Chapter 13
Bone Marrow Stem Cell-Stimulating Factors and Brain Recovery After Stroke

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Abstract Stroke is a major cause of death and long-term neurological disability in adults worldwide. In the United States alone, stroke presents a serious public health problem, and it has created heavy public and personal financial burdens. By contrast to the severity of stroke in public health, the treatment of stroke is very limited. Currently, a clot-dissolving drug (rtPA) is the only treatment available for ischemic stroke. The majority of stroke patients are not able to receive this treatment due to the narrow therapeutic window: 4.5 h after stroke onset. Developing new treatment that fits the majority of stroke patients is a huge challenge for stroke research. Over the past decade, numerous studies have shown the therapeutic potential of stem cell factor (SCF) and granulocyte-colony-stimulating factor (G-CSF), the two essential hematopoietic growth factors, in acute, subacute, and chronic stroke. In this chapter, we have reviewed the biological function of SCF and G-CSF in both the hematopoietic system and central nervous system, summarized the progress of SCF and G-CSF research in adult ischemic stroke in both basic and clinical studies, and discussed the directions for future studies.

Keywords Stroke • SCF • G-CSF • Neuroprotection • Neurorestoration • Cerebral ischemia

Abbreviations

AKT	Serine/threonine kinase or protein kinase B
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
BFU-E	Erythroid burst-forming units
BrdU	Bromodeoxyuridine (5-bromo-2′-deoxyuridine)

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K. Jin et al. (eds.), *Bone Marrow Stem Cell Therapy for Stroke*,
DOI 10.1007/978-981-10-2929-5_13

CFU-GM	Granulocyte-macrophage colonies
CNS	Central nervous system
ERK	Extracellular signal-regulated kinase
FDA	Food and Drug Administration
G-CSF	Granulocyte-colony-stimulating factor
G-CSFR	Granulocyte-colony-stimulating factor receptor
GFP	Green fluorescent protein
HMGB1	High mobility group protein B1
HPCs	Hematopoietic progenitor cells
HSCs	Hematopoietic stem cells
IL-1	Interleukin-1
i.p.	Intraperitoneal
i.v.	Intravenous
LTP	Long-term potentiation
MEK	Mitogen-activated protein/extracellular signal-regulated kinase
MRI	Magnetic resonance imaging
M-type	Mushroom type
NF-kB	Nuclear factor kappa beta
NMDA	N-Methyl-D-aspartate
NPCs	Neural progenitor cells
NSCs	Neural stem cells
PI3K	Phosphatidylinositol-3 kinase
PSD-95	Postsynaptic density protein 95
rtPA	Recombinant tissue plasminogen activator
s.c.	Subcutaneous
SCF	Stem cell factor
SGZ	Subgranular zone
SHRs	Spontaneously hypertensive rats
STAT3	Signal transducer and activator of transcription 3
SVZ	Subventricular zone
TNF	Tumor necrosis factor
U-type	Uncertain type
YFP	Yellow fluorescent protein

13.1 Introduction

Stroke is the fifth leading cause of death in the United States, and it remains the number one cause of long-term disability in the world. Over the past two decades, major advances have been made in the understanding of the pathophysiology of stroke, while there has not been much progress in the development of stroke treatment [26].

Of all strokes, 87 % are ischemic [61]. Most stroke studies, therefore, target ischemic stroke. Although great efforts have been made in developing treatments for ischemic stroke, only one drug, recombinant tissue plasminogen activator (rtPA) for thrombolysis, has been approved by the Food and Drug Administration (FDA) for treatment of acute ischemic stroke. This therapeutic approach must be initiated within 4.5 h after stroke onset (1995; [31]). Because of the narrow time window for treatment and the potential risk of intracerebral hemorrhage, in fact, only 1–3 % of stroke patients are able to receive this treatment [90]. As a result, more than 97 % of stroke patients lack a specific treatment. Thus, developing new therapeutic strategies to save a patient's life and improve their functional recovery is a major challenge for stroke research.

Stem cell factor (SCF) and granulocyte-colony-stimulating factor (G-CSF) are the essential hematopoietic growth factors that govern the growth, survival, differentiation, and mobilization of bone marrow stem cells [91, 102, 103]. Since 2003, the therapeutic effects of SCF and G-CSF on neuroprotection and neurorestoration in ischemic stroke have been frequently studied. Here we have briefly reviewed current understanding for the biological function of SCF and G-CSF in both the hematopoietic system and central nervous system (CNS), summarized the progress of SCF and G-CSF research in adult ischemic stroke, and discussed the directions for future studies.

13.2 The Origin and Biological Function of the Stem Cell Factor and Granulocyte-Colony-Stimulating Factor

SCF and G-CSF are the key members of the hematopoietic growth factor family and play important roles in regulating hematopoietic stem cell (HSC) proliferation, differentiation, and mobilization [20, 30, 55, 91, 102]. Since the discovery of SCF and G-CSF, great advancements have been made in understanding of the biological function of SCF and G-CSF and in developing pharmaceutical intervention of SCF and G-CSF to treat hematopoietic diseases and to repopulate and mobilize HSCs. Accumulating evidence has shown that SCF and G-CSF are not only crucially involved in the hematopoietic system [9, 53], but they also play a role in the CNS [95].

13.2.1 The Origin and Biological Function of SCF and G-CSF in the Hematopoietic System

SCF exists as a dimeric glycoprotein with a molecular weight of approximately 45 kDa [55]. SCF is classified into two forms: a membrane-bound form and a soluble form. Both the soluble and the transmembrane forms of SCF are biologically active. SCF is produced by the endothelial cells and fibroblasts [50]. Bone marrow

stromal cells also produce SCF, which regulates the hematopoietic cell development in endocrine and paracrine manners. It has been revealed that HSCs and hematopoietic progenitor cells (HPCs) contain SCF mRNA; therefore, the growth and survival of HSCs/HPCs may also be regulated by autocrine synthesis of SCF [9, 67]. The production of SCF is increased by inflammatory stimuli such as interleukin-1 (IL-1) or tumor necrosis factor (TNF) [9].

