Platelet-Derived Growth Factor-BB Lessens Light-Induced Rod Photoreceptor Damage in Mice

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PURPOSE. Platelet-derived growth factor (PDGF)-BB is known to have neuroprotective effects against various neurodegenerative disorders. The purpose of this study was to determine whether PDGF-BB could be neuroprotective against light-induced photoreceptor damage in mice.

METHODS. Mice were exposed to 8000-lux luminance for 3 hours to induce phototoxicity. Two hours before light exposure, the experimental mice were injected with PDGF-BB intravitreally, and the control mice were injected with phosphate-buffered saline. The light-exposed PDGF-BB–injected mice and saline-injected mice were evaluated electroretinographically and histologically. The site and expression levels of PDGFR-β and PDGF-BB were determined by immunostaining and Western blotting, respectively. The effect of PDGF-BB on rod and cone photoreceptor damage was also evaluated in vitro in 661W cells, a murine cone photoreceptor cell line, and in primary retinal cell cultures.

RESULTS. An intravitreal injection of PDGF-BB significantly reduced the decrease in the amplitudes of the electroretinograms (ERGs) and the thinning of the outer nuclear layer (ONL) induced by the light exposure. It also reduced the number of TUNEL-positive cells in the ONL. PDGFR-β was expressed in the rod outer segments (OSs) but not the cone OSs. The levels of PDGF-BB and PDGFR-β were decreased after light irradiation. In addition, PDGF-BB had protective effects against light-induced damage to cells of rod photoreceptors but had no effect on the 661W cells in vitro.

CONCLUSIONS. These findings indicate that PDGF-BB reduces the degree of light-induced retinal damage by activating PDGFR-β in rod photoreceptors. These findings suggest that PDGF-BB could play a role in the prevention of degeneration in eyes susceptible to phototoxicity.

Keywords: PDGF-BB, AMD, light damage

Photoreceptor degeneration is associated with visual disability and blindness. It is a principal pathology seen in various retinal degenerative disorders such as nonexudative age-related macular degeneration (AMD). Although the pathogenesis of AMD is still not well understood, intense visible light exposure is considered one of the environmental risk factors for nonexudative AMD. There is much evidence that intense or continuous light exposure can damage the photoreceptor and worsen nonexudative AMD. In the laboratory, remarkable similarities have been found between retinal damage from intense light exposure and anatomic changes in advanced nonexudative AMD. Furthermore, many researchers have reported the efficacy of administration of antioxidants and neurotropic factors on light-induced photoreceptor cell death. However, effective treatments preventing or ameliorating the loss of photoreceptors and the progression of nonexudative AMD have not been determined.

The platelet-derived growth factor (PDGF) family is made up of dimeric proteins that play important roles in the development of neural, glial, and vascular cells. This growth factor family includes PDGF-A, -B, -C, and -D, which form disulfide-linked homo- or heterodimers. There are two types of tyrosine kinase receptors for the PDGFs: PDGFR-α and PDGFR-β. In the eye, PDGFR-α is expressed in Müller cells and astrocytes while PDGFR-β is expressed in vascular endothelial cells, retinal pigment epithelial (RPE) cells, and pericytes. The PDGF-B chain can interact with both PDGFR-α and PDGFR-β, but the PDGF-A chain can bind only to PDGFR-α. The PDGF-C and PDGF-D chains have long N-terminal CUB (complement protein C1r/C1s, Uegf, and Bmpl) domains, which must be cleaved before they can interact with the receptors. Thus, PDGF-B appears to be the only fully active ligand for PDGFR-β in the eye.

