Involvement of SDF1a and STAT3 in Granulocyte Colony-Stimulating Factor Rescues Optic Ischemia-Induced Retinal Function Loss by Mobilizing Hematopoietic Stem Cells

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PURPOSE. Granulocyte colony-stimulating factor (G-CSF) has been applied clinically for several years. In this study, we used G-CSF to induce the mobilization of hematopoietic progenitor cells into peripheral blood in an ischemia-induced retinal degeneration model.

METHODS. Male Sprague-Dawley rats received G-CSF treatment for 5 days following optic ligation. Histologic and functional evaluations were performed and results were compared with those from untreated rats. Real-time PCR, Western blotting, and immunohistochemical analyses were used to evaluate the expression of retinal cell markers and other substances.

RESULTS. Retinal histology showed that transient optic ligation induced retinal cell loss. Postischemia, animals that received G-CSF treatment had a higher retinal cell survival rate than that of control animals. Analysis of apoptosis showed that retinas from G-CSF–treated animals exhibited fewer apoptotic cells than those from control retinas. Immunoblotting analyses indicated the presence of greater numbers of CD34+, but less chemokine receptor type 4 (CXCR4), and stromal cell-derived factor 1 alpha (SDF1α)-positive cells in the G-CSF–treated ischemic retinas than in ischemic retinas without treatment 14 days after ischemia. The ischemic retinas from G-CSF–treated animals displayed upregulated Thy1 and opsin expression compared with the retinas from untreated animals. Electrotetroretinography indicated superior retinal function in animals treated with G-CSF than in untreated animals postischemia, and that STAT3 might play an important role.


Neurodegenerative diseases of the inner retina are major causes of blindness worldwide. These include ischemic retinopathies, which can occur in various conditions such as acute retinal vascular occlusion or carotid artery disease.1 Retinal occlusion displays similarities to vascular occlusive diseases occurring elsewhere in the body, such as stroke and coronary artery disease, and is second only to diabetic retinopathy as a cause of visual loss from retinal vascular disease. Two types of retinal vascular occlusion can occur: retinal artery occlusion and retinal vein occlusion. Retinal artery occlusion and retinal vein occlusion are similar in pathogenesis but different in clinical nature, with each having unique etiologies, and differential diagnosis, management, and prognosis.2 Retinal artery occlusion is caused by an internal blockage of the arteries that reduces the blood supply to the retina. Retinal vein occlusion occurs when the circulation of a retinal vein becomes obstructed by an overlying blood vessel.2 This reduces blood drainage and can cause hemorrhages in the retina within an area encompassing the inner retinal ganglion cells to the outer photoreceptors. Within time, the blood vessels in the retina might close, leading to further loss of vision and the possible development of new abnormal blood vessels (neovascularization).3–5 Neovascularization can also cause neovascular glaucoma, potentially leading to total blindness.

Because retinal degeneration usually results in irreversible blindness, an effective therapy for retinal degenerative diseases is urgently needed. However, as yet, no reliable treatment exists that can prevent loss of vision or restore vision once lost. One potential therapy is to induce the formation of new retinal cells from stem cells, thus replacing the faulty cells. Several studies have identified that stem cell transplantation reduces neural damage in neurodegenerative diseases such as stroke,5,7 Parkinson’s and Alzheimer’s diseases,8,9 spinal cord injury,10 and retinal degenerative diseases.11–13 However, stem cell transplantation requires surgical intervention and is associated with a higher mortality rate than that of conventional treatments. The identification of more effective methods for therapeutic stem cell use is, therefore, needed. Granulocyte colony-stimulating factor (G-CSF) has been applied clinically for more than 10 years as a treatment for neutropenia and for hematopoietic cell mobilization.14 It accelerates neutrophil recovery following bone marrow transplantation, and stimulates the mobilization of hematopoietic progenitor cells into the peripheral blood.15,16 In addition to bone marrow cells, investigators have used hematopoietic stem cells (HSCs) in transplantation to promote the regeneration of nonhematopoietic tissues such as skeletal muscle and the heart17 and neural18 and hepatic cells.19,20 HSCs are, therefore, regarded as multipotent stem cells and an ideal cell source for stem cell therapy. In this study, we used an animal model of optic ischemia to investigate the potential therapeutic application of G-CSF, a stimulator of HSC release, in retinal ischemic degenerative diseases, with the aim of providing a safe and effective therapy for such diseases.

