Retinal Astrocytes and GABAergic Wide-Field Amacrine Cells Express PDGFRα: Connection to Retinal Ganglion Cell Neuroprotection by PDGF-AA

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PURPOSE. Our previous experiments demonstrated that intravitreal injection of platelet-derived growth factor-AA (PDGF-AA) provides retinal ganglion cell (RGC) neuroprotection in a rodent model of glaucoma. Here we used PDGFRα-enhanced green fluorescent protein (EGFP) mice to identify retinal cells that may be essential for RGC protection by PDGF-AA.

METHODS. PDGFRα-EGFP mice expressing nuclear-targeted EGFP under the control of the PDGFRα promoter were used. Localization of PDGFRα in the neural retina was investigated by confocal imaging of EGFP fluorescence and immunofluorescent labeling with a panel of antibodies recognizing different retinal cell types. Primary cultures of mouse RGCs were produced by immunopanning. Neurobiotin injection of amacrine cells in a flat-mounted retina was used for the identification of EGFP-positive amacrine cells in the inner nuclear layer.

RESULTS. In the mouse neural retina, PDGFRα was preferentially localized in the ganglion cell and inner nuclear layers. Immunostaining of the retina demonstrated that astrocytes in the ganglion cell layer and a subpopulation of amacrine cells in the inner nuclear layer express PDGFRα, whereas RGCs (in vivo or in vitro) did not. PDGFRα-positive amacrine cells are likely to be Type 45 gamma-aminobutyric acidergic (GABAergic) wide-field amacrine cells.

CONCLUSIONS. These data indicate that the neuroprotective effect of PDGF-AA in a rodent model of glaucoma could be mediated by astrocytes and/or a subpopulation of amacrine cells. We suggest that after intravitreal injection of PDGF-AA, these cells secrete factors protecting RGCs.

Keywords: PDGF-AA, glaucoma, PDGFRα, neuroprotection, retina, retinal ganglion cells, astrocytes, amacrine cells

There are several risk factors associated with glaucoma, including elevated intraocular pressure (IOP), age, family history, ethnic origin, and certain medical conditions. The only clinically proven treatment of glaucoma is a pharmacologic or surgical reduction of IOP.1,2 However, IOP reduction does not always provide sufficient therapeutic effects, and there are multiple efforts to develop new neuroprotective therapies for glaucoma.3–5 Delivery of neurotrophic factors and inhibitors of apoptosis by intravitreal injection6–9 or viral vectors encoding different proteins,10–12 intravitreal injection of various types of stem cells,11–14 suppression of oxidative stress, and modulation of immune response have all been investigated.15

Intravitreal injection of mesenchymal stem cells (MSCs) isolated from bone marrow or umbilical cord blood has been tested by several laboratories.13,14,16,17 Such injections provided different degrees of retinal ganglion cell (RGC) neuroprotection in ocular hypertensive rodent glaucoma models14 as well as in an acute optic nerve damage model such as optic nerve crush (ONC).15,17,18 Neuroprotective effects of MSC injections were observed even without a direct contact of MSCs with the retina, implying that MSCs may secrete neuroprotective factors. Several such factors have been identified, and two of these factors, platelet-derived growth factor-AA (PDGF-AA) and PDGF-AB, were studied in greater detail using retinal explant cultures and an ocular hypertensive rat glaucoma model.19,20 In a rat glaucoma model, intravitreal injection of recombinant PDGF-AA or PDGF-AB efficiently blocked IOP-induced RGC death. However, the molecular mechanisms of the PDGF-mediated RGC neuroprotection remained elusive.

