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Anti-apoptotic effect of Granulocyte-Colony Stimulating Factor after focal cerebral ischemia in the rat

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Abstract

We investigated the molecular mechanisms of the anti-apoptotic properties of Granulocyte-Colony Stimulating Factor (G-CSF) on neurons and whether G-CSF affects glial cell survival following focal cerebral ischemia in rats. Sprague-Dawley rats were subjected to a transient 90min middle cerebral artery occlusion (MCAO) by the intraluminal occlusion technique. Rats were treated with either a single dose of G-CSF (50 µg/kg, subcutaneously) at the onset of reperfusion or G-CSF (50 µg/kg body weight, subcutaneously) was administered starting at the onset of reperfusion and followed by the administration of the same dose per day for an additional 2 days. Brains were harvested either 24hrs, 72hrs or 2 weeks after reperfusion for assays of infarct volume, immunohistological studies and Western Blot analysis for phosphorylated signal transducer and activator of transcription 3 (pSTAT3), Pim-1, bcl-2, Bax, cytochrome c, cellular inhibitor of apoptosis protein 2 (cIAP2), and cleaved caspase-3 levels. G-CSF significantly reduced infarct volume and ameliorated the early neurological outcome. G-CSF treatment significantly up-regulated pSTAT3, Pim-1, bcl-2 expression, and down-regulated cytochrome c release to the cytosol, Bax translocation to the mitochondria, and cleaved caspase-3 levels in neurons. The activation of the STAT3 pathway was accompanied by increased cIAP2 expression in glial cells. After MCAO, G-CSF treatment increased both neuronal and glial survival by effecting different anti-apoptotic pathways which reflects the multifactorial actions of this drug. These changes were associated with remarkable improvement in tissue preservation and behavioral outcome.

Keywords

Apoptosis; Bcl-2; Glia; Pim-1; STAT; Stroke

Granulocyte-Colony Stimulating Factor (G-CSF) is a member of the hematopoietic growth factor family, which orchestrates the proliferation, differentiation, and survival of

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hematopoietic progenitor cells (Demetri and Griffin, 1991). It has been widely used in clinical practice for the treatment of such conditions as neutropenia, associated with cytotoxic therapy.

However, growing evidence has suggested that G-CSF also has important non-hematopoietic functions in other tissues including the central nervous system (CNS). Recent studies have shown the presence of the G-CSF-receptor (G-CSFR) in a wide variety of cells in the brain, including neurons and glial cells (Schneider et al., 2005). G-CSF and its receptor are co-expressed in neurons and are upregulated in response to neural injury, suggesting an autocrine protective signaling mechanism (Kleinschnitz et al., 2004; Schneider et al., 2005). Moreover, experimental studies have shown that exogenous G-CSF is a promising neuroprotective agent (see review) (Solaroglu et al., 2006).

An anti-apoptotic action is one of the proposed mechanisms of G-CSF related neuroprotection after CNS injury. The Janus kinase2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway has been suggested as a mediator of the anti-apoptotic actions of G-CSF (Komine-Kobayashi et al., 2005; Schabitz et al., 2003; Schneider et al., 2005); however, the precise molecular mechanisms have not been entirely explored, especially under *in vivo* experimental settings.

Pim-1, a member of the serine/threonine kinase proto-oncogene family, is one of STAT3's target genes, which has been shown to be induced by G-CSF in hematopoietic cell lines (Lilly et al., 1992). Pim-1 increases cell survival through the regulation of bcl-2 proteins (Lilly et al., 1999). In the present study, we investigated whether the anti-apoptotic effects of G-CSF was at least partially regulated by STAT3- induced Pim-1 expression, and whether G-CSF inhibits the mitochondria-dependent apoptotic pathway in neurons after focal cerebral ischemia.

