A Potential Role for PDGF-C in Experimental and Clinical Proliferative Vitreoretinopathy

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Purpose. Proliferative vitreoretinopathy (PVR) is a disorder characterized by the formation of cellular membranes on both surfaces of the retina and within the vitreous cavity. It occurs in 5% to 10% of patients who undergo retinal reattachment surgery. In the rabbit model of the disease, the platelet-derived growth factor α receptor (PDGFRα) is dramatically more capable of promoting PVR than is closely related PDGFRβ. To test the ligand hypothesis (i.e., that this phenomenon can be explained by a predominance of PDGFRα-specific ligands) this study was conducted to determine the profile of PDGF ligands expressed by cells that induce PVR and in the vitreous of rabbits that have PVR. In addition, we examined which PDGF isoforms were present in the vitreous of patients with PVR, to assess the relevance of the rabbit model to the clinical setting.

Methods. PDGF isoforms were detected and quantified by Western blot analysis and ELISA. An assay was performed of conditioned medium from mouse embryo fibroblasts expressing the PDGFRα (Fα) and rabbit conjunctival fibroblasts (RCFs), both of which cause PVR in the experimental model, and from human retinal pigment epithelial cells (ARPE-19). Because PDGF-C is secreted in a latent form and must be proteolytically processed to become biologically active, a PDGF-C processing assay was established, and conditioned medium was tested from these cells lines, for processing activity. Vitreous specimens, from control and PVR rabbits and from patients undergoing vitrectomy surgery, either to repair retinal detachment or for other reasons, were also tested for PDGF isoforms and for PDGF-C processing activity.

Results. PDGF isoforms that activate PDGFRβ (PDGF-B and -D) were either undetectable or were present at very low levels in all the samples tested. Relatively low levels of PDGF-A and -AB were detected, whereas PDGF-C was the predominant isoform. Fα, RCFs, and ARPE-19 cells accumulated PDGF-C in the conditioned medium at an average rate of 2.0 ± 0.2, 2.9 ± 0.3, and 71.3 ± 6.0 ng/mL per day, respectively. Although there was no detectable PDGF-C in the vitreous of control rabbits ($n = 8$), there was an average of 1784 ± 1150 ng/mL latent PDGF-C in the vitreous from rabbits with PVR ($n = 14$). Of the patients with PVR, eight of nine contained PDGF-C (range, 50–1000 ng/mL). In contrast, PDGF-C was detected in only 1 of 16 of the patients without PVR. In both conditioned medium and vitreous samples, the latent (instead of the active) form of PDGF-C was detected, even though processing activity was present in all the samples tested.

Conclusions. The predominance of PDGF isoforms that activate PDGFRα support the ligand hypothesis as an explanation of why PDGFRα is more capable of inducing PVR than is PDGFRβ. Furthermore, the profile of PDGF isoforms observed in the rabbit model accurately reflected the clinical specimens from patients with PVR. Finally, these findings implicate one of the new PDGF family members as an important contributor to experimental and clinical PVR. (*Invest Ophthalmol Vis Sci.* 2007;48:2335–2342) DOI:10.1167/iovs.06-0965

Proliferative vitreoretinopathy (PVR) is defined as the growth and contraction of cellular membranes on both inner and outer surfaces of the retina after rhegmatogenous retinal detachment. The PVR membrane consists of extracellular matrix proteins and cells originating from retinal pigment epithelium (RPE), retinal glial cells, fibroblasts, and inflammatory macrophages. The growth of this membrane and its contraction can produce retinal distortions and tractional detachment. A funnel-shaped detachment of the entire retina is characteristic of advanced PVR. PVR is the primary cause of visual failure after surgical therapy for rhegmatogenous retina detachment and remains a major obstacle to improving the long-term outcome of retinal detachment surgery.[1–4]

There is both indirect and direct evidence supporting the idea that growth factors play a key role in PVR. Indirect evidence includes the fact that growth factors promote cellular responses that are integral to PVR, such as proliferation, migration, and contraction. In addition, growth factors accumulate in the vitreous of patients with PVR, and the cells within the PVR membrane secrete and/or respond to these growth factors.[5–17]

Direct support of the role of growth factors comes from work in animal models of PVR. Immortalized mouse embryo fibroblasts failed to induce PVR effectively, unless they expressed receptors for platelet-derived growth factor (PDGF).[18] Systematic comparison of cells harboring different PDGF receptors revealed that cells expressing the PDGF α receptor (PDGFRα) induced PVR, whereas PDGF β receptor (PDGFRβ)–expressing cells did not. Thus, PDGF is essential in experimental PVR. In addition, these findings revealed that the type of receptor expressed by cells can determine their potential to induce PVR. These studies also established an experimental model to investigate why expression of different PDGFRs have such a profound impact on the manifestation of PVR.

In the PDGF family, there are four gene products that assemble into five dimeric isoforms: PDGF-A, -AB, -B, -C, and -D.[19,20] PDGF-A, -AB and -B undergo intracellular processing and activation during transport in the exocytic pathway, whereas the novel PDGFs, PDGF-C and -D, are secreted in a latent state that requires activation by extracellular proteases. The proteases responsible for activating the new PDGF family

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members are actively being investigated. Whereas plasmin is capable of producing active PDGF-C, tissue plasminogen activator (tPA) appears to the physiologically relevant activator of PDGF-C.[19,21–23] The proteases known to process PDGF-D are plasmin and urokinase plasminogen activator.[20,24,25]

The inherent selectivity of PDGF isoforms for different PDGFRs is the basis for the ligand hypothesis as a potential explanation of why cells expressing PDGFRα have a much higher PVR potential than do cells harboring PDGFRβ. Cells that have only PDGFRβ can be activated by PDGF-B and -D.[19] In contrast, PDGFRα-expressing cells can be activated by all PDGF ligands except for PDGF-D, which is specific for the PDGFRβ.[19,20] The ligand hypothesis states that PDGFRα-expressing cells are better at causing PVR because one or more of the ligands that preferentially activate PDGFRα (PDGF-A, -C or -AB) predominate in the vitreous of the PVR state.

