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Neuroprotection of G-CSF in cerebral ischemia

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1. ABSTRACT

In several experimental studies of cerebral ischemia, G-CSF exerts neuroprotective effects through different mechanisms, including mobilization of hematopoietic stem cells, anti-apoptosis, neuronal differentiation, angiogenesis and anti-inflammation. Hence, G-CSF not only inhibits neuron death, but also generates "new" neural tissue formation. A small pilot trial reports on the safety and feasibility of G-CSF therapy in stroke patients. According to this evidence, we can speculate that G-CSF, being used either alone or in combination with another agent, should be an effective strategy in the treatment of cerebral ischemia.

2. INTRODUCTION

Stroke is a leading cause of death and disability worldwide and the number of patients afflicted with cerebral ischemia is on the increase at present, with no effective clinical treatment that enhances recovery. Currently, some thrombolytic agents such as tissue plasminogen activator and urokinase are available to patients who have suffered from acute ischemic stroke, but treatment with tissue-plasminogen activator is limited by side effects and by the fact that it must be initiated within a short window of time, only a small percentage (10-18%) of ischemic stroke patients can undergo thrombolysis (1-3). Therefore, the attention has focused on neuroprotective

strategy that could potentially expand the therapeutic time window in patients with acute ischemic stroke. Numerous neuroprotective strategies aiming at important targets such as glutamate toxicity or free radical formation have failed due to lack of efficacy or intolerable side effects. It is believed that a successful strategy should be well tolerated, not interfere with essential brain physiology. The understanding of the mechanisms involved in the brain plasticity and their modulation, together with the possibility of restoring functional deficits by encouraging endogenous neurogenesis or by cell therapy, opens up new directions in the treatment of stroke patients.

Granulocyte-colony stimulating factor (G-CSF), a member of cytokine family of growth factors, mainly stimulates the proliferation, survival, and maturation of the neutrophilic granulocyte lineage and is used to treat neutropenia. Recently, a series of studies have demonstrated the neuroprotective effect of G-CSF in cerebral ischemia (4-7). However, the precise mechanisms of the neuroprotective effect of G-CSF are not entirely known. Here, we have summarized the biological properties, clinical applications, and novel mechanisms of G-CSF as a potential therapeutic agent in cerebral ischemia.

3. G-CSF AND NEUROPROTECTION

3.1. Evidence for neuroprotection of G-CS in cerebral ischemia

G-CSF displays a significant neuroprotection after cerebral ischemia. Administration of G-CSF reduced infarction volume (5,6,8) and mortality rate was significantly declined in animals treated with G-CSF compared with control animals (8,9). G-CSF-treated experimental models showed the better functional recoveries from 2 weeks through 5 weeks after ischemia compared to the cerebral ischemia-only controls (6,9). G-CSF given in the subacute phase (days 11 to 20) effectively improved not only motor performance but also higher brain function, compared with acute-phase treatment (days 1 to 10) (10). Neutrophilic blood count was significantly increased after 24 hours in G-CSF–treated animals compared with controls (8). G-CSF injection also showed a reduction in hemispheric atrophy at 35 days after cerebral ischemia and a significantly lower level of Evans blue dye extravasation compared to cerebral ischemia-only at 3 days, indicating a reduced blood-brain barrier (BBB) disruption (6). G-CSF administration after transient ischemia also led to a decrease in the amount of edematous tissue present as measured by both structural magnetic resonance imaging (MRI) and brain water content (11).

In a randomized controlled trial, 7 patients with acute ischemic stroke received subcutaneous G-CSF injections (15 μg/kg per day) for 5 days within 7 days of onset. At 12-month follow-up, patients who had received G-CSF showed significant improvement in neurologic functions according to the clinical scales. The mean environmental management system score in the G-CSF-treated patients showed a significant increase over that of the control patients from the sixth month after therapy. MRI scans revealed no anatomic or structural changes, including cerebral hemorrhage. There was no significant difference with regard to infarction size at baseline and at 12-month follow-up (12). Taken together, G-CSF offers hope for therapy of stroke patients possibly through mobilization of endogenous stem cells (13).

