

# Systemic injection of recombinant human erythropoietin after focal cerebral ischemia enhances oligodendroglial and endothelial progenitor cells in rat brain

Young Jae Kim<sup>1</sup>, Yong-Wook Jung<sup>2</sup>

<sup>1</sup>Department of Laboratory Medicine, Masansamsung Medical Center, School of Medicine, Sungkyunkwan University, Masan, <sup>2</sup>Department of Anatomy, College of Medicine, Dongguk University, Gyeongju, Korea

**Abstract:** Erythropoietin (EPO) has been demonstrated the ability of recombinant human erythropoietin (r-Hu-EPO), when administered intracerebro-ventricularly, to improve stroke outcome through the reduction of stroke damage. In a brain ischemic model, however, systemic administration of r-Hu-EPO has not been intensely investigated given that in general, large glycosylated molecules have been deemed incapable of crossing the blood-brain barrier. In this study, administration of r-Hu-EPO for 4 days, intraperitoneally after ischemia-reperfusion (I-R) increased the number of bromodeoxyuridine (BrdU)-positive cells in the penumbra ( $10.1 \pm 1.4$ ,  $n=5$ ,  $P<0.05$ ) and in the subventricular zone (SVZ) of the lateral ventricle (LV) ( $25 \pm 2.7$ ,  $n=5$ ,  $P<0.05$ ) as compared with those of I-R (penumbra:  $2.5 \pm 0.7$ ; SVZ of LV:  $3.8 \pm 1.5$ ). A significant increase of BrdU-positive cells in these areas was coincident with a strong immunoreactivity of oligodendrocyte progenitor cell marker (2', 3'-cyclic nucleotide 3'-phosphodiesterase). Furthermore, r-Hu-EPO administration increased the number of BrdU-positive cells in the choroid plexus ( $7.8 \pm 2.3$ ,  $n=5$ ,  $P<0.05$ ) and in cerebral blood vessels ( $3.5 \pm 1.3$ ,  $n=5$ ,  $P<0.05$ ) when compared with those of I-R (choroid plexus:  $1.2 \pm 0.5$ ; cerebral blood vessels:  $0.6 \pm 0.1$ ). These results suggest that, even when systemically administered, r-Hu-EPO may have therapeutic potential for stroke via the proliferation of oligodendroglial and endothelial progenitor cells.

**Key words:** Erythropoietin, systemic administration, oligodendroglial and endothelial progenitors

Received February 5, 2010; Revised April 30, 2010; Accepted May 3, 2010

## Introduction

Erythropoietin (EPO) was first described as a cytokine which acted as a major regulator of erythropoiesis (Moritz *et al.*, 1997). However, the biological role of EPO is not limited only to haematopoiesis (Digicaylioglu *et al.*, 1995; Morishita *et al.*, 1997). Vitellaro-Zuccarello *et al.* (2008) described that

acute administration of recombinant human erythropoietin (r-Hu-EPO) in a rat model of spinal cord injury reduces the lesion size, attenuates gliosis and microglial/macrophage activation, enhances myelinogenesis and improves locomotor outcome. In addition, Kadota *et al.* (2009) reported that continuous low dose infusion of EPO exerted neuroprotective/rescue effects with neurogenic potential in a rat Parkinsonian model.

It has been demonstrated that focal ischemic injury stimulates progenitor proliferation and increases the size of the adult rodent forebrain subventricular zone (SVZ) of the lateral ventricle (LV) (Arvidsson *et al.*, 2002; Parent *et al.*, 2002). Further, Wang *et al.* (2004) reported that

### Corresponding author:

Yong-Wook Jung  
Address: 707, Sukjang-dong, Gyeongju, Korea [780-714]  
Tel: +82-54-770-2404, Fax: +82-54-770-2447, E-mail: jungyw@dongguk.ac.kr

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intracerebroventrically injected EPO enhanced neurogenesis in the SVZ of the LV after stroke in the adult rodent. However, other studies have reported depletion of neuronal stem cells in the SVZ after hypoxia-ischemia (Levison *et al.*, 2001; Skoff *et al.*, 2001). Additionally, neuronal and/or astroglial progenitors also reside in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampal formation (Gage *et al.*, 1998). Despite strong evidence for the neuroprotective benefits of EPO in the treatment of acute stroke (Sakanaka *et al.*, 1998; Sirén *et al.*, 2001), it is unclear whether the neuroprotective effects of EPO mediate proliferation of neuronal and/or glial progenitor cells in germinative areas such as the SVZ of the LV and the SGZ of the DG. Moreover, the neuroprotective mechanism of systemically (intraperitoneally) administered EPO remains poorly understood.

Cultivated mature endothelial cells also express EPO and erythropoietin receptor (EPOR) and endothelial cell proliferation has been shown to be induced by EPO (Vogel *et al.*, 1997). In practical, endothelial cells and hematopoietic cells are believed to be derived from the same mesenchymal precursor, the so called hemangioblast. This may explain why endothelial cells carry the EPOR and can be stimulated by EPO (Marti *et al.*, 2000). However, there has been a lack of reports regarding endothelial proliferation in the ischemic rat brain under EPO treatment despite the fact that neuronal/glial proliferation and angiogenesis is known to be coupled in the brain and that both are cooperative in the treatment of the ischemic brain (Leventhal *et al.*, 1999; Kim *et al.*, 2009).

