Growth Factors Outside the PDGF Family Drive Experimental PVR

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Purpose. Proliferative vitreoretinopathy (PVR) is a recurring and problematic disease for which there is no pharmacologic treatment. Platelet-derived growth factor (PDGF) in the vitreous is associated with experimental and clinical PVR. Furthermore, PDGF receptors (PDGFRs) are present and activated in epiretinal membranes of patient donors, and they are essential for experimental PVR. These observations suggest that PVR arises at least in part from PDGF/PDGFR-driven events. The goal of this study was to determine whether PDGFs were a potential therapeutic target for PVR.

Methods. Experimental PVR was induced in rabbits by injecting fibroblasts. Vitreous specimens were collected from experimental rabbits or from patients undergoing vitrectomy to repair retinal detachment. A neutralizing PDGF antibody and a PDGF Trap were tested for their ability to prevent experimental PVR. Activation of PDGFR was monitored by antiphosphotyrosine Western blot analysis of immunoprecipitated PDGFRs. Contraction of collagen gels was monitored in vitro.

Results. Neutralizing vitreal PDGFs did not effectively attenuate PVR, even though the reagents used potently blocked PDGF-dependent activation of the PDGF α receptor (PDGFRα). Vitreal growth factors outside the PDGF family modestly activated PDGFRα and appeared to do so without engaging the ligand-binding domain of PDGFRα. This indirect route to activate PDGFRα had profound functional consequences. It promoted the contraction of collagen gels and appeared sufficient to drive experimental PVR.

Conclusions. Although PDGF appears to be a poor therapeutic target, PDGFRα is particularly attractive because it can be activated by a much larger spectrum of vitreal growth factors than previously appreciated. (*Invest Ophthalmol Vis Sci.* 2009; 50:3394–3403) DOI:10.1167/iovs.08-3042

Proliferative vitreoretinopathy (PVR) occurs as a complication in 3.9% to 13.7% of patients undergoing surgery to reattach a detached retina.[1,2] This is a blinding disease in which the retina re-detaches because of the contraction of a fibroproliferative membrane that forms on the surface of the retina.[3–6] Vitreal growth factors are thought to promote formation and contraction of the membrane, which is populated by several cell types, including retinal pigment epithelial cells, fibroblasts, glial cells, and macrophages.[7–11] Platelet-derived growth factor (PDGF) is among the long list of vitreal growth factors implicated in contributing to PVR.[9,12–25] Additional evidence for the role of PDGF/PDGFR in PVR are the observations that cells within the fibroproliferative membrane isolated from patient donors express PDGF and PDGFRs and that the PDGFRs are activated.[9,26,27] Furthermore, in an experimental model of PVR, functional PDGFRs are a prerequisite for disease formation.[28–30] Given that key components of PVR (proliferation of myofibroblasts and increased synthesis of extracellular matrix) are common to fibrosis in other organs, it is likely that the insights gleaned from the study of any one of these settings will be at least in part applicable to the other pathologic settings.

The most common animal models of PVR involve the injection of cells into the vitreous and subsequent observation of the formation of a membrane, which contracts and thereby induces retinal detachment.[31] Several groups have found that PVR is substantially attenuated if PDGFRs of the injected cells were missing or inhibited.[28–30] The source of PDGF to activate these receptors appears to initially be from the coinjected, platelet-rich plasma. At later time points, there are high levels of PDGF-C in the vitreous, coming at least in part from the injected cells that naturally produce this isoform of PDGF.[15] The presence of PDGF-C in the vitreous of rabbits mirrored the clinical situation. PDGF-C was observed in the vitreous of most patients with PVR, but no PDGF-C was detected in most patients without PVR.[15] Taken together, these findings suggest that neutralizing PDGF-C could prevent experimental PVR and could be a potential therapy for patients with PVR.

The PDGF family is composed of five ligands that assemble dimeric receptors consisting of homodimer or heterodimer combinations of the two PDGF receptor subunits.[32–34] There are several mechanisms by which PDGFRs are activated (i.e., undergo tyrosine phosphorylation) and thereby initiate intracellular signaling events that culminate in various cellular responses. The most extensively studied mechanism involves PDGF-dependent dimerization of receptor subunits that increases the receptor's intrinsic kinase activity and results in extensive autophosphorylation.[35,36] Certain agonists of G protein–coupled receptors, autoantibodies in the blood of patients with scleroderma, and certain agents within the bone marrow (but are probably not PDGFs) also promote tyrosine phosphorylation of PDGFR.[37–44] Finally, signaling events induced by polypeptide growth factors outside the PDGF family (non-PDGFs) are greater in cells that express PDGFRs than in nonexpressing cells,[45] suggesting that non-PDGFs are capable of engaging PDGFRs. Together these data indicate that activation of PDGFRs is not restricted to the direct PDGF-dependent route, suggesting that PDGFRs may act independently of PDGFs to contribute to cellular responses and even disease manifestation.

While investigating the role of PDGF/PDGFR in PVR, we discovered that experimental PVR was more dependent on

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PDGFRα than the PDGF isoforms that activate this receptor. Moreover, non-PDGFs activated PDGFRα—that is, they increased tyrosine phosphorylation of PDGFRα and potentiated contraction of collagen gels. Finally, activation of PDGFRα by non-PDGFs was sufficient to induce experimental PVR.

Materials and Methods

Cell Culture

F and Fα cells were previously described.[28] Briefly, they are mouse embryo fibroblasts derived from mice null for both *pdgfr* genes and immortalized with SV40 T antigen. Fα cells are F cells in which we expressed full-length human PDGFRα. FαΔX cells are F cells in which we expressed a PDGFRα mutant that is missing most of the extracellular domain and, hence, cannot bind PDGF. ARPE19 (RPE19) cells are a human retinal pigment epithelial cell line purchased from American Type Culture Collection (Manassas, VA). RPE19α cells were generated by expressing human PDGFRα in the parental RPE19 cells,[15] which naturally express a low level of PDGFRα. Primary rabbit conjunctiva fibroblasts (RCFs) were isolated from rabbit conjunctiva as previously described.[46] F, Fα, FαΔX, and RCFs were maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose; Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gemini Bio Products, Calabasas, CA), 500 U/mL penicillin, and 500 μg/mL streptomycin. RPE19α cells were cultured in a 1:1 mixture of DMEM and Ham F12 medium (Gibco-BRL) supplemented with 10% FBS, 500 U/mL penicillin, and 500 μg/mL streptomycin.