There is good evidence that PDGF-BB has neuroprotective effects against various neurodegenerative disorders, for example, N-methyl-D-aspartate–induced hippocampal neuronal cell death and ischemia. In addition, the results of an earlier study showed that PDGF-BB plays an important role in the normal development of retinal vessels and neurons. However, it has not been determined whether PDGF-BB has neuroprotective effects on light-induced damage to photoreceptors. Thus, the purpose of this study was to determine whether PDGF-BB has a protective effect against light-induced photoreceptor damage. To accomplish this, we exposed mice to intense light, and examined the mice functionally and morphologically. PDGF-BB was injected intravitreally 2 hours before light exposure in the experimental mice, and saline was injected in the control mice.
MATERIALS AND METHODS

Animals

All experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. Male adult ddY albino mice (8 weeks old), female ddY pregnant mice, and neonatal mice (Japan SLC Ltd., Hamamatsu, Japan) were housed in an air-conditioned room maintained at 22 ± 2°C under controlled lighting conditions of a 12/12-hour light/dark cycle. The level of ambient illumination in the animal quarters was approximately 250 lux. The mice had free access to a standard diet (CLEA Japan, Inc., Tokyo, Japan) and tap water.

Exposure to Light

Mice were placed in a dark room to adapt to scotopic conditions for 24 hours before the light exposure. The pupils of the mice were then dilated with topical 1% cyclopentolate hydrochloride (Santen Pharmaceuticals Co., Ltd., Osaka, Japan) 30 minutes before light irradiation. Nonanesthetized mice were exposed to 8000 lux of visible light emitted by white fluorescent lamps (FL30W; Toshiba, Tokyo, Japan) for 3 hours in cages (one mouse per cage) whose interiors were painted white to reflect the light. The experiments were started at 9:00 AM and the mice, except for the normal group, were exposed to light at the same time. The temperature during light exposure was maintained at 25 ± 1.5°C. After 3-hour exposure, all mice were placed in a dark room for 24 hours and then returned to the previous lighting conditions.

PDGF-BB and PDGF-AA Injection

Mice were anesthetized with isoflurane (Merck Hoei Ltd., Osaka, Japan), and 2 µL 10 or 100 ng recombinant murine PDGF-BB (Peprotech, Rocky Hill, NJ, USA) or 100 ng recombinant murine PDGF-AA (Peprotech) was injected intravitreally into the left eyes 2 hours before light exposure. Control mice were injected with the same volume of 0.01 M phosphate-buffered saline (PBS) into the left eyes. All solutions were injected into the vitreous with a 10-µL Hamilton glass syringe (701N; Hamilton Co., Reno, NV, USA) fitted with a 34-G nanopass needle (Terumo, Tokyo, Japan). After the intravitreal injection, 5 µL 0.01% levofloxacin ophthalmic solution (Santen Pharmaceuticals Co., Ltd.) was applied topically.

Electroretinographic Analyses

Electroretinograms (ERGs) were recorded 5 days after the light exposure as described in detail previously. 22 Mice were placed in a dark room for 24 hours before the ERG recordings. They were then anesthetized with an intraperitoneal injection of a mixture of ketamine (120 mg/kg; Daiichi-Sankyo, Tokyo, Japan) and xylazine (6 mg/kg; Bayer Health Care, Tokyo, Japan). The pupils were dilated with 5 µL 1% tropicamide and 2.5% phenylephrine (Santen Pharmaceuticals Co., Ltd.). Flash ERGs were recorded (PowerLab/8SP and LabChart software; AD Instruments, New South Wales, Australia) from the left eyes of the mice. The ERGs were recorded with a golden ring corneal electrode (Mayo, Aichi, Japan) and a reference electrode (Nihon Kohden, Tokyo, Japan) on the tongue. A needle was inserted subcutaneously near the tail for the ground electrode. The animals were prepared for the recordings under dim red light, and the mice were kept on a heating pad to maintain a constant body temperature during the ERG recordings. Light flashes of 3.98 cd/s/m² and 0.3-ms duration were delivered through a g anzfeld stimulator (Mayo). The ERGs were elicited by stimuli of −2.92, −1.92, 1.02, 0.02, and 0.98 log cd s/m². The digital band-pass filters were set at 0.3 to 500 Hz to isolate the a- and b-waves. The a-wave amplitude was measured from the baseline to the trough of the a-wave, while the b-wave was measured from the trough of the a-wave to the highest b-wave peak.