C-kit has been demonstrated to be the receptor of SCF [6]. C-kit encodes a transmembrane tyrosine protein kinase receptor, and it has been found to express in HSCs/HPCs [2, 54, 89]. SCF/C-kit binding is a key process for SCF to regulate hematopoiesis. SCF acts directly on an enriched population containing HSCs/HPCs to accelerate their entry into the cell cycle [48] and enhance HSC/HPC expansion and survival in vitro [5, 92]. SCF is also crucially involved in the generation of white blood cells and red blood cells. It has been demonstrated that SCF is a key player for stimulating CD34-positive HSCs to form granulocyte-macrophage colonies (CFU-GM) and macroscopic erythroid burst-forming units (BFU-E) [5].

G-CSF is an approximately 24 kDa hydrophobic glycoprotein containing a neuraminic acid moiety, which regulates biological activity of G-CSF [16]. There are two recombinant forms of G-CSF, one is a non-glycosylated form and the other is a glycosylated form. Both of the two forms have similar biological activities and bioavailability when administered subcutaneously or intravenously. Many cells produce G-CSF after appropriate stimulation. Monocytes are the most prominent source of G-CSF [88]. Mesothelial cells, fibroblasts, and endothelial cells have also been found to produce G-CSF [44, 47, 101].

The receptor of G-CSF (CD114; G-CSFR) is a typical cytokine receptor with one transmembrane domain, an intracellular signal transduction domain, and homo-oligomerizes upon ligand binding [16]. G-CSF receptors are expressed on HSCs/HPCs [54] and mature neutrophilic granulocytes, monocytes, and platelets [7, 77]. The function of G-CSF has been demonstrated to play a vital role in directing the commitment of HSCs/HPCs to common myeloid lineage [68].

Numerous studies have determined the effects of the SCF and G-CSF combination (SCF+G-CSF) on hematopoiesis and HSC/HPC mobilization. It has been shown that SCF+G-CSF synergistically enhances the proliferation, differentiation, and survival of HSCs/HPCs [19]. The synergistic effect of SCF+G-CSF in hematopoiesis may be partially regulated by phosphatidylinositol-3 kinase (PI3K) and ERK signaling [19]. SCF+G-CSF has also been demonstrated to have synergistic effects in the mobilization of HSCs/HPCs from the bone marrow to the bloodstream in both laboratory animals and humans [3, 8, 20, 35, 84].

13.2.2 The Biological Function of SCF and G-CSF in the Central Nervous System

In addition to the effects of SCF and G-CSF in the hematopoietic system, increasing evidence shows that SCF and G-CSF also play a role in the CNS. Receptors for SCF and G-CSF have been found to express in the brain [38, 52], particularly in the

neural stem cells/neural progenitor cells (NSCs/NPCs) [39, 64, 73, 96], and in cerebral neurons [73, 96] of adult mice and rats. It has been demonstrated that both SCF and G-CSF can pass through the blood-brain barrier [73, 100]. These findings suggest that hematopoietic growth factors, SCF and G-CSF, may have biological function in the CNS.

The role of SCF and G-CSF in directing NSCs/NPCs to give rise to neurons has been illustrated in both in vitro and in vivo studies. In cultured NSCs/NPCs, G-CSF has been shown to promote differentiation of NSCs/NPCs into neurons in a dose-dependent manner [73]. Infusing SCF into the cerebrolateral ventricle results in increases of newborn neurons in the neurogenic region, the subventricular zone [39]. When adding SCF and G-CSF during the proliferating stage of NSCs/NPCs, SCF in combination with G-CSF (SCF+G-CSF) shows a dual function in directing cell cycle arrest and promoting neuronal fate commitment through the enhancement of neurogenin 1 activity [64]. Together, these studies reveal that SCF and G-CSF are involved in neurogenesis.

Numerous in vitro and in vivo studies have examined the contribution of SCF and G-CSF in neuronal survival and neuronal plasticity. SCF selectively enhanced the survival of cultured embryonic chick dorsal root ganglia neurons [11]. SCF protects cultured neurons from apoptosis through the regulation of MEK/ERK or PI3K/AKT/NF-kB/Bcl-2 pathways [17]. Using cultured cortical neurons, G-CSF has been demonstrated to counteract programmed neuron death via PI3K mediation [73]. SCF acts as a neurotrophic factor supporting neuron survival during the development of the peripheral nervous system [11, 37]. SCF enhances neurite outgrowth in embryonic dorsal root ganglia [36, 37]. SCF+G-CSF synergistically promotes neurite outgrowth and network formation of cultured cortical neurons through PI3K/AKT/NF-kB/BDNF pathway [82]. Mice deficient in either SCF [60] or C-kit [43] display impaired long-term potentiation (LTP) and spatial learning and memory. G-CSF knockout mice show cognitive impairments, LTP reduction, and impairments in neural network formation in the hippocampus [18]. Collectively, these research data suggest that SCF and G-CSF, the two hematopoietic growth factors, act as neurotrophic factors to regulate neuron survival and neural plasticity. These findings provide insights into the potential role of SCF and G-CSF on neuroprotection and neurorestoration in the treatment of stroke.