GPG293 cells[47] were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 μg/mL tetracycline (Sigma, St. Louis, MO), 2 μg/mL puromycin (Sigma), 0.3 mg/mL G418 (Sigma), and 16.7 mM HEPES (Invitrogen, Carlsbad, CA). The medium used during virus collection was DMEM supplemented with 10% FBS, 2 mM L-glutamine, 16.7 mM HEPES. All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Major Reagents

The neutralizing PDGF-C antibody is a mouse monoclonal IgG1, HH1–57 (clone 57) produced at ZymoGenetics (Seattle, WA) as follows: Balb/c mice were immunized with recombinant growth factor domain (also called core domain[48]) of human PDGF-C.[49] Splenocytes and lymphocytes from two mice that had high titers toward the immunogen were fused with the X63-Ag8.653 mouse myeloma cell line[50] according to published procedures.[51] The resultant hybridoma line was cloned twice, grown in perfusion fermentation, and IgG purified by protein A-Sepharose chromatography.[52] Monoclonal antibody HH1–57 was selected for its ability to inhibit PDGF-CC (the growth factor domain of PDGF-C) mitogenic activity on rat liver stellate cells (data not shown). This monoclonal antibody showed no cross-reactivity with other PDGF family members (data not shown).

Trap (PDGFRα-Fc5; construct PDGFRα Fc5 pZMP42) is a fusion between the entire extracellular domain of human PDGFRα and human Fc5. It was produced at ZymoGenetics in CHO cells and was purified by protein A-Sepharose chromatography.[52] The resultant material was evaluated for neutralization of PDGF-A, -B, and -C mitogenic activity using a 3T3 fibroblast mitogenesis assay.[53]

Recombinant human PDGF-C core domain (also called growth factor domain[49]) was prepared as previously described.[15] The anti-PDGFRα (27P) antibody was produced and characterized as previously described.[54] The two antiphosphotyrosine antibodies, 4G10 and PY20, were purchased from Upstate Biotechnology (Charlottesville, VA) and BD Transduction Laboratories (Madison, WI), respectively. Recombinant human PDGF-A and -B, human hepatocyte growth factor (HGF), and mouse bFGF were purchased from PeproTech, Inc. (Rocky Hill, NJ). Antibodies that recognized PDGFRα and fibroblast growth factor receptor 1 (FGFR1) were purchased from Cell Signaling (Danvers, MA). The horseradish peroxidase (HRP)-conjugated goat anti–rabbit IgG, goat anti–mouse IgG secondary antibodies, and anti–phospho-PDGFRα (phosphoY720) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescent substrate for detection of HRP was from Pierce Protein Research Products (Rockford, IL). Insulin and epithelial growth factor (EGF) were purchased from Calbiochem (San Diego, CA).

Construction of αΔX

Construction of αΔX was completed in two steps. First, the portion of the human *PDGFRα* cDNA encoding the transmembrane and the intracellular domains was excised as a *Pst*I/*Bam*HI fragment and subcloned into *pBluescript II SK+* to generate *pBluescript-PDGFRα*. Second, the full-length human *PDGFRα* cDNA was used as a template to PCR amplify the signal peptide sequence with the use of *Pfu* polymerase (Stratagene, La Jolla, CA) under standard conditions. Primers used were as follows: sense, 5′-GCGAAGCTTGTTTTGGGGACGTGGTGGCCAGCGCCTTCC including a *Hin*dIII site; antisense, 5′-GGACTGCAGCAGCCACCGTGAG GGATGAATTCAGCTGCACAAC including a *Pst*I site. These primers were synthesized by the Massachusetts General Hospital (MGH; Cambridge, MA) DNA core facility. PCR products were digested with *Hin*dIII and *Pst*I and were subcloned into *pBluescript-PDGFRα* that had been precut with the same set of restriction enzymes. The resultant construct was termed *pBluescript-PDGFRαΔX* and was verified by nucleotide sequencing at the MGH DNA core facility. The *PDGFRαΔX* insert was subcloned as a *Hin*dIII-*Not*I fragment into the *pLHDCX*[3] retroviral vector, and the resultant construct was termed *pLHDCX*[3]*-PDGFRαΔX*. The predicted amino acid sequence of αΔX includes the first 44 amino acids that encompass the signal peptide. The rest of the extracellular domain is missing. Amino acid 45 in this receptor corresponds to leucine 525, which is the beginning of the transmembrane domain. The remainder of this receptor includes the rest of the transmembrane domain and the entire intracellular domain.

We generated cell lines expressing αΔXPDGFRs as follows: To make the virus, the *pLHDCX*[3]*-PDGFRαΔX* construct was transfected into 293GPG cells with reagent (Lipofectamine Plus; Invitrogen). Virus-containing medium was collected for 5 days and then was concentrated (25,000*g*, 90 minutes, 4°C).[47] F cells were infected by incubation with the concentrated retrovirus in DMEM supplemented with 10% FBS and 8 μg/mL polybrene (hexadimethrine bromide; Sigma) for 24 hours. Successfully infected cells were selected in histidine-free DMEM supplemented with 0.5 mM histidinol (Sigma). Resultant cell lines were termed FαΔX; the level of the truncated PDGFRα was determined by Western blot analysis with an anti–PDGFRα antibody that recognizes the receptor's intracellular domain.

Immunoprecipitation and Western Blot Analysis

Cells were grown to 90% confluence and then incubated for 24 hours in DMEM or DMEM/F12 without serum. Unless indicated otherwise, the cells were exposed to growth factors for 10 minutes at a concentration of 50 ng/mL for PDGF, 29 ng/mL for insulin, and 100 ng/mL for all others. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in extraction buffer (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, 1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged for 15 minutes at 13,000*g*, 4°C, and PDGFRα was immunoprecipitated from clarified lysate as previously described.[15] The immunoprecipitating antibody was a crude rabbit polyclonal (27P). The blotting antibody was a 1:1 mixture of antiphosphotyrosine antibodies (4G10/PY20). The primary blot membrane was stripped and reprobed with 27P or the PDGFRα antibody from Cell Signaling. At least three independent experiments were performed. Signal intensity was determined by densitometry (Quantity One; Bio-Rad, Hercules, CA) and was normalized for the amount of PDGFRα in each sample.[15]

