

# Beneficial Effects of Hematopoietic Growth Factor Therapy in Chronic Ischemic Stroke in Rats

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**Background and Purpose**—Stroke is the leading cause of adult disability worldwide. Currently, there is no effective treatment for stroke survivors. Stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) are the growth factors regulating hematopoiesis. We have previously observed that SCF and G-CSF have neuroprotective and functional effects on acute brain ischemia. In the present study, the beneficial effects of SCF and G-CSF on chronic brain ischemia were determined.

**Methods**—SCF, G-CSF, or SCF+G-CSF was administered subcutaneously to rats 3.5 months after induction of ischemic stroke by middle cerebral artery occlusion. Neurological deficits were evaluated by limb placement test and foot fault test over time. Field-evoked potential was performed 19 weeks after treatment. Infarct volume was histologically determined using serial coronal sections.

**Results**—Significant functional improvement was seen in SCF+G-CSF-treated rats 1, 5, and 17 weeks after injections. SCF alone also improved functional outcome, but it did not show as stable improvement as SCF+G-CSF. No functional benefit was seen in G-CSF-treated rats. Field-evoked potential studies further confirmed the behavioral data that the normal pattern of neuronal activity was reestablished in the lesioned brain of the rats with good functional outcome. Interestingly, infarction volume was also significantly reduced in SCF+G-CSF-treated rats.

**Conclusion**—These data provide first evidence that functional restoration in chronic brain ischemia can be attained using hematopoietic growth factors. (*Stroke*. 2007;38:2804-2811.)

**Key Words:** animal models ■ cerebral infarction ■ chronic stroke ■ functional recovery ■ treatment

Stroke is ranked the number one cause of persistent disability in adults worldwide. In addition to being an enormous public health problem, stroke also represents a serious public financial burden. In the United States, annual costs of health care for stroke rose to \$62.7 billion in 2006, and each stroke victim is burdened with a lifetime cost of \$140 048.<sup>1</sup> Currently, no effective therapy is available for treatment of chronic stroke.

Stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) are the growth factors naturally produced in the body and regulating blood cell production. SCF and its receptor, cKit, are important in hematopoiesis, gametogenesis, and melanogenesis.<sup>2,3</sup> G-CSF binds its specific receptor regulating neutrophilic granulocyte regeneration.<sup>4</sup> SCF+G-CSF synergistically mobilizes CD34<sup>+</sup> progenitor cells from the bone marrow to the bloodstream.<sup>5</sup> However, substantial evidence has shown that SCF and G-CSF may also play roles in the central nervous system. Systemic administration of SCF and G-CSF reportedly increases bone marrow-derived neurons in the intact adult mouse brain.<sup>6</sup> We and others have

observed that the receptors for SCF and G-CSF are expressed in the neurogenic regions and neurons in the adult rodent brain, and SCF and G-CSF stimulate neural progenitor cell proliferation and augment neurogenesis.<sup>7-11</sup> SCF and cKit mutant mice show deficits in spatial learning and memory and long-term potentiation.<sup>12,13</sup> SCF/cKit binding protects cortical neurons from apoptosis and excitotoxicity in vitro.<sup>14</sup> G-CSF has recently been shown to have a neuroprotective effect on focal brain ischemia.<sup>15-17</sup> We previously observed that subcutaneous injections of SCF and G-CSF during the acute phase (3 hours to 7 days) after focal brain ischemia reduced infarction size, improved functional outcome, and increased neural progenitor cell proliferation.<sup>7</sup> In addition, both SCF and G-CSF were able to pass through the blood-brain barrier in intact rats.<sup>18</sup> Interestingly, Kawada et al<sup>19</sup> reported that systemic administration of SCF+G-CSF during 11 to 20 days (subacute phase) after induction of focal brain ischemia reduced infarction size and improved functional outcome. However, it is not known whether SCF and G-CSF have therapeutic effects on chronic brain ischemia. Generally,

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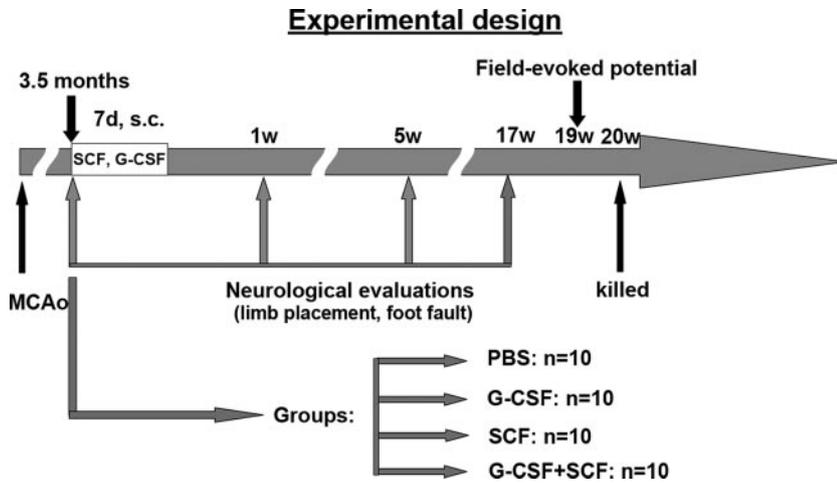
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**Figure 1.** Schematic diagram of experimental design.

