Brain Repair by Hematopoietic Growth Factors in a Rat Model of Stroke

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Background and Purpose—Stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) are essential growth factors in hematopoiesis. We determined whether receptors for SCF and G-CSF exist in the brain and whether exogenous SCF and G-CSF are beneficial to brain repair after brain ischemia.

Methods—A well-established rat model of experimental stroke was used in this study. SCF, G-CSF, SCF+G-CSF, or saline was subcutaneously administered 3 hours to 7 days after brain ischemia. Bromodeoxyuridine was administered simultaneously. Sensorimotor function was evaluated with a limb placement test and foot fault test over time.

Results—We observed that receptors for SCF and G-CSF were expressed in both neurogenic regions and neurons. SCF-treated rats showed the best functional restoration at 1 week that was maintained 4, 7, and 10 weeks after the final injection. G-CSF-induced functional recovery was limited and unstable. Interestingly, stable but delayed functional improvement was seen in SCF+G-CSF-treated rats. Infarction size was significantly reduced in all growth factor-treated rats. In addition, SCF and SCF+G-CSF enhanced neural progenitor cell proliferation in the subventricular zone bilaterally, whereas G-CSF and SCF+G-CSF treatment increased bromodeoxyuridine-positive cells in perifarct areas.

Conclusions—SCF and G-CSF are neuroprotective and beneficial to functional restoration when administered during the acute phase after brain ischemia, indicating hematopoietic growth factors play a role in brain repair. (Stroke. 2007;38: 2584-2591.)

Key Words: focal ischemia ■ functional recovery ■ hematopoietic growth factor ■ neural stem cells ■ treatment

Stroke is the third leading cause of death and a leading cause of long-term disability in adults worldwide. Currently, recombinant tissue plasminogen activator with a 3-hour treatment window is the only drug approved by the US Food and Drug Administration for treatment of acute stroke.1

Stem cells are the cells with the capability of self-renewal and multiple cell lineage differentiation. Convincing evidence shows that stem cells/progenitor cells reside in many adult organs, including the brain and the bone marrow. Adult neural stem cells/neural progenitor cells (NSC/NPC) are located in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus.2 Brain ischemia induces NPC proliferation3 and enhances neurogenesis, which has been proposed to be associated with brain repair and functional recovery.4 Bone marrow stem cells have been shown to share the phenotypes of neural tissue5 and give rise to neurons through cell fusion6 and noncell fusion7 in the brain.

Stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) are hematopoietic growth factors (HGFs) that play an important role in regulating hematopoiesis. SCF (also termed master cell growth factor, kit ligand, and steel factor) binds to its receptor cKit, a tyrosine kinase transmembrane receptor and mediates cell proliferation, differentiation, and migration in hematopoiesis, gametogenesis, and melanogenesis.8 G-CSF controls the neutrophilic granulocyte proliferation and maturation through binding to the specific G-CSF receptor (G-CSFR).9 SCF+G-CSF exhibits a synergistic effect on mobilizing CD34+ cells from bone marrow to blood.10 CD34+ cells are known as a heterogeneous population of multipotent progenitors.

Recent findings have shown that SCF and G-CSF may also have effects on the central nervous system. SCF mutant mice showed a deficit in spatial learning and memory.11 Long-term potentiation and spatial learning were impaired in cKit mutant mice.12 SCF protects cortical neurons from camptothecin-induced apoptosis and glutamate excitotoxicity in vitro.13 G-CSF has been shown to have a neuroprotective...
effect on brain ischemia.\textsuperscript{14–16} SCF in combination with G-CSF promotes neuron production from bone marrow in intact animals.\textsuperscript{17} The aims of the present study were to determine whether receptors for SCF and G-CSF were expressed in the brain and whether systemic administration of SCF and G-CSF was beneficial to cerebral ischemia. We also investigated which of the factors, alone or combination, was the optimal choice for treatment of acute stroke.

Methods

Focal Brain Ischemia
Male spontaneously hypertensive rats, weighing 300 to 320 g, were anesthetized with methohexital sodium (50 mg/kg intraperitoneally) and then subjected to cortical brain ischemia. The right common carotid artery was ligated with a 3–0 silk suture, and the right middle cerebral artery was permanently ligated distal to the striatal branches with a 10–0 nylon suture.\textsuperscript{18–20} Sham-operative rats were manipulated with the same manner except ligation of the common carotid artery and middle cerebral artery. The animal protocol was approved by the Institutional Animal Care and Use Committee.