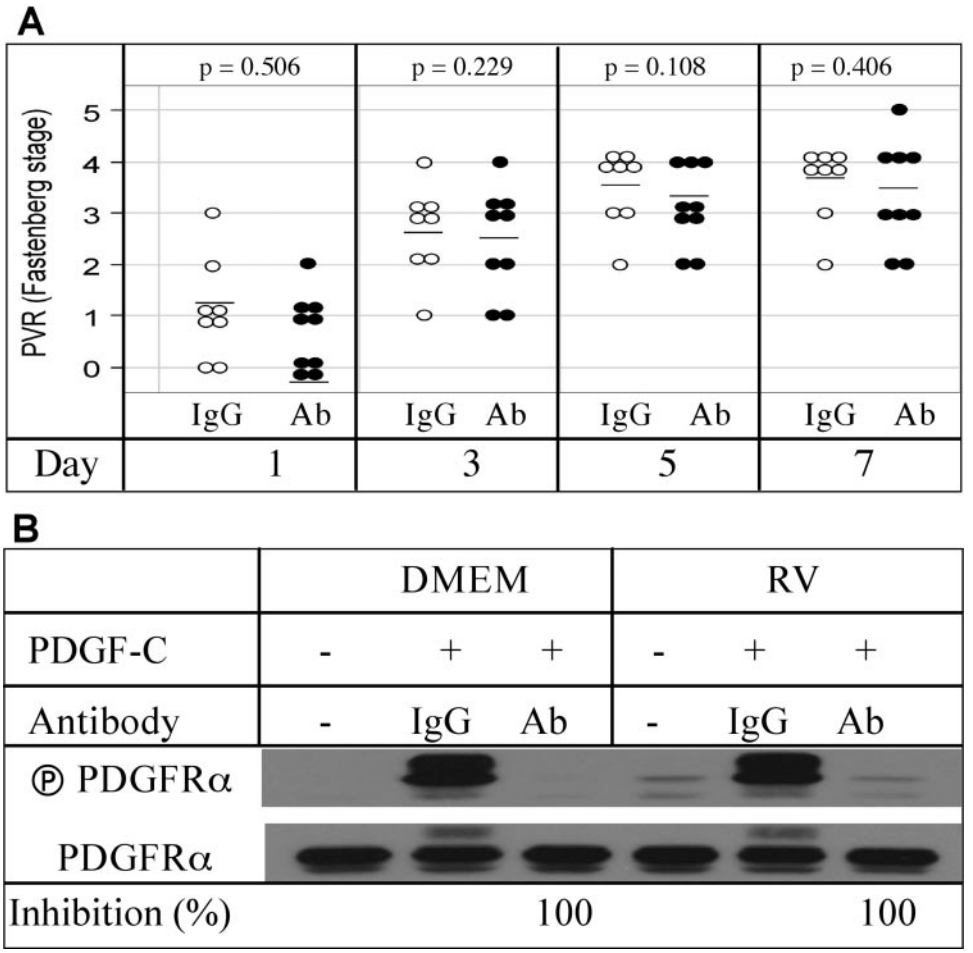

FIGURE 1. PDGF-C–neutralizing antibody did not prevent PVR. (**A**) PVR was induced in rabbits. Along with the cells (rabbit conjunctival fibroblasts), rabbits were injected with neutralizing PDGF-C antibody (Ab) or isotype-matched IgG (IgG). The concentration of antibody was calculated as follows: At day 28, there was an average of 1784 ± 150 ng PDGF-C in the 1 mL vitreous of a rabbit with PVR.[15] Assuming a linear increase from a starting point of 0.0,[15] there would be 446 ng (5.4×10^{12} molecules) PDGF-C at week 1, which would be neutralized by 0.4 μg (2.7×10^{12} molecules) of anti–PDGF-C antibody. A pilot experiment (five rabbits injected with the anti–PDGF-C Ab and five with the control IgG) showed that 0.5 μg antibody did not prevent PVR. Consequently, we increased the dose 400-fold; the results of those experiments are shown in this figure. Rabbits were observed on the indicated days. Stage 0, no disease; stage 1, membrane formation; stage 2, vitreoretinal traction, no retinal detachment; stage 3, retinal detachment, up to two quadrants; stage 4, retinal detachment, more than two quadrants but not complete detachment; stage 5, complete retinal detachment. *Horizontal bar*: mean of each group. There was no statistically significant difference between the two groups at any of the time points; consequently, the experiment was stopped at day 7. (**B**) Serum-deprived RPE19α cells were treated as indicated for 10 minutes at 37°C (PDGF-C: 100 ng/mL; IgG: isotype-matched IgG [5 μg/mL]; Ab: neutralizing PDGF-C monoclonal antibody [5 μg/mL]). Antibody and PDGF were preincubated in the presence of DMEM or healthy rabbit vitreous (RV) for 30 minutes at room temperature and then were added to cells. Cleared lysates were subjected to immunoprecipitation using a PDGFRα antibody, and resultant samples were subjected to Western blot analysis with antiphosphotyrosine (*top*) or anti–PDGFRα (*bottom*) antibodies. These data show that the neutralizing PDGF-C antibody effectively prevented PDGF-C–dependent activation of PDGFRα, and the vitreous did not interfere with this function of the antibody. These results are representative of two independent experiments.

Collagen I Contraction Assay

Contraction assay was performed as previously described.[55,56] Briefly, cells were suspended in 1.5 mg/mL neutralized collagen I (pH 7.2; INAMED, Fremont, CA) at a density of 10^6 cells/mL and were transferred to a 24-well plate (Falcon, Franklin Lakes, NJ) that had been preincubated with PBS + 5 mg/mL bovine serum albumin overnight. The gel was solidified by incubation at 37°C for 90 minutes and then overlaid with 0.5 mL DMEM ± 20 ng/mL bFGF. Media were replaced every day. The gel diameter was measured on days 1, 2, 3, and 4; it was initially 15 mm. Area was calculated using the formula $3.14 \times (\text{diameter}/2)^2$. Each experimental condition was assayed in duplicate, and at least three independent experiments were performed. A photograph of a representative contraction assay is shown (see Fig. 6A).

Rabbit Model for PVR and Preparation of Rabbit Vitreous

Pigmented or Dutch Belt rabbits were purchased from Covance (Denver, PA). PVR was induced in the right eye of each rabbit as previously described.[28,46] Briefly, gas vitrectomy was performed by injecting 0.1 mL perfluoropropane (C_3F_8; Alcon, Fort Worth, TX) into the vitreous cavity 4 mm posterior to the corneal limbus. One week later, each rabbit received three injections, as follows: 0.1 mL platelet-rich plasma; 0.1 mL DMEM containing 2×10^5 of the desired cells; and 0.1 mL buffer, or 200 μg antibody (anti–PDGF-C or IgG), or 386 μg Trap. Retinal status was evaluated with an indirect ophthalmoscope fitted with a +30 D fundus lens at days 1, 3, 5, 7, 14, 21, and 28 after surgery. PVR was graded according to the Fastenberg classification from 0 through 5.[57] On Day 28, the animals were killed, and the eyes were enucleated and frozen at −80°C. In some experiments the animals were killed at day 7. All surgeries were performed under aseptic conditions and in accordance with 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.