To test the ligand hypothesis, we sought to determine which isoforms of PDGF are secreted by cells associated with PVR and accumulate in the vitreous of rabbits with PVR. Both fibroblasts and RPE cells secreted PDGFRα-specific ligands, especially PDGF-C. In addition, there were large amounts of PDGF-C in the vitreous of rabbits with PVR. These observations strongly support the ligand hypothesis. Finally, our findings obtained with this experimental model were consistent with the clinical setting. PDGF-C was present in the vitreous of nearly all patients with PVR, whereas PDGF-C was detected in only a minority of patients undergoing retinal surgery for reasons unrelated to PVR.

Materials and Methods

Cell Culture

Mouse embryo fibroblasts derived from PDGFR-knockout mice that did not express either of the two PDGFR genes (F cells) and F cells in which PDGFRα had been re-expressed (Fα cells) were derived as previously described.[18] The human retinal pigment epithelial cell line ARPE-19 (RPE) was purchased from American Type Culture Collection (ATCC, Manassas, VA). Primary rabbit conjunctiva fibroblasts (RCFs) were isolated from rabbit conjunctiva as previously described.[18] The Fα cells and RCFs were maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose; Invitrogen-Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gemini Bio Products, Calabasas, CA), 500 U/mL of penicillin, and 500 μg/mL of streptomycin. RPE cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium (Invitrogen-Gibco) supplemented with 10% FBS, 500 U/mL of penicillin, and 500 μg/mL of streptomycin. The cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

The following protocol was used to produce conditioned medium (CM). After the cells grew to approximately 90% confluence, they were rinsed once with phosphate-buffered saline (PBS), and the medium was replaced with serum-free DMEM or a 1:1 mixture of DMEM and Ham's F12. The general viability of the cells was monitored by observation under a light microscope, and medium was collected at different time points (days 1, 2, and 5 and weeks 1 and 2) from cells that appeared healthy. The harvested medium was centrifuged for 10 minutes at 2000 rpm and the supernatant was frozen until analysis.

Reagents

The rabbit anti-PDGF-C core domain antibody was produced by immunizing New Zealand White rabbits with the peptides 3-3 (residues 299-326) and purified with a peptide-affinity column.[22] Goat anti-PDGF-D and anti-PDGF-C antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The goat anti-PDGF-A, -AB, and -B antibody was from Upstate Biotechnology (Lake Placid, NY). The two anti-PDGFRα antibodies (27P and 80.8) were produced and characterized as previously described.[26] The two anti-phosphotyrosine antibodies, 4G10 and PY20, were purchased from Upstate and BD-Transduction Laboratories (Lexington, KY), respectively. Tissue plasminogen activator (tPA) was purchased from Sigma-Aldrich (St. Louis, MO); a tPA inhibitor (tPA-STOP) was from American Diagnostica, Inc. (Stamford, CT). Recombinant human PDGF-A and -B were purchased from Peprotech, Inc. (Rocky Hill, NJ), and recombinant human PDGF-C core domain was prepared as previously described.[22]

Rabbit Model of PVR and Preparation of Rabbit Vitreous

Rabbits (Dutch belted) were purchase from Covance (Denver, PA). PVR was induced in the right eye, as previously described.[18,27] Briefly, gas vitrectomy was performed by injecting 0.1 mL of perfluoropropane (C_3F_8) into the vitreous cavity 4 mm posterior to the corneal limbus. One week later, all rabbits were injected with 0.1 mL of PRP (platelet-rich plasma). PRP was prepared as described previously.[18] In addition, the rabbits were injected with 0.1 mL DMEM (8 rabbits; control group) or 2×10^5 RCFs in 0.1 mL DMEM (14 rabbits, PVR group). The retinal status was evaluated with an indirect ophthalmoscope fitted with a +30-D fundus lens at days 1, 4, 7, 14, 21, and 28 after surgery. PVR was graded according to the Fastenberg classification from 0 through 5.[28] On day 28, the animals were killed, the eyes were enucleated and frozen at −80°C. All surgeries were performed in aseptic conditions and pursuant to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol for the use of animals was approved by the Schepens Animal Care and Use Committee.

To prepare the rabbit vitreous, we dissected it from the eyeball while it was still frozen, permitted it to thaw, and then centrifuged it at 4°C for 5 minutes at 10,000*g*. The resulting supernatant was used for all analyses.

Patient Vitreous

All human vitreous specimens were obtained from patients of Schepens Retina Associates who were undergoing vitrectomy surgery at Massachusetts Eye and Ear Infirmary (MEEI), in Boston, Massachusetts. IRB approval to perform these studies was obtained (protocol 05-03-019X, "Assay of Human Vitreous for Activity that Processes PDGF-C") before undertaking any of the experiments. The reasons for surgery were varied (e.g., PVR, macular hole, vitreous hemorrhage, dropped lens, retinal detachment, and retinoschisis), and patients provided consent for specimen donation in a consecutive fashion, regardless of preoperative diagnosis. Those patients who did not have PVR ($n = 16$) served as control subjects for those who did ($n = 9$). Vitreous biopsies were obtained at the outset of vitrectomy surgery, immediately after the vitrectomy cutting instrument was inserted, but before the infusion was turned on. For the eyes that had already undergone vitrectomy, vitreous fluid of 0.2 mL was drawn from the central portion of the vitreous cavity by using a TB syringe with a 30-gauge needle before the infusion cannula was opened. The samples thus obtained represent the undiluted initial material obtained from a core vitrectomy. This method did not significantly alter the normal procedure, nor did the patients assume any additional risk by their donation of the specimen. The research adhered to the tenets of the Declaration of Helsinki.

Expression and Purification of GST-PDGF-C

The full-length human *PDGF-C* cDNA (1038 bp) was amplified by polymerase chain reaction (PCR) using *Pfu* polymerase (Stratagene, La Jolla, CA) under standard conditions. Primers used for amplifying *PDGF-C* were: sense 5′ CTTTGGATCCGCTCCTCCTCGGCCTCCTC, including a *Bam*HI site for in-frame cloning, and antisense 5′ ACTAGAATTCTTACCCTCCTGCGTTCC, including an *Eco*RI site. These primers were synthesized by MWG Biotech (High Point, NC). The amplified fragments were digested with *Bam*HI and *Eco*RI and cloned into prokaryotic expression vector pGEX-1 (GE Healthcare, Piscataway, NJ). Constructs were verified by nucleotide sequencing at the Massachusetts General Hospital DNA core facility (Cambridge, MA). The glutathione *S*-transferase (GST)-PDGF-C fusion protein was induced with isopropyl-β-D-thiogalactoside (IPTG; 0.5 mM), and it accu-

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mulated in inclusion bodies. GST-PDGF-C was purified and refolded as previously described.[29] The purified fusion proteins were dialyzed into PBS at 4°C overnight. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the resultant gels were stained with Coomassie brilliant blue; the purity of GST-PDGF-C was no less than 95%.

