**ORIGINAL ARTICLE**

**G-CSF Suppresses Edema Formation and Reduces Interleukin-1β Expression After Cerebral Ischemia in Mice**

Claire L. Gibson, PhD, Nigel C. Jones, PhD, Malcolm J. W. Prior, PhD, Philip M. W. Bath, MD, FRCP, and Sean P. Murphy, PhD

**Abstract**

Granulocyte-colony stimulating factor (G-CSF) is reported to be neuroprotective after transient cerebral ischemia with respect to decreasing lesion volume and enhancing functional recovery. We investigated whether G-CSF is neuroprotective after permanent ischemia and the possible mechanisms underlying this neuroprotection. Mice underwent permanent or 60-minute middle cerebral artery occlusion (MCAO) and received G-CSF (50 μg/kg) or vehicle at the onset or 1 hour post-MCAO. Forty-eight hours after transient MCAO, structural magnetic resonance imaging revealed a significant reduction (50%) in the amount of edematous tissue present in G-CSF-treated mice (p < 0.05). G-CSF treatment also prevented a significant increase in ipsilateral brain water content that was present in vehicle-treated mice (p < 0.05). Using real-time polymerase chain reaction, we found that G-CSF treatment significantly suppressed (p < 0.05) the injury-induced upregulation of IL-1β mRNA while having no effect on TNFα and NOS-2 mRNA expression. This suggests that part of the neuroprotection may be attributed to the ability of G-CSF to reduce the inflammatory response.

**Key Words:** Granulocyte-colony stimulating factor (G-CSF), Inflammation, Interleukin-1β, Ischemia, Magnetic resonance imaging, Middle cerebral artery occlusion.

**INTRODUCTION**

Growth factors can exert neuroprotective and neurotrophic effects on mature neurons in vitro, and some of these growth factors also display neuroprotective properties after their exogenous administration after stroke (1). Granulocyte colony-stimulating factor (G-CSF), a member of the cytokine family of growth factors, has been reported to reduce lesion volume and improve functional recovery after transient ischemia in mice (2) and rats (3–5). The main actions of G-CSF are mediated by binding to a G-CSF receptor present on hematopoietic, neuronal, and glia cells (3). G-CSF has been reported to possess antiapoptotic properties through activation of various intracellular signaling cascades, including the activation of bcl-2 (6). In response to a variety of infectious states, G-CSF can also mediate antiinflammatory effects (7–9), which may be attributed to an ability to modulate the levels of proinflammatory cytokines. G-CSF can also stimulate the release of bone marrow stem cells into the peripheral circulation (10) and is a common treatment after hematologic disease or chemotherapy treatment for cancer when white blood cell counts tend to be dangerously low and there is risk of infection. Interestingly, treatment with G-CSF can increase the rate of “homing” of bone marrow cells to the central nervous system (11). However, little insight has been gained into the mechanisms of G-CSF neuroprotection after cerebral ischemia.

The induction of focal cerebral ischemia triggers a complex series of events, including excitotoxicity, inflammation, apoptosis, and necrosis, which all contribute to the pathology (12). Edema formation after stroke is a significant contributor to the resulting pathology. We report that a single dose of G-CSF given after the onset of ischemia reduces edema formation and lesion volume after both transient and permanent middle cerebral artery occlusion (MCAO) in mice. In addition, we investigated the effect of G-CSF on the expression of IL-1β, TNFα, and NOS-2. The results suggest that at least part of the mechanism of G-CSF neuroprotection may be attributed to a reduction in the expression of IL-1β.

