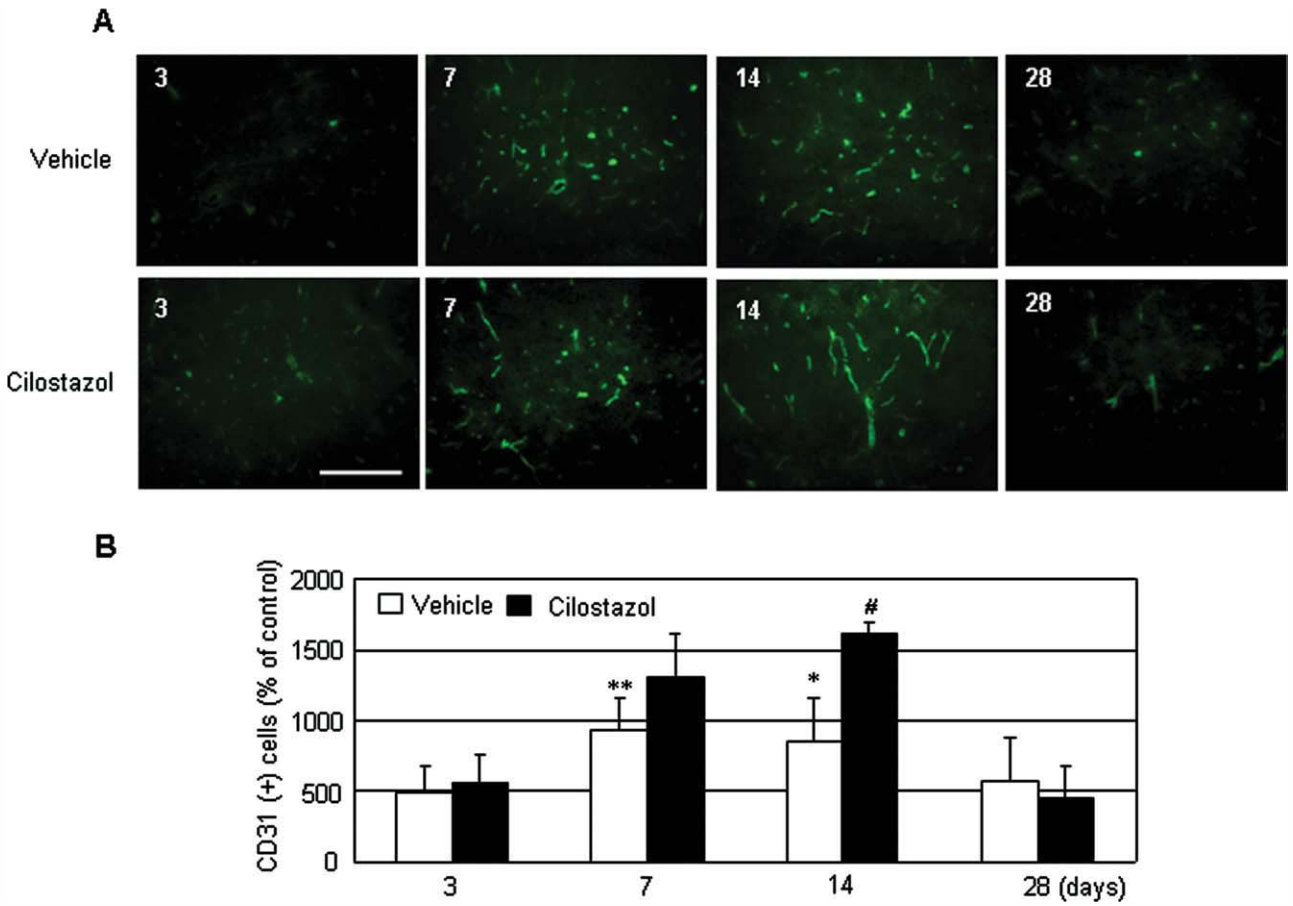

Fig. 6. **A**: Representative photographs showing immunostaining of CD31 in the hippocampus of vehicle- and cilostazol-treated groups 3, 7, 14, and 28 days after ischemia. CD31-positive cells were significantly increased by cilostazol treatment compared with vehicle group at day 14 after ischemia. **B**: Quantitative analysis of the CD31-positive cells expressed as the mean ± SEM of five mice/group. $^{\star}P < 0.05$ and $^{\star\star}P < 0.01$ vs. sham-operated group and $^{\#}P < 0.05$ vs. vehicle. Scale bar = 200 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