Besides neuronal apoptosis, apoptotic cell death in glial cells has also been implicated in the pathophysiology of ischemic stroke. Glial cells (especially astrocytes) provide structural, trophic and metabolic support for neurons under normal conditions as well as during post-ischemic conditions (Nedergaard and Dirnagl, 2005). It has been shown that the preservation of astrocytic metabolism and survival is essential for neuronal survival and is a predictor for recovery *in vivo* models of focal cerebral ischemia (Bambrick et al., 2004; Haberg et al., 2006; Xia et al., 2004). Hence, improvement of glial cell survival is suggested to be one crucial pathway to protect neurons against ischemic injury (Bambrick et al., 2004; Nedergaard and Dirnagl, 2005; Trendelenburg and Dirnagl, 2005). Although G-CSFR has also been detected in glial cells, whether G-CSF affects glial survival after focal cerebral ischemia remains unknown. The present study was also designed to clarify the role of G-CSF in the survival of glial cells after transient focal cerebral ischemia.

Experimental procedures

All experiments were approved by the Loma Linda University Animal Care and Use Committee.

Transient Middle Cerebral Artery Occlusion (MCAO)

Fifty-five adult male Sprague-Dawley rats weighing between 290 and 330g were randomly allocated in 3 groups: Sham, MCAO and G-CSF-treated (MCAO+G-CSF) groups. Anesthesia was induced intraperitoneally with ketamine (80mg/kg) and xylazine hydrochloride (8mg/kg) followed by atropine at a dose of 0.1mg/kg (subcutaneously). The left femoral artery was cannulated for continuous monitoring of mean arterial blood pressure, heart rate and blood sampling for analysis of arterial blood gases and glucose levels measured before, during and after the MCAO. Rats were intubated and respiration was maintained with a small animal respirator (Harvard Apparatus, MA). Rats were subjected to a MCAO as described by Yin et

al (Yin et al., 2003), with modifications. Briefly, under an operating microscopic field, the left common carotid artery, internal carotid artery (ICA) and external carotid artery (ECA) were surgically exposed. The ECA was coagulated and a 4-0 nylon suture with silicon (Docol Co., NM) was inserted into the ICA through the ECA stump to occlude the MCA. The core temperature was maintained at $37\pm0.5^{\circ}\text{C}$. After 90min of MCAO, the suture was carefully removed from the ICA. The neck incision was closed and the rats were allowed to recover. The body temperature was carefully monitored during the post-operative period until the complete recovery of the animal from the anesthetic. The animals were housed individually and had free access to food and water until sacrificed.

Treatment Schedules

Animals were divided into two different treatment schedules (Figure 1):

1. **Schedule A:** Rats received a single dose of $50\mu\text{g/kg}$ (subcutaneously) of G-CSF (Neupogen, Amgen Inc.) at the onset of reperfusion. The MCAO group received the same volume of saline. Rats were sacrificed 24hrs after reperfusion.
2. **Schedule B:** G-CSF ($50\mu\text{g/kg}$ body weight, subcutaneously) was administered at the onset of reperfusion and again administered with the same dosage daily for an additional 2 days. The MCAO group received the same volume of saline. Rats were sacrificed either 72hrs or 2 weeks after reperfusion.

Neurological Scores

The neurological scores were evaluated by using a scoring system reported by Garcia et al., in a blinded fashion (Garcia et al., 1995).

Determination of Infarction Volume

Samples from sham ($n=3$), MCAO ($n=6$) and G-CSF-treated ($n=6$) groups were used to evaluate the infarction volume after 24hrs. Briefly, at the end of the reperfusion period, general anesthesia was reintroduced and the rats were perfused transcardially with ice-cold PBS. The brain was removed and sectioned coronally into 2mm thick slices starting from the frontal pole. Slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, Germany) for 30mins at 37°C in the dark. When the stain had developed, the tissue blocks were removed into 10% formalin overnight. Five coronal sections per animal were then photographed. TTC stains both neuronal and glial cells with a deep red pigment. In areas where neuronal loss occurs TTC does not stain and tissue remains white. Hence, the area of unstained tissue (the infarcted areas) were demarcated and were analyzed by Image J software (NIH), version 1.32. To compensate for the effect of brain edema, the corrected infarct volume was calculated as described previously (Schabitz et al., 2003). To evaluate the infarction volume at 72hrs after reperfusion samples from the sham ($n=3$), MCAO ($n=5$), and G-CSF-treated ($n=5$) groups underwent the same TTC staining procedure.