**MATERIALS AND METHODS**

**Focal Cerebral Ischemia**

This study was conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986. All mice were adult male C57 BL/6, weighing between 24 and 32 g at the time of surgery. In total, 81 mice were used in the current study. A total of 14 mice underwent transient MCAO; 2 died and 2 were excluded as a result of inadequate occlusion/reperfusion. A total of 52 mice underwent permanent MCAO, 7 of which died and one was excluded as a result of inadequate occlusion. Anesthesia was induced by inhalation of 4% isoflurane (in a NO2/O2 70%/30% mixture) and maintained by inhalation of 1.5% isoflurane. Body temperature was monitored throughout surgery (through a rectal probe) and maintained...
at 37.0 ± 0.6°C using a heating blanket (Harvard Apparatus Ltd., Kent, U.K.). Laser Doppler flowmetry (Moor Instruments, Devon, U.K.) was used to monitor relative cerebral blood flow (CBF) for 5 minutes before and 5 minutes after MCAO, and for transient MCAO immediately before and after reperfusion. At the beginning of surgery, a small incision was made in the skin overlying the temporalis muscle, and then a 0.7-mm flexible laser Doppler probe (model P10) was secured on the superior portion of the temporal bone (6 mm lateral and 2 mm posterior from bregma). Focal cerebral ischemia was induced by occlusion of the right middle cerebral artery as previously described (2, 13). During the 60-minute period of MCAO, mice were randomly assigned to receive either G-CSF or vehicle. After 60-minute MCAO, mice were reanesthetized, and the occluding filament was withdrawn gently back into the common carotid artery to allow reperfusion to take place. Relative CBF was monitored for a further 5 minutes, during which time drug was administered, before the wound being sutured and mice were allowed to recover from anesthesia. For mice undergoing permanent MCAO, Doppler monitoring continued for 5 minutes after the onset of MCAO, during which period drug treatment was randomly assigned and delivered. The Doppler probe was then removed, the wound sutured, and mice recovered from anesthesia. For mice undergoing permanent MCAO, Doppler monitoring continued for 5 minutes after the onset of MCAO, during which period drug treatment was randomly assigned and delivered. The Doppler probe was then removed, the wound sutured, and mice recovered from anesthesia. Sham-operated mice (n = 15) underwent the same surgical procedure, except that the filament was not advanced far enough to occlude the middle cerebral artery.

**Drug Treatment**

Mice subjected to MCAO were randomly assigned to receive either G-CSF or vehicle. After transient MCAO, one group (n = 5) received G-CSF (Amgen, The Netherlands) dissolved in saline and injected subeutaneously (50 μg/kg) at the onset of reperfusion (i.e. 1 hour post-MCAO). Mice in another group (n = 5) underwent the same experimental protocol, except that they received vehicle only. For mice undergoing permanent MCAO, the G-CSF group (n = 26) received the same dose of G-CSF (50 μg/kg) and by the same route as mice undergoing transient MCAO, except the G-CSF was injected at the onset of MCAO. Mice in the vehicle group (n = 22) underwent the same experimental protocol, except that they received vehicle only. The experimenter was blinded to the treatment that mice received before all subsequent analyses.

**Structural Magnetic Resonance Imaging**

All mice undergoing transient MCAO were subjected to structural magnetic resonance imaging (MRI) 48 hours after surgery (G-CSF n = 5; vehicle n = 5). Anesthesia was induced by inhalation of 4% isoflurane (in a N2O/O2 70%/30% mixture) and maintained by inhalation of 1.5% isoflurane. Throughout the MRI procedure, the breathing rate was monitored. TURBO RARE 3D coronal images (RARE factor = 16) were obtained on a Bruker Avance Biospec imaging system (Bruker Biospin MRI, Coventry, U.K.) at 2.35 T using an effective TE of 62.9 ms, TR of 4136 ms, with an image resolution of 0.2 × 0.2 × 0.2 mm. The resulting images of animals scanned 48 hours after injury depicted the formation of spreading edema as defined by hyperintense (white) regions. The volume of edema was calculated using the “region of interest” tool on Paravision (Bruker Biospin MRI), isolating areas of T2-weighted abnormality. Because each voxel represented a volume of 0.08 mm³, the volume of edema for each animal could be estimated.