3.2. The expression of G-CSF and G-CSF receptor in central nervous system

A variety of cell types express G-CSF following appropriate stimulation. Cells of the monocyte/macrophage lineage are among the most prominent sources of G-CSF, but vascular endothelial cells, fibroblasts, and mesothelial cells can also produce G-CSF. In addition, G-CSF is expressed by neurons in all brain regions including the hippocampus CA3 field, the hilus and subgranular zone of the dentate gyrus, neurons in the entorhinal cortex, neurons in the olfactory bulb, several cerebellar and brainstem nuclei where its receptor was expressed, which implies important new functions of this protein within the central nervous system (CNS) (7). Besides neurons, G-CSF is expressed in astrocyte cultures after stimulation (14-17). However, Schneider et al. did not detect any astrocytic G-CSF expression in vivo, even in the acute cerebral ischemia. Our results showed that G-CSF was expressed in astrocytes, even in the resting state (data not published). It is certainly possible that G-CSF expression in astrocytes might be evoked in vivo by other stimuli or occurs under different ischemic conditions or at different postischemic time points. No information reveals that microglia express G-CSF.

Importantly, G-CSF transcripts was induced 485-fold at 4 hours and 65-fold at 16 hours in ischemic lesions after middle cerebral artery occlusion (MCAO) compared with control brains (18). Furthermore, an increase in G-CSF mRNA expression was not only seen in the ischemic lesion but also in the nonischemic frontal cortex after focal cerebral ischemia (18). Similarly, there was a dramatic upregulation of G-CSF (more than 100-fold) on the ipsilateral hemisphere 2 h after MCAO model, which was accompanied by induction of G-CSF on the contralateral hemisphere. At 6 hours following ischemia, this induction became more specific to the ischemic hemisphere, and it was no longer detectable at 20 hours of reperfusion (7). We found that LPS, but not IFN-gamma, can induce the expression and secretion of G-CSF in cultured astrocytes. The upregulation of G-CSF was accompanied by a more modest induction of the G-CSF receptor after cerebral ischemia, more prominent in the ipsilateral than the contralateral hemisphere (7). Immunohistochemistry demonstrated the co-expression and up-regulation of G-CSF and its receptor in neurons after MCAO and reperfusion, revealing that G-CSF and its receptor likely function as an autocrine adaptive system within the CNS.

3.3. Penetration of the blood-brain barrier

A prerequisite for effect of G-CSF within the CNS is to penetrate the blood-brain barrier (BBB). The amount of iodinated G-CSF (^{131}I–G-CSF) in brain and serum was measured at 1, 4, and 24 hours after intravenous injection in non-injured rats and the brain/serum ratios of

^{131}I–G-CSF and ^{131}I-albumin was calculated as an index of BBB permeability. Injection of G-CSF showed a higher brain/serum ratio at different time points, which indicated passage of G-CSF through the intact BBB (7), indicating that systemically given G-CSF is able to pass the intact BBB. The most striking effect of peripherally administered G-CSF on the brain was seen in the dentate gyrus, where G-CSF increased the number of newly generated neurons under ischemic conditions but also in non-ischemic, sham-operated animals. These results reveal that G-CSF-induced neuroprotection is mediated through a G-CSF receptor signaling pathway on cerebral microvessels following the receptor-mediated endocytosis.

4. POSSIBLE MECHANISMS OF G-CSF NEUROPROTECTION

4.1. G-CSF mobilizes hematopoietic stem cells

G-CSF treatment leads to a significant reduction in infarct size and improves neural plasticity. These effects may be related to the mobilization of autologous hemopoietic stem cells into circulation, enhancing their translocation into ischemic brain, and thus significantly improving lesion repair. Administration of G-CSF is known to mobilize hematopoietic stem cells from the bone marrow into the peripheral blood. Hematopoietic stem cells have been used in place of bone marrow cells in transplantation for the regeneration of non-hematopoietic tissues. Our previous results have demonstrated that subcutaneous injection of G-CSF increased the mobilization of circulating $CD34^+$ cells that migrated into the brain (9). Other studies also showed that ischemic brain specifically attracted peripheral transplanted bone marrow stromal cells (BMSC) (19-21). Which signaling molecules attract peripheral $CD34^+$ cells and direct their migration to damaged areas? Although only 5% of steady-state peripheral blood $CD34^+$ cells were found to express chemokine receptor CXCR4 (22,23), a increased proportion of hematopoietic stem cells, mobilized by G-CSF, expressed CXCR4 receptors on their cell surface. Cerebral ischemia causes an increase in CXCR4 receptor ligand stromal-derived factor-1 (SDF-1) expression in regions adjacent to the infracted area, indicating that SDF-1 within the brain could be a chemoattractant for peripheral $CD34^+$ cells. A marked increase in expression of CXCR4 was detected in ischemic region of G-CSF-treated rats compared with contralateral non-ischemic side or normal healthy controls (24), suggesting that hematopoietic $CD34^+$ cells undergo directional migration toward SDF-1 in regions adjacent to the infracted area.