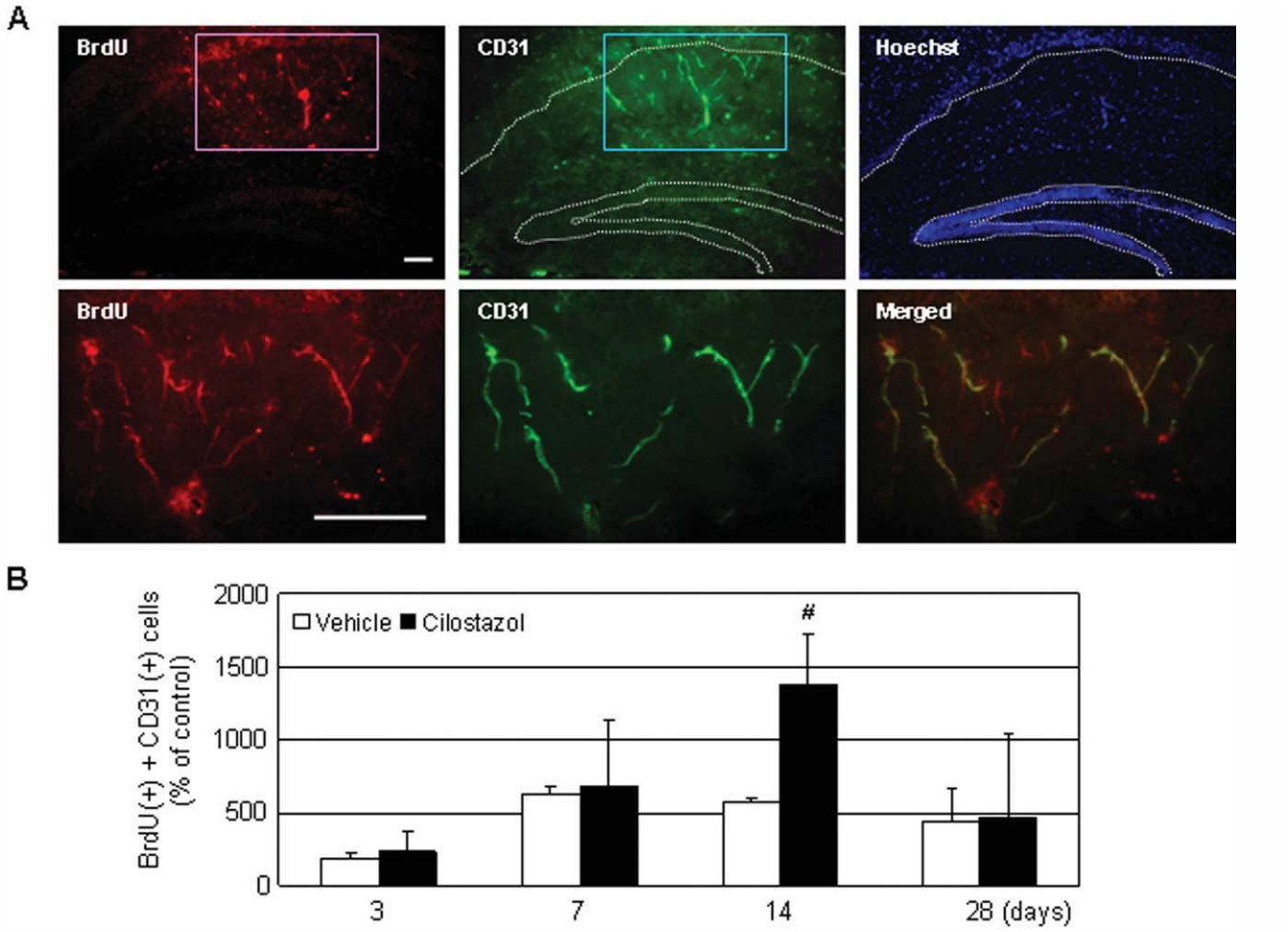

Fig. 7. **A**: Representative photographs showing double immunostaining of BrdU-positive cells (red) and CD31-positive cells (green) in the hippocampus of cilostazol-treated mice 14 days after ischemia. **B**: The number of cells coexpressing BrdU and CD31 was quantified. Quantitative analysis is expressed as the mean ± SEM of five mice/group. $^{\#}P < 0.05$ vs. vehicle. Scale bars = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

bone marrow and circulating peripheral blood at days 7 and 14. These observations are consistent with cilostazol-mediated enhanced EPC recruitment to the bone marrow and peripheral blood. Cilostazol-induced increased recruitment of EPCs that promotes tissue neovascularization may be, in part, ascribed to increased VEGF (Asahara et al., 1999). This postulation provides a basis for a drug therapy after transient forebrain ischemia, in that a sufficient number of EPCs, mobilized by angiogenic factors together with cilostazol, can target ischemic-injured hippocampal sites to promote neovascularization. It remains unclarified whether the Sca-1/VEGFR2-positive cells represent a homogenous cell population, because EPC differentiation is not restricted to the endothelial lineage, and endogenous murine bone marrow cells may give rise to endothelial cells, microglial cells, and a limited number of NeuN-positive cells (Hess et al., 2002).

SDF-1α, a powerful chemoattractant for both human and murine hematopoietic stem cells, has been implicated as a chemokine for EPCs, and the CXCR4 chemokine receptor contributes to the mobilization and targeting of stem cells into regions of ischemic brain injury (Hill et al., 2004). We found that the number of SDF-1α-positive cells was significantly increased in the molecular layer by cilostazol treatment at day 14 following ischemia, and most SDF-1α-positive cells colocalized with the CD31-positive cells, suggesting a close interaction of endothelial cells with injured tissues.

