The Role of Thrombin in Proliferative Vitreoretinopathy

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Submitted: May 19, 2014
Accepted: June 25, 2014

Citation: Bastiaans J, van Meurs JC, Mulder VC, et al. The role of thrombin in proliferative vitreoretinopathy. *Invest Ophthalmol Vis Sci*. 2014;55:4659–4666. DOI:10.1167/iovs.14-14818

Purpose. To determine the role of thrombin in the development of proliferative vitreoretinopathy (PVR).

Methods. Vitreous was collected from patients undergoing a vitrectomy (macular holes and puckers, $n = 11$ [controls]; retinal detachment without PVR development following vitrectomy, $n = 15$ [RRD1]; retinal detachment with PVR development within 6 months after vitrectomy, $n = 11$ [RRD2]; and established PVR, $n = 14$ [PVR]). Thrombin activity in vitreous was determined using a thrombin-specific chromogenic substrate. ARPE-19 cells were stimulated with 8× diluted vitreous samples in the presence and absence of hirudin. The samples were analyzed at $t = 0$ and $t = 24$ hours for the presence of 27 cytokines/chemokines and growth factors using a multiplex approach. In comparable studies, ARPE-19 cells were stimulated for 2 hours, and mRNA expression levels for *CCL2*, *CXCL8*, *GMCSF*, *IL6*, and *PDGFB* were determined by real-time quantitative (RQ)-PCR.

Results. Thrombin activity was significantly ($P < 0.05$) higher in vitreous of the PVR group compared to the other groups. Proliferative vitreoretinopathy vitreous stimulated the production of chemokine (C-C motif) ligand (CCL)2, chemokine (C-X-C motif) ligand (CXCL)8, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, and platelet-derived growth factor (PDGF)-BB by ARPE-19 to significantly ($P < 0.05$) higher levels than vitreous from the RRD1 and RRD2 groups. These effects of PVR vitreous were significantly ($P < 0.05$) reduced by hirudin. These data were confirmed by mRNA studies.

Conclusions. Thrombin activity is increased in vitreous of patients with established PVR and is involved in the activation of proinflammatory and profibrotic pathways in RPE cells. Inhibition of thrombin activity may therefore represent a potential treatment option for proliferative vitreoretinopathy.

Keywords: thrombin, rhegmatogenous retinal detachment (RRD), proliferative vitreoretinopathy (PVR), inflammation, fibrosis, retinal pigment epithelium (RPE), cytokines, chemokines, growth factors, vitreous

Proliferative vitreoretinopathy (PVR) is an inflammatory fibrotic disorder that can develop after rhegmatogenous retinal detachment, and is the most common failure of retinal detachment repair. Proliferative vitreoretinopathy development is characterized by the formation of subretinal, intraretinal, and/or epiretinal fibroproliferative membranes that cause the retina to detach due to the contractile properties of myofibroblasts that are abundantly present in these membranes.[1,2] Retinal pigment epithelial (RPE) cells contribute to the formation of these fibroproliferative membranes through the secretion of cytokines and growth factors, proliferation, and dedifferentiation into extracellular matrix–producing myofibroblasts.[1,3,4] However, our current knowledge of the underlying pathobiological processes in PVR is still limited, which may explain why no clear improvement in medical therapy has been achieved during the last decades.

Proliferative vitreoretinopathy development is associated with blood–retinal barrier breakdown and activation of the coagulation system, as evidenced by fibrin deposition in retina and vitreous from PVR patients.[5–7] Thrombin is the terminal coagulation enzyme that converts soluble fibrinogen into the insoluble fibrin clot, and subretinal fluids from retinal detachment patients have been described as containing high capacity to generate thrombin activity.[6,8] Besides its central role in coagulation, thrombin stimulates many cellular processes involved in inflammation, wound repair, and fibrosis in a variety of cell types, including RPE cells, by activating the high-affinity thrombin receptor protease-activated receptor (PAR)1.[5,9–13] Recently we demonstrated that thrombin stimulates human RPE cells to produce a wide variety of cytokines, chemokines, and growth factors, among which chemokine (C-C motif) ligand (CCL)7, chemokine (C-X-C motif) ligand (CXCL)8, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6, platelet-derived growth factor (PDGF)-AA, and PDGF-BB[9,10] are associated with PVR development.[14–17] Although these data suggest that thrombin can contribute to PVR development, no data are present on thrombin activity in

Table 1. Patients and Controls

Group	Description	Sex, Male/Female	Age, y
Controls	Macular holes and macular puckers	6/5	74.73 ± 6.07
RRD1	Rhegmatogenous retinal detachment (no PVR development; 6 months after vitrectomy)	10/4	57.33 ± 6.89
RRD2	Rhegmatogenous retinal detachment (with PVR development; 6 months after vitrectomy)	11/4	64.64 ± 19.82
PVR	Established PVR	6/5	59.36 ± 11.24

vitreous samples and its contribution to RPE cell activation in PVR.

Here we examined intravitreal thrombin activity in PVR. This study is the first to demonstrate that vitreous from PVR patients contains increased thrombin activity that contributes to vitreous-induced production of cytokines/chemokines and growth factors by RPE cells. Our data indicate that elevated intravitreal thrombin activity contributes to PVR development, at least by inducing proinflammatory and profibrotic responses in RPE cells. Intravitreal thrombin activity may represent a novel biomarker for PVR development, and thrombin inhibition may be considered as a therapeutic option in the treatment of PVR.

