Sodium Orthovanadate Enhances Proliferation of Progenitor Cells in the Adult Rat Subventricular Zone after Focal Cerebral Ischemia

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ABSTRACT

Neuronal progenitor cells able to produce new neuron and glia persist in the adult central nervous system (CNS). Their proliferation is up-regulated by growth factors or cytokines under some pathological conditions, including ischemia. Because sodium orthovanadate (SOV), a protein tyrosine phosphatase inhibitor, can up-regulate tyrosine kinase-linked growth factor receptor signaling via the inhibition of tyrosine residue dephosphorylation, it may be capable of enhancing progenitor cells. To investigate the effect of SOV on progenitor cells in the subventricular zone (SVZ), we injected rats intraperitoneally with 50 mg/kg bromodeoxyuridine (BrdU) and 12.5 or 25 mM SOV or BrdU and saline (control) on days 1 to 7 after middle cerebral artery occlusion. The density of BrdU-positive cells in the ipsilateral SVZ showed a significant SOV dose-dependent increase. This effect was found only in the ipsilateral and not contralateral SVZ, and it was not found in nonischemic rats. Double immunolabeling with BrdU and double cortin, a marker of migrating neuroblast, revealed that the density of double-positive cells increased significantly in an SOV dose-dependent manner. Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining suggested that the SOV-induced increase was not due to antiapoptotic effects. Treatment with SOV also significantly increased the density of cells positive for BrdU and phosphorylated Akt and BrdU and phosphorylated extracellular signal-regulated kinase (ERK). We postulate that ischemia triggers off the proliferation of SVZ cells by bioactive factors such as growth factors and that SOV enhances the proliferation of only triggered-off SVZ cells with Akt and ERK activation. Our findings suggest that SOV may aid in the self-repair of the postischemic CNS.

Vanadium, a transition metal found in relative abundance in nature, is of widely varied biological and physiological significance (Elberg et al., 1994; Brichard and Henquin, 1995). Because vanadate, the +5 oxidation state of vanadium, seems to act as a phosphate analog (Bevan et al., 1995; Huyer et al., 1997) mimicking the transition state (Zhang et al., 1997), it behaves as a competitive inhibitor of protein tyrosine phosphatases, including intracellular signal transduction systems. We previously reported that in gerbils with transient forebrain ischemia, sodium orthovanadate (SOV) and insulin-like growth factor (IGF-1) lead to Akt activation through phosphatidylinositol 3-kinase (PI3K) activation and to extracellular signal-regulated kinase (ERK) activation. Since both Akt and ERK underlie survival in various cells, SOV treatments indeed rescue hippocampal CA1 neurons from delayed neuronal cell death (Kawano et al., 2001, 2002). Similarly, SOV had neuroprotective effects on rat middle cerebral artery (MCA) occlusion model (Hasegawa et al., 2003). However, the time window of SOV-induced neuroprotective effects was limited only in the early phase (within few hours) after stroke (Hasegawa et al., 2006). Since the Akt pathway is believed to be a key component in proliferation or differentiation of neuronal progenitor cells, it is valuable to

ABBREVIATIONS: SOV, sodium orthovanadate; IGF, insulin-like growth factor; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; MCA, middle cerebral artery; CNS, central nervous system; SVZ, subventricular zone; MAPK, mitogen-activated protein kinase; BrdU, 5-bromo-2′-deoxyuridine; PBS, phosphate-buffered saline; PFA, paraformaldehyde; Dcx, double cortin; RT, room temperature; NeuN, neuronal nuclei; GFAP, glial fibrillary acidic protein; p-, phosphorylated; RMS, rostral migratory stream; OB, olfactory bulb; i.p., intraperitoneal.
use SOV in promotion of neuronal self-repair mechanisms after acute ischemic phase.

In stroke patients, it is difficult to administer SOV during the acute ischemic phase, and their progression to cerebral infarction indicates that the opportunity to rescue neurons from cell death has been missed. Our rat experiments were designed to examine the possibility of reducing the infarct area during, and of promoting central nervous system (CNS) self-repair after, the acute phase, because CNS regeneration would improve neural function in stroke patients. We focused on the effect on CNS self-repair of SOV delivered after the acute ischemic phase.

