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·Original Article·

Time-dependent effect of combination therapy with erythropoietin and granulocyte colony-stimulating factor in a mouse model of hypoxic-ischemic brain injury

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ABSTRACT

Erythropoietin (EPO) and granulocyte colony-stimulating factor (G-CSF) are likely to play broad roles in the brain. We investigated the effects of combination therapy with EPO and G-CSF in hypoxic-ischemic brain injury during the acute, subacute, and chronic phases. A total of 79 C57BL/6 mice with hypoxic-ischemic brain injury were randomly assigned acute (days 1–5), subacute (days 11–15) and chronic (days 28–32) groups. All of them were treated with G-CSF (250 μg/kg) and EPO (5 000 U/kg) or saline daily for 5 consecutive days. Behavioral assessments and immunohistochemistry for angiogenesis, neurogenesis, and astrogliosis were performed with an 8-week follow-up. Hypoxia-inducible factor-1 (HIF-1) was also measured by Western blot analysis. The results showed that the combination therapy with EPO and G-CSF in the acute phase significantly improved rotarod performance and forelimb-use symmetry compared to the other groups, while subacute EPO and G-CSF therapy exhibited a modest improvement compared with the chronic saline controls. The acute treatment significantly increased the density of $CD31^+$ (PECAM-1) and α-smooth muscle $actin^+$ vessels in the frontal cortex and striatum, increased $BrdU^+$/PSA-$NCAM^+$ neurogenesis in the subventricular zone, and decreased astroglial density in the striatum. Furthermore, acute treatment significantly increased the HIF-1 expression in the cytosol and nucleus, whereas chronic treatment did not change the HIF-1 expression, consistent with the behavioral outcomes. These results indicate that the induction of HIF-1 expression by combination therapy with EPO and G-CSF synergistically enhances not only behavioral function but also neurogenesis and angiogenesis while decreasing the astroglial response in a time-dependent manner.

Keywords: erythropoietin; granulocyte colony-stimulating factor; hypoxia-inducible factor-1; hypoxic-ischemic brain injury

INTRODUCTION

Erythropoietin (EPO) and granulocyte colony-stimulating factor (G-CSF) are likely to play broad roles in the central nervous system as well as the blood-forming system because receptors for both are expressed in the brain[1-3]. EPO is the major growth regulator of the erythroid cell lineage, and its main biological function is to regulate the proliferation, maturation, and survival of erythroid

progenitor cells in the bone marrow[4]. It is also a promising candidate drug for various neurological diseases[5]. Its beneficial effects have been attributed to a multitude of neuroprotective mechanisms, including anti-apoptosis, anti-oxidant actions, and restoration of blood-brain barrier integrity, as well as stimulation of neurogenesis and angiogenesis[6-8]. However, EPO treatment of patients with ischemic stroke has recently failed in a double-blind, randomized clinical trial, although preclinical findings and a clinical pilot study suggested that recombinant human EPO has beneficial effects[9,10]. Therefore, the EPO and other neuroprotective or neurotrophic agents need to be combined to produce the optimal functional recovery.

G-CSF is a humoral factor affecting the survival, proliferation, and differentiation of hematopoietic cells[11,12]. G-CSF has been approved for the prevention and treatment of chemotherapy-induced neutropenia and apheresis in hematopoietic transplantation to mobilize bone marrow cells into systemic circulation[13,14]. It has also been shown to exhibit several beneficial effects in animals with stroke[14-16]. In particular, it improves functional recovery after stroke even when the treatment is initiated after a delay[17].

Recent studies reported that G-CSF upregulates EPO expression *via* the induction of hypoxia-inducible factor-1 (HIF-1) activity and that combination therapy of G-CSF and EPO synergistically enhances neuronal survival and angiogenesis in animal models of cerebral ischemia[18,19]. As a regulatory gene, HIF-1 controls critical pro-angiogenic genes such as vascular endothelial growth factor (VEGF) and EPO[20,21]. G-CSF has been shown to enhance HIF-1 activity. Activated HIF-1 binds to the EPO promoter to turn on EPO expression[22]. Both EPO and G-CSF have been shown to possess angiogenic and neuroprotective properties[23-25], and are regarded as therapeutic agents in acute stroke models[25,26]. However, there is no evidence on the therapeutic time-window of EPO and G-CSF. Therefore, this study investigated the effects of EPO and G-CSF combination therapy on functional recovery in a mouse model of hypoxic-ischemic brain injury during the acute, subacute, and chronic phases.

MATERIALS AND METHODS

Animals

Seventy-nine male C57BL/6 mice at 12 weeks of age were used: 43 for behavioral testing and immunohistochemistry at 8 weeks post-treatment; 18 for immunohistochemistry at 2 weeks post-treatment; and 18 for Western blot analysis. After permanent ischemic brain damage by unilateral right carotid artery ligation, hypoxic brain injury (8% O_2 for 45 min) was induced. Body temperature was maintained at 37 ºC in a hypoxic chamber. All animals were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and were given food and water *ad libitum* under alternating 12-h light/dark cycles.

Ethics Statement

All procedures were in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental procedures were approved by the Animal Care and Use Committee of Yonsei University Health System (permit number: 2011-0272). All animals were maintained in a temperature-controlled animal care facility according to the animal protection regulations. All efforts were made to minimize suffering and the number of animals used.

Animal Grouping and Treatment

After induction of hypoxic-ischemic brain injury, mice were randomly assigned to the acute saline (n = 8), acute EPO + G-CSF (n = 9), subacute saline (n = 7), subacute EPO + G-CSF (n = 7), chronic saline (n = 6), and chronic EPO + G-CSF (n = 6) groups for behavioral assessment and immunohistochemistry at 8 weeks post-treatment. In addition, the other mice were equally allocated to the groups (n = 3 each) for immunohistochemistry at 2 weeks post-treatment, and for Western blot analysis the day after completion of 5-day EPO+G-CSF treatment. The mice received subcutaneous injections of 5 000 U/kg of recombinant human EPO and 250 μg/kg of recombinant human G-CSF, or saline once daily for 5 consecutive days (acute phase, days 1–5; subacute phase, days 11–15; chronic phase, days 28–32). These doses were based on a previous report that each has a sufficient effect[19]. Before hypoxic-ischemic brain injury, additional 16 mice were also recruited as a no brain injury group to provide a more comprehensible understanding of the extent of functional recovery. A schematic timeline of this experiment from surgery to 8 weeks post-treatment is provided in Fig. 1A.