Methods

Vitreous Fluids

Vitreous samples varying from 0.6 to 1.2 mL from the following groups of patients who underwent a standard three-port vitrectomy were used for this study: (1) macular holes, $n = 3$, and idiopathic macular puckers, $n = 8$, which served as controls; (2) rhegmatogenous retinal detachment without PVR development after vitrectomy (RRD1, $n = 15$); (3) rhegmatogenous retinal detachment with PVR development within 6 months following vitrectomy (RRD2, $n = 11$); and (4) established PVR (PVR, $n = 14$). The vitreous samples were collected from 2010 to 2013, centrifuged at 1500*g*, and stored at −20°C for a maximum of 7 days before long-term storage at −80°C. All subjects gave their consent for the use of rest material for research; storage and use of the vitreous for further studies were according to the guidelines of the Medical Ethics Committee of the Erasmus MC, University Medical Center, Rotterdam and were performed in accordance with guidelines established by the Declaration of Helsinki. These studies were approved by the human studies committee of the Schepens Eye Research Institute (Boston, MA, USA) and were performed in accordance with guidelines established by the Declaration of Helsinki. Patient characteristics are given in Table 1.

Cell Cultures

The human retinal pigment epithelial cell line ARPE-19 was obtained from American Type Culture Collection (Manassas, VA, USA). ARPE-19 cells were cultured in RPE medium Dulbecco's modified Eagle's medium [DMEM]/Ham's F-12 1:1 medium; HyClone, Logan, UT, USA) containing 10% heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin (all from BioWhittaker, Verviers, Belgium) and between passages 23 and 30 when used for experiments. The cells were maintained under standard cell culture conditions at 37°C in humidified air with 5% CO_2.

Thrombin Activity Measurement in Vitreous Fluids

Thrombin activity in vitreous was determined by using a thrombin-specific substrate as we and others have done previously for bronchoalveolar lavage fluid.[18,19] In short, 25 μL vitreous fluid was diluted in a 96-well microtiter plate with 25 μL Tris-buffered saline (TBS pH 8.3) or TBS containing 0.1 U/mL of the specific thrombin inhibitor hirudin (Sigma Aldrich Corp., St. Louis, MO, USA). A thrombin (Calbiochem, La Jolla, CA, USA) standard curve ranging from 50 to 0.78 mU/mL was prepared in TBS or TBS containing 0.1 U/mL hirudin. All solutions were incubated for 30 minutes at 37°C to allow formation of thrombin–hirudin complexes. Thereafter, 50 μL of a 1 mM solution of the thrombin-specific substrate Tos-Gly-Pro-Arg-pNA (Sigma Aldrich Corp.), dissolved in 1.5 mM HCl, was added to the (diluted) samples and incubated at 37°C. The optical density (OD) was measured at 405 nm after 8 hours. Thrombin activity in vitreous fluid was quantified based on the difference in OD between vitreous fluid with and without hirudin and the reference curve and expressed as mU/mL.

Determining the Effect of Different Vitreous Dilutions on IL6 and PDGFB mRNA Expression by ARPE-19

ARPE-19 cells were seeded in 12-well plates at a density of 3×10^5 cells/well in RPE medium containing 10% FCS and allowed to adhere overnight. Subsequently the medium was refreshed twice a week until the cells were grown to 100% confluence. Prior to stimulation, the cells were serum starved in RPE medium containing 1% FCS for 24 hours followed by an additional 24 hours in serum-free RPE medium. Thereafter, cells were stimulated for 1, 2, or 4 hours with vitreous of four PVR patients diluted 1/4 and 1/8 in serum-free RPE medium. Ribonucleic acid was isolated using a GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich Corp.) and reverse transcribed into cDNA.[10] Transcript levels of *IL6* and *PDGFB* mRNA were determined by real-time quantitative (RQ)-PCR (7700 PCR system; Applied Biosystems, Foster City, CA, USA). Expression levels of the analyzed gene transcripts were normalized to the control gene *ABL* (Abelson).[10] Primer and probe combinations used are listed in Table 2.

Measurement of Vitreous-Induced Cytokine, Chemokine, and Growth Factor Secretion by ARPE-19

ARPE-19 cells were seeded in 12-well plates at a density of 3×10^5 cells/well in RPE medium containing 10% FCS and allowed to adhere overnight. Subsequently the medium was refreshed twice a week until the cells were grown to 100% confluence. Prior to stimulation, the cells were serum starved in RPE medium containing 1% FCS for 24 hours followed by an additional 24 hours in serum-free RPE medium. Thereafter cells were preincubated with fresh serum-free RPE medium with or without 7.5 U/mL hirudin for 60 minutes. Subsequently the cells were stimulated for 24 hours with serum-free medium containing 1/8 diluted vitreous (average thrombin concentration per sample for RRD1: 0.699 ± 0.14 mU/mL; RRD2: 0.917 ± 0.38 mU/mL; PVR: 5.678 ± 1.88 mU/mL) or 5 U/mL thrombin with or without 7.5 U/mL hirudin. Following the

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TABLE 2. Primer and Probe Sequences

