G-CSF and neuroprotection: a therapeutic perspective in cerebral ischaemia

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Abstract
In several experimental studies of cerebral ischaemia, G-CSF (granulocyte colony-stimulating factor) exerted neuroprotective effects through different mechanisms, including mobilization of haemopoietic 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 have a dual activity beneficial both to acute neuronal protection and long-term plasticity after cerebral ischaemia, thus proposing that G-CSF is an ideal new drug for stroke and neurodegenerative diseases.

Introduction
Stroke is a leading cause of death and disability worldwide and the number of patients afflicted with cerebral ischaemia 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 ischaemic 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 ischaemic stroke patients can undergo thrombolyisis [1–3]. Therefore the attention has focused on a neuroprotective strategy that could potentially expand the therapeutic time window in patients with acute ischaemic 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 and 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.

G-CSF (granulocyte colony-stimulating factor), a member of the 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 ischaemia [4–7]. Because of the expression of G-CSF and its receptor in the CNS (central nervous system), G-CSF may have an autocrine-protective signalling mechanism in response to neural injury in the CNS. The expression of G-CSF receptor on cerebral microvessels provides the possibility that iodinated G-CSF ($^{131}$I-G-CSF) defines the passage of G-CSF through the intact blood–brain barrier following the receptor-mediated endocytosis. However, the precise mechanisms of the neuroprotective effect of G-CSF are not entirely known. Here, we have summarized possible mechanisms of G-CSF as a potential therapeutic agent in cerebral ischaemia.

G-CSF exhibits neuroprotective effect after cerebral ischaemia
G-CSF displays a significant neuroprotection after cerebral ischaemia. Administration of G-CSF reduced infarction volume [5,6,8] and mortality rate was significantly decreased in animals treated with G-CSF compared with controls [8,9]. G-CSF-treated experimental models showed better functional recoveries from 2 weeks to 5 weeks after ischaemia compared with the cerebral ischaemia-only controls [6,9]. G-CSF given in the subacute phase (days 11–20) effectively improved not only motor performance but also higher brain function, compared with acute-phase treatment (days 1–10) [10]. Our results indicate that subcutaneous injection of G-CSF (10 µg/kg per day) for 5 days decreases mortality rate, reduces infarction volume and improves neurological behaviour after cerebral ischaemia (Figure 1).

In a randomized controlled trial, seven patients with acute ischaemic stroke received subcutaneous G-CSF injections (15 µg/kg per day) for 5 days within 7 days of onset.
12-month follow-up, patients who had received G-CSF showed significant improvement in neurological deficits and functions. 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 (magnetic resonance imaging) scans revealed no anatomical or structural changes, including cerebral haemorrhage. There was no significant difference with regard to infarction size at baseline and at 12-month follow-up [11]. Taken together, G-CSF offers hope for therapy of stroke patients possibly through mobilization of endogenous stem cells [12].

**G-CSF mobilizes HSCs (haemopoietic stem cells) to the injured brain**

Administration of G-CSF is known to mobilize HSCs from the bone marrow into the peripheral blood. It has been reported that G-CSF results in a significant decrease in infarct volumes and enhances survival rates, which may be mediated by the mobilization of autologous HSCs in experimental cerebral ischaemia [13,14]. Our results have demonstrated that subcutaneous injection of G-CSF increased the mobilization of circulating CD34+ cells that were seen around the perivascular spaces in ischaemic hemisphere, indicating that CD34+ cells mobilized with G-CSF can home into ischaemic brain tissues through blood circulation [9]. Other studies also showed that ischaemic brain specifically attracted peripheral transplanted BMSC (bone marrow stromal cells) [15–17].

Which signalling molecules attract peripheral CD34+ cells and direct their migration to damaged areas? Cerebral ischaemia causes an increase in CXCR4 (CXC chemokine receptor 4) receptor ligand SDF-1 (stromal-derived factor-1) expression in regions adjacent to the infarcted area, indicating that SDF-1 within the brain could be a chemoattractant for peripheral CD34+CXCR4+ cells [18]. A marked increase in expression of CXCR4 was detected in the ischaemic region of G-CSF-treated rats compared with the contralateral non-ischaemic side or normal healthy controls [19], suggesting that haemopoietic CD34+ cells undergo directional migration towards SDF-1 in regions adjacent to the infarcted area.