Evaluation of Neurological Deficits
The limb placement test and the foot fault test were blindly examined before brain ischemia, before treatment, and 1, 4, 7, and 10 weeks after the final injection of HGFs. The limb placement test has been described elsewhere.\textsuperscript{18} Briefly, forelimb and hindlimb placements were evaluated at 8 different conditions. The maximum score was 16 for each side of the body. The foot fault test was modified from a previous study.\textsuperscript{22} The hindlimbs of animals were gently held up, and the forelimbs were placed on a wire grid. The animal's ability to walk across the grid was evaluated, and the number of slippages of the affected paw between grids was recorded as a foot fault.

Groups and Drug Delivery
Neurological deficit-matched rats were randomly assigned into 4 groups: saline control, SCF, G-CSF, and SCF+G-CSF (n=10). Sham-operative rats (n=6) served as intact controls. An equal volume of saline or growth factors was injected subcutaneously (recombinant rat SCF, 200 µg/kg; recombinant human G-CSF, 50 µg/kg, provided by Amgen) for 7 days starting 3 hours after cortical brain ischemia. To determine if SCF and G-CSF promote NPC proliferation, we conducted a second experiment. Ischemic rats were randomized to receive subcutaneous injections of phosphate-buffered saline (PBS), SCF, G-CSF, or SCF+G-CSF (n=4) 3 hours to 7 days postischemia. Bromodeoxyuridine (BrdU; 50 mg/kg per day, dissolved in PBS, intraperitoneally; Sigma), for labeling dividing cells, was administered simultaneously to all rats for 7 days. The rats were then killed 1 day after injections.

Determination of Infarction Size
Rats were killed 12 weeks after brain ischemia and were perfused with 4% formaldehyde. The brains were cut into 9 pieces with a rat brain matrix (2-mm intervals). The brain blocks were embedded in paraffin and brain sections from each piece were stained with hematoxylin and eosin. Infarct volumes were calculated according to the method described previously in a blind manner.\textsuperscript{19} The infarction size was presented as a percentage of the volume of the contralateral cortex.

Immunohistochemistry
Cryostat brain sections were fixed with 4% formaldehyde in PBS. The brains perfused with 4% formaldehyde were processed for the free-floating method. For BrdU immunohistochemical staining, the sections were pretreated with 1 mol/L HCl 65°C. Nonspecific binding was blocked with 10% normal serum diluted in 1% bovine serum albumin (IgG-free; Jackson ImmunoResearch) and 0.25% Triton X-100. Sections were then incubated with primary antibodies, rabbit anti-c-Kit, goat anti-cKit (1:50; Santa Cruz Biotechnology), mouse anti-BrdU (1:50; Roche), and mouse anti-NeuN (1:500; Chemicon) at 4°C overnight. Sections were incubated with fluorescent- (Cy2 or Cy3) conjugated secondary antibodies (Jackson ImmunoResearch) in the dark at room temperature or biotinylated secondary antibody (Vector Laboratories) for BrdU immunostaining. Counterstaining was performed with DAPI (1:5000; Sigma). BrdU immunoreactivity was detected with an avidin-biotin-complex method (Vector Laboratories) and visualized with diaminobenzidine (Vector Laboratories).

Counting Bromodeoxyuridine-Positive Cells
Three to 5 coronal sections from each brain were selected based on the coordinates of bregma +1.0 mm (SVZ), and bregma −3.0 mm (SGZ).\textsuperscript{3} BrdU-positive cells were counted under high power on a Nikon microscope with a Magnifire digital camera, and the cell counting was performed blindly.

Statistical Analysis
Neurological evaluation data were analyzed with Kruskal-Wallis nonparametric analysis with a post hoc multiple comparisons. Data collected from infarction size measurement, body weight, and cell counting were tested by a one-way analysis of variance with post hoc multiple comparisons. Data are presented as means±SEM. Statistical significance was defined at $P<0.05$.

Results

Receptors for Stem Cell Factor and Granulocyte-Colony Stimulating Factor in the Neurogenic Regions
By using the immunofluorescent approach, cKit, the receptor for SCF (Figure 1A–C) and G-CSFR (Figure 1D–F) were both observed on the ependymal cells and in the SVZ in adult rats. Moreover, we noticed that immunofluorescent staining for both cKit and G-CSFR in the SVZ was located in the membrane of the neural progenitor cells (Figure 1 insets). In addition, both cKit and G-CSFR were colabeled with BrdU in the ependymal cells and the SVZ (Figure 1G–H), indicating cKit and G-CSFR-labeled cells were neural progenitor cells.

