Growth Factors, Cytokines, and Cell Cycle Molecules

A Novel Strategy to Develop Therapeutic Approaches to Prevent Proliferative Vitreoretinopathy

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Proliferative vitreoretinopathy (PVR) thwarts the repair of rhegmatogenous retinal detachments. Currently, there is no effective prevention for PVR. Platelet-derived growth factor receptor α (PDGFR α) is associated with PVR in humans and strongly promotes experimental PVR driven by multiple vitreal growth factors outside the PDGF family. We sought to identify vitreal factors required for experimental PVR and to establish a potential approach to prevent PVR. Vitreous was obtained from normal rabbits or those in which PVR was either developing or stabilized. Normal vitreous contained substantial levels of growth factors and cytokines, which changed quantitatively and/or qualitatively as PVR progressed and stabilized. Neutralizing a subset of these agents in rabbit vitreous eliminated their ability to induce PVR-relevant signaling and cellular responses. A single intravitreal injection of neutralizing reagents for this subset prevented experimental PVR. To identify growth factors and cytokines likely driving PVR in humans, we subjected vitreous from patients with or without PVR to a similar series of analyses. This analysis accurately identified those agents required for vitreous-induced contraction of cells from a patient PVR membrane. We conclude that combination therapy encompassing a subset of vitreal growth factors and cytokines is a potential approach to prevent PVR. (*Am J Pathol* 2011, 179:2931–2940; DOI: 10.1016/j.ajpath.2011.08.043)

Proliferative vitreoretinopathy (PVR) remains the most serious sight-threatening complication in patients recovering from surgery to repair retinal detachment.^{1–3} Although the incidence of PVR after primary reattachment repair is 5% to 10%, this number rises to 25% in patients whose retinal detachment occurred after severe ocular trauma.^{4,5} The need for a pharmacological treatment to

prevent PVR and thus improve the success of long-term recovery is paramount. Repeated retinal reattachment surgery is currently the only treatment option for individuals afflicted with PVR. Consequentially, almost half of patients suffering PVR after primary reattachment surgery will experience recurring PVR and near or total vision loss.^{6,7}

Although the etiology of PVR is not completely understood, the dislocation of cells to vitreous during retinal detachment is widely believed to be a contributing factor. Cells found in the epiretinal membrane (ERM) are retinal pigment epithelial (RPE) cells, fibroblasts, fibroblast-like cells (which may be dedifferentiated RPEs), glial cells, and to a much lesser extent, macrophages.^{8–12} Substantial evidence indicates that PVR is driven by growth factors and cytokines present in the vitreous (see Supplemental Table S1 at <http://ajp.amjpathol.org>), which promote cellular responses intrinsic to PVR such as cell survival, proliferation, and migration.^{8–28} These growth factors and cytokines are also capable of inducing production of extracellular matrix proteins that are a major component of ERMs. Finally, growth factors and cytokines drive contraction of the retina-associated ERM, which generates traction that can open treated retinal breaks and/or create new ones and thereby compromise vision.

The most common of the 26 animal models of PVR involves intravitreal injection of fibroblasts²⁹ that organize into an ERM, which contracts and thereby detaches the retina. In the context of this model, platelet-derived growth factor (PDGF) receptor α (PDGFR α), expressed by the injected fibroblasts, is a key determinant of PVR.^{30–32} PDGFR α is expressed and activated in ERMs from human donors, indicating an association of PDGFR α with clinical PVR.^{27,31,33}

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Table 1. Antibodies and Neutralizing Reagents Used in This Study