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METHODS

Male Sprague-Dawley rats, weighing 200 to 250 g, were maintained on a 12/12-hour light cycle in a pathogen-controlled environment with free access to food and water. The animals were housed in the animal care facility at Fujien Catholic University according to standard animal care guidelines. All animal protocols were in accordance with the Association for Research in Vision and Ophthalmology’s “Statement for the Use of Animals in Ophthalmic and Vision Research” and were approved by the Institutional Animal Care and Use Committee of Fujien Catholic University.

Optic Ischemia Animal Model

Methods used for induction of transient optic ischemia were modified from those of Faberowski et al.22 and Rosenbaum et al.22 Animals were anesthetized by administering chloral hydrate intraperitoneally (IP) (400 mg/kg; Sigma, St. Louis, MO), and their pupils were dilated using 1% tropicamide (Alcon, Puurs, Belgium). Following lateral conjunctival peritomy and disinsertion of the lateral rectus muscle, the optic sheath was exposed and then incised using an upward 2-0 nylon suture was then passed around the optic sheath and tightened until the cessation of blood flow in all retinal vessels. The absence of blood perfusion was confirmed by viewing under a dissection microscope. The suture was removed 90 minutes postligation and retinal reperfusion was confirmed using microscopy. Some animals received G-CSF (100 μg/kg in saline; Chugai Pharmaceutical Co., Tokyo, Japan) subcutaneously for 5 days following the ischemic operation. Other animals remained untreated. In addition, some experimental rats were injected with bromodeoxyuridine (BrdU) (50 mg/kg; Sigma) daily for 14 days prior to euthanasia.

Morphologic Analyses

Both eyes of the animals were collected 14 days after the ischemic operation. The specimens were fixed with 4% paraformaldehyde and sectioned into 5-μm sections using a cryostat. Some preparations were stained with hematoxylin and eosin for morphologic observation. The method of estimating the retinal thickness was modified from that reported by LaVail et al.23 and Bok et al.24 Thicknesses of the outer nuclear layer (ONL) and inner nuclear layer (INL) were measured in nine sets of five measurements in each nasal and temporal hemisphere. The first set was made of 250 μm from the optic nerve, and subsequent sets were made of 250 μm and were measured more peripherally. An upright microscope (Leica DM2500; Leica Microsystems) with a digital image camera (Leica DFC4200; Leica Microsystems) was used to examine retinal thickness and morphology.

Bromodeoxyuridine Immunoreactivity

Retinal cryostat sections were brought to room temperature and washed with a phosphate-buffered saline (PBS). A solution containing a 2% saline-sodium citrate buffer (SSC; Sigma) and 50% formamide (Amersham Biosciences, Buckinghamshire, UK) was applied to the specimens prior to incubation for 2 hours at 65°C. After rinsing with the PBS, the specimens were preincubated with a blocking solution and then incubated overnight at 4°C with a mouse monoclonal anti-BrdU antibody (SC-20045; Santa Cruz Biotechnology, Santa Cruz, CA). Following the primary antibody incubation, the sections were visualized using an antibody conjugated with a fluorophore, such as Alexa Fluor 488 or Alexa Fluor 555 (Molecular Probes, Carlsbad, CA), for 30 minutes. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma).

Electroretinogram

Functional analysis of the retina was performed using a scotopometric electroretinogram (ERG). In this study, we used a commercial ERG system (RETIsport; Acrivet, Hennigsdorf, Germany). The ERG recording methods were adapted from those of Bok et al.24 and Bayer et al.20 with minor modifications. Prior to ERG recordings, the animals were adapted to the dark for at least 2 hours and then anesthetized with chloral hydrate IP (400 mg/kg; Sigma). Under dim red light, their pupils were dilated using 1% tropicamide (Alcon, Puurs, Belgium), and a gold-foil electrode was placed on the lateral side of the corneal surface. Following the ischemic operation, the ERG was examined weekly for 5 weeks, and the a-waves and b-waves were recorded as references of retinal function.

Statistical Analysis

Data were analyzed using commercial software (SPSS software, version 18; SPSS Inc., Chicago, IL). One-way ANOVA with Bonferroni or Fisher’s least significant difference (LSD) multiple comparisons was used to determine the significance of differences between group means.
RESULTS