Receptors for Stem Cell Factor and Granulocyte-Colony Stimulating Factor on the Neurons
In addition to neurogenic regions, we also observed that cKit and G-CSFR were expressed on the cortical neurons as well as the neurons in the hippocampus (Figure 2) of the adult rat brain. In the neurons, cKit immunofluorescent staining was found in the cell membrane (Figure 2A–E). This is consistent with the function of cKit as a tyrosine kinase transmembrane receptor. In contrast to cKit, G-CSFR was abundantly expressed in the nuclei of the cortical neurons (Figure 2F–I), hippocampal pyramidal neurons, and the granular neurons in the dentate gyrus (Figure 2J–M).
Functional Improvements by Hematopoietic Growth Factor Treatment

In the limb placement test (Figure 3A), there were no differences among the groups before brain ischemia and before starting treatment. Rats in all ischemic groups (saline, SCF, G-CSF, and SCF+G-CSF) showed significantly more severe neurological deficits than sham-operative controls ($P<0.01$) 3 hours after brain ischemia. Ischemic rats that received SCF treatment showed best functional performance in comparison with other ischemic rats that received injection of saline ($P<0.01$), G-CSF, or SCF+G-CSF ($P<0.05$) 1 week after treatment. SCF-treated rats also showed better performance than saline controls ($P<0.01$), G-CSF alone (4 weeks: $P<0.05$, 7 and 10 weeks: $P<0.01$) at 4, 7, and 10 weeks after injections. SCF+G-CSF treatment led to a trend toward functional improvement compared with saline controls 1 week after injections but did not reach the level of statistical significance. However, 4 weeks after treatment, SCF+G-CSF-treated rats displayed a significant functional restoration when compared with the rats treated with saline or G-CSF ($P<0.01$), and the functional improvement was also seen 7 and 10 weeks postinjection. G-CSF-treated rats displayed better performance than saline controls 1 week and 10 weeks after treatment ($P<0.05$).

A similar pattern of functional improvement was also observed in the foot fault test (Figure 3B). One week after HGF injections, SCF induced a significant reduction in foot faults when compared with saline, G-CSF, or SCF+G-CSF ($P<0.01$), and SCF-induced recovery continued to 10 weeks after treatment (SCF versus saline: 4 weeks, $P<0.05$; 7 and 10 weeks, $P<0.01$; SCF versus G-CSF: 7 and 10 weeks, $P<0.01$). SCF+G-CSF-treated rats showed significantly fewer foot slips than saline controls, G-CSF (4, 7, and 10 weeks: $P<0.01$), or SCF alone (4 weeks: $P<0.05$).

Infarction Size Reduction by Hematopoietic Growth Factor Treatment

Eleven months after brain ischemia, animals were killed for infarction size determination by using an indirect measurement to avoid overestimating infarction size attributable to secondary tissue loss. $^{31}$ Infarct volume was calculated as percentage of contralateral cortex or hemisphere. Rats that received 7-day injections of SCF, G-CSF, or SCF+G-CSF showed a significant reduction in cortical infarction size (Figure 4) when compared with saline controls ($P<0.01$).

Neural Progenitor Cell Proliferation by Hematopoietic Growth Factor Treatment

Immunoperoxidase staining showed that SCF and SCF+G-CSF led to a significant increase in the number of BrdU incorporated cells in the SVZ bilaterally when compared with PBS controls ($P<0.01$, Figure 5A). A large number of BrdU-labeled cells in the ipsilateral SVZ were observed in SCF-treated rats in comparison with the rats in the groups of PBS, G-CSF, and SCF+G-CSF ($P<0.05$).