The present study sought out to explore the potential for the proliferation of neuronal/glial and endothelial progenitor cells after ischemia-reperfusion (I-R) in subjects treated intraperitoneally with r-Hu-EPO. We selected a model of I-R for this study rather than one of permanent ischemia because it more closely mimicked the clinical situation of an acute cerebral infarction with early reperfusion, producing a necrotic core surrounded by a substantial penumbra.

## Materials and Methods

### Reagents

The r-Hu-EPO (Epoetin beta, 2000 IU/UI/0.3 ml, Roche) used was a human 165-aa glycoprotein manufactured using recombinant DNA technology and containing the identical amino acid sequence to isolated natural EPO as well the same biological activity (Egrie *et al.*, 1986; Pardridge 1997). R-Hu-

EPO is approximately 80% homologous to rodent EPO, and it has been shown to be biologically active in rodents for erythropoietic as well as neurotrophic functions. Although an immune response against human antigens can be elicited in rodents, it requires several weeks to obtain even a weak response and thus is not important in the context of these short-term studies. All experiments were performed by using r-Hu-EPO, which was formulated as a sterile, colorless liquid in isotonic sodium chloride/sodium citrate or sodium chloride/ sodium phosphate buffered saline (PBS) with 1.25% human albumin.

### Induction of I-R

Studies were carried out in 9-week-old male Sprague-Dawley rats (250~280 g, n=13) each having access to drinking water and standard rodent food pellets ad libitum. All experimental protocols were reviewed and approved by the Animal Care and Use Committee of Dongguk University (IRB: 09~35). Animal care and use were in accordance with the guidelines of the National Institutes of Health (Bethesda, MD).

Focal cerebral I-R was induced by occlusion of the left middle cerebral artery as described previously (Hasegawa *et al.*, 1994). Briefly, anesthesia was induced with 3% isoflurane in a mixture of oxygen/nitrous oxide (30 : 70), and rats were maintained with 1% isoflurane in the oxygen/nitrous oxide gas mixture. A catheter was inserted and positioned in the femoral artery. Arterial blood pressure was measured and recorded continuously throughout the procedure, and body temperature was maintained between 36.5°C and 37.0°C with the aid of a heating pad and heating lamp. The left middle cerebral artery was occluded using a 4~0 mono filament (3 cm in length) coated with a mixture of silicone resin. For reperfusion, the nylon filament was withdrawn 2 h after middle cerebral artery occlusion (MCAO) and then perfused for 14 days.

### EPO and BrdU treatment after I-R

To examine whether treatment with systemically administered r-Hu-EPO stimulated the proliferation of neural and endothelial progenitor cells in the ischemic brain, r-Hu-EPO at a dose of 5,000 units/kg was intraperitoneally injected daily for 4 days into rats, starting reperfusion after MCAO. Doses of r-Hu-EPO were selected based on previous studies (Sirén *et al.*, 2001; Calvillo *et al.*, 2003). To achieve labeling of proliferating cells, bromodeoxyuridine (BrdU,

100 mg/kg, Sigma Aldrich Corp., St Louis, MO, USA) was injected intraperitoneally daily for 4 days into rats starting 10 days after reperfusion. After completing the I-R protocols, animals were anesthetized with isoflurane and the brains were removed and subject to 2% 2,3,5-triphenyltetrazolium chloride (TTC: sigma Aldrich Corp., St Louis, MO, USA) staining and immunohistochemical studies.

### TTC staining of infarction

Rats (n=3) were killed and their brains were quickly removed and sectioned into 2-mm-thick slices starting at the frontal pole using a Brain Matrix Slicer (Vibratome Co.). Slices were then immersed in TTC in a petri-dish and incubated at 37°C for 20 minutes. Slices were flipped at the 10-minute mark for consistent staining of anterior and posterior faces and then scanned using an Epson perfection 1,200 U scanner and Adobe Photoshop software.

### Tissue processing

The brains from the I-R (n=5) and EPO-treated I-R rats (n=5) were fixed with a transcardiac infusion of 4% paraformaldehyde and post-fixed in the same fixative for 12 h. Perfused brains were then paraffin-embedded and 5 µm thick serial coronal sections were obtained at the level of dorsal third ventricle (bregma -2.30 mm). Paraffin wax was removed in xylene over night at room temperature (RT) and the sections were rehydrated with ethanol (99%, 96%, 70%). After washing in distilled water, the sections were then stained using a DAKO kit for immunohistochemistry.

### Pretreatment for BrdU immunohistochemistry

After washing in distilled water, the sections were then incubated in 50% formamide/2XSSC buffer (0.3M NaCl/ 0.03 M sodium citrate) at 65°C for 2 h, rinsed in 2XSSC, incubated in 2 M HCl for 30 min, and rinsed in 0.1 M borate buffer pH 8.5 for 10 minutes.