beyond 3 to 6 months after stroke onset is considered the chronic phase.<sup>20,21</sup> Therefore, the aim of the present study was to determine whether administration of SCF and G-CSF in the setting of chronic stroke (14 weeks after induction of brain ischemia) could restore impaired function.

## Materials and Methods

The animal experiments and the numbers of animals used in this study were approved by the Animal Care and Use Committee at Northwestern University. All procedures were performed in accordance with the standards of National Institutes of Health guidelines for the care and use of laboratory animals.

### Animal Model of Stroke

Six- to 7-month-old male spontaneously hypertensive rats were used for this study. After anesthesia (50 mg/kg methohexital sodium intraperitoneally; Monarch Pharmaceuticals), cortical brain ischemia was induced in accordance with the method described elsewhere.<sup>22</sup> Briefly, an incision was made in the midline on the neck. The right common carotid artery was dissected and ligated with a 3-0 silk surgical suture. After an incision cut between the right ear and eye, the middle cerebral artery was coagulated with a cauterizer (World Precision Instrument) distal to the striatal branch under a microscope.

### Experimental Design

Fourteen weeks after brain ischemia, neurological deficits were evaluated. Neurological score-matched rats were randomized into 4 groups: phosphate-buffered saline (PBS, treatment control), SCF, G-CSF, and SCF+G-CSF ( $n=10$ /group). Recombinant rat SCF (200  $\mu\text{g}/\text{kg}$ ) and/or recombinant human G-CSF (50  $\mu\text{g}/\text{kg}$ ) (both growth factors provided by Amgen) and equal volumes of PBS were subcutaneously injected for 7 days.

Neurological deficits were assessed with the limb placement test and the foot fault test before therapy and at 1, 5, and 17 weeks after the final injection. Neural activities in the intact and affected somatosensory cortex were recorded by field-evoked potential 19 weeks after treatment. The rats were euthanized 20 weeks post-therapy (34 weeks after brain ischemia), and the infarction volume was measured based on a series of brain sections. The behavioral assessments, field-evoked potential recording, and infarction size determination were all performed and interpreted in a blind fashion (Figure 1).

### Evaluation of Neurological Deficits

In the limb placement test, rats were graded from 0 to 2 in each of the eight subtests<sup>23,25</sup> as follows: score 0, unable to place limb; score 1, partial or delayed (>2 seconds) placement; and score 2, immediate placement. The foot fault test<sup>24</sup> was slightly modified from the

original protocol. The rats were gently held and allowed to walk with the forepaws on a grid field. Ten steps were allowed, and the number of falls or slips was recorded. The intact and affected forepaws were tested individually and each test was repeated twice.

### Recording of Field-Evoked Potentials

The rats were anesthetized with 1% to 1.5% isoflurane. Two recording electrodes were placed on the ipsilateral and the contralateral skull (A: 1.0 mm rostral to the bregma, 2.0 mm lateral to the midline; B: 3.0 mm caudal to the bregma, 1.5 mm lateral to the midline). A stimulating needle was inserted under the skin of a digit, and the parameters of the stimulation were: 2 Hz, 0.4 mA, 500-ms interval, and 300- $\mu\text{s}$  duration. The stimulation lasted for 5 minutes and was repeated twice.

### Determination of the Infarction

At the end of the experiment, the rats were anesthetized (50 mg/kg pentobarbital sodium intraperitoneally) and then transcardially perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer. The brains were cut into 9 pieces, 2-mm thick, with a rat brain matrix. The sections were photocopied and the infarction size was indirectly measured using a software package<sup>25</sup> (Scion Image, Version Beta 4.0.2; Scion Corp). The infarction was presented as a percentage of the contralateral hemisphere.