Granulocyte Colony-Stimulating Factor Reduces Retinal Cell Loss Postischemia

In this study, we used a retinal ligation ischemia and reperfusion model to evaluate the effects of G-CSF on retinal ischemia-induced cell death. Adult rats received optic trunk ligation in one eye for 90 minutes prior to G-CSF administration. Figure 1 shows the histologic morphology of retinas 14 days postischemia in animals with or without G-CSF treatment. The retina has several cell layers with ganglion cells (GCs) distributed in the inner layer. Figure 1 shows the histologic morphology of retinas 14 days postischemia in animals with or without G-CSF treatment. The retina has several cell layers with ganglion cells (GCs) distributed in the inner layer. The inner nuclear layer (INL) contains the nuclei of bipolar cells, amacrine cells, and horizontal cells, whereas the outer nuclear layer (ONL) contains the nuclei of photoreceptors. Figure 1 shows the histologic morphology of retinas 14 days postischemia in animals with or without G-CSF treatment. The retina has several cell layers with ganglion cells (GCs) distributed in the inner layer. The inner nuclear layer (INL) contains the nuclei of bipolar cells, amacrine cells, and horizontal cells, whereas the outer nuclear layer (ONL) contains the nuclei of photoreceptors.
horizontal cells, whereas the outer nuclear layer (ONL) contains the nuclei of photoreceptors. Fourteen days postischemia, retinas from ischemic eyes were absent of GCs and displayed reduced INL and ONL compared with the retina from the contralateral nonischemic eye of the same animal (Figs. 1A, 1B, 1E, 1F). Figures 1E and 1F also indicated that G-CSF reversed the decrease of ONL and INL thickness after ischemia.

The observation of an enlarged subretinal space in ischemic eyes indicated possible detachment of the retina postischemia (Fig. 1B). As shown in Figure 1D, 14 days after the ischemic operation, we observed greater numbers of ganglion cells, inner nuclear cells, and photoreceptors in G-CSF–treated ischemic retinas than in untreated ischemic retinas (Fig. 1B). It is possible that G-CSF might prevent ischemia-induced retinal cell loss by reducing apoptosis, or restore retinal cells postischemia by mobilizing hematopoietic stem cells from the bone marrow to sites of injury.

**Granulocyte Colony-Stimulating Factor Increases the Numbers of Dividing Cells in the Ischemic Retina**

Immunostaining for BrdU, a marker of active cell division, identified proliferating cells in the area of ischemia-induced retinal injury. We observed scanty BrdU-labeled cells in the contralateral nonischemic eyes. Fourteen days postischemia, the G-CSF–treated injured eyes displayed marked increases in the numbers of BrdU-labeled cells (Fig. 2D) compared with the injured eyes without G-CSF treatment (Fig. 2B). We observed BrdU-labeled cells in the subretinal space and GC layer, suggesting that these proliferating cells might be transported to sites of injury through blood vessels in the choroid (subretinal region) and the optic artery (inner retinal region). It is possible that the proliferating cells in the injured retinas are HSCs released from the bone marrow and transported through blood vessels to sites of injury, and that G-CSF stimulates the release of these HSCs.

**Analysis of Cell Apoptosis Using TUNEL Methods**

We evaluated cell apoptosis in ischemic retinas using TUNEL analysis in Figure 3. As shown in Figure 3E, normal and G-CSF–only retinas were void of apoptotic cells. There were many apoptotic cells observed in the retinas treated with and without G-CSF 12 hours after ischemia (Figs. 3A, 3B, 3E), and there was no significant difference between these two groups. However, 24 hours postischemia, we observed numerous apoptotic cells in the ONL and subretinal space (Figs. 3C, 3E). We also observed reduced numbers of apoptotic cells in the G-CSF–treated retinas (Figs. 3D, 3E), as indicated by fewer TUNEL-labeled cells in the subretinal space. These findings indicated that G-CSF treatment reduced cell apoptosis caused by retinal ischemia.

**Quantification of Surviving Retinal Cells**

In this study, we used real-time PCR to evaluate cell survival in different conditions by analyzing the expression of the retinal cell markers Thy1 (ganglion cells) and opsin (rod cells). As
shown in Figure 4, expression of Thy1 and opsin was reduced in ischemic eyes, indicating that retinal ischemia induced cell apoptosis. However, G-CSF treatment increased Thy1 and opsin expression. This suggested that G-CSF might prevent cell loss, or regenerate retinal cells, following ischemic retinal degeneration.

Functional Evaluation Using Electroretinogram

We evaluated the visual function of animals using a scotopic ERG by recording the a-wave and b-wave amplitudes of experimental and contralateral eyes (Fig. 5). Results indicated reduced retinal function as early as 7 days postischemia. Both a-wave and b-wave amplitudes were reduced significantly (46% for a-wave and 26% for b-wave) in the ischemic eyes compared with the contralateral control eyes. Administration of G-CSF reduced functional deterioration in the retina (Fig. 5). After 3 weeks, the a-wave amplitudes in the G-CSF-treated ischemic eyes increased significantly compared with those of untreated ischemic eyes. We observed the same effects on b-wave...
amplitudes after 1 week. The contralateral eyes of the control and G-CSF–treated animals displayed nonsignificant differences. These results indicated that G-CSF treatment can preserve, or reduce the loss of, retinal function postischemia.