Gene	Sequence Forward Primer, 5′–3′	Sequence Reverse Primer, 5′–3′	Sequence Probe, 5′–3′
ABL	TGGAGATAACATCTAAGCATAACTAAAGGT	GATGTAGTTGCTTGGGACCCA	FAM-CCATTTTTGGTTTGGGCTTCACACCATT-TAMRA
CXCL8	GGCCGTGGCTCTCTTGG	GGGTGGAAAGGTTTGGAGTATGT	FAM-TGTGTGAAGGTGCAGTTTTGCCAAGGA-TAMRA
GMCSF	CCGCCTGGAGCTGTACAA	AGGGGATGACAAGCAGAAAGTC	FAM-TTGCACAGGAAGTTTCCGGGTTG-TAMRA
IL6	TAGCCGCCCCACACAGA	GTGCCTCTTTTGCTGCTTTCAC	FAM-AGCCACTCACCTCTTCAGAACGAATTGACA-TAMRA
PDGFB	TCCCGAGGAGCTTTATGAGATG	CGGGTCATGTTCAGGTCCAAC	FAM-AGTGACCACTCGATCCGCTCCTTTG-TAMRA

stimulation period of 24 hours (T24), culture supernatants were harvested for cytokine, chemokine, and growth factor analysis. In addition, an aliquot of 125 μL of the prepared 1/8 diluted vitreous samples ($t = 0$ hours; T0) was stored at −20°C for determination of basal cytokine, chemokine, and growth factor levels.

T0 samples and T24 culture supernatants were analyzed with a Bio-Plex Pro Human Cytokine, Chemokine, and Growth Factor Assay (Bio-Rad, Hercules, CA, USA) allowing simultaneous detection of the following cytokines, chemokines, and growth factors: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, FGFb, eotaxin, granulocyte colony-stimulating factor (G-CSF), GM-CSF, IFNγ, interferon gamma-induced protein (IP)-10/CXCL10, monocyte chemotactic protein (MCP)-1/CCL2, macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, PDGF-BB, regulated on activation, normal T cell expressed and secreted (RANTES), TNF-α, VEGF-A (see Supplementary Table S1 for the detection limits). The assay was performed according to manufacturer's instructions.

Measurement of PVR Vitreous-Induced Cytokine, Chemokine, and Growth Factor mRNA Expression Levels in ARPE-19

ARPE-19 cells were seeded in 12-well plates at a density of 3×10^5 cells/well in RPE medium containing 10% FCS and allowed to adhere overnight. Subsequently the medium was refreshed twice a week until the cells were grown to 100% confluence. Prior to stimulation, the cells were serum starved in RPE medium containing 1% FCS for 24 hours followed by an additional 24 hours in serum-free RPE medium. Thereafter, cells were preincubated with serum-free RPE medium with or without 7.5 U/mL hirudin for 60 minutes and subsequently stimulated for 2 hours with serum-free medium containing 1/8 diluted PVR vitreous (average thrombin concentration per sample: 4.211 ± 0.60 mU/mL) with or without 7.5 U/mL hirudin. Ribonucleic acid was isolated and reverse transcribed into cDNA.[10] Transcript levels of *CCL2*, *CXCL8*, *GMCSF*, *IL6*, and *PDGFB* were determined by RQ-PCR. Expression levels of the analyzed gene transcripts were normalized to the control gene *ABL*.[10] *CCL2* transcript levels were determined using a commercially available Taqman Gene Expression assay (Applied Biosystems). Other primer and probe combinations used are listed in Table 2.

Statistical Analysis

Messenger RNA and protein data were analyzed using the Kruskal-Wallis (one-way ANOVA) test followed by the Mann-Whitney U test when applicable. A P value < 0.05 was considered significant.

RESULTS

Thrombin Activity in Vitreous

Thrombin activity was hardly present in the vitreous of patients from the control group (2.48 ± 1.25 mU/mL, mean value ± standard error of the mean [SEM], Fig. 1). Vitreous samples from patients with rhegmatogenous retinal detachment without PVR development in a later stage (RRD1) displayed slightly, but not significantly, higher thrombin activity (9.93 ± 1.86 mU/mL) than the control group. Thrombin activity in vitreous samples of patients with rhegmatogenous retinal detachment that did develop PVR within 6 months after vitrectomy (RRD2) showed a clear

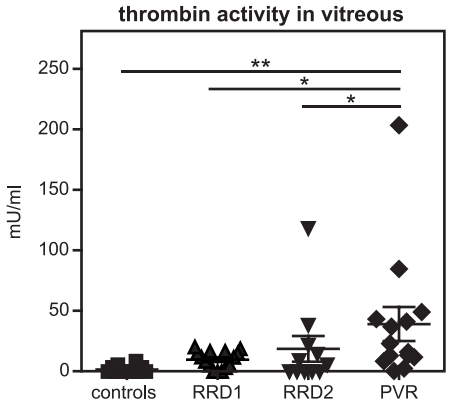

FIGURE 1. Thrombin activity in vitreous samples (controls: macular holes, $n = 3$, and macular puckers, $n = 8$; rhegmatogenous retinal detachment with no PVR development after vitrectomy [RRD1], $n = 15$; rhegmatogenous retinal detachment with PVR development after vitrectomy [RRD2], $n = 11$; and established PVR [PVR], $n = 14$) was determined with the thrombin-specific chromogenic substrate Tos-Gly-Pro-Arg-pNA in the absence and presence of the thrombin-specific inhibitor hirudin. Statistical analysis was performed by using a Kruskal-Wallis (one-way ANOVA) test followed by the Mann-Whitney *U* test. $P < 0.05$ was considered significant. $^*P < 0.05$ and $^{**}P < 0.01$.

trend toward increased thrombin activity (18.93 ± 10.61 mU/mL) compared to the control and RRD1 groups, but this was not statistically significant. Thrombin activity (39.30 ± 14.04 mU/mL) was significantly ($P < 0.05$) elevated in the vitreous of patients with established PVR compared to all other groups (Fig. 1). There were no statistical differences between the sex or age of the patients in the different groups (Table 1).