Rabbit vitreous was prepared by dissection from the eyeball while it was still frozen, permitted to thaw, and centrifuged at 4°C for 5 minutes at 10,000*g*. The resultant supernatant was used for all analyses.

Patient Vitreous

All human vitreous specimens were obtained from patients at Schepens Retina Associates who were undergoing vitrectomy surgery at Massachusetts Eye and Ear Infirmary (Boston, MA). Institutional review board approval to perform these studies was obtained (protocol 05–03-019X, "Assay of Human Vitreous for Activity That Processes PDGF-

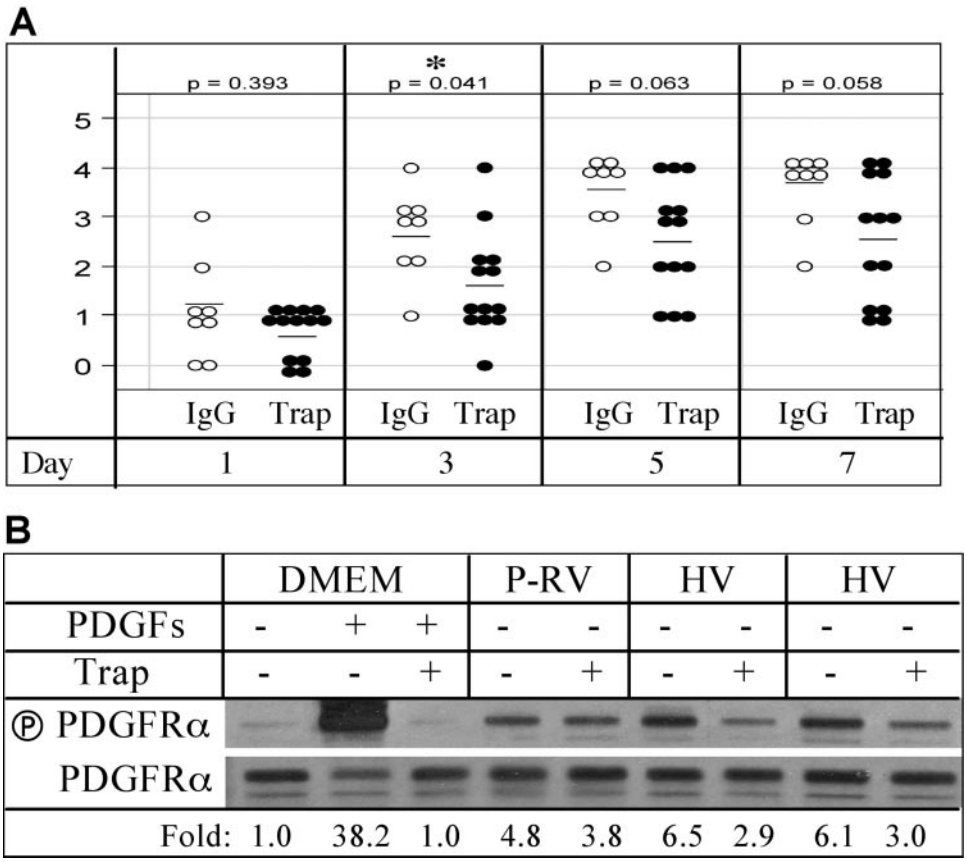

FIGURE 2. Blocking all vitreal PDGFs did not prevent PVR. (**A**) PVR was induced in the right eyes of 21 rabbits by injecting platelet-rich plasma and RCFs (rabbit conjunctival fibroblasts); 13 of the rabbits were also injected with 386 μg PDGFRα-Fc (Trap), and the other eight were injected with a nonimmune IgG. This amount of Trap is roughly 280-fold greater than the amount of all PDGFs present at week 1 (see Fig. 1 legend). The clinical status of each rabbit was evaluated at the indicated times and plotted in the graph. *Horizontal bar*: mean of each group. Data were subjected to Mann-Whitney *U* test for assessment of whether differences were statistically significant. *P* values are indicated at the tops of the figures. *Asterisk*: the difference between the two experimental groups achieved statistical significance on day 3. (**B**) RPE19α cells were deprived of serum for 24 hours, exposed for 10 minutes to a mixture of PDGFs (PDGF-A, -B, -C, each at 50 ng/mL) in the presence or absence of Trap (386 μg/mL). Vitreous from rabbits with stage 5 PVR (P-RV) or PVR patient donors (HV) was used in place of the PDGF mixture, as indicated. Cell lysates were immunoprecipitated with a PDGFRα antibody (27P).[54] Resultant samples were subjected to Western blot analysis using antiphosphotyrosine antibodies (a 1:1 mixture of 4G10 and PY20) and then were stripped and reprobed with 27P. The normalized ratio of the two signals is presented at the *bottom* of the figure. These data show that Trap effectively prevented PDGF-dependent activation of PDGFRα, vitreous activated PDGFRα poorly, and only a fraction of this activity was attributed to PDGF. Data presented are representative of at least three independent experiments.

C") before any experiments were undertaken. The research adhered to the tenets of the Declaration of Helsinki.

Statistical Analysis

Results from the rabbit studies were subjected to Mann-Whitney *U* test analysis, whereas as all other data were analyzed with the unpaired *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Blocking PDGFs Did Not Prevent PVR

To test the hypothesis that PDGFs are a suitable therapeutic target for experimental PVR, we coinjected rabbits with a neutralizing PDGF-C or a control antibody along with the combination of rabbit conjunctival fibroblasts and platelet-rich plasma. Surprisingly, even a vast excess of the antibody was unable to attenuate experimental PVR (Fig. 1A). Biochemical characterization of the antibody indicated that it effectively prevented PDGF-C–dependent activation of PDGFRα, even in the presence of rabbit vitreous (Fig. 1B).

Although PDGF-C was by far the predominant PDGF isoform in the vitreous of rabbits with PVR, PDGF-A, -AB, and -B were also present.[15] Consequently, we tested whether PDGFRα-Fc (Trap), which neutralizes all the vitreal PDGF isoforms, was able to prevent experimental PVR. As shown in Figure 2A, PVR was partially mitigated in rabbits injected with PDGF Trap. However, Trap did not protect from PVR nearly as well as receptor-directed strategies.[28–30] These observations raised the intriguing possibility that PDGFRα was activated by vitreal agents other than PDGFs.

One explanation for the inability of the antibody and Trap to protect against PVR was that it was either absent from the vitreous or unable to neutralize PDGFs. To address these possibilities, we harvested the vitreous from the killed animals and determined the amount of antibody and Trap present. As shown in Figures 3A and B, both neutralizing agents were present at the end of the experiment. Quantification of the amount of these agents indicated that it was sufficient to neutralize all the vitreal PDGFs (see the Fig. 3 legend for calculations).

