Treatment of Stroke With Erythropoietin Enhances Neurogenesis and Angiogenesis and Improves Neurological Function in Rats

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Background and Purpose—Erythropoietin (EPO) promotes proliferation and differentiation of erythroid progenitors and the survival of maturing erythroid cells. Here, we investigated the role of EPO in brain repair after stroke.

Methods—Rats were treated with recombinant human EPO (rhEPO) at 24 hours after the onset of embolic stroke. An array of behavior tests was performed. Rats were euthanized 28 days after stroke for measurements of infarct volume, angiogenesis, and neurogenesis. In vitro, neurospheres derived from the subventricular zone (SVZ) of the rat and cerebral endothelial cells derived from the mouse were treated with rhEPO. Capillary-like tube formation and neuronal differentiation were measured.

Results—Treatment with rhEPO significantly improved functional recovery, along with increases in density of cerebral microvessels at the stroke boundary and numbers of BrdU, doublecortin, and nestin immunoreactive cells in the SVZ. rhEPO treatment significantly increased brain levels of vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF). In vitro, rhEPO enhanced capillary tube formation of cerebral endothelial cells, which was inhibited by a specific VEGF receptor 2 antagonist (SU1498). Incubation of neurospheres derived from stroke SVZ with anti-EPO neutralizing antibody inhibited neurogenesis, whereas incubation of stroke-derived neurospheres with rhEPO enhanced neurogenesis.

Conclusion—Our data suggest that EPO-increased VEGF and BDNF may be involved in angiogenesis and neurogenesis, which could contribute to functional recovery.

Key Words: § erythropoietin § neurogenesis § angiogenesis

Erythropoietin (EPO) is a hematopoietic cytokine that promotes proliferation and differentiation of erythroid progenitors and the survival of maturing erythroid cells. Treatment of forebrain neural stem cells with EPO amplifies the production of neuronal progenitors in vitro. Furthermore, EPO enhances angiogenesis both in vitro and in vivo. Angiogenesis and neurogenesis are coupled in the brain. Despite strong evidence for the neuroprotective benefits of EPO in the treatment of acute stroke, the effects of EPO on angiogenesis, neurogenesis, and neurological function during stroke recovery have not been investigated.

Materials and Methods
All experimental procedures were approved by the IACUC of Henry Ford Hospital.

Animal Model
The middle cerebral artery (MCA) of male Wistar rats (The Jackson Laboratory, Bar Harbor, Maine) was occluded by placement of an embolus at the origin of the MCA.

In Vivo Experimental Protocol
To examine whether treatment with EPO promotes functional recovery, angiogenesis and neurogenesis, rhEPO (epoetin alpha, AMGEN) was administered (intraperitoneally) at a dose of 5000 (n=14) or 10 000 (n=14) units/kg daily for 7 days starting at 24 hours after MCAo. Doses of rhEPO were selected based on previous studies. Ischemic rats (n=14) treated with the same volume of saline were used as a control group. For labeling proliferating cells, bromodeoxyuridine (BrdU, 50 mg/kg; Sigma Chemical) was injected (intraperitoneally) daily for 13 days into rats starting 1 day after MCAo. All rats were euthanized 28 days after stroke. To examine the effect of rhEPO on brain levels of VEGF and brain-derived neurotrophic factor (BDNF), rhEPO (5000 U/kg) or saline was administered to ischemic rats (n=3 for each group) with the identical paradigm. These rats were euthanized 7 days after stroke for enzyme-linked immunosorbent assay (ELISA).

Behavioral Tests and Measurements of Infarct Volume
Corner and foot-fault tests were measured at 1, 3, 7, 14, 21, and 28 days after MCAo, as previously described. Infarct volume was measured from 7 hematoxylin and eosin (H&E)-stained coronal sections, as previously described.
Neurosphere Culture
SVZ cells were dissociated from normal (n=6) and ischemic (n=6) rats.14 The cells were plated at a density of 10 000 cells per milliliter in medium containing 20 ng/mL of epidermal growth factor (EGF; R&D system) and basic fibroblast growth factor (bFGF). Passage 1 cells were used in the study.