What are the mechanisms or factors that G-CSF-induced $CD34^+$ cells increase survival, and improve Neurological Severity Score (NSS) after cerebral ischemia? One possibility is that G-CSF-mobilized $CD34^+$ cells integrate into the tissue, replace damaged cells, and reconstruct neural circuitry. Another reasonable hypothesis is that the interaction of $CD34^+$ cells with the host parenchymal cells in ischemic tissue may lead parenchymal cells to produce trophic factors that contribute to the recovery of neural functions (25). The level of fibronectin in brain of rats treated with G-CSF was enhanced compared with control rats (9). It has been noted that fibronectin promotes survival and migration of primary neural stem cells transplanted into the traumatically injured mouse brain (26). Fibronectin-deficient mice increased neuronal apoptosis and infarction area following transient focal cerebral ischemia (27,28). To mimic the ischemia-reperfusion injury in experimental animals, we employed hippocampal slice cultures that were first treated with oxygen and glucose deprivation (OGD) and then with oxygen-glucose re-supply, finding that fibronectin significantly increased the neurite outgrowth of OGD hippocampal slices, upregulated the expression of Bcl-2 protein, and ameliorated the ultrastructure damage of OGD hippocampal slices (9). Blockade of fibronectin in situ with an anti-fibronectin antibody dramatically decreased outgrowth of neurites (29), suggesting that the critical interaction between regrowing axons and astrocyte-associated fibronectin may be an additional factor in axon regeneration.

4.2. G-CSF activates antiapoptotic pathways

G-CSF protected neurons against programmed cell death caused by the apoptosis inducer, which appeared to be mediated via the neuronal G-CSF receptor, as an antibody against G-CSF receptor was able to abolish the protection (7). G-CSF exerted a neuroprotective effect through the direct activation of anti-apoptotic pathway by up-regulating Stat3, pStat3, and Bcl-2 in transient focal ischemia of mice (5). Another study also found that the neuroprotective role of G-CSF was manifested through the JAK/ Stat signaling pathway and subsequent activation of Bcl-2 (30), in which overexpression of Bcl-2 protected against postischemic cerebral neuronal death (31). Schabitz et al showed that G-CSF receptor existed not only on hematopoietic cells but also on neurons and glial cells, and that the neuroprotective effect of G-CSF is dependent on G-CSF receptor-mediated activation of the JAK/Stat pathway, especially increased Stat3 expression in the ischemic penumbra (7). Under oxygen and glucose deprivation of human cerebral-neuroblastoma hybrid cell line, G-CSF prevented caspase-3 activation and subsequent cell death (4). Using enhanced green fluorescent protein chimera mice, G-CSF decreased the migration of Iba-1/EGFP-positive bone marrow-derived monocytes/macrophages and increased intrinsic microglia/macrophages at ischemic penumbra, suggesting that bone marrow-derived monocytes/macrophages are not involved in G-CSF-induced neuroprotection after ischemic injury, and that G-CSF exerts a neuroprotective effect through the direct activation of anti-apoptotic pathway (5).