The Effect of Different Vitreous Dilutions on *IL6* and *PDGFB* mRNA Expression by ARPE-19

Vitreous enhanced *IL6* mRNA expression by ARPE-19 cells after 1 and 2 hours of stimulation, which returned to baseline level again after 4 hours of stimulation (Fig. 2). *PDGFB* mRNA expression level by ARPE-19 was also enhanced by vitreous stimulation with equal levels of induction after 1, 2, and 4 hours of stimulation (Fig. 2). No differences between the 1/4 and 1/8 vitreous dilution were seen for either *IL6* mRNA or *PDGFB* mRNA induction in ARPE-19 (Fig. 2). Based on these data, further studies were undertaken with vitreous dilutions of 1/8.

The Effects of Vitreous on Cytokine, Chemokine, and Growth Factor Production by ARPE-19

Stimulation of ARPE-19 cells with RRD1, RRD2, and PVR vitreous samples for 24 hours significantly ($P < 0.05$) stimulated CCL2, CXCL8, IL-6, IL-12 (p70), and VEGF-A production by ARPE-19 cells (Fig. 3). Proliferative vitreoretinopathy vitreous stimulated CCL2, CXCL8, and IL-6 production by ARPE-19 to significantly ($P < 0.05$) higher levels than RRD1 and RRD2 vitreous samples, while no differences were observed between the groups for vitreous-induced IL-12 (p70) and VEGF-A production by ARPE-19. Proliferative vitreoretinopathy vitreous stimulated G-CSF and IL-17A production by ARPE-19, albeit not to a statistically significant level. Production of PDGF-BB by ARPE-19 was significantly ($P < 0.05$) enhanced by PVR vitreous samples, but not by RRD1 and RRD2 vitreous samples (Fig. 3). CCL4 levels were significantly higher ($P < 0.05$) in the T0 samples from RRD2 and PVR patients compared to T0 samples from RRD1 patients, while

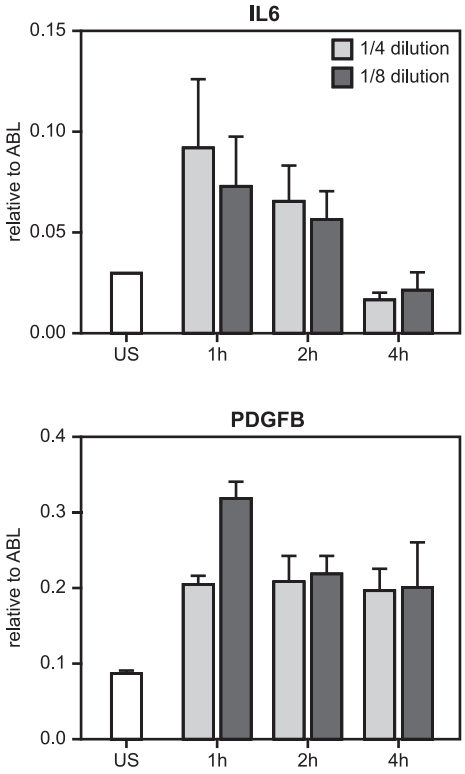

FIGURE 2. ARPE-19 cells were stimulated for 1, 2, and 4 hours with vitreous of patients with established PVR (4× and 8× diluted), and *IL6* (*top*) and *PDGFB* (*bottom*) mRNA expression levels were determined by RQ-PCR and normalized against the control gene *ABL*. Data are presented as the mean value from four independent experiments ± SEM. US, unstimulated.

CXCL10 was significantly ($P < 0.05$) higher in the T0 samples from PVR patients compared to T0 samples from RRD1 and RRD2 patients (Fig. 3). CCL4 and CXCL10 levels did not increase after stimulating the ARPE-19 cells for 24 hours with vitreous from either the RRD1, RRD2, or PVR groups (Fig. 3). In all groups, basic fibroblast growth factor (FGFb) levels significantly ($P < 0.05$) declined after 24 hours of ARPE-19 stimulation when compared to the levels detected in the T0 samples (Fig. 3). After 24 hours of ARPE-19 stimulation, GM-CSF levels declined significantly ($P < 0.05$) in the RRD1 and the RRD2 groups but not in the PVR group, with GM-CSF levels being significantly ($P < 0.05$) higher in the PVR group compared to RRD1 and RRD2 samples at T24. All other factors measured by the used multiplex assay were not detected. For a total summary see Supplementary Table S1.