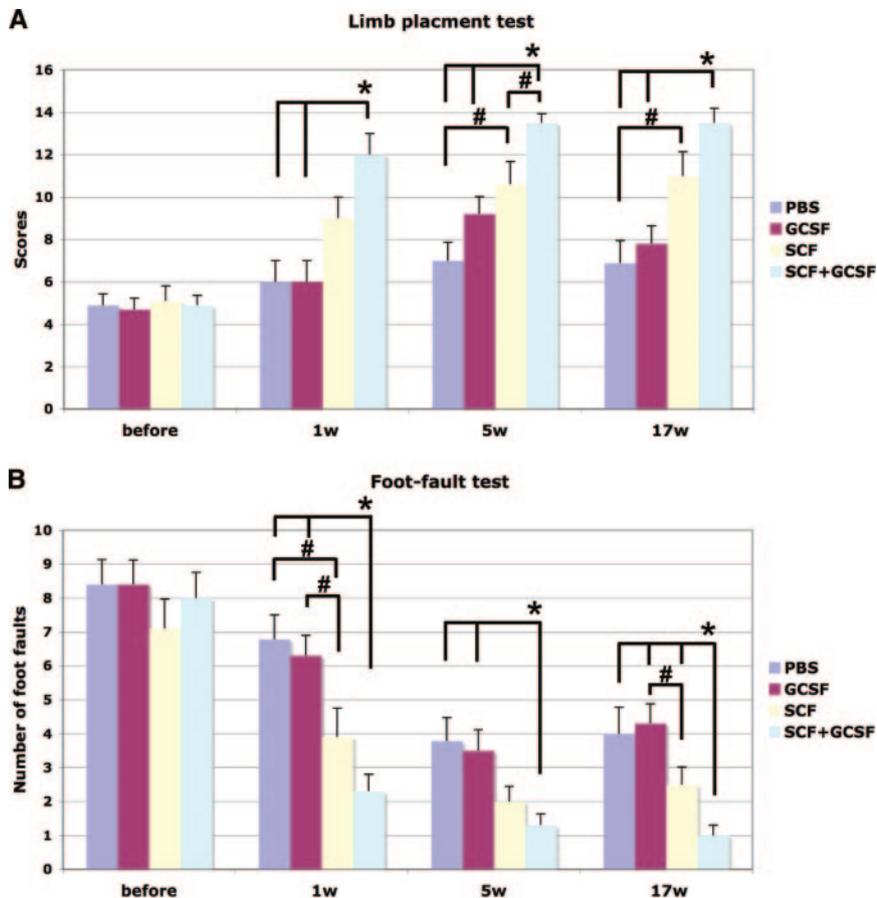
### Statistical Analysis

The behavioral data and the measurements of infarction size (percent of the contralateral hemisphere) were tested with a Kruskal-Wallis nonparametric analysis and adjusted with Bonferroni correction. A Mann-Whitney nonparametric analysis was used for further determination of differences between 2 groups. The differences in total tissue loss ( $\text{mm}^3$ ) and the duration of evoked potential were examined using a one-way analysis of variance with the correction of multiple comparisons. Paired Student *t* test (2-tailed) was used to determine differences in evoked potential between the left hemisphere and the right hemisphere. Data were presented as means  $\pm$  SE.  $P < 0.05$  was considered to be significant.

## Results

### Neurological Deficits Were Ameliorated by SCF+G-CSF Intervention

Rats treated with either PBS or G-CSF alone showed no significant changes in performance on the limb placement test (Figure 2A) at any time over the next 17 weeks. However, rats that received the combination of SCF and G-CSF showed a marked, significant improvement after 1 week of treatment with the growth factors when compared with PBS controls



**Figure 2.** Neurological deficits evaluated by the limb placement test (A) and foot fault test (B). Note that SCF+G-CSF treatment resulted in a stable, longlasting, and significantly functional restoration among the groups ( $n=9$  to  $10/\text{group}$ ). SCF alone also showed functional improvement when compared with PBS and/or G-CSF alone.  $*P<0.01$ ,  $\#P<0.05$ .

and G-CSF alone treatment ( $P<0.01$ ). At 5 weeks after the treatment, the best functional improvement was also observed in SCF+G-CSF-treated rats among the groups (SCF+G-CSF versus PBS and G-CSF,  $P<0.01$ ; SCF+G-CSF versus SCF,  $P<0.05$ ). SCF+G-CSF-induced functional restoration persisted at 17 weeks after the treatment in comparison with PBS controls and G-CSF alone ( $P<0.01$ ). Animals treated with SCF alone also showed significant improvement at 5 and 17 weeks when compared with PBS controls ( $P<0.05$ ).

Results with the foot fault test (Figure 2A) were similar to the findings with the limb placement test in all groups. Animals treated with SCF+G-CSF showed significantly fewer foot slippages than PBS controls and G-CSF alone (1, 5, and 17 weeks after injections) and SCF alone (17 weeks) ( $P<0.01$ ). Animals treated with SCF alone also showed significant reductions in the number of foot slippages at 1 week after the injection in comparison with PBS controls and G-CSF alone ( $P<0.05$ ) and 17 weeks postinjection when compared with G-CSF alone ( $P<0.05$ ).