In Vitro Experimental Protocol
To examine the effects of EPO on neurosphere differentiation, neurospheres (80 μm in diameter) were plated directly onto laminin-coated glass coverslips in serum-free medium containing rhEPO (0, 1, 5, and 10 U/mL). Neurospheres were incubated with rabbit IgG (3 μg/mL; R&D Systems) as a control group. To examine the specificity of rhEPO effects, neurospheres were treated with an anti-EPO neutralizing antibody (3 μg/mL; R&D System). Incubation was terminated at 7 or 14 days after plating and immunostaining for neuronal and astrocyte markers were performed for evaluation of differentiation. To examine whether EPO promotes angiogenesis, a capillary tube formation assay was performed. Mouse brain-derived endothelial cells (2×10^4 cells)14 were incubated in Matrigel (Becton Dickinson) for 3 hours in Dulbecco Modified Eagle medium (DMEM) containing rhEPO (1.25, 2.5, 5, 10, or 20 U/mL), rhEPO (10 U/mL), and anti-EPO neutralizing antibody (3 μg/mL; R&D system), or rhEPO (10 U/mL) and a specific VEGF receptor 2 antagonist (SU1498, 5 μmol/L; LC Laboratories).16

Immunohistochemistry
Immunofluorescent staining for brain tissue and culture cells was performed on paraffin-embedded coronal sections (6 μm) or on culture cells, as previously described.15 17 Primary antibodies used were: mouse anti-BrdU (1:1000; Boehringer Mannheim), rabbit anti-EPO receptor (1:500; Santa Cruz Biotechnology), mouse anti-β-tubulin III (TuJ-1, 1:1000; Novus Biologicals Inc), goat anti-doublecortin (1:200; Santa Cruz Biotechnology Inc), rabbit anti-glial fibrillary acidic protein (GFAP, 1:200; Dako), and rabbit anti-von Willebrand factor (vWF, 1:200; Dako). Culture cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (Vector Laboratories),16 Immunofluorescent staining for brain tissue and culture cells was performed, and controls were performed using commercially available kits R&D Systems and Promega Corporation respectively.

Hematocrit
A blood sample for hematocrit determination was drawn via a tail vein before and weekly up to 35 days after rhEPO treatment (Readacrit Centrifuge; Clay Adams).

Statistical Analysis
One-way analysis of variance (ANOVA) followed by Student-Newman–Keuls test was used. The data were presented as means±SE. A value of P<0.05 was taken as significant.

Results
Treatment With rhEPO Improves Neurological Outcome
Similar neurological impairments were detected 24 hours after stroke (Figure 1). Treatment with rhEPO significantly (P<0.05) improved neurological outcome on the foot fault (Figure 1A) and corner (Figure 1B) tests compared with control rats, although infarct volume was not significantly (P>0.05) reduced in rats treated with 5000 (34±2.6% of the contralateral hemisphere, n=8) or 10 000 U/kg (33±3.2%, n=8) compared with the control group (39±1.7%, n=8).

EPO Enhances Angiogenesis In Vivo and In Vitro
Treatment with rhEPO significantly (P<0.05) enlarged vascular diameters (Figure 2A and 2D) around the ischemic lesion but not in the contralateral hemisphere (Figure 2C), compared with the ipsilateral vessels in the control rats (Figure 2B). Moreover, a significant (P<0.05) increase in vascular density was detected around the ischemic lesion in rhEPO treated rats (Figure 2E and 2H) compared with the control group (Figure 2F and 2H). Three-dimensional measurements of FITC-dextran perfused vessels revealed that treatment with rhEPO significantly (P<0.05) increased VEGF levels at 5000 U/kg (n=8) and 10 000 U/kg (n=8) compared with the control group. The data indicate that EPO enhances angiogenesis in stroke brain. rhEPO treatment (5000 U/kg) significantly (P<0.05), increased VEGF levels at the ischemic boundary regions from 6.75±0.6 pg/mg in the control group (n=3) to 10.27±2.1 pg/mg in the treated group (n=3). To complement the in vivo effects of EPO on angiogenesis and VEGF, a significant increase in capillary-like tube formation was detected when endothelial cells were incubated with rhEPO (Figure 2K and 2N), compared with the endothelial cells incubated with DMEM (Figure 2L and 2N). EPO induced capillary-like tube formation was completely inhibited when the endothelial cells were incubated with rhEPO (10 U/mL) in the presence of anti-EPO neutralizing antibody (Figure 2M and 2N), indicating that EPO increases angiogenesis. To examine whether EPO enhances angiogenesis via increases in VEGF, the endothelial cells were incubated for 3 hours in the presence of rhEPO (10 U/mL) and VEGF levels were measured using ELISA. Treatment with rhEPO increased VEGF levels (P<0.05) from 145±10.5 pg/mg in the control group to 194±11.8 pg/mg in the rhEPO-treated group. In addition, when the endothelial cells were incubated in the presence of rhEPO (10 U/mL) and SU1498, capillary-like tube formation was sig-
Figure 2. EPO enhances angiogenesis. A to C, Cerebral vessels immunostained with the antibody against BrdU at 28 days after stroke. A, B, and C, BrdU immunoreactive endothelial cells (arrows) at the ischemic boundary region of a representative rat treated with rhEPO at 5000 U/kg, BrdU-positive endothelial cells (arrow) of a representative rat from the stroke-only group rat and BrdU-negative endothelial cells of the contralateral hemisphere, respectively. D, Quantitation of vascular perimeters. E through G, Cerebral blood vessels immunostained with the antibody against von Willebrand factor (vWF). Numbers of cerebral vessels increased in the ischemic region in rats treated with rhEPO at 5000 U/kg (E) compared with the stroke-only rats (F). Numbers of cerebral vessels did not change in the contralateral hemisphere in rats treated with EPO (G). H, Quantitative data of vascular density at the ischemic boundary regions. I and J, FITC-dextran perfused vessels at ischemic boundary regions of rhEPO-treated (I) or stroke-only rat (J). Incubation of cerebral endothelial cells with rhEPO induces capillary tube formation (K and N) compared with control (L and N). However, incubation of cerebral endothelial cells with rhEPO at 10 U/mL in the presence of anti-EPO antibody (M and N) or a specific VEGF receptor 2 antagonist, SU1498, (N) blocks rhEPO-induced capillary tube formation. *P<0.05 versus control and #P<0.05 versus EPO 10 U/mL. Numbers 1.25 to 20 represent EPO doses at U/mL. Bar in C=10 μm for panels A to C and bar in E=25 μm for panels E to G. Image size was 276×276×25 μ for panels I and J.
nificantly (P<0.05) reduced (Figure 2N). Together, these data indicate that EPO increases VEGF that mediates rhEPO-induced angiogenesis.