Partial Proteolysis

We compared the native and recombinant GST-PDGF-C by partial proteolysis. The source of native PDGF-C was RPE CM (60 μL/sample). The same volume of CM from RPE cells in which PDGF-C expression, suppressed with small interfering (si)RNA oligos, was added to 18 ng of GST-PDGF-C. Proteases (0.1 μg of trypsin or chymotrypsin) were added and incubated at 37°C for 5 minutes, and then the samples were subjected to a PDGF-C Western blot using the antibody that recognizes the core domain.

To suppress PDGF-C expression, we used siRNA sequence selector software (BD Biosciences, Palo Alto, CA), and selected three target sequences specific for human PDGF-C (GenBank accession NM_016205) (1) GCTTGAAGACCCAGAAGAT (bp 775-793), (2) GCCACAATTCACAGAAGCT (bp 982-1000), and (3) TGCACACCTCGTAACTTCT (bp 1238-1256). The corresponding oligonucleotides, flanked with *Bam*HI and *Eco*RI, were subcloned into a retroviral vector (RNAi-Ready pSIREN-RetroQ; BD Biosciences). The siRNA retroviruses were generated as described.[30] As judged by anti-PDGF-C Western blot analysis of the CM, PDGF-C expression was suppressed by 93.5% ± 1.5% in the cells that stably expressed the siRNA oligo 1.

PDGF-C Processing Assay

PDGF-C processing activity was assayed as follows. Samples to be tested (CM, 20 μL; rabbit vitreous, 1 μL; and patient vitreous, 5 μL) were incubated with 50 ng GST-PDGF-C in PBS for 0.5, 2, 4, 8, 12, and 20 hours at 37°C. The total reaction volume was 40 μL. The positive control for these experiments was tPA (3 μL, 0.5 μM). The input consisted of an equivalent amount of GST-PDGF-C that was treated identically, except that it was not incubated with CM or vitreous. After the incubations, the proteins were resolved by reducing SDS-PAGE and then were immunoblotted with a goat anti-PDGF-C core domain antibody (Santa Cruz Biotechnology). These pilot experiments indicated that a 12-hour incubation period was optimal, which was used in all processing assays, unless indicated otherwise.

To assess the extent to which tPA contributed to the processing activity, we performed all assays in the presence or absence of the tPA inhibitor (tPA-STOP; American Diagnostica). Experimental samples were preincubated with the tPA inhibitor (50 μM) for 2 hours before the standard processing assay was performed.

Immunoprecipitation and Immunoblot Analysis

Fα cells were grown to 90% confluence and then incubated for 24 hours in DMEM without serum. The desired agents were added for 5 minutes at 37°C, the cells were washed twice with H/S (20 mM HEPES [pH 7.4], 150 mM NaCl), and then lysed in EB (10 mM Tris-HCl [pH 7.4], 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 20 μg/mL aprotinin, 2 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged for 15 minutes at 13,000*g*, and PDGFRα was immunoprecipitated from 1.5 mg clarified lysate, as previously described,[26] except that protein A Sepharose was used to collect the immune complexes instead of *Staphylococcus aureus* membranes. The immunoprecipitating antibody was a crude rabbit polyclonal (27P). The blotting antibody was the mixture of anti-phosphotyrosine antibodies (4G10/PY20). The primary blot was stripped and reprobed with a mixture of two PDGFRα antibodies (27P and 80.8). The extent of phosphorylation was analyzed by densitometry (Quantity One software; Bio-Rad, Hercules, CA) and normalized for the amount of PDGFRα in each sample.

The amount of PDGF-C, -A, -AB, -B, and -D in the CM, rabbit vitreous, and human vitreous was determined by Western blot by comparison with positive control samples (5, 10, 20, 40 ng) of GST-PDGF-C, GST-PDGF-D, and PDGF-A, and -B.

Experimental samples (40 μL of unconcentrated [RPE, RCF] or concentrated [Fα] CM, 5 μL of rabbit vitreous, and 20 μL patient vitreous) were subjected to reducing (PDGF-C and -D) or unreducing (PDGF-A, -AB, and -B) SDS-PAGE and then immunoblotted with the PDGF antibodies described earlier. The resultant data were quantified densitometrically (Quantity One; BD Bioscience). A nanogram level of the amount of PDGF was determined by comparison with the signal intensity of known quantities of PDGF that were analyzed in parallel.

Enzyme-Linked Immunosorbent Assay

ELISAs were developed to detect and quantify PDGF-A, -AB, -B, -C, and -D. The antibodies were tested for cross-reactivity to other PDGF isoforms before their use in the ELISA assay. The monoclonal antibodies against PDGF-C reacted only with the core domain of PDGF-C, whereas the antibody against PDGF-D recognized both the full-length and the core domain of PDGF-D. Isoform-specific monoclonal antibodies were coated onto 96-well microtiter plates at 1 μg/mL in 0.1 M Na_2HCO_3 (pH 9.6) and incubated overnight at 4°C. The plates were washed with PBS containing 0.05% Tween-20, then blocked with blocking buffer (PBS containing 1% BSA and 0.05% Tween-20) for 2 hours at 37°C. PDGF standards and test samples were diluted in blocking buffer, added to appropriate plates and incubated for 1 hour at 37°C. For PDGF-C and -D ELISA, the plates were washed and incubated with a ligand-specific, biotinylated secondary monoclonal antibody at 0.5 μg/mL for 1 hour at 37°C. The plates were washed and incubated with streptavidin conjugated to horseradish peroxidase (Pierce, Rockford, IL) at 0.5 μg/mL, diluted in blocking buffer. For PDGF-A and -AB ELISAs, a rabbit anti-A chain polyclonal antibody was added at 1 μg/mL to wells for 1 hour at 37°C. For the PDGF-B ELISA, a rabbit anti-B chain polyclonal antibody was added at 1 μg/mL to wells for 1 hour at 37°C. The plates were washed and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Biosource, Camarillo, CA). After a final wash, all plates were incubated with TMβ substrate (BioFx, Owings Mills, MD) for 5 minutes at ambient temperature. The reaction was blocked by addition of a blocking reagent (TMβ 450; BioFx) and read at an absorbance of 450 nm in an ELISA plate reader (SpectraMax 340; Invitrogen-Molecular Devices, Sunnyvale, CA).

