Granulocyte-Colony Stimulating Factor Delays PWI/DWI Mismatch Evolution and Reduces Final Infarct Volume in Permanent-Suture and Embolic Focal Cerebral Ischemia Models in the Rat

Bernt T. Bråtane, MSc; James Bouley, BSc; Armin Schneider, MD; Birgul Bastan, MD; Nils Henninger, MD; Marc Fisher, MD

Background and Purpose—Granulocyte-colony stimulating factor (G-CSF) is used clinically to attenuate neutropenia after chemotherapy. G-CSF acts as a growth factor in the central nervous system, counteracts apoptosis, and is neuroprotective in rodent transient ischemia models.

Methods—We assessed the effect of G-CSF on ischemic lesion evolution in a rat permanent-suture occlusion model with diffusion- and perfusion-weighted magnetic resonance imaging and the neuroprotective effect of G-CSF in a rat embolic stroke model.

Results—With a constant perfusion deficit, vehicle-treated animals showed an expanding apparent diffusion coefficient lesion volume that matched the perfusion deficit volume at \( t = 3 \) hours, with the 24-hour infarct volume equivalent to the perfusion deficit. In G-CSF–treated rats, the apparent diffusion coefficient lesion volume did not increase after treatment initiation, and the infarct volume at 24 hours reflected the initial apparent diffusion coefficient lesion volume. In the embolic model, we observed a significant decrease in infarct volume in G-CSF–treated animals compared with the vehicle-treated group.

Conclusions—These results confirm the potent neuroprotective activity of G-CSF in different focal ischemia models. The magnetic resonance imaging data demonstrate that G-CSF preserved the perfusion/diffusion mismatch. (Stroke. 2009; 40:3102-3106.)

Key Words: G-CSF ■ growth factors ■ stroke ■ cerebral ischemia ■ diffusion-weighted imaging ■ perfusion-weighted imaging ■ penumbra ■ mismatch

Granulocyte-colony stimulating factor (G-CSF) was originally identified as a hematopoietic factor in the myeloid lineage and is responsible for the generation of neutrophilic granulocytes. Recently, the presence and activity of this factor in the central nervous system have been identified. G-CSF and its receptor are upregulated after cerebral ischemia, and G-CSF acts antiapoptotically on neurons, passes the intact blood–brain barrier, and reduces infarct size in experimental stroke models. This led to a number of smaller clinical trials in acute ischemic stroke patients. However, most experiments were performed in transient ischemic models, and there are no published data regarding an embolic model.

Thrombolysis with tissue-type plasminogen activator (t-PA) remains the only approved therapy for acute stroke. Unfortunately, the use of t-PA is limited by a relatively narrow time window. Efficacy was recently demonstrated to increase over time. The biologic reason for the reduced therapeutic efficiency over time likely lies in the progressive deterioration of cell viability with ongoing ischemia/hypoxia in hypoperfused brain areas. This may be accompanied by generation of free radicals during reperfusion (ie, reperfusion injury). Clinically, this concept is supported by data that suggest that the presence of a perfusion/diffusion (perfusion-weighted imaging/diffusion-weighted imaging; PWI/DWI) mismatch on magnetic resonance imaging (MRI) identifies patients in whom thrombolysis may be efficacious later in the therapeutic time window. The PWI/DWI mismatch provides a volumetric estimate of the putative ischemic penumbra and the duration of its temporal existence. The PWI/DWI mismatch may represent potentially salvageable ischemic tissue at risk for infarction and is thus a target for acute stroke therapies. Extending the time window for thrombolysis might be one strategy to protect the ischemic penumbra. We

Received March 27, 2009; final revision received May 29, 2009; accepted June 8, 2009.

From the Department of Neurology (B.T.B., J.B., B.B., N.H., M.F.), University of Massachusetts Medical School, Worcester, Mass, and Molecular Neurology (A.S.), Sygnis Bioscience GmbH & Co KG, Heidelberg, Germany.

Correspondence to Bernt Tore Bratane, Center for Comparative Neuroimaging, University of Massachusetts Medical School, 303 Belmont St, Worcester, MA 01604. E-mail Bernt.Bratane@umassmed.edu

© 2009 American Heart Association, Inc.

