Thrombin-Induced VEGF Expression in Human Retinal Pigment Epithelial Cells

Zong-Mei Bian, Susan G. Elner, and Victor M. Elner

Purpose. The purpose of the present study was to investigate the effects of thrombin and thrombin in combination with other proangiogenic factors on VEGF expression in hRPE cells.

Methods. hRPE cells were stimulated with thrombin TNF-α, monocytes, and TGF-β2. After stimulation, conditioned medium and lysed cells were subjected to ELISA, Western blot analysis, immunocytochemistry, and RT-PCR analyses. Inhibitors specific for various signal transduction pathways were used to determine the signaling pathways involved.

Results. Treatment of RPE cells with thrombin resulted in dose- and time-dependent increases in VEGF mRNA levels and protein production. hRPE VEGF expression is predominantly protease-activated receptor (PAR)-1 dependent. Approximately 80% of thrombin-induced VEGF secretion was abrogated by inhibitors of MAPK/ERK kinase (MEK), p38, c-Jun NH2-terminal kinase (JNK), protein tyrosine kinase (PTK), phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), nuclear factor-κB (NF-κB), and reactive oxygen species (ROS). Analyses of VEGF protein production and mRNA synthesis revealed that VEGF induction by thrombin plus TNF-α or coculture with monocytes was additive, whereas that by co-incubation with TGF-β2 was synergistic. The costimulated VEGF production by TGF-β2 plus thrombin was an average of three times higher than the sum of that induced by each agent alone. Furthermore, BAPTA [bis-(*o*-aminophenoxy)ethane-*N*,*N*′,*N*′-tetraacetic acid], a calcium chelator, blocked the VEGF secretion induced by thrombin and thrombin plus TGF-β2 by 65% and 20%, respectively, but had no effect on that induced by TGF-β2 alone.

Conclusions. Thrombin alone and in combination with TNF-α, monocytes, and TGF-β2 potently stimulated VEGF expression in hRPE cells via multiple signaling pathways. The thrombin-induced calcium mobilization may play an important permissive role in maximizing TGF-β2–induced VEGF expression in RPE cells. (*Invest Ophthalmol Vis Sci.* 2007;48:2738–2746) DOI:10.1167/iovs.06-1023

Retinal pigment epithelial (RPE) cells, strategically located between neuroretinal photoreceptors and choriocapillaris of choroidal tissue, form the outer blood–retinal barrier. In addition to supporting normal photoreceptor function, RPE cells secrete a variety of cytokines, growth factors, and extracellular matrix components that enable RPE cells to participate actively in retinal and choroidal neovascularization.[1,2] Neovascularization is a pathologic process common to many ocular diseases of diverse etiology such as proliferative diabetic retinopathy (PDR), proliferative vitreoretinopathy (PVR), and age-related macular degeneration (AMD). Retinal and choroidal neovascularization is the most common and important complication of these diseases and often leads to blindness.[3]

Vascular endothelial growth factor (VEGF) is a multifunctional cytokine strongly implicated in angiogenesis. Similar to pericytes, endothelial cells, Müller cells, and astrocytes, hRPE cells also secrete VEGF.[4] There is considerable evidence that VEGF secretion by RPE cells leads to neovascularization in the posterior segment of the eye. Several therapies to control angiogenesis involve antagonizing VEGF or its receptors.[5] Thus, identifying precise molecular mechanisms, upstream of VEGF gene expression in RPE cells, may be useful for developing treatment that is even more effective in controlling ocular neovascularization.