Reagent	Source/specificity	Type*	Effective dose <i>in vitro/in vivo</i> [†]	Producer	Additional notes
TRAP	h/h,m,rb	sRfrg	5/250 μ g/mL	ZymoGenetics (Seattle, WA)	PDGFR α (ecd)/IgG-Fc chimera (Wong et al ³⁷)
sEGFR	h/h,m,rb	sRfrg	4/200 μ g/mL	R&D Systems	EGFR(ecd)/IgG-Fc chimera (traps EGF and TGF- α)
α -HGF	g/h,rb	nAb	Manu./50 $\times$	R&D Systems	Also used for quantification by IB
α -FGF-2	m/h,rb	nAb	Manu./50 $\times$	Millipore	Neutralizes FGF-2 (basic FGF) isoform
α -TGF- β	rb/h,rb	nAb	Manu./50 $\times$	R&D Systems	Detects all isoforms (TGF- β 1, - β 2, and - β 3)
α -IGF-1	m/h,rb	nAb	Manu./50 $\times$	Millipore	Also used for quantification by IB
α -IFN γ	m/h,rb	nAb	Manu./50 $\times$	Millipore	
α -VEGF-A	m/h,rb	nAb	Manu./50 $\times$	R&D Systems	
α -IL-8	m/h,rb	nAb	Manu./50 $\times$	Millipore	
α -IL-6	m/h,rb	nAb	Manu./50 $\times$	Millipore	
α -G-CSF	rb/h,rb	nAb	Manu./50 $\times$	Millipore	
α -PDGF-C	h/h,rb	IB-Ab	1:2000 (serum)	ZymoGenetics	α -PDGF core, used for IB quantification ²⁶
α -PDGFR α	rb/h,m,rb	IB-Ab	1:1000 (serum)	See Lei et al, ²⁶ Wong et al ³⁷	Designated "27P"
α -pPDGFR α	rb/h,m,rb	IB-Ab	200 ng/mL	Santa Cruz Biotechnology	Recognizes pY720
α -pPDGFR α	rb/h,m,rb	IB-Ab	1:2000 (serum)	See Robbins et al ³³	Recognizes pY742
α -Akt	rb/h,m	IB-Ab	100 ng/mL	Cell Signaling	
α -pAkt	rb/h,m	IB-Ab	200 ng/mL	Cell Signaling	
α -p53	m/h,m	IB-Ab	200 ng/mL	See Lei et al, ²⁶ Robbins et al, ³³ Wong et al ³⁷	
α -RasGAP	rb/h,m	IB-Ab	1:2000 (serum)	Kazlauskas lab	
α -CTGF	g/h,m,rb	IB-Ab	200 ng/mL	Santa Cruz Biotechnology	Recognizes both N- and C-terminal CTGF (fragments)
HRP- α -m-IgG	g/m	2nd	Manu.	Santa Cruz Biotechnology	See footnote [‡]
HRP- α -rb-IgG	g/rb	2nd	Manu.	Santa Cruz Biotechnology	See footnote [‡]
isotype IgGs	m,rb,g/con	con	NA	Santa Cruz Biotechnology	

*2nd indicates the secondary antibody for Western blot analysis.

[†]The first (or only) value shown is the concentration used in cell-based experiments; the second value is concentration present in rabbit eyes initially following injection.

[‡]Enhanced chemiluminescent substrate for horseradish peroxidase detection is from Pierce (Rockford, IL).

50 $\times$, 50-fold higher than recommended (manu.) dose; Ab, antibody; con, non-immune isotype control IgGs; ecd, extracellular domain of receptor; g, goat; h, human; IB, immunoblot; m, mouse; Manu., set according to manufacturer's instructions; NA, not applicable; nAb, neutralizing antibody; pY, phosphotyrosine; rb, rabbit; serum, serum antibody; sRfrg, soluble receptor fragment.

Although PDGF isoforms that activate PDGFR α abound in both human and rabbit PVR vitreous, they do not appear to be responsible for activating PDGFR α .^{26,34} An indirect mechanism predominates in which vitreal growth factors outside of the PDGF family promote an intracellular route to activate PDGFR α .³⁵ This indirect mode results in chronic activation of PDGFR α because it circumvents receptor internalization and degradation.³⁶ Importantly, indirectly activated PDGFR α engages a characteristic set of signaling events and cellular responses that are tightly associated with PVR.³⁶ The goal of this study was to identify the subset of vitreal growth factors and cytokines that was required for experimental PVR, and to develop potential therapeutic approaches to protect patients from developing PVR.

Materials and Methods

Growth Factors, Antibodies, and Neutralization Reagents

Recombinant human growth factors were either used directly to treat cells or as standards to quantify vitreal growth factors by Western immunoblot analysis. All antibodies and

neutralizing reagents used in this study are detailed in Table 1. Recombinant human PDGF-A, hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), interleukin (IL)-8, tumor growth factor- α (TGF- α), TGF- β 1, interferon (IFN)- γ , and connective tissue growth factor (CTGF) were purchased from Peprotech (Rocky Hill, NJ), whereas recombinant human epidermal growth factor (EGF), fibroblast growth factor (FGF)-2, and vascular endothelial growth factor (VEGF)-A were from the National Cancer Institute.

Quantifying Growth Factors and Cytokines in Vitreous

A panel of 24 growth factors and cytokines were quantified in individual vitreous samples from rabbits and humans with or without PVR. Multiplex assays were performed to measure 20 of 24 of the growth factors/cytokines, whereas quantitative Western immunoblot analysis was performed to measure the remaining 4.