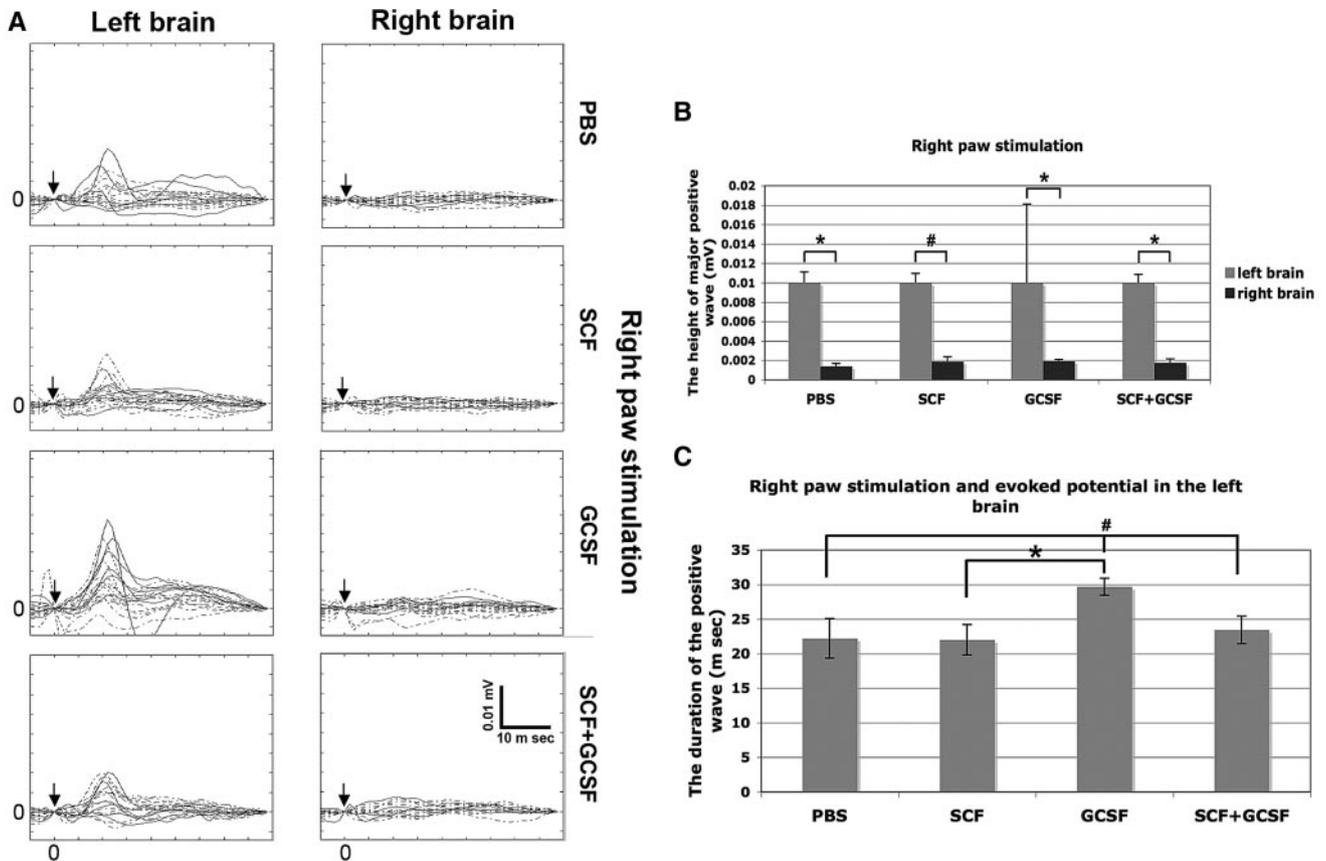
Overall, the combination of SCF and G-CSF resulted in significant functional improvements, which was significantly superior to PBS controls and SCF and G-CSF alone treatment.

#### Field-Evoked Potentials Were Normalized by SCF+G-CSF Intervention

Field-evoked potentials were recorded from the somatosensory cortex in both cerebral hemispheres after unilateral forepaw stimulation. In all groups, when the intact (right)

forepaw was stimulated, prominent evoked potentials were detected in the contralateral hemisphere (left intact hemisphere) (Figure 3A). The neuronal activity between the 2 hemispheres showed significant differences in all groups (Figure 3B) (SCF:  $P<0.05$ ; PBS, G-CSF, and SCF+G-CSF:  $P<0.01$ ). Among the 4 groups, G-CSF-treated rats showed a trend toward increase in the height of the major positive waves in the intact brain but did not reach the statistically significant level (Figure 3B). However, the duration of the evoked potential in G-CSF-treated rats was significantly longer than other groups (Figure 3C) ( $P<0.05$  compared with PBS and SCF+G-CSF,  $P<0.01$  compared with SCF).