Thrombin stimulates signaling pathways via interactions with G-protein-coupled protease-activated receptors (PARs). Human thrombin receptor is a protein with seven transmembrane domains and a large extracellular amino-terminal extension. Thrombin cleaves within this extension, thereby creating a new receptor amino terminus that functions as a tethered ligand and activates the receptor. At least three subtypes of PARs—PAR-1, -3, and -4—are activated by thrombin. PAR-1 and -3 are constitutively expressed in RPE cells, but PAR-1 is most likely the major form of PAR that mediates thrombin activation in RPE cells.[6,7]

Thrombin generation is usually triggered when disruption of vascular integrity allows coagulation factors in the plasma to contact extravascular tissue factor (tissue thromboplastin; coagulation factor III). Prothrombin in 1 mL of human plasma, when fully converted, forms 15 to 38 U of thrombin, which is concentrated in the resultant clot.[8,9] Thrombin is formed from prothrombin in areas of increased vascular permeability and hemorrhage at sites of blood–retinal barrier breakdown in many retinal diseases, such as ocular trauma, PVR, PDR, and AMD. Thrombosis is also enhanced by localized increases in thrombin concentration in the retinal circulation during retinal ischemia that results in increased leakage of serum components, including prothrombin.[10] Thrombin itself may induce gap formation between endothelial cells, further enhancing its own formation.[11] Thrombin stimulates VEGF in many cell types[12–14] and interplays with VEGF synergistically in promoting angiogenesis.[15] Despite the likely exposure of RPE cells to thrombin in retinal diseases, no study has shown that thrombin induces RPE cells to produce VEGF. Therefore, it is of pathophysiological importance to assess the role of thrombin in stimulating VEGF expression in RPE cells. Moreover, thrombin is also likely to coexist with other proangiogenic factors such as TNF-α and TGF-β, which have been detected in diseased ocular tissues.[16–19] Histopathological studies of choroidal neovascular membranes from patients with AMD have demonstrated the presence of various growth factors, including TGF-β.[20] Nevertheless, interactions of thrombin with other proangiogenic factors in stimulating VEGF secretion have not

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been reported. Apart from its central role in blood coagulation, thrombin also regulates other cellular functions, including those involved in wound healing and inflammation. One example is thrombin's mitogenic effects on RPE cells.[21] The latter finding has led to thrombin's use as a therapeutic agent.[22,23] As a mitogen of retinal cells, thrombin has been used in intraocular surgery for diabetic retinopathy and ocular trauma to control bleeding and to close macular holes. However, thrombin treatment frequently causes significant inflammation. This clinical observation is consistent with our previous observations showing the potential proinflammatory role of thrombin, TNF-α, and monocytes by stimulating RPE cells to secrete chemokines.[24–26] These observations underscore the potential importance of investigating whether thrombin at sites of blood–retinal barrier breakdown works in concert with leukocytes to stimulate VEGF gene expression in ocular tissues. In this study thrombin, working additively with TNF-α and monocytes and synergistically with TGF-β2, triggered multiple signaling pathways and led to enhanced VEGF gene expression in human RPE cells.

Materials and Methods

Human thrombin was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). TNF-α and TGF-β2 were purchased from R&D Systems (Minneapolis, MN); U0126 from Promega (Madison, WI); SB202190 from Calbiochem (San Diego, CA); CAPE (caffeic acid phenethyl ester) from Biomol (Plymouth Meeting, PA); and Nac (*N*-acetylcysteine), DPI (diphenyleneiodonium chloride) and BAPTA [1,2-bis (*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] and all other reagents from Sigma-Aldrich (St. Louis, MO). Human thrombin receptor 1 (PAR-1)-activating peptide (SFLLRNPNDKYEPF, H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-OH) and human thrombin receptor 3 (PAR-3)-activating peptide (TPRGAP, H-Thr-Phe-Arg-Gly-Ala-Pro-OH) were obtained from Bachem Americas (Torrance, CA). SCH79797 was obtained from Tocris Cookson, Inc. (Ellisville, MO).

The signal transduction inhibitors used in this study and their molecular targets are summarized in Table 1.

Cell Isolation and Culture

Human RPE cells were isolated within 24 hours of death from donor eyes that were obtained from the Midwest Eye Bank, as previously described.[27] In brief, the sensory retina tissue was separated gently from the RPE monolayer, and the RPE cells were removed from Bruch's membrane with papain (5 U/mL). The RPE cells were cultured in Dulbecco's modified essential medium (DMEM) containing 15% fetal bovine serum, penicillin G (100 U/mL), streptomycin sulfate (100 μg/mL), and amphotericin B (0.25 μg/mL) in culture plates (Falcon Primaria; BD Biosciences, Bedford, MA) to inhibit fibroblast growth. The human RPE monolayers exhibited typical hexagonal arrays with uniform immunohistochemical staining for fibronectin, laminin, cytokeratin 8/18, and type IV collagen in the chicken-wire distribution characteristic of these epithelial cells. The identity of RPE cells in the culture was also confirmed by apical immunohistochemical staining of Na^+-K^+-ATPase.[28] Cells were subcultured, grown to reach near confluence, and used for experiments. The cells were in culture for up to four to six passages.