Multiplex Assay and Analysis

Multiplex bead analysis was performed using two different immunoassay kits obtained from Millipore (Billerica,

MA). One, the Milliplex MAP customized 17-Plex Human Cytokine/Chemokine assay was used to measure the vitreal levels of EGF, FGF-2, granulocyte colony-stimulating factor (G-CSF), IFN γ , IL-1 β , IL-6, IL-8, IL-10, monocyte chemoattractant protein-1 (MCP-1), TGF- α , tumor necrosis factor- α (TNF- α), TNF- β , VEGF, granulocyte-macrophage colony-stimulating factor (GM-CSF), PDGF-A, PDGF-AB, and PDGF-B. Two, the Milliplex MAP pan-TGF- β 1, -2, -3 (3-Plex) assay measured the vitreal levels of activated TGF- β 1, TGF- β 2, and TGF- β 3. Immunoassays were performed based on the manufacturer's instructions. Briefly, vitreous samples were added in triplicate wells on a 96-well plate and incubated overnight with a mixture of monoclonal antibody-coated capture beads. Beads were then washed and incubated with biotin-labeled polyclonal anti-human growth factor/cytokine antibodies for 1 hour, after which streptavidin-phycoerythrin was added for 30 minutes. Fluorescent emissions distinct to each growth factor or cytokine were simultaneously measured using the BioPlex Detection System and analyzed using BioPlex software (Bio-Rad, Hercules, CA). Growth factor/cytokine concentrations were determined based on a series of standards run in parallel. Results from individual samples ($n = 10$ for rabbits, $n = 5$ for humans) belonging to the same group were compiled and statistically analyzed.

Quantitative Western Immunoblot and Analysis

Quantitative Western immunoblot analysis was performed to measure the vitreal levels of HGF, IGF-1, CTGF, and PDGF-C since the multiplex platform is not yet available to measure these factors. An aliquot of the same samples that were subjected to multiplex analysis was used for quantitative Western immunoblot analysis. Vitreous was run on an 8% to 12% SDS-PAGE gel alongside recombinant human growth factor/cytokine standards. Proteins were transferred to PVDF membranes and blotted with α -HGF, α -IGF-1, α -CTGF, or α -PDGF-C detection antibodies (Table 1). Signal intensity was determined by densitometry using Quantity One (Bio-Rad) and growth factor/cytokine concentrations determined based on the known concentrations of standards run on the same gel. In some cases, membranes were stripped and re-probed to quantify multiple growth factors and cytokines from a single gel.

Cell Culture

Primary mouse embryonic fibroblasts (MEFs) were obtained at third passage from American Type Culture Collection (Manassas, VA). Retinal pigment epithelial cells from human PVR membranes (RPEM cells) were derived from a surgically removed membrane of a PVR patient³³; these cells were used at passage 4 or 5. Primary rabbit conjunctiva fibroblasts were isolated as described previously.³⁸

MEFs and rabbit conjunctiva fibroblasts were maintained in high glucose-containing Dulbecco's modified Eagle's medium (hg-DMEM; Gibco BRL/Invitrogen, Carlsbad, CA). RPE cells were maintained in a 1:1 mixture of hg-DMEM and Ham's F12 medium (Gibco BRL/Invitrogen). All cells were incubated at 37°C in a humidified 5% CO₂ atmosphere, and

cultured in medium supplemented with 10% FBS, 500 U/mL penicillin, and 500 μ g/mL streptomycin.

Cell Treatment

For *in vitro* experiments, near-confluent cultures of cells were starved of serum overnight and treated the next morning. Vitreous used in cell treatments was always an equal-volume mix of several individual samples. For treatment, vitreous was added directly to cells after removal of media. For vitreous treatments involving neutralizations, neutralizing agents were preincubated with vitreous for 30 minutes at room temperature before addition to cells.

Western Blot Analysis

After treatment, cells were washed twice using ice-cold phosphate-buffered saline (PBS), and then lysed by addition of sample buffer [50 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 1% β -mercaptoethanol, 10 mmol/L EDTA, and 0.02% bromophenol blue]. Lysates were incubated on ice for 15 minutes, heated to 95°C for 5 minutes, then clarified by centrifugation at 13,000 $\times$ *g*, 4°C, for 15 minutes. The supernatant was run on an 8% to 12% SDS-PAGE gel. Data presented for all immunoblots in this study are representative of three independent experiments unless otherwise noted. Signal intensity was determined by densitometry using Quantity One (Bio-Rad), standardized to the background and then normalized for loading.

Growth Factor/Cytokine Neutralization

All neutralizing antibodies and ligand traps used in this study are detailed in Table 1.

In Vitro Neutralization Assays

A 2- to 25-fold excess of neutralization reagent was used, based on the concentration of its intended growth factor/cytokine target in the vitreous. An equivalent amount of isotype-matched IgG was used as a control.

Neutralization of Growth Factors/Cytokines in Rabbit Eyes

In rabbits, a 50-fold molar excess over the amount used *in vitro* was used for each neutralization reagent making up the neutralizing reagent solution rNb. A 14-fold excess of neutralization antibody and a 21-fold excess of ligand Trap persists in vitreous at 1.5 weeks when a 50-fold excess of these molecules are initially injected.³⁴ Control treatments were isotype-matched with equal amounts of IgG.