13.3 The Role of SCF and G-CSF on Neuroprotection and Neurorestoration in Adult Ischemic Stroke

After a stroke, brain tissue that is located in and outside the infarct area undergoes significant changes including primary neuron loss, secondary neuron loss, neuroinflammation, neuron functional reorganization, neural network rewiring, and blood vessel regeneration. Based on the pathological progression and timing poststroke, stroke is classified into three clinical phases: the acute, subacute, and chronic phase. The duration and pathological severity of the three phases vary between individuals and depend on the infarction size, infarct location, cerebrovascular collateral

response, patient's age, and medical comorbidities. Generally, the acute phase of stroke is the first 48 h after stroke, and the subacute phase of stroke is the period between 48 h and 6 weeks or to 3 months poststroke, whereas the chronic phase starts 3–6 months after stroke [33, 42, 63]. The primary neuron loss in the infarct core and penumbra zone [87] occurs during the acute phase of stroke, and the secondary neuron loss outside the infarct area mainly happens in the subacute phase of stroke [33]. In contrast to the pathological features of neuron loss in the acute and subacute phases [63], in the chronic phase, a stroke patient's neurological status becomes relatively stable, and the surviving neurons establish new networks in an effort to take over the function of the dead neurons [10, 13, 85, 95].

As stated above, the pathological features of the three phases of stroke are different. Therefore, the therapeutic strategies for each phase should be specific to the pathological alterations. The challenge of the specific treatment for each phase of stroke, however, is that the precise boundary among the three phases is difficult to identify and distinguish. It is often seen that some targeting molecules, such as NMDA receptor, matrix metalloproteinases, and intracellular mediator HMGB1, may have neuroprotective benefits in the acute phase of stroke but they may also risk negatively influencing the process of brain repair in the later recovery phase [59]. By contrast to the targeting molecules, increasing evidence has shown that administration of SCF and G-CSF in any of the stroke phases appears to be beneficial. Systemic administration of SCF and G-CSF in the acute or subacute phase of experimental stroke displays neuroprotective benefits; when administering during the chronic phase, SCF and G-CSF show neurorestorative effects in enhancing brain recovery.

13.3.1 The Effects of SCF and G-CSF in Acute Stroke

The effects of SCF and G-CSF in acute stroke have been extensively investigated. There is a large body of publications studying the role of SCF and G-CSF in neuroprotection in the phase of acute stroke in both animal models and stroke patients.

13.3.1.1 The Effects of SCF in Acute Stroke

C-kit, the receptor for SCF, has been shown to be increased in the neurogenic regions (the subventricular zone (SVZ) and the subgranular zone (SGZ)) of adult rats 24 h after cerebral ischemia [39]. Intraventricular delivery of SCF for 3 days post-cerebral ischemia [39] or subcutaneous daily injections of SCF during the period of 3 h and 7 days after induction of cortical ischemia [96] result in increases in the number of BrdU-labeled neural progenitor cells in the SVZ in rat models of focal cerebral ischemia. Intraventricular delivery of SCF for 3 days after focal cerebral ischemia enhances neurogenesis in the neurogenic regions [39]. Subcutaneous daily injection of SCF beginning at 3 h and ending 7 days after cerebral cortical

ischemia shows a robust improvement in sensory motor function 1 week posttreatment. The SCF-induced functional improvement lasts more than 10 weeks after treatment, and the infarction size is reduced by SCF treatment [96]. These findings suggest neuroprotective and neuroregenerative effectiveness of SCF treatment in the acute phase of stroke.

13.3.1.2 The Effects of G-CSF in Acute Stroke

In comparison to SCF, G-CSF has been extensively studied in acute stroke.

Several studies have revealed the role of endogenous G-CSF in neuroprotection after stroke. Using rat models of focal cerebral ischemia, Schneider and coworkers [73] reported that both G-CSF and its receptor, G-CSFR, were widely expressed in the neurons throughout the brain. Two and 6 h after focal cerebral ischemia, G-CSF and G-CSFR were strongly increased in the neurons adjacent to the infarct area. In addition to rodents, G-CSFR is also robustly expressed in the peri-infarct neurons of human brain in the acute phase of ischemic stroke [34]. In G-CSF-deficient mice, the infarct volume is increased, and cerebral ischemia-induced neurological deficits are exacerbated as compared to wild-type mice. Systemic injections of G-CSF to the mice lacking of G-CSF before and 2 days after focal cerebral ischemia prevent G-CSF deficiency-induced enlarged infarction size and worsened neurological outcome [76]. These studies suggest that endogenous G-CSF and G-CSFR in neurons play an important role in neuroprotection.

The efficacy of exogenous administration of G-CSF in the acute phase of stroke has been largely examined in rat models [49, 72–74, 78, 80, 96] or mouse models (C57BL mice) of focal cerebral ischemia [27, 46, 79]. G-CSF treatment is initiated at the time points ranging from 30 min to 48 h after induction of ischemia with either single injection [27, 46, 73, 74, 79] or daily injections up to 10 days post-ischemia [49, 72, 73, 78, 80, 96]. Systemic administration of G-CSF (s.c., i.v., or i.p.) with treatment dosages of 50 μg/kg or 60 μg/kg shows beneficial effects in reducing infarction size and ameliorating neurological deficits [27, 46, 49, 72–74, 78–80, 96]. These research findings indicate that exogenous administration of G-CSF in the acute phase of stroke protects neurons from ischemic injury.

Understanding the mechanism underlying G-CSF treatment-induced neuroprotection in the acute phase of cerebral ischemia remains incomplete. Accumulating evidence shows that G-CSF treatment in the acute phase of focal cerebral ischemia reduces the disruption of the blood-brain barrier [49], reduces brain edema [28], decreases glutamate release in the infarcted striatum [32], suppresses pro-inflammatory cytokines and inflammatory mediators in the peri-ischemic areas [28, 74], and inhibits peripheral inflammatory cell infiltration to the ischemic hemisphere [49]. Anti-apoptosis may be involved in G-CSF-induced neuroprotection in acute ischemic stroke. Activation of STAT3/Bcl2 signaling, which plays a role in inhibiting apoptosis, is increased in the ipsilesional hemisphere by G-CSF treatment in the acute phase of cerebral ischemia [46, 70, 80]. G-CSF treatment in acute ischemic stroke reduces the number of cleaved caspase-3 expressing neurons in the

injured cerebral cortex [80]. In addition, BrdU-labeled proliferating cells are increased in the ipsilesional hemisphere [78] or peri-infarct areas [96] after G-CSF treatment in acute cerebral ischemia. Neurogenesis and angiogenesis in the ipsilesional hemisphere are also enhanced by G-CSF treatment in acute ischemic stroke [49, 75]. However, these studies demonstrate the correlation between G-CSF-induced neuroprotection and G-CSF treatment-related cellular and molecular events such as anti-inflammation, anti-apoptosis, and pro-angiogenesis. The causal link mechanism of G-CSF-induced neuroprotection remains to be determined.