The Effect of Thrombin Inhibition on PVR Vitreous-Induced Cytokine, Chemokine, and Growth Factor Production by ARPE-19

Stimulation of ARPE-19 cells with PVR vitreous samples for 24 hours significantly ($P < 0.05$) stimulated CCL2, CXCL8, GM-CSF, IL-6, and PDGF-BB production by ARPE-19 cells compared to the RRD1 and RRD2 vitreous samples (Fig. 3; Supplementary Table S1). Thrombin (5 U/mL), used as positive control, also significantly ($P < 0.05$) stimulated CCL2, CXCL8, GM-CSF, IL-6, and PDGF-BB production by ARPE-19, which was completely abolished by the direct thrombin inhibitor hirudin ($P < 0.05$) (Fig. 4; Supplementary Table S1). The stimulatory effect of RRD1 and RRD2 vitreous on the production of these factors by ARPE-19 was not affected by hirudin (Fig. 4; Supplementary

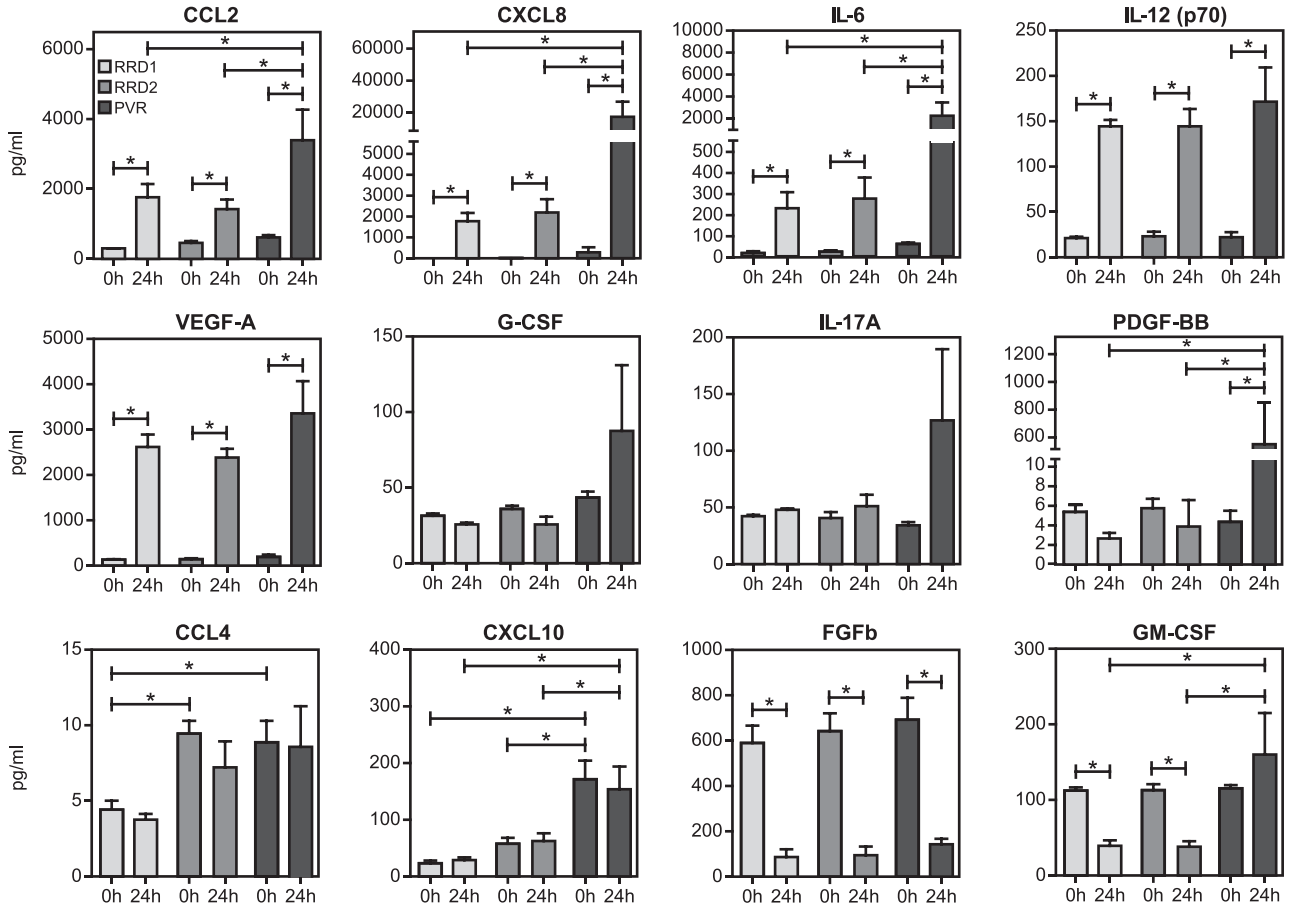

FIGURE 3. ARPE-19 cells were stimulated for 24 hours with 8× diluted vitreous from the RRD1, RRD2, and PVR patient groups. Culture supernatants were analyzed by a Bio-Plex Pro Human Cytokine, Chemokine, and Growth Factor Assay, allowing the detection of 27 cytokines, chemokines, and growth factors simultaneously. Data are presented as the mean value from four individual vitreous samples per group ± SEM. Statistical analysis was performed with the Kruskal-Wallis (one-way ANOVA) test followed by the Mann-Whitney *U* test when applicable. $P < 0.05$ was considered significant. $^{*}P < 0.05$.