Cell Contraction Assay

This assay relates to the ability of cells in the ERM to exert traction on the retina (which causes detachment) and is one way to assess the effects of various treatments on the contractility of cells. The assay was performed as previously described.^{39,40} Briefly, a cell suspension was prepared

containing 1.5 mg/mL of neutralized collagen I at pH 7.2 (INAMED, Fremont, CA) and 1×10^6 cells/mL, and transferred to a 24-well plate pre-incubated with PBS plus 5 $\mu\text{g}/\mu\text{L}$ bovine serum albumin (BSA) for at least 6 hours. Collagen gels were solidified by incubating them at 37°C for 90 minutes. Solidified gels were overlaid with 0.5 mL of DMEM or DMEM plus whatever treatment was used. Medium containing the desired treatment was changed daily. The gel diameter was measured on days 0, 1, 2, and 3. At day 0, the diameter of the gel equals the diameter of the well. Triplicates were performed in each experiment. For each assay, no less than two independent experiments were performed.

Rabbit PVR Model

Adult female (3 to 6 month) Dutch-belted rabbits were purchased from Covance (Denver, PA). PVR was induced in the right eye of rabbits as previously described.^{30,38} Briefly, after 1 week of acclimatization, gas vitrectomies were performed in rabbits by injecting 0.1 mL of perfluoropropane (C_3F_8) (Alcon, Fort Worth, TX) into the vitreous cavity 3 mm posterior to the corneal limbus. A week later, each rabbit was injected with 0.1 mL of platelet-rich plasma (PRP) and 0.1 mL DMEM containing 2×10^5 rabbit conjunctival fibroblasts. PRP was included to enhance the severity and consistency of the response to injected cells.³⁰ We also injected 0.1 mL of balanced saline solution containing either the neutralizing reagents ("rNb"), or isotype-matched control immunoglobulin ("IgGs").

Intraocular pressure was monitored, and retinal status evaluated using an indirect ophthalmoscope and a +30-D fundus lens at days 1, 3, 5, 7, 14, 21, and 28 after injection. PVR was graded across five stages according to the Fastenberg scale of classification⁴¹: stage 0, no disease; stage 1, epiretinal membrane formation; stage 2, vitreoretinal traction without retinal detachment; stage 3, localized retinal detachment (of one to two quadrants); stage 4, extensive retinal detachment (of two to four quadrants without complete detachment); stage 5, complete retinal detachment. Signs of toxicity, including intraocular inflammation and retinal hemorrhages, were assessed during each fundus examination. Animals were sacrificed at day 7 or day 28; eyes were enucleated and frozen at -80°C . All surgeries were performed aseptically and in conformance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The Schepens Animal Care and Use Committee approved the protocol used for these animal experiments.

Preparation of Rabbit Vitreous

Rabbit vitreous was extracted from frozen eyes, thawed at room temperature, and centrifuged at 4°C for 5 minutes at $10,000 \times g$. The resulting clarified supernatants were used for all subsequent analyses.

Patient Vitreous

All patients underwent a standard three-port vitrectomy, with removal of vitreous before *pars plana* infusion.⁴² A total of 24 human vitreous samples (12 non-PVR and 12 PVR) were obtained, with individual sample volumes ranging from 0.6 to 1.3 mL. Samples came from two independent sources: 12 (6 PVR and 6 non-PVR) from patient donors at the Ocular Angiogenesis Group, Department of Ophthalmology Academic Medical Center in Meibergdreef, Amsterdam; and 12 (6 PVR and 6 non-PVR) from patient donors at the Vancouver Hospital in association with the University of British Columbia. "Non-PVR" vitreous was obtained from patients with either macular holes or macular puckers (ie, retinal/ocular conditions unrelated to PVR). In all cases, informed written consent was first obtained from patient donors. Institutional review board approval to perform these studies (protocol S-226-0212, "Vitreous Factors in Proliferative Vitreoretinopathy") was obtained before conducting any experiments. Research involving human specimens adhered to the tenets of the Declaration of Helsinki.

Statistical Analysis

Data were analyzed using either the unpaired *t*-test, or Mann-Whitney analysis; *P* values less than 0.05 were considered statistically significant.

Results

Quantification of Growth Factors in Experimental PVR Vitreous

To ascertain which growth factors and cytokines were important contributors to PVR development, multiplex and Western blot analyses were used to quantify the levels of 24 growth factors and cytokines implicated in experimental or clinical PVR (see Supplemental Table S1 at <http://ajp.amjpathol.org>). Figure 1 illustrates the growth factors and cytokines present in healthy rabbit vitreous and in platelet-rich plasma (PRP); the sum of these agents represents what injected cells are initially exposed to in our model. Presumably, these are the factors that contribute to initiation of PVR via survival, proliferation, and production of extracellular matrix proteins.⁴³ Furthermore, those factors present at the highest levels likely make the greatest contribution.