4.3. G-CSF drives neuronal differentiation

Cerebral ischenmia contains various states of cells undergoing apoptosis or necrosis. Neuronal death after ischemia might involve a combination of apoptotic and necrotic processes even at the level of the individual neuron. This raises a question how G-CSF induces neuroprotective effect for apoptotic or necrotic neurons. It has been defined that the adult mammalian forebrain has neural stem cells and neural progenitor cells in the anterior subventricular zone (SVZ), rostral migratory stream, olfactory bulb core, and dentate gyrus (DG). However, G-

CSF can induce bone marrow stem cells proliferation and mobilization, and activate endothelial cell proliferation, which might help to establish a vascular niche for neural stem cells (4). Importantly, G-CSF and its receptor were expressed in neurons of the SVG and the DG (7). G-CSF dose-dependently induced activity of the promoter of the mature neuronal marker β-III-tubulin with a maximal induction greater than that reached by the most standard neuronal induction protocol, including markers for neuronal differentiation (beta-III-tubulin and NSE) and markers for mature glial cells (PLP and GFAP). Further observation found that G-CSF led to an increase in the population of cells expressing mature neuronal markers, indicating that G-CSF has a function to regulate the differentiation of adult neural stem cells (7). Similarly, G-CSF stimulated neurogenesis through reciprocal interaction with vascular endothelial growth factor (VEGF) and Stat activation (4). Administration of hematopoietic cytokines in the subacute phase after cerebral infarction is effective for functional recovery facilitating proliferation of intrinsic neural stem/progenitor cells (10). Taken together with the recent evidences that G-CSF can rescue dying neurons (7,8), G-CSF might potentially serve to promote brain recovery and repair. In addition, G-CSF also enhanced the recruitment of progenitor cells from the lateral ventricular wall into the ischemic area of the neocortex and increased hippocampal neurogenesis not only in ischemic animals but also in the intact, nonischemic region (7). Based on these evidences, G-CSF may enhance structural repair and function even in healthy subjects or may offer a novel therapeutic strategy for the treatment of chronic stroke patients.

4.4. G-CSF enhances angiogenesis

Concomitant with an increase in neutrophil numbers in circulation, G-CSF increased plasma VEGF from neutrophils in vivo (32). Local G-CSF administration into ischemic tissue elevated capillary density and provided a functional vasculature and contributed to neovascularization of ischemic tissue (6). Blockade of the VEGF pathway abrogated G-CSF-induced angiogenesis, suggesting that G-CSF-induced angiogenesis is VEGF-dependent (32). The vascular surface area, the vascular branch points, the vascular length, and the number of BrdU+ endothelial cells were significantly increased in the G-CSF-treated group compared with the ischemia-only group. On the other hand, there is compelling evidence that circulating angiogenic cells are able to home to sites of vascular injury and further stimulate angiogenesis. However, the number of angiogenic cells in the blood is very low, limiting their accumulation to sites of ischemia. Capoccia et al observed that G-CSF stimulated angiogenesis through the mobilization of monocytes into the blood with their subsequent recruitment to sites of ischemia and stimulation of angiogenesis through a paracrine mechanism (33). Ohki et al found that G-CSF also augmented the number of circulating VEGF receptor-2 endothelial progenitor cells compared with untreated controls. These data clearly show that G-CSF modulates angiogenesis by increasing myelomonocytic cells (VEGFR1+ neutrophils) and their release of VEGF (32). One week after unilateral hindlimb ischemia, administration of G-CSF significantly increased the laser Doppler blood perfusion index, number of angiographically detectable collateral vessels (angiographic score), and capillary density (34). G-CSF injection starting at 1 day induced larger endothelial proliferation compared with injection starting at 7 days, providing evidences that G-CSF enhances the angiogenesis and reduces the ischemic damage, which promotes the long-term functional recovery (6). Question is whether G-CSF augments the differentiation of BMSC into endothelial cells of blood vessels. The differentiation of BMSC into endothelial cells of blood vessels was increased in G-CSF-treated animals through VEGF, resulting in early recovery of blood flow in the ischemic limbs (35). Recently, we found that G-CSF can stimulate astrocytes to secrete VEGF that may directly promote angiogenesis within the CNS by paracrine pathways (data not published).