Table S1). Hirudin did significantly ($P < 0.05$) reduce the capacity of PVR vitreous to stimulate CCL2, CXCL8, GM-CSF, IL-6, and PDGF-BB production by ARPE-19 after 24 hours of stimulation (Fig. 4; Supplementary Table S1). This inhibition was not complete and reached the levels induced by the RRD1 and RRD2 vitreous samples (Supplementary Table S1). Thrombin (5 U/mL) significantly ($P < 0.05$) stimulated *CCL2*, *CXCL8*, *GMCSF*, *IL6*, and *PDGFB* mRNA expression by ARPE-19 after 2 hours of stimulation, which was significantly ($P < 0.05$) reduced by hirudin (Fig. 5). Proliferative vitreoretinopathy vitreous samples also enhanced *CCL2*, *CXCL8*, *GMCSF*, *IL6*, and *PDGFB* mRNA expression levels by ARPE-19 after 2 hours of stimulation, which was significant ($P < 0.05$) for *CXCL8*, *IL6*, and *PDGFB* mRNA (Fig. 5). Addition of hirudin reduced the capacity of the PVR vitreous samples to stimulate *CCL2*, *CXCL8*, *GMCSF*, *IL6*, and *PDGFB* mRNA production by ARPE-19, which was significant ($P < 0.05$) for *CXCL8*, *IL6*, and *PDGFB*.

DISCUSSION

Breakdown of the blood–retinal barrier is associated with PVR, and a role for direct RPE activating effects of thrombin has been proposed to contribute to the pathobiology of PVR. Our study is the first to demonstrate increased thrombin activity in vitreous from patients with established PVR and to show that this stimulates RPE cells to produce proinflammatory cytokines and chemokines such as CCL2, CXCL8, GM-CSF, and IL-6, as well as the profibrotic mediator PDGF-BB.

In this study we measured thrombin activity in vitreous from patients with macular hole development, idiopathic macular pucker formation, rhegmatogenous retinal detachment without PVR development after vitrectomy, rhegmatogenous retinal detachment with later PVR development after vitrectomy, and established PVR. In macular hole and macular pucker patients the blood–retinal barrier is intact, and we detected no to low thrombin activity in these vitreous samples, with levels comparable to that reported in two recently published studies.[20,21] Compared to this, thrombin activity was significantly elevated in vitreous from patients with established PVR, while a trend toward increased thrombin activity was seen in vitreous from patients who developed PVR within 6 months following vitrectomy. Our study therefore supports that PVR development is a gradual process in which later stages of disease are associated with increased intravitreal thrombin activity. Whether this increase is a cause or consequence of disease progression is so far unclear. Nevertheless, measurement of intraocular thrombin activity might represent a new biomarker for PVR development that warrants further studies.

Inflammation is an important component of PVR pathogenesis, and RPE cells contribute to ocular inflammation via the production of proinflammatory mediators. Thrombin has been