Stroke is available at http://stroke.ahajournals.org DOI: 10.1161/STROKEAHA.109.553958
previously demonstrated a proof of concept for this hypothesis with normobaric hypoxia treatment \( ^{13} \) and stimulation of the sphenopalatine ganglion. \( ^{14} \)

Using serial PWI and DWI, we explored the effects of G-CSF on the spatiotemporal evolution of the ischemic penumbra. In addition, we studied for the first time the effects of G-CSF in a rat embolic stroke model.

**Materials and Methods**

All procedures used in this study were performed in accordance with institutional guidelines. Spontaneously breathing male Wistar rats (n = 35; Taconic Farms, Hudson, NY) weighing 230 ± 19 g were anesthetized with isoflurane (5% for induction, 2% for maintenance) in room air. PE-50 polyethylene tubing was inserted into the femoral artery for monitoring of mean arterial blood pressure (MABP) and for obtaining blood samples to measure blood gases (pH, PaO\(_2\), PaCO\(_2\)), electrolytes (Na\(^+\), K\(^+\), Ca\(^{2+}\)), and plasma glucose before and 30, 60, 90, 120, and 180 minutes after stroke. PE-50 tubing was placed into the femoral vein to allow for intravenous infusion of G-CSF or vehicle. Body temperature was monitored continuously with a rectal probe and maintained at 37.0 ± 0.3°C with a thermostatically controlled heating lamp.

**Middle Cerebral Artery Occlusion**

Permanent suture-occlusion of the right middle cerebral artery (sMCAO) was performed in 15 animals randomized 1:2 to either vehicle (n = 10) or drug (n = 5) with the placement of 4-0 silicone-coated nylon filament sutures, as previously described in detail. \( ^{14} \)

Embolic stroke was performed in 20 animals randomized to either vehicle (n = 10) or drug (n = 10), as described previously in detail. \( ^{13} \)

Laser Doppler flowmetry was used to monitor occlusion success in the embolic stroke animals. \( ^{15} \) Neurologic scoring was performed at 24 hours, as previously described (rating scale: 0 = no deficit, 1 = failure to extend the left forepaw, 2 = decreased grip strength of left forepaw, 3 = circling to parietic side by pulling the tail, 4 = spontaneous contralateral circling, and 5 = death). \( ^{16} \)

**Design of the Embolic Permanent-Occlusion Study**

**Experiment 1**

G-CSF was administered 60 minutes and 4 hours after induction of cerebral ischemia. Animals remained anesthetized during the first 120 minutes of the experiment. Rats were randomized to receive either 120 \( \mu \)g/kg G-CSF at each injection time point (AX200, Sygnis Bioscience, Heidelberg, Germany) or vehicle (0.25 mol/L sorbitol, 0.004% Tween-80, and 0.01 mol/L sodium acetate buffer; pH 4). The first injection was given intravenously over 30 minutes 60 minutes after occlusion, the second, as an intraperitoneal bolus 4 hours after occlusion. Infarct volumes were measured 24 hours after stroke on 2.35-triphenyltetrazolium chloride (TTC)-stained sections after correction for edema. \( ^{17} \) Animals that died prematurely (between 16 and 24 hours after stroke onset) were included in this data analysis. \( ^{18} \)

**Design of the MRI Permanent-Occlusion Study**

**Experiment 2**

Calculation of the quantitative apparent diffusion coefficient (ADC) and cerebral blood flow (CBF) maps allows a rater-independent lesion volume assessment derived from the DWI and PWI, respectively. \( ^{17} \) Using previously validated thresholds for ischemia, we evaluated the effects of G-CSF on ADC- and CBF-derived spatiotemporal lesion evolution. \( ^{17} \) Immediately after sMCAO, animals were placed into the magnet. G-CSF was administered in the same way as in experiment 1. The first injection of G-CSF was started just before obtaining the MRI data at 60 minutes, and animals remained anesthetized until the end of imaging at 180 minutes. At 24 hours after sMCAO, brains were removed and sectioned coronally into seven 1.5-mm-thick slices (corresponding to the MRI slices) and stained with TTC. All histologic analyses were conducted by an author who was blinded to the experimental protocol (B.B.).