As PVR developed, the profile of growth factors/cytokines in the vitreous changed (Figure 1). Some of the growth factors/cytokines (such as EGF) that were exclusive to PRP were scarcely detectable at day 7. This is consistent with our previous observation that the level of proteins as large as antibodies declined by over 95% from the starting level within 1 week.³⁴ Other growth factors/cytokines (eg, HGF) increased, suggesting that the injected cells and/or the host boosted their production. The level of many growth factors/cytokines (FGF-2, TGF- β s, PDGFs) changed little. The growth factors and cytokines present/abundant in day 7 PVR vitreous repre-

Figure 1. The level of growth factors and cytokines in rabbit vitreous with or without PVR. Vitreous or platelet-rich plasma (PRP) was isolated from rabbits and subjected to either multiplex or Western blot analysis to quantify the level of 24 growth factors or cytokines. Healthy vitreous indicates vitreous isolated from uninjected eyes; PVR vitreous designates vitreous isolated from rabbits that were subjected to the PVR protocol and developed a PVR score of stage 3 or higher. The levels of G-CSF, IL-6, IL-10, MCP-1, TNF- α , TNF- β , and GM-CSF were all below detection (ie, <0.1 ng/mL) and not shown in the graph. Only the active forms of the three TGF- β isoforms were quantified. The bars represent the mean, whereas error bars indicate SD. Vitreous from 10 to 11 rabbits was used in this analysis; within each group, 5 or 6 were used for multiplex analysis and the remainder for Western blot analysis. PRP from three rabbits was used for multiplex analysis and from a second group of three rabbits for Western blot analysis. Three scales, contiguously aligned, were used along the y axis to span the entire range of concentrations measured.

sent those most likely to be contributing to the early/progressive stage of PVR.

As PVR ran its course and stabilized, there were further changes to the profile of growth factors and cytokines (Figure 1). At day 28, IFN γ , IL-8, CTGF, and VEGF were at their highest level. These agents may contribute to maintenance/stability of the PVR membrane.

These observations demonstrate that the profile of vitreal growth factors and cytokines changes as PVR develops. Healthy vitreous contains many growth factors and cytokines that contribute to PVR (eg, FGF-2, TGF- β s, HGF).^{11,13,23,34,35,40} As PVR develops, the levels of these agents persist or increase, and additional growth factors and cytokines appear. Based on our profiling experiments, we identify a “set of 9” non-PDGFRs that are most abundant during initiation, progression, or maintenance of PVR. This set of 9 consists of EGF, FGF-2, IFN γ , IL-8, TGF- α , VEGF, TGF- β , HGF, and IGF-1.

Vitreal Growth Factors Induced PVR-Relevant Signaling Events

We investigated the ability of the set of 9 to induce signaling events that are associated with experimental PVR, including prolonged activation of PDGFR α and Akt, and suppression of p53 levels.³⁶ For these studies, we used primary fibroblasts because their p53 pathway is intact. Both PVR vitreous and the set of 9 (at a concentration that corresponded to the maximal levels observed in PVR vitreous) activated PDGFR α and triggered prolonged activation of Akt (Figure 2). The greater activation of PDGFR α and Akt by vitreous at the 10-minute time point can be attributed to the PDGFs that are present in PVR vitreous (Figure 1) and activate PDGFR α more strongly at early time points than do non-PDGFRs.³⁶

Both types of agonists reproducibly induced a $>50\%$ decline in the level of p53 at the 120-minute time point (Figure 2). Individual members of the set of 9

provoked only modest activation of PDGFR α when used at a concentration corresponding to their peak level in PVR vitreous (data not shown). At higher concentrations, some of the non-PDGFRs (FGF-2, TGF- α , EGF, and HGF) stimulated phosphorylation of PDGFR α to the level attained using the set of 9 (data not shown). These studies indicate that multiple members of the set of 9 are capable of activating PDGFR α and engaging the characteristic signaling events of PVR. Furthermore, their collective efforts are necessary to induce the full response.

Figure 2. Signaling events induced by PVR vitreous or the set of 9 non-PDGFRs. Serum-starved MEFs were treated for the indicated times with either day 7 rabbit PVR vitreous, or the set of 9 non-PDGFRs. Each agent in this set was used at the concentration measured in day 7 rabbit vitreous: FGF-2 (9.5 ng/mL), IGF-1 (8.3 ng/mL), TGF- β 1 (7.7 ng/mL), TGF- α (0.5 ng/mL), EGF (0.12 ng/mL), IFN- γ (1.0 ng/mL), IL-8 (0.12 ng/mL), VEGF (1.0 ng/mL), and HGF (15.0 ng/mL). Cells were lysed and subjected to Western blot analysis using the indicated antibodies. Ratios representing normalized band intensities are shown under each pair of immunoblots. The data shown are representative of three independent experiments. TCL, total cell lysate.

