Neuroprotective Effects of Granulocyte Colony-Stimulating Factor on Ischemia-Reperfusion Injury of the Retina

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Key Words
Ischemia reperfusion / Granulocyte colony-stimulating factor / Neuroprotection / Phosphorylated AKT / Antiapoptosis / Inner retinal layer / TUNEL assay

Abstract
Purpose: It has been reported that granulocyte colony-stimulating factor (G-CSF) provides neuroprotection in models in which neuronal cell death is induced. This research was designed to investigate the effects of G-CSF on neurodegeneration of the inner retinal layer in a rat model of ischemic reperfusion (I/R) injury. Materials and Methods: Retinal ischemia was induced by increasing the intraocular pressure to 110 mm Hg for 45 min in the left eyes of the rats. A sham operation was carried out on the right eyes. G-CSF (100 μg/kg/day in 0.3 ml saline) or the same volume of saline was intraperitoneally injected just before the operation and continued for 4 consecutive days (a total of 5 consecutive days). Morphological examinations, including the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, were performed 7 days after I/R induction. The expression of phosphorylated AKT in the retina was examined by Western blot analysis and immunohistochemistry. Results: Cell loss in the ganglion cell layer was more significantly reduced in the I/R-induced eyes of the G-CSF-injected rats than in the I/R-induced eyes of the saline-injected rats (20.3 vs. 6.6%). The inner retinal thickness ratios, such as the inner plexiform layer to the inner limiting membrane/outer nuclear layer and the inner nuclear layer/outer nuclear layer, were significantly better preserved in the I/R-induced eyes of the G-CSF-injected rats than in the I/R-induced eyes of the saline-injected rats. TUNEL assays showed fewer apoptotic cells in the retinal sections of the I/R-induced eyes of the G-CSF-injected rats. The phosphorylation of AKT (p-AKT/AKT) was upregulated in the retinas of the I/R-induced eyes of the G-CSF-injected rats. Conclusion: Our results demonstrated that systemic injection of G-CSF can protect retinal ganglion cells and inner retinal layers from I/R injury. The effects could be associated with the activation of AKT.

Introduction
Granulocyte colony-stimulating factor (G-CSF) has previously been used extensively for treating chemotherapy-induced neutropenia, as well as for reconstituting bone marrow and mobilizing stem cells, and has a favorable safety profile [1]. G-CSF promotes the differentiation, proliferation and survival of neutrophilic lineage cells [2] and mobilizes hemopoietic stem cells to move...
from bone marrow into peripheral blood [3]. Moreover, it has been shown that G-CSF has antiapoptotic effects [4]. It has also been reported that the G-CSF receptor is widely expressed in the central nervous system and the retina [5–7], and that G-CSF is able to cross the blood-brain barrier [5]. Recently, antiapoptotic and neuroprotective effects of G-CSF in the central nervous system were identified using models for cerebral ischemia [8, 9], Parkinson’s disease [10], Alzheimer’s disease [11], spinal cord injury [12], and amyotrophic lateral sclerosis [13]. G-CSF is currently in phase I/II trials for ischemic stroke in humans [14]. In addition, the neuroprotective effects of G-CSF for retinal diseases have been reported using models for light-induced damage [6], optic nerve crush [15, 16] and oxygen-induced retinopathy [17]. Moreover, there have been reports of the functional neuroprotective effects of the retinal ischemia reperfusion (I/R) model by G-CSF [18]. However, the mechanisms by which G-CSF prevents retinal damage have remained unclear.

Pressure-induced I/R injury has been reported to be a model of central retinal artery occlusion and acute angle closure glaucoma because of similarities in the pathological changes that take place in those diseases, such as cell death of the ganglion cells and amacrine cells through both apoptosis and necrosis [23]. Of the endogenous components, glutamate, oxygen-free radicals, nitric oxide and calcium could be related to cell death in ischemic injury [19, 20].

In this study, we investigated whether G-CSF has a neuroprotective effect on pressure-induced I/R injury histologically, and the mechanisms by which G-CSF prevents retinal damage.