Rotarod Performance

A rotarod test was used to assess motor coordination and

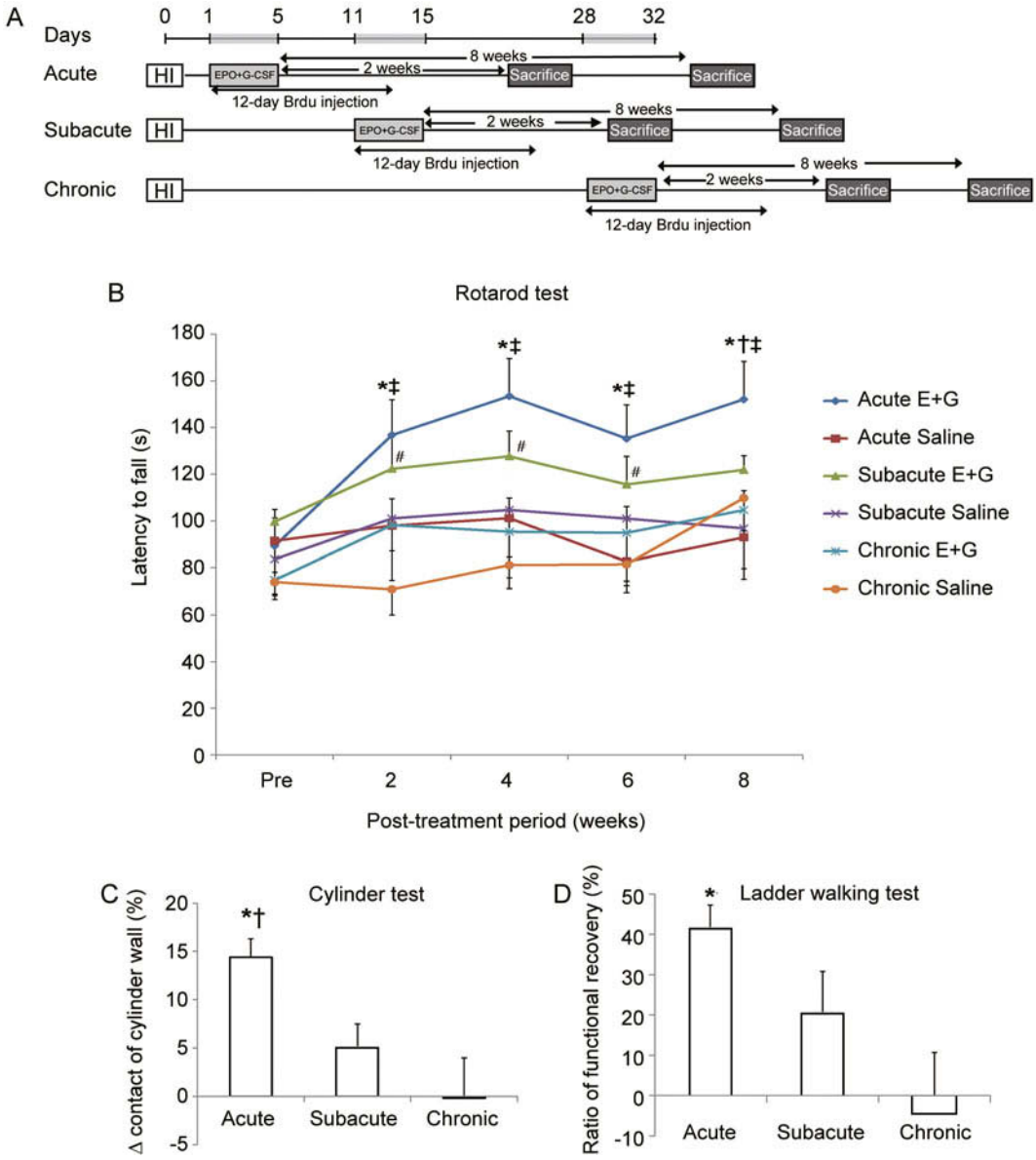

Fig. 1. Experimental design and behavioral assessment. A: Schematic of experimental protocols. B: The rotarod test demonstrated that the acute E+G treatment group showed a significantly longer latency than the other groups, except for the subacute E+G group. Especially, the acute E+G group exhibited a longer latency than the subacute E+G group at 8 weeks (P* <0.05 *vs* acute saline group; †*P* <0.05 *vs* subacute E+G group; ‡*P* <0.05 *vs* subacute saline and chronic groups). On the other hand, the subacute E+G group showed a modest improvement compared to the chronic saline controls during 2–6 weeks post-treatment (#*P* <0.05 *vs* chronic saline group), whereas the chronic E+G group showed no change in performance throughout the experimental period. (C) In the cylinder test, the difference (Δ) relative to control group in the percentage of wall contacts with the contralateral limb in the acute E+G group increased (**P* <0.05 *vs* chronic group; †*P* <0.05 *vs* subacute group). (D) In the ladder-walking test, the acute E+G group showed a functional recovery, whereas the chronic E+G group did not improve (**P* <0.05 *vs* chronic group). E+G: mice treated with combination of EPO and G-CSF.**

balance. All animals received a pre-treatment performance evaluation the day after surgery, and at 2, 4, 6 and 8 weeks after treatment, using an acceleration (4–80 rpm) paradigm. The latency to fall from the rod was measured twice during each test, and individual tests were terminated at a maximum latency of 300 s.

Forelimb-use Asymmetry Test

To evaluate functional asymmetry resulting from the unilateral brain lesion, the cylinder test and the ladder-walking test were performed at 8 weeks post-treatment. In the cylinder test, the number of times each forelimb contacted the cylinder wall as the mouse reared on its hind limbs was evaluated over a period of 5 min. The percentage of cylinder wall contacts with the hemiplegic forelimb was evaluated by the following formula[28,29]: [contacts with the contralateral limb + 1⁄2 (contacts with both limbs)] / (contacts with the ipsilateral limb + contacts with the contralateral limb + contacts with both limbs)×100%. The difference (Δ) relative to the control group in the percentage of cylinder wall contacts by the contralateral limb was calculated in mice treated with EPO and G-CSF.

In the ladder-walking test, the mice were required to walk a distance of 1 m three times on a horizontal ladder with metal rungs differing distances apart. The number of slips from the transverse rungs with each forelimb was measured by videotape analysis. The difference (Δ) relative to the control group in the percentage of slips on the transverse rungs of the ladder to the total number of steps taken by the hemiplegic forelimbs was also calculated.

Immunohistochemistry

Mice received bromodeoxyuridine (BrdU, 50 mg/kg, i.p.) once a day for 12 days from the beginning of drug treatment to evaluate endogenous cell genesis. Then, they were sacrificed at 2 or 8 weeks after treatment under ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia and given an intracardial perfusion of 4% paraformaldehyde, and the brains were harvested.