4.5. G-CSF inhibits inflammatory mediators

G-CSF has been used as an anti-inflammatory agent in murine endotoxemia. Thus, a therapeutic approach that reduces inflammation may protect against cerebral ischemic injury. G-CSF protected against death in a nonseptic model of ischemia/reperfusion injury and concluded that such beneficial effect is the consequence of either reduction of TNF-alpha or inhibition of iNOS activity (36,37). Other studies reported that G-CSF decreased the levels of inflammatory IL-1beta, IL-6, and IL-8 under several conditions (38). Analysis of iNOS Western blot and immunohistochemistry clearly indicated that G-CSF significantly reduced iNOS levels and decreased the activation of microglia expressing iNOS (5). However, Gibson et al observed that G-CSF treatment only suppressed the up-regulation of IL-1beta mRNA while having no effect on TNF-alpha and iNOS mRNA expression (11). Our results demonstrated that G-CSF reduced NO production from cultured astrocytes. Based on these results, one neuroprotective mechanism for G-CSF may be induced partly through its anti-inflammatory mechanism.

5. WEIGHING THE PERSPECTIVE OF G-CSF TREATMENT

Recent studies have demonstrated that G-CSF administration achieved a significant neuroprotective effect in cell culture and after cerebral ischemia model through different mechanisms (Figure 1). Distinctions between global vs. focal cerebral ischemia, permanent vs. temporary focal ischemia, and acute phase vs. recovery phase are being investigated. A small pilot trial is the first clinical study reporting on the safety and feasibility of G-CSF therapy in stroke patients. Efficacy and further confirmatory safety data will need to come from larger phase II studies that are randomized and blinded. Despite these successes, it should be noted that this was a preliminary study and, because of the small number of participating patients, any inferences are tentative. Thus, critical analyses, well-designed preclinical studies and limited clinical trials of the safety, toxicity, optimal drug dosage, route and timing of delivery post-stroke will ultimately determine whether or not we are ready to