PDGF-AA and PDGF-AB are the ligands for PDGF receptors, which are members of the receptor tyrosine kinase family.21–22 In vertebrates, there are four PDGF genes encoding PDGF-A, PDGF-B, PDGF-C, and PDGF-D and two genes encoding PDGF receptors, PDGFRα and PDGFRβ.23 PDGF-A and PDGF-B form homo- or heterodimers. PDGF-AA is a specific ligand for PDGFRα, while PDGF-AB can interact with both PDGFRα and PDGFRβ.22 PDGF-AA/PDGFRα signaling affects a number of critical cellular functions including cell survival, proliferation, and differentiation.23

By using conventional and conditional PDGFRα knockout mice, the functions of PDGFRα in different tissues have been examined.24 Mice with a null mutation in PDGFRα die between embryonic day 8 (E8) and E16, displaying a variety of organ defects.25 The expression pattern of PDGFRα was investigated by in situ hybridization26 and immunostaining with corresponding antibodies.27–30 Information about the pattern of
PDGF-AA and Retinal Ganglion Cell Neuroprotection

PDGFRRz expression in the eye and especially in the retina is somewhat controversial, mainly due to the quality of PDGFRRz antibodies used. The elucidation of the PDGFRRz pattern of expression in the retina is critical for understanding the molecular mechanisms involved in RGC neuroprotection by PDGF-AA. Mice have been generated in which the histone H2B-enhance green fluorescent protein (EGFP) fusion protein reporter construct was knocked into the PDGFRRz locus (αGFPrz). Although EGFP expression in the retina has not been analyzed in heterozygous αGFPrz/+ mice, EGFP expression faithfully reproduced the PDGFRRz expression pattern in several analyzed tissues.4

In this report, we investigated the pattern of PDGFRRz expression in the retina using αGFPrz/+ mice and wild-type (WT) mice. We identified cells expressing PDGFRRz in the ganglion cell layer (GCL) as astrocytes, and in the inner nuclear layer (INL) as a subpopulation of amacrine cells. These data suggest an indirect mechanism of RGC neuroprotection by PDGF-AA in a rodent model of glaucoma.

METHODS

Animals

Mice were maintained in accordance with guidelines set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, using protocols approved by the National Eye Institute Committee on the Use and Care of Animals. PDGFRRz-EGFP mice were purchased from The Jackson Laboratory (B6.129S4-PDGFRRztm11(EGFP)Sor/J, Stock #007669; Bar Harbor, ME, USA).

RGC Primary Cultures

Purification of RGCs was performed as described previously.31,32 Briefly, retinas were isolated from postnatal 1- to 10-day-old mice and dissociated with papain. Microglia cells were immunodepleted with anti-CD11b–conjugated Dynabeads (Life Technologies, Carlsbad, CA, USA). The suspension of retinal cells was immunopanned on plates preconjugated with anti-Thy1.2 antibody (Serotec, clone F7D5; Raleigh, NC, USA) and goat anti-mouse IgM (Jackson ImmunoResearch, West Grove, PA, USA) at room temperature. After extensive washing, RGCs were released from the plate by 0.025% trypsin, counted, and seeded at a density of 10,000 per well in 96-well plates or 50,000 cells per well in 24-well plates in the media composed of Neurobasal (Life Technologies), B27, N2 supplement, L-glutamine, forskolin, and penicillin/streptomycin. PDGF-AA (50 ng/mL), BDNF (50 ng/mL), and ciliary neurotrophic factor (CNTF) (50 ng/mL) or PDGF-AA, BDNF, and CNTF together were added to cultures where indicated. These concentrations of added proteins were selected following our previous studies.31,32 Cells were cultured in a CO2 incubator at 37°C for 1 to 5 days.

RGC Viability Assay

RGC viability in culture was evaluated using a CellTiter-Glo assay kit (Promega, Madison, WI, USA). Briefly, primary RGCs were seeded onto a 96-well cell culture plate at a density of 10,000 cells/well. One to five days after seeding, cells were lysed with 50 μL 1× passive lysis buffer (Promega), and the luminescence was measured using a plate reader (1420 Multilabel Counter; Perkin Elmer, Shelton, CT, USA).