Immunohistochemical Analysis of Retinal Cells

We used epifluorescent immunohistochemistry to evaluate the expression of specific proteins and cell differentiation in the retina. The CD34 molecule is a marker of HSC. In this study, ischemic retinas displayed CD34 positivity (Figs. 6B, 6C). We observed greater numbers of CD34-positive cells in the ischemic eyes of G-CSF–treated animals (Figs. 6C, 6J, 6K) than in the ischemic eyes of untreated animals (Figs. 6B, 6J, 6K). We noted CD34 positivity in the subretinal space in ischemic eyes (Fig. 6B), and in the outer plexiform layer (OPL) in G-CSF–treated ischemic eyes (Fig. 6C). The retinas of eyes contralateral to the ischemic eyes in G-CSF–treated and untreated animals were absent of CD34-positive cells (Figs. 6A, 6J, 6K). These findings indicated that G-CSF increased the mobilization of hematopoietic progenitor cells to sites of retinal injury postischemia.

Cells from injured areas can release cytokines, such as SDF1α, which attract hematopoietic cells to the site of injury for repair or renewal.27,28 In this study, we observed that the subretinal space in ischemic retinas expressed SDF1α and its receptor CXCR4 (Figs. 6E, 6H), and that G-CSF–treated animals displayed some SDF1α and CXCR4 expression in the same location (Figs. 6E, 6I). The results of immunoblotting are shown in Figures 6J and 6K, showing downregulation of SDF1α and CXCR4 expression in the G-CSF–treated retinas 14 days after ischemia.

Signal transducers and activators of transcription (STAT) are transcription factors that mediate the induction of cytokines and growth factors in various biological responses.29 They regulate apoptotic signals and can also modulate cell repair or protection signals, depending on the stimulus and cell type. To ascertain the possible mechanisms involved in the effects of G-CSF on repair of apoptotic retinal cells, we evaluated retinal STAT expression and phosphorylation. Fourteen days postischemia, the retinas of both eyes of the two groups of animals displayed STAT3 expression (Fig. 7). However, only the ischemic eyes showed phosphorylated STAT3 (pSTAT3) expression (Figs. 7F, 7N), with expression predominantly observed in the subretinal space. The numbers of pSTAT3–positive cells were increased in the ischemic retinas of G-CSF–treated animals compared with the control, and untreated ischemic eyes (Figs. 7Q, 7R). This pattern is similar to the one for CD34–positive cells in Figure 6, which indicated that phosphorylation of STAT3 plays an important role in the mediation of protection of retinal cells against ischemic damage or their repair postischemia caused by G-CSF.

**Discussion**

Cytokines and chemokines secreted by damaged tissues have crucial roles in the mobilization of stem cells to sites of...
FIGURE 6. Immunohistochemical analyses of retinas 14 days postischemia. Red fluorescence indicates CD34-positive cells in (A), (B), and (C); SDF1a-positive cells in (D), (E), and (F); CXCR4-positive cells in (G), (H), and (I); and immunoblotting in (J) and (K). Cell nuclei were stained with DAPI. (B), (E), and (H) retinal sections from an eye receiving retinal ischemic operation; (A), (D), and (G) retinal sections from control eyes; (C), (F), and (I) images of G-CSF–treated eyes following retinal ischemic operation. Occasional CD34-, CXCR4-, and SDF1a-positive cells are observed in the subretinal spaces in ischemic retinas. The subretinal spaces of G-CSF–treated ischemic eyes display increased CD34 expression. CD34-positive cells are shown in the OPL in G-CSF–treated eyes (C). The results of immunoblotting indicate that G-CSF treatment decreases the expression of SDF1a and CXCR4 and induces the migration of hematopoietic progenitor (CD34-positive) cells to sites of injury in ischemic retinas. These hematopoietic progenitor cells might also graft into the inner retina in G-CSF–treated eyes. n = 6 in each group for immunoblotting. Scale bar: 50 μm. One-way ANOVA with the LSD test was used for statistical analysis. *P < 0.05, compared with the control group; #P < 0.05, compared with the ischemic-only group.
FIGURE 7. Immunohistochemical analyses of retinas 14 days postischemia. Red fluorescence indicates pSTAT3 expression and green fluorescence indicates STAT3 expression. Cell nuclei were stained with DAPI. (E–H) Retinal sections from an eye receiving retinal ischemic operation, and (A–D) sections from the contralateral untreated eye of the same animal. (M–P) An ischemic eye and (I–L) the contralateral untreated eye of a G-CSF-treated animal postischemia. (Q, R) The results of immunoblotting for STAT3, pSTAT3, and the percentage of pSTAT3/STAT3 are shown. Normal retinal morphology in control and G-CSF-only eyes, where STAT3 phosphorylation is absent. Occasional pSTAT3-labeled cells are shown in the subretinal space of an ischemic retina. Strong pSTAT3 expression is observed in the ischemic retina of a G-CSF-treated animal. The result of immunoblotting suggests G-CSF increases the phosphorylation of STAT3 in retinas after ischemia. These findings indicated that G-CSF treatment...
reduces retinal cell loss postischemia and that STAT3 plays an important role in this effect. Scale bar: 100 μm. n = 6 in each group for immunoblotting. One-way ANOVA with the LSD test was used for statistical analysis. *P < 0.05, compared with the control group; #P < 0.05, compared with the ischemic-only group.