Controversial results have been reported regarding the neuroprotective role of G-CSF in acute stroke. Using a mouse model of permanent occlusion of the middle cerebral artery in CB-17 mice, Taguchi and colleagues [83] reported a negative result of G-CSF in acute ischemic stroke. Subcutaneous (s.c.) delivery of G-CSF (0.5, 5, 50, or 250 μg/kg) beginning at 1 h or 24 h poststroke and continuing up to 3 days or 7 days resulted in no change in infarction size at 3 days poststroke but increases in brain atrophy at 35 days poststroke in all tested doses except for the lowest one (0.5 μg/kg). The G-CSF treatment also impaired neurobehavioral function and increased infiltration of inflammatory cells (CD11b expressing cells and F4/80 positive cells) into the peri-infarct area. This negative study raises a caution flag on the neuroprotective effectiveness of G-CSF in acute stroke.

Two studies of meta-analysis have assessed the effects of G-CSF on acute stroke using animal models of focal cerebral ischemia. These studies have revealed that (1) G-CSF treatment initiating within 6 h or later than 6 h post-ischemic induction reduces infarction size and enhances functional recovery and the effectiveness of treatment within 6 h poststroke shows a dose-dependent manner [56] and (2) G-CSF treatment reduces motor impairments and death, while G-CSF reduces infarction size in transient but not in permanent models of focal ischemic stroke [22].

Clinical trials of G-CSF intervention for acute stroke have been carried out. In a phase IIa study, intravenous delivery of G-CSF to acute ischemic patients at doses of 30–180 μg/kg over the course of 3 days was reported to be safe and well tolerated [71]. In a small patient-sized phase I clinical trial, G-CSF was also proven safe and well tolerated in acute ischemic stroke patients, and G-CSF intervention showed improvement of neurological function [58]. However, the positive results do not display in a large phase IIb trial [69]. In this multinational, multicenter, randomized, and placebo-controlled trial (NCT00927836), G-CSF (135 μg/kg, i.v. over 72 h) was administered within 9 h post-ischemic stroke to the patients with an infarct location in the middle cerebral artery territory. However, G-CSF intervention failed to reduce the infarct size and improve functional outcome.

13.3.1.3 The Effects of SCF+G-CSF in Acute Stroke

Using a cerebral cortical ischemia model of stroke in spontaneously hypertensive rats (SHRs), in addition to SCF or G-CSF alone treatment [96], we have determined the therapeutic effects of SCF+G-CSF in acute stroke [96]. In this study, SCF+G-CSF was subcutaneously injected for 7 days beginning at 3 h post-ischemia. Similar

to treatments of SCF or G-CSF alone, the combined SCF+G-CSF treatment led to reduction of infarction size. The BrdU-labeled cells in the peri-infarct area were also increased by SCF+G-CSF treatment. In contrast to the robust effects of SCF alone treatment in somatosensory and motor function recovery at 1 week posttreatment, SCF+G-CSF-treated rats did not show functional improvement at this early time point. However, the SCF+G-CSF-treated rats displayed a delayed but long-lasting improvement of somatosensory and motor function, which was detected at 4, 7 and 10 weeks after treatment. Toth and coworkers [86] examined the efficacy of SCF+G-CSF treatment in acute stroke using a cerebral cortical ischemia model of stroke in C57BL mice. SCF+G-CSF treatment, which initiated at immediately after ischemia followed by daily injections (i.p.) for 5 days, reduced infarct volume, promoted homing of BrdU/GFP co-expressing bone marrow-derived progenitor cells into the ischemic brain, increased $CD31^{+}/GFP^{+}$ endothelial cells in the ipsilesional hemisphere, and increased angiogenesis. Using the same mouse model of ischemic stroke as stated above [86], SCF+G-CSF was administered daily (s.c.) during the period of 1–10 days after induction of cerebral ischemia. In addition to reducing infarction size, SCF+G-CSF treatment increased bone marrow-derived neurons in the peri-infarct area and improved spatial learning and memory [45]. These studies shed light on the involvement of bone marrow-derived progenitor cells in SCF+G-CSF-induced beneficial effects in acute ischemic stroke.

13.3.2 The Effects of SCF and G-CSF in Subacute Stroke

There are even fewer studies targeting the therapeutic effects of SCF and G-CSF in subacute stroke than the studies of acute stroke. Using a rat model of transient focal ischemia, Lee and coworkers [49] determined the efficacy of G-CSF in subacute stroke. G-CSF was injected daily (i.p.) for 3 days beginning at 4 or 7 days post-ischemia. G-CSF treatment initiated at 4 days post-ischemia showed better functional improvement and greater reduction in hemispheric atrophy when compared to the treatment that was initiated at 7 days post-ischemia. Using a mouse (C57BL) model of cerebral cortical ischemia, Kawada and colleagues [45] examined the effects of SCF+G-CSF in both acute stroke (treatment during 1–10 days post-ischemia) and subacute stroke (treatment during 11–20 days post-ischemia). They observed that the treatment in both acute and subacute stroke reduced infarction size; however, SCF+G-CSF treatment in the subacute phase of cerebral ischemia showed better improvement in functional recovery and greater increases in bone marrow-derived neurons in the ipsi-infarct hemisphere and in NPC proliferation in the neurogenic region (the SVZ). In addition, they also found that SCF+G-CSF displayed a synergistic effect in promoting proliferation of NPCs in the SVZ as compared to SCF or G-CSF alone treatment. A study from the same group of Kawada showed that SCF+G-CSF treatment in the subacute phase of cerebral ischemia upregulated IL-10, an anti-inflammatory cytokine, on a much greater scale than the treatment in the acute phase [57]. Overall, it appears that the optimal timing