Western Blotting

Western blot analyses were performed as described previously.33 Briefly, isolated tissues or cells were homogenized in a lysis buffer (10 mM Tris-HCL, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 10% glycerol, and protease inhibitor cocktail) by repeated pipetting and then incubated for 20 minutes on ice. Following centrifugation, the soluble fraction was collected; 5 to 15 μg extracted proteins were separated on a 4% to 12% SDS-PAGE gel (Invitrogen, Carlsbad, CA, USA) and transferred to a polyvinylidene fluoride (PVDF) membrane (Invitrogen). Membranes were blocked with 5% Western Blocking Reagent/TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5) for 60 minutes and then incubated with indicated antibodies followed by incubation with anti-mouse or -rabbit IgG antibody conjugated to horseradish peroxidase (HRP) (1:10,000 dilution; GE Healthcare, Pittsburgh, PA, USA). The HRP signals were developed using a chemiluminescence detection kit (SuperSignal Femto Dura Extended Duration Substrate; Pierce, Rockford, IL, USA) and detected using FluorChem M (Protein Simple, San Jose, CA, USA).

Figure 1. Expression pattern of αGFPrz in a mouse eye. (A, A’) Transverse sections of 1-month-old mouse eye. (A’) includes DAPI staining. (B–D, B’–D’) Sections through the cornea, eye drainage structures (iris, trabecular meshwork and ciliary body), and retina. (B’–D’) display images of the same areas shown in (B–D) obtained using differential interference contrast (DIC) microscopy for better visualization of the tissues. These include DAPI staining. RPE, retinal pigmented epithelium. (E–E”) Sections through the optic nerve. The wild-type optic nerve (E”) is shown for comparison. Scale bars: 500 μm (A, A’); 50 μm (B’–E”).
Amacrine Cell Injection and Immunocytochemistry

Patch electrodes were pulled on a P-97 Micropipette Puller (Sutter, Novato, CA, USA) with a resistance of 12 to 18 MΩ. GFP-positive amacrine cells in the inner plexiform layer were targeted for recording and neurobiotin injection. The electrodes with solution contained 118 mM K-D-glucionate, 12 mM KCl, 0.5 mM MgCl₂, 10 mM HEPES, 0.5 mM K-EGTA, 3 mM ATP, 1 mM GTP, and 1 mM neurobiotin (Vector Laboratories, Burlingame, CA, USA). pH of the media was adjusted to 7.4 with KOH. Slices were mounted on a Zeiss Examiner 1D microscope (Thornwood, NY, USA). They were superfused continuously with Ames' solution and bubbled continuously with 95%O₂/5%CO₂ at room temperature. Retina explants flat-mounted onto filter paper were sectioned into 220-μm-thick sections. Neurobiotin diffusion into single EGFP-positive amacrine cells was achieved by using the ZAP function in Axopatch 200B amplifiers (Molecular Devices, Sunnyvale, CA, USA). The electrode was left on the cell for approximately 5 minutes.

Retinal slices containing neurobiotin-injected amacrine cells were fixed for 20 minutes in 4% paraformaldehyde (PFA). The tissues were washed with standard solution (0.1 M sodium phosphate buffer plus 0.5% Triton X-100 and 0.1% NaN₃, pH 7.4) at room temperature, blocked overnight with standard solution containing 4% normal donkey serum at 4°C. The tissues were then incubated with anti-Choline Acetyltransferase antibody (ChAT, 1:50 dilution; Millipore, Bedford, MA, USA) in the standard solution containing 1% donkey serum for 2 hours, followed by Cy5-conjugated donkey anti-goat (1:1000 dilution; Jackson ImmunoResearch). Neurobiotin was visualized with Alexa Fluor Cy3-conjugated streptavidin (1:1000 dilution; Molecular Probes, Eugene, OR, USA). Images were acquired with a Zeiss LSM-510 confocal microscope and contrast enhanced equally with Photoshop CS6 (Adobe Systems, San Jose, CA, USA).