In this study, we used BrdU-labeling to identify proliferating cells, observing significantly higher numbers of BrdU-labeled cells in the subretinal space and GC layer of G-CSF-treated ischemic retinas compared with untreated ischemic retinas. Stromal cell-derived factor-1 release other trophic factors, thus, contributing to the cells might have induced HSC, or cells near the lesions, to of hematopoietic progenitor/stem cells with ischemic retinal retinal progenitor cells. It is also possible that the interaction and induces their transdifferentiation into specific retinal or poietic progenitor/stem cells to damaged areas of the retina. Adamus et al.44 further identified STAT3 involvement in retinal ganglion cell degeneration and repair. Adamus et al.44 also showed that STAT3 plays an important role in the protection of photoreceptors from apoptotic death using a retinal degenerative model. In our study, G-CSF significantly upregulated pSTAT3 expression in the ischemic retina, suggesting that STAT3 phosphorylation is dependent on G-CSF signaling. The mobilization of HSC to the retina is considered the principal mechanism by which G-CSF ameliorates retinal function following ischemic injury. The binding of GCSF to its receptor induces Jak-1 and Jak-2 activation, and might also promote STAT phosphorylation.59 Phosphorylated STAT might translocate to the nucleus to initiate transcription of its target genes, which exert pleiotrophic effects on several common processes that regulate cell fate.29,45 Our observations indicated that the effects of G-CSF on reducing retinal cell loss and preserving retinal function postischemia are dependent on its direct action on retinal cells through a STAT3 pathway induced by G-CSF receptor activation.

The pathologic features of retinal damage following retinal ischemia indicate a selective pattern. The inner layers of the retinal sheet represent the most vulnerable retinal region. Noninvasive ERG is an important functional assessment method with which to evaluate retinal damage in injury models. In an ERG, the a-wave is derived from the activation of photoreceptors, whereas the b-wave is considered to directly reflect the depolarization of ganglion and bipolar cells, and indirectly reflect the activation of secondary neurons of the inner layer of the retina. Although ischemia affects both ERG a-wave and b-wave amplitudes, several studies have shown reduced ERG b-wave amplitude to be a particularly sensitive index of ischemic injury, as demonstrated in human and experimental models of retinal ischemia.46–47 In our study, ERG a-wave and b-wave amplitudes were markedly decreased in the ischemic eyes 7 days postischemia. When we analyzed these retinas using microscopy on day 14, we observed almost absent GC, inner plexiform layer (IPL), and INL layers and slight reductions in the thickness of the OPL layer. Electrotoretinographic evaluation also showed that G-CSF treatment reduced the magnitudes of the reductions in a-wave and b-wave amplitudes in ischemic-injured eyes, and that the GC, IPL, and INL layers remained largely preserved in the G-CSF–treated group. In this study, G-CSF administration, therefore, exerted neuroprotective effects on the photoreceptors and damaged neurons of the inner retinal layer, and SDF1α and STAT3 played important roles in these effects.

Ischemic retinal diseases, such as central or branch retinal vessel occlusion, ischemic optic neuropathy, and glaucoma, can result in loss of sight and severe retinal cell apoptosis. An effective clinical therapy for these diseases currently remains lacking. Stem cell transplantation could represent a potential therapeutic method. However, cell transplantation procedures are complex multistep processes involving the isolation of stem cells. The complexity of such techniques might, therefore, limit their success rates. The treatment strategies described in our study could eliminate the need for surgical procedures and reduce the risk of unwanted immune
responses. Our results indicated that administration of G-CSF postischemia can mobilize progenitor cells to sites of injury, provide a possible means of generating specific retinal cell types, and also increase the functional recovery of the ischemic retina.

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References