Materials and Methods

Animals
Adult male Sprague-Dawley rats (7–8 weeks old), weighing 210–290 g, were purchased from SLC (Shizuoka, Japan) for this experiment. The animals were treated according to the regulations in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The rats had free access to food and water and were maintained in cages in an environmentally controlled room with a temperature of 22 ± 2°C, and a 12-hour light-dark cycle (light period: 8:00 to 20:00).

Induction of Retinal I/R
Retinal I/R was induced as previously described [24], but with slight modifications. Briefly, the rats were anesthetized by isoflu-
phorylated AKT (p-AKT) was performed by standard protocols. The peripheral blood 7 days after induction of G-CSF was analyzed (n = 6 in each group). * p < 0.05.

**Western Blot Analysis of Phosphorylated AKT**
Seven days after I/R induction, Western blot analysis of phosphorylated AKT (p-AKT) was performed by standard protocols [26] with slight modifications. Rat retinas were isolated and sonicated in Laemmli sample buffer (Bio-Rad Laboratories, Calif., USA) and protease inhibitor cocktail (Bio-Rad Laboratories). The lysate was centrifuged at 14,000 rpm for 30 min and the supernatant was prepared. The same amount of samples was prepared using a standard concentration of bovine serum albumin. Protein samples containing 10 μg of protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gels (Oriental Instruments Ltd., Tokyo, Japan) with a size marker (Amersham, UK) and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). The membranes were incubated in blocking buffer (Roche Diagnostics GmbH, Mannheim, Germany) for 60 min to block nonspecific binding. Primary antibodies were added and the preparations were incubated overnight at 4°C. The primary antibodies included anti-rabbit p-AKT (Ser473, rabbit IgG 1:2,000; Cell Signaling Inc., Danvers, Mass., USA), AKT (pan, C67E7, rabbit IgG 1:1,000; Cell Signaling) and β-actin (1:5,000; Abcam, Cambridge, Mass., USA) antibodies. The membranes were washed with washing buffer (Roche Diagnostics) followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:5,000; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA) for 1 h at room temperature. The blot was then washed with washing buffer. Protein bands detected by the antibody were visualized by ECL plus reagent (GE Healthcare, Chalfont St. Giles, UK) on a luminescent image analyzer (RAS 4000mini; Bucher Biotech, Basel, Switzerland). Quantification was performed on a computer using Image J software (National Institutes of Health, Bethesda, Md., USA). To determine the activated AKT, the percentage of activated AKT was defined as p-AKT/total AKT. To confirm equal protein loading, the expression of β-actin was examined in each Western blot.

**Immunohistochemistry of p-AKT in the Retina**
To detect p-AKT protein expression in the retina, p-AKT immunostaining was performed according to the manufacturer’s protocol 7 days after I/R induction. Paraffin-embedded sections as described in Histological Examination were deparaffinized with xylene and dehydrated through a graded ethanol series. They were then boiled for 20 min in citrate buffer (pH 6.0) for antigen retrieval and incubated with 3% hydrogen peroxide solution for 10 min at room temperature to inhibit endogenous peroxidase activity. Primary antibody of p-AKT (1:25; Cell Signaling) was added and incubated at 4°C overnight. The samples were then washed and exposed to Histofine Simple Stain MAX PO (MULTI; Nichirei Bioscience, Tokyo, Japan) secondary antibody for 1 h at room temperature. Finally, they were visualized by the use of DAB. Hematoxylin was used for counterstaining. Positive control sections (Cell Signaling) were stained to confirm the applicability of this system, while the solution for dilution (Cell Signaling) was used as negative control.

**Statistical Analysis**
All measurements in this study were performed in a masked fashion. Mean values with standard deviations (SDs) were obtained and are presented here. Significant differences among the groups were examined using Student’s t test. Statistical significance was declared if the p value was <0.05.

**Results**

**Administration of G-CSF and Peripheral WBC Counts**
First, we examined whether intraperitoneal administration of G-CSF could augment the number of WBCs in the peripheral blood 7 days after I/R induction. When G-CSF was administered, the number of WBCs was significantly increased compared to the saline group. The table below shows the WBC counts for both groups:

<table>
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<tr>
<th>WBC × 10^3/μl</th>
<th>Physiologic saline</th>
<th>G-CSF</th>
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<td>2.6 ± 0.3</td>
<td>6.8 ± 1.2</td>
<td>*</td>
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* p < 0.05
CSF (100 μg/kg) was intraperitoneally injected into Sprague-Dawley rats for 5 consecutive days, the number of WBCs in the peripheral blood of the rats reached $8.58 \pm 1.63 \times 10^3 / \mu L$. On the other hand, when saline was injected instead of G-CSF, the number of WBCs was $4.46 \pm 1.59 \times 10^3 / \mu L$ (fig. 1). The numbers of erythrocytes and platelets did not change significantly (data not shown). These results suggest that G-CSF can specifically augment the number of WBCs in the peripheral blood.