**FIGURE 2.** Expression pattern of a²GFP in the retina. (A–A’) Whole-mount retina from 1-month-old a²GFP/⁺ mouse. Two boxed areas in (A) are shown at higher magnification at the right (A’–A’’). (B, C) Relative intensity of EGFP fluorescence in the GCL and INL. Right images in (B) were pseudocolored for better demonstration of differences in the intensity of fluorescence in the GCL and INL. Four fields were counted for each layer. Scale bars: 500 μm (A–A’'); 50 μm (B).
Immunofluorescent Microscopy

Cells were washed with cold phosphate-buffered saline (PBS) and then fixed with 2% to 4% paraformaldehyde in PBS for 15 minutes at room temperature. After washing with PBS, cells were permeabilized with 0.25% Triton X-100 in PBS for 5 minutes at room temperature. Cells were incubated with a blocking solution (5% normal donkey serum/TBST) for 60 minutes at room temperature and then incubated with primary antibodies overnight at 4°C. After washing with PBS + Triton X-100 (PBST), cells were incubated with fluorescently labeled secondary antibodies according to the host specificity of primary antibodies and 4',6-diamidino-2-phenylindole (DAPI) at room temperature for 1 hour. Specimens were washed with PBST and mounted in a slow-fade diamond reagent (Invitrogen). For whole-mount staining, retinas were dissected and fixed in 4% PFA for 20 minutes. Triton X-100 (0.4%) containing PBS was used for permeating the retina. Nonspecific binding sites in the tissue were blocked with 4% normal serum in PBST from the same host species as the labeled secondary antibody, and primary antibodies with appropriate dilution in the serum-containing PBST buffer were added. Whole-mount retinal tissues were incubated for 4 to 7 days and then washed with PBST at least five times for 20 minutes each time. Fluorescent secondary antibodies were diluted in PBST and added to retinal whole-mount tissues overnight. Then the tissues were washed in PBST and mounted. Samples were analyzed using a Zeiss LSM 780 confocal microscope and ZEN software. For whole-mount retinas, images were acquired from the four fields in the superior, inferior, temporal, and nasal quadrants.

For the quantification of RGCs, specimens were immunostained with antibodies against RNA binding protein with multiple splicing (RBPMS) (PhosphoSolutions, Aurora, CO, USA) or Brn3a (Millipore). For the quantification of the percentage of GFAP-positive/EGFP-positive cells in each layer, whole-mount retinal image stacks were obtained from three PDGFRα-EGFP mice. Images corresponding to GCL and INL were then separated and merged into one single image or separate images for GCL or INL (see Supplementary Figs. S1A, S1B). EGFP-positive cells in GCL and INL were counted first and then GFAP-positive cells were counted in the population of EGFP-positive cells to calculate the percentage of GFAP+/EGFP+ cells.

Open-Source Software and Statistical Analysis

All the imaging data were analyzed by ImageJ. Statistical analysis was performed using the R statistical computing language and its package tidyverse. Statistical comparisons of paired data were performed using Student’s t-test. Multigroup data were analyzed using a 1-way analysis of variance (ANOVA) with subsequent post hoc pairwise t-test with Bonferroni’s adjustment. Data are shown as mean ± standard error of the mean (SEM). P values < 0.01 were considered to be statistically significant.

RESULTS

**PDGFRα Expression in the Mouse Eye**

To investigate the expression of PDGFRα in the mouse retina we used αGFP+ mice. One of the advantages of this line is a nuclear localization of EGFP that allowed easy distinction between

![Figure 3. Colocalization of EGFP fluorescence and immunostaining with PDGFRα antibodies in the mouse retina.](image)
EGFP-positive cells and surrounding EGFP-negative cells. Although EGFP expression in the retina has not been investigated for this line, published results demonstrated that EGFP expression reproduced the expression pattern of PDGFRα in all tested tissues.24 In the adult mouse eye, EGFP-positive cells were observed in lens epithelial cells and differentiating lens fibers, corneal stroma, iris, drainage structures, retina, optic nerve, retinal pigmented epithelium, and sclera (Fig. 1).

Expression pattern of PDGFRα in the retina was investigated in more detail. Analysis of the whole-mount retina demonstrated that the highest density of EGFP-positive cells was observed in the optic nerve head with a relatively even distribution through the rest of the retina (Figs. 2A–A’). EGFP-positive cells were located mainly in the GCL and INL with fluorescence intensity two times higher for the cells located in the GCL compared to the INL (Figs. 2B, 2C).