**Brain Water Content**

This was calculated 48 hours post-MCAO using the wet–dry method. Mice subjected to transient (G-CSF, n = 5; vehicle, n = 5; shams, n = 5) and permanent MCAO (G-CSF, n = 6; vehicle, n = 5; shams, n = 6) were killed by cervical dislocation. Brains were removed, separated into ipsilateral and contralateral hemispheres, and gently blotted with tissue paper to remove small quantities of adsorbent CSF. All hemispheres were then weighed with a basic precision scale to within 0.1 mg, giving the wet weight. Samples were placed in an oven at 100°C for 24 hours, after which they were again weighed (dry-weight). The percentage of water of each sample was calculated as follows: percent brain water = wet weight – dry weight/wet weight × 100.

**Lesion Volume**

Mice undergoing permanent MCAO were killed 48 hours after surgery for lesion volume analysis (G-CSF, n = 6; vehicle, n = 6). After cervical dislocation, brains were removed and sectioned into 5 × 2-mm coronal slices using a mouse matrix (ASI Instruments, Houston, TX). To quantitate ischemic damage, slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, Gillingham, U.K.) in saline for 30 minutes at room temperature in the dark and stored at 4°C in 10% formalin before analysis. TTC is a marker for mitochondrial function and has been shown to be a reliable indicator of ischemic areas for up to 3 days postischemia (13–15). Digital photographs of all stained slices were taken and the unstained area of infarction was measured on the posterior surface of each coronal section using Scion Image Software (Frederick, MD). Infarct areas were calculated by an indirect method, whereby overestimation of the infarct area as a result of the contribution of edema is avoided (16). Briefly, the infarcted area of the ischemic hemisphere was determined by subtracting the noninfarcted area of the ischemic hemisphere from the total area of the uninfarcted hemisphere. Total infarct volume was calculated by multiplying the area of infarct for each slice by the slice thickness and then summing the slices.

**Histology**

To assess histologic damage after permanent MCAO mice were anesthetized 48 hours post-MCAO with an overdose of sodium pentobarbitone and transcardially perfused using 20 mL 0.9% saline followed by 1 mL/g body weight buffered fixative solution (4% paraformaldehyde and 15% picric acid in phosphate-buffered saline adjusted to pH 7.4). Brains were removed, postfixed in the same solution for 2 hours, and transferred into 30% buffered sucrose solution overnight at 4°C. Coronal cryostat sections (25 μm) were cut, mounted onto APES-coated slides, and left to air dry at room temperature for 2 hours before being Nissl-stained using cresyl violet solution. Slides were then dehydrated, cleared, and coverslipped using Entellan (Merck, Hoddesdon, U.K.). Digital
photographs were taken using AxioVision (Carl Weiss, Welwyn, U.K.).

Real-Time Polymerase Chain Reaction (TaqMan) After Permanent Ischemia

A subset of mice subjected to permanent ischemia were used for gene analysis 6 hours (G-CSF, n = 6; vehicle, n = 4) and 24 hours (G-CSF, n = 6; vehicle, n = 5) post-MCAO. Sham-operated controls were also included for each time point. Mice were killed by cervical dislocation, brains were removed, separated into ipsilateral and contralateral hemispheres, snap-frozen in liquid nitrogen, and stored at –80°C until use. Total RNA was isolated from brain samples using the SV Total RNA isolation system (Promega, Southampton, U.K.) according to the manufacturer’s instructions. First-strand cDNA was synthesized using random primers (Promega) and Moloney murine leukemia virus reverse transcriptase under the following conditions: 70°C for 5 minutes, 42°C for 60 minutes, and 75°C for 10 minutes. Additional reactions were performed in which the reverse transcriptase was omitted to allow for assessment of genomic DNA contamination. Multiplex real-time polymerase chain reaction (PCR) was carried out using an ABI prism 7000 sequence detector (Applied Biosystems, Warrington, U.K.) with 2 µL cDNA, 18 µM each primer, 5 µM probe, and Universal TaqMan 2 × PCR Mastermix (Applied Biosystems) to a final volume of 25 µL. All samples were run in triplicate. Primers and MGB TaqMan probes (labeled with the fluorescent reporter FAM) for IL-1β, TNF-α, and NOS-2 were designed by Applied Biosystems (Assay-on-Demand) to avoid genomic amplification. Cyclophilin E, labeled with the fluorescence dye VIC, was designed by Applied Biosystems (Assay-by-Design). The thermal cycling conditions used during the PCR were as follows: 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. A standard curve was obtained for relative expression of the gene of interest (FAM-labeled) and endogenous cyclophilin (VIC-labeled), and a linear relationship was observed over > 500-fold range. Cycling threshold (Ct) values of the target gene were normalized to Ct values of the endogenous cyclophilin, and final results were calculated according to the formula 2−ΔΔCt. Cyclophilin mRNA levels were not altered in response to either injury or drug treatment. In addition to each sample being run in triplicate, the value of mRNA expression for each gene in each sample was expressed as a ratio of the expression to each relevant hemisphere of the 4 shams. Thus, for each sample, 4 levels of mRNA expression for one gene were obtained and the mean of these was calculated for each sample.