**Histopathological Examination**

Figure 2 shows the changes in cell numbers in the GCL and the IPL-ILM/ONL and INL/ONL ratios after I/R induction. We histologically evaluated neural cell damage using these cell numbers and ratios. The thickness of the different retinal layers was measured in both the superior and inferior retina at a fixed distance (750–1,000 μm) from the edge of the optic disc. Means ± SDs of the data are shown (n = 7 in each group). *p < 0.05.

CSF (100 μg/kg) was intraperitoneally injected into Sprague-Dawley rats for 5 consecutive days. Seven days after I/R injury, the rats were sacrificed and the eyes were examined. Specimens of the sham-operated eyes of saline-injected rats (sham + saline), the I/R-induced eyes of the saline-injected rats (I/R + saline) and the I/R-injected eyes of the G-CSF-injected rats (I/R + G-CSF) were prepared, and RGC cell numbers, IPL-ILM/ONL and INL/ONL ratios were calculated, as described in Materials and Methods. Representative data are shown. ×400. b The number of morphologically identified viable cells in the GCL was counted in triplicate in both the superior and inferior retina at a fixed distance (750–1,000 μm) from the edge of the optic disc. Means ± SDs of the data are shown (n = 7 in each group). *p < 0.05. c The thickness of the different retinal layers was measured in both the superior and inferior retina at a fixed distance (750–1,000 μm) from the edge of the optic disc. Means ± SDs of the data are shown (n = 7 in each group). *p < 0.05.

Fig. 2. Histological examination of I/R injury and inhibitory effect of G-CSF on retinal damage. a G-CSF, or the same volume of saline, was injected into the rats just before I/R induction, and G-CSF or saline was injected into the rats for 5 consecutive days. Seven days after I/R injury, the rats were sacrificed and the eyes were examined. Specimens of the sham-operated eyes of saline-injected rats (sham + saline), the I/R-induced eyes of the saline-injected rats (I/R + saline) and the I/R-injected eyes of the G-CSF-injected rats (I/R + G-CSF) were prepared, and RGC cell numbers, IPL-ILM/ONL and INL/ONL ratios were calculated, as described in Materials and Methods. Representative data are shown. ×400. b The number of morphologically identified viable cells in the GCL was counted in triplicate in both the superior and inferior retina at a fixed distance (750–1,000 μm) from the edge of the optic disc. Means ± SDs of the data are shown (n = 7 in each group). *p < 0.05. c The thickness of the different retinal layers was measured in both the superior and inferior retina at a fixed distance (750–1,000 μm) from the edge of the optic disc. Means ± SDs of the data are shown (n = 7 in each group). *p < 0.05.
Examination of Apoptotic Cells by TUNEL Assay

It has been reported that the time course of cell death is different in the various retinal layers. For example, cell death occurs first in the GCL and last in the ONL. In the inner retinal layers, the number of TUNEL-positive cells reached a peak at 24 h after I/R induction, followed by cell loss at day 7. In the ONL, TUNEL-positive cells reached a peak at 48 h [27, 28]. Therefore, we carried out the TUNEL assay 1 day and 7 days after I/R induction. DNA fragmentation was detected using the TUNEL technique in each layer. No TUNEL-positive cells were detected in retinal tissue sections obtained from the sham-operated eyes of the saline-injected rats. TUNEL-positive cells were detected mainly in the INL at 24 h after reperfusion (fig. 3a). Seven days after I/R induction, TUNEL-positive cells were detected mainly in the ONL (fig. 3b). The mean number of TUNEL-positive cells in the INL-GCL at 24 h after I/R induction was 14 cells/200 μm in the I/R-induced eyes of the saline-injected rats and 3.1 cells/200 μm in the I/R-induced eyes of the G-CSF-injected rats, respectively (p = 0.008). In the ONL, the number of TUNEL-positive cells at 24 h was 3.58 cells/200 μm in the