Human monocytes were freshly isolated from the peripheral blood of healthy volunteers, using modification of the methods by Yoshida et al.[24] Mononuclear cells were separated by density gradient centrifugation. The cells were washed and then layered onto density gradient (Fico-Lite monocytes, 1.068 g/mL; Linaris, Bettingen, Germany) for the enrichment of monocytes. The purity of the cells was greater than 97%.

Table 1. Inhibitors and Their Targets

Inhibitor	Protein Target
U0126	MEK
SB202190	P38
Sp600125	JNK
AG490	Jak2
Genistein	PTK
Ro318220	PKC
CAPE	NF-κB
Ly294002	PI3K
Nac	ROS
DPI	ROS
BAPTA/AM	Calcium chelator

Enzyme-Linked Immunosorbent Assay

The levels of antigenic VEGF in the serial dilutions of human RPE supernatants were quantitated by modification of a double-ligand ELISA method, as previously described.[28] Standards included 0.5 log dilutions of recombinant VEGF (R&D Systems) from 5 pg to 100 ng/well.

Quantifying mRNA of VEGF

Nearly confluent human RPE cells seeded in 60-mm dishes were treated with thrombin, TGF-β2, or thrombin plus TGF-β2, with or without the thrombin inhibitor hirudin, for 6 hours. Quantikine mRNA colorimetric quantification kits (R&D Systems) were used to quantitate RPE VEGF mRNA. In brief, after removal of excess medium, the cell lysates were obtained by adding cell lysis diluent to the cell monolayers. Then 150 μL diluent was hybridized in a hybridization plate with 50 μL of VEGF gene-specific, biotin-labeled capture oligonucleotide probes in a 65°C water bath for 1 hour. Next, 150 μL of hybridization solution was transferred to a streptavidin-coated microplate and incubated for 60 minutes at room temperature on a shaker. After the plates were washed, 200 μL of anti-digoxigenin conjugate was added to each well and incubated for 60 minutes. After the unbound conjugate solution had been washed away, 50 μL of substrate solution and 50 μL of substrate were in turn added and incubated for 60 or 30 minutes. The chromogen was generally developed for 30 minutes, but was monitored depending on the amount of VEGF present in the experimental samples and the samples used for standard calibration. The absorbance at 490 nm with correction of 690 nm was determined after the addition of 50 μL of stop solution. The concentration of VEGF mRNA was calculated by interpolation of a standard calibration curve. All samples and calibrations were in duplicate.

Semiquantitative Reverse Transcription–Polymerase Chain Reaction

The total cellular RNA was isolated from nearly confluent cultures of human RPE cells (TRIzol; Invitrogen, Carlsbad, CA), according to the reagent manufacturer's procedure. The cDNA synthesis reaction was set up according to the protocol for a reverse transcription system (Invitrogen). Briefly, 5 μg of RNA was added to the reaction mixture with M-MLV (Moloney murine leukemia virus) reverse transcriptase (100 U/μL) and 1 μL random primers, for a total volume of 20 μL PCR for each product was performed with three different cycles (15, 25, and 35). The PCR reactions were accepted as semiquantitative when each sample was carried out in the midlinear portion of the response curve. Under the experimental conditions in our study, the linear range for the PCR reaction for β-actin was observed between 15 and 30 cycles. Thus, we chose the lower midlinear portion of the response curve (20 PCR cycles) for β-actin. Specific cDNA was amplified by 28 (1 μL cDNA) and 20 cycles (1 μL cDNA) for VEGF, and β-actin, respectively. The following conditions were used in PCR reaction for VEGF and β-actin: denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, and extension at 72°C for 2 minutes. The reaction was initiated by adding 0.15 μL of *Taq* DNA polymerase (5 U/μL) to a final volume of 20 μL. The synthetic proprietary oligonucleotide primers for human VEGF were obtained from R&D (catalog no. RDP-33-025). To ensure that an equal amount of templates was used in each amplifica-