To test whether this was indeed the case, we determined whether the vitreous from these animals was unable to activate PDGFRα. As shown in Figure 3C, vitreous from rabbits without PVR (N-V) activated PDGFRα poorly. Activation was no better when using vitreous from rabbits that developed PVR and were injected with agents to neutralize PDGFs (Fig. 3C; compare the first three lanes). These results indicate that the antibody and Trap effectively blocked vitreal PDGFs. Furthermore, there was sufficient quantity of the neutralizing agents to prevent the activation of exogenously added PDGFs (Fig. 3C; compare lanes 4 and 5 with 6 and 7). Together the data in Figure 3 indicate that the antibody and Trap were present and capable of blocking PDGF-dependent activation of PDGFRα.

In the course of characterizing Trap, we noted two unusual properties of the PVR rabbit vitreous. First, though it contained a saturating dose of PDGFs, it routinely induced only weak phosphorylation of PDGFRα (12.6% of control in the represen-

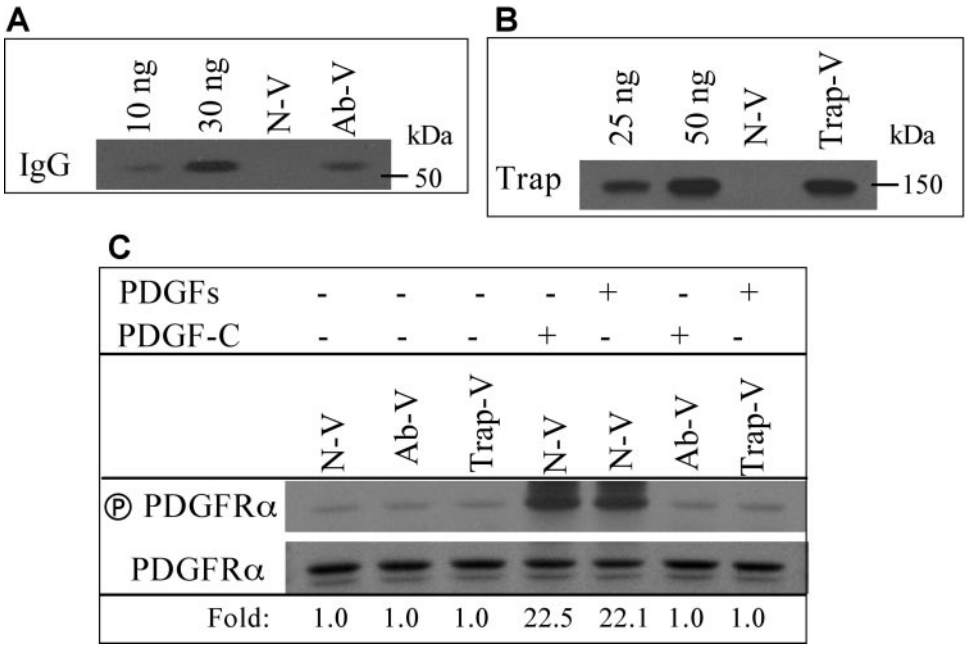

FIGURE 3. Antibody and Trap were present and active. Rabbits used in the experiments shown in Figures 1 and 2 were killed on days 7 and 28, respectively, and the vitreous was subjected to Western blot analysis for assessment of the amount of the injected antibody or Trap that persisted until the end of the experiment. (**A**) A total of 3 μL vitreous (N-V, normal rabbit vitreous; Ab-V, antibody-injected vitreous) was subjected to Western blot analysis using an HRP-conjugated goat anti–mouse antibody. Several doses of purified mouse IgG (10 ng, 30 ng) were included to quantify the amount of antibody in the vitreous. Although this panel depicts results of a representative experiment, quantifying the results from three rabbits indicated that 6.9 ± 0.1 ng/μL of the injected anti–PDGF-C antibody was present in the vitreous at day 7. Assuming that the volume of the rabbit vitreous was 1 mL, we calculated that 3.5% of the injected antibody was present after 1 week; 3.5% of a 400-fold excess of the amount of antibody needed to neutralize the PDGF-C (see Fig. 1A legend) should have been able to block 14 times the amount of PDGF-C present. (**B**) A total of 10 μL vitreous was analyzed was subjected to Western blot analysis using an HRP-conjugated goat anti–human antibody (the Fc portion of the Trap is human). N-V, normal rabbit vitreous; Trap-V, trap-injected vitreous. Several doses of purified Trap (25 ng, 50 ng) were included to quantify the amount of Trap in the vitreous. Although this panel depicts results of a representative experiment, quantifying the results from three rabbits indicated that there was 3.8 ± 0.5 ng/μL of the Trap in the vitreous at day 28. Assuming that the volume of the rabbit vitreous was 1 mL, we calculated that 0.98% of the Trap persisted until week 4. The initial dose of Trap was 280-fold in excess of the amount needed to block all PDGFs present at week 1 (see Fig. 2A legend). Thus, there appeared to be a sufficient dose of Trap in the vitreous at week 1 because there was a 2.7-fold excess of Trap at week 4. (**C**) Serum-starved RPE19α cells were exposed to vitreous exactly as described in (**A**) and (**B**). In some cases the vitreous was supplemented with PDGF (as described in Fig. 1B). Cells were lysed, and cleared lysates were subjected to sequential Western blot analysis with an anti–phospho-PDGFRα antibody (*top*), followed by an anti–pan-PDGFRα antibody (*bottom*). Neutralizing agents were capable of preventing vitreal PDGFs from activating PDGFRα (*lanes 1–3*). The capacity of the neutralizing agents was sufficiently high to prevent the activation of PDGFRα by exogenously added PDGFs (*lanes 4–7*). Results shown are representative of three independent experiments.

tative experiment shown in Fig. 2B, lane 2). The discovery that vitreal PDGFs were impotent was consistent with the finding that neutralizing them did not prevent PVR (Figs. 1, 2). Second, while Trap efficiently blocked phosphorylation of PDGFRα in response to a cocktail of PDGF isoforms, it reproducibly failed to completely prevent vitreous-stimulated phosphorylation of PDGFRα (Fig. 2B; compare lanes 2 and 3 with lanes 4 and 5). As with the vitreous from PVR rabbits, vitreous from patients with PVR induced only weak phosphorylation of PDGFRα, and this response was only partially inhibited by Trap (Fig. 2B). These data suggested that PDGFs in the vitreous were not performing to their full capacity and that the vitreous contained agents outside the PDGFR family that could trigger the phosphorylation of PDGFRα.