### Immunohistochemistry

Following deparaffinization, sections were stained with a DAKO kit. Endogenous peroxidase activity was blocked by 5-min incubation in 3.0% H<sub>2</sub>O<sub>2</sub>. After washing in PBS, the sections were reacted with rat anti-BrdU (Sigma, St. Louis, Mo, USA, 1 : 100), rat anti-neuronal nuclei (NeuN) monoclonal antibody (Chemicon, Temecula, CA, USA, 1 : 100), rat anti-gliar fibrillary acidic protein (GFAP) monoclonal antibody (Boehringer, Mannheim, Germany, 1 : 200), rat

anti-2', 3'-cyclic nucleotide 3'-phosphodiesterase monoclonal antibody (CNPase) (Chemicon, Temecula, CA, USA, 1: 100) for 16~18 h at 4°C, and consequently with biotinylated universal anti-mouse, -goat, and -rabbit immunoglobulins in PBS for 30 min. After washing in PBS, the sections were incubated with streptoavidin conjugated to horseradish peroxidase (HRP) in PBS for 30 min. Finally, the sections were reacted with a solution containing diaminobenzidine (DAB) and hydrogen peroxide (0.001%). The sections were then counterstained with Mayer's hematoxylin to visualize cell nuclei. BrdU-positive cells in each group of 5 animals were observed at the level of bregma -2.30 mm. At least five brain sections from each group were observed under 20X magnification field. All BrdU-positive cell counting was performed in a blind and randomized manner. Values were presented as means±SE. Comparisons between groups were made using un-paired Student *t*-test and *P* values less than 0.05 were considered significant. Quantification of the relative optical density of the immunoreactivity for NeuN, GFAP and CNPase was performed using Scion Image software (version 1.59).

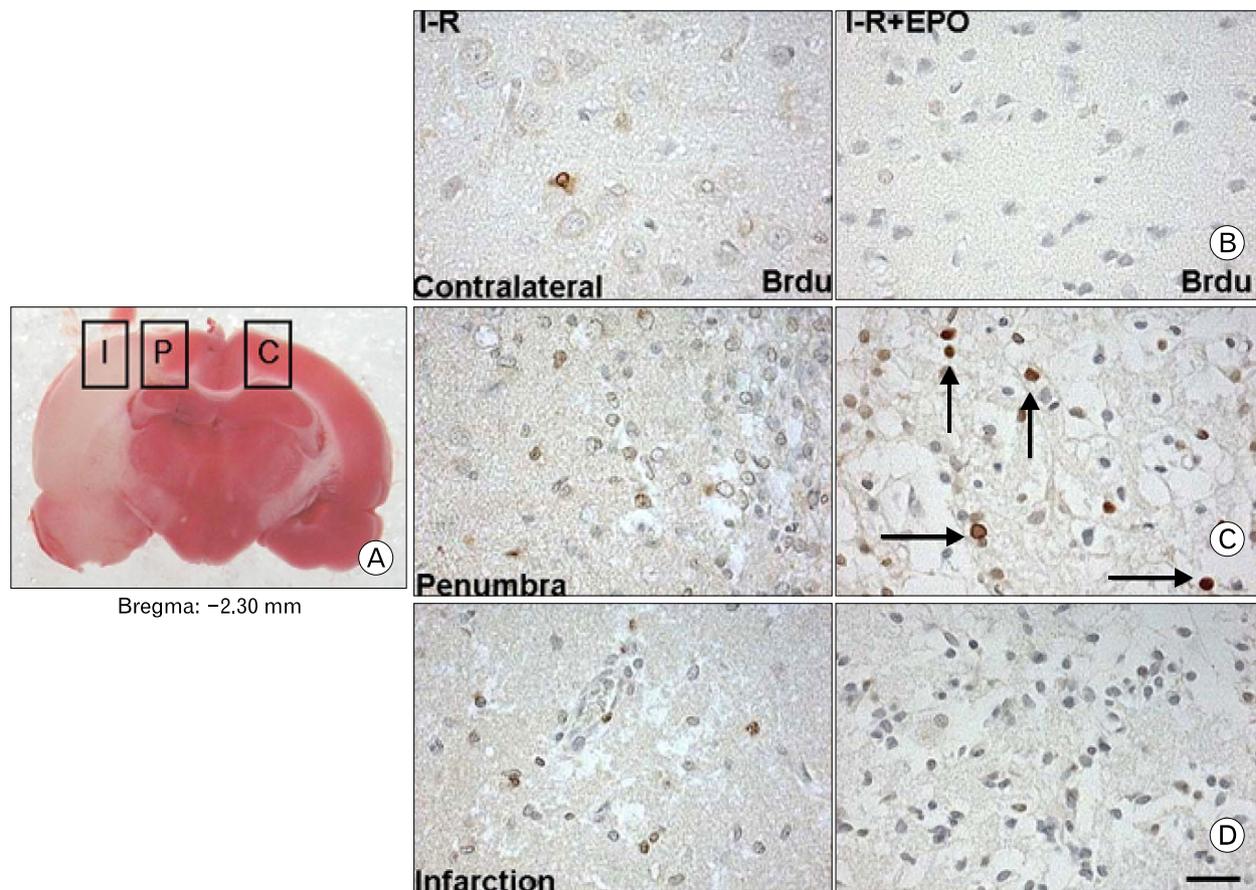
## Results

### Establishment of I-R

In agreement with a previous study (Yan *et al.*, 2001), extensive infarction was detected in the frontal and parietal cortical and subcortical areas, including the basal ganglia, over a series of brain sections after I-R. However, r-Hu-EPO treatment after I-R did not affect the infarction size as compared with that of I-R (data not shown).