Statistics

All data are expressed as mean ± standard error of mean. Survival data were analyzed by applying the Kaplan-Meier curve, followed by the Mantel-Haenszel log-rank test to identify differences according to treatment. Edema volume obtained by structural MRI was analyzed using Student t-test for differences according to treatment. Data obtained from percentage of brain water content was analyzed using one-way analysis of variance (ANOVA) for differences according to treatment. Two-way ANOVA was applied to the lesion volume data (to identify differences according to treatment and location of lesion) and the gene expression data (to identify differences according to treatment and time). Post hoc analyses were carried out with Bonferroni’s test. All data were analyzed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). In all instances, statistical significance was defined as p < 0.05.

RESULTS

After MCAO, a total of 9 mice died. To determine the effect of G-CSF on survival rate, the data were analyzed by applying the Kaplan-Meier curve followed by the Mantel-Haenszel log-rank test (χ² = 1.068). There were no statistically significant differences in survival rate between treatment groups (p = 0.301).

![FIGURE 1. (A) T2-weighted images 48 hours after transient middle cerebral artery occlusion reveal the presence of edematous tissue, as represented by areas of hyperintensity in the right hemisphere. (B) Measurements of the volume of edematous tissue revealed a decrease after granulocyte-colony stimulating factor (G-CSF) compared with vehicle treatment (*, p < 0.05). Scale bar = 1 mm.](image-url)

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Cerebral Blood Flow

Doppler monitoring showed that in all mice subjected to transient MCAO, relative CBF was reduced to 22.44% (±1.03) and 19.15% (±3.56) of preischemic values within 5 minutes of advancing the filament in mice that subsequently received G-CSF or vehicle, respectively.

**Volume of Edematous Tissue Present After Transient Middle Cerebral Artery Occlusion**

Figure 1A depicts T2-weighted MRI images obtained 48 hours after transient MCAO in mice that had received G-CSF or vehicle. An area of hyperintense activity reveals the presence of edematous tissue. Measurement of edema volume (Fig. 1B) indicated a significant difference between G-CSF and vehicle treatment (p = 0.041).

The amount of edematous tissue was also measured by calculating the percent brain water content (Fig. 2A). After transient MCAO, one-way ANOVA revealed a significant effect of treatment on the amount of ipsilateral edema (p = 0.027). Post hoc analysis revealed this increase was significant in vehicle-treated mice (p < 0.05) compared with shams, whereas G-CSF-treated mice did not differ significantly from shams. One-way ANOVA also revealed a significant effect of treatment (p = 0.0008) on the ipsilateral edema formation after permanent MCAO (Fig. 2B). Post hoc analysis revealed a significant increase in ipsilateral edema after vehicle treatment compared with shams (p < 0.001), whereas G-CSF-treated mice did not differ significantly from shams. Treatment had no effect on the amount of contralateral edema after either transient (p = 0.921) or permanent MCAO (p = 0.8261).