The ELISA sensitivity approximations are 1 ng/mL for all isoforms except PDGF-B, for which the lower limit of detection was 0.1 ng/mL. Each experimental sample was analyzed at two dilutions in duplicate.

Results

Fα Cell Secretion and Processing of PDGF-C

We have reported that immortalized mouse embryo fibroblasts isolated from mice lacking both PDGFR genes (F cells) fail to induce PVR in the standard rabbit model of PVR.[18] Furthermore, the PVR potential of these cells was dramatically improved by expressing PDGFRα, but not PDGFRβ.[18] The explanation provided by the ligand hypothesis is that F cells secrete and/or the vitreous accumulates PDGF isoforms that are specific for PDGFRα and thereby establish an autocrine loop in F cells expressing PDGFRα (Fα cells). To test this hypothesis, we used two different experimental approaches (Western blot analysis and ELISA) to assess the profile of PDGF isoforms secreted by Fα cells. PDGF-C was the only isoform that we detected by either approach (Fig. 1, Table 1, and data not shown), and it accumulated in the medium of Fα cells at an average rate of 2.2 ± 0.2 ng/mL per day. It is possible that other PDGF isoforms were present, yet below the level of detection, which was 25 ng for PDGF-A, 20 ng for PDGF-B, and 10 ng for PDGF-D for the Western blot. For the ELISA the detection limit was approximately 1 ng/mL for PDGF-A, AB, -C, and -D and 0.1 ng/mL for PDGF-B. Our observation, that under basal conditions fibroblasts secreted PDGF-C but not the other

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PDGF isoforms, is consistent with reports from several other laboratories.[21,22,31]

Since PDGF-C is secreted as a latent, inactive precursor,[19,20] we wanted to know whether PDGF-C detected in the CM of Fα cells was latent or active. The molecular mass of PDGF-C indicated that it was the latent form (Fig. 1A). We detected the core domain (the active form) in only two of six experiments. The probable reason for this is instability of the core domain in CM (see below). We conclude that the latent form of PDGF-C accumulated in the CM of Fα cells. This conclusion was indirectly supported by the fact that PDGF-C was not detected in the ELISA, which does not recognize the latent form of PDGF-C.

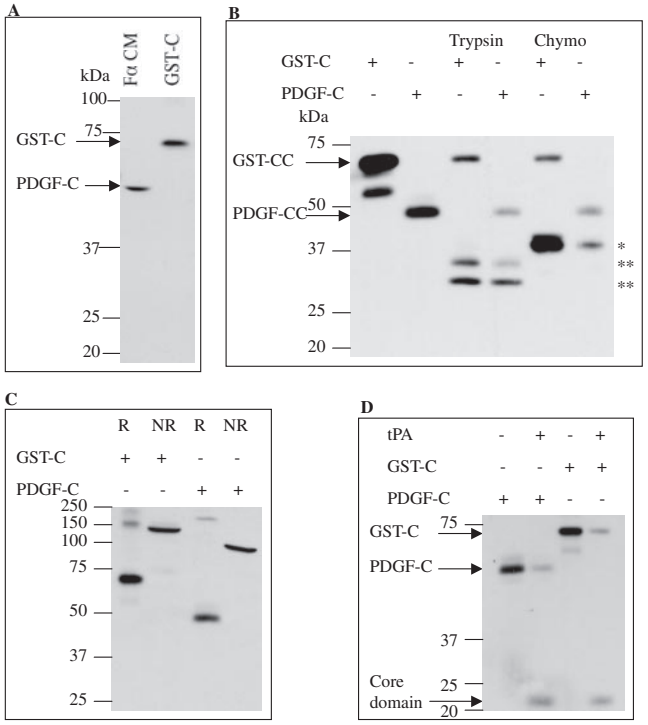

FIGURE 1. Fα cells secreted PDGF-C; characterization of GST-PDGF-C. (**A**) Fα cells were grown to 90% confluence in serum-containing medium, washed, and incubated for 5 days in serum-free medium. This medium (Fα CM) was collected, centrifuged, concentrated, and subjected to Western blot analysis with an anti-PDGF-C antibody directed to the core domain. Full-length GST-PDGF-C (GST-C) served as a positive control. In three independent experiments, PDGF-C accumulated at an average of 2.2 ± 0.2 ng/mL per day. (**B**) Partial proteolysis of native (PDGF-C) and recombinant (GST-C) full-length PDGF-C. Native and recombinant PDGF-C were incubated for 5 minutes at 37°C in the absence (the *two left lanes*) or presence of 0.1 μg of the indicated protease. The samples were then subjected to a PDGF-C Western blot with the antibody that recognizes the core domain. *Arrows*: full-length proteins; recombinant PDGF-C has a higher molecular mass than does native PDGF-C because it is fused with the 26-kDa GST domain. Both of the proteases reduced the amount of the full-length species and generated smaller fragments (* or **). When digested with the same protease, all (with trypsin) or a subset (with chymotrypsin [Chymo]) of the proteolytic fragments generated from native and recombinant PDGF-C had the same molecular mass. Similar results were obtained on three independent occasions. (**C**) The molecular mass of PDGF-C is sensitive to reducing agents. Native or recombinant PDGF-C was analyzed by reducing (R) or nonreducing (NR) SDS-PAGE, followed by PDGF-C Western blot analysis. In three independent experiments, the molecular mass of PDGF-C (either native or recombinant) was approximately two times greater under nonreducing conditions, which is consistent with the fact that PDGF-C is a disulfide-bonded dimer.[19,20] (**D**) Processing of native (PDGF-C) or recombinant (GST-C) full-length PDGF-C. Native or recombinant PDGF-C (36 ng) was incubated at 37°C for 12 hours in the presence or absence of tPA (68 ng). The samples were then subjected to a PDGF-C Western blot with the antibody that recognizes the core domain. The amount of core domain liberated from either source was similar in three independent experiments.