Neutralizing Non-PDGFs Prevented Vitreous-Dependent Signaling Events and Cellular Responses

To determine whether the growth factors and cytokines under investigation encompassed *all* agents that contributed to vitreous-dependent signaling, we tested whether a cocktail of reagents that neutralized them could completely inhibit PVR vitreous from inducing PVR-relevant signaling events. The minimal neutralizing set that most effectively blocked PVR-relevant signaling events (denoted “rNb”) contained reagents that neutralized EGF, TGF- α , FGF-2, IL-8, TGF- β s, HGF, IGF-1, and PDGFs (Figure 3, A and B). These results indicate that we identified all vitreal agents contributing to stimulation of PVR-relevant signaling events in cells. One of the cellular responses associated with PVR is contraction, which can be modeled in an *in vitro* setting, and is potentially driven by PVR vitreous.^{40,44} As shown in Figure 3C, rNb effectively blocked contraction induced by PVR vitreous. The results from these experiments identify the vitreal agents responsible for driving both PVR-relevant signaling events and cellular responses.

In this series of experiments, we routinely included agents to neutralize PDGFs. Although we observed only a minor contribution of vitreal PDGFs to signaling events or cellular responses using vitreous alone,³⁴ we neutralized PDGFs because it is possible that manipulating vitreous could unleash their bioactivity.

Blocking a Subset of Vitreal Growth Factors and Cytokines Prevented Experimental PVR

The *in vitro* studies presented in Figures 1, 2, and 3 predict that rNb should prevent or at least attenuate experimental PVR. To test this idea, we treated rabbits with a single injection of either rNb or nonimmune IgG at the time that PVR was initiated. Rabbits were monitored over 28 days from initial injection and scored for PVR stage at various times within this interval (Figure 4). The control group (treated with nonimmune IgG) developed PVR with the expected kinetics and frequency: at day 28, 67% of rabbits had retinal detachments (stage 3 PVR or higher). The remaining 33% developed stage 2 PVR that appeared poised to advance to retinal detachment. In contrast, none of the experimental group (treated with rNb) developed retinal detachments. One quarter of the rabbits had no discernable pathology whatsoever, and 42% developed only an epiretinal membrane. Although 33% of the rNb-treated rabbits advanced to stage 2, none of them appeared to be on the brink of retinal detachment, as was the case with stage 2 rabbits from the control group. Neutralization of multiple growth factors and cytokines by the injection of rNb did not result in intraocular inflammation (as visaged by the absence of vitreal and anterior-chamber inflammatory cells), retinal hemorrhages, or degeneration. Furthermore, histological analysis of a retina isolated from an rNb-treated rabbit (PVR stage 0) revealed no observable retinal damage when compared to the retina from the noninjected eye of the

Figure 3. Neutralizing growth factors and cytokines inhibited PVR vitreous-induced signaling events and gel contraction. **A:** rNb prevented PVR vitreous-induced signaling. Serum-starved MEFs were treated for the indicated duration at 37°C with DMEM alone, day 7 PVR vitreous supplemented with either isotype-control IgG, or one of the four neutralization mixtures (rNa, rNb, rNc, or rNd) listed in B. Cells were harvested, and the resulting samples processed as described in Figure 2. All immunoblots shown are representative of three independent experiments. TCL, total cell lysate. **B:** Table of neutralization components (left column) and their respective growth factor/cytokine targets (right columns). **C:** rNb prevented PVR vitreous-induced contraction of collagen gels. MEFs were subjected to the collagen gel contraction assay (Materials and Methods) in the presence of either DMEM (—), or day 7 PVR vitreous (vit) supplemented with IgG or rNb. Gel diameter was measured manually, and data presented are a mean of three independent experiments $\pm$ SD (shown as error bars). Images of triplicate wells from a single representative experiment at day 2 are shown below the graph.

Figure 4. Neutralizing a subset of vitreal growth factors and cytokines prevented retinal detachment in rabbits. One week after a gas vitrectomy, rabbits received three separate 0.1-mL injections of primary rabbit conjunctival fibroblasts, platelet-rich plasma, and either a 50-fold molecular excess of rNb neutralization mixture (12 rabbits, **pentagons**), or the equivalent amount of isotype control IgG (12 rabbits, **triangles**). Rabbits were examined and scored for PVR at the indicated times. Horizontal bars represent the mean of each group. Statistically significant differences between the two groups at each time point were determined by Mann-Whitney analysis (P values are indicated above each time point). Fundus photographs were not recorded because the morphology for a given stage was essentially the same as previously reported.⁴⁴

same rabbit (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). We conclude that the rNb cocktail completely prevented retinal detachment, and this treatment is well tolerated in rabbits. Furthermore, our *in vitro* approach to characterize and neutralize vitreal bioactivity accurately predicted the efficacy of an *in vivo* therapy.