New neurons were evaluated in the subventricular zone (SVZ, n = 3 mice). Sections were stained for the cell proliferation marker BrdU (1:400, Abcam, Cambridge, UK), and the early neuronal marker polysialylated neuronal cell adhesion molecule (PSA-NCAM, 1:400, Chemicon, Pemecula, CA). Double-labeled cells were then assessed by confocal imaging (LSM700, Zeiss, Gottingen, Germany). The area of the SVZ was obtained using the Meta Morph Imaging System (Molecular Device, Sunnyvale, CA) and converted to volume by multiplying the area by the section thickness (16 μm). The total numbers of newly generated cells and new neurons were then estimated as the densities of $BrdU^+$ cells and $BrdU^+$/$PSA\text{-}NCAM^+$ cells (/mm^3).

Individual sections were also co-stained for βII-tubulin (1:400, Covance, Princeton, NJ), neuronal nuclei (NeuN, 1:400, Chemicon, Pemecula, CA), or glial fibrillary acidic protein (GFAP, 1:400, Chemicon) to confirm the densities (/mm^3) of specific neural-lineage cells. In addition, immunostaining was performed with CD31 (PECAM-1, 1:400, Abcam) or α-smooth muscle actin (α-SMA, 1:400, Abcam) to confirm the vessels density (/mm^3) of the damaged frontal cortex and striatum, and with anti-chondroitin sulfate antibody (CS-56, 1:400, Abcam) to confirm the glial scar density (/mm^3) of the damaged striatum.

Western Blot Analysis

Cytosol and nucleus proteins in brain tissues from the damaged hemisphere were prepared using a protein extraction kit (Millipore, Billerica, MA) according the manufacturer's protocol. For electrophoresis, 50 μg of extracted protein was dissolved in sample buffer (60 mmol/L Tris-HCl, pH 6.8, 14.4 mmol/L β-mercaptoethanol, 25% glycerol, 2% SDS, and 0.1% bromophenol blue), boiled for 5 min, and separated on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel. Separated cytosol and nucleus proteins were transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, UK) using a trans-blot system (Bio-Rad). Blots were blocked for 1 h in Tris-buffered saline (TBS) containing 5% non-fat dry milk at room temperature, washed three times with TBS, and incubated at 4 ºC overnight with anti-HIF-1 antibody (1:1 000, Abcam) in TBST. The next day, blots were washed three times with TBST and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (1:3 000, Santa Cruz, CA) at room temperature. After washing three times with TBST, the protein was visualized with an ECL detection system (Amersham Pharmacia Biotech).

Statistical Analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) version 18.0. The effects of the combination of EPO and G-CSF on the endogenous repair process and functional recovery after hypoxic-ischemic brain injury were evaluated. Namely, the density of $CD31^+$, $\alpha\text{-}SMA^+$, $\beta II\text{-}tubulin^+$, $NeuN^+$, $GFAP^+$, and $CS56^+$ cells (/mm^3), the numbers of $BrdU^+$ and $BrdU^+$/$PSA\text{-}NCAM^+$ cells (/mm^3), and HIF-1 in the cytosol and nucleus, as well as the results of behavioral tests were

analyzed using one-way ANOVA followed by a *post-hoc* Tukey comparison. A statistically significant level was defined when P <0.05.

RESULTS

Combined Treatment with EPO and G-CSF in the Acute Phase Improves Rotarod Performance

The rotarod test with acceleration (4–80 rpm) was performed bi-weekly. There was no significant difference among the groups in performance prior to treatment. However, at 2 weeks post-treatment, mice treated with EPO and G-CSF in the acute phase showed a significant improvement (136.76 ± 15.09 s), compared to the other groups, except for the subacute EPO + G-CSF group (F = 4.426, P = 0.001) (Fig. 1B). This improvement in the acute EPO + G-CSF group was maintained to 8 weeks post-treatment (152.80 ± 14.61 s) (F = 4.021, P = 0.003). Moreover, the acute EPO + G-CSF group exhibited a longer latency than the subacute EPO + G-CSF group (119.88 ± 5.06 s) at 8 weeks post-treatment (P = 0.022) (Fig. 1B). On the other hand, the subacute EPO + G-CSF group exhibited a modest improvement compared to the chronic saline controls during 2–6 weeks post-treatment (P <0.05). The performance of the chronic EPO + G-CSF group did not change throughout the experimental period (Fig. 1B).

Acute Phase Treatment Attenuates Forelimb-use Asymmetry

In the cylinder test, the difference (∆) relative to the control group in the percentage of wall contacts with contralateral limb was higher in mice treated with EPO and G-CSF in the acute phase (14.50 ± 1.73%) than those treated in the subacute phase (5.17 ± 2.24%) and the chronic phase (−0.33 ± 3.83%) (F = 8.875, P = 0.002) (Fig. 1C). In the ladder-walking test, mice treated in the acute phase (1.67 ± 0.33%) and the subacute phase (2.0 ± 0.68%) showed a significant reduction in the difference (∆) relative to the control group in the percentage of slips among total steps with the hemiplegic forelimb compared to mice treated in the chronic phase (−0.50 ± 0.50%) (F = 6.678, P = 0.008). We also calculated the ratio of motor function (%) relative to the no brain injury group and estimated the extent of functional recovery (Δ) by comparison with the respective controls 8 weeks after treatment, The acute and subacute EPO + G-CSF groups showed functional improvement by 41.71 ± 5.40% and 20.57 ± 8.91% respectively, whereas the chronic EPO + G-CSF group did not show any improvement (–4.41 ± 13.83%) (F = 4.821, P = 0.029). Particularly, the acute EPO + G-CSF group showed better functional recovery than the chronic EPO + G-CSF group (P = 0.009) (Fig. 1D). Taken together, the cylinder and ladder walking performance results suggested that only the acute EPO + G-CSF group had a significant attenuation of the forelimb-use asymmetry, while the subacute EPO + G-CSF group showed modest improvement compared to saline controls.

Acute Phase Treatment Significantly Increases Vessel Density

To determine the endogenous angiogenesis and arteriogenesis, blood vessels were quantified in both the frontal cortex and the striatum. $CD31^+$ ($PECAM\text{-}1^+$) endothelial cell densities in the frontal cortex and the striatum were higher in the acute EPO + G-CSF group than in the other groups at 2 weeks (frontal cortex F = 7.340, P <0.001; striatum F = 9.350, P <0.001) and 8 weeks post-treatment (frontal cortex F = 5.907, P = 0.001; striatum F = 7.421, P <0.001) (Fig. 2A–C). The subacute EPO + G-CSF group also showed a tendency to increase $CD31^+$ endothelial cell density in the striatum but not in the frontal cortex. Particularly, they showed an increase compared to the chronic EPO + G-CSF group and saline controls at 8 weeks post-treatment (P <0.05) (Fig. 2A–C). On the other hand, $\alpha\text{-}SMA^+$ vessel density in the frontal cortex was increased in the acute EPO + G-CSF group compared to the other groups at 8 weeks post-treatment (F = 9.412, P <0.001), and that of the striatum also increased relative to the subacute and chronic groups (F = 3.589, P = 0.012) (Fig. 2D−F). The results suggest that combination therapy of EPO and G-CSF during the acute phase increases arteriogenesis in addition to angiogenesis in the frontal cortex and the striatum.