tion reaction, human β-actin sense (5′-GTGGGGCGCCCCAGGCACCA-3′) and antisense (5′-GCTCGGCCGTGGTGGT GAAGC-3′) primers were used in parallel. Each PCR product was analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Immunocytochemistry

Immunochemical staining was performed according to the manufacturer's protocol (ABC kit; Vector, Burlingame, CA). Nearly confluent human RPE cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature. The cells were incubated with primary rabbit polyclonal antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to VEGF at 37°C for 1 hour. The cell-bound antibody complexes were then visualized by development in the substrate solution containing 3-amino-9-ethylcarbazole (AEC) to yield a red reaction product, as previously described.[27] The same concentration of nonspecific rabbit IgG replaced the primary anti-VEGF antibody, and immunostaining of unstimulated RPE cells served as the control.

Statistical Analysis

The mean VEGF concentration ± SEM was determined for each assay condition. Various assay conditions were compared by two-way ANOVA (Statview; SAS Cary, NC).

Results

Human RPE VEGF Protein Production and mRNA Expression

To determine stimulation of VEGF secretion, human RPE cells were challenged by 10 U/mL of thrombin. The growth medium was harvested after 8, 24, and 48 hours of stimulation. As shown in Figure 1A, thrombin at 10 U/mL induced time-dependent increases in VEGF secretion. The induction of VEGF was noted within 8 hours of stimulation, was more evident at 24 hours, and showed sustained increases from 24 to 48 hours, reaching 3.5- and 4.8-fold higher than nonstimulated RPE cells. This stimulation by thrombin was dose dependent. Thrombin at 10 U/mL resulted in approximately 40% more VEGF secretion than did the 1 U/mL concentration. To ensure that stimulation of VEGF by thrombin was specific, two approaches were used. We first used hirudin, a potent and specific thrombin inhibitor, to examine whether co-incubation with hirudin could inhibit thrombin's action. As shown in Figure 1A, the enhanced VEGF secretion was reduced by 50% by co-incubation with hirudin (40 U/mL). Second, we used specific agonist peptides to determine which forms of PARs mediate thrombin-induced VEGF expression. Specific agonist peptides have been developed as useful tools to validate which forms of PARs are present in various cell types.[29] To find out whether the observed thrombin-mediated effects on RPE VEGF secretion may be due to specific activation of PAR-1 and -3, the two PAR forms previously shown on human RPE cells, we used specific thrombin receptor agonist peptides for RPE treatment. As shown in Figure 1B, thrombin receptor 1 agonist peptide induced dose-dependent RPE VEGF secretion. At 100 μM the PAR-1-specific thrombin receptor activated peptide (TRAP) produced levels of VEGF secretion nearly identical with that produced by 10 U/mL thrombin, whereas PAR-3 (50 and 100 μM)-specific TRAP did not induce measurable VEGF production. In addition, SCH79797, a potent nonpeptide antagonist for PAR-1,[30] induced dose-dependent reduction in hRPE VEGF expression. SCH79797 inhibited hRPE VEGF reduction by 66% at 30 μM and 37% at 3 μM (Fig. 1C). These results suggest that thrombin-induced human RPE VEGF secretion is predominantly mediated by PAR-1.