Histologic Analyses

The mice were killed by cervical spine dislocation, and the left eyes were enucleated. The eyes were fixed in 4% paraformaldehyde (PFA) for at least 24 hours at 4°C and then embedded in paraffin. Six paraffin-embedded sections (5 µm) were cut through the optic disc of each eye, prepared in the standard manner, and stained with hematoxylin and eosin. The damage induced by the light exposure was evaluated in three sections from each eye. The sections were examined and photographed with a fluorescence microscope (BZ-X710; Keyence, Osaka, Japan), and the thickness of the outer nuclear layer (ONL) was measured at 240-µm intervals from the optic disc to the periphery of photographs with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

TUNEL Staining and Immunostaining

After cervical spine dislocation, the eyes were enucleated, fixed in 4% PFA for at least 24 hours at 4°C, and then immersed in 25% sucrose with 0.01 M PBS for 2 days. The eyes were then embedded in optimal cutting temperature (OCT) compound (Sakura Finetechanical Co., Ltd., Tokyo, Japan). Ten-micrometer sections were cut with a cryostat and immediately frozen with liquid nitrogen and stored at −80°C. These sections were mounted on slides (MAS COAT; Matsunami Glass Ind., Ltd., Osaka, Japan) and prepared for TUNEL staining and immunohistochemistry.

The TUNEL staining was performed according to the manufacturer’s protocol (In Situ Cell Death Detection kit; Roche Biochemicals, Mannheim, Germany) to detect dead photoreceptor cells. For this, sections were rinsed in PBS three times and incubated with 0.1% sodium citrate aqueous containing 0.1% Triton X-100 for 10 minutes. They were then placed in TUNEL reaction mixture, 10% terminal deoxyribo-nucleotidyl transferase (TdT) enzyme solution diluted in fluorescein–dUTP mixture solution, at 37°C for 1 hour. Sections were washed in PBS three times at room temperature. Three sections of each eye were photographed, and the number of TUNEL-positive cells in the ONL was counted. The average of the three images was used as the number of TUNEL-positive cells per eye.

For immunostaining, the retinal sections were blocked in nonimmune goat serum or horse serum (Vector Labs, Burlingame, CA, USA) for 1 hour, and then incubated with the primary antibody at 4°C overnight. For the mouse antibody, M.O.M. immunodetection kits (Vector Labs) were used for blocking and solvent. After incubating overnight, the sections were covered with a secondary antibody for 1 hour and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000; Biotium, Inc., Hayward, CA, USA) for 5 minutes. They were then treated with Trueblack Lipofuscin Autofluorescence Quencher (Biotium, Inc.) and mounted in Fluoromount (Diagnostic BioSystems, Pleasanton, CA, USA).

The following antibodies were used: rabbit anti-PDGF-β (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA); goat anti-OPN51W (1:50; Santa Cruz Biotechnology); mouse anti-rhodopsin (1:1000; Merck Millipore, Billerica, MA, USA); Alexa Fluor 546 donkey anti-rabbit IgG, Alexa Fluor-488 donkey anti-goat IgG, Alexa Fluor 488 goat anti-rabbit IgG, and Alexa Fluor...
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546 goat anti-mouse IgG (1:1000; Invitrogen, Carlsbad, CA, USA).

The sections were examined and photographed with a confocal microscope (FLUOVIEW FV10i; Olympus, Tokyo, Japan) or BZ-X710 (Keyence). For quantitative analyses, images were taken 500 μm superior of the optic disc.