Acute Phase Treatment Increases Neuronal Density

To determine the endogenous neurogenesis and neural-lineage cell density, the expression of neural markers was assessed in both the SVZ and the striatum. $\beta\text{II-tubulin}^+$ neuronal density in the SVZ and striatum was higher in the acute than in the chronic EPO + G-CSF group at 2 weeks post-treatment (F = 2.797, P = 0.034) and in the other groups at 8 weeks post-treatment (F = 3.570, P = 0.012) (Fig.

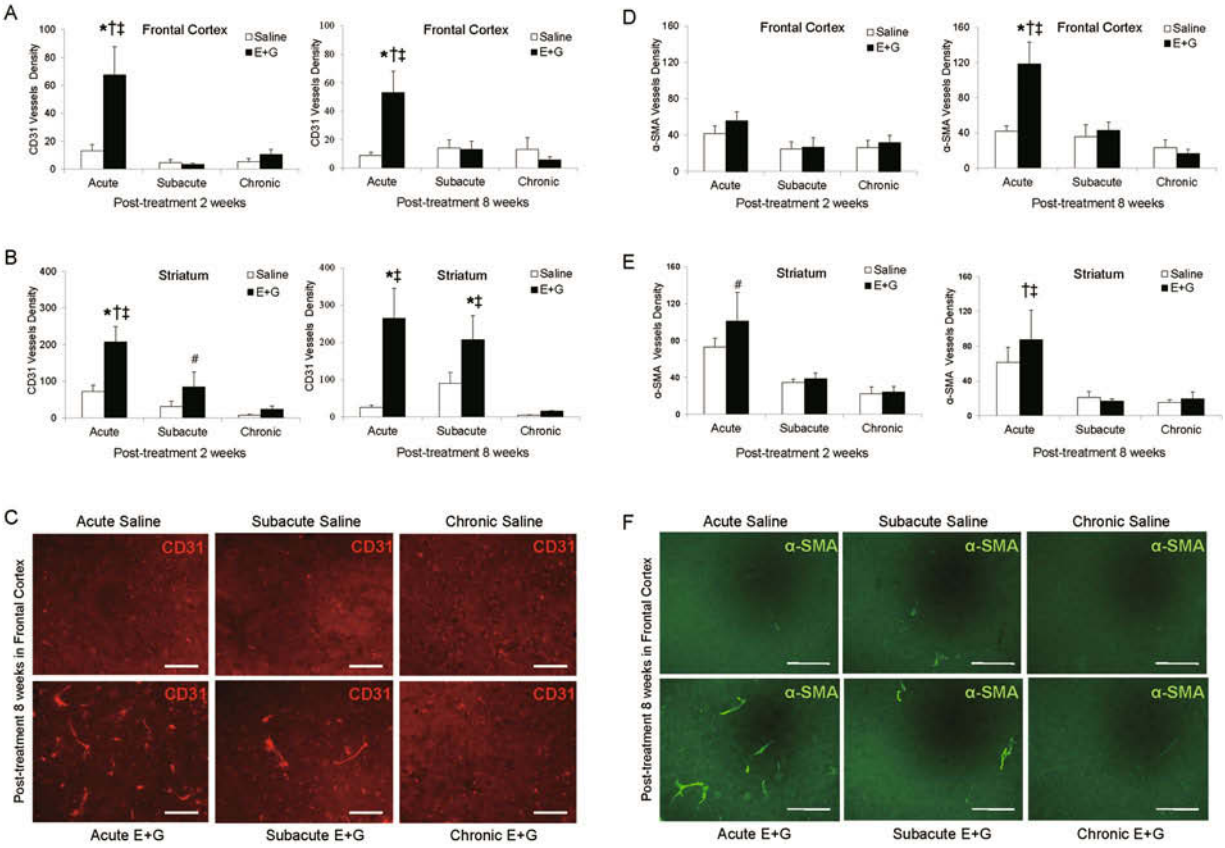

Fig. 2. EPO and G-CSF treatment in the acute phase significantly increases vessels density. (A) CD31 (PECAM-1)$^+$ endothelial cell density of the frontal cortex was significantly greater in the acute E+G group than in the other groups at 2 and 8 weeks post-treatment (P* <0.05 *vs* acute saline group; †*P* <0.05 *vs* subacute E+G group; ‡*P* <0.05 *vs* subacute saline and chronic groups). (B) The endothelial cell density in the striatum was greater in the acute E+G group at 2 and 8 weeks post-treatment (**P* <0.05 *vs* acute saline group; †*P* < 0.05 *vs* subacute E+G group; ‡*P* <0.05 *vs* subacute saline and chronic groups). The subacute E+G group also showed an increase of the endothelial cell density in the striatum compared to the chronic E+G group and saline controls (**P* <0.05 *vs* acute saline group; ‡*P* <0.05 *vs* subacute saline and chronic groups; $^\#$*P* <0.05 *vs* chronic groups). (C) Representive images of CD31 staining at 8 weeks post-treatment in the frontal cortex. Scale bars, 50 μm. (D) α-SMA$^+$ vessel density in the frontal cortex was increased in the acute E+G group at 8 weeks post-treatment (**P* <0.05 *vs* acute saline group; †*P* <0.05 *vs* subacute E+G group; ‡*P* <0.05 *vs* subacute saline and chronic groups). (E) The vessel density in the striatum also showed an increase at 2 and 8 weeks post-treatment (†*P* <0.05 *vs* subacute E+G group; ‡*P* <0.05 *vs* subacute saline and chronic groups; $^\#$*P* <0.05 *vs* chronic groups). (F) Representive images of α-SMA staining at 8 weeks post-treatment in the frontal cortex. Scale bars, 150 μm. E+G: mice treated with combination of EPO and G-CSF.**

3A, B). However, the expression of NeuN, a mature neuronal marker showed no significant difference among the groups at 2 and 8 weeks post-treatment (Fig. 3C). On the other hand, newly-generated cells stained with BrdU were higher in the acute EPO + G-CSF group than the other groups at 2 weeks (F = 9.488, P <0.001), but not at 8 weeks (Fig. 3D). When PSA-NCAM, an early neuronal and migrating neuroblast marker, was co-stained with BrdU, the number of BrdU$^+$/PSA-NCAM$^+$ cells in the SVZ was also greater in the acute EPO + G-CSF group than in the other groups at 2 weeks post-treatment (F = 10.125, P <0.001) (Fig. 3E, F).