Bromodeoxyuridine-Labeled Cells in Periinfarct Areas by Hematopoietic Growth Factor Treatment

The brain sections crossing the SVZ (as shown in Figure 5B) and hippocampus (as shown in Figure 5C) were collected to
determine whether HGFs increased the number of BrdU-labeled cells in periinfarct areas. We observed that the number of BrdU-incorporated cells in the brain areas surrounding the infarction was significantly larger in G-CSF- and SCF/H11001 G-CSF-treated rats than PBS controls (Figure 5B–C). In the intact regions of the frontal–parietal cortex (Figure 5B–C-a) bordering the infarct, a dramatic increase in the number of BrdU-positive cells was observed in SCF+G-CSF-treated rats when compared with those of PBS-, SCF-, and G-CSF-treated rats (P<0.05). The number of BrdU-labeled cells in the ventral cortex periinfarct (Figure 5C-c) was also significantly larger in SCF+G-CSF-treated rats than PBS controls (P<0.05). G-CSF significantly increased BrdU-positive cells in the frontal cortex (Figure 5B-a) and the

**Figure 2.** Receptors for SCF and G-CSF in the neurons of the adult rat brain. A–E, SCF receptors (cKit, red) are expressed on the membrane of neurons. A–C, Cortical pyramidal neurons. D, E, Granular neurons in the dentate gyrus. F–M, G-CSFR is expressed in the nuclei of neurons. F–I, The nuclei of cortical neurons were triple labeled with G-CSFR (red), NeuN (green), and nuclei (DAPI, blue). J–M, G-CSFR (red) is coexpressed with nuclear dye (DAPI) in the granular neurons of the dentate gyrus (J, K) and the pyramidal neurons of CA3 in the hippocampus (L, M). Insets: Magnifications of the areas indicated by white boxes or arrows. Bar in C (indicator for A–C) and bar in M (indicator for J–M)=40 μm. Bar in E (indicator for D, E) and bar in I (indicator for F–I)=20 μm.
ventral cortex (Figure 5C-c) (G-CSF versus PBS, P<0.05). Both SCF+G-CSF treatment and G-CSF-alone treatment resulted in a significant increase in BrdU-incorporated cells in the corpus callosum surrounding the infarct in comparison with PBS controls (P<0.05, Figure 5B-b). However, SCF did not affect BrdU-positive cells in any regions bordering the infarct.

Discussion

The present study shows that receptors for SCF and G-CSF, are expressed in the neurogenic areas and on the mature neurons. Systemic administration of SCF and G-CSF during the first week after focal brain ischemia reduces infarct volume and improves functional restoration. SCF and G-CSF alone or in combination resulted in a difference in functional outcome, NPC-dividing, and proliferating cells in periinfarct areas.

SCF and G-CSF bind their receptors in the bone marrow, playing important roles in regulating hematopoiesis.9,10,23 In this study, we observed that receptors for SCF and G-CSF were also expressed in neurons and neurogenic regions. Similar to our data, Jin and coworkers24 also found that cKit was expressed in cultured neurons and neurogenic regions in vivo. ckit immunoreactivity has been observed in the interneurons of cerebellum25 as well as in a subpopulation of dorsal root ganglion neurons.26 In addition, G-CSFR was expressed in cortical pyramidal neurons and adult stem cells in vitro.27 Although exact physiological and pathological functions of cKit and G-CSFR in the central nervous system remain unelucidated, our data observed in the current study suggest that they are involved in neuroprotection and functional recovery after brain ischemia.

Systemic administration of SCF and G-CSF during the acute phase of focal brain ischemia led to infarction size reduction, indicating both SCF and G-CSF had neuroprotective effects on brain ischemia. It has been shown that SCF acts as a neurotrophic factor, supporting neuron survival during development of the peripheral nervous system.28,29

### Body Weight 1 Week After SCF and G-CSF Injections

<table>
<thead>
<tr>
<th>Groups</th>
<th>Before o.p.</th>
<th>1 Week</th>
<th>4 Weeks</th>
<th>7 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (n=6)</td>
<td>315.0±5.6</td>
<td>326.3±5.1</td>
<td>341.7±6.7</td>
<td>343.3±6.2</td>
</tr>
<tr>
<td>Saline (n=10)</td>
<td>310.0±3.9</td>
<td>323.0±5.0</td>
<td>344.0±4.5</td>
<td>352.0±6.3</td>
</tr>
<tr>
<td>G-CSF (n=10)</td>
<td>308.0±2.0</td>
<td>301.0±3.8</td>
<td>335.0±3.0</td>
<td>345.0±3.1</td>
</tr>
<tr>
<td>SCF (n=10)</td>
<td>314.4±5.6</td>
<td>317.8±7.2</td>
<td>348.9±7.0</td>
<td>355.6±6.3</td>
</tr>
<tr>
<td>Both (n=10)</td>
<td>308.2±3.0</td>
<td>317.3±3.8</td>
<td>337.7±4.0</td>
<td>351.0±2.8</td>
</tr>
</tbody>
</table>