Nissl staining

Samples from the MCAO ($n=3$) and G-CSF-treated ($n=3$) groups that were subjected to the Schedule B treatment protocol were used for histological assessment. Briefly, 2 weeks after the MCAO, the rats were anesthetized and transcardially perfused with ice-cold PBS followed by 10% paraformaldehyde. The brains were quickly removed and post fixed in 10% paraformaldehyde and 30% sucrose for 3 days. The brains were cryoprotected and then rapidly frozen by 2-methylbutane chilled in liquid nitrogen. Coronal tissue sections $10\mu\text{m}$ thick were cut with the aid of a cryostat (Leica LM3050S).

For Nissl staining, the slices were hydrated in 0.1% cresyl violet for 3 min. Then they were dehydrated in ethanol and cleaned with xylenes. The slides were then examined with light microscopy and pictures were taken with a digital camera (OLYMPUS BX51).

Immunohistochemical Staining

Samples from the three groups (n=3 for each group) were used for immunohistochemistry. At 24hrs after the reperfusion, general anesthesia was reintroduced and the rats were perfused transcardially with ice-cold PBS, followed by 10% phosphate buffered formalin. Double fluorescent staining was performed as previously described (Yin et al., 2003). Briefly, frozen tissue sections (10µm thick) were incubated with primary antibodies at 4°C overnight. The primary antibodies used for double fluorescent staining were as follows; G-CSFR, pSTAT3, cytochrome c, Pim-1 and cIAP2 (Santa Cruz Inc., 1:50), NeuN and GFAP (Chemicon, 1:400). After rinsing with PBS, the sections were incubated for 1hr in FITC-, AMCA-, or Texas red-conjugated secondary antibodies (Jackson Immuno Research, 1:100). The sections were then visualized using a fluorescent microscope (Olympus) and digital photographs were taken. For negative controls, either the primary or the secondary antibodies were omitted and the same staining procedures were followed.

For immunohistochemistry, the brain sections were incubated overnight at 4°C with cleaved caspase-3 antibody (Cell Signaling Technology, Inc., 1:500) and sections were treated with the ABC staining kit (Santa Cruz Inc.). Analysis of the pictures was performed using MagnaFire SP 2.1B software.

Subfractionation of cellular proteins for Western blots (WB)

Rats were deeply anesthetized with ketamine 24hrs after reperfusion and then decapitated. The brains were rapidly removed and the ipsilateral brain cortexes were separated and flash frozen in liquid nitrogen. Samples were stored at -80°C until analysis. Protein samples (n=4 for each group) for WB analysis were prepared as described with some modifications (Matsumori et al., 2005). Whole-cell lysates were obtained by homogenizing the brain sample with a homogenizer in 5 volumes of buffer A (20mM HEPES, 1.5mM MgCl₂, 10mM KCl, 1mMEDTA, 1mM EGTA, 250mM sucrose, 0.1mM PMSF, 1mM dithiothreitol (DTT) and proteinase inhibitor cocktail tablets; pH 7.9). Samples were further centrifuged at 750g at 4°C for 15mins to separate the sample into supernatant A and pellet A. Pellet A, containing the nuclear fraction, was resuspended in 90µl of buffer B (20mM HEPES, 1.5mM MgCl₂, 20mM KCl, 0.2mMEDTA, 0.5mM EGTA, 0.2mM PMSF, 0.5mM DTT and proteinase inhibitor cocktail tablets; pH 7.9) and mixed with 30µl of buffer C (20mM HEPES, 1.2M KCl, 0.2mM EDTA, 0.2mM PMSF, 0.5mM DTT and proteinase inhibitor cocktail tablets; pH 7.9). The samples were placed on ice for 30mins during the extraction and then centrifuged at 12,000g. Supernatants containing the nuclear fraction were transferred and stored at -80°C. Supernatant A, containing the cytosolic/mitochondrial protein, was further centrifuged at 16,000g for 30mins at 4°C to separate supernatant B from pellet B. Supernatant B was used as the cytosolic fraction and pellet B was used as the mitochondrial fraction after resuspension in buffer A. Protein concentrations were then determined by the DC protein assay.