### Enhancement of progenitor cells in ischemic penumbra and SVZ of LV treated with r-Hu-EPO

I, P, and C in the rectangles from Fig. 1A indicate infarction, penumbra, and the contralateral cortical area. R-Hu-EPO treatment after I-R increased the number of BrdU-positive cells in the penumbra (10.1±1.4, n=5, *P*<0.05) compared with that of I-R (2.5±0.7, n=5) (Fig. 1C). However, treatment with r-Hu-EPO did not increase the numbers of BrdU positive cells in the infarction area or contralateral non-ischemic cortex compared with that of the penumbra even though both showed altered neuronal morphology (Figs. 1B and D). Black and white arrows from Fig. 2A indicate penumbra and infarction cortical areas.



**Fig. 1.** Immunohistochemical staining of bromodeoxyuridine (BrdU)-positive cells after ischemia-reperfusion (I-R) and I-R with r-Hu-EPO treatment (I-R+EPO) in the rat brain. (A) 2,3,5-triphenyltetrazolium chloride (TTC) staining of brain slices at bregma  $-2.30$  mm after I-R. The rectangles indicate the infarction area I, ischemic penumbra P, and contralateral non-ischemic cortex C. (B~D) Treatment with r-Hu-EPO after I-R did not increase the number of BrdU-positive cells in the infarction and contralateral non-ischemic areas (B, D, I-R+EPO). However, the number of BrdU-positive cells in the ischemic penumbra of I-R+EPO rat brain was increased compared with that of the I-R (C, I-R+EPO, arrows). Scale bar,  $50 \mu\text{m}$ .

In contrast to infarction area, large amount of NeuN- and GFAP- immunoreactive cells in the penumbra were remained after I-R (Figs. 2B and C, I-R). The immunoreactivity of CNPase was almost faded in the penumbra as well as infarction area after I-R (Fig. 2D, I-R). In the penumbra, the immunoreactivity of NeuN and GFAP was unchanged after r-Hu-EPO treatment (Figs. 2B and C, I-R+EPO). However, the immunoreactivity of CNPase in the penumbra was strongly expressed after treatment with r-Hu-EPO as compared with that of I-R (Fig. 2D, I-R+EPO). The rectangle in Fig. 3A indicates the ipsilateral LV. There was a significant increase in BrdU-positive cells in the ipsilateral SVZ of the LV after r-Hu-EPO treatment ( $25 \pm 2.7$ ,  $n=5$ ,  $P < 0.05$ ) compared with that of the I-R ( $3.8 \pm 1.5$ ,  $n=5$ ) (Fig. 3B, I-R+EPO). The immunoreactivity of NeuN and GFAP in the ipsilateral SVZ of the LV after r-Hu-EPO treatment was not significantly

different from the I-R (data not shown). However, a strong increase in immunoreactivity of CNPase was detected in the ipsilateral SVZ of the LV after r-Hu-EPO treatment as compared with that of I-R (Fig. 3C, I-R+EPO). In the SGZ of the DG, r-Hu-EPO treatment did not increase the number of BrdU-positive cells in the upper and lower blade as compared with that of LV (data not shown). The increased number of BrdU-positive cells and dense labeling of CNPase in the penumbra and SVZ of the LV after r-Hu-EPO treatment are increasing the possibility that EPO selectively enhances the proliferation of oligodendrocyte progenitor cells (OPCs).

#### Enhancement of endothelial cell proliferation in ischemic brains treated with r-Hu-EPO

The letters B, C, D, and E in the rectangles indicates alphabetical numbers of panels (Fig. 4A). Panel B and