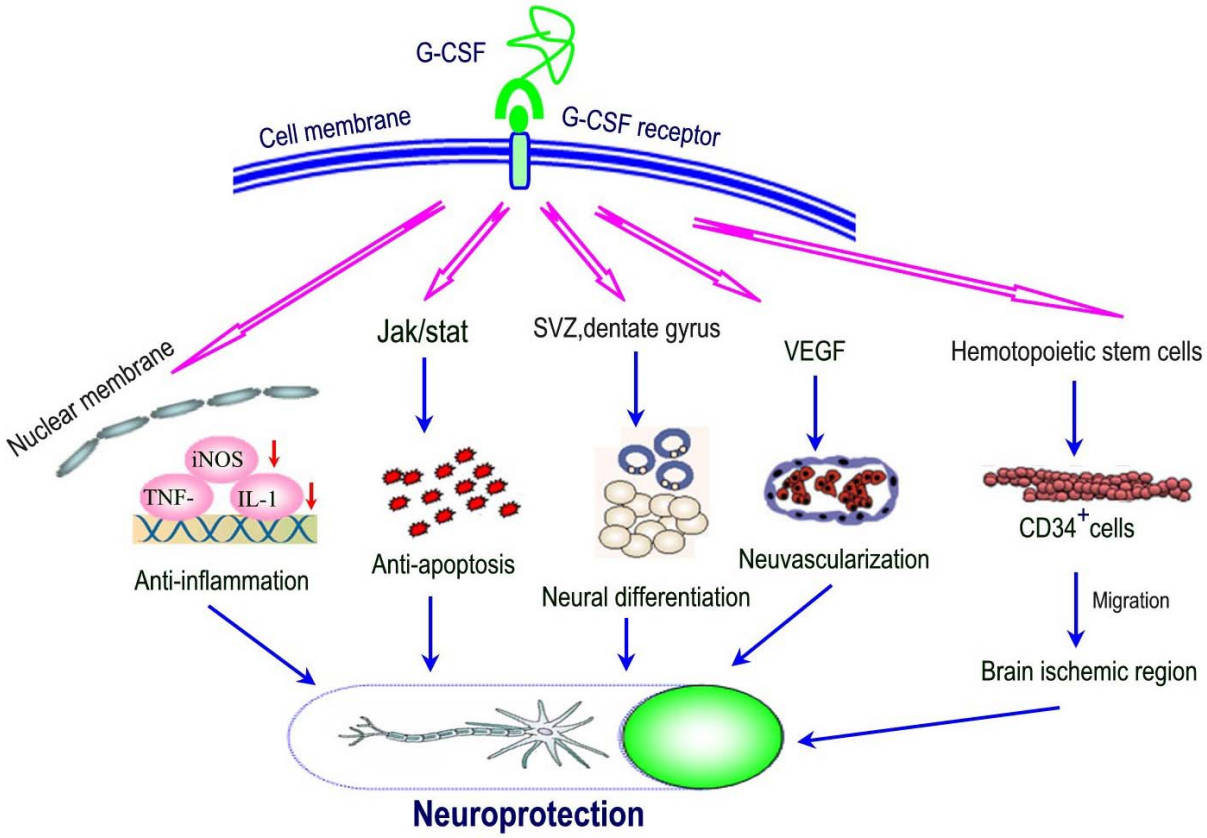

Figure 1. Possible mechanisms for neuroprotective effect of G-CSF in cerebral ischemia. G-CSF displays neuroprotective roles through different mechanisms: 1. anti-inflammation. G-CSF inhibits the up-regulation of TNF-α, IL-1β, iNOS, IL-6, and IL-8; 2. anti-apoptosis. G-CSF mediates anti-apoptotic pathway through the JAK/ Stat signaling pathway and subsequent activation of Bcl-2; 3. neuronal differentiation. The adult animal has neural stem cells and neural progenitor cells in the anterior subventricular zone (SVZ) and dentate gyrus (DG). G-CSF has a function to regulate the differentiation of adult neural stem cells; 4. angiogenesis. G-CSF elevates capillary density and provides a functional vasculature and contributes to neovascularization of ischemic tissue through the VEGF pathway; 5. the mobilization of autologous hemopoietic stem cells. G-CSF triggers the mobilization of autologous hemopoietic stem cells that migrate into ischemic brain, and thus significantly improve lesion repair.

advance G-CSF therapy into definitive large-scale clinical application for stroke, making G-CSF an ideal drug candidate for expansion of the therapeutic time window in patients with cerebral ischemia.

6. CONCLUSIONS AND PERSPECTIVES

The presence of the G-CSF/G-CSF–receptor in the brain and its role in neuroprotection have been investigated in many recent studies. The successful cerebral ischemic model and the small number of phase II trial in human stroke, as well as the multiple mechanisms by which G-CSF is active in neuroprotection of cerebral ischemia, suggest that the use of G-CSF will likely translate successfully into human trials. Besides further randomized, double-blinded and placebo-controlled trials, a potential problem in the use of G-CSF for cerebral ischemia will be the undesirable side effect of granulopoiesis, particularly following multiple doses. The identification and separation of the structural determinants of the granulopoiesis, neuroprotective and angiogenic activities within the G-CSF molecule may provide alternative ways to minimize side effects. The strategy to develop derivatives of G-CSF lacking activity of granulopoiesis, but retaining neuroprotective potential may allow for chronic usage of G-CSF in cerebral ischemia. Construction of polypeptides that retain only the neuroprotective activity of the molecule could also have further considerable value. Further work is needed to better understand the precise mechanisms of G-CSF–induced neuroprotection.

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Abbreviations: G-CSF: granulocyte-colony stimulating factor, MRI: magnetic resonance imaging, MCAO: middle cerebral artery occlusion, LPS: lipopolysaccharide, BBB: blood-brain barrier, BMSC: bone marrow stromal cells, CXCR4: chemokine (CXC motif) receptor 4, SDF-1: stromal cell–derived factor–1, NSS: neurological severity score, OGD: oxygen and glucose deprivation, STAT3: signal transducer and activator of transcription 3, Iba-1/EGFP: ionized calcium-binding adapter molecule 1/enhanced green fluorescent protein, SVZ: subventricular zone, DG: dentate gyrus, NSE: neuron-specific enolase, PLP: proteolipid protein, GFAP: glial fibrillary acidic protein, VEGF: vascular endothelial growth factor, iNOS: inducible nitric oxide synthase

Key Words: Granulocyte-colong stimulating factor, G-CSF, Cerebral ischemia, Neuroprotection, Stroke, Inflammation, Apoptosis, Differentiation, Angiogenesis, Review

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