Western Blot Analysis

WB analysis was performed as previously described (Miao et al., 2001). Equal amounts of protein (50 µg) were loaded in each lane of polyacrylamide-SDS gels. The gels were electrophoresed, followed by a protein transfer to a nitrocellulose membrane. The membrane was then blocked with a blocking solution and probed with primary antibodies overnight at 4°C. The primary antibodies and concentrations were as follows; pSTAT3 (Abcam Inc., 1:1200), Pim-1, bcl-2, cytochrome c, Bax, and cIAP2 (Santa Cruz Inc., 1:200), cleaved caspase-3 (BD

Pharmingen, 1:300). Immunoblots were next processed with secondary antibodies (Santa Cruz Inc., 1:2000) for 1hr at room temperature. Immunoblots were then probed with an ECL+Plus chemiluminescence reagent kit (Amersham) to visualize the signal followed by exposure to X-ray film. Films were scanned and the optical density was determined with Image J. The optical density values were determined with repeated measurements on 3 independent experiments normalized to loading control and expressed as relative intensities (% sham). Actin (Santa Cruz Inc, 1:2000), COX IV (Abcam Inc., 1:5000), and Histone H1 (Santa Cruz Inc., 1:1000), were blotted on the same membrane as an internal control for the cytosolic, mitochondrial, and nuclear fractions, respectively.

Statistical Analysis

One way analysis of variance (ANOVA) test was used for comparing the differences between the groups. When the analysis of variance showed significant differences, the post hoc multiple comparison test (Tukey) was applied. The neurological scores were compared by Kruskal-Wallis one way ANOVA followed by multiple comparison procedures by Dunn's method. Comparisons between the 2 groups for the infarction volume and the neurological scores were assessed by the unpaired t-test. The data was expressed as means $\pm$ standard error of mean (SEM), and $p < 0.05$ was accepted as statistically significant.

Results

Physiological Data

No statistically significant differences were noted among the experimental groups for any of the physiological parameters including body temperature, heart rate, mean arterial pressure, blood gases and glucose levels (data not shown).

G-CSF treatment attenuates infarction volume and neurological deficits

Representative coronal brain sections from the MCAO and G-CSF-treated rats (**Schedule A**) stained with 2% TTC after 24hrs of reperfusion are shown in Figure 2A. The mean infarction volume in the MCAO group was 287.3 ± 10.3 versus 186.8 ± 7.2 mm³ in the G-CSF-treated group 24hrs after reperfusion (Figure 2B), demonstrating that G-CSF significantly reduced the infarction volume. Representative coronal brain sections from the MCAO and the G-CSF-treated rats (**Schedule B**) stained with 2% TTC after 72hrs of reperfusion are shown in Figure 2C. At 72hrs after reperfusion, the mean infarction volume in the MCAO and the G-CSF groups were 316.4 ± 17.9 and 161.2 ± 20.1 mm³, respectively (Figure 2D).

The neurological scores evaluated 24, 48 and 72hrs after reperfusion in the MCAO and the G-CSF-treated groups are shown in Figure 2E, demonstrating that G-CSF significantly attenuated the early neurological deficits following MCAO.

Figure 2F shows representative Nissl staining of the coronal brain sections from the MCAO and the G-CSF treated groups (**Schedule B**), 2 weeks after the injury. There was significant tissue loss (arrows) in the brains from the MCAO group compared to the G-CSF group.

G-CSFR is expressed in neurons and increased STAT3 phosphorylation by G-CSF treatment mediates increased Pim-1 levels

Double immunofluorescence staining for NeuN and G-CSFR revealed the expression of G-CSFR in neurons 24hrs after reperfusion (Figure 3A). There was also an increased number of pSTAT3 and NeuN colocalized cells in the ipsilateral cortex of the G-CSF-treated rats (Figure 3B). Double immunofluorescence staining for NeuN and Pim-1 revealed the expression of Pim-1 in neurons (Figure 3C). Western blot analyses of pSTAT3 from the cytosolic fraction

and Pim-1 from the nuclear fraction further revealed that G-CSF treatment significantly elevated the phosphorylation of STAT3 and nuclear Pim-1 levels as compared with the MCAO group 24hrs after reperfusion ($p<0.05$) (Figure 3D, E).