What are the mechanisms or factors by which G-CSF-induced CD34+ cells increase survival and improve Neurological Severity Score after cerebral ischaemia? 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 ischaemic tissue may lead parenchymal cells to produce trophic factors that contribute to the recovery of neural functions [20]. We found that 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 [21]. Fibronectin-deficient mice had increased neuronal apoptosis and infarction area following transient focal cerebral ischaemia [22,23]. To mimic the ischaemia/reperfusion injury in experimental animals, we employed hippocampal slice cultures that were first treated with OGD (oxygen and glucose deprivation) and then with oxygen–glucose re-supply, finding that fibronectin significantly increased the neurite outgrowth of OGD hippocampal slices and ameliorated the ultrastructure damage of OGD hippocampal slices [9]. Blockade of fibronectin in situ with an anti-fibronectin antibody...
G-CSF activates anti-apoptotic 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 the anti-apoptotic pathway by up-regulating STAT3 (signal transducer and activator of transcription 3), pSTAT3 and Bcl-2 in transient focal ischaemia of mice [5]. Another study also found that the neuroprotective role of G-CSF was manifested through the JAK (Janus kinase)/STAT signalling pathway and subsequent activation of Bcl-2 [25], in which overexpression of Bcl-2 protected against post-ischaemic cerebral neuronal death [26]. Schäbitz and co-workers [7] showed that G-CSF receptor existed not only on haemopoietic 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, particularly increased STAT3 expression in the ischaemic penumbra. Under OGD of human cerebral–neuroblastoma hybrid cell line, G-CSF prevented caspase 3 activation and subsequent cell death [4]. Using enhanced green fluorescent protein chimaera mice, G-CSF decreased the migration of Iba-1/EGFP (enhanced green fluorescent protein)-positive bone marrow-derived...
Figure 3 | Expression of nestin, vWF and MAP-2 expression in brain sections
The immunohistochemistry of nestin (A, B), vWF (C, D) and MAP-2 (E and F) was performed in brain slices that were obtained from G-CSF-treated rats and control rats at day 7 after MCAO.

monocytes/macrophages and increased intrinsic microglia/macrophages at ischaemic penumbra, suggesting that bone marrow-derived monocytes/macrophages are not involved in G-CSF-induced neuroprotection after ischaemic injury, and that G-CSF exerts a neuroprotective effect through the direct activation of anti-apoptotic pathway [5]. We found that the expression of Bcl-2 protein and MAP-2 (microtubule-associated protein-2) protein (Figures 3E and 3F) within brain after ischaemia was increased in rats receiving G-CSF treatment compared with control rats, indicating that G-CSF-mediated neuron survival may be related to the Bcl-2-mediated anti-apoptotic pathway.

G-CSF enhances angiogenesis
Concomitant with an increase in neutrophil numbers in circulation, G-CSF increased plasma VEGF from neutrophils in vivo [27]. Local G-CSF administration into ischaemic tissue elevated capillary density and provided a functional vasculature and contributed to neovascularization of ischaemic tissue [6]. Blockade of the VEGF pathway abrogated G-CSF-induced angiogenesis, suggesting that G-CSF-induced angiogenesis is VEGF-dependent [27]. The vascular surface area, the vascular branch points, the vascular length and the number of BrdU-positive endothelial cells were significantly increased in the G-CSF-treated group compared with the ischaemia-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 ischaemia. Capoccia et al. [28] observed that G-CSF stimulated angiogenesis through the mobilization of monocytes into 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 method, including markers for neuronal differentiation (β-III-tubulin and NSE (neuron-specific enolase)) and markers for mature glial cells [PLP (proteolipid protein) 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 VEGF (vascular endothelial growth factor) and STAT activation [4]. Administration of haemopoietic cytokines in the subacute phase after cerebral infarction is effective for functional recovery, facilitating proliferation of intrinsic neural stem/progenitor cells [10]. In accordance with these results, our previous studies revealed that nestin-positive cells were elevated in the marginal zone of the infarction of rats receiving G-CSF injection (Figures 3A and 3B), suggesting that G-CSF may promote the differentiation of neural stem cells after ischaemia/reperfusion lesion. Taken together with the recent evidence that G-CSF can rescue dying neurons [7,8], G-CSF might potentially serve to promote brain recovery and repair by promoting the differentiation of neural stem cells. In addition, G-CSF also enhanced the recruitment of progenitor cells from the lateral ventricular wall into the ischaemic area of the neocortex and increased hippocampal neurogenesis not only in ischaemic animals but also in the intact, non-ischaemic region [7]. Based on these lines of evidence, 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.