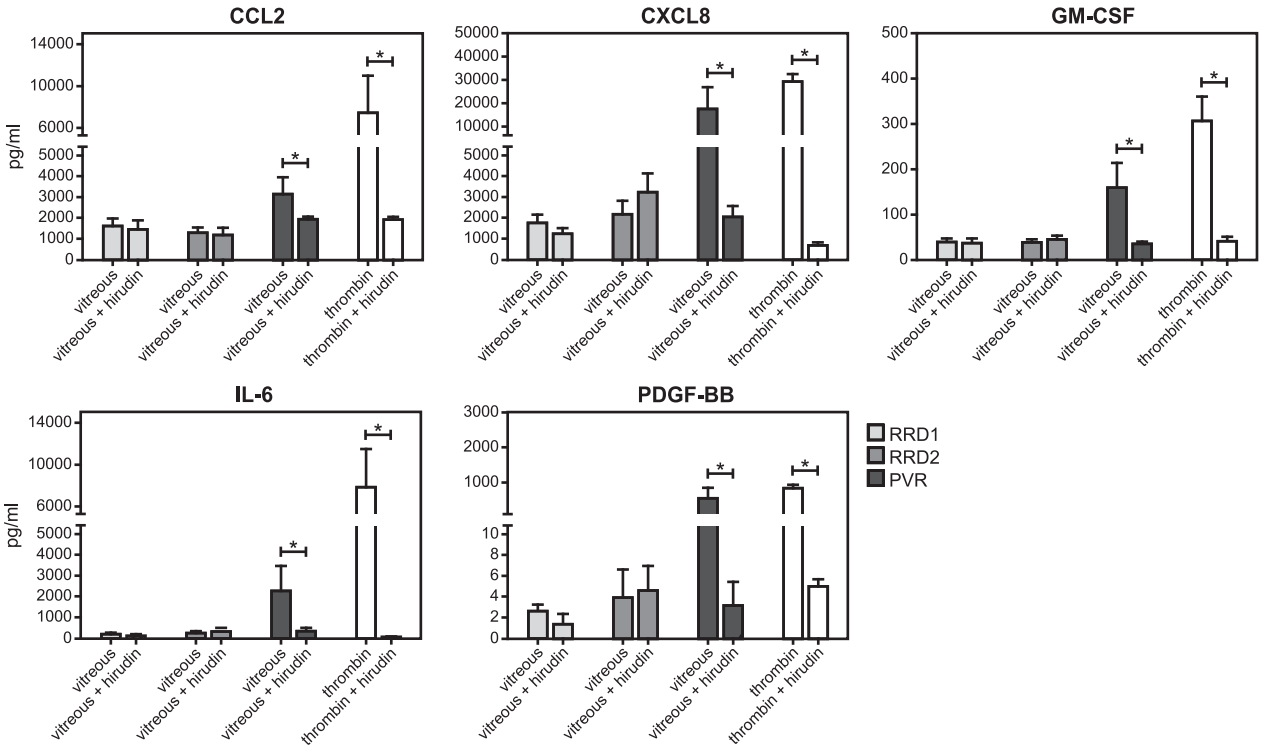

FIGURE 4. ARPE-19 cells were stimulated for 24 hours with 8× diluted vitreous from the RRD1, RRD2, and PVR patient groups or thrombin (5 U/mL) in the absence or presence of the thrombin-specific inhibitor hirudin (7.5 U/mL). Culture supernatants were analyzed by a Bio-Plex Pro Human Cytokine, Chemokine, and Growth Factor Assay, allowing the detection of 27 cytokines, chemokines, and growth factors simultaneously. Factors that showed significant differences between PVR, RRD1, and RRD2 after 24 hours of stimulation (Fig. 3) are shown. Data are presented as the mean value from four individual samples per group ± SEM. Statistical analysis was performed with the Kruskal-Wallis (one-way ANOVA) test followed by the Mann-Whitney *U* test when applicable. $P < 0.05$ was considered significant. $^{*}P < 0.05$.

described as a potent inducer of cytokine/chemokine and growth factor production by RPE cells via activation of PAR1.[10,12] Here we found that vitreous from the RRD1, RRD2, and PVR groups significantly stimulated the production of CCL2, CXCL8 (IL-8), IL-6, IL-12 (p70), and VEGF-A by RPE cells. Vitreous of patients with established PVR stimulated the production of CCL2, CXCL8, and IL-6 by RPE cells to significantly higher levels than vitreous from the RRD1 and RRD2 groups. Thrombin inhibition only inhibited the capacity of PVR vitreous to induce CCL2, CXCL8, and IL-6 production by ARPE-19; but, although significant, this was not complete and reached the levels induced by the RRD1 and RRD2 vitreous samples (Supplementary Table S1). Thus in established PVR, intravitreal thrombin activity is a major, but not the sole, factor that stimulates production of the proinflammatory mediators CCL2, CXCL8, and IL-6 by RPE, while in RRD patients without PVR (RRD1) development or PVR development later on (RRD2), the induction of these factors appears to be largely independent of intravitreal thrombin activity. The vitreous-induced production of VEGF-A and IL-12 (p70) was equal between the three groups and not reduced by thrombin inhibition (Supplementary Table S1), suggesting no or a limited role for intravitreal thrombin in inducing the production of these factors by ARPE-19. Thrombin (5 U/mL) did, however,

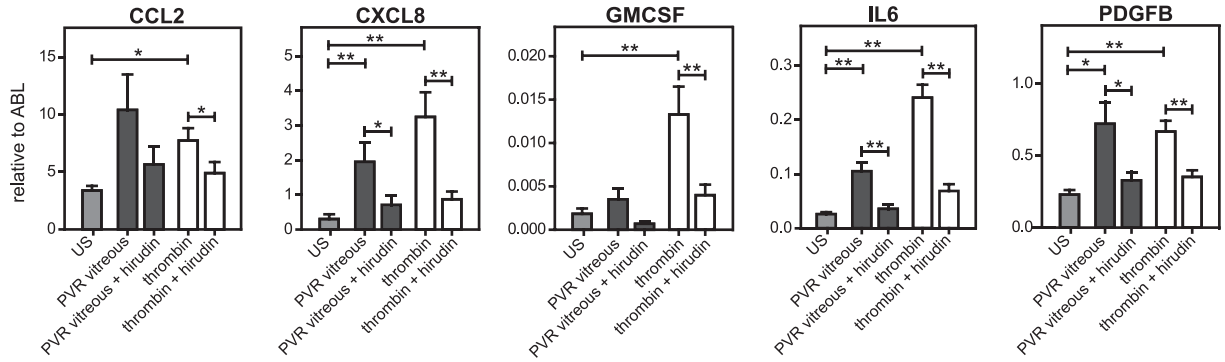

FIGURE 5. ARPE-19 cells were stimulated for 2 hours with 8× diluted vitreous from PVR patients ($n = 7$) or thrombin (5 U/mL) in the absence or presence of the thrombin-specific inhibitor hirudin (7.5 U/mL). *CCL2*, *CXCL8*, *GMCSF*, *IL6*, and *PDGFB* mRNA expression levels were determined by RQ-PCR and normalized against the control gene *ABL*. Data are presented as the mean value ± SEM. Statistical analysis was performed with the Kruskal-Wallis (one-way ANOVA) test followed by the Mann-Whitney *U* test when applicable. $P < 0.05$ was considered significant. $^{*}P < 0.05$ and $^{**}P < 0.01$. US, unstimulated.