TABLE 1. ELISA Results for PDGF Isoforms in CM and Rabbit Vitreous

Samples	A	AB	B	C*	D
CM					
F	0	0	0	0	0
ARPE-19	5.1 ± 4.2	0.4 ± 0.6	0	3.1 ± 3.6	0
RCF	0	0	0	0	0
Vitreous					
Control	0	0	0	0	0
PVR	40.4 ± 4.0	59.7 ± 6.6	2.9 ± 1.0	0	0

Data are expressed as the mean nanograms/milliliter ± SD. For the CM experiments the data are from three independent experiments. For the vitreous experiments the control group consisted of 8 rabbits, whereas there 14 rabbits in the PVR group. All samples that were subjected to ELISA analysis were tested at two dilutions and in duplicate. The data are the mean of three independent experiments (CM samples) or replicate animals (vitreous samples). Note that PDGF-A, -AB, and -B are secreted in their active form (i.e. they do not undergo extracellular processing, as do PDGF-C and -D). Consequently, the molecular mass for PDGF-A and -AB are not routinely reported because only the active form is secreted.[21]

* The ELISA was unable to detect latent PDGF-C.

Because fibroblasts express tPA, which processes PDGF-C,[19,20,23] we investigated whether Fα cells were capable of processing latent PDGF-C to its active form. For these studies we generated and characterized recombinant, full-length PDGF-C in the context of a GST fusion protein. Because the recombinant PDGF-C was fused with the 26-kDa GST domain, it had a higher mass than the native protein (Fig. 1). Despite this difference in mass, partial proteolysis of the two proteins resulted in fragments of the same size (Fig. 1B, asterisks). This was true when using either of two proteases. This result indicated that the two proteins had the same sensitivity to proteolysis and strongly suggested that they were folded comparably. Furthermore, the molecular mass of PDGF-C was approximately two times greater under nonreducing versus reducing conditions (Fig. 1C), which is consistent with the disulfide-mediated dimeric nature of PDGF-C.[19,20,23] Additional support for the idea that recombinant GST PDGF-C was properly folded was the fact that it was not recognized in the active form-specific PDGF-C ELISA until it was processed (data not shown) and that it activated the PDGFRα after processing (Fig. 2C). We then proceeded to use the GST-PDGF-C in the processing assay. As expected, tPA reduced the amount of the full-length GST-PDGF-C, and processing was efficiently inhibited by a tPA inhibitor (tPA-STOP). Under these conditions, we detected the core domain, and similar amounts were produced from either native or recombinant GST-PDGF-C (Fig. 1C). We repeated the processing assay with recombinant GST-PDGF-C as a substrate, by using Fα CM instead of purified tPA, and observed comparable results (Fig. 2B). The only difference was that the tPA blocking agent (tPA STOP; American Diagnostica) stopped an average of 63.7% ± 4.5% of the processing (instead of 97.5% ± 0.6%) suggesting that the CM contained proteases other that tPA that are capable of processing PDGF-C. Taken together, these data revealed that recombinant GST-PDGF-C was a suitable substrate for the processing assay, that Fα cells were capable of processing PDGF-C, and that most of the activity in the CM of Fα cells was tPA.

Although the core domain was observed in the experiments shown in Figs. 2A and 2B, we routinely found it difficult to detect this species. To investigate the possible reason for this observation, we tested the stability of the core domain in CM.

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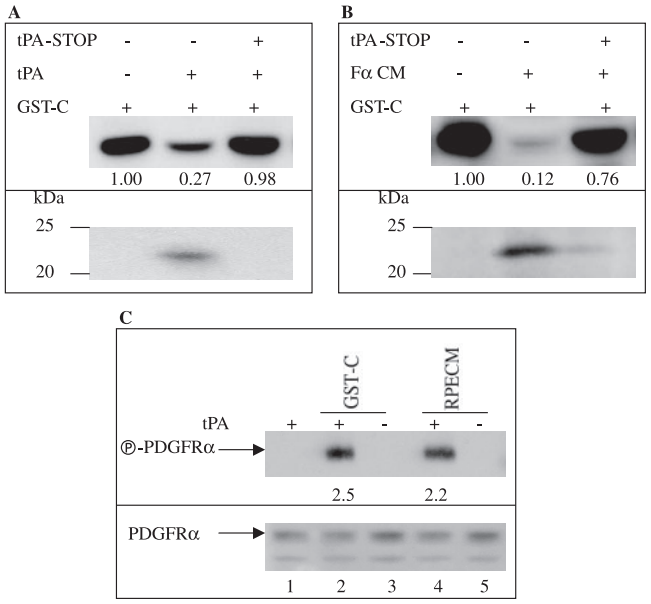

FIGURE 2. Fα CM contained tPA, which processed PDGF-C. (**A**) *Top*: tPA was preincubated with or without a tPA inhibitor (STOP, an inhibitor of tPA; American Diagnostica, Inc., Stamford, CT) for 2 hours at room temperature and then incubated with GST-PDGF-C (GST-C) for 12 hours at 37°C. The samples were subjected to PDGF-C Western blot analysis, as described in Figure 1. Under these conditions, 73% of the latent PDGF-C was processed, which was calculated by subtracting the amount of latent PDGF-C remaining at the end of the processing assay from the amount at the start of the assay (1.0 – 0.27 = 0.73). As expected, the tPA inhibitor stopped nearly all (97%) of the processing activity. This value was calculated as the ratio of the actual inhibition (0.98 – 0.27)/maximum possible inhibition (1.0 – 0.27) × 100. *Bottom*: the core domain that was generated. This experiment was repeated three independent times, and the average ± SD for processing and extent to which the tPA blocked was 65.6% ± 9.0% and 97.5% ± 0.6%, respectively. (**B**) *Top*: CM from Fα cells (Fα CM) was tested for processing activity as described in (**A**). Fα CM processed 88% of the latent PDGF-C. *Bottom*: the core domain. Inclusion of the tPA blocking agent stopped 72% of the processing, which indicated that tPA was responsible for the bulk of the processing activity in the CM. This experiment was repeated three independent times, and the average ± SD for processing and extent to which tPA was blocked was 81.4% ± 6.0% and 63.7% ± 4.5%, respectively. (**C**) To test functionally whether PDGF-C had been processed, we determined whether it could stimulate tyrosine phosphorylation of PDGFRα. Latent PDGF-C (recombinant [GST-C] or native [PDGF-C]) was processed as described in the legend of Figure 1D and then added to PDGFRα-expressing cells for 5 minutes. Controls for this experiment included unprocessed PDGF-C (*lanes 3* and *5*) and tPA (*lane 1*). The cells were lysed, and PDGFRα was immunoprecipitated and subjected to an anti-phosphotyrosine Western blot (*top*) followed by anti-PDGFRα Western blot (*bottom*). *Arrow*: the mature form of PDGFRα, which undergoes tyrosine phosphorylation. The signal intensities were quantified, and the resultant ratios are given. The results show that the potency of the native and recombinant PDGF-C was comparable. Similar results were observed in three independent experiments.