Although the adult mammalian CNS was previously considered to be incapable of significant self-repair or regeneration (Bjorklund and Lindvall, 2000), progenitor cells with the ability to differentiate new neuron and glia have been found to persist in the adult CNS. Temple (2001) reported that neural stem cells, characterized by long-term self-renewal and multipotentiality, are present throughout life in various mammalian species, including humans and that the adult CNS contains a range of progenitors with limited growth- and differentiation potential. These adult progenitors are abundant in the periventricular areas, including the subventricular zone (SVZ), and they are distributed throughout the parenchyma of various CNS regions (Palmer et al., 1999). They proliferate in vivo in response to growth factor stimulation by fibroblast growth factor-2, epidermal growth factor, IGF-1, and vascular endothelial growth factor. The proliferation of neural progenitor cells is up-regulated in a variety of pathological conditions, including ischemia (Dempsey et al., 2003). In adult rats, exogenous IGF-1 increased the proliferation of cells induced by focal ischemia (Dempsey et al., 2003).

Most growth factors bind to their receptors and activate ERK in the cytosol. ERK are members of the mitogen-activated protein kinase (MAPK) family involved in the transduction of extracellular signals into intracellular responses, and the stimulation of ERK phosphorylation by growth factors mediates cell proliferation (Harada et al., 2004; Zhou et al., 2004). We reported elsewhere that SOV activated downstream signals of IGF-1 receptor and that SOV activated ERK and Akt by receptor activation (Kawano et al., 2001). Because many growth factor receptors are activated by autophosphorylation of tyrosine residue, SOV can activate or maintain the activity of these receptors by inhibiting the dephosphorylation of tyrosine residue.

We hypothesized that the administration of SOV after brain ischemia may induce progenitor cell activation and adult CNS regeneration. To assess neuronal regeneration after cerebral infarction, we produced transient MCA occlusion in rats and investigated the effect of SOV on progenitor cell activation in the ipsilateral SVZ. SOV delivered during the acute phase is neuroprotective (Hasegawa et al., 2003); in the current study, we injected SOV after the acute phase. We found that consecutive i.p. administrations of SOV after acute ischemic phase promoted the proliferation of progenitor cells in the SVZ. Our findings suggest that SOV, delivered after the acute ischemic phase, has the potential to promote neural self-repair or regeneration via the induction of SVZ progenitor cell proliferation.

Materials and Methods

Experimental Animals. All experiments were approved by The Animal Care and Use Committee of Kumamoto University (Kumamoto, Japan). Adult male Wister rats weighing 250 to 300 g were maintained under constant environmental conditions (temperature 22 ± 2°C, humidity 55 ± 5%, and 12:12-h light/dark cycle) in the Animal Research Center of Kumamoto University. They had free access to food and water before and after all procedures. Anesthesia was induced with 4% halothane and maintained with 2 to 2.5% halothane, 30% oxygen, and 70% nitrous oxide via a face mask. Their rectal temperature was monitored and maintained at 37.5 ± 0.5°C throughout the surgical procedure using a feedback-regulated heating system (Small Animals Heat Controller, Unique Medical, Tokyo, Japan).

Induction of Focal Ischemia (MCA Occlusion). We induced reversible focal ischemia by a modification of the method of Koizumi et al. (1986) as described by Hasegawa et al. (2003). After exposing the left common, external, and internal carotid arteries through a midline incision in the neck, 20 mm of a 3-0 monofilament nylon suture coated with poly-L-lysine (Belayev et al., 1996) were inserted into the left common carotid artery into the lumen of the internal carotid artery. After 1.5 h, the suture was carefully withdrawn to allow MCA reperfusion. Neurological findings were scored 10 min before reperfusion using a modification of the neurological score of Bederson et al. (1986); grade 0 was recorded in the absence of observable deficits; grade 1 was scored if there was forelimb flexion; grades 2 and 3 were scored if there was decreased resistance to a lateral push in the absence or presence of circling, respectively; and grade 4 was assigned to comatose animals. Rats with grade 0 and 4 were excluded from further experiments.