**MRI Measurements**

MRI experiments were performed on a 4.7-T/40-cm horizontal magnet equipped with a Biospec Bruker console (Billerica, Mass) and a 20-G/cm gradient insert (ID = 120 mm, 120- \( \mu \)s rise time). A surface coil (ID = 23 mm) was used for brain imaging, and an actively decoupled neck coil was used for perfusion labeling. \( ^{17} \) Animals were imaged at 25, 45, 60, 90, 120, 150, and 180 minutes after sMCAO. DWI and PWI were acquired as previously described in detail. \( ^{18} \)

**Calculation of In Vivo Lesion Size**

Images were analyzed with QuickVol II (http://www.quickvol.com) \( ^{19} \) software. Quantitative CBF and ADC maps and their corresponding threshold-derived lesion volumes were calculated as described previously. \( ^{17,18} \) The thresholds used to define respective abnormal ADC and CBF regions were a reduction to 0.53 × 10^{-3} mm²/s and 0.3 mL/g/min, as previously validated. \( ^{17} \)

**Statistical Analysis**

All experiments were performed in a blinded, randomized manner. Data are presented as mean ± SEM unless stated otherwise. Statistical comparisons were performed with a repeated-measures ANOVA with a post hoc Tukey-Kramer test for multiple comparisons and 2-tailed Student t test, where appropriate. For comparison of ADC lesion volume progression over time and relative to CBF lesion volume and treatment, a multiple linear-regression model was used. Calculations were performed with NCSS 2007 (NCSS, Kaysville, Utah) or JMP 7.01 (SAS Institute, Cary, NC). \( P < 0.05 \) was considered significant.

**Results**

**Experiment 1: Embolic Model**

Blood gases, electrolytes, and glucose were not significantly affected by treatment assignment, and values remained within physiologic limits throughout the study (data not shown). MABP was not influenced by treatment (\( P > 0.05 \)); however, there was a significant group-independent decline in MABP at 30 minutes, after which BP increased again (supplemental Figure 1A, available online at http://stroke.ahajournals.org). Twelve of 20 animals died prematurely between 16 and 24 hours after embolic stroke (5 in the vehicle group and 7 in the G-CSF group) and were included in all analyses. Infarct volumes determined by postmortem TTC staining were 295 ± 20 mm³ (vehicle) versus 206 ± 16 mm³ G-CSF (\( P = 0.003 \); Figure 1). Neurologic scores at 24 hours did not show any difference between treatments (vehicle, 4.0 ± 1.3 vs G-CSF, 4.2 ± 1.3, mean ± SD; Table).

![](image.png)

**Figure 1.** TTC-defined infarct volumes 24 hours after induction of embolic stroke. G-CSF treatment resulted in significantly smaller infarcts compared with the vehicle group (\( P < 0.05 \)).
Table. Neurologic Scores

<table>
<thead>
<tr>
<th>Group</th>
<th>4-h Menzies Score, Mean ± SD (Min, Max, Range)</th>
<th>24-h Menzies Score, Mean ± SD (Min, Max, Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1 Vehicle (n=10)</td>
<td>...</td>
<td>4.0 ± 1.3 (2, 5, 3)</td>
</tr>
<tr>
<td>G-CSF (n=10)</td>
<td>...</td>
<td>4.2 ± 1.3 (2, 5, 3)</td>
</tr>
<tr>
<td>Experiment 2 Vehicle (n=5)</td>
<td>3.0 ± 0.0 (3, 3, 0)</td>
<td>3.0 ± 1.2 (2, 5, 3)</td>
</tr>
<tr>
<td>G-CSF (n=10)</td>
<td>2.5 ± 0.9 (1, 3, 2)</td>
<td>2.8 ± 1.0 (1, 5, 4)</td>
</tr>
</tbody>
</table>

Neurologic scores were obtained from experiments 1 and 2. There was no significant difference between vehicle- and G-CSF–treated animals in either experiment.