**Western Blotting**

After cervical dislocation, the eyes were enucleated, and the retinas were removed and rapidly frozen in liquid nitrogen. To extract the proteins, the tissue was homogenized in cell lysis buffer with a homogenizer (Microtec Co., Ltd., Chiba, Japan). The lysate was centrifuged at 12,000g for 20 minutes, and the supernatant was used for the Western blotting. The protein concentration was measured by comparison with known concentrations of BSA with a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL, USA). Equal parts of an aliquot of protein and sample buffer with 10% 2-mercaptoethanol were used to separate the proteins by 15% SDS-PAGE. The separated protein was then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Merck Millipore). The membranes were blocked for 30 minutes at room temperature with 5% Block One-P (Nacalai Tesque, Inc., Kyoto, Japan) in 10 mM Tris-buffered saline with 0.05% Tween 20, and then incubated overnight at 4°C with the primary antibody. The primary antibodies used were rabbit anti-PDGFR-β (1:200; Santa Cruz Biotechnology), rabbit anti-phospho PDGFR-β (1:200; Abcam, Cambridge, MA, USA), rabbit anti-PDGF-BB (1:400; Abcam), rabbit anti-Akt (1:1000; Cell Signaling Technology, Inc.), rabbit anti-phospho Akt (Ser473, 1:1000; Cell Signaling Technology, Inc.), and mouse anti-β-actin (1:5000; Sigma-Aldrich Corp., St. Louis, MO, USA). The secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1000; Pierce Biotechnology, Rockford, IL, USA) and HRP-conjugated goat anti-mouse IgG (1:2000; Pierce Biotechnology) used for 1 hour at room temperature. The immunoreactive bands were made visible with Immunostar LD (Wako Pure Chemical, Osaka, Japan) and then processed with the LAS-400 Mini digital imaging system (Fuji Film Co., Ltd., Tokyo, Japan).

**In Vitro Studies**

661W cells, a murine cone photoreceptor cell line derived from murine retinal tumors, were a kind gift from Muayyad R. Al-Ubaidi (Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA). The 661W cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Corp.) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Meiji Seika Kaisha Ltd., Tokyo, Japan). The cells were then suspended in neurobasal medium containing L-glutamine, B27 supplement (Invitrogen), and antibiotics. Cells were plated onto poly-D-lysine/laminin-coated glass chamber slides at 1.5 × 10^5 cells/mL. After incubation for 24 hours, the medium was changed to neurobasal medium containing L-glutamine, B27 minus antioxidants (Invitrogen), and antibiotics. After the cells were isolated, the medium was changed. The cells were used for immunostaining after 2 days.

**Light Irradiation–Induced Cell Death Assay In Vitro**

To examine the effects of PDGF-BB on light-induced cell death, the 661W cells were seeded at 3 × 10^5 cells/well in 96-well plates, or the primary retinal cells were plated at 7.5 × 10^5 cells/well into poly-D-lysine/laminin-coated glass chamber slides. They were incubated for 24 hours and then the medium was changed. After 1-hour incubation, PDGF-BB, dissolved in PBS, was added to each well, and the cells were then exposed to 2500 lux of white fluorescent light (Nikon, Tokyo, Japan) for 24 hours under 5% CO_2 at 37°C.

Nuclear staining assays were performed with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) and propidium iodide (PI) (Molecular Probes). They were added to the culture medium for 15 minutes at final concentrations of 8.1 and 1.5 μM, respectively. Hoechst 33342 stains the nuclei of viable and apoptotic or necrotic cells. PI is a membrane-impermeable dye that is generally excluded from viable cells. Fifteen minutes after adding the stains, the wells were photographed with an Olympus IX70 inverted epifluorescence microscope. The total number of cells was counted and the percentage of PI-positive cells calculated.

**In Vitro Immunostaining**

The primary retinal cultures were fixed with 4% PFA at room temperature for 15 minutes. The cells were incubated with 0.2% Triton X-100 in PBS for 10 minutes and 50 mM glycine (Wako Pure Chemical) in PBS for 15 minutes. The cells were blocked with 10% goat serum (Vector Labs) for 30 minutes and then incubated with the primary antibody overnight at 4°C. The cells were then incubated for 1 hour with secondary antibody and counterstained with Hoechst 33342 (Molecular Probes). The antibodies used were mouse anti-rhodopsin (1:1000; Merck Millipore) and Alexa Fluor 546 goat anti-mouse IgG (1:1000; Invitrogen). Finally, the cells were mounted in Fluoromount (Diagnostic BioSystems) and examined and photographed with a confocal microscope (FLUOVIEW FV10i). The total number of immunoreactive cells was counted within the entire area of the image (211.968 × 211.968 μm). The number was calculated as number/mm.