Administration of SOV. Starting at 24 h after MCA occlusion, the rats received daily i.p. injections (2 ml/kg) of 12.5 or 25 mM SOV (Na3VO4; Wako Pure Chemicals, Osaka, Japan) in saline or saline alone for seven consecutive days (Fig. 1A)

Bromodeoxyuridine Labeling. BrdU (Sigma-Aldrich, St. Louis, MO), the thymidine analog that incorporated into the DNA of dividing cells during S phase, was used for mitotic labeling. For cumulative labeling of proliferating cells during the 7 days following MCA occlusion, we injected BrdU (50 mg/kg i.p.) daily for seven consecutive days starting at 24 h after MCA occlusion (Fig. 1A); the rats (15/group) were sacrificed at 24 h after the last injection. Unoperated rats served as controls; the BrdU plus SOV and BrdU plus saline injection protocols were as in the experimental groups; these rats (15/group) were sacrificed 24 h after the last injection. All rats were transcardially perfused with ice-cold phosphate-buffered saline followed by 4% ice-cold phosphate-buffered paraformaldehyde (PFA). Then, their brains were removed, fixed overnight in 4% PFA, and consecutive 30-μm-thick coronal sections between bregma levels +3 and −1 mm were cut with a vibratome (Leika Microsystems, Tokyo, Japan). The sections were subjected to H&E and Nissl staining and immunohistochemical study.

Measurement of the Area of Ischemic Brain Injury. On the eighth day after transient MCA occlusion, the rats (15/group) were fixed by transcardial perfusion with 4% ice-cold PFA, immersed overnight in fixative, postfixed in 4% PFA, and 30-μm-thick sections were cut in the coronal plane between bregma levels +3 mm and −1 mm on a vibratome. These sections were H&E- and Nissl-stained (Fig. 1B, showing only Nissl staining). The area of ischemic brain injury was measured as reported previously (Hasegawa et al., 2003) using the image analysis software (Scion Image Beta 4.02; Scion Corporation, Frederick, MD). The area of infarction was corrected to compensate for edema formation by subtracting the area of the intact ipsilateral hemisphere from that of the intact contralateral hemisphere. The area of the cortex and caudate putamen on each slice was calculated separately, and the total infarct area and the area of the cortex and caudate putamen on each slice were added and multiplied by the slice thickness to obtain the infarction area.
Immunohistochemistry. The sections were then double-stained for BrdU and double cortex (Dcx) or various neuronal markers. Dcx is a microtubule-associated protein expressed by migrating neuroblasts in the developing and adult nervous system (Francis et al., 1999; Gleeson et al., 1999). Free-floating coronal sections (30 μm) were washed three times (30 min each) in PBS at room temperature (RT), and denatured by incubation in 2 mol/l hydrochloric acid (90 min at RT) before BrdU staining. They were washed for 30 s with PBS, pH 8.5, and then three more times (5 min each at RT) in PBS. Nonspecific binding sites were blocked for 1 h in blocking solution (PBS in 3% bovine serum albumin) containing 1% Triton X-100. The sections were then double-stained for BrdU labeling and SOV or saline administration. Following 90-min MCA occlusion and reperfusion (day 0), the rats received i.p. injections of BrdU and SOV or saline on days 1 to 7. Their brains were transcardially harvested on day 8. B, coronal sections (bregma level + 1 mm) prepared on day 8 were Nissel-stained to evaluate the area of infarction. Scale bar, 5 mm.

Quantitative analysis of infarct area coronal sections from rats treated with 25 or 12.5 mM SOV and the saline controls. Analysis was on day 8; each group consisted of 15 rats. Values are the mean ± S.D. (millimeter²).

We cut the brain with a vibratome and obtained many serial 30-μm thick coronal sections at a level of bregma +3 to −1 mm. From these sections, we have selected the serial sections in which SVZ was clearly observed, based on an anatomical structure, and we have performed immunostaining. All BrdU-positive nuclei in the SVZ were counted. BrdU-positive nuclei in these areas are presented in Fig. 1.