for treatment is quite different between the treatment of G-CSF alone and SCF+G-CSF combination treatment: for G-CSF, the earlier the better, while for SCF+G-CSF, the later the better. The mechanism underlying this timing sensitivity for G-CSF or SCF+G-CSF treatment remains largely unknown. It provides an insightful notion, however, in directing future therapeutic studies for stroke: treatment efficacy depends on the timing of the intervention.

A phase IIb single-center, randomized, and placebo-controlled clinical trial for assessing the safety of G-CSF in subacute stroke has been completed. In this clinical study, G-CSF (10 μg/kg) or placebo was given subcutaneously for 5 days to 60 stroke patients. The treatment was initiated 3–30 days after stroke. G-CSF treatment showed a trend toward the reduction of the infarct volume in magnetic resonance imaging (MRI) results but did not cause significant adverse effects as compared to placebo controls, suggesting that G-CSF is safe when administered in the subacute phase of stroke [23].

13.3.3 The Effects of SCF and G-CSF in Chronic Stroke

Over the past decade, our research team has demonstrated the efficacy and possible mechanisms of SCF and G-CSF on stroke recovery in the chronic phase of stroke using rodent models of cerebral cortical ischemia.

SCF and G-CSF intervention in chronic stroke has been tested and validated to be effective when administered 3.5–6 months after cerebral cortical ischemia in both SHRs, C57BL mice, or transgenic mice with C57BL genetic background [13, 14, 51, 65, 66, 99].

First, we determined the efficacy of SCF and G-CSF treatment on stroke recovery in the chronic phase using a cerebral cortical ischemia model in SHRs.

The rationale for using SHRs is that hypertension is the most important risk factor for stroke in humans [41]. Chronic hypertension causes extensive changes in the cerebrovascular bed [4, 40]. Occlusion of the middle cerebral artery distal to the striatal branch and/or of the ipsilateral common carotid artery in SHRs leads to a more consistent and larger infarction in the cortex than in normotensive rats because of inadequate blood flow through collateral vessels [4, 12, 21, 29, 81, 94, 96–99]. In addition to the consistent infarction, this model also induces permanent deficits in somatosensorimotor function that last up to the chronic phase of stroke. Further, this model has no problem for long-term survival [62, 81, 94, 96–99]. Using the cortical ischemia model in SHRs, SCF (200 μg/kg), G-CSF (50 μg/kg), or SCF+G-CSF was subcutaneously administered for 7 days beginning at 3.5 months post-ischemic stroke. We found that only the SCF+G-CSF combination treatment led to a stable and long-term (17 weeks) improvement in somatosensory motor function. SCF alone treatment resulted in functional improvement but the improvement did not present as stable as the SCF+G-CSF combination treatment. G-CSF alone treatment did not show functional benefits. In addition, field-evoked potentials further validated the neurobehavioral findings and revealed that a normal pattern of somatosen-

sory pathways was reestablished by SCF+G-CSF treatment. In addition to the functional improvement, the infarct cavity was reduced in SCF+G-CSF-treated SHRs [99], suggesting that neural regeneration may be involved in brain repair by SCF+G-CSF treatment in the chronic phase. This study provides first evidence that functional restoration in the chronic phase of stroke is possible through the SCF+G-CSF combination treatment.

We have also assessed the safety and effectiveness of the SCF+G-CSF combination treatment on stroke recovery in the chronic phase using aged animals [65], because stroke has the highest incidence in those over the age of 60 [61]. Aged male SHRs (11–13 months) and C57BL mice (16–18 months) were subjected to focal cerebral cortical ischemia. These ages of SHRs and C57BL mice are equivalent to 61–72 years in humans based on their differences in average lifespan [65]. Six dosages were examined examined in the chronic phase of ischemic stroke in the aged SHRs and C57BL mice: (1) SCF+G-CSF at 200 μg/kg for SCF and 50 μg/kg for G-CSF, (2) SCF+G-CSF at 100 μg/kg for SCF and 25 μg/kg for G-CSF, (3) SCF+G-CSF at 50 μg/kg for SCF and 25 μg/kg for G-CSF, (4) SCF+G-CSF at 20 μg/kg for SCF and 10 μg/kg for G-CSF, (5) SCF+G-CSF at 10 μg/kg for SCF and 5 μg/kg for G-CSF, and (6) SCF+G-CSF at 5 μg/kg for SCF and 2.5 μg/kg for G-CSF. Subcutaneous injections of SCF+G-CSF were given for 5 days beginning at 3–4 months after induction of cerebral ischemia. We observed that all six tested dosages did not cause either acute or chronic toxicity to the livers and kidneys, demonstrating that SCF+G-CSF treatment for chronic stroke is safe for the aged population. When determining the effects of SCF+G-CSF in mobilizing bone marrow stem cells into the blood, the three higher dosages of SCF+G-CSF showed significant elevation of C-kit-expressing stem cells in the blood. In a somatosensory motor testing (limb placement test), two higher dosages of SCF+G-CSF (100/25 μg/kg, and 50/25 μg/kg) led to stable and long-term functional improvement. The intermediate dose of SCF+G-CSF (20/10 μg/kg) showed a short-term improvement, whereas the two lower dosages did not improve somatosensory motor function in the chronic phase of stroke in aged SHRs. These findings suggest that the SCF+G-CSF combination treatment for chronic stroke recovery is a safe and effective approach for the aged population. SCF+G-CSF combination treatment in chronic stroke mobilizes bone marrow stem cells and improves functional recovery in a dose-dependent manner.