Interestingly, when the affected (left) forepaw was stimulated, neuronal activities recorded in both hemispheres were quite different among the groups. In the PBS controls and G-CSF-treated rats, field-evoked potentials were detected in both hemispheres (Figure 4A). Quantification data showed that the evoked potentials between the right and the left brain in the PBS or G-CSF group were no different (Figure 4B). In marked contrast, rats that received injections of SCF+G-CSF showed prominent evoked potentials only in the contralateral (affected) hemisphere (Figure 4A), and the height of the major positive waves in the lesioned brain was significantly higher than in intact brain (Figure 4B) ( $P<0.05$ ). In addition, the duration of the major positive waves in the affected brain were significantly shorter in SCF+G-CSF-treated rats than the rats in other groups (Figure 4C) ( $P<0.05$  compared with PBS and SCF,  $P<0.01$  compared with G-CSF), and the



**Figure 3.** Right paw stimulation induced evoked potential in the somatosensory cortex. A, Field-evoked potential recorded in the left and right somatosensory cortex after electrostimulation on the right intact paw (n=9 to 10/group). B, Quantification of the height of the major positive waves (mV). Note that the neuronal activity was recorded in the left unaffected hemisphere of the rats in all groups, which was significantly higher than the right hemisphere. C, Quantification of the duration of the major positive waves in the left hemisphere after the right paw stimulation. Note that the duration of the evoked potential was significantly longer in G-CSF-treated rats than the rats in other groups. Arrows in A indicate the start time of the simulation. #*P*<0.05, \**P*<0.01.

duration was similar to the evoked potential in the intact brain when the unaffected paw was stimulated. These data indicate that SCF+G-CSF-treated rats showed a normal evoked potential and a normal neuronal activity pattern in the lesioned brain when the affected paw was stimulated. The rats injected with SCF alone also showed prominent evoked potentials in the lesioned hemisphere (Figure 4A). In SCF-treated rats, the height of the major positive waves in the lesioned brain showed a trend toward increase in comparison with the waves in the intact brain, but it did not reach the levels of statistical significance (Figure 4B).

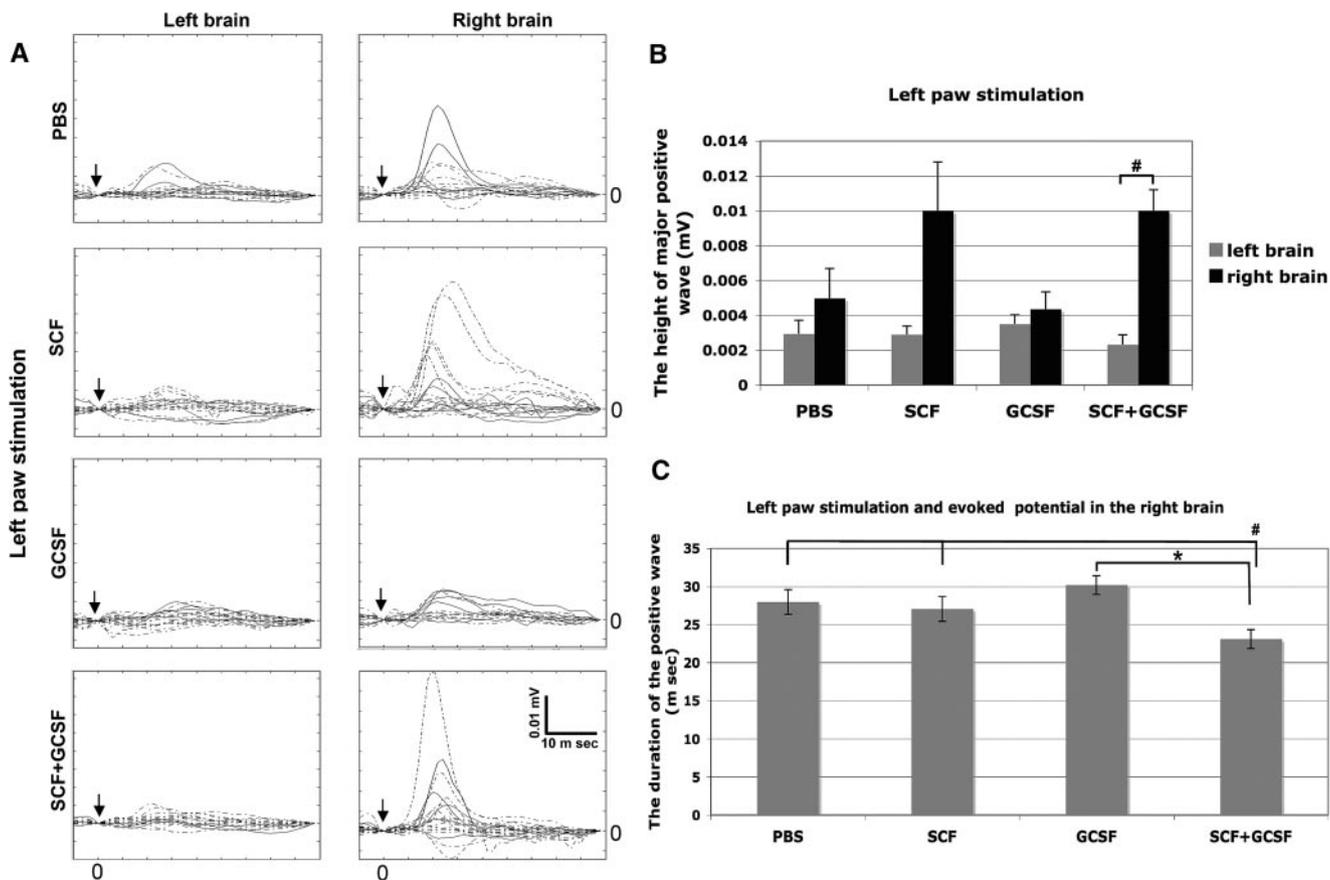
**Infarct Volume Was Reduced by SCF+G-CSF Intervention**