To confirm that EGFP expression in the retina, similar to other analyzed tissues,24 reproduced the expression pattern of PDGFRα, we tested several available PDGFRα antibodies and selected antibodies from Cell Signaling (Beverly, MA, USA) that were successfully used in previous publications dealing with mouse tissues.29–30 This antibody produced a strong staining in some cells in both GCL and INL, and these cells were EGFP positive. A weaker punctuated staining was also observed in some cells, and these cells were EGFP negative (Fig. 3). The pattern of weak punctuated staining in GCL did not reflect a typical distribution of RGCs.

### Identification of Retinal Cells Expressing PDGFRα

Several antibodies that recognize RGCs (Brn3a and RBPMS) or both RGCs and amacrine cells (Pax6 and NeuN) have been used to identify EGFP-positive cells in the GCL. Staining of transverse retinal sections demonstrated that EGFP-positive cells were not costained with any of these markers and that these cells were located outside of the collagen 4-positive blood vessels (Fig. 4A). Staining of the whole-mount retina confirmed that EGFP and Brn3a signals do not colocalize. Instead, 98 ± 1% of EGFP-positive cells in the GCL and 1.2 ± 1.2% of cells in the INL were costained with antibodies against GFAP, a known glial marker, as judged by counting of three independent samples (Fig. 4B, Supplementary Fig. S1). These data suggested that PDGFRα-expressing cells in the GCL are astrocytes.

Several markers have been used to identify EGFP-positive cells in the INL. Transverse retinal sections were stained with antibodies against Pax6 and Syntaxin 1, markers that stain most of the identified subtypes of amacrine cells.37–40 Good colocalization of EGFP fluorescence and immunostaining has been observed for both markers (Fig. 5A). At the same time, no colocalization of EGFP fluorescence and immunostaining with antibodies against microglia/macrophage-specific protein Iba1 was observed (Fig. 5A). These data suggested that EGFP-positive cells in the INL represent one of the amacrine cell types. EGFP-positive cells in the INL were mainly observed in the sublayer of INL close to the GCL. This localization of EGFP-positive cells in the INL suggested that they do not belong to the non-gamma-Aminobutyric acidergic (non-GABAergic) non-glycinergic (nGnG) type of amacrine cell, since the nuclei of the nGnG type of amacrine cells are localized at the outermost edge of the amacrine cell zone, proximal to the region of bipolar cell nuclei.41 The absence of EGFP fluorescence and immunostaining with antibodies against Sox2 and GLYT1, the known markers of cholinergic and glycinergic amacrine cells, respectively, suggested that EGFP-positive amacrine cells belong to one of the GABAergic types of amacrine cells (Figs. 5A, 5B). Staining of the whole-mount retina with GAD67 antibodies demonstrated that only a fraction of GABAergic types of amacrine cells express PDGFRα (Fig. 5C).

In mouse retina, GABAergic neurons compose approximately 40% of amacrine cells.42 To identify one or more subtypes of GABAergic amacrine cells expressing EGFP, we performed single-cell dye injections. We targeted EGFP-positive soma in the inner plexiform layer (IPL) for neurobiotin injection in freshly cut retinal slices or whole-mount pieces (n = 5, Fig. 6). We then fixed the tissues and processed them to visualize the morphology of the amacrine cells. The stratification of starburst amacrine cells (ChAT immunolabeling) has been used as a reference marker to localize the ramifications of the dendrites from the GFP-positive amacrine cells. We found that these amacrine cells extend dendrites through IPL and narrowly ramify right beneath the ON ChAT band at approximately 65% depth of the IPL (Figs. 6B, 6D). The dendritic field of the analyzed amacrine cells is approximately 150 to 200 μm in diameter (Fig. 6D), consistent with being a type of GABAergic wide-field amacrine cell. According to the electron microscopy reconstruction results,43 the analyzed amacrine cells are likely to be Type 45. In addition, the amacrine cells appear to be coupled to each other as.
neurobiotin diffuses from the injected cell to neighboring cells that are also EGFP positive (Figs. 6B, 6C, 6E, 6F).