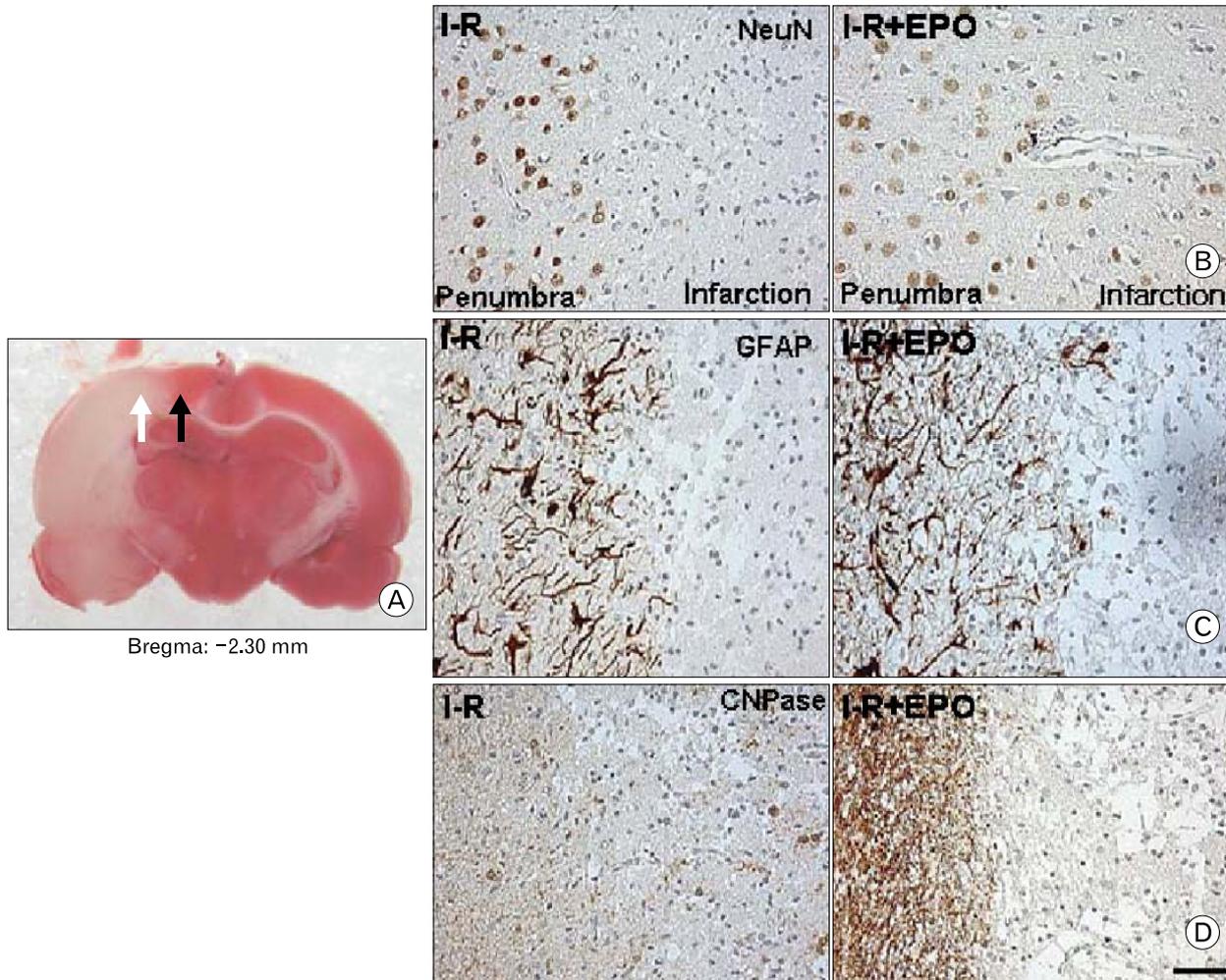


Fig. 2. Immunohistochemical staining of neuronal nuclei (NeuN), glial fibrillary acidic protein (GFAP), and 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) in infarction and penumbra areas after I-R and I-R+EPO. (A) TTC staining of brain slices at bregma  $-2.30$  mm after I-R. White and black arrows indicate infarction and penumbra areas. There were not seen NeuN-, GFAP-, and CNPase-immunoreactive cells in the infarction areas after I-R and I-R+EPO (B~D). The numbers of NeuN- and GFAP-immunoreactive cells in the penumbra of the I-R+EPO were not changed compared with those of I-R (B, C, I-R+EPO). However, CNPase-immunoreactive cells in the penumbra were increased under the treatment with r-Hu-EPO after I-R compared with that of I-R (D, I-R+EPO). Scale bar,  $50 \mu\text{m}$ .

C indicate the locations of the choroid plexus (CP) and capillary plexus of third ventricle (CP-TV) while panel D and E indicate the locations of the pia mater (PM) and blood vessels in the hippocampus. Treatment with r-Hu-EPO after I-R increased the number of BrdU-positive cells in the endothelial cells of the CP ( $7.8 \pm 2.3$ ,  $n=5$ ,  $P < 0.05$ ) and CP-TV ( $15.5 \pm 2.4$ ,  $n=5$ ,  $P < 0.05$ ) compared with those of the I-R (CP:  $1.2 \pm 0.5$ ; CP-TV:  $4.8 \pm 1.3$ ,  $n=5$ , respectively) (Figs. 4B and C). A significant increase of BrdU-positive cells in the I-R treated with r-Hu-EPO was also detected in the endothelial cells of the PM ( $10.5 \pm 2.1$ ,  $n=5$ ,  $P < 0.05$ ) compared with that of the I-R ( $3.2 \pm 1.2$ ,  $n=5$ ) (Fig. 4D). Furthermore, the number of BrdU-positive cells in the blood vessels of the hippocampus

increased after r-Hu-EPO treatment ( $3.5 \pm 1.3$ ,  $n=5$ ,  $P < 0.05$ ) compared with that of the I-R ( $0.6 \pm 0.1$ ,  $n=5$ ) (Fig. 4E).