G-CSF treatment preserves mitochondrial bcl-2 levels, reduces Bax translocation into the mitochondria, cytochrome c release from the mitochondria, and protects neurons against apoptotic cell death

Triple immunofluorescent staining revealed that cleaved caspase-3 and cytochrome c expressions were colocalized in the neurons 24hrs after reperfusion (Figure 4A). An increased number of cleaved caspase-3 stained astrocytes were observed in the MCAO group compared to the G-CSF group as shown by double fluorescent staining in yellow color (Figure 4B). Mitochondrial bcl-2 levels were significantly reduced following MCAO ($p<0.05$), however, G-CSF treatment significantly restored the levels of mitochondrial bcl-2 24hrs after reperfusion ($p<0.05$) (Figure 4C). MCAO, also, dramatically increased the Bax content in the mitochondrial fraction ($p<0.05$), and significantly reduced cytosolic Bax levels ($p<0.05$), whereas the administration of G-CSF reduced the MCAO-induced rise in mitochondrial Bax levels and restored cytosolic Bax levels ($p<0.05$) (Figure 4D, E). Immunohistochemistry revealed that there were a reduced number of cleaved caspase-3 expressing neurons in the injured cerebral cortex of the G-CSF-treated animals 24hrs after reperfusion (Figure 5A). Furthermore, the MCAO rats showed a massive accumulation of cytochrome c in the cytoplasm and a significant increase in the activation of caspase-3 in the ischemic cortex as compared to the sham rats 24hrs after the reperfusion ($p<0.05$). Conversely, with G-CSF treatment, cytochrome c release from the mitochondria and caspase-3 activation were drastically reduced 24hrs after reperfusion ($p<0.05$) (Figure 5B, C, D).

G-CSF treatment upregulates STAT3 phosphorylation and cIAP2 expression in glial cells

There was an extensive foci of pSTAT3 and GFAP colocalized cells in the ipsilateral cortex of the G-CSF-treated rats 24hrs after reperfusion (Figure 6A). Double immunofluorescence staining for GFAP and cIAP2 revealed an increased expression of cIAP2 in glia in the G-CSF-treated group compared to the MCAO group (Figure 6B). Western blot analyses of cIAP2 from the cytosolic fraction revealed that G-CSF treatment significantly elevated the cIAP2 levels as compared with the MCAO group ($p<0.05$) (Figure 6C).

Discussion

Recent studies have clearly demonstrated that G-CSF exerts an anti-apoptotic effect on neurons both *in vivo* and *in vitro* (Komine-Kobayashi et al., 2005; Park et al., 2005; Schabitz et al., 2003; Schneider et al., 2005). A role for the JAK2/STAT3 signaling pathway has been suggested as a mediator of the anti-apoptotic actions of G-CSF which is mediated via binding to the cell surface receptor G-CSFR in neurons (Schabitz et al., 2003; Komine-Kobayashi et al., 2005; Schneider et al., 2005). Consistent with these previous reports, our results showed that G-CSFR is expressed by neurons and that G-CSF treatment markedly increased STAT3 phosphorylation in the injured brain. Hence, we investigated the levels of one of STAT3's target genes Pim-1, which has been shown to have anti-apoptotic properties in hematopoietic cell lines (Lilly et al., 1999), and has been shown to be induced by G-CSF (Lilly et al., 1992).