Evaluation of the density of BrdU-positive cells. A to D, laser-scanning confocal microscopic images of immunohistochemical staining for BrdU in the SVZ on the eighth day after MCA occlusion. A and B, ischemic rats. C and D, nonischemic rats. A and C, SOV-treated. B and D, saline-treated. Scale bar, 100 μm. MCAO, middle cerebral artery occlusion. E, quantitative analysis of the density of BrdU-positive cells in the ipsilateral and contralateral SVZ of ischemic rats treated with 25 or 12.5 mM SOV or with saline. Each group consisted of 15 rats. *, p < 0.05 versus other groups; #, p < 0.05 versus contralateral. F, quantitative analysis of the density of BrdU-positive cells in the ipsilateral SVZ of nonischemic rats treated with 25 mM SOV or with saline. G, quantitative analysis of the density of BrdU-positive cells in the ipsilateral caudate putamen of ischemic rats treated with 25 or 12.5 mM SOV or with saline. H, quantitative analysis of the density of BrdU-positive cells in ipsilateral parietal cortex of ischemic rats treated with 25 or 12.5 mM SOV or with saline.
terms of cell density per squared millimeter. The cell density in seven examined sections per rat was averaged to obtain a mean density value for each animal.

Double-immunohistochemical-stained sections were analyzed with a laser-scanning confocal imaging system mounted on a Fluoview FV300 laser confocal microscope (Olympus Optical Co.). Emissions for BrdU and Dcx, BrdU and NeuN, BrdU and GFAP, BrdU and p-Akt, and BrdU and p-ERK antibodies, green (fluorescein for BrdU immunoreactivity) and red (rhodamine for Dcx, Texas Red for NeuN, GFAP, p-Akt, and p-ERK immunoreactivity), were elicited with a laser beam at 488 and 647 nm. Emissions were sequentially acquired with two separate photomultiplier tubes through 522- and 680-nm emission filters. Images were stored on the computer, and the protocol used for BrdU-positive cell counting was applied to determine the number of cells with double immunoreactivity. The density of these cells in the SVZ was evaluated.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Staining. DNA fragmentation and apoptotic bodies were detected with the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) method using an in situ apoptosis detection kit (Takara Bio Inc., Shiga, Japan). TUNEL signals were amplified with streptavidin Alexa Fluor-488 conjugate (1:200; Invitrogen) using the TSA Biotin System (PerkinElmer Life and Analytical Sciences, Boston, MA).

Statistics. All values reported here were expressed as means ± S.D. Overall statistical significance for differences among groups was tested by one-way analysis of variance, followed by multiple comparisons between each group or between control and other groups using Dunnett’s multiple comparison test. \( p < 0.05 \) was considered significant.

Results

On Nissel-stained sections, the infarct area in rats treated with 25 mM SOV, 12.5 mM SOV, or saline at 24 h after the induction of 90-min MCA occlusion was 6.91 ± 0.29, 6.52 ± 0.12, and 6.31 ± 0.15 mm², respectively, and these values were...
were not significantly different (Fig. 1C). In each group, the area of infarction included the caudate putamen with some extension into the parietal cortex (Fig. 1B). This finding coincides with our previous observation (Hasegawa et al., 2003) that SOV injected more than 24 h after ischemia induction did not inhibit ischemic neuronal injury.

As shown in Fig. 2, A, B, and E, in rats exposed to 90-min MCA occlusion and injected for the next 7 days with BrdU, the ipsilateral SVZ clearly contained BrdU-positive cells. The density of these cells per squared millimeter was 5564 ± 131, 3569 ± 249, and 1734 ± 55 in rats treated with 25 mM SOV, 12.5 mM SOV, or saline, respectively. The density of BrdU-positive cells in the ipsilateral SVZ was significantly greater than that in SOV-treated animals ($p < 0.05$) and was dose-related. At 25 and 12.5 mM SOV, the density of BrdU-positive cells in the contralateral SVZ was 1156 ± 40 and 1143 ± 22; it was 1134 ± 43 in rats injected with saline. The differences were not statistically significant (Fig. 2E). But in each group, the density of BrdU-positive cells in the ipsilateral SVZ was significantly greater than that in the contralateral SVZ (Fig. 2E). We also found no significant differences when we examined the density of BrdU-positive cells in the ipsilateral caudate putamen and parietal cortex of rats treated with 25 or 12.5 mM SOV or injected with saline (Fig. 2, G and H). Our data indicate that SOV, delivered in the subacute phase after MCA occlusion, dose dependently enhanced cell proliferation in the ipsilateral SVZ of adult rats. There was no significant difference in the density of BrdU-positive cells in the SVZ of nonischemic rats treated with SOV or saline (Fig. 2, C, D, and F). The caudate putamen and parietal cortex of these animals did not contain BrdU-positive cells (data not shown).