**Lesion Volume After Permanent Middle Cerebral Artery Occlusion**

Figure 3A shows representative brain slices stained with TTC 48 hours after permanent MCAO in G-CSF- and vehicle-treated mice. The lesion is located within the striatal and cortical areas of the brain, as indicated by the white area.

**Cerebral Blood Flow**

Doppler monitoring showed that in all mice subjected to transient MCAO, relative CBF was reduced to 22.44% (±1.03) and 19.15% (±3.56) of preischemic values within 5 minutes of advancing the filament in mice that subsequently received G-CSF or vehicle, respectively. After 60 minutes MCAO and withdrawal of the filament, relative CBF was increased to at least 50% of preischemic values for the mice to be included in the study. There were no significant differences in the increase in relative CBF after withdrawal of the filament in the G-CSF (106.69% ± 14.2) compared with the vehicle group (93.43% ± 7.61). For mice undergoing permanent MCAO, relative CBF was reduced to 20.07% (±1.74) and 22.07% (±1.72) of preischemic values within 5 minutes of advancing the filament in mice that subsequently received G-CSF or vehicle, respectively.
Measurements from TTC sections (Fig. 3B) indicated an overall reduction in lesion volume after G-CSF treatment at 48 hours post-MCAO ($F_{(1,12)} = 6.87, p = 0.0137$). Post hoc analysis revealed a significant reduction in cortical lesion volume present after G-CSF treatment ($p < 0.05$), whereas there was no affect on striatal lesion volume.

**Histology After Permanent Middle Cerebral Artery Occlusion**

Nissl staining revealed the extent of cell loss 48 hours after permanent MCAO (Fig. 4). In contralateral cortical regions (Fig. 4A), cells of various morphologies can be identified organized into layers. However, in the occluded hemisphere, this organization has been lost and there appears to be an overall decrease in cell density (Fig. 4B). In addition, the complexity of cell shapes and sizes observed in the contralateral hemisphere (Fig. 4C) has been replaced by cells of homogeneous morphology (Fig. 4D) and resembling infiltrating inflammatory cells. In striatal regions, the cell density apparent contralaterally (Fig. 4E) has been greatly reduced on the occluded side (Fig. 4F).

**Gene Expression After Permanent Middle Cerebral Artery Occlusion**

Real-Time PCR revealed the levels of expression of mRNA for IL-1β, TNFα, and NOS-2 in both hemispheres at 6 and 24 hours after permanent MCAO normalized to internal cyclophilin mRNA (Fig. 5). There was a significant upregulation

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**FIGURE 4.** Representative Nissl stained sections reveal cell density in cortical and striatal regions after permanent middle cerebral artery occlusion. In the hemisphere contralateral to injury, cortical areas consist of a variety of cell types and sizes with a distinctive layered organization (A). On the occluded side, this layered organization is absent (B), and the variety of cell morphologies (C) has been replaced with cells of very similar morphology (D). In striatal regions, the cell density shown in the contralateral hemisphere (E) is greatly reduced in the occluded hemisphere (F). Scale bars = (A, B) 100 μm; (C–F) 50 μm.
of IL-1β mRNA in the ipsilateral compared with the contralateral hemisphere in mice that had received either vehicle (F(1,17) = 20.73, p = 0.0005) or G-CSF (F (1,23) = 11.36, p = 0.003). The increase in ipsilateral IL-1β mRNA was significantly reduced after G-CSF (F(1,20) = 5.51, p = 0.0313, Fig. 5A). G-CSF treatment did not affect the level of contralateral IL-1β mRNA expression (p = 0.812, data not shown).

After permanent MCAO, there was a significant increase in the expression of TNFα mRNA in the injured hemisphere in mice that had received either vehicle (F (1,17) = 61.52, p < 0.0001) or G-CSF (F(1,23) = 11.45, p = 0.0031). However, this increase in TNFα mRNA was not affected by G-CSF, either in the ipsilateral (p = 0.539, Fig. 5B) or contralateral hemisphere (p = 0.75, data not shown).