The serine/threonine kinase proto-oncogene family is one of the genes induced by the activation of the STAT3 signaling pathway, and Pim-1 is a member of this family (Shirogane et al., 1999). Recent studies focused on the role of Pim kinases in promoting cell proliferation and preventing cell death including hematopoietic and cancer cell lines (Chen et al., 2005; Lilly et al., 1992; Stout et al., 2004). However, relatively little is known about the regulation and function of Pim kinases in the CNS. Pim-1 expression has been shown to increase in the dentate

gyrus of the hippocampus after systemic kainic acid administration in rats (Feldman et al., 1998) and has also been reported to be induced by plasticity-producing stimulation in the brain (Konietzko et al., 1999). The present study demonstrates that G-CSF treatment significantly increased nuclear Pim-1 expression in the injured cortex after focal cerebral ischemia. Hence, G-CSF mediated Pim-1 induction may play a protective role in the brain after focal ischemic injury.

Recent studies have suggested that G-CSF via STAT3 signaling leads to an increase in the levels of anti-apoptotic Bcl-2 protein family members, bcl-X_L and bcl-2 (Komine-Kobayashi et al., 2005; Schneider et al., 2005). Bcl-2 is also a target protein of Pim-1 and mediates part of the anti-apoptotic effects of Pim-1 in hematopoietic cell lines (Lilly et al., 1999). The results obtained in the present study are consistent with previous studies which showed that the administration of G-CSF markedly increased STAT3 phosphorylation and returned mitochondrial bcl-2 levels in the injured cortex toward control values. It is well established that bcl-2 and bcl-X_L prevent apoptosis by regulating the mitochondrial membrane potential and thereby blocking cytochrome c release into the cytosol (Shimizu et al., 1999; Yang et al., 1997), where cytochrome c plays a key role in the initiation of apoptosis through the activation of caspase-3. On the other hand, the release of cytochrome c can also occur subsequent to the translocation of Bax to the mitochondria. Bax is a pro-apoptotic member of the Bcl-2 protein family and it has been suggested that it forms a mitochondrial channel which is permeable to cytochrome c (Jurgensmeier et al., 1998). In the present study, we demonstrated that Bax translocation to the mitochondria and cytochrome c release from the mitochondria, as well as caspase-3 activation were attenuated significantly by G-CSF treatment. It can be suggested that the inhibition of the mitochondrial-dependent activation of caspase-3, by inhibiting Bax translocation and/or increasing the levels of anti-apoptotic bcl-2, is likely to be one of the mechanisms underlying the neural anti-apoptotic effect of G-CSF.

Furthermore, it can be suggested that increased Pim-1 expression is at least partially involved in this anti-apoptotic machinery. However, there is the usual problem of an inherent interconnection between the effects of a drug on tissue salvage and the protein signals observed in an *in vivo* study. To confirm these results *in vitro* studies are needed.

Although the anti-apoptotic effect of G-CSF has focused primarily on neurons in previous studies, it is well established that focal cerebral ischemia also causes damage to glial cells. To our knowledge, the effect of G-CSF on glial cell survival has not been studied before. Increased glial survival is important for the support and the production of trophic factors in the CNS and may play a key role in conferring resistance to ischemic damage. Consistent with previous reports, we showed glial apoptosis in the ipsilateral cortex following MCAO (Xia et al., 2004). However, G-CSF treatment reduced the expression of cleaved caspase-3 in glia, hence reducing glial apoptosis. Moreover, we showed that G-CSF increased the activation of the STAT3 pathway in glial cells which was accompanied by increased cIAP2 expression which is a member of the inhibitor of apoptosis proteins (IAPs) family. Although the mechanism of action of the IAPs in the apoptotic cascade is not well-elucidated, these molecules appear to regulate the activity of both initiator (caspase-9) and effector caspases (caspase-3 and -7) (Ekert et al., 2001; Liston et al., 2003; Roy et al., 1997; Salvesen and Duckett, 2002). IAPs have been shown to be expressed by glial cells and enhance the malignant potential of gliomas by suppressing apoptotic cell death (Chakravarti et al., 2002; Kim et al., 2004). Previously, cIAP2 has been shown to be selectively upregulated by G-CSF through the activation of the STAT3 pathway in hematopoietic cells (Hasegawa et al., 2003). Perhaps the STAT3 pathway may also play a role in G-CSF-mediated glial cell survival, at least in part, by up-regulating cIAP2 levels. Taking these data together, we conclude that G-CSF increases both glial and neuronal survival by effecting different anti-apoptotic pathways which reflect the multifactorial actions of this drug. However, *in vitro* studies are needed to show the expression

of the G-CSFR in astrocytes and demonstrate the protective action of G-CSF on glial cell survival. Hence, it is not clear at present whether anti-apoptotic signaling in astrocytes is directly caused by potential presence of the G-CSFR in glial cells.