To characterize the proliferating cells, we stained tissue sections for Dcx, a marker of migrating neuroblasts (Fig. 3). According to Nacher et al. (2001), in the normal adult rats, Dcx is expressed in the SVZ and rostral migratory stream (RMS), but only a few Dcx-expressing cells were detected in the striatum. We found a similar Dcx expression in nonischemic rats (Fig. 4, D and F). Conversely, Dcx-labeled cells were abundant in rats exposed to transient MCA occlusion (Fig. 3, B, E, H, and T). As shown in Fig. 3F, the density (cells per millimeter$^2$) of BrdU-Dcx double-positive cells in the SVZ after MCA occlusion was 3201 ± 28 and 2061 ± 24 in rats treated with 25 and 12.5 mM SOV, respectively; it was 1175 ± 47 in rats injected with saline. The density of BrdU-Dcx double-positive cells was significantly higher in SOV-treated ischemic rats ($p < 0.05$), and the increase was SOV dose-dependent. In contrast, the ratio of BrdU-Dcx double-positive cells among BrdU-positive cells was not significantly different; it was 57.5, 57.8, and 67.8% in rats treated with 25 mM SOV, 12.5 mM SOV, or saline (Fig. 3U).

In our study, none of the BrdU-positive cells in the ipsilateral SVZ, cortex, and subcortex exhibited NeuN immunoreactivity (data not shown). Approximately 5% of BrdU-positive cells were also positive for GFAP in ischemic rats sacrificed 24 h after the last injection of SOV or saline (Fig. 3, J–R).

Next, we performed immunostaining of Nestin, which is a class VI intermediate filament protein, that has been used as a biological marker to identify neural stem cells (Lendahl et al., 1990; Reynolds et al., 1992). Nestin-labeled cells were abundant in rats exposed to transient MCA occlusion (Fig. 5, B, E, G, and J). As shown in Fig. 5M, the density (cells per millimeter$^2$) of BrdU-Nestin double-positive cells in the SVZ after MCA occlusion was 2990 ± 339 in rats treated with 25 mM SOV, and it was 1133 ± 44 in rats injected with saline. The density of BrdU-Nestin double-positive cells was significantly higher in SOV-treated ischemic rats ($p < 0.05$). Furthermore, the density (cells per millimeter$^2$) of Nestin-Dcx double-positive cells in the SVZ after MCA occlusion was 1923 ± 107 in rats treated with 25 mM SOV, and it was 1525 ± 88 in rats injected with saline. The density of Nestin-Dcx double-positive cells was significantly higher in SOV-treated ischemic rats ($p < 0.05$) (Fig. 5N). Alternatively, the ratio of BrdU-Nestin double-positive cells among BrdU-positive cells was not significantly different; it was 60.6 and 64.5% in rats treated with 25 mM SOV and saline, respectively (Fig. 5O).

These data indicate that SOV does not enhance the proliferation of only neuroblasts. Rather, it may enhance the proliferation of total progenitor cells in the SVZ ipsilateral to the site of MCA occlusion.

To estimate apoptotic change in the SVZ, we used TUNEL staining (Fig. 6, A and B). The density (cells per millimeter$^2$)
of TUNEL-positive cells in the SVZ after MCA occlusion was 52 ± 12 in rats treated with 25 mM SOV, and it was 49 ± 3 in rats injected with saline. The density of TUNEL-positive cells was not significantly different in rats treated with 25 mM SOV or saline (Fig. 6E). According to these data, it was suggested that the major effect of SOV is activation of SVZ cells, rather than inhibition of apoptosis.