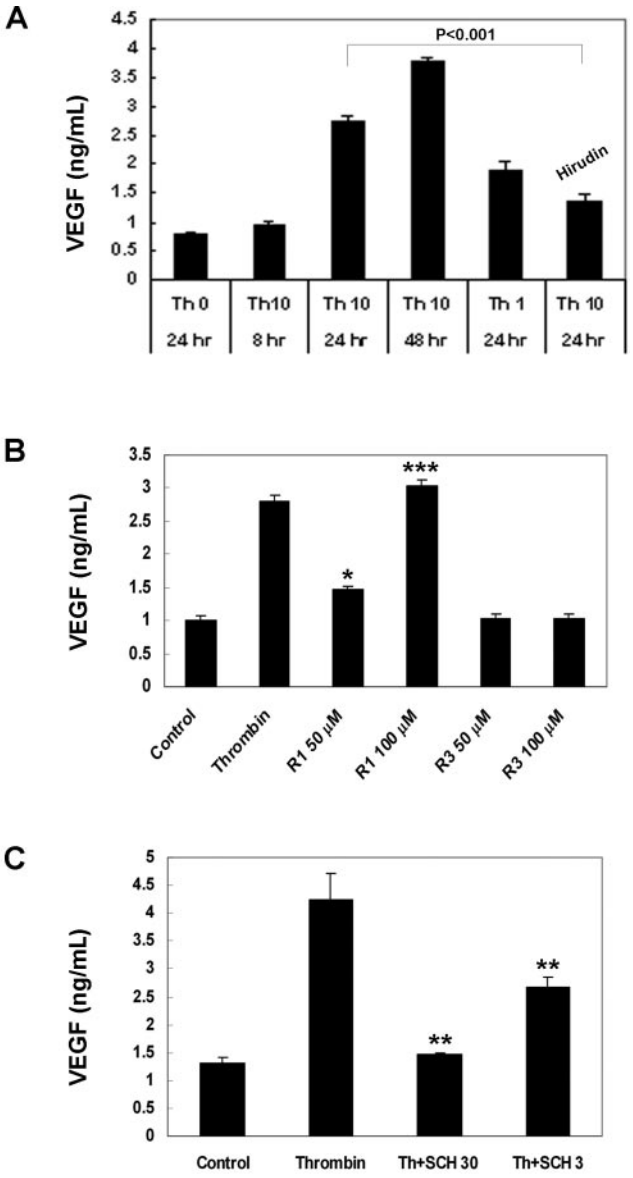

Figure 1. Stimulation of human RPE VEGF secretion. (**A**) Time- and dose-dependent stimulation of RPE VEGF secretion by thrombin. Human RPE cells were stimulated by thrombin (0, 1, or 10 U/mL; Th), with or without hirudin and SCH79797 for 8, 24, and 48 hours. (**B**) Stimulation of RPE VEGF secretion by TRAPs. The RPE cells were stimulated with thrombin or TRAPs for thrombin receptors PAR-1 and -3. $^{*}P < 0.05$, $^{***}P < 0.001$ versus the control. (**C**) Inhibition of thrombin-stimulated human RPE VEGF production by SCH79797. Unstimulated RPE cells were the control. The conditioned media was assayed for VEGF by ELISA. $^{**}P < 0.01$ versus the control. The data are expressed as the mean ± SEM ($n = 3$). Th, Thrombin; R1, PAR-1; R3, PAR-3; SCH30, SCH79797 at 30 μM; and SCH3, SCH79797 at 3 μM.

To determine whether the stimulation of VEGF secretion by thrombin occurs at transcriptional and/or translational levels, steady state VEGF mRNA was quantified by semiquantitative RT-PCR. As seen in Figure 2A, induction of VEGF mRNA expression by thrombin was evident as early as 1 hour after stimulation, peaked at 2 hours, and sustained for at least 20 hours. Consistent with the results by ELISA, induction of VEGF mRNA expression by thrombin was greater than around 1.5-fold of that found in untreated RPE cells ($P < 0.01$) and was significantly abolished by 40 U/mL of hirudin (Fig. 2B).