*P<0.01 (G-CSF versus sham, saline, and both).
†P<0.05 (G-CSF versus SCF).
Both indicates SCF+G-CSF; o.p., operation.
Recently, it has been shown that SCF/cKit binding protects cortical neurons from apoptosis and excitotoxicity in vitro, and that the neuroprotective effect is mediated by MEK/ERK and P13K/Akt signal transduction pathways. G-CSF has been reported to protect neurons against ischemic injury. The neuroprotective effect of G-CSF is regulated through an activation of STAT3, ERK, and P13K/Akt pathways to promote neuron survival and inhibit apoptosis. Taken together, SCF and G-CSF alone or in combination, induced infarction size reduction related to their direct neuroprotective effects and/or neurotrophic effects.

Although SCF and G-CSF alone or in combination reduced infarction size, their effects on functional restoration were different. Interestingly, Kawada and coworkers also observed a similar phenomenon. In their study, SCF+G-CSF was administered during the acute or subacute phase of cortical brain ischemia, and both acute and subacute treatment induced a reduction in infarction size. However, the number of bone marrow-derived neurons and functional rehabilitation in subacute treatment were superior to acute treatment. In the current study, SCF showed a quick and long-lasting sensorimotor functional recovery, SCF+G-CSF led to a delayed but long-lasting functional improvement, and G-CSF alone induced a partial and unstable functional outcome. This reflects that the biological functions of SCF and G-CSF under the condition of acute brain ischemia are different. It has been shown that in patients with stroke, the number of red blood cells in the bloodstream decreased, and white blood cells increased. SCF is an essential growth factor regulating erythropoiesis. In addition, we observed that SCF promoted angiogenesis in the cortex perinfarct areas when administered during the acute phase of brain ischemia as well as by matrigel assay in vitro (unpublished observation). Therefore, we postulate that SCF-induced early functional recovery might be associated with the regeneration of blood supply to the neural tissue under ischemia. Functional improvement seen in SCF-treated rats may also be related to enhancement of brain plasticity. It has been documented that brain plasticity plays an important role in functional rehabilitation after brain ischemia. Several lines of evidence have shown that SCF and ckit participate in neuronal plasticity. Neurons with synaptic connections coexpress SCF and cKit. Long-term potentiation and spatial learning and memory were impaired in cKit and SCF mutant mice. In contrast to SCF, G-CSF stimulates neutrophil proliferation, differentiation, and survival. The role of neutrophils in brain ischemia remains controversial. It has been shown that neutrophil-released proteolytic enzymes and free radicals are neurotoxic. Although we noted that G-CSF reduced infarction size, we also observed that body weight was significantly lost in G-CSF-treated rats. G-CSF-induced sickness may negatively influence functional restoration, resulting in unstable functional improvement in G-CSF-treated rats. Interestingly, both SCF- and SCF+G-CSF-treated rats showed long-lasting functional recovery and was accompanied with increase of NPC proliferation in the SVZ. Consistent with this data, previous studies have shown that an enriched environment improves functional outcome and also leads to NPC proliferation in the SVZ after cortical brain ischemia. Stem cells have been found to release many neurotrophic factors and promote axonal outgrowth after spinal cord injury. We postulate that SCF- and SCF+G-CSF-induced NPC proliferation in the SVZ may support neuronal network reestablishment, contributing to a stable and long-term functional improvement after cortical brain ischemia. SCF+G-CSF has been shown to have a synergistic effect on mobilizing CD34 progenitor cells into the bloodstream and CD34 cells contribute to neurovascularization and neuronal regeneration. In addition, systemic administration of SCF+G-CSF 24 hours to 10 days postischemia led to functional improvement and an increase in the number of bone marrow-derived neurons in the perinfarct areas 4 weeks postischemia.

In summary, SCF and G-CSF are beneficial to brain ischemia. Among the experimental groups, SCF alone was an ideal treatment in the acute phase of brain ischemia. Our data suggest that hematopoietic growth factors are actively involved in brain repair. Hematopoietic growth factors may have dual effects on both the bone marrow and the brain, which make them more effective to repair brain injury. Based on previous studies and our current findings, we postulate that
the functional benefits of the HGFs are associated with their effects on neuroprotection, neurogenesis, and neuronal plasticity. However, the precise mechanisms of the HGF-related brain repair after brain ischemia need further studies to elucidate.

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Disclosures
Amgen provided both SCF and G-CSF for this study.

References
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