Because we previously found that SOV exerts its neuroprotective effect via activation of Akt and ERK (Kawano et al., 2001; Hasegawa et al., 2003), we investigated their phosphorylation in BrdU-positive cells using anti p-Akt and p-ERK antibodies. We compared the density of immunoreactive cells in the SVZ of rats injected with 25 mM SOV or saline and found that most of the BrdU-positive cells were immunostained for p-Akt and p-ERK antibodies (Figs. 7 and 8). The density (cells per millimeter²) of BrdU/p-Akt double-positive cells in the SVZ of ischemic rats treated with 25 mM SOV (3409 ± 63) was significantly higher than that in rats injected with saline (1620 ± 67; p < 0.05) (Fig. 8, A–D, and I). As for BrdU/p-ERK, the ratio of BrdU/p-ERK double-positive cells among BrdU-positive cells was not significantly different; it was 51.0 or 69.2% in rats treated with 25 mM SOV or saline, respectively (Fig. 8J), and there was no difference between nonischemic rats treated with 25 mM SOV or saline (Fig. 8, E–H, and K). Few GFAP-positive cells were immunostained for p-Akt and p-ERK. We have observed many double-positive cells of p-Akt and p-ERK, p-Akt and Dcx, and p-ERK and Dcx. Thus, we considered that Akt and ERK were activated in Dcx-positive cells after ischemia (see Supplemental Figure). It was concluded that activation of Akt and ERK correlated with activation of SVZ cells by SOV.

**Discussion**

**Proliferating Cells in SVZ in the Adult Mammalian Brain.** Cells in the SVZ give rise to most neurons and glial cells in the embryonic forebrain (Gage et al., 1998; Garcia-Verdugo et al., 1998), and neuroepithelial SVZ cell populations contain multipotent progenitor cells that give rise to neurons and glial cells throughout life (Garcia-Verdugo et al., 1998). In the normal mouse brain, proliferating cells constitute an average of 10% of the cell population in the SVZ.
treated with 25 mM SOV or saline. Thus, because the effect of SOV was restricted to the ischemic hemisphere, we suggest that the ischemic insult triggered-off the proliferation of SVZ cells and that SOV treatment induced the proliferation of these cells in a dose-dependent manner. SOV behaves as a competitive inhibitor of protein tyrosine phosphatase (Bevan et al., 1995; Huyer et al., 1997; Zhang et al., 1997) and increases the tyrosine phosphorylation levels of intracellular proteins (Morinville et al., 1998). Following ligand binding, some growth factor receptors are activated by tyrosine residue autophosphorylation, and SOV can activate or prolong the activity of these receptors. Indeed, we reported that the administration of SOV and IGF-1 increased Akt activation via IGF-receptor activation and that SOV is neuroprotective by delayed neuronal cell death of gerbil hippocampal CA1 neurons (Kawano et al., 2001). We also found that it exerts antiapoptotic effects via Akt and ERK activation after transient MCA occlusion (Hasegawa et al., 2003). The observed increase in BrdU-positive cells may be reflection of decreased apoptosis. However, because we found that a few cells in the SVZ of SOV-treated ischemic rats were TUNEL-positive, we postulate that SOV administered during the subacute phase affected cell proliferation rather than apoptosis.

Fig. 6. Apoptotic cells in SVZ and caudate putamen. MCA occlusion-induced apoptosis in the SVZ (A and B) and caudate putamen (C and D). Sequential apoptotic changes in the ipsilateral SVZ were observed by TUNEL (green) and propidium iodide (PI) staining (red). The photographs were taken on the eighth day after MCA occlusion and are representative of rats treated with 25 mM SOV (A and C) or saline (B and D). Scale bar, 100 μm. CP, caudate putamen. E, quantitative analysis of the density of TUNEL-positive cells in the ipsilateral SVZ of ischemic rats treated with 25 mM SOV or saline.

(Morshead and van der Kooy, 1992). We identified BrdU-positive cells in the adult rat SVZ, their characteristics were similar to those reported for SVZ progenitor cells (Morshead and van der Kooy, 1992; Gage et al., 1998; Garcia-Verdugo et al., 1998).