CXCR4 expressions was up-regulated by cilostazol in mouse bone marrow-derived EPC; this up-regulation was inhibited by and KT5720 (a selective protein kinase A inhibitor), as it was by AMD3100 (a CXCR4 chemokine receptor antagonist), suggesting that elevated cAMP activates CXCR4 expression in the endothelium (Goichberg et al., 2006). Collectively, the results

strongly support the suggestion that cilostazol may be important in targeting or homing in bone marrow-derived stem cells to injured tissues for repair and remodeling. These speculations are further supported by the facts that dibutyryl cAMP treatment increases mRNA expression of angiogenic cytokines VEGF and SDF-1α in wound tissue, in vivo, and in cultured fibroblasts and macrophages in vitro (Asai et al., 2006).

In the present study, BrdU-positive cells, an early marker of cell proliferation, were detected mainly in the molecular layer between the hippocampus and the upper blade of the dentate gyrus. The proportion of BrdU-positive cells colabeled with CD31-positive cells in the molecular layer was significantly increased by day 14 after ischemia in the cilostazol-treated group, and CD31-positive cells running perpendicularly to the pyramidal layer of the CA1 area were observed in the inner molecular layer. In line with these findings, vWF immunostaining showed cells with a vascular phenotype within the molecular layer and dentate gyrus at day 14 in the cilostazol-treated group (data not shown), suggesting that there was an increase in newly generated microvessels. This is consistent with the observed chronologic elevation in angioneurins, including VEGF and BDNF, with increased eNOS activation. Whether cilostazol enhances neurogenesis in the injured hippocampus area remains to be determined and will require examination of the proliferation, migration, and differentiation of the neural progenitor cells.

In conclusion, the results from a mouse model of transient forebrain ischemia support our suggestion that neovascularization is markedly enhanced in the hippocampal CA1 area by cilostazol treatment. Elucidation of the mechanisms may clarify the role of vascular niche in stimulation of neurogenesis and offer new insight into the mechanism of functional recovery in association with activation of endogenous neural stem cells.

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