Non-PDGFs Induced Tyrosine Phosphorylation of PDGFRα

We considered the possibility that growth factors outside the PDGF family (non-PDGFs) induced the phosphorylation of PDGFRα because vitreous contains many non-PDGFs,[9,12–14,16–25] and expression of PDGFRs can enhance certain signaling events induced by non-PDGFs.[45] We found that four different vitreal non-PDGFs reproducibly triggered low-level phosphorylation of PDGFRα (Fig. 4A). In three independent experiments, the fold differences were 4.0 ± 0.3, 3.4 ± 0.2, 1.8 ± 0.1, and 2.6 ± 0.2 for bFGF, EGF, insulin, and HGF, respectively. These data revealed that vitreal non-PDGFs were capable of increasing tyrosine phosphorylation of PDGFRα.

We also investigated whether PDGF promoted tyrosine phosphorylation of FGFR. Repeated attempts failed to detect a PDGF-dependent increase in the phosphotyrosine content of FGFR1 (Fig. 4B). Thus, though non-PDGFs promoted tyrosine phosphorylation of PDGFRα, the reciprocal relationship did not appear to exist.

Extracellular Domain of PDGFRα Was Not Required for Its Phosphorylation by Non-PDGFs

We considered whether non-PDGFs increased the tyrosine phosphorylation of PDGFRα through PDGF-C because the cell types used in these studies secreted latent PDGF-C,[15] which can be proteolytically activated.[15] To this end, we tested whether non-PDGFs lost their ability to increase tyrosine phosphorylation when PDGFRα was without its ligand-binding domain. Although PDGFRα that lacked the ligand binding domain (αΔX) failed to respond to PDGF-A, it underwent tyrosine phosphorylation in response to bFGF (Fig. 5A). The extent of bFGF-induced tyrosine phosphorylation in the full-length and truncated PDGFRs was comparable (Fig. 5A), as were their expression levels (Fig. 5B). We concluded that the extracellular domain of PDGFRα was dispensable for these non–PDGF-initiated events.

Indirect Activation of PDGFRα Promoted Cellular Events Intrinsic to PVR

We considered whether activation of PDGFRα by non-PDGFs was sufficient to promote cellular events intrinsic to

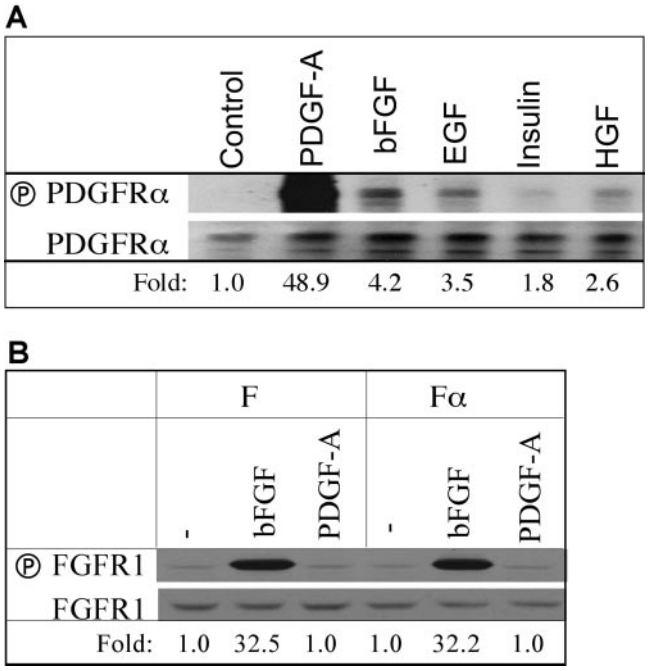

FIGURE 4. Non-PDGFs promoted the tyrosine phosphorylation of PDGFRα. (**A**) RPE19α cells were deprived of serum for 24 hours and were treated for 10 minutes with PDGF-A (50 ng/mL), bFGF (100 ng/mL), EGF (100 ng/mL), HGF (100 ng/mL), or insulin (29 ng/mL). Cells were lysed, and PDGFRα was immunoprecipitated and subjected to Western blot analysis using antiphosphotyrosine (*top*) or anti–PDGFRα antibodies. Data were quantified and are expressed as a ratio of stimulated to unstimulated. Results show that non-PDGFs promoted tyrosine phosphorylation of PDGFRα. Data are representative of three independent experiments. (**B**) F and Fα cells were cultured to 90% confluence and then starved for 24 hours in serum-free medium. Cells were left untreated (−) or were exposed to bFGF (100 ng/mL) or PDGF-A (50 ng/mL) for 10 minutes at 37°C and lysed, and FGFR was immunoprecipitated with an anti–FGFR1 antibody. Resultant samples were subjected to Western blot analysis using a combination of PY20 and 4G10 antiphosphotyrosine antibodies. The membrane was subsequently reprobed with an anti–FGFR1 antibody. Results show that PDGF did not promote tyrosine phosphorylation of FGFR1. Data are representative of two independent experiments.

PVR. In these experiments we focused on the contraction of cells in a collagen gel, which models the sight-robbing phase of PVR in which the cells in the membrane contract and result in retinal detachment. A representative non-PDGF (bFGF) triggered modest contraction in cells that did not express PDGFRα (Figs. 6A, B). This response was greater in cells expressing either full-length, or αΔX PDGFRα (Figs. 6A, B). Thus, indirect activation of PDGFRα greatly enhanced the ability of cells to contract collagen gels in response to non-PDGFs such as bFGF.

Indirect Activation of PDGFRα Was Sufficient for PVR

Given that indirect activation of PDGFRα promoted cellular events intrinsic to PVR, we tested whether it was sufficient to drive PVR itself. Therefore, we compared the PVR potential of F cells expressing no PDGFRs, the full-length PDGFRα, and the αΔX truncated receptor. Consistent with our previous findings,[28,58,59] cells that expressed no PDGFRs induced PVR poorly; that is, none of the rabbits underwent retinal detachment (Fig. 6C). Expression of PDGFRα dramatically increased the PVR potential of these cells such that greater than 50% of the rabbits experienced at least partial retinal detachment (stage 3 or higher) by day 3, and 100% of the rabbits detached at later time points (Fig. 6C). This was a more robust response than we previously observed,[28,58,59] and it might have occurred because more cells (250,000 instead of 200,000) were injected in this series of experiments. The PVR potential of cells expressing the truncated receptor was weaker than the full-length receptor at the earliest time points (Fig. 6C). This observation is consistent with the finding that Trap slowed the progression of PVR in rabbits injected with RCFs (Fig. 2). It appears that direct activation of PDGFRα is important at the beginning of the disease; intense, direct activation of PDGFRα may result from the presumably high level of PDGFs supplied by the coinjected platelet-rich plasma. Importantly, most of the rabbits injected with cells expressing the truncated receptor also experienced retinal detachment at the later time points (Fig. 6C). There was no statistically significant difference in PVR score between the Fα and αΔX groups at any of the time points beyond day 3.