**Experiment 2: G-CSF Halts the Evolution of ADC-Derived Lesion Volumes in the Presence of a Permanent Perfusion Deficit**

Blood gases, electrolytes, blood glucose, and MABP did not differ between the 2 groups (data not shown). There was a group-independent significant rise in MABP over the course of the experiment (*P*<0.05; supplemental Figure IB). Two of 15 animals died between 16 and 24 hours (1 in each group) and were included in the data analysis. Neurologic scores at 4 and 24 hours did not show any difference between treatments (Table).

Figure 2 summarizes the spatiotemporal evolution of threshold-derived CBF and ADC lesion volumes. The CBF lesion volume did not differ between groups (vehicle and G-CSF) and remained relatively constant over time (Figure 2A). The ADC-derived lesion volume in vehicle-treated animals increased over time in a linear fashion up to 120 minutes, at which point the curve flattened. The final infarct volume determined at 24 hours by TTC staining was not significantly different from the last ADC lesion volume measured at 180 minutes after occlusion (*P*>0.05; Figure 2B).

In G-CSF–treated animals, the ADC lesion volume increased from 25 to 45 minutes after occlusion, identical to the trend in the vehicle group. However, at the 60-minute time point, just after initiation of G-CSF administration, the increase began to attenuate. At 90 minutes, the ADC lesion volume in G-CSF–treated animals was significantly smaller than in vehicle-treated rats (repeated-measures ANOVA, *P*<0.0001). At subsequent time points, the ADC lesion volume remained stable in the G-CSF group (132±23 mm³ at 180 minutes). TTC-defined infarct volumes were significantly smaller in G-CSF–treated compare with vehicle-treated animals (G-CSF, 124±19 vs vehicle, 223±7 mm³; *P*=0.007) and corresponded well to the 3-hour ADC lesion volumes in both groups and to the 3-hour CBF-derived lesion volumes in the vehicle group.

Figure 2C indicates the absolute mismatch between CBF- and ADC-derived lesion volumes. Relative to vehicle-treated animals, the ADC/CBF mismatch lesion volumes were significantly larger starting at 90 minutes after occlusion (*P*<0.05) in the G-CSF group. Finally, in a multiple linear-regression model (factors including CBF lesion volume, animal [random factor], treatment, time, time×treatment interaction), the ADC lesion volume progression over time was compared with the corresponding CBF lesion volumes as well as treatment. According to this analysis, the treatment effect became significant at 84 minutes after sMCAO.

**Discussion**

**G-CSF Demonstrates Efficacy in 2 Cerebral Ischemia Models**

Our results demonstrate the neuroprotective effects of G-CSF in a rat permanent suture-occlusion model as well as an embolic clot model. Although the neuroprotective effects of G-CSF were previously confirmed by several independent investigators using various models as well as a recent meta-analysis, almost all of the prior studies assessing the putative neuroprotective properties of G-CSF used transient-occlusion models. To our knowledge, only one publication explicitly described the use of a mouse permanent-occlusion model.

Although infarct volume reductions in our study were substantial (30% in the embolic model at 24 hours and 44% in the permanent suture model), this was not reflected in changes on the neurologic score. This may be due to the insensitivity of the scale and its ordinal nature, in combina-
tion with the relatively small number of animals used in these complex experiments as well as the large number of early deaths in the embolic model.

Given the severity of the models, the dose of G-CSF was doubled in comparison with previous experiments because the protective effect of G-CSF appears to be dose-dependent, with higher doses providing greater protection, and with the aim of providing a similar dose under clinical investigation. Based on the observation that G-CSF has a half-life of $\approx 3.5$ hours, a second dose was given at 4 hours after stroke induction, thus sustaining its therapeutic plasma concentrations for an extended period and potentially providing improved cytoprotection.

Our data add important information about G-CSF in 2 severe stroke models that may have greater relevance to human stroke and add to the existing database on G-CSF activity in cerebral ischemia, broadening confidence in G-CSF's brain-protective potential.

**G-CSF ‘Froze’ the Penumbra**

The present data demonstrate for the first time that G-CSF can “freeze” the ADC/CBF mismatch region in the presence of an ongoing, constant, perfusion deficit (Figure 3). The extent of penumbral protection was dramatic, because the initial ADC lesion volume demonstrated at 25 minutes after occlusion did not increase over time and was correlated with the final infarct volume at 24 hours, as deduced from TTC staining.