induce IL-12 (p70) and VEGF-A by ARPE-19 (Supplementary Table S1), while others demonstrated a dose-dependent effect of thrombin on VEGF-A production.[12] In our experiments we used diluted vitreous (1/8), which may have obscured effects of intravitreal thrombin activity on the production of factors such as IL-12 (p70) and VEGF-A, as well as others, by the ARPE-19 cells. Incubation of the ARPE-19 cells with vitreous from RRD1 and RRD2 patients resulted in a decline of GM-CSF levels in the culture media. This might be related to binding of GM-CSF to its receptor, which is ubiquitously expressed by epithelial cells.[22] This decline in GM-CSF was not observed when PVR vitreous was added to the ARPE-19 cells. Moreover, thrombin did stimulate GM-CSF secretion by ARPE-19 (Supplementary Table S1), while PVR vitreous enhanced GM-CSF mRNA levels in RPE cells, as did thrombin alone, which was blocked by hirudin. This indicates that intravitreal thrombin activity present in PVR vitreous stimulates GM-CSF production by RPE cells. Our data therefore clearly imply that intravitreal thrombin activity is involved in driving the production of CCL2, CXCL8, GM-CSF, and IL-6 by RPE in PVR. CCL2, CXCL8, GM-CSF, and IL-6 are potent activators and chemoattractants for immune cells such as monocytes, macrophages, neutrophils, and B-lymphocytes that are present in PVR membranes and vitreous, while GM-CSF also stimulates differentiation of monocytes into macrophages.[10,23–26]

Retinal fibrotic contractile membranes represent the final stage of PVR, but their formation requires a complex process of proliferation, migration, dedifferentiation, and extracellular matrix deposition by different cell types, among them RPE, that is driven by profibrotic mediators.[1] Basic fibroblast growth factor (FGFb) is a profibrotic factor proposed to contribute to PVR.[27] We found a reduction of FGFb after vitreous incubation with ARPE-19 while thrombin alone did not induce FGFb production by ARPE-19 (Supplementary Table S1).[10] This suggests that the reduction in FGFb might be due to binding to its receptor, which is expressed by RPE,[28] and illustrates that thrombin does not stimulate FGFb production by RPE cells. Platelet-derived growth factor is considered an important profibrotic mediator in PVR that induces proliferation and dedifferentiation of RPE cells into contractile myofibroblasts that are abundantly present in PVR membranes and represent the main cellular subset responsible for excessive collagen production in fibrosis.[4,27–30] Recently, we demonstrated that thrombin induces dedifferentiation of RPE cells into collagen-producing myofibroblasts via autocrine release of PDGF-BB.[9] Here we found that vitreous from PVR patients strongly stimulated the production of PDGF-BB by ARPE-19 in a thrombin-dependent manner. Consequently, elevated intravitreal thrombin activity can activate profibrotic processes in PVR, at least via autocrine PDGF-BB release and signaling in RPE cells.

Vitreous samples did not stimulate production of the chemokines CCL4 and CXCL10 by ARPE-19 cells. CCL4 and CXCL10 were, however, present at higher levels in the prepared 1/8 diluted vitreous samples (T0; Fig. 3; Supplementary Table S1) from the patients who later on developed PVR (RRD2) or had established PVR at the time of vitrectomy. Vitreous from established PVR contained higher levels of CXCL10 than RRD2 vitreous, implying that CXCL10 has a more profound role in later stages of disease. These observations are in line with previous reports and support a role for these chemokines in the inflammatory response in PVR[31–33]; they suggest that CCL4 and CXCL10 may represent ocular biomarkers for PVR development.

Polarity is an important determinant for RPE function in the maintenance of ocular homeostasis.[34] For our studies we used nonpolarized ARPE-19 monolayers. It has, however, been reported that polarized RPE can react differently under inflammatory conditions than nonpolarized cells with regard to pigment epithelial-derived factor and VEGF production, which is related to opposing activity levels of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and c-Jun N-terminal kinase (JNK) pathway.[34,35] Previously, we demonstrated that thrombin-induced cytokine/chemokine/growth factor production by ARPE-19 and primary RPE was mediated via PAR1-induced NF-κB signaling, while others demonstrated that thrombin activates multiple signaling pathways post PAR1, including NF-κB and JNK.[10,11] Therefore we cannot exclude the possibility that polarized ARPE-19 cells would have responded differently with regard to vitreous-induced cytokine/chemokine/growth factor production compared to nonpolarized ARPE-19 cells, for instance, for VEGF production. However, RPE dedifferentiation and loss of polarity is part of PVR development and progression.[36]

In conclusion, this study demonstrates that PVR development is associated with an increase in intravitreal thrombin activity, especially during the later stage of PVR, and supports a role for intravitreal thrombin activity in stimulating inflammatory and fibrotic pathways in RPE. We propose a comparable role for thrombin in other inflammatory proliferative vitreoretinal disorders with blood–retinal barrier breakdown, such as exudative age-related macular degeneration, proliferative diabetic retinopathy, and retinal vein occlusion; among these, the latter was recently found to be associated with increased intravitreal thrombin activity.[21] Inhibition of thrombin activity may therefore represent a potential treatment option for proliferative vitreoretinal diseases like PVR.

Acknowledgments

The authors thank Sandra de Bruin-Versteeg for her help with the illustrations. The research for this study was (in part) performed within the framework of the Erasmus Postgraduate School Molecular Medicine.

Supported by Combined Ophthalmic Research Rotterdam (CORR-Project code: 3.1.0) and the International Retinal Research Foundation, Birmingham, Alabama, United States.

Disclosure: **J. Bastiaans**, None; **J.C. van Meurs**, None; **V.C. Mulder**, None; **N.M.A. Nagtzaam**, None; **M. Smits-te Nijenhuis**, None; **D.C.M. Dufour-van den Goorbergh**, None; **P.M. van Hagen**, None; **H. Hooijkaas**, None; **W.A. Dik**, None

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