## Discussion

### Intraperitoneal injection of r-Hu-EPO following I-R

Central and systemic EPO systems are separate, a concept which has been further reinforced by the impermeability of the blood brain barrier (BBB) to most plasma proteins. However, recent studies have reported that r-Hu-EPO, systemically injected into rats subjected to kainate-induced seizures, may be able to cross the BBB using receptor-

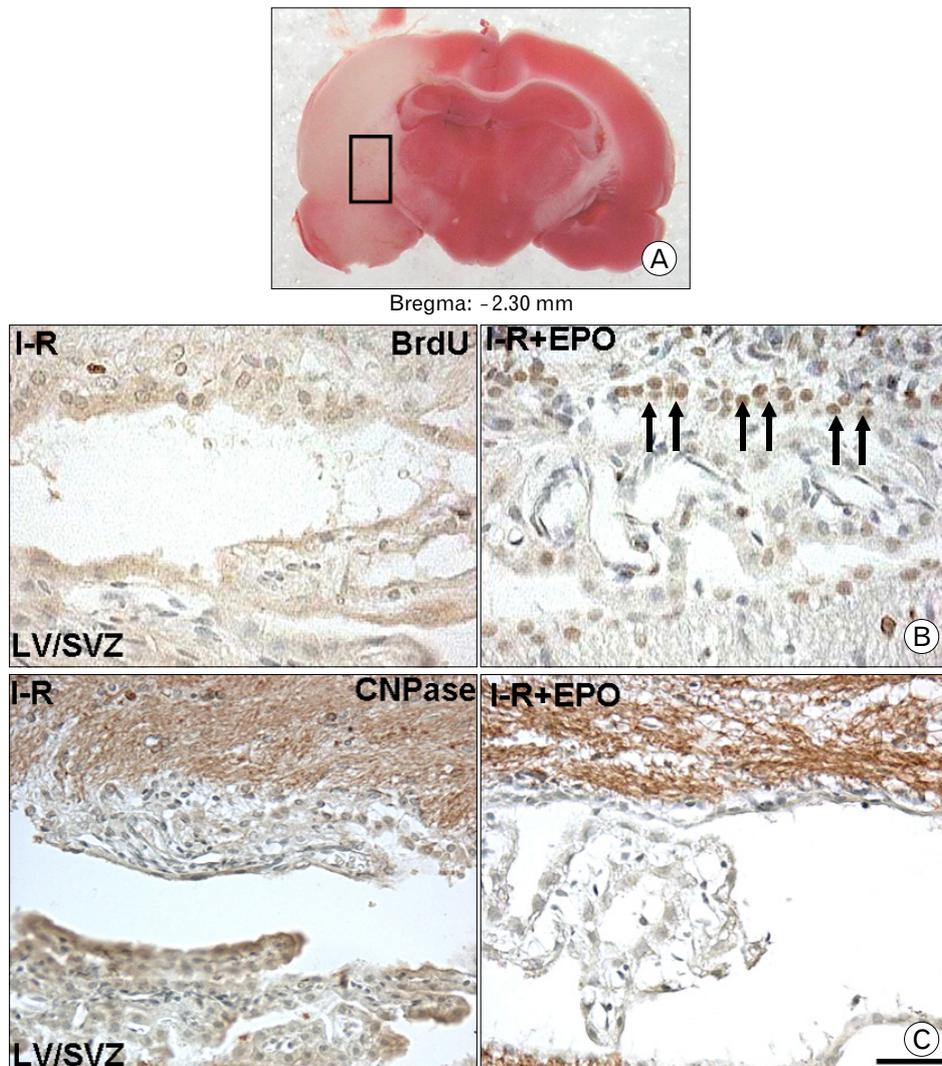


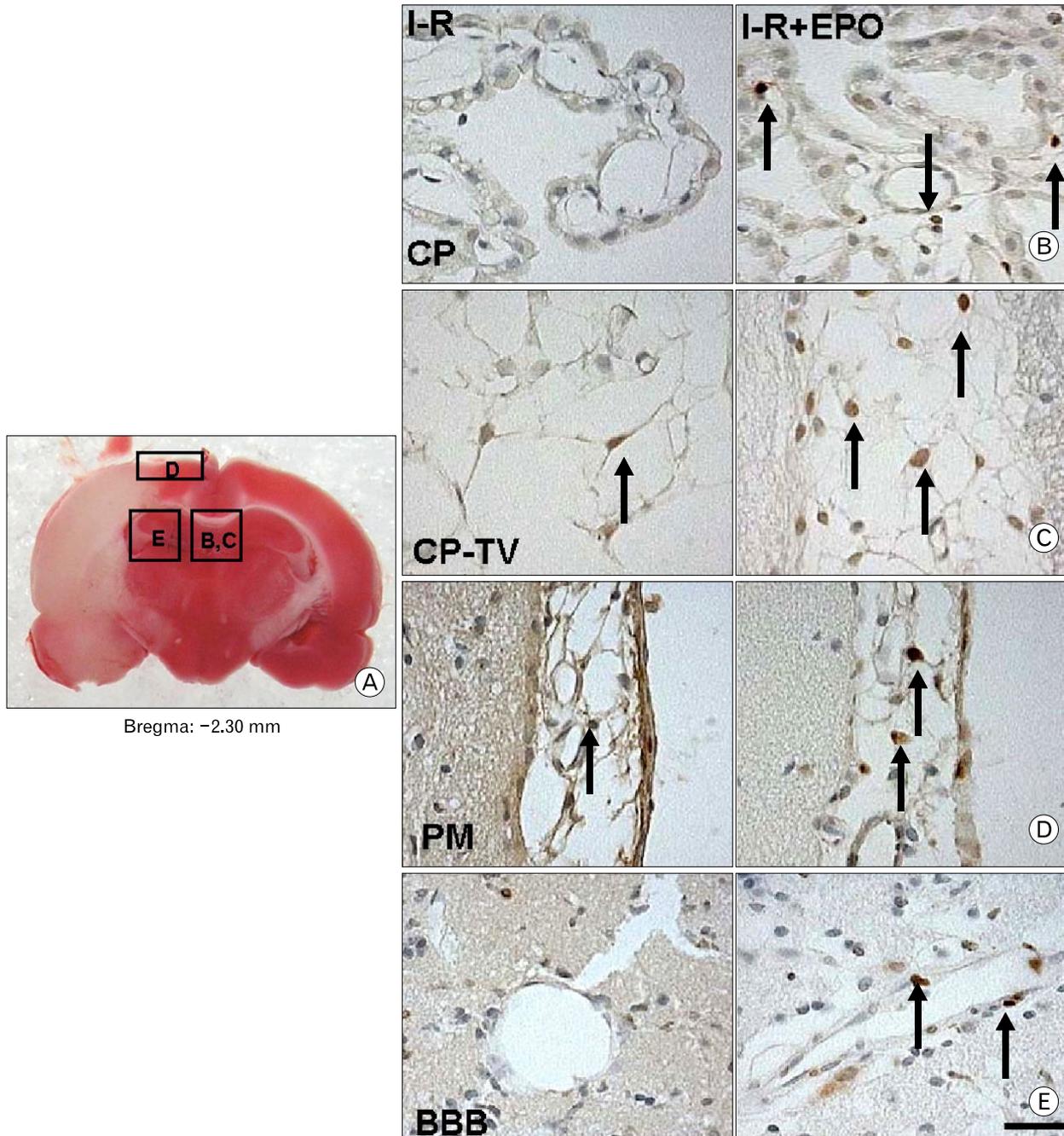
Fig. 3. Immunohistochemical staining of BrdU-positive cells in the subventricular zone (SVZ) of the lateral ventricle (LV) after I-R and I-R+EPO. (A) TTC staining of brain slices at bregma -2.30 mm after I-R. The rectangle indicates the ipsilateral LV. BrdU-positive cells in the ipsilateral SVZ of the LV under r-Hu-EPO treatment following I-R were significantly increased (B, I-R+EPO, arrows) compared with that of I-R. Immunohistochemical staining for CNPase in the ipsilateral SVZ of the LV was increased in the r-Hu-EPO treated ischemic rat as compared with that of I-R (C, I-R+EPO). Scale bar, 50  $\mu$ m.

dependent endocytosis followed by translocation into the brain (Brines *et al.*, 2000; Grasso *et al.*, 2002), thereby lessening seizure severity, elongate seizure latencies, and reduce neuronal damage (Brines *et al.*, 2000). The data in the present study has demonstrated that systemic administration of r-Hu-EPO is also effective in stimulating the proliferative potentials of progenitor cells in the ischemic penumbra and SVZ of LV in a similar fashion to intracerebro-ventriculally administered EPO. Marti *et al.* (2000) suggested the mechanism of effectiveness of systemically administered EPO in the ischemic brain, whereby ischemia produces leaks in the BBB and therefore brain cells are able to easily access blood-borne EPO in ischemic infarcted areas.

### Increased oligodendrocyte progenitor cells in the I-R brain treated with r-Hu-EPO

Our data indicate that systemic administration of r-Hu-EPO after I-R significantly increased progenitors, especially in the penumbra. However, the contralateral cortex and infarction area did not show a similar increase. EPO is required for normal brain development and regulates neurogenesis and glial cell proliferation in the adult mouse brain (Shingo *et al.*, 2001; Sugawa *et al.*, 2002), whereas stroke up-regulates endogenous EPO and its receptor (Bernaudin *et al.*, 1999). Thus, our data suggest the possibility that proliferation of progenitors in the penumbra was due to the selective increase of endogenous EPO in the penumbra in addition to the systemically administered r-Hu-EPO.