At the completion of the experiment, the animals were euthanized, and infarct volume was determined. Infarction size was expressed as a percentage of the volume of the contralateral hemisphere. There were no differences in the infarct volumes among the control, G-CSF-, or SCF-treated groups. However, the rats treated with both SCF and G-CSF showed a significant reduction in infarction size when compared with PBS control animals (SCF+G-CSF: 9±2%, PBS: 16±2%, *P*<0.05; Figure 5). The combination of SCF and G-CSF treatment reduced the size of infarction by approximately 44% compared with the PBS control animals.

**Discussion**

This study demonstrates that the combined administration of SCF and G-CSF at 14 weeks (3.5 months) after brain ischemia results in improved functional outcome and reduced infarction size. To our knowledge, this is the first evidence that pharmaceutical intervention as late as 3.5 months after stroke can ameliorate neurological deficits and reduce the size of infarcts.

Significant functional improvement occurred within 1 week of administration of the cytokines. The rapidity of the improvement suggests that it involves the stimulation of intact neurons and synaptic reorganization rather than a regenerative process. Substantial evidence supports a role for SCF signaling in brain development and function. Mice with a heterozygous (Sl/Sl) mutation of SCF display a deficit in spatial learning and memory.<sup>12</sup> A homozygous (Ws/Ws) mutation of cKit results in an impairment of long-term potentiation in the hippocampal mossy fiber-CA3 pathway and a deficit in performance in the Morris water maze task.<sup>13</sup> In addition, we previously observed that cKit was expressed on the membrane of neurons and G-CSF receptor was abundantly expressed in the neuronal nuclei in the adult brain of rats,<sup>7</sup> and both SCF and G-CSF crossed the blood-brain barrier, penetrating to the intact brain.<sup>18</sup> Therefore, we postulate that administration of SCF+G-CSF in the chronic