Quantification of Growth Factors and Cytokines Present in Human PVR Vitreous

We used multiplex and Western blot analysis to quantify the level of growth factors and cytokines in vitreous from patient donors that either had PVR, or a non-PVR retinal condition (either a macular hole or pucker). We observed that 14 of the 24 agents quantified were present at a concentration greater than 0.1 ng/mL (Figure 5). Our subsequent efforts focused on a large subset of vitreal growth factors/cytokines that were detected in PVR vitreous; these included FGF-2, G-CSF, IL-6, TGF- α , VEGF, TGF- β s, HGF, IGF-1, and PDGFs.

Neutralizing a Subset of Vitreal Agents Present in PVR Patients Quenched Vitreous-Mediated Promotion of PVR-Relevant Signaling Events and Cellular Responses

A cocktail containing reagents to neutralize the majority of growth factors and cytokines present in PVR vitreous prevented PVR vitreous-induced activation of PDGFR α , prolonged activation of Akt, and the decline in the level of p53 (see Supplemental Figure S1, A and B, at <http://ajp.amjpathol.org>). Neutralizing just seven of the nine was equally effective, whereas only a partial block was observed when five of the nine were neutralized (see Supplemental Figure S2, A and B, at <http://ajp.amjpathol.org>). Furthermore, the minimal cocktail that completely blocked PVR vitreous-induced signaling in primary fibroblasts, denoted "hNb," also suppressed the contraction of these cells in collagen (see Supplemental Figure S2C at <http://ajp.amjpathol.org>). These results identified the

Figure 5. The level of growth factors and cytokines in vitreous of patients with or without PVR. Same as Figure 1 except that the analysis was performed on vitreous from patient donors. Non-PVR ocular pathologies included macular holes or macular pucker. Both the non-PVR and PVR groups consisted of 12 patients; data represent the mean values for each group, whereas error bars show SD. The levels of EGF, IFN- γ , IL-1 β , IL-10, TNF- α , TNF- β , GM-CSF, and PDGF-AB/BB were all below detection (ie, <0.1 ng/mL) in all samples and thus omitted from the graph. Only the active forms of the three TGF- β isoforms were quantified. Statistically significant differences ($P < 0.05$) existed between non-PVR and PVR vitreal levels for the following growth factors/cytokines: FGF-2, G-CSF, IL-6, TGF- α , VEGF, CTGF, TGF- β s, HGF, IGF-1, and PDGFs. Two scales were used along the y axis to adequately span the entire range of concentrations.

vitreal agents that were required for human vitreous to trigger signaling events and cellular responses relevant to PVR.

To move one step closer to the clinical setting, we tested the ability of hNb to block PVR vitreous-induced signaling and gel contraction using retinal pigment epithelial cells derived from a RPEM. Addition of hNb to human PVR vitreous effectively blocked PVR-relevant signaling events in RPEMs (Figure 6A). Furthermore, contraction of RPEMs in collagen gels was inhibited when incubated with human PVR vitreous containing hNb, compared to vitreous containing control IgGs (Figure 6B). Thus, the same neutralizing cocktail selected using fibroblasts also works to neutralize PVR relevant signaling in RPE cells derived from a PVR membrane. Taken together, these findings identify the growth factors and cytokines that are required for PVR pathogenic activity in human vitreous, and suggest that neutralizing this relatively small set of agents is a novel prophylactic option to reduce the incidence of PVR among patients undergoing surgery to repair a detached retina.

Discussion

In this study, we identified a subset of vitreal growth factors and cytokines that were essential for driving experimental PVR. In rabbit vitreous, the combination of eight classes of growth factors/cytokines was required for eliciting PVR-relevant outcomes in cells: TFG- α , EGF, HGF, FGF-2, TGF- β s, IL-8, IGF-1, and PDGFs (Figure 3). Neutralizing this set of factors effectively protected rabbits from developing retinal detachment (Figure 4). We therefore established a series of *in vitro* assays that accurately predict therapeutic efficacy in an animal model, and developed a new therapy to prevent experimental PVR. In humans, we found that the combination of seven classes of growth factors and cytokines was essential for PVR-relevant outcomes in cells: TFG- α , HGF, FGF-2, TGF- β s, IL-6, IGF-1, and PDGFs (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). Furthermore, neutralizing them prevented PVR-relevant signaling and contraction of collagen gels containing human epithelial cells derived from a PVR membrane (Figure 6). Therefore, the same neutralization strategy that prevented PVR in rabbits also prevented human PVR vitreous from inducing PVR-relevant responses.