We have carried out several mechanistic studies to understand how SCF+G-CSF combination treatment repairs a stroke-damaged brain in the chronic phase. Using a bone marrow transplantation approach to track bone marrow-derived cells, our study revealed that bone marrow-derived endothelial cells and bone marrow-derived neurons were involved in SCF+G-CSF-enhanced angiogenesis and neurogenesis in the brain of chronic stroke [66]. To determine the effects of SCF+G-CSF in regulating dynamics of synaptic circuits in the chronic phase of experimental stroke, we used two-photon microscopy to scan the brain area adjacent to the infarct cavity before and after SCF+G-CSF treatment in aged Thy-1-YFPH mice (C57BL background) [13] (Fig. 13.1). The Thy-1-YFPH mice express yellow fluorescent protein (YFP) only in the layer V pyramidal neurons [24]. Before treatment, the number of

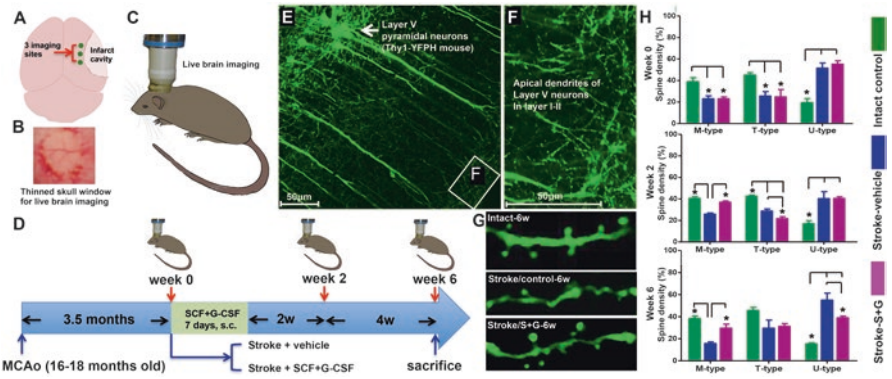


Fig. 13.1 Rebuilding synaptic circuits in the peri-infarct cavity cortex are enhanced by SCF+G-CSF treatment in the chronic phase of experimental stroke in aged mice. (**a**) Schematic diagram showing the three imaging sites adjacent to the infarct cavity. (**b**) Schematic diagram showing a thinned skull window that was prepared for live brain imaging. (**c**) Schematic diagram of the live brain imaging. (**d**) Schematic diagram of experimental design. (**e**) Layer V pyramidal neurons in the cortex of Thy-1-YFPH mice. Note that *yellow* fluorescent protein (YFP) is only expressed in the layer V pyramidal neurons. Boxed area is the layer I–II of cortex enlarged in the panel F. (**f**) The apical dendrites and dendritic spines of the layer V pyramidal neurons distributing in the layer I–II of cortex of Thy-1-YFPH mice. (**g**) Representative live brain images of the apical dendrites and dendritic spines in the brains of intact controls, stroke-vehicle controls, and stroke-SCF+G-CSF-treated mice at 6 weeks posttreatment. Note that the majority of dendritic spines in the cortex of intact controls are mushroom type of spines (M-type), while the majority of dendritic spines in the cortex of stroke-vehicle controls are uncertain type of spines (U-type). The mushroom type of spines appears to be increased in the cortex of stroke-SCF+G-CSF-treated mice. (**h**) Dynamics of dendritic spines before and after treatment. Note that M-type spines and thin-type of spines (T-type) are reduced in the two stroke groups, while the U-type spines are increased in the two stroke groups before treatment. These findings suggest that synaptic degeneration or reduced synaptic circuits occurs in the peri-infarct cavity cortex in the chronic phase of experimental stroke. Two weeks after treatment, stroke-SCF+G-CSF-treated mice show increases in M-type spines and decreases in T-type spines, suggesting the reestablishment of synaptic circuits in the peri-infarct cavity cortex. Six weeks after treatment, M-type spines are increased, and the U-type spines are reduced in the stroke mice treated with SCF+G-CSF. These data indicate that SCF+G-CSF treatment rebuilds synaptic circuits in the peri-infarct cavity cortex. This figure summarizes the results of the study published elsewhere [13]