**EPO Enhances Neurogenesis In Vivo and In Vitro**

Stroke increased the numbers of BrdU (Figure 3A), nestin, a marker of progenitor cells (Figure 3E), and doublecortin (Figure 3I) immunoreactive cells, a marker of migrating neuroblasts, in the ipsilateral SVZ compared with the immunoreactive cells in the contralateral SVZ (Figure 3C, 3G, and 3K). However, treatment with rhEPO significantly (P<0.05) increased the numbers of BrdU (Figure 3B and 3D), nestin (Figure 3F and 3H), and doublecortin (Figure 3J and 3L) immunoreactive cells in the ipsilateral SVZ compared with the numbers in the ipsilateral SVZ of the control group. Furthermore, EPO enhanced migration of doublecortin immunoreactive cells into the ischemic boundary of the cortex (Figure 3L and 3N) and striatum (Figure 3P) compared with doublecortin-positive cells in control animals (Figure 3M and 3O). These data suggest that treatment with rhEPO increases neurogenesis.

Treatment of rats with rhEPO significantly (P<0.05) increased BDNF levels (11.0±0.61 ng/mg) compared with the control group (6.35±0.15 ng/mg).

Complementing the in vivo indication of neurogenesis, neurospheres derived from stroke rats exhibited a significant (P<0.05) increase in the number of TuJ1 (neuronal marker)-positive cells (Figure 4B and Figure 4D) but not the number of GFAP (astrocyte marker) positive cells compared with the corresponding numbers from nonstroke rats (Figure 4A and 4D). To examine whether endogenous EPO contributes to stroke-induced neurogenesis, neurospheres were incubated with the anti-EPO neutralizing antibody or the antibody against rabbit IgG. Treatment with the anti-EPO antibody significantly (P<0.05) reduced the number of TuJ1-positive cells compared with the number in the control group (Figure 4C and 4D). In addition, neurospheres exhibited EPO receptor immunoreactivity (Figure 4E). These data suggest that endogenous EPO regulates stroke-induced neurogenesis.

To test whether exogenous EPO instructs neurospheres to differentiate to neurons, cultured neurospheres derived from stroke or normal SVZ were immunostained for neuronal (TuJ1) or astrocyte (GFAP) markers and with DAPI to determine total cell number. EPO significantly (P<0.05) increased the percentage of neurons but not astrocytes generated by the stroke and normal neurospheres in a dose-dependent manner (Table). The percentage of neurons generated by stroke neurospheres was significantly (P<0.05) greater than that generated by normal neurospheres (Table). However, when neurospheres were incubated with rhEPO (10 U/mL) in the presence of an anti-EPO neutralizing antibody,
the percentage of neurons was significantly ($P<0.05$) reduced compared with the control group (Table).

We then examined whether EPO directly stimulates neurospheres to generate BDNF. Neurospheres were incubated with rhEPO (10 U/mL), and BDNF in the supernatant was measured with an ELISA kit. BDNF was not detected by ELISA, indicating that EPO does not increase neurosphere BDNF.