Combined Treatment with EPO and G-CSF Decreases Astroglial Density

To assess endogenous astrogliosis, GFAP, an astrocyte marker, and CS-56, a glial scar marker, were stained in the striatum. GFAP expression in the acute EPO + G-CSF group was lower than that in the acute saline group at 2 weeks post-treatment (P = 0.032) (Fig. 4A). The subacute EPO + G-CSF group also showed a decrease in GFAP$^+$

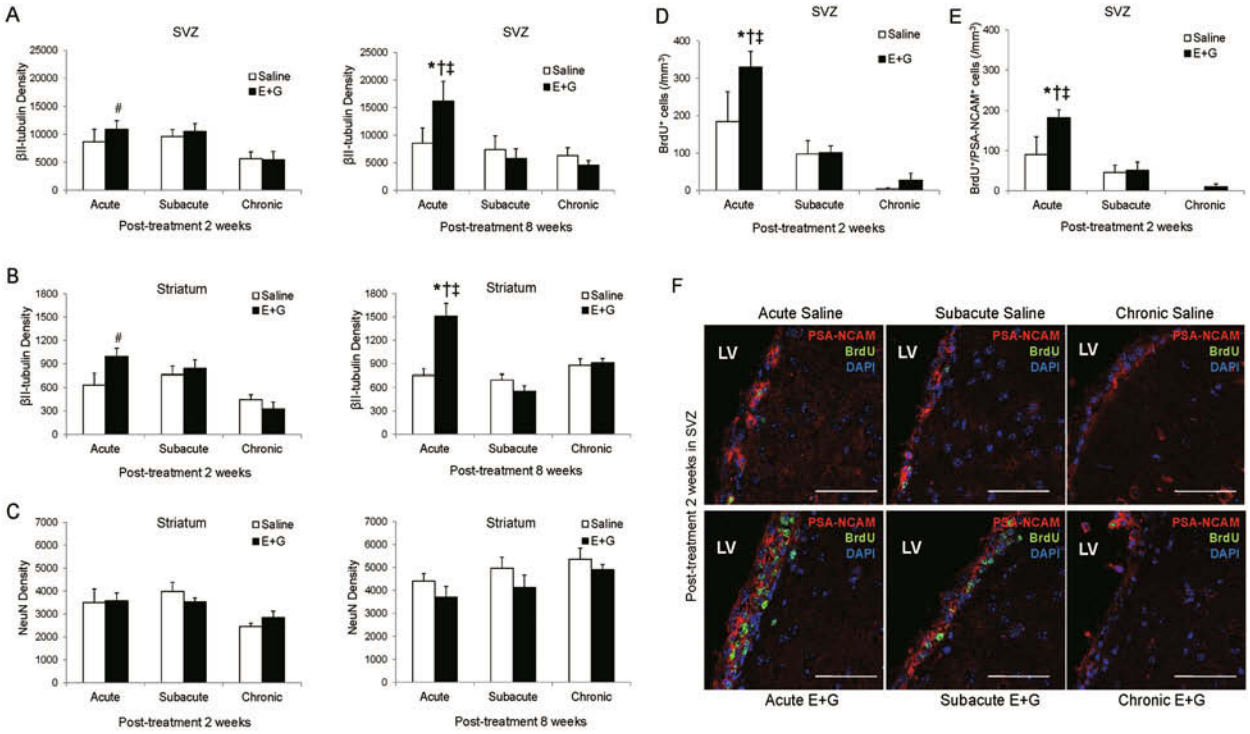

Fig. 3. Acute phase treatment increases neurogenesis and neuronal density. (A, B) βII-tubulin$^+$ neuronal density in the SVZ (A) and striatum (B) was higher in the acute E+G group than in the chronic groups at 2 weeks post-treatment ($^{\#}P$ <0.05 *vs* chronic groups) and the other groups at 8 weeks post-treatment ($^*P$ <0.05 *vs* acute saline group; $^{\dagger}P$ <0.05 *vs* subacute E+G group; $^{\ddagger}P$ <0.05 *vs* subacute saline and chronic groups). (C) The expression of NeuN, a mature neuronal marker, showed no significant difference among the groups at 2 and 8 weeks post-treatment. (D, E) The numbers of BrdU$^+$ and BrdU$^+$/PSA-NCAM$^+$ cells were greater in the acute E+G group than the other groups at 2 weeks post-treatment ($^*P$ <0.05 *vs* acute saline group; $^{\dagger}P$ <0.05 *vs* subacute E+G group; $^{\ddagger}P$ <0.05 *vs* subacute saline and chronic groups). (F) Representive images of BrdU$^+$/PSA-NCAM$^+$ staining at 2 weeks post-treatment in the SVZ. Scale bars, 50 μm. LV, lateral ventricle; SVZ, subventricular zone; E+G, mice treated with combination of EPO and G-CSF.

density compared to the subacute saline control (P <0.001) (Fig. 4A). However, these differences did not persist to 8 weeks post-treatment. On the other hand, CS-56 expression in the acute EPO + G-CSF group was decreased compared to the acute saline group at 8 weeks post-treatment (P = 0.031) (Fig. 4B). Taken together, mice treated with EPO and G-CSF in the acute and subacute phases showed a similar pattern of decreased GFAP expression in the neostriatum, suggesting that combination therapy with EPO and G-CSF reduces astroglial cell fate determination (Fig. 4C).

HIF-1 Expression Increases in the Acute Phase Treatment Group

To measure the expression of HIF-1 after treatment in the three phases, Western blot analysis was performed. The acute EPO + G-CSF group showed an increase in HIF-1 expression compared to the other groups, except for the subacute EPO + G-CSF group in the cytosol (F = 3.968, P = 0.007) and in the nucleus (F = 2.483, P = 0.048). The subacute EPO + G-CSF group also had a clear tendency for increased HIF-1 expression in the cytosol and nucleus compared to the respective saline group. Moreover, total HIF-1 expression increased in the acute phase after combination therapy compared to the other groups, including the subacute EPO + G-CSF group (F = 3.537, P = 0.012). On the other hand, the chronic EPO + G-CSF group did not differ from the respective saline group (Fig. 5).