G-CSF drives neuronal differentiation
Cerebral ischaemia contains various groups states of cells undergoing apoptosis or necrosis. Neuronal death after ischaemia might involve a combination of apoptotic and necrotic processes even at the level of the individual neuron. This raises the question of how G-CSF induces a 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 SVZ (subventricular zone), rostral migratory stream, olfactory bulb core, and DG (dentate gyrus). However, G-CSF can induce bone marrow stem cell proliferation and mobilization, and activate
Figure 4 | Possible mechanisms for neuroprotective effect of G-CSF in cerebral ischaemia

G-CSF displays a neuroprotective role through different mechanisms. (i) Anti-inflammation: G-CSF inhibits the up-regulation of TNFα, IL-1β, iNOS, IL-6, and IL-8. (ii) Anti-apoptosis: G-CSF mediates anti-apoptotic pathway through the JAK/STAT signalling pathway and subsequent activation of Bcl-2. (iii) Neuronal differentiation: the adult animal has neural stem cells and neural progenitor cells in the anterior SVZ and DG. G-CSF has a function to regulate the differentiation of adult neural stem cells. (iv) Angiogenesis: G-CSF elevates capillary density and provides a functional vasculature and contributes to neovascularization of ischaemic tissue through the VEGF pathway. (v) The mobilization of autologous HSCs: G-CSF triggers the mobilization of autologous HSCs that migrate into ischaemic brain, and thus significantly improves lesion repair.

the blood, with their subsequent recruitment to sites of ischaemia and stimulation of angiogenesis through a paracrine mechanism. Ohki et al. [27] found that G-CSF also augmented the number of circulating VEGF receptor-2 (VEGFR2) EPCs (endothelial progenitor cells) compared with untreated controls. These results clearly show that G-CSF modulates angiogenesis by increasing myelomonocytic cells (VEGFR1-positive neutrophils) and their release of VEGF [27]. One week after unilateral hindlimb ischaemia, administration of G-CSF significantly increased the laser Doppler blood perfusion index, number of angiographically detectable collateral vessels (angiographic score), and capillary density [29]. G-CSF injection starting at 1 day induced larger endothelial proliferation compared with injection starting at 7 days, providing evidence that G-CSF enhances the angiogenesis and reduces the ischaemic damage, which promotes the long-term functional recovery [6]. The 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 ischaemic limbs [30]. The levels of VEGF expression correlated with the degree of neovascularization, as defined by vWF (von Willebrand factor) levels. In in vivo experiments, our results also show that administration of G-CSF enhanced the numbers of vWF-positive cerebral microvessels in the marginal zone of the infarction after ischaemia/reperfusion lesion (Figures 3C and 3D). Although the role of G-CSF in neovascularization has been convincingly shown by several groups, the question remains how G-CSF improves neovascularization. For instance, G-CSF, which is typically used for mobilization of CD34 cells in patients, also increased the levels of circulating EPCs. Recruitment and incorporation of EPCs into ischaemic tissue require a co-ordinated multistep process including mobilization, chemoattraction, adhesion, transmigration, migration, tissue invasion and in situ differentiation. Recently, we found that G-CSF can stimulate astrocytes to secrete VEGF that may directly promote angiogenesis within the CNS by paracrine pathways (Z.-H. Huang, B.-G. Xiao and C.-Z. Lu, unpublished work).

G-CSF inhibits inflammatory mediators

G-CSF has been used as an anti-inflammatory agent in murine endotoxaemia. Thus a therapeutic approach that reduces inflammation may protect against cerebral ischaemic injury. G-CSF protected against death in a non-septic model of ischaemia/reperfusion injury, and studies concluded that such a beneficial effect is the consequence of either reduction of TNFα (tumour necrosis factor α) or inhibition
of iNOS (inducible nitric oxide synthase) activity [31,32]. Other studies reported that G-CSF decreased the levels of inflammatory IL-1β (interleukin-2), IL-6, and IL-8 under several conditions [33]. 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. [34] observed that G-CSF treatment only suppressed the up-regulation of IL-1β mRNA while having no effect on TNFα and iNOS mRNA expression. 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.

**Weighing the neuroprotection of G-CSF treatment**

Recent studies have demonstrated that G-CSF administration achieved a significant neuroprotective effect in cell culture and after cerebral ischaemia in a model system through different mechanisms (Figure 4). Distinctions between global and focal cerebral ischaemia, permanent versus temporary focal ischaemia, and acute phase versus 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 double 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. However, a variety of unresolved questions remain to be answered (Table 1). 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 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 ischaemia.

**Conclusion and perspectives**

The presence of the G-CSF/G-CSF-receptor in the brain and its role in neuroprotection has been investigated in many recent studies. The successful cerebral ischaemic model and the small number of pilot studies in stroke patients, as well as the multiple mechanisms by which G-CSF is active in neuroprotection of cerebral ischaemia, suggest that the use of G-CSF will probably 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 ischaemia 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 ischaemia. Construction of polypeptides that retain only the neuroprotective activity of the molecule could also have further considerable value. Much additional work is needed to clarify the precise mechanisms of G-CSF-induced neuroprotection. It is anticipated that G-CSF is an endogenous ligand in the CNS that has a dual activity beneficial both to acute neuronal degeneration and long-term plasticity after cerebral ischaemia, thus suggesting that G-CSF is an ideal new drug for stroke and neurodegenerative diseases.

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## References


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