**PDGF-AA Does Not Protect RGCs in Primary Cultures**

To confirm that PDGF-AA does not protect RGCs through direct interaction, we used RGC primary cultures. RGCs were isolated using a standard immunopanning protocol and showed typical neuronal morphology in culture (Fig. 7A). RGCs in culture, similar to RGCs in the retina, did not express PDGFRα as shown by Western blotting experiments (Fig. 7B). Addition of PDGF-AA to RGC cultures did not protect RGCs, while addition of a mixture of known neuroprotective factors, BDNF and CNTF, improved survival of RGCs (Fig. 7C). These results support the idea that PDGF-AA neuroprotective action on RGCs occurs through indirect mechanisms.
DISCUSSION

The PDGF family of proteins and their receptors has multiple functions in embryonic development, cell growth, migration, differentiation, neurogenesis, osteogenesis tumor growth, and metastasis. PDGF-AA, one of the four family members, is mainly synthesized and secreted by epithelial cells, promoting mesenchyme expansion and angiogenesis. PDGF-AA/PDGFR α signaling affects a number of critical cellular functions including cell survival, proliferation, and differentiation.

Previously, we identified PDGF-AA as a novel neuroprotective factor protecting RGCs in retinal explant cultures and in a laser-induced ocular hypertension model of glaucoma. The blockage of PDGFR signaling with a small-molecule inhibitor of PDGF receptor kinase, AG-1296, or with a small-molecule inhibitor of PI3 kinase that lies downstream in the PDGF receptor signaling cascade eliminated a neuroprotective effect of PDGF-AA. The nature of cells reacting to PDGF-AA in the retina was not elucidated in our previous study. A recent report demonstrated that intravitreal injections of PDGF-AB in rats with laser-induced elevation of IOP increased microglia and monocyte-derived macrophage population in the eye and protected intraretinal synapses from degeneration. Because PDGFRα is the only known receptor for PDGF-AA and because data about its expression in different types of retinal cells are controversial, here we reexamined PDGFRα expression in the retina to better understand possible mechanisms of its neuroprotective action. As a tool, we used PDGFRα-EGFP mice in which nuclear-targeted EGFP is expressed under the control of the PDGFRα promoter. Although cells showing the strongest immunostaining signals with antibodies against PDGFRα were also EGFP positive, some cells showing less intensive immunostaining were EGFP negative. We believe that this reflects not perfect quality of PDGFRα antibodies rather than restricted pattern of EGFP reporter expression that was knocked into the PDGFRα locus.

Analyzing the distribution of EGFP immunofluorescence in the retina, we demonstrated that PDGFRα is not expressed in RGCs in vivo or in vitro, implicating that PDGF-AA provides neuroprotection of RGCs not through direct interaction with
these cells. We identified astrocytes in the GCL and a subpopulation of amacrine cells in the INL as cells expressing PDGFRα in the neural retina. These observations are supported by a single-cell RNA sequencing analysis. According to these data, PDGFRα is a marker of a distinct cluster of amacrine cells and is also expressed in astrocytes. Our present data suggest that retinal astrocytes in the GCL and/or subpopulation of amacrine cells in the INL play a role in the previously demonstrated RGC neuroprotective role of PDGF-AA in a rodent model of glaucoma. PDGFAA-treated retinal astrocytes and/or subpopulation of amacrine cells may secrete factors that provide neuroprotection of RGCs. It is well documented that retinal astrocytes produce multiple factors under different normal and pathologic conditions. Some of these factors may enhance RGC survival, while others are efficient in the promotion of axon growth. One of such PDGF-AA-induced factors could be astrocyte-derived neurotrophic factor that promotes retinal repair and regeneration. Astrocytes play a key role in synapse formation, maintenance, and function. It was shown that intravitreal PDGF-AB injection also protects intraretinal synapses and dendrites from degeneration in eyes with experimental IOP elevation. We believe that PDGF-AA interaction with PDGFRα in retinal astrocytes may contribute to synapse protection.

In conclusion, PDGFRα-EGFP mice represent a useful tool for isolation of cells expressing PDGFRα and elucidation of molecular changes induced in these cells after PDGF-AA treatment. The identification of factors secreted from activated astrocytes and/or subpopulation of amacrine cells after PDGF-AA treatment may lead to the development of appropriate RGC neuroprotection mechanisms.

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