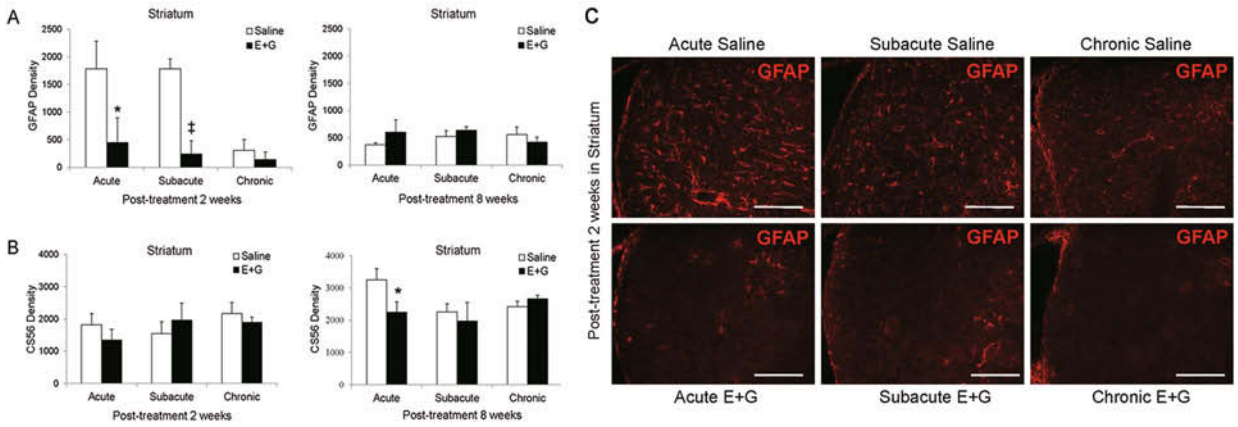

Fig. 4. EPO and G-CSF treatment decreases astroglial density. (A) Combination therapy with EPO and G-CSF during the acute and subacute phases decreased the GFAP expression at 2 weeks post-treatment in the damaged striatum, compared with the other groups at 2 weeks post-treatment (P* <0.05 *vs* acute saline group; ‡*P* <0.05 *vs* subacute saline group). (B) The acute E+G group showed decreased CS-56 expression in the damaged striatum, compared with the respective saline controls at 8 weeks post-treatment (**P* <0.05 *vs* acute saline group). (C) Representative images of GFAP staining in the striatum at 2 weeks post-treatment. Scale bars, 150 μm. E+G group: mice treated with combination of EPO and G-CSF.**

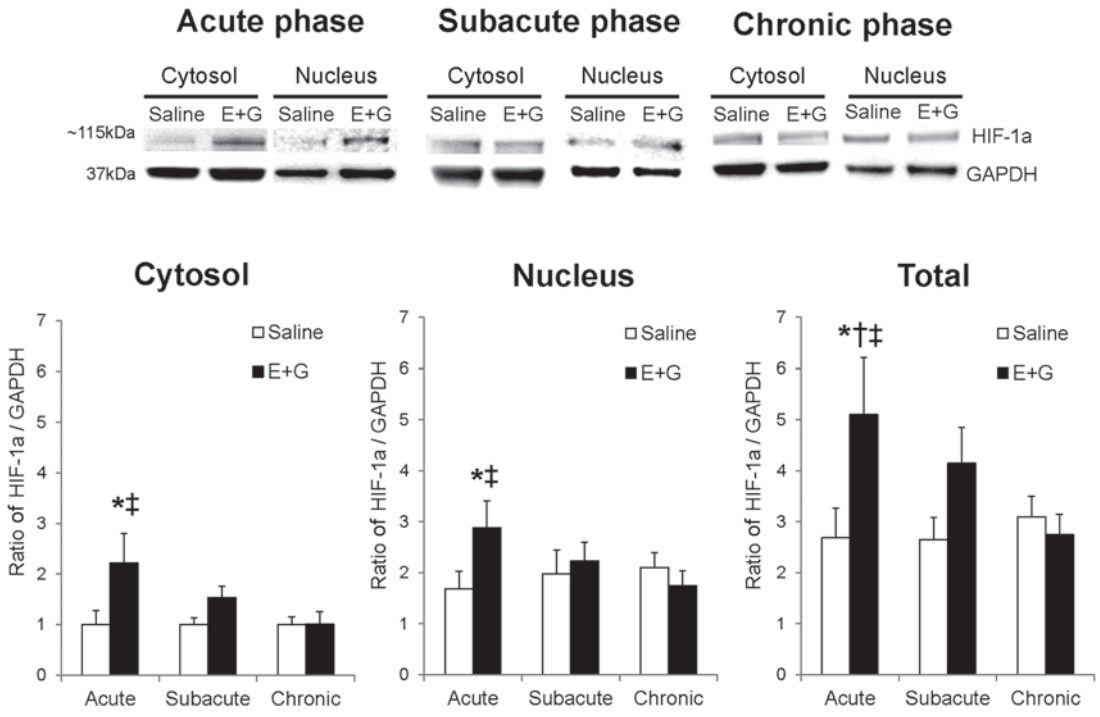

Fig. 5. HIF-1 expression increases in the acute phase treatment group. The acute E+G group showed increased HIF-1 expression in the cytosol and nucleus compared to the other groups, except for the subacute E+G group (P* <0.05 *vs* acute saline group; ‡*P* <0.05 *vs* subacute saline and chronic groups). The subacute E+G group also had a clear tendency for increased HIF-1 expression in the cytosol and nucleus. Moreover, total HIF-1 expression increased in the acute phase compared to the other groups, including the subacute E+G group (**P* <0.05 *vs* acute saline group; †*P* <0.05 *vs* subacute E+G group; ‡*P* <0.05 *vs* subacute saline and chronic groups). On the other hand, the chronic E+G group did not differ from the respective saline group. E+G: mice treated with combination of EPO and G-CSF.**

DISCUSSION

EPO affects the properties of endothelial cells in culture, such as proliferation and motility, and is associated with neovascularization as an angiogenic factor[30]. G-CSF has also been shown to induce an angiogenic response in endothelial cells as well as endothelial cell growth, and enhances arteriogenesis in ischemic stroke[31,32]. Moreover, a recent study has shown that EPO and G-CSF together synergistically enhance angiogenesis and arteriogenesis to promote tissue plasticity[19]. The aim of this study was to investigate the particular time-window of the effects, rather than the efficacy, of the combination therapy.

In this study, we also evaluated the peripheral human EPO levels using ELISA in each treatment group, and found a clear elevation in peripheral blood 1 day after the final injection (day 5 of injection) whereas human EPO was almost undetectable in the control group treated with saline, confirming systemic action by the subcutaneous injection of the growth factors (Fig. S1).