<table>
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<tr>
<th>24 h after ischemia</th>
<th>7 days after ischemia</th>
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<tr>
<td>Sham + saline</td>
<td>I/R + saline</td>
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<tr>
<td>I/R + G-CSF</td>
<td>I/R + G-CSF</td>
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Fig. 3. G-CSF can reduce the apoptosis of RGC after I/R injury. TUNEL staining of retinal sections at 24 h and 7 days after I/R injury. a In the sham-operated eyes of the saline-injected rats (sham + saline), there were no TUNEL-positive cells. In the I/R-induced eyes of the saline-injected rats (I/R + saline), small numbers of TUNEL-positive cells were detected in the GCL and INL at 24 h after I/R induction. Representative data are shown. b In the eyes, TUNEL-positive cells were detected mainly in the ONL at 7 days after I/R induction. Representative data are shown. c, d Number of TUNEL-positive cells in each retinal layer. TUNEL-positive cells per 200 μm of each retinal layer were counted, as described in Materials and Methods. At least 24 images from 4 eyes were prepared per group. Means ± SDs of the data are shown (n = 4 in each group). * p < 0.05.
I/R-induced eyes of the saline-injected rats and 1.5 cells/200 μm in the I/R-induced eyes of the G-CSF-injected rats, respectively (p = 0.005; fig. 3c). On the other hand, 7 days after I/R induction, the mean number of TUNEL-positive cells in the INL-GCL was 2.17 in the I/R-induced eyes of the saline-injected rats, and 0.17 in the I/R-induced eyes of the G-CSF-injected rats, respectively (p = 0.043). The number of TUNEL-positive cells in the ONL was 11.1 cells/200 μm in the I/R-induced eyes of the saline-injected rats and 3.00 cells/200 μm in the I/R-induced eyes of the G-CSF-injected rats, respectively (p = 0.005; fig. 3d).

Fig. 4. Analysis of p-AKT expression in the rat retina at 7 days after I/R induction. a, b Quantitative data of AKT phosphorylation by Western blot analysis. At 7 days after I/R induction, 10 μg of retinal protein was examined by Western blot analysis, as described in Materials and Methods. Means ± SDs of the data are shown (n = 4 in each group). * p < 0.05. c Immunostaining of paraffin sections of the retina for p-AKT at 7 days after I/R induction. Immunostaining of the eyes at 7 days after I/R induction was carried out as described in Materials and Methods. Representative data are shown from 4 independent experiments. ×400.
These results demonstrated that administration of G-CSF had a significant anti-apoptotic effect on I/R injury.

**Western Blot Analysis of p-AKT**

To examine the effect of retinal I/R induction and the injection of G-CSF on AKT activity, we carried out Western blot analysis of the p-AKT.

Seven days after I/R induction, phosphorylation of AKT increased in the I/R-induced eyes of the G-CSF-injected rats in comparison with the other groups, whereas AKT expression remained unchanged as compared with the other groups (fig. 4a). The p-AKT/AKT ratio was 0.26 in the sham-operated eyes of the saline-injected rats, 0.27 in the I/R-induced eyes of the saline-injected rats, and 0.81 in the I/R-induced eyes of the G-CSF-injected rats (fig. 4b). The expression of p-AKT in the I/R-induced eyes of the G-CSF-injected rats was about 3-fold higher than in the other groups (p = 0.0154).

**Immunohistochemistry of p-AKT in the Retina**

No positive cells in immunostaining for p-AKT were found in any sections without primary antibody. There were few p-AKT-positive cells in the sham-operated eyes of the saline-injected rats. On the other hand, there were some positive cells for p-AKT in the I/R-induced eyes of the saline-injected rats, and the positive cells significantly increased in the I/R-induced eyes of the G-CSF-injected rats (fig. 4c). Most of the p-AKT-positive cells in the I/R-induced eyes of the G-CSF-injected rats existed in the inner part of the retina and, particularly, in the GCL as compared with the I/R-induced eyes of saline-injected rats.

