Brain Repair by Hematopoietic Growth Factors in a Rat Model of Stroke
Li-Ru Zhao, Seema Singhal, Wei-Ming Duan, Jayesh Mehta and John A. Kessler

*Stroke*. 2007;38:2584-2591; originally published online July 26, 2007;
doi: 10.1161/STROKEAHA.106.476457

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/38/9/2584

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org/subscriptions/
Brain Repair by Hematopoietic Growth Factors in a Rat Model of Stroke

Li-Ru Zhao, MD, PhD; Seema Singhal, MD; Wei-Ming Duan, MD, PhD; Jayesh Mehta, MD; John A. Kessler, MD

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 periinfarct 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.

\textbf{Methods}

\textbf{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.

\textbf{Determination of Infarction Size}

Counts were performed blindly. 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).

\textbf{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.

\textbf{Results}

\textbf{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.

\textbf{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).

\textbf{Immunohistochemistry}

Cryostat brain sections were fixed with 4% formaldehyde in PBS and then permeabilized in 0.2% Triton X-100. The sections were then incubated in 1% bovine serum albumin (IgG-free; Jackson ImmunoResearch) for 1 hour 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).
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-treated rats (4, 7, and 10 weeks: P<0.01), or SCF alone (4 weeks: P<0.05).

Although G-CSF led to functional improvement in the limb placement test 1 week and 10 weeks after treatment, overall SCF- and SCF+G-CSF-treated rats showed better functional outcome than those of G-CSF-treated rats. In addition, significant reduction of body weight was observed in G-CSF-treated rats when compared with saline controls and SCF treatment (P<0.05) and SCF+G-CSF treatment (P<0.01) (Table).

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. 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 perinfarct 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/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

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

![Figure 3. Evaluation of functional restoration after HGF treatment. A, Limb placement test (score 0, severe neurological deficits; score 16, no neurological deficits). Seven-day injections of HGFs initiated 3 hours postischemia resulted in significant functional improvement in the rats treated with SCF and G-CSF. Three-hours after middle cerebral artery occlusion: *$P<0.01$ (sham versus saline, GCSF, SCF, and GCSF+SCF); 1 week: *$P<0.01$ (SCF versus saline), †$P<0.05$ (SCF versus GCSF and GCSF+SCF, GCSF versus saline); 4 weeks: *$P<0.01$ (SCF versus saline, GCSF+SCF versus saline and GCSF), †$P<0.05$ (SCF versus GCSF); 7 weeks: *$P<0.01$ (SCF and GCSF+SCF versus saline and GCSF), †$P<0.05$ (GCSF versus saline). B, Evaluation of functional recovery by the foot fault test. Ischemia-induced foot slips were significantly ameliorated by SCF and G-CSF+G-CSF treatment. 1 week: *$P<0.01$ (SCF versus saline, GCSF, and GCSF+SCF); 4 weeks: *$P<0.01$ (GCSF+SCF versus saline and GCSF), †$P<0.05$ (SCF versus saline, GCSF+SCF versus SCF); 7 weeks and 10 weeks: *$P<0.01$ (SCF and GCSF+SCF versus saline and GCSF). Data are presented as means±SEM and tested by Kruskal-Wallis nonparametric analyses with multiple comparison corrections.](http://stroke.ahajournals.org/)

### Table: 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>350.1±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 PI3K/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 PI3K/Akt pathways to promote neuron survival and inhibit apoptosis. 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 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 neovascularization and neuronal regeneration. In addition, systemic administration of SCF+G-CSF 24 hours to 10 days posts ischemia led to functional improvement and an increase in the number of bone marrow-derived neurons in the perinfarct areas 4 weeks posts ischemia. In the present study, SCF+G-CSF administered 3 hours to 7 days after induction of brain ischemia increased the number of BrdU-positive cells in the cortex bordering the infarct 1 day after the final injection, suggesting some of these BrdU-positive cells may be bone marrow-derived progenitors. The process of the progenitor cells to differentiate into neurons takes approximately 4 weeks; this may be part of the reason that SCF+G-CSF induced a delayed functional improvement.

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.

Acknowledgments

We thank Chunshu Piao and Wenping Guo for their work on double-labeling of cKit/BrdU and G-CSFR/BrdU. We thank Louisiana Gene Therapy Research Consortium for supporting this study (to L.R.Z., W.M.D.).
Sources of Funding
This work was supported by NIH grants (to J.A.K.) R01 NS20778, R01 NS20013, and R01 NS34758; and by AHA grant (to L.-R.Z.) 0665522B.

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

References