Consistent with previous findings that the combination therapy of EPO and G-CSF promotes angiogenesis and functional recovery in ischemic models[19], we showed significant improvements in behavioral performance (rotarod and forelimb-use asymmetry tests) and increases in expression of the endothelial cell marker CD31 and the smooth muscle cell marker α-SMA after combined treatment. The functional and histological improvement was evident in mice treated in the acute phase, but not in the chronic phase, while mice treated in the subacute phase showed modest improvement. The combination therapy of EPO and G-CSF in the acute phase also increased newly-generated $BrdU^+$ cells and $BrdU^+$/$PSA\text{-}NCAM^+$ cells in the SVZ defined as endogenous cell genesis and neurogenesis at 2 weeks post-treatment, but these cells did not survive to 8 weeks. These results are consistent with previous findings that proliferation of neural progenitor cells residing in the ipsilateral SVZ following ischemic injury or following the treatment returned to near control level 2–4 weeks later[3, 19, 33, 34].

In the present study, combination therapy with EPO and G-CSF during the acute and subacute phases significantly decreased $GFAP^+$ astroglial density at 2 weeks post-treatment, and acute treatment decreased CS-56 expression in the damaged striatum at 8 weeks. In response to central nervous system injury, astrocytes change their morphology, proliferate, and migrate to the injury site to form a scar. These reactive astrocytes display hypertrophic morphology and express high levels of GFAP[35]. The glial scar forms a physical barrier to neurite outgrowth and produces inflammatory mediators that cause further tissue damage[36-39]. Accordingly, reducing scar formation has been suggested to overcome the physical barrier to axonal growth. Reactive astrocytes also have an unintended toxic effect by inducing glutamate release that augments neuronal death in the acute phase after hypoxic-ischemic brain injury, which causes the coincidence of hypoxia and inflammation[40]. In contrast, EPO is known to increase the level of neurogenesis while decreasing the glial response, favoring a neurogenic response in the damaged striatum after neonatal stroke[34]. Therefore, the shift of direct cell fate from astrogliosis toward neurogenesis shown in this study might be associated with long-term behavioral outcomes and functional improvement, as suggested in previous studies[40,41].

EPO and its receptor system with HIF-1-responsive proteins stimulate red blood cell production to increase O_2 transport in the blood and O_2 supply to the brain[42]. The HIF-1 expression is able to act as a mechanism for neural plasticity[43, 44]. A recent study reported that G-CSF upregulates EPO expression *via* the induction of HIF-1 activity and the translocation of HIF-1 into the nucleus. Consequently, neurogenesis and angiogenesis enhanced by HIF-1 activity contribute to functional recovery[18, 19]. Similarly, our results showed that the acute-phase treatment induced HIF-1 expression in the cytosol and nucleus compared with the other groups, whereas the chronic-phase treatment did not make any difference. This finding is clearly related to the functional recovery after the combination therapy with EPO and G-CSF.

As a limitation of this study, this experiment designed saline groups as the only controls during the different periods, and the effect of EPO alone or G-CSF alone was not evaluated as positive control groups. Since a previous study already determined that combination therapy with EPO and G-CSF synergistically enhances functional recovery[19], we mainly focused on the time-window of the effect by this therapy.

In conclusion, the results of this study suggest that combination therapy with EPO and G-CSF produced functional recovery in a mouse model of hypoxic-ischemic

brain injury in a time-dependent manner, and the underlying mechanisms may be the induction of HIF-1 activity in both cytosol and nucleus, and an early change in the cell fate determination from astrogliosis toward neurogenesis. Moreover, acute phase treatment might contribute more to functional recovery than chronic-phase treatment in hypoxic-ischemic brain injury.

SUPPLEMENTAL DATA

Supplemental Data include one figure and can be found online at http://www.neurosci.cn/epData.asp?id=141.

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REFERENCES

[1] Hasselblatt M, Jeibmann A, Riesmeier B, Maintz D, Schabitz WR. Granulocyte-colony stimulating factor (G-CSF) and G-CSF receptor expression in human ischemic stroke. Acta Neuropathol 2007, 113: 45–51.

[2] Schlager GW, Griesmaier E, Wegleiter K, Neubauer V, Urbanek M, Kiechl-Kohlendorfer U, *et al*. Systemic G-CSF treatment does not improve long-term outcomes after neonatal hypoxic-ischaemic brain injury. Exp Neurol 2011, 230: 67–74.

[3] Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med 2002, 8: 963–970.

[4] Sprigg N, Bath PMB. Colony stimulating factors (blood growth factors) are promising but unproven for treating stroke. Stroke 2007, 38: 1997–1998.

[5] Digicaylioglu M. Erythropoietin in stroke: quo vadis. Expert Opin Biol 2010, 10: 937–949.

[6] Siren AL, Fratelli M, Brines M, Goemans C, Casagrande S, Lewczuk P, *et al*. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. Proc Natl Acad Sci U S A 2001, 98: 4044–4049.

[7] Leist M, Ghezzi P, Grasso G, Bianchi R, Villa P, Fratelli M, *et al*. Derivatives of erythropoietin that are tissue protective but not erythropoietic. Science 2004, 305: 239–242.

[8] Wang L, Zhang Z, Wang Y, Zhang R, Chopp M. Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. Stroke 2004, 35: 1732–1737.

[9] Ehrenreich H, Hasselblatt M, Dembowski C, Cepek L, Lewczuk P, Stiefel M, *et al*. Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med 2004, 8: 495–505.

[10] Ehrenreich H, Weissenborn K, Prange H, Schneider D, Weimar C, Wartenberg K, *et al*; EPO Stroke Trial Group. Recombinant human erythropoietin in the treatment of acute ischemic stroke. Stroke 2009, 40: e647–656.

[11] Burgess AW, Metcalf D. Characterization of a serum factor stimulating the differentiation of myelomonocytic leukemic cells. Int J Cancer 1980, 26: 647–654.

[12] Sachs L. The molecular control of blood cell development. Science 1987, 238: 1374–1379.

[13] Cavallaro AM, Lilleby K, Majolino I, Storb R, Appelbaum FR, Rowley SD, *et al*. Three to six year follow-up of normal donors who received recombinant human granulocyte colony-stimulating factor. Bone Marrow Transplant 2000, 25: 85–89.

[14] Toth ZE, Leker RR, Shahar T, Pastorino S, Szalayova I, Asemenew B, *et al*. The combination of granulocyte colony-stimulating factor and stem cell factor significantly increases the number of bone marrow-derived endothelial cells in brains of mice following cerebral ischemia. Blood 2008, 111: 5544–5552.

[15] Beck H, Voswinckel R, Wagner S, Ziegelhoeffer T, Heil M, Helisch A, *et al*. Participation of bone marrow-derived cells in long-term repair processes after experimental stroke. J Cereb Blood Flow Metab 2003, 23: 709–717.