Using purified tPA and full-length recombinant GST PDGF-C, we generated the core domain and added it to the CM. Western blot analysis revealed that the core domain disappeared within 12 hours (data not shown). These observations suggest that the core domain was unstable, hence difficult to detect in CM.

To test whether the core domain produced by processing of full-length PDGF-C was functionally active, we tested its ability to induce tyrosine phosphorylation of PDGFRα. We included both native and recombinant GST-PDGF-C in this series of experiments. Cells expressing PDGFRα were exposed to processed or unprocessed PDGF-C. PDGFRα was immunoprecipitated and subjected to an anti-phosphotyrosine Western blot analysis. As shown in Figure 2C, full-length PDGF-C was unable to promote phosphorylation of PDGFRα, regardless of whether it was native or recombinant (lanes 3 and 5). In contrast, processed PDGF-C activated PDGFRα, and the native and recombinant forms had similar potency (Fig. 2C, compare lanes 2 and 4). We conclude that Fα cells secrete both PDGF-C and the proteases necessary to process it to its active form.

RPE and RCF Cell Secretion and Processing of PDGF-C

We extended our analysis of PDGF isoforms to two additional cell types that are highly relevant to PVR. RPE cells, which are the most abundant cell type in human PVR membranes, and rabbit conjunctival fibroblasts (RCFs), the most commonly used cell type in the rabbit PVR model. As shown in Figure 3, the properties of these two cell types were very similar to those of the Fα cells. They secreted PDGF-C and the latent form accumulated in the media. RPE and RCF cells accumulated PDGF-C at an average rate of 71.3 ± 6.0 and 2.9 ± 0.3 ng/mL per day, respectively. Furthermore, there was processing activity in the CM, and the majority (although not all of it) was blocked by the tPA blocking agent (tPA-STOP; American Diagnostica). The other PDGF isoforms were either below the level of detection or present much lower levels than PDGF-C (Table 1 and data not shown).

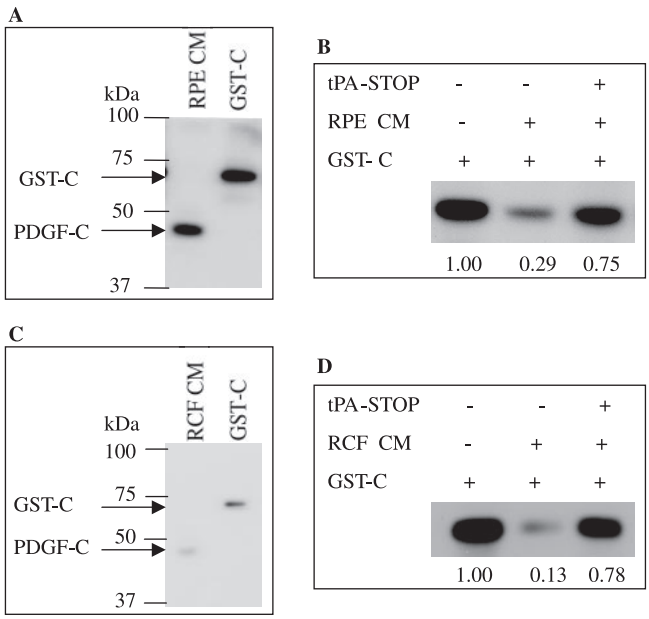

FIGURE 3. RPE and RCF cells secreted PDGF-C. (**A**) CM from RPE cells (RPE CM) was subjected to PDGF-C Western blot analysis, as described in Figure 1. In three independent experiments, there was an average of 999.7 ± 108.5 ng/mL latent PDGF-C in RPE CM. The CM was from a 14-day incubation, and the rate of accumulation was 71.3 ± 6.0 ng/mL per day. (**B**) RPE CM was tested for processing activity as described in Figure 2. Quantifying the results of these experiments indicated that RPE CM processed 71% of the GST-C; tPA blocker inhibited 65% of this activity. In three independent experiments, we found that the average processing and extent to which tPA was blocked was 65.3% ± 5.5% and 60.0% ± 4.5%, respectively. (**C**, **D**) Same as (**A**) and (**B**), except with RCFs. (**C**) Three independent experiments showed an average of 20.3 ± 4.5 ng/mL latent PDGF-C in RPE CM. The CM was from a 7-day incubation, and the rate of accumulation was 2.9 ± 0.3 ng/mL day. (**D**) RCF CM processed 87% of the PDGF-C, and 74% of the activity was stopped by a tPA blocker (tPA-STOP; American Diagnostica, Inc., Stamford, CT). In three independent experiments we found that the average processing and extent to which tPA was blocked was 82.0% ± 5.6% and 70.3% ± 4.7%, respectively.