After MCAO, G-CSF treatment increased both neuronal and glial survival which was associated with a remarkable improvement in tissue preservation and behavioral outcome. Although the administration of G-CSF significantly improved the early neurological outcome, this beneficial effect can be mediated not only by caspase-altering mechanisms but also may involve other mechanisms such as the inhibition of the inflammatory response, as shown previously (Gibson et al., 2005b; Komine-Kobayashi et al., 2005; Zavala et al., 2002). Besides the early-neurological improvement, recently, Gibson et al. clearly demonstrated the long-term benefits using the same dose of G-CSF (50µg/kg) on both motor and cognitive outcome up to 3 weeks after an experimental stroke (Gibson et al., 2005a). The dose (Schedule A) used in the present study is a pharmacological dose that has already been shown to cause neuroprotection in previous experimental studies (Gibson et al., 2005b; Gibson et al., 2005a; Schneider et al., 2005). Moreover, this dose is also in the range of G-CSF doses that has been used in human clinical trials (Gabrilove et al., 1988). In the present study, we also investigated the neuroprotective effect of administering G-CSF with a dose of 50µg/kg/day for 3 days (Schedule B). This treatment was also associated with decreased infarction volume at 3 days and the preservation of tissue loss at 2 weeks after MCAO.

More importantly, G-CSF has been shown to be a safe drug, as many patients have received it over the last decade after cytotoxic therapies. It is therefore imperative to determine the mechanisms of neuroprotection of G-CSF which may be a novel neuroprotectant for future use.

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Abbreviations

cIAP2	cellular inhibitor of apoptosis protein 2
CNS	central nervous system
DTT	dithiothreitol
ECA	external carotid artery
G-CSF	Granulocyte-Colony Stimulating Factor
G-CSFR	G-CSF-receptor
IAPs	inhibitor of apoptosis proteins
ICA	internal carotid artery
JAK2	Janus kinase2
MCAO	middle cerebral artery occlusion

STAT3

signal transducer and activator of transcription 3

Figure 1.

Figure shows treatment schedules used in the experiment. In **Schedule A**, rats received a single dose of 50 μ g/kg (subcutaneously) of G-CSF at the onset of reperfusion. The MCAO group received the same volume of saline. Rats were sacrificed 24hrs after reperfusion. In **Schedule B**, G-CSF (50 μ g/kg body weight, subcutaneously) was administered at the onset of reperfusion and again administrated with the same dosage daily for an additional 2 days. The MCAO group received the same volume of saline. Rats were sacrificed either 72hrs or 2 weeks after reperfusion.

Figure 2.

Representative coronal brain sections from the MCAO and G-CSF-treated rats stained with 2% TTC after 24hrs (n=6 per group) (A) and 72hrs (n=5 per group) (C) of reperfusion showing infarction. Quantitative analysis (B, D) revealed that both schedule A (a single dose of 50µg/kg of G-CSF at the onset of reperfusion) and schedule B (G-CSF 50 µg/kg body weight at the onset of reperfusion and the same dosage daily for an additional 2 days) treatments produced a significant reduction in the infarction volume (*p<0.05 vs. MCAO) (E) MCAO resulted in a significant decrease in the neurological scores and G-CSF treatment produced a significant improvement in the neurological score (*p<0.05 vs. MCAO). (F) Rats that were subjected to schedule B treatment sacrificed 2 weeks after the injury. G-CSF significantly reduced tissue loss in brains after a MCAO.

Figure 3.