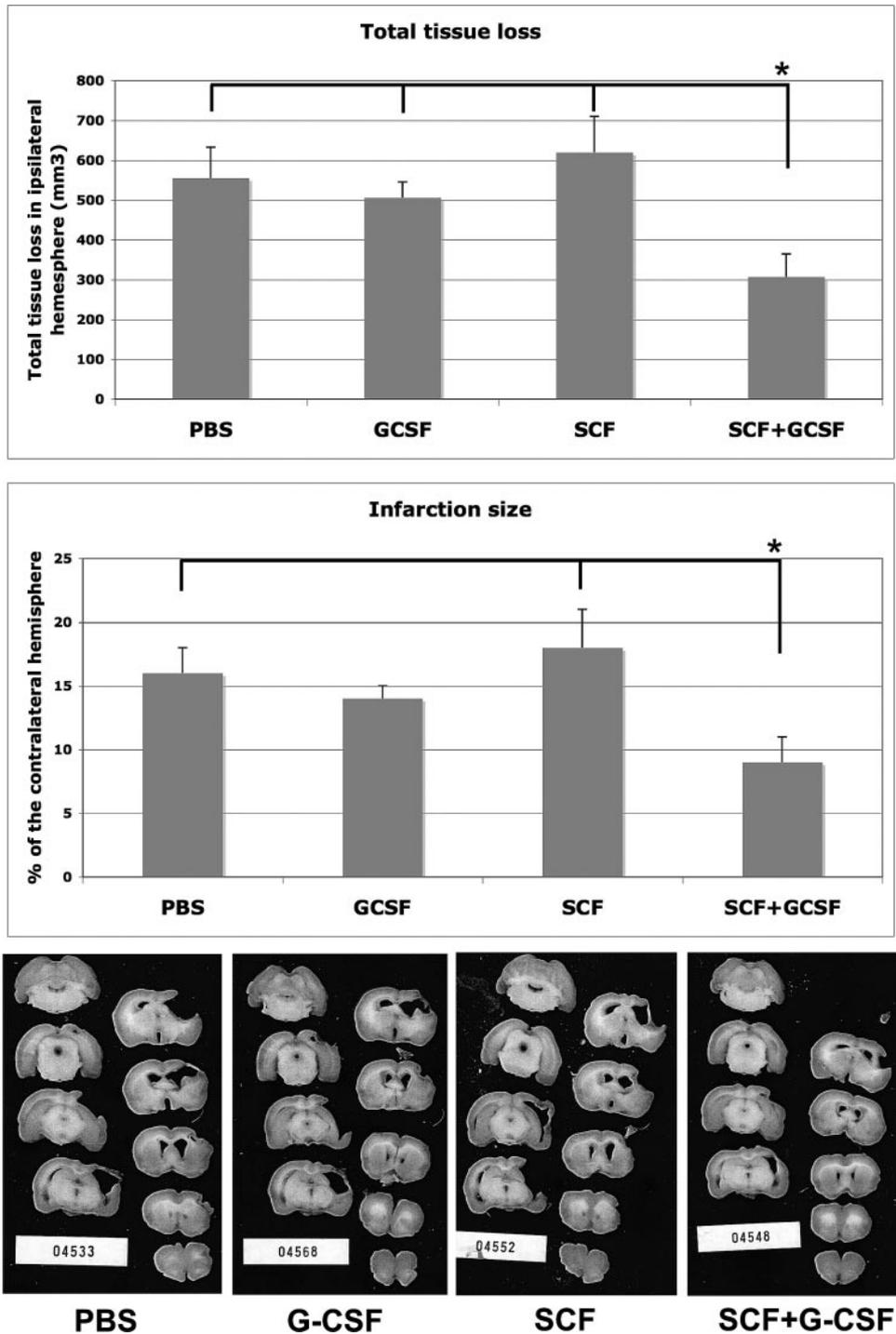
**Figure 4.** Left paw stimulation induced evoked potential in the somatosensory cortex. A, Field-evoked potential recorded in the left and right somatosensory cortex after electrostimulation at the left affected paw ( $n=9$  to  $10$ /group). B, Quantification of the height of the major positive waves (mV). Note that the prominent neuronal activity in response to the stimulation was recorded in the right affected hemisphere of the rats that received SCF or SCF+G-CSF treatment. Neuronal activity of the right brain was significantly increased in SCF+G-CSF-treated rats in comparison to the left brain. C, Quantification of the duration of the major positive waves in the right hemisphere after the left paw stimulation. Note that the duration of the field-evoked potential was significantly shorter in SCF+G-CSF-treated rats than other 3 groups. Arrows in A indicate the start time of the stimulation. # $P<0.05$ , \* $P<0.01$ .

phase of brain ischemia has a direct effect on neuronal plasticity, which participates in functional improvement.

The field-evoked potentials observed in the present study further supported that the growth factors facilitated functional reorganization within the damaged hemisphere. In the clinical studies, using noninvasive brain imaging techniques, investigators found that functional reorganization occurred in both the affected and unaffected hemispheres of patients with stroke.<sup>26,27</sup> In the rat model of stroke, the proteins related to synaptic reorganization were expressed in both hemispheres.<sup>28</sup> However, recent clinical evidence suggests that prominent neuronal activity in the lesioned hemisphere contributes to good functional recovery in patients with stroke,<sup>29–32</sup> whereas neuronal activity in the contralateral hemisphere (unaffected) after brain ischemia may be related to having a large infarction, early response to brain ischemia, and/or compensatory reliance on using the unaffected limb.<sup>32,33</sup> In brain ischemic rats, increase of synaptogenesis in the unaffected brain was evidenced as the result of overusing the unimpaired forelimb, and this compensatory reliance might also exacerbate learned nonuse of the impaired forelimb.<sup>33</sup> Our data further support the current notion that good and long-lasting functional recovery occurs in the lesioned

hemisphere. In the present study, after brain ischemia, unilateral stimulation of an affected digit in PBS control animals elicited neuronal activity in both hemispheres, whereas digit stimulation in the SCF+G-CSF-treated animals elicited prominent neuronal activity only in the appropriate contralateral hemisphere. These results suggest restoration of the normal pattern of neuronal activity in the brains of the SCF+G-CSF-treated animals. Although SCF-treated rats also showed prominent evoked potential in the affected brain, the duration was longer than SCF+G-CSF-treated animals. This difference might be part of the reason that SCF-induced functional recovery was less stable than SCF+G-CSF. Interestingly, G-CSF-treated rats showed a large evoked potential with a longer duration in the unaffected brain when the unimpaired paw was stimulated. Whether this is due to the compensatory reliance on the unaffected limb needs further study to elucidate.