We conclude that though direct activation of PDGFRα may be important to accelerate the onset of the disease, it was not

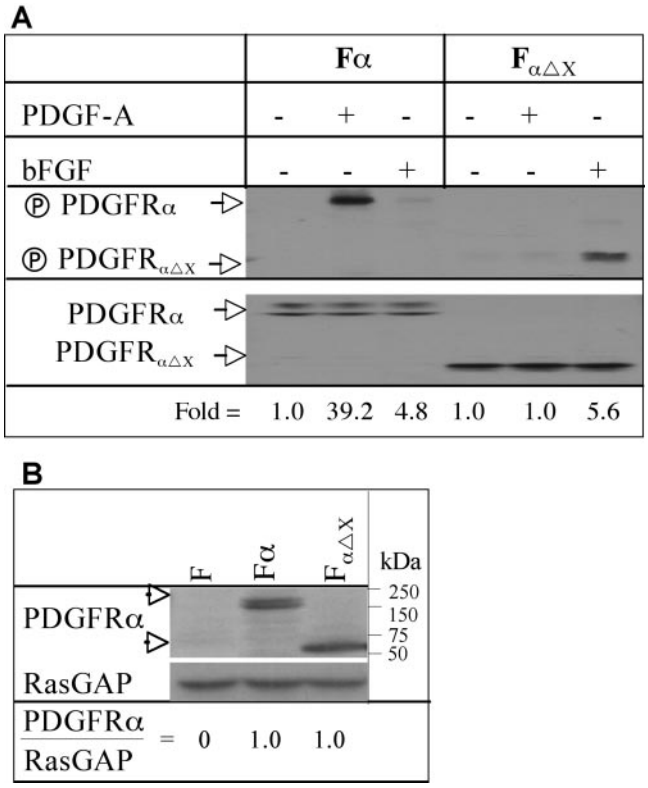

FIGURE 5. Indirect activation of PDGFRα was independent of its ligand-binding domain. (**A**) Serum-starved F cells expressing full-length PDGFRα (Fα) or the truncated PDGFRα ($F_{\alpha\Delta x}$) were exposed to bFGF or PDGF-A for 10 minutes. PDGFRα was immunoprecipitated from cleared lysates using the 27P antibody, and the resultant samples were subjected to Western blot analysis using anti–phosphotyrosine antibodies (*top*) or an anti–PDGFRα antibody (*bottom*). The extent of PDGFRα phosphorylation was normalized to the amount of PDGFRα immunoprecipitated and was expressed as a ratio of stimulated to unstimulated. bFGF induced comparable phosphorylation of the full-length and truncated receptors. Data are representative of at least three independent experiments. (**B**) Cleared lysates from the indicated cell lines were subjected to Western blot analysis using an anti–PDGFRα or an anti–RasGAP antibody. Results show similar levels of PDGFRα within the various panels of cell lines. These experiments were repeated on at least two independent occasions.