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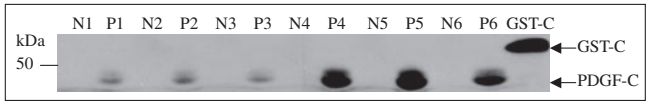


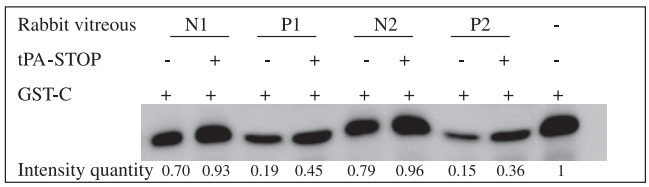
FIGURE 4. PVR greatly increased the amount of PDGF-C in the vitreous of rabbits. Vitreous (5 μL) from randomly selected control (N1–N6) and PVR (P1–P6) rabbits was subjected to PDGF-C Western blot analysis. The positive control was 18 ng GST-PDGF-C (GST-C). Quantification of the signal intensities indicated that there was 497, 665, 495, 2500, 3070, or 1960 ng/mL latent PDGF-C in the P1–P6 samples, whereas no PDGF-C was detected in any of the control samples. The average ± SD for 14 PVR and 8 control rabbits was 1784 ± 1150 and 0 ± 0 ng/mL, respectively.

Effect of PVR on the Amount of PDGF-C in the Vitreous of Rabbits

We also examined the level of PDGF isoforms in the vitreous of control and PVR rabbits. The control animals (n = 8) were injected with PRP and failed to develop PVR, whereas the experimental rabbits (n = 14) were injected with a combination of PRP and RCFs and advanced to stage-5 PVR. All rabbits were killed, and the vitreous was harvested at day 28. Western blot analysis revealed that there was an average of 1784 ± 1150 ng/mL PDGF-C in the vitreous of rabbits that developed PVR, whereas none was detected in the control group (Fig. 4). A relatively small amount of PDGF-A and -AB was detected in the PVR rabbits by ELISA (Table 1). Even though these levels were above the detection limit for the Western blot, we did not routinely detect these PDGF isoforms by the Western approach (data not shown). Furthermore, the PDGFRβ ligands (PDGF-B and -D) were either undetectable or were present at very low levels (Table 1 and data not shown). These studies indicate that PVR led to accumulation of several PDGF isoforms in the vitreous that activate the PDGFRα, and that PDGF-C was the most dramatically increased.

It was the latent form of PDGF-C that accumulated in the vitreous of rabbits with PVR. This prompted us to determine whether the vitreous also contained processing activity. Although both the control and PVR vitreous had readily detectable processing activity, there was as an average of 2.4-fold more activity in the vitreous of the PVR rabbits (Fig. 5). Furthermore, tPA-STOP blocked a smaller fraction of the processing activity in the vitreous from rabbits with PVR (68.5% ± 14.2% versus 30.0% ± 2.9%). Because the vitreous contained processing activity and the apparent instability of the core domain, our values probably underestimate the amount of PDGF-C that was present. These findings also indicate that PVR induced proteases in addition to tPA that could process PDGF-C.

A



B

	Normal	PVR
Processing activity	29.9 ± 8.6%	72.0 ± 10.5%
Extent of inhibition by tPA-STOP	68.5 ± 14.2%	30.0 ± 2.9%

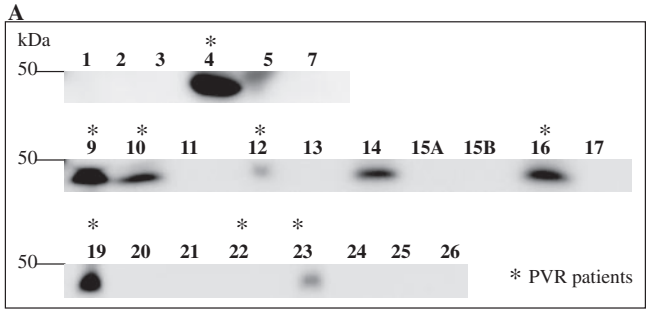
FIGURE 5. Rabbit vitreous processed PDGF-C. (**A**) Rabbit vitreous (1 μL) was subjected to the processing assay described in the legend of Figure 2. The results show that the vitreous from control and PVR rabbits processed 25.5% and 83% of the PDGF-C, respectively. The processing activity was blocked 78% and 28% by tPA inhibitor (tPA-STOP; American Diagnostica, Inc., Stamford, CT) in the healthy and PVR rabbit vitreous, respectively. (**B**) Summary of the findings from 8 control and 14 PVR rabbits.

PDGF-C in the Vitreous of Patients with PVR

The results from the rabbit model indicate that PVR resulted in a dramatic increase of PDGF-C in the vitreous. To determine whether this is also the case in human disease, we determined the level of PDGF-C in a cohort of patients who were undergoing retinal surgery to correct PVR or for unrelated reasons. All specimens were analyzed before their clinical status was known. We found that the vitreous in eight of nine of the patients with PVR contained PDGF-C in the vitreous. In all cases, the PDGF-C was latent, and ranged from 50 to 1000 ng/mL (Fig. 6). In contrast, only 1 of the 16 patients who did not have PVR had a detectable level of PDGF-C. Finally, none of the other PDGF isoforms (-A, -AB, -B, and -D) were detected in any of the samples (data not shown). Because of the limited amount of specimens, we had enough material to perform the replicate analysis only by Western blot analysis. Thus, it is possible that there were low levels of the other PDGF isoforms in the patients' vitreous that we could not detect by this method of analysis.

We also tested a subset of the human vitreous specimens for processing activity. Regardless of the clinical status, processing activity was detected in all specimens (Fig. 7). The range in the PVR samples was 68% to 85% (average, 79% ± 7.3%) and 44% to 67% (average, 56% ± 16.2%) in the non-PVR group (Fig. 7). Furthermore, the tPA blocking agent (tPA STOP; American Diagnostica, Inc.) blocked an average of 34% ± 5.8% of the processing activity in the vitreous from patients with PVR, indicating that tPA constitutes a minority of the proteases that process PDGF-C. Finally, there was a similar degree of inhibition (35% ± 7.1%) with the tPA inhibitor in the specimens from

A



B

Clinical status	PVR	Non-PVR
Patient #	9	16
PDGF-C	8	1

FIGURE 6. PDGF-C was present in the vitreous of patients with PVR (*). (**A**) Vitreous (20 μL) was collected and analyzed for the presence of PDGF-C by Western blot analysis. The amount of PDGF-C was 1000, 480, 212, 50, 240, 360, 0, and 80 ng/mL in patients 4, 9, 10, 12, 16, 19, 22, and 23, respectively. The active form of PDGF-C was routinely not detected. There was 265 ng/mL latent PDGF-C in non-PVR patient 14. (**B**) Summary of the findings. Of the patients with PVR, 8 of 9 had PDGF-C in the vitreous, whereas only 1 of 16 of the non-PVR patients had a detectable level of PDGF-C. Data are not included for an independently analyzed patient (patient 27), who had PVR and 192 ng/mL latent PDGF-C in the vitreous.