BrdU, an analog of thymidine, is incorporated into DNA of cells during the S phase; it has been used to investigate cell proliferation (Dolbeare, 1996; Kuhn et al., 1996). Zhang et al. (2001) reported that proliferating progenitor cells in the adult rodent brain increase after MCA occlusion. We found that progenitor cells in the ipsilateral SVZ of rats increased after 90-min MCA occlusion; their proliferating pattern was similar to that reported by Zhang et al. (2001). On the basis of our findings, we concluded that in adult rats, SVZ cells proliferate in response to an ischemic insult.

SOV Enhances Proliferating Progenitor Cells in the SVZ after Ischemia. In ischemic rats treated with SOV, the density of BrdU-positive cells in the SVZ was dramatically increased. Interestingly, SOV enhanced only the proliferation of progenitor cells in the ipsilateral SVZ; it had no effect on cells in the contralateral SVZ or in the normal rat brain. Thus, because the effect of SOV was restricted to the ischemic hemisphere, we suggest that the ischemic insult triggered-off the proliferation of SVZ cells and that SOV treatment induced the proliferation of these cells in a dose-dependent manner. SOV behaves as a competitive inhibitor of protein tyrosine phosphatase (Bevan et al., 1995; Huyer et al., 1997; Zhang et al., 1997) and increases the tyrosine phosphorylation levels of intracellular proteins (Morinville et al., 1998). Following ligand binding, some growth factor receptors are activated by tyrosine residue autophosphorylation, and SOV can activate or prolong the activity of these receptors. Indeed, we reported that the administration of SOV and IGF-1 increased Akt activation via IGF-receptor activation and that SOV is neuroprotective by delayed neuronal cell death of gerbil hippocampal CA1 neurons (Kawano et al., 2001). We also found that it exerts antiapoptotic effects via Akt and ERK activation after transient MCA occlusion (Hasegawa et al., 2003). The observed increase in BrdU-positive cells may be reflection of decreased apoptosis. However, because we found that a few cells in the SVZ of SOV-treated ischemic rats were TUNEL-positive, we postulate that SOV administered during the subacute phase affected cell proliferation rather than apoptosis.

Dempsey et al. (2003) showed that in adult rats, exogenous IGF-1 increased the proliferation of cells induced by focal ischemia. We postulated that SOV activated the proliferation of progenitor cells via the activation of growth factor receptors such as the IGF receptor. This hypothesis explains why SOV had an effect only on the ipsilateral side and only after ischemic insult. Ischemia may result in an increase in some growth factors in a restricted area (ipsilateral side), which, in turn, may lead to proliferation of progenitor cells in the SVZ, and SOV may enhance the effect of these growth factors.

Zhang et al. (2001) reported that in untreated ischemic rats, the number of BrdU-positive cells in the SVZ significantly increased during the first 14 days; the increase peaked on day 7, and the number of dividing cells returned to the control level at 3 to 5 weeks after ischemia induction. Thus, we examined the eighth day after ischemic insult, and the peak cell density in SOV-treated rats was approximately 3 times greater than in our saline-treated controls. We found that the effects of SOV were dose-dependent. Our observations suggest that the effect of SOV is ascribable to its enhancement of progenitor cell proliferation.

Characteristics of Proliferating Cells in SVZ. Because cells in the SVZ possess proliferative potential and neural stem cell characteristics (Chiasson et al., 1999), we stained sections for Dcx, a marker for migrating neuroblasts. Nacher et al. (2001) reported that in the normal adult rat brain, Dcx was expressed in the SVZ and RMS; however, only a few Dcx-positive cells were detected in the striatum. We found a similar Dcx expression pattern in our sham-operated rats (Figs. 2 and 4). Although the number of Dcx-positive cells was significantly higher in SOV-treated rats, the ratio of BrdU/Dcx double-positive cells among BrdU-positive cells in SOV-treated and saline-treated rats was not significantly different from our ischemic rats (Fig. 3).