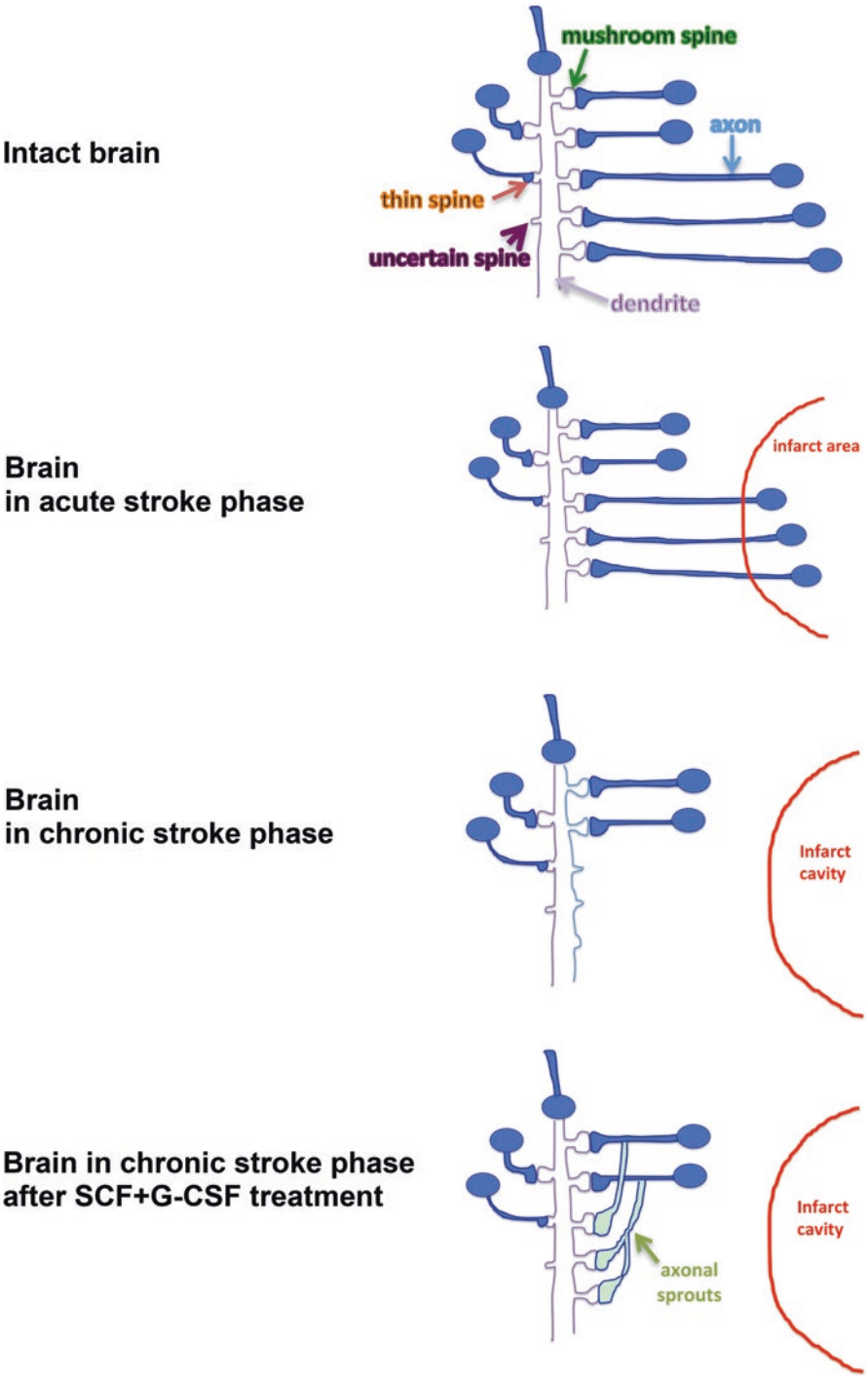
mushroom-type (M-type) spines in the layer V pyramidal neurons was reduced, and the uncertain-type (U-type) spines, which cannot build synapses with other neurons, were increased in the stroke mice. This observation suggests that reduced synaptic circuits occur in the peri-infarct cavity cortex in chronic stroke brain. Six weeks after SCF+G-CSF treatment, however, the M-type spines were significantly increased, and the U-type spines were significantly reduced in the layer V pyramidal neurons adjacent to the infarct cavity. In addition to the two-photon live brain imaging, immunohistochemistry data showed significant increases of postsynaptic density protein 95 (PSD-95) puncta and dendritic branches in the peri-infarct cavity cortex 6 weeks after SCF+G-CSF treatment. These findings suggest that SCF+G-CSF treatment in the chronic phase of stroke enhances synaptic network regenera-

tion in the peri-infarct cavity cortex (Fig. 13.2). This study advances the current knowledge of stroke recovery: an aged brain damaged by ischemic stroke is reparable by a pharmaceutical approach, SCF+G-CSF.

Our follow-up studies have clarified that neural network rewiring in the peri-infarct cavity cortex is required for SCF+G-CSF-enhanced functional improvement in the chronic phase of experimental stroke. In an in vitro study, we have demonstrated that SCF+G-CSF synergistically enhances neurite outgrowth and neural network formation through NF-kB mediation [82]. In an in vivo study [14], we sought to use an approach for blocking the NF-kB-mediated neural network rewiring to determine the causal link between the SCF+G-CSF-promoted neural network regeneration and SCF+G-CSF-enhanced functional improvement in the chronic phase of experimental stroke. In this study, NF-kB inhibitor was infused into the lateral ventricle in the contralesional cortex before and during the 7-day subcutaneous injections of SCF+G-CSF. Motor function was evaluated before treatment as well as 2 and 6 weeks after treatment. Our data revealed that SCF+G-CSF treatment in the chronic phase of stroke increased axonal sprouting, synaptogenesis, and angiogenesis specifically in the peri-infarct cavity cortex but not in the contralesional cortex. NF-kB inhibitor completely abolished the SCF+G-CSF-increased axonal sprouting, synaptogenesis and angiogenesis in the peri-infarct cavity cortex 10 weeks after treatment. In addition, the SCF+G-CSF-improved motor function at 2 and 6 weeks posttreatment was also eliminated by NF-kB inhibitor. This study demonstrates a key role of neural network rewiring in the peri-infarct cavity cortex in the SCF+G-CSF-enhanced motor function recovery in the chronic phase of stroke.

To further identify whether the SCF+G-CSF-enhanced synaptic network rewiring and the SCF+G-CSF-enhanced motor function recovery occurred simultaneously, we carried out an independent study. In this study [15], using a combination approach through live brain imaging, whole brain imaging, molecular manipulation, synaptic and vascular assessments, and motor function examination, we further validated our findings that the SCF+G-CSF-enhanced motor function recovery in the chronic phase of stroke was linked to neural network rewiring in the peri-infarct cavity cortex. Thy-1-YFPH mice were also used for this study. SCF+G-CSF treatment was initiated at 6 months post-experimental stroke. Similar to the earlier study [14], infusion of NF-kB inhibitor was used for blocking the SCF+G-CSF-enhanced neural network rewiring in the peri-infarct cavity cortex.