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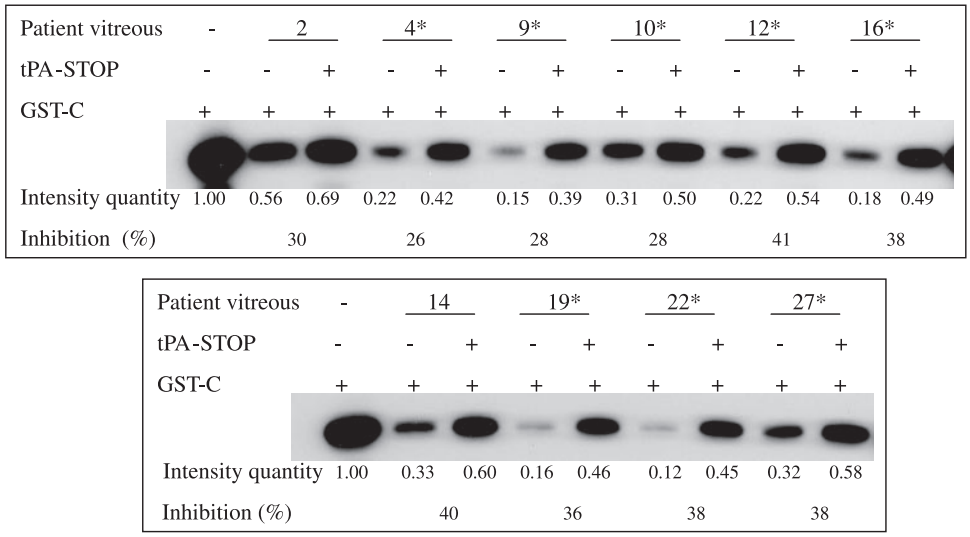

FIGURE 7. Patients' vitreous processed PDGF-C. The vitreous from patients who did (*) or did not have PVR was tested for processing activity, as described in the legend of Figure 2. The results show that the vitreous from all the patients processed PDGF-C and a tPA blocker (tPA-STOP; American Diagnostica, Inc., Stamford, CT) partially inhibited processing (34% ± 5.8% in PVR vitreous and 35% ± 7.1% in non-PVR vitreous).

patients without PVR. These findings demonstrate that PDGF-C was present in most of the patients with PVR. In addition, although tPA contributed to the processing of PDGF-C, other proteases appeared to constitute most of the processing activity in PVR patients.

DISCUSSION

In this study, we investigated the ligand hypothesis as a possible explanation for why expression of growth factor receptors can determine the PVR potential of cells. Our findings provide strong support for this possibility. Cell lines associated with PVR secreted ligands that activate the receptors that promote PVR. Furthermore, these same ligands were present in the vitreous from rabbits with severe PVR, and in the vitreous from most of the patients with PVR.

Although our findings clearly promote the ligand hypothesis, they do not address the possibility that there are differences in the signaling output of PDGFRα and PDGFRβ, which contribute to the increased PVR potential of PDGFRα. Although the intracellular domain of these two receptors are highly homologous and engage many of the same signaling pathways,[32,33] they arise from different genes and are not fully interchangeable during development.[34] Thus, it is possible that being awash in PDGF-C is not the only reason Fα cells induce PVR significantly better than do Fβ cells. PDGFRα may also trigger signaling events that promote PVR. We plan to address this signaling hypothesis by testing the PVR potential of cells expressing a PDGFR chimera that responds to PDGFRα ligands and signals through a PDGFRβ cytoplasmic domain.

Numerous groups have reported that PDGF is elevated in the vitreous of patients with PVR and/or is expressed by RPE and glial cells within PVR membranes.[7–12,14–17] These previous studies focused on the original members of the PDGF family (PDGF-A, -AB, and -B). The findings in this study are consistent with these earlier reports and extend the analysis to include PDGF-C and-D, the newly appreciated family members. We found that there was much more PDGF-C than any of the other isoforms of PDGF. Although we cannot exclude the possible involvement of the other forms of PDGF (or other growth factors in the vitreous and membrane), the predominance of the PDGF-C isoform identifies it as the most logical therapeutic target.

We were surprised to find such high levels of PDGF-C in the vitreous of rabbits with severe PVR. It takes 10 to 20 ng/mL of PDGF-C to induce responses such as contraction or proliferation, and the level detected in experimental and clinical PVR was typically in vast excess of this value. The presence of processing activity indicates that at least some of this pool of latent PDGF-C was being converted to the active form. Therefore, the amount of latent PDGF-C being synthesized was probably even greater than the amount observed. At the present time we do not know if all PDGF-C is coming from the injected cells. Although we originally expected that the platelet-rich plasma (which is co-injected with the cells to induce PVR) would be a major source of PDGF, PDGF-C was not detected in the vitreous from control rabbits, which were injected only with PRP. In light of our findings, we speculate that the injected cells are the primary source of PDGF-C; ongoing experiments to stably silence the expression of PDGF-C in cells before injecting them will provide a better understanding of this issue.

Of note is the observation that the amount and nature of the processing activity was changed by PVR in rabbits. A simple explanation of this phenomenon is that PVR increased the amount of tPA, which would increase the overall activity and overwhelm the tPA blocking agent added to the reactions. However, pilot experiments indicated that we were using saturating amounts of inhibitor, and that the amount of tPA present in vitreous samples was very low (data not shown). These observations suggest that PVR induces the expression and/or activity of PDGF-C-processing proteases that are not tPA.

Finally, our studies beg the question of whether interfering with the production, processing or function of PDGF-C could prevent PVR. Our ongoing studies are directed toward addressing this question.

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