[16] Six I, Gasan G, Mura E, Bordet R. Beneficial effect of pharmacological mobilization of bone marrow in experimental cerebral ischemia. Eur J Pharmacol 2003, 458: 327–328.

[17] Schneider A, Wysocki R, Pitzer C, Kruger C, Laage R, Schwab S, *et al*. An extended window of opportunity for G-CSF treatment in cerebral ischemia. BMC Biol 2006, 4: 36.

[18] Sadamoto Y, Igase K, Sakanaka M, Sato K, Otsuka H, Sakaki S, *et al*. Erythropoietin prevents place navigation disability and cortical infarction in rats with permanent occlusion of the middle cerebral artery. Biochem Biophys Res Commun 1998, 253: 26–32.

[19] Liu SP, Lee SD, Lee HT, Liu DD, Wang HJ, Liu RS, *et al*. Granulocyte colony-stimulating factor activating HIF-1alpha acts synergistically with erythropoietin to promote tissue plasticity. PLoS One 2010, 5: e10093.

[20] Levesque JP, Hendy J, Takamatsu Y, Williams B, Winkler IG, Simmons PJ. Mobilization by either cyclophosphamide or granulocyte colony-stimulating factor transforms the bone marrow into a highly proteolytic environment. Exp Hematol 2002, 30: 440–449.

[21] Levesque JP, Takamatsu Y, Nilsson SK, Haylock DN, Simmons PJ. Vascular cell adhesion molecule-1 (CD106) is

cleaved by neutrophil proteases in the bone marrow following hematopoietic progenitor cell mobilization by granulocyte colony-stimulating factor. Blood 2001, 98: 1289–1297.

[22] Ke Q, Costa M. Hypoxia-Inducible Factor-1 (HIF-1). Mol Pharmacol 2006, 70: 1469–1480.

[23] Pierelli L, Perillo A, Greggi S, Salerno G, Panici PB, Menichella G, *et al*. Erythropoietin addition to granulocyte colony-stimulating factor abrogates life-threatening neutropenia and increases peripheral-blood progenitor-cell mobilization after epirubicin, paclitaxel, and cisplatin combination chemotherapy: results of a randomized comparison. J Clin Oncol 1999, 17: 1288.

[24] Olivieri A, Offidani M, Cantori I, Ciniero L, Ombrosi L, Masia MC, *et al*. Addition of erythropoietin to granulocyte colony-stimulating factor after priming chemotherapy enhances hemopoietic progenitor mobilization. Bone Marrow Transplant 1995, 16: 765–770.

[25] Pierelli L, Menichella G, Scambia G, Teofili L, Iovino S, Serafini R, *et al. In vitro* and *In vitro* effects of recombinant human erythropoietin plus recombinant human G-CSF on human haemopoietic progenitor cells. Bone Marrow Transplant 1994, 14: 23–30.

[26] Brines ML, Ghezzi P, Keenan S, Agnello D, de Lanerolle NC, Cerami C, *et al*. Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. Proc Natl Acad Sci U S A 2000, 97: 10526–10531.

[27] Cheer SM, Wagstaff AJ. Epoetin Beta: a review of its clinical use in the treatment of anaemia in patients with cancer. Drugs 2004, 64: 323–346.

[28] Im SH, Yu JH, Park ES, Lee JE, Kim HO, Park KI, *et al*. Induction of striatal neurogenesis enhances functional recovery in an adult animal model of neonatal hypoxic-ischemic brain injury. Neuroscience 2010, 169: 259–268.

[29] MacLellan CL, Auriat AM, McGie SC,Yan R, Huynh HD, De Butte MF, *et al*. Gauging recovery after hemorrhagic stroke in rats: implications for cytoprotection studies. J Cereb Blood Flow Metab 2006, 26: 1031–1042.

[30] Ribatti D, Presta M, Vacca A, Ria R, Giuliani R, Dell'Era P, *et al*. Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization *in vivo*. Blood1999, 93: 2627–2636.

[31] Lee ST, Chu K, Jung KH, Ko SY, Kim EH, Sinn DI, *et al*. Granulocyte colony-stimulating factor enhances angiogenesis after focal cerebral ischemia. Brain Res 2005, 1058: 120–128.

[32] Sugiyama Y, Yagita Y, Oyama N, Terasaki Y, Omura-Matsuoka E, Sasaki T, *et al*. Granulocyte colony-stimulating factor enhances arteriogenesis and ameliorates cerebral damage in a mouse model of ischemic stroke. Stroke 2011, 42: 770–775.

[33] Zaidi AU, Bessert DA, Ong JE, Xu H, Barks JD, Silverstein FS, *et al*. New oligodendrocytes are generated after neonatal hypoxic-ischemic brain injury in rodents. Glia 2004, 46: 380–390.

[34] Gonzalez FF, McQuillen P, Mu D, Chang Y, Wendland M, Vexler Z, *et al*. Erythropoietin enhances long-term neuroprotection and neurogenesis in neonatal stroke. Dev Neurosci 2007, 29: 321–330.

[35] Pekny M, Nilsson M. Astrocyte activation and reactive gliosis. Glia 2005, 50: 427–434.

[36] Askalan R, Deveber G, Ho M, Ma J, Hawkins C. Astrocytic-inducible nitric oxide synthase in the ischemic developing human brain. Pediatr Res 2006, 60: 687–692.

[37] Bush TG, Puvanachandra N, Horner CH, Polito A, Ostenfeld T, Svendsen CN, *et al*. Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. Neuron 1999, 23: 297–308.

[38] Silver J, Miller JH. Regeneration beyond the glial scar. Nat Rev Neurosci 2004, 5: 146–156.

[39] Sofroniew MV. Reactive astrocytes in neural repair and protection. Neuroscientist 2005, 11: 400–407.

[40] Vangeison G, Rempe DA. The Janus-faced effects of hypoxia on astrocyte function. Neuroscientist 2009, 15: 597–588.

[41] Desclaux M, Teigell M, Amar L, Vogel R, Gimenez YRM, Privat A, *et al.* A novel and efficient gene transfer strategy reduces glial reactivity and improves neuronal survival and axonal growth *in vitro*. PLoS One 2009, 4: e6227.

[42] Jelkmann W, Effects of erythropoietin on brain function. Curr Pharm Biotechnol 2005, 6: 65–79.

[43] Chen ZY, Warin R, Noguchi CT. Erythropoietin and normal brain development: receptor expression determines multi-tissue response. Neurodegener Dis 2006, 3: 68–75.

[44] Buemi M, Cavallaro E, Floccari F, Sturiale A, Aloisi C, Trimarchi M, *et al.* The pleiotropic effects of erythropoietin in the central nervous system. J Neuropathol Exp Neurol 2003, 62: 228–236.