A previous study reported that motor activity in a Rota-Rod could modify dendritic spine formation [93]. To prevent altering dendritic spines by repeated motor function tests with a Rota-Rod, the chronic stroke mice without behavioral tests were used for live brain imaging and whole brain imaging to identify SCF+G-CSF per se induced remodeling of synaptic circuits in the cortex adjacent to the infarct cavity and/or in the contralesional cortex and to determine whether the synaptic circuit rewiring in the peri-infarct cavity cortex simultaneously happens when motor function is improved by SCF+G-CSF treatment. Our findings showed that SCF+G-CSF treatment at 6 months poststroke improved motor function recovery. SCF+G-


Intact brain
mushroom spine
axon
thin spine
uncertain spine
dendrite
Brain
in acute stroke phase
infarct area
Brain
in chronic stroke phase
Infarct
cavity
Brain in chronic stroke phase
after SCF+G-CSF treatment
Infarct
cavity
axonal
sprouts

CSF promoted mushroom spine formation, enlarged postsynaptic membrane size, and increased postsynaptic PSD-95 accumulation and blood vessel density in the peri-infarct cavity cortex but not in the contralesional cortex. When two-photon live brain imaging showed SCF+G-CSF-enhanced synaptic circuit regeneration in the peri-infarct cavity cortex 2 and 6 weeks posttreatment, motor functional improvement was also seen in the SCF+G-CSF-treated mice 2 and 6 weeks posttreatment.

Once the SCF+G-CSF-increased synaptic network regeneration in the peri-infarct cavity cortex was blocked by NF-kB inhibitor, the SCF+G-CSF-improved motor function was also eliminated. This study has further confirmed that the enhanced neural network formation in the peri-infarct cavity cortex via NF-kB regulation is crucially involved in the SCF+G-CSF-improved motor function in chronic stroke.

A double-blinded, randomized, and placebo-controlled clinical trial for examining the safety and efficacy of G-CSF in chronic stroke has been conducted. Forty-one ischemic stroke patients (>4 months after stroke) were included in this trial. G-CSF (10 μg/kg, s.c.) was given for 10 days. The results showed that the G-CSF treatment was safe to the chronic stroke patients, whereas the improved functional outcome was not seen in G-CSF-treated patients. Authors discussed including more patients in future studies to increase the power of statistical analysis [25].

13.4 Concluding Remarks

Unlike rtPA therapy that has a limited therapeutic window within 4.5 h after ischemic stroke onset, SCF and G-CSF appear to have broad therapeutic potential for acute, subacute, and chronic stroke according to the basic studies using animal models of focal ischemic stroke. The majority of these studies used one-dose and one-time treatment, and some of the studies examined different dosages and treatment time points. Based on the findings, the optimal time for G-CSF treatment appears to be the acute phase or earlier subacute phase of stroke; for SCF, the best treatment time may be the acute phase of stroke; and for SCF+G-CSF combination treatment, the optimal treatment time appears to be the later stage – the subacute and chronic

Fig. 13.2 Schematic diagram of synaptic networks in different conditions. In intact brain, the majority of dendritic spines are the mushroom-type spines. Thin-type spines are the flexible spines that either grow into large mushroom spines or shrink/disappear in response to microenvironment changes. The uncertain-type spines are the spines under degeneration, and this type of spine cannot form synaptic connections with other neurons. During the acute phase of stroke, neurons in the infarct area die due to lack of blood supply. As a result, the post-synapses of the dead neurons undergo degeneration (mushroom spines shrink to uncertain-type spines). SCF+G-CSF treatment in the chronic phase of experimental stroke promotes axonal sprouting and dendritic branching and enhances mushroom spine formation and synaptogenesis. Thus, the SCF+G-CSF treatment enhances rebuilding of synaptic circuits and neural networks in the peri-infarct cavity cortex in the chronic phase of experimental stroke. The schematic diagram shown in this figure is based on our previous studies published elsewhere ([13, 14], 2016)

phases of experimental stroke. However, it remains largely unknown why the treatments of SCF and G-CSF alone or combination treatment have different optimal timings and how SCF and G-CSF alone or combination treatment protects neurons from ischemic injury or restores/repairs neuron function after stroke.

Before moving to clinical trials, several crucial questions need to be addressed: Do we use the most clinically relevant animal models of stroke to determine the therapeutic effects? Do we clarify the precise pathological features for acute, subacute, and chronic phases of stroke? Do we demonstrate the optimal intervention timing, dosage, and delivery route for SCF and G-CSF alone or combination treatment? Do we validate the research findings using different animal models especially in nonhuman primates?

Although clinical trials have proven the safety of using G-CSF in treating acute, subacute, and chronic stroke patients, the efficacy of G-CSF in functional recovery has not yet been demonstrated positively. It is worth noting that keeping the infarct type/size and treatment time point uniform is relatively easier in basic science research using animal models than clinical studies using stroke patients. The variation in infarction size and location, cerebrovascular collateral response, patient's age, sex, race, and medical comorbidities as well as differences in intervention timing may cause robust increases in standard deviation of research data. As a result, significant increases of sample sizes (number of stroke patients) are required for reaching the levels of statistical difference in clinical trials.

Overall, SCF and G-CSF research brings new hope for developing a new treatment for stroke as these hematopoietic growth factors show therapeutic potential for acute, subacute, and chronic stroke. Many open questions, however, need to be addressed in the future for both basic research and clinical trials.

Acknowledgments The authors would like to thank Sandy McGillis for her assistance in artworks for the live brain imaging and in editing the manuscript. This work was partially supported by the National Institutes of Health and National Institute of Neurological Disorders and Stroke (NINDS)(R01 NS060911) in the United States.

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