**Discussion**

In this study, we show that retinal ganglion cell (RGC) death caused by high IOP-induced transient ischemia was suppressed by G-CSF administration histologically. Moreover, we found that one of the mechanisms could be the antiapoptosis effect via the activation of AKT. Our results showed that the cell numbers in the GCL and INM-IPL/ONL and INL/ONL ratios of the I/R-induced eyes of the G-CSF-treated rats were greater than those of the I/R-induced eyes of saline-injected rats 7 days after the I/R injury. In the TUNEL assay, the G-CSF injection reduced the number of apoptotic cells. We found that G-CSF significantly augmented the expression of p-AKT in Western blot analysis.

The mechanisms underlying the neuroprotective effects of G-CSF are unclear. However, there are several hypotheses: the first is that there are direct effects, including anti-apoptotic and anti-inflammatory processes, at the site of injury [15]. Anti-inflammatory effects occur via the inhibition of the inducible nitric oxide synthase, the suppression of tumor necrosis factor-α and the reduction in interleukin-1β expression [9, 29]. The second hypothesis is that there are indirect effects, including the stimulation of neurogenesis, recruitment of bone marrow stromal cells [5, 30], and the promotion of angiogenesis [31]. All these might act in synergy to induce the neuroprotective response observed in a variety of different retinal injury models when G-CSF has been administered.

However, it is likely that the neuroprotective effects afforded by G-CSF administration are induced through G-CSF receptors in the direct effects, since previous studies have shown that G-CSF reduced the migration of bone marrow-derived monocytes/macrophages and augmented the intrinsic microglia/macrophages at the ischemic site by using bone marrow chimera mice expressing enhanced green fluorescent protein [9]. Optic nerve crush studies have shown that G-CSF has potent neuroprotective effects for preventing RGC death induced by optic nerve crush when G-CSF was administered systemically or topically. The antiapoptotic effect could be directly mediated via the G-CSF receptor expressed on neural cells [5, 16]. It has also been reported that G-CSF receptor expression is induced upon cerebral ischemia, suggesting that an autocrine protective signaling mechanism is at work in ischemia [5, 32, 33]. Thus, we hypothesized that the direct effect, especially the antiapoptotic effect from G-CSF administration, may be involved in I/R injury. In this study, we have observed that the administration of G-CSF had a significant anti-apoptotic effect in I/R injury.

G-CSF combines with the G-CSF receptor and activates signal pathways such as Jak/Stat, PI3K/AKT and MAPK/Akt [5, 6, 8, 9]. Among these pathways, activation of PI3K/AKT is believed to have the most powerful anti-apoptotic effects upon administration of G-CSF [5]. AKT is one of the components of the PI3K pathway. It has been reported that the AKT pathway is activated after optic nerve clamping [34], intravitreal injection of N-methyl-D-aspartate [35, 36], episcleral vein cauterization [37, 38] and translimbal photocoagulation [39], suggesting that it is a common response of the retina to detrimental stimuli. Thus, we examined the activated AKT to investigate the mechanisms of the antiapoptotic effects by G-CSF administration.

In this study, we have observed that PI3K/AKT was activated not only just after I/R induction but also 7 days...
after I/R induction. These results suggest that the status of AKT activation induced by I/R injury continues long-term and works for neuroprotection. Moreover, we have observed that the activated p-AKT is found mainly in the GCL. These observations suggest that activation of AKT may prevent apoptotic death after I/R injury and, also, that it may explain the selective protection of the inner retina as well. However, it is still unclear how G-CSF affects retinal AKT 3 days after its injection, and whether other mechanisms, including other signal pathways, mediate these effects. These questions need to be addressed in future studies.

Only a few side effects of G-CSF have been reported. Moreover, it has been reported that leukocytosis by G-CSF was restricted to the vessel compartment and that it had no deleterious effects on lesion function and recovery [40]. Therefore, we believe that G-CSF could be used safely for neuroprotection even in human beings. G-CSF is used clinically and has a good safety profile, suggesting that it could rescue RGC from death in ophthalmic disorders such as glaucoma, optic neuropathies and various retinovascular diseases. Therefore, systemic G-CSF administration could be a useful strategy in the treatment prevention of ophthalmic diseases.

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Disclosure Statement

The authors report no conflicts of interest.

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