**Statistical Analyses**

The data are presented as the mean ± standard error of the mean (SEM). Statistical comparisons for the in vitro and in vivo experiments were done with Student’s t-tests or Dunnett’s test with SPSS Statistics (IBM, Armonk, NY, USA) software. A P < 0.05 was taken to be statistically significant.

**RESULTS**

**Protective Effect of PDGF-BB Against Light-Induced Retinal Damage**

To evaluate the effects of PDGF-BB pretreatment against light-induced damage of retinal function, ERGs were recorded from...
FIGURE 1. Electroretinograms and photomicrographs of retina obtained after an intravitreal injection of PDGF-BB on light-induced damage to the photoreceptors of mice. (A) Representative dark-adapted ERG responses of the a- and b-wave recorded 5 days after light exposure. (B) Mean amplitudes of a- and b-waves of the ERGs of mice (normal, $n=10$; vehicle treated, $n=7$; 10 ng/eye PDGF-BB, $n=8$ and 100 ng/eye PDGF-BB, $n=9$). (C) The mean thickness of the outer nuclear layer (ONL) of the retina of mice after light exposure. Representative photomicrographs of hematoxylin- and eosin-stained retinal sections (normal, $n=9$; vehicle treated, $n=7$; 10 ng/eye PDGF-BB, $n=9$ and 100 ng/eye PDGF-BB, $n=9$). Scale bar: 50 μm. (D) Thickness of ONL 5 days after light exposure. (E) Representative photomicrographs of TUNEL-stained retinal sections at 48 hours after light exposure and without light exposure (normal, $n=8$; vehicle treated, $n=7$; and 100 ng/eye PDGF-BB, $n=6$). Scale bar: 50 μm. (F) Quantitative analysis of the number of TUNEL-positive cells in the ONL. Data are the mean ± standard error of the mean (SEM). *$P < 0.05$ versus normal group (Student’s $t$-tests), **$P < 0.01$ versus normal group (Student’s $t$-tests).
10 or 100 ng PDGF-BB–injected eyes, the vehicle-injected eyes, and normal eyes at 5 days after light exposure. The amplitudes of both the a- and b-waves were significantly reduced at 5 days after light exposure compared with the normal eyes. However, the decrease in the a- and b-wave amplitudes was significantly less in the PDGF-BB–treated group than in the vehicle-treated group in a concentration-dependent manner (Figs. 1A, 1B).

Histologically, the thickness of the ONL was markedly reduced in the vehicle-treated group, and prior injection with PDGF-BB suppressed the decrease in the thickness of the ONL compared with the vehicle-treated group (Figs. 1C, 1D).

In the same conditions, the effect of PDGF-AA was also evaluated. One hundred nanograms recombinant PDGF-AA was administrated intravitreally 2 hours before light exposure, which concentration was the same as for the effective dose of PDGF-BB. Both functionally and histologically, pretreatment with PDGF-AA did not show protective effect on light-induced retinal damage (Supplementary Fig. S1).

TUNEL staining at 48 hours after light exposure showed that there were only a few TUNEL-positive cells in normal mouse retinas. In contrast, the number of TUNEL-positive cells markedly increased in the ONL of the light-treated group at 48 hours after light exposure. A prior injection of PDGF-BB significantly suppressed the increase in the number of TUNEL-positive cells (Figs. 1E, 1F).

Location of PDGFR-β Expression in Murine Retina

To determine the location of PDGFR-β in the retina, we performed immunostaining with anti-PDGFR-β antibody in normal mouse retinas. Our results showed that PDGFR-β was expressed in the outer segments (OSs) of the photoreceptors (Figs. 2A, 2B). We examined whether the PDGFR-β was expressed in the rod and cone OSs by double staining with PDGFR-β and rhodopsin or s-opsin, which are specific markers for rod photoreceptors or cone photoreceptors, respectively (Fig. 2A). Our results showed that PDGFR-β–positive cells were colocalized with rhodopsin-positive cells, but not colocalized with s-opsin–positive cells (Fig. 2B).