None of the BrdU-positive cells exhibited NeuN immunoreactivity in the ipsilateral SVZ, cortex, and subcortex in the present study (data not shown). About 5% of BrdU-positive cells were immunoreactive for GFAP, and the ratio of BrdU/GFAP double-positive cells was not significantly different between SOV-treated and control rats. Although the number
of BrdU/Nestin double-positive cells significantly higher in SOV-treated rats, the ratio of BrdU/Nestin double-positive cells among BrdU-positive cells in SOV-treated and saline-treated rats was not significantly different in our ischemic rats (Fig. 5). We found a similar expression pattern as that observed with Dcx. On the basis of these observations, we concluded that SOV has the potential to increase the total number of progenitor cells in the SVZ after MCA occlusion and that its effect is not cell type-specific.

**SOV Effect Is Correlated to Activation of Akt and ERK in the SVZ.** Mammalian neurogenesis is regulated by an interaction between intrinsic genetic mechanisms and extrinsic cues, such as growth factors and their downstream signaling pathways. PI3K/Akt signaling has been shown to play an important role in neurogenesis (Li et al., 2001). In addition, Akt is critical mediators of cellular responses to growth factors. The MAPK cascade is a highly conserved signaling system through which cells respond to a variety of extracellular stimuli (Marshall, 1995), and MAPK signaling promotes neurogenesis (Li et al., 2001; Zhao et al., 2003). Both PI3K and MAPK pathways are implicated in the proliferation of progenitor cells (Jin et al., 2005).

We previously reported that SOV induced the activation of Akt and ERK, which resulted in an antiapoptotic effect (Kawano et al., 2001, 2002); however, it remained unclear whether their activation contributes to cell proliferation. To investigate the mechanisms underlying the actions of SOV, we performed immunohistochemical studies using antibodies against p-Akt and p-ERK. In BrdU-positive cells of SOV-treated rats, the immunoreactivity of p-Akt and p-ERK was significantly increased. Thus, the activation of Akt and ERK correlates to SVZ cell proliferation by SOV. It is possible that activated Akt and ERK may enhance cell proliferation. However, the causal relationship of SOV, Akt/ERK activation, and SVZ cell activation is still unclear.

**CNS Self-Renewal Potential after Ischemia.** Under physiological conditions, cells originating in the SVZ migrate along the RMS via chain migrations to the olfactory bulb (OB) where they differentiate into granule and periglomerular neurons (Garcia-Verdugo et al., 1998). Progenitor migra-
tion from the SVZ to the forebrain ceases by the second postnatal week (Levison and Goldman, 1993). However, after focal cerebral ischemia, an increase in the number of BrdU-labeled cells in the SVZ results in an increase in the number of BrdU-labeled cells in the OB (Zhang et al., 2001). Therefore, some BrdU-labeled cells detected in the ipsilateral cortex and subcortex after an ischemic insult may derive from cells in the SVZ.

The RMS, along which progenitor cells migrate from the SVZ to the OB, is located near the peri-infarct zone. Gu et al. (2000) suggested that the BrdU/microtubule-associated protein-positive cells they observed in the ischemic cortex of adult rats may have been SVZ cells that escaped the RMS. Parent et al. (2002) showed that some newly proliferated SVZ cells migrated into the striatum and differentiated into a region-specific phenotype, and Arvidsson et al. (2002) reported that after stroke, some newly formed neurons and neuroblasts migrated from the SVZ into damaged striatal areas where they matured into medium-sized spiny neurons. These observations suggest that after ischemia, neuronal cells are regenerated from cells migrating from the SVZ, although their density may be low.

Indeed, region-specific neuronal regeneration has been observed several weeks after ischemia (Gu et al., 2000; Arvidsson et al., 2002; Gritti et al., 2002). Because we studied the effect of SOV on the eighth day after transient MCA occlusion, we were unable to assess the presence of newly formed neurons in the damaged area. Studies are underway in our laboratory to determine whether SOV treatment of rats with transient MCA occlusion results in the presence of newly formed neurons in the chronic phase.

In summary, we demonstrated that in rats exposed to transient MCA occlusion, SOV induces an increase in the proliferation of progenitor cells in the ipsilateral SVZ. SOV had no effect on the contralateral side or in nonischemic rats. We postulate that the delivery of SOV after an ischemic insult may enhance subsequent CNS self-repair.

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References


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