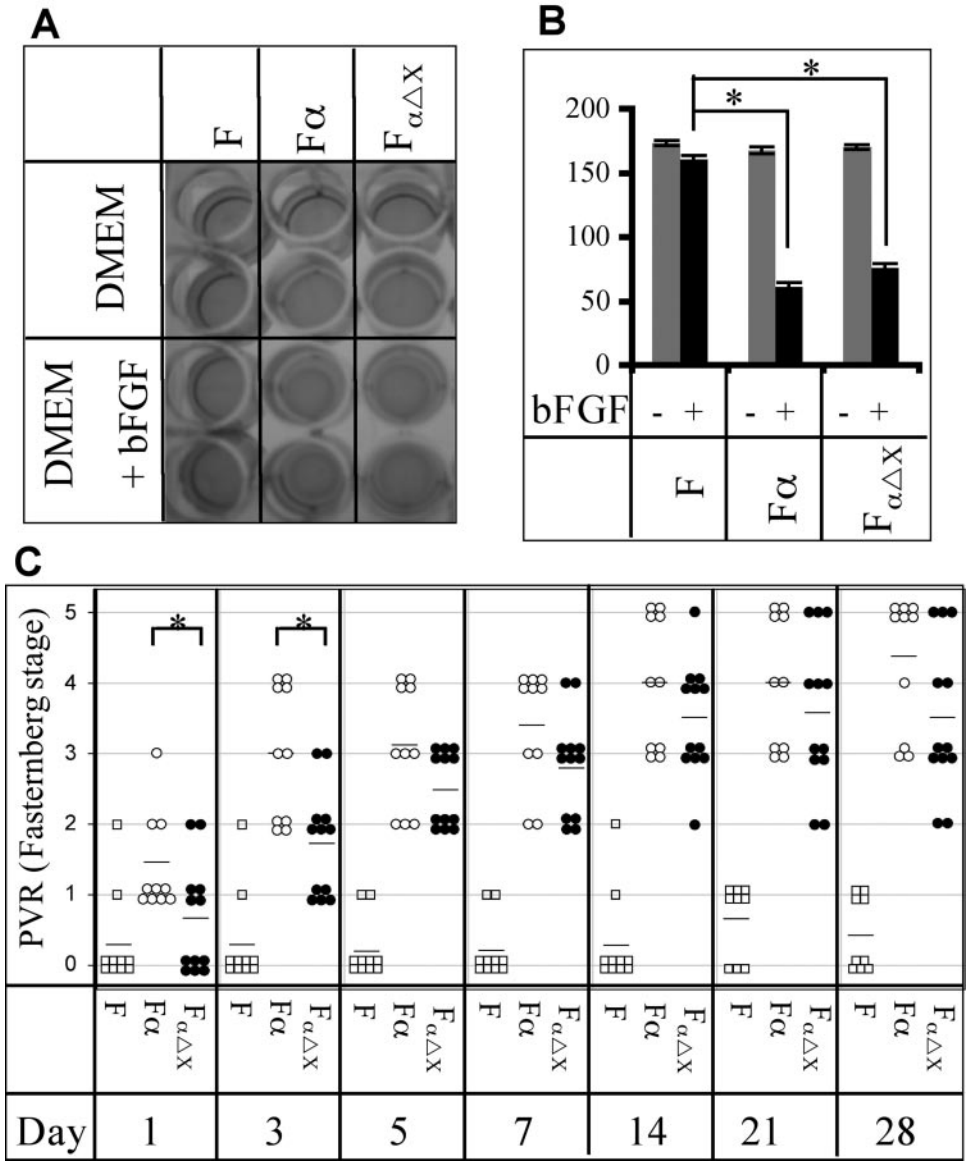

FIGURE 6. Expression of PDGFRα promoted non–PDGF-driven contraction and PVR. (**A**, **B**) F, Fα, or FαΔX cells were embedded in a collagen matrix at a density of 10^6 cells/mL and aliquoted into a 24-well plate (300 μL cells/well). After the collagen matrix was solidified, serum-free medium was added that was (+) or was not (−) supplemented with 20 ng/mL bFGF. Media were replaced every day. Contraction of the collagen gel was measured on day 3, and the raw data (in mm) are presented. *Gray bars*: response in the absence of bFGF. *Black bars*: response in the presence of bFGF (20 ng/mL). Mean ± SD of three independent experiments is shown. $^*P < 0.05$ using an unpaired *t*-test. bFGF-induced fold changes in contraction for F, Fα, and FαΔX were 0.92 ± 0.06, 0.37 ± 0.03, and 0.45 ± 0.02, respectively. bFGF promoted only weak contraction in cells that expressed no PDGFRα. Expressing full-length or truncated PDGFRα dramatically improved the ability of cells to contract in response to non-PDGFs. (**C**) PVR was induced in rabbits by injecting F, Fα, or FαΔX cells along with platelet-rich plasma. None of the rabbits injected with F cells developed retinal detachment. Although there was a statistically significant difference in the Fα and FαΔX groups at days 1 and 3, both groups experienced retinal detachment at the later time points. One of the rabbits in the F group developed inflammation after day 14 and was no longer included in the later time points. Results show that though PDGF-dependent activation of PDGFRα accelerated the onset of disease, it was not required for experimental PVR.

required for retinal detachment. Furthermore, the data suggest that PDGFRα expression potently promoted PVR because it can be activated by a wide spectrum of vitreal growth factors, not only those within the PDGF family.

DISCUSSION

The findings described in this report redefine our working hypothesis of how PDGFRα promotes experimental PVR (Fig. 7). When cells are coinjected with platelet-rich plasma into the vitreous of healthy rabbits, non-PDGFs engage not only their own receptors but PDGFRα as well. This mode of indirect activation of PDGFRα boosts cellular responses such as contraction and thereby tips the balance toward disease progression. Although direct activation of PDGFRα by vitreal PDGFs accelerates the onset of PVR, this traditional route for PDGFRα activation is not a prerequisite for disease formation.

There are several reasons to suspect that non-PDGFs act through PDGF-C to activate PDGFRα. The cell types used in these studies secrete PDGF-C.[15] Although this PDGF-C is latent and requires proteolytic processing, the cells also produce the proteases capable of activating PDGF-C.[15] Furthermore, there is precedent for protease-based cross-talk between receptors. Activation of G protein–coupled receptors increases the activity of metalloproteases that release bound EGF (HB-EGF), which activates the EGF receptor.[60] However, the fact that non-PDGFs activated PDGFRα even when it lacked the extracellular ligand-binding domain indicated that the mechanism was independent of direct activation of PDGFRα via PDGF-C.

We observed activation of PDGFRα by non-PDGFs in all five cell lines we have examined to date. These cell types include A-RPE19 cells (human), primary rabbit conjunctival fibroblasts, and three lines of mouse embryo species fibroblasts.[61] Thus, activation of PDGFRα by non-PDGFs was not restricted by cell type or whether the cells had been immortalized. Although these findings using cultured cells do not necessarily extend to the behavior of cells that are responsible for clinical PVR, several observations are consistent with the possibility that non-PDGFs promote the activation of PDGFRα in this context as well. PDGFRα is expressed and activated in membranes from patients with PVR,[27] and the vitreous from such donors contains non-PDGFs that activate PDGFRα (Fig. 2B).

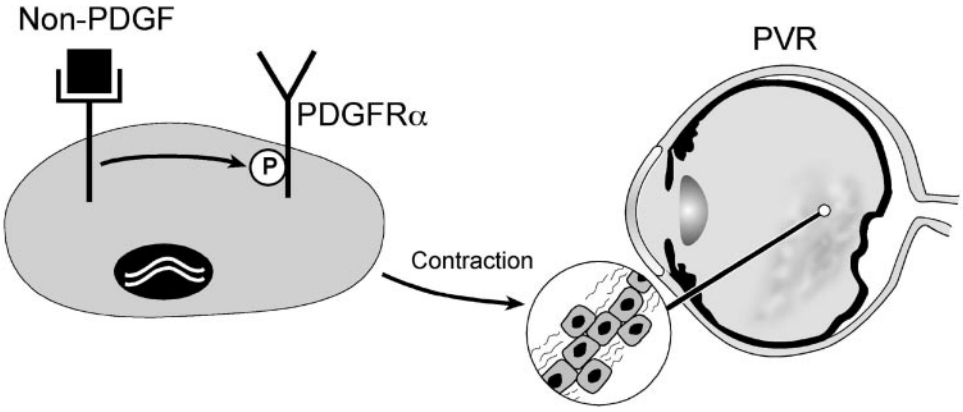

FIGURE 7. A model for how non-PDGFs drive experimental PVR through PDGFRα. Entry of cells into the vitreous (by injection in the context of the experimental model or as the result of retinal tearing/trauma in the patient) exposes the cells to many growth factors outside the PDGF family. Acting through their own receptors, these non-PDGFs trigger signaling events that are insufficient to precipitate PVR. If the cells express PDGFRα, the non-PDGFs activate it and thereby boost cellular responses relevant to PVR, such as contraction. Potentiating these types of cellular responses tips the balance in favor of disease progression and results in PVR.

This newly described mechanism by which PDGFRα promotes pathology differs from the three previously reported mechanisms. First, excessive, direct activation of PDGFRα by PDGF-based paracrine or autocrine loops participates in the manifestation of numerous cancers, including glioblastomas and ovarian tumors.[62–64] Second, gastrointestinal stromal tumors harbor PDGFRα with activating mutations,[65] whereas a variety of translocations are present in certain myeloid disorders and leukemias.[66–68] Third, activating autoantibodies engage PDGFRα in the context of systemic sclerosis.[44] Indirect activation of PDGFRα by non-PDGFs, as we describe herein, represents a fourth mechanism by which PDGFRα can be recruited for pathologic purposes.

The discovery that neutralizing vitreal PDGFs did not profoundly attenuate experimental PVR is an important advance in our search for potential therapeutic targets for PVR. The data presented in this report indicate that PDGFRα may be the Achilles heel of PVR because blocking its activity reduces the pathologic input of numerous vitreal growth factors. Evaluating US Food and Drug Administration–approved agents that inhibit PDGFRα for their capacity to block experimental PVR seems a viable strategy to quickly identify agents that are most likely to be useful to treat patients with PVR.

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