(A) Double immunofluorescence staining for NeuN (red) and G-CSFR (green) shows the expression of G-CSFR in neurons (merge, yellow) in the ipsilateral cortex from the MCAO group. Scale bar represents 10 μ m. (B) Double immunofluorescence staining for NeuN (green) and pSTAT3 (red) in the rat brain cortex from the MCAO and G-CSF-treated groups. Notice that there is an increased number of pSTAT3 and NeuN colocalized cells (merge, yellow) in the G-CSF-treated group. Scale bar represents 30 μ m. Insets show negative controls. (C) Double immunofluorescence staining for NeuN (green) and Pim-1 (red) shows Pim-1 expression in a neuron from a G-CSF-treated brain. Scale bar represents 10 μ m. (D, E) Western Blot analysis shows that G-CSF treatment significantly increased phosphorylated STAT3 (*p<0.05 vs. sham, †p<0.05 vs. MCAO) and nuclear Pim-1 levels in the injured cortex compared to the MCAO group 24hrs after reperfusion (*p<0.05 vs. sham). Representative bands from sham, MCAO, and G-CSF-treated groups as well as the corresponding β -actin and Histone H1 bands as loading controls are shown in the panels.

Figure 4.

(A) Triple immunofluorescence staining was performed for NeuN (red), cleaved caspase-3 (green) and cytochrome c (blue) in the ischemic cortex of the rat brain. Merge images are shown in white demonstrating an apoptotic neuron (arrow, high magnification sample window). (B) Double immunofluorescence staining was performed for GFAP (green) and cleaved caspase-3 (red) in the ischemic cortex of the rat brain. Merge images are shown in yellow demonstrating apoptotic astrocytes in the MCAO group (arrows). Upper windows show negative controls. Scale bar represents 30 μ m. (C–E) Western blot analysis shows the mitochondrial bcl-2, as well as cytosolic and mitochondrial Bax expressions 24hrs after reperfusion. Representative bands from sham, MCAO, and G-CSF-treated groups, as well as the corresponding β -actin and COX IV bands as loading controls are shown in the panels. A significant reduction in cytosolic Bax and a significant increase in mitochondrial Bax, suggesting Bax translocation to the mitochondria, were found in the MCAO group 24hrs after reperfusion (* p <0.05 vs. sham). G-CSF treatment significantly reduced the translocation of Bax to the mitochondria after injury ($\dagger p$ <0.05 vs. MCAO).

Figure 5.

(A) Immunostaining for cleaved caspase-3 in the cortex of the ipsilateral hemisphere 24hrs after reperfusion. There are a reduced number of cleaved caspase-3 positive cells in the G-CSF-treated group. Scale bar represents 30μm. (B-D) Western blot analysis shows the cytosolic and mitochondrial cytochrome c and cleaved caspase-3 expressions 24hrs after reperfusion. Representative bands from sham, MCAO, and G-CSF-treated groups, as well as the corresponding β-actin and COX IV bands as loading controls are shown in the panels. A significant accumulation of cytochrome c in the cytosolic fraction was found in the MCAO group, whereas G-CSF treatment significantly reduced the accumulation of cytochrome c in the cytoplasm (*p<0.05 vs. sham, †p<0.05 vs. MCAO). The level of cleaved caspase-3 significantly increased 24hrs after reperfusion. G-CSF prevented an increase in cleaved caspase-3 levels, even though the cleaved caspase-3 levels after G-CSF treatment were significantly higher than sham samples (*p<0.05 vs. sham, †p<0.05 vs. MCAO).

Figure 6.

(A) Double immunofluorescence staining was performed for GFAP (green) and pSTAT3 (red). At 24hrs, an increased pSTAT3 immunostaining is seen in glia in samples from the G-CSF-treated group. Glia expressing pSTAT3 appeared yellow (arrows). (B) Double immunofluorescence staining for GFAP (red) and cIAP2 (green) revealed an extensive expression of cIAP2 in glial (merge, arrows) in the G-CSF-treated group comparing to the MCAO group. Scale bar represents 30 μ m. (C) Representative Western Blot analysis showing cIAP2, as well as β -actin as a loading control, expression in brain tissues from sham, MCAO, and G-CSF-treated groups 24hrs after reperfusion. Quantification of Western Blot analysis showed significantly increased cIAP2 levels in the G-CSF group compared with the MCAO group (*p<0.05 vs. sham and MCAO).