Surprisingly, the administration of SCF+G-CSF also reduced the volume of the infarcts. It has been thought that infarction size is manifested and fixed at 48 hours after focal brain ischemia.<sup>34</sup> Therefore, any treatments afterward should not influence infarction size. However, there is evidence that systemic administration of SCF+G-CSF during the subacute



**Figure 5.** Volumes of infarction influenced by SCF and G-CSF treatment. A, Total tissue loss in the ipsilateral hemisphere (mm<sup>3</sup>); B, the infarction size expressed as a percentage of the contralateral hemisphere; C, representative brain sections from each group. Note that SCF+G-CSF significantly reduced infarction size (\**P*<0.05).

phase of brain ischemia (day 11 to 20) reduces infarction size.<sup>19</sup> It has been shown that secondary tissue loss continues up to the chronic phase of brain ischemia. Hara and coworkers<sup>35</sup> have reported that after focal brain ischemia, secondary tissue loss was observed in the ipsilateral cortex, striatum, substantia nigra, and thalamus from 7 days through 9 months. They also found that expressed as a percentage of the contralateral hemisphere, the remaining tissue in the ipsilateral ischemic hemisphere was 94% at 7 days, 87% at 1 month,

68% at 3 months, and 65% at 9 months. Substantial evidence shows that SCF and G-CSF have neuroprotective effects. Dhandapani et al reported that SCF/cKit binding protects cortical neurons from apoptosis and excitotoxicity in vitro, and the neuroprotective effect is mediated by MEK/ERK and PI3K/Akt signal transduction pathways.<sup>14</sup> Administration of G-CSF during the acute phase after focal brain ischemia leads to a reduction in infarction size.<sup>15-17</sup> The neuroprotective effect of G-CSF was regulated through an

activation of STAT3, ERK, and P13K/Akt pathways, which promotes neuron survival and inhibits apoptosis.<sup>9</sup> Therefore, some of the reduction in infarct size after the growth factor treatment may reflect prevention of secondary tissue loss. However, the 44% reduction in infarct size in the present study is probably too large to be explained solely by prevention of secondary tissue loss and may reflect recruitment of cells to the damaged area as well.

There are a number of different ways that SCF and G-CSF treatment may have helped to recruit new cells to the area of infarction. Both cKit and G-CSF receptor are expressed in the neurogenic regions of the adult rat brain.<sup>7–9</sup> We have observed that systemic administration of SCF+G-CSF during 3 hours to 7 days after brain ischemia promoted neural progenitor cell proliferation in the subventricular zone.<sup>7</sup> Moreover, administration of SCF+G-CSF during the subacute phase of brain ischemia dramatically increased neural progenitor cell proliferation in the subventricular zone and also led to improved functional outcome.<sup>19</sup> In the subventricular zone, enhanced neural progenitor cell proliferation, neuronal fate differentiation, and migration into the infarct areas have been suggested to be involved in functional restoration.<sup>36,37</sup> Recently, Thored et al reported that brain ischemia-induced neuroregeneration persisted up to the chronic phase (4 months after focal brain ischemia), and newly generated neuroblasts continuously migrated into the infarct areas during the chronic phase of brain ischemia.<sup>38</sup> Moreover, both SCF and G-CSF were able to pass through the blood–brain barrier in intact animals.<sup>18</sup> Therefore, it is plausible to postulate that systemic administration of SCF+G-CSF at 3.5 months after brain ischemia promotes neural progenitor cell proliferation, differentiation, and migration into the infarct area, which participates in infarction size reduction. Alternatively, it is possible that treatment with SCF+G-CSF mobilizes circulating hematopoietic stem cells, which enter into the brain and give rise to neural cells. In support of this interpretation, systemic administration of SCF+G-CSF has been shown to increase bone marrow-derived neurons in both the intact<sup>6</sup> and ischemic<sup>19</sup> adult mouse brain. Regardless of the source of the cells, treatment with SCF+G-CSF significantly reduced the volume of the infarcts. Infarction size is known to correlate with the degree of neurological deficit in rats,<sup>39</sup> and the reduction in infarction size in the present study correlated well with functional improvement. Taken together, longlasting function improvement by SCF+G-CSF treatment may be associated with neuroprotection and neural regeneration.

In summary, administration of both SCF and G-CSF in the chronic phase of stroke reduced infarct volume and improved neural function. The benefits appear to reflect both functional reorganization of the damaged hemisphere and recruitment of cells to the area of infarction. Although the precise mechanisms underlying the regenerative effects of the growth factors are not yet known, our findings demonstrate a pharmacological approach to facilitate repair of a chronically damaged brain.

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### Disclosures

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