Expression Levels of PDGF-BB and PDGFR-β After Light Exposure

To assess the level of expression of PDGF-BB and PDGFR-β in the retina after light exposure, the quantity of these proteins was determined by Western blotting. The levels of both PDGF-BB and PDGFR-β in the retina were reduced after light exposure in a time-dependent manner. In particular, the concentrations of these proteins were significantly reduced at 48 hours after light exposure (Figs. 3A, 3B). The fluorescent intensity of PDGFR-β in the OSs was significantly decreased at 48 hours after light exposure (Figs. 3C, 3D).

Effects of PDGF-BB Against Light-Induced Cone and Rod Photoreceptor Damage

To evaluate the protective effect of PDGF-BB against light-induced photoreceptor damage, PDGF-BB was added to the 661W cell media and the cells were then exposed to 2500 lux visible light for 24 hours. Representative images of Hoechst 33342- and PI-stained samples are shown in Figure 4A. Exposure to the light led to a significant increase in the death of 661W cells, and PDGF-BB treatment did not suppress the light-induced cell death (Fig. 4B).

To confirm the specific effect of PDGF-BB on rod photoreceptors, we studied primary retinal cell cultures as described in detail previously. Twenty-four hours after seeding the primary cells, PDGF-BB was added to the media. The cells were then irradiated with intense light in the same manner as 661W cells. The number of rhodopsin-positive cells was decreased after the irradiation by intense light, and the reduction was significantly suppressed by prior exposure to PDGF-BB (Figs. 4C, 4D).
Activation of PDGFR-β After Intravitreal Injection of PDGF-BB

PDGF-BB binds to and activates PDGFR-β. To demonstrate whether the protective effect of PDGF-BB on light-induced retinal damage is actually mediated by PDGFR-β signaling, phosphorylation of PDGFR-β in the retina was determined by Western blotting. The level of phosphorylation of PDGFR-β was significantly increased 30 minutes after intravitreal injection of PDGF-BB (Fig. 5A). In a previous report, it was shown that PDGF-BB protects neurons from oxidative stress through the activation of PI3K/Akt and MAPK pathway. Therefore, we also determined the phosphorylation of Akt in the retina. Phosphorylated Akt was significantly upregulated 30 minutes after intravitreal injection of PDGF-BB, and the increased level was still maintained at 2 hours after injection (Figs. 5B, 5C).
DISCUSSION

The results showed that an injection of PDGF-BB prior to exposure to light lessened the light-induced decrease in the amplitude of the ERGs and also the degree of thinning of the ONL in vivo. We also found that PDGFR-β was expressed on rod but not cone OSs. The expression levels of PDGF-BB and PDGFR-β were decreased after intense light irradiation, and PDGF-BB had protective effects against the light-induced death of primary rod photoreceptors but not in 661W cells. Furthermore, the levels of phosphorylation of PDGFR-β and Akt in the retina were significantly increased after PDGF-BB injection.

The results of earlier studies showed that an intravitreal injection of some growth factors and neurotrophins, such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and acidic fibroblast growth factor (aFGF), protected photoreceptors from light-induced damage. These proteins activate the corresponding tyrosine kinase receptors and then have neuroprotective effects against...
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FIGURE 5. Level of PDGFR-β tyrosine phosphorylation and its downstream signaling activation after intravitreal injection of recombinant PDGF-BB. (A) Phospho PDGFR-β expression level in the retina at 30 minutes after intravitreal injection of recombinant PDGF-BB. Phospho PDGFR-β was significantly increased at 30 minutes after intravitreal injection. (B) Phospho Akt expression level in the retina. Phospho Akt was significantly increased at 30 minutes and 2 hours after intravitreal injection. Data are the mean ± SEM (n = 5). **P < 0.01 versus control (Student’s t-tests).

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