Although neutralizing a subset was sufficient to prevent retinal detachment, it did not cause overt retinal toxicity, and the vast majority of the rabbits developed membranes (Figure 4 and data not shown). This suggests that vitreous contained additional growth factors and cytokines. Indeed, vitreous-driven proliferation of fibroblasts was unimpeded by the rNb cocktail (see Supplemental Figure S3 at <http://ajp.amjpathol.org>; and data not shown). These findings suggest that there are more growth factors and cytokines in vitreous than the 24 we considered in this study, and that targeting those that are required for retinal detachment does not completely negate the bioactivity of vitreous. Finally, the existence of functional relationships between growth factors (eg,

Figure 6. A neutralizing cocktail inhibited human PVR vitreous-induced signaling events and gel contraction. **A:** Neutralization of a subset of vitreal growth factors and cytokines prevented PVR vitreous-induced signaling. Subconfluent cultures of human RPE cells isolated from a PVR membrane were serum starved overnight and then treated for the indicated time at 37°C with either DMEM, or human PVR vitreous supplemented with nothing (—), nonimmune IgG, or hNb (composition described in Supplemental Figure S2B available at <http://ajp.amjpathol.org>). After stimulation, cells were lysed, and the resulting samples were processed as described in Figure 2. All immunoblots shown are representative of three independent experiments. TCL, total cell lysate. **B:** hNb prevented PVR vitreous-induced contraction of collagen gels. Human RPE cells isolated from a PVR membrane were subjected to the collagen gel contraction assay (Materials and Methods) in the presence of either DMEM (—), or vitreous (vit) from a PVR patient donor supplemented with IgG or hNb. Gel diameter was measured manually and data presented are a mean of three independent experiments $\pm$ SD (shown as error bars). Images of duplicate wells from a single representative experiment at day 2 are shown below the graph. Human vitreous used in this experiment was taken from the same pool used in Supplemental Figure S2 (available at <http://ajp.amjpathol.org>).

TGF- β promotes expression of CTGF^{45,46}) leaves open the possibility that the growth factors targeted in this study do not encompass all vitreal agents that promote PVR.

The two major proliferative vitreoretinal disorders—PVR and proliferative diabetic retinopathy (PDR)—are both characterized by an increased vitreal level of pathological mediators, which are important in driving disease progression.⁸ In experimental PVR, multiple growth factors and cytokines need to be targeted to effectively protect rabbits from developing disease, and the same is likely true for humans (Figures 4 and 6).^{3–5} In PDR, retinal ischemia induces the production and release of potent vasoproliferative factors into the vitreous such as VEGF and erythropoietin.^{47–49} Treatment for PDR has focused on neutralization of VEGF alone, which is effective in only a subset of PDR patients.⁵⁰ Perhaps combinatorial therapy with additional neutralization would benefit a wider spectrum of PDR patients.

The success of a neutralization-based therapy depends on the efficacy of the reagents injected, their biological duration, and any potential toxicity arising from these reagents. In rabbits, a single injection (of rNb) at day 0 prevented retinal detachment in all animals ($n = 12$) over the course of 1 month (Figure 4). Although the outlook is encouraging, it would be useful to test the effectiveness of this treatment on alternate models of retinal detachment.²⁹ It is also worth considering a combinatorial approach to therapy. The antioxidant *N*-acetylcysteine (NAC) prevents retinal detachment in rabbits by blocking the intracellular processes by which non-PDGFs lead to PDGFR α activation.⁴⁴ A combined therapy involving the neutralization approach presented in this work and NAC treatment would doubly target PVR-driving mechanisms at both the extracellular and intracellular levels.

In as complex a biological environment as vitreous, precise determination of the growth factors and cytokines essential for PVR necessitate both profiling methods (Figures 1 and 5) and *in vitro* neutralization bioassays (Figures 3 and 6, and Supplemental Figure S2 at <http://ajp.amjpathol.org>). Although profiling is an important first step to identify what factors are present and their quantities, use of the neutralization bioassays allows us to focus on the relevant contributors by systematically eliminating nonessential factors. The use of bioassays also addresses the difficult question of whether factors not being profiled are contributing to disease. Using a combination of profiling and bioassays is thus a powerful strategy to effectively identify the relevant agents present in complex biological samples.

The dispensability of vitreal PDGFs in PVR remains an enigma. Although functional PDGFR α s are essential to disease, PDGF does not appear to mediate receptor activation, despite the abundance of PDGFR α -activating isoforms of PDGF in both rabbit and human PVR vitreous.^{26,34} The preference for the indirect route of PDGFR α activation suggests that vitreous contains inhibitors that sequester PDGF or block its interaction with PDGFR α . Further investigation will be essential to elucidate the reason why vitreal PDGFs appear to be underperforming.

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