Progenitor cells that reside in the adult SVZ of the LV



**Fig. 4.** Immunohistochemical staining of BrdU-positive cells in the endothelium of the choroid plexus (CP), capillary plexus of the third ventricle (CP-TV), pia mater (PM), and blood vessels of the hippocampus after I-R and I-R+EPO treatment. (A) TTC staining of brain slices at bregma  $-2.30$  mm after I-R. B to E in the rectangle indicates the label for each panel. (B~E) A few BrdU-positive cells were found in endothelial cells of the CP-TV and PM after I-R (C, D, I-R, arrows). Treatment with r-Hu-EPO significantly increased the number of BrdU-positive cells in the CP and CP-TV compared with those of I-R (B, C). BrdU-positive cells in the PM of the ischemic cortex under r-Hu-EPO treatment increased compared with that of I-R (D). In the hippocampus, the numbers of BrdU-positive cells in the blood vessels under r-Hu-EPO treatment increased compared with that of I-R (E). Scale bar,  $50 \mu\text{m}$ .

include a subpopulation of cells that exhibit the fundamental properties of neural stem cells: long-term self-renewal and multipotentiality (Potten & Loeffler, 1990; Weiss *et al.*, 1996)

whereas the progenitor cells that reside in the SGZ of the adult DG exhibit less self-renewal ability and unipotentiality (Kuhn *et al.*, 1996; Weiss *et al.*, 1996; Palmer *et al.*, 2000).

Furthermore, focal cerebral ischemia induced significant increases in numbers of BrdU labeled cells in the ipsilateral cortex, subcortex, SVZ and olfactory bulb in contrast to global ischemia, which induced in BrdU cells only in the DG (Liu *et al.*, 1998). Therefore, a selective increase of BrdU-positive cells in the SVZ of the LV indicates that r-Hu-EPO exhibit distinct profiles of cellular proliferation to specific regional progenitors depending on the type of ischemia.

The result of present study also showed that increased BrdU-positive cells in ischemic penumbra and SVZ of the LV in r-Hu-EPO-treated ischemic rats are associated with significantly increased CNPase-immunoreactive cells which are identified as OPCs (Ness *et al.*, 2005). CNPase is found almost exclusively in oligodendrocytes lineage cells, the cells that form myelin in the central nervous system. This finding is in line with previous observations showing that systemic delivery of r-Hu-EPO in an experimental model of autoimmune encephalomyelitis decreases demyelination and increases integral membrane proteoglycan NG2-positive OPCs (Zhang *et al.*, 2005). Since OPCs are able to differentiate in myelinating oligodendrocytes both *in vitro* (Shi *et al.*, 1998) and *in vivo* (Bu *et al.*, 2004), their enhanced proliferation likely contributes to the restoration of oligodendrocyte. If the increased immunoreactivity of CNPase means the enhanced numbers of OPCs, increased OPCs may be beneficial because these cells express the excitatory amino acid cotransporter 1 and are consequently involved in the removal of the excess of the neurotransmitter from the extracellular space (Gottlieb *et al.*, 2000), thus limiting its neurotoxic effects (Profyris *et al.*, 2004). However, it remains unclear that enhanced OPCs in the penumbra and SVZ of the LV of the ischemic brain integrate into the infarcted cortical architecture and/or improve the pathological and/or neurological changes.

### Increased endothelial cell proliferation in the I-R brain treated with r-Hu-EPO

The present data demonstrated that treatment with r-Hu-EPO increased the BrdU-positive cells in the CP, CP-TV, PM, and blood vessels in the hippocampus following I-R which means r-Hu-EPO strongly augments angiogenesis in the ischemic brain. EPO has been shown to induce proangiogenic effects in cultivated endothelial cells and in the chick embryo chorioallantoic membrane assay (Carlini *et al.*, 1995; Ribatti *et al.*, 1999). Recently, other studies demonstrated that EPO not only affected mature endothelial cells in postnatal neovascularization, but also profoundly increased the number

of circulating endothelial progenitor cells by mobilizing bone marrow-derived hematopoietic stem cells (Asahara *et al.*, 1999; Takahashi *et al.*, 1999). In addition, as in the case with erythroid precursor cells, EPO also seemed to be a survival factor for endothelial cells through the prevention of cell injury and DNA fragmentation by activating AKT1 and inhibiting cytochrome c release and caspase activity (Chong *et al.*, 2002). Therefore, systemically administered r-Hu-EPO might contribute to increased angiogenesis as well as to reduced vascular damage after I-R. However, it is still unclear as to whether increased angiogenesis and reduced vascular damage in blood vessels of the ischemic brain can recover the vessels' barrier function given that the reduction of oxygen supply (ischemic stress), as well as post-ischemia reoxygenation, clearly induced profound alterations in the cytoskeleton organization in vascular endothelial cells (Crawford *et al.*, 1996).

Angiogenesis and proliferation of OPCs are linked in the adult brain via vascular endothelial growth factor (VEGF) and brain derived neurotrophic factor (BDNF) (Zhang *et al.*, 2000). Administration of r-Hu-EPO increased VEGF levels in the ischemic penumbra, which was able to block using SU1498, a specific VEGF receptor 2 antagonist (Wang *et al.*, 2004). In addition, cerebral endothelial cells express EPOR which may represent targets for intraperitoneal administration of r-Hu-EPO. Further, increased endothelial cells produce more BDNF that enhances oligodendrocyte proliferation and differentiation (Pincus *et al.*, 1998; Girard *et al.*, 2005; Zhang *et al.*, 2006). Therefore, our data suggest that r-Hu-EPO may act directly on cerebral endothelial cells that secrete more and more VEGF and BDNF thereby, accelerating angiogenesis and the production of OPCs via a paracrine pathway.

### Acknowledgements

This work was supported by Dongguk Research Fund, Dongguk University (2010).

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