**Effects of rhEPO on Hematocrit**

Treatment with rhEPO for 7 days significantly ($P<0.05$) increased hematocrit levels at 7 days, with a peak at 14 days of treatment onset (Figure 5). Thereafter, hematocrit levels gradually decreased approaching pretreatment levels at 35 days after treatment onset (Figure 5).

**Discussion**

Our data indicate that treatment with rhEPO beginning 24 hours after stroke significantly improved functional recovery and concomitantly enhanced angiogenesis and neurogenesis. Furthermore, our data indicate that EPO induces angiogenesis via regulation of VEGF.

EPO treatment provides neuroprotection when initiated soon after injury,7 with beneficial effects largely attributed to decreases in neuronal apoptosis.7 However, mice overexpressing erythropoietin had enlarged infarct volume after permanent occlusion of the middle cerebral artery when hematocrit levels increased to 80%.18 We show that treatment of stroke with EPO initiated 24 hours after stroke improves functional recovery without reducing infarct volume and that treatment enhances angiogenesis and neurogenesis, both in vivo and in vitro. Neurogenesis and angiogenesis may promote neurological function.19,20 Because we have not demonstrated that neuroblasts in the ischemic boundary regions integrate into the cerebral architecture and have mature neuronal electrophysiological properties, the data associating neurogenesis with functional recovery should be interpreted with caution.

EPO is required for normal brain development and regulates neurogenesis in the adult mouse brain.2 Stroke upregulates EPO and its receptor.21 EPO binds to its receptor to promote proliferation, differentiation, and maturation of erythroid progenitor cells.22 Here, we demonstrate that EPO...

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**Effect of EPO on Numbers of Tuj1-Positive and GFAP-Positive Cells**

<table>
<thead>
<tr>
<th>EPO (U/mL)</th>
<th>% of Tuj1-Positive Cells</th>
<th>% of GFAP-Positive Cells</th>
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<tr>
<td></td>
<td>7 DIV</td>
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<tr>
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<tr>
<td>10</td>
<td>21±2.6*</td>
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<tr>
<td>10+ anti-EPO</td>
<td>26±3.6*</td>
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**Figure 5.** Changes in hematocrit before, during, and after rhEPO treatment. Zero time point represents before rhEPO treatment. *$P<0.05$ vs zero time point; $+P<0.05$ versus 5000 U of rhEPO.
significantly increased numbers of BrdU-positive cells in the ipsilateral SVZ and neuroblasts in the ischemic boundary regions of the adult rat brain. In parallel, our in vitro data show that blocking EPO by an EPO-neutralizing antibody completely inhibited stroke-induced neurogenesis by multipotent neural stem cells, whereas incubation with rhEPO enhanced neurogenesis by neurospheres derived from normal and stroke SVZ. Thus, our data indicate that endogenous EPO mediates neurogenesis in response to stroke and exogenous EPO can further enhance neurogenesis.

Intraventricular infusion of BDNF or a viral vector encoding BDNF induces neurogenesis in adult mouse brain.23 Our data show that treatment with EPO in vivo significantly increased brain levels of BDNF and neurogenesis, suggesting that BDNF may stimulate neurogenesis. However, incubation of neurospheres derived from the SVZ with EPO enhanced neurogenesis but did not induce BDNF, indicating that SVZ cells are not a source of EPO-induced BDNF. Cerebral endothelial cells express EPO receptors that may be targets of intravenous administration of EPO, and endothelial cells produce BDNF.24 Together, our data suggest that EPO acts directly on cerebral endothelial cells that secrete BDNF, and vascular BDNF stimulates neurogenesis via a paracrine pathway.6

VEGF mediates stroke-induced angiogenesis.20 EPO stimulates migration, proliferation, and angiogenesis of endothelial cells derived from various organs other than brain.3 In the present study, we show that EPO enhances angiogenesis at the ischemic boundary and EPO induces capillary-like tube formation on cerebral endothelial cells. Furthermore, administration of rhEPO increased cerebral VEGF levels and EPO-induced capillary-like tube formation was blocked by a specific VEGF receptor 2 antagonist (SU1498), suggesting that VEGF mediates EPO-enhanced angiogenesis.

Angiogenesis and neurogenesis are linked in the adult brain via VEGF and BDNF.5,6 Neurogenesis occurs within an angiogenic niche.5 After angiogenic stimulation, endothelial cells secrete BDNF, which induces neurogenesis.6 In addition to its angiogenic role, VEGF stimulates neurogenesis.25 Our data show that EPO increased neuroblasts for at least 28 days in the ischemic boundary regions and EPO-enhanced angiogenesis and neurogenesis are associated with increases in cerebral BDNF and VEGF levels. Therefore, besides its role on neural protection, EPO provides a permissive microenvironment for neural plasticity during stroke recovery.

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References
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