Permanent MCAO also resulted in a significant upregulation in the expression of NOS-2 mRNA in the ipsilateral compared with the contralateral hemisphere, and this was significant in mice that had received either vehicle (F(1,17) = 33.19, p = 0.0001) or G-CSF (F(1,23) = 16.00, p = 0.0007). The expression of NOS-2 mRNA was not affected by G-CSF treatment in the ipsilateral (p = 0.679, Fig. 5C) or contralateral hemisphere (p = 0.2518, data not shown).

DISCUSSION

The present study demonstrates that G-CSF administration after transient ischemia leads to a decrease in the amount of edematous tissue present as measured by both structural MRI and brain water content. The neuroprotective potential of G-CSF treatment was also investigated for the first time after permanent ischemia, where it was found to reduce edema formation and lesion volume. The effect of G-CSF on reducing lesion volume was restricted to cortical areas and it had no effect on striatal lesion volume. In terms of inflammatory gene expression, G-CSF treatment significantly reduced the injury-induced upregulation of IL-1β while having no effect on TNFα and NOS-2 expression.

In the current study, we administered G-CSF at a dose (50 μg/kg) that has previously been shown to be effective at reducing lesion volume and improving functional recovery after transient MCAO (2–5). Apart from relative cerebral blood flow immediately after reperfusion, we did not monitor the effect of G-CSF on physiological parameters. However, others have reported no effects in the rat of a similar dose of G-CSF on rectal temperature, pH, PCO₂, PO₂, hematocrit, blood glucose, heart rate, and mean arterial pressure over the subsequent 24-hour period (3).

The observation that G-CSF reduces edema and lesion formation after ischemia may be explained by its antiinflammatory and antiapoptotic properties. However, we only measured edema formation and lesion volume 48 hours post-ischemia and cannot rule out the possibility that G-CSF treatment delayed, rather than prevented, the development of either. The antiapoptotic properties of G-CSF have been shown to be mediated through binding to a G-CSF receptor present on hematopoietic, neuronal, and glial cells (3). Once activated, the G-CSF receptor can activate proteins such as STAT3, which, in turn, activate antiapoptotic proteins such as bcl-2 (6, 17).

G-CSF has been reported to mediate antiinflammatory effects after a variety of infections (7–9). This may be mediated through an ability to decrease the levels of proinflammatory cytokines such as TNFα and IL-1β, which has been demonstrated in vitro (18). In addition, G-CSF has been shown to reduce the expression of NOS-2 mRNA and subsequent NO synthesis in vitro (19). In the current study, we observed that G-CSF significantly reduced the in vivo expression of IL-1β mRNA but had no effect on TNFα and NOS-2 expression.
However, our study provides no evidence for a direct effect of G-CSF on IL-1β expression, and it is possible that suppression may occur through indirect mechanisms. This suppression of IL-1β may account, at least in part, for the ability of G-CSF to reduce lesion volume and edema formation.

Exogenous administration of IL-1β has been shown to exacerbate ischemic damage (20), whereas administration of an endogenous IL-1β receptor antagonist (21, 22) or a neutralizing antibody (23) reduced brain damage and edema when administered before a stroke in rats. The reduced cytokine activation by G-CSF may also reduce both neutrophil activation and subsequent infiltration, which are contributing factors to ischemic damage. In fact, G-CSF has been shown to reduce neutrophil infiltration in a model of splanchnic ischemia and reperfusion (24).

We have provided evidence for a neuroprotective effect of G-CSF administration after both transient (2) and permanent focal cerebral ischemia, and propose that this may be attributed to the ability of G-CSF to reduce edema formation. To investigate the possible mechanisms of this G-CSF effect, we chose to concentrate on permanent ischemia. Although the 2 models of transient and permanent ischemia differ in the respect that one permits reperfusion, it is interesting to observe that G-CSF reduced edema formation in both models. We attribute at least part of this effect to the ability of G-CSF to reduce IL-1β gene expression.

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