Using a regression analysis, we observed significant protection by G-CSF relative to vehicle treatment as early as 84 minutes after occlusion. This is important, because it provides new insight into potential mechanisms of action. Typically, necrosis is the predominant mechanism that follows acute, permanent vascular occlusion, whereas in milder injury, cell death is predominantly due to apoptosis. This delayed (programmed) cell death occurs predominantly in the penumbral region and is thus temporally and spatially different from the faster neuronal and glial cell death (necrosis) in the ischemic core. It has been suggested that the early upregulation of proapoptotic proteins may modulate the final ischemic injury in the subacute phase, accounting for up to $\approx 10\%$ in lesion volume reduction. Therefore, inhibition of apoptosis by G-CSF in the current model is not expected to halt hyperacute ischemic lesion evolution. However, it was previously demonstrated that G-CSF protects against glutamate-induced excitotoxicity in cell culture. Glutamate-induced excitotoxicity is one of the major factors involved in the initiation of acute ischemic necrosis and affects lesion evolution during focal cerebral ischemia. Hence, it appears very likely that the observed attenuation of ADC-derived lesion evolution (penumbral freezing) is at least in part mediated by inhibition of glutamate-induced excitotoxicity. In addition, excitotoxic mechanisms can also initiate molecular events that ultimately trigger apoptosis, inflammation, and other subacute processes contributing to ischemic injury, which might have been blunted by G-CSF.

In addition to antiexcitotoxic mechanisms, we and others have identified that antiapoptosis contributes to the acute and subacute neuroprotective actions of G-CSF. Induction of antiapoptotic cascades in vitro is immediate, with phosphorylation and activation of v-Akt murine thymoma viral oncogene homolog within 5 minutes after addition of G-CSF to the neurons. Antiapoptotic effects of G-CSF were observed in a variety of different neuron types or neuronal cell lines, such as dopaminergic cells, motoneurons, and primary cortical and hippocampal neurons. Mechanisms include induction of signal transducers and activators of transcription 3 signaling; phosphorylation of extracellular signal–regulated protein kinase-1, -2, and –5; and activation of Akt kinase, the last of which appears to be responsible for $\approx 70\%$ of the efficacy of G-CSF in vitro, as previously demonstrated by inhibition experiments. Finally, although it has been proposed that G-CSF may cause bone marrow–derived cells to invade the brain and provide indirect protection, possibly associated with the release of protective factors, this mechanism is unlikely to be rapid enough for the observed acute and subacute brain protection.

The findings observed in our experiments suggest novel clinical applications for G-CSF. G-CSF is safe in acute ischemic stroke patients, and at least in animal models, there...
is no indication that it might cause intracerebral hemorrhage or increase the risk of systemic bleeding. G-CSF might be considered as an emergency drug that could be given in the preclinical setting to prolong the time window for, and possibly improve outcome after, thrombolysis with t-PA. The safety and efficacy of the combination of G-CSF and t-PA must be explored in additional preclinical experiments and clinical trials. Also, additional doses of G-CSF should be tested in combination with hyperacute MRI to create a clinical trials. Also, additional doses of G-CSF should be explored in additional preclinical experiments and safety and efficacy of the combination of G-CSF and t-PA possibly improve outcome after, thrombolysis with t-PA. The preclinical setting to prolong the time window for, and volume, and differentially affects neuronal cell death pathways after suture middle cerebral artery occlusion in rats. J Cereb Blood Flow Metab. 2007;27:1632–1642.


Granulocyte-Colony Stimulating Factor Delays PWI/DWI Mismatch Evolution and Reduces Final Infarct Volume in Permanent-Suture and Embolic Focal Cerebral Ischemia Models in the Rat
Bernt T. Bråtane, James Bouley, Armin Schneider, Birgul Bastan, Nils Henninger and Marc Fisher

Stroke. 2009;40:3102-3106; originally published online July 30, 2009; doi: 10.1161/STROKEAHA.109.553958

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/40/9/3102

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/