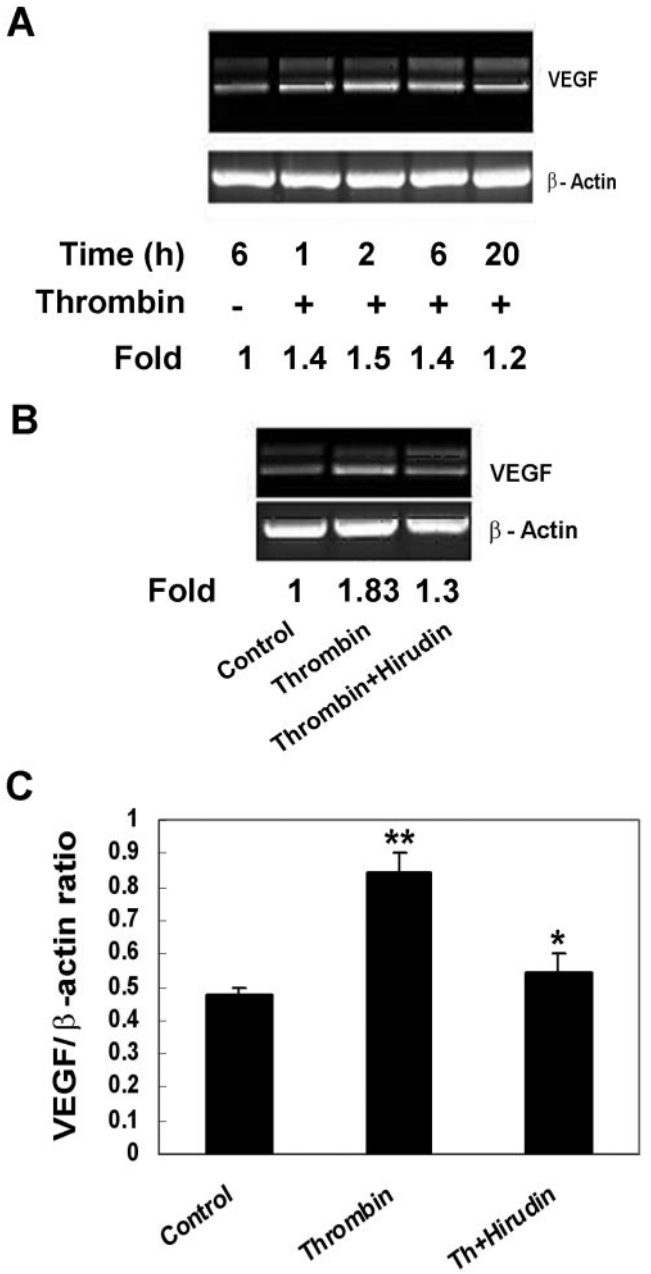

FIGURE 2. Stimulation of human RPE VEGF mRNA expression by thrombin. The cells were treated with thrombin (0 or 10 U/mL) for 1, 2, 6, and 20 hours (**A**) and with thrombin (10 U/mL), with or without hirudin (40 U/mL) for 6 hours (**B**). Total mRNA was isolated and subjected to RT-PCR analysis. The data shown in (**A**) and (**B**) represent results from a typical experiment. The changes were expressed as ratios of the normalized densities of VEGF PCR products by β-actin between treated and untreated samples (**A**, **B**). Statistical analysis of thrombin-induced VEGF mRNA expression in parallel samples. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control.

Multiple Signal Transduction Pathways Involved in Thrombin-Induced Human RPE VEGF Expression

Although the signaling pathways for thrombin have been well documented in many cell types,[31] those for thrombin-induced VEGF production in human RPE cells remain unknown. To assess the signal transduction pathways involved in thrombin-induced VEGF gene expression in RPE cells, various inhibitors specific for signal mediators were used (Table 1). The inhibitors included Ly294002 (100 μM), a PI3K inhibitor; U0126 (20 μM), an inhibitor of MEK, the signal molecule upstream from ERK; SB202190 (30 μM), a p38 inhibitor; Sp600125 (20 μM), a JNK inhibitor; CAPE (25 μg/ mL), a NF-κB inhibitor; AG490 (50 μM), a Jak2 inhibitor; genistein (25 μg/mL), an inhibitor of tyrosine kinase; Ro318220 (10 μM), a PKC inhibitor; and Nac (25, 10, and 1 mM) and DPI (25 μM), blockers of reactive oxygen species. hRPE cells exposed to various inhibitors at maximum doses in this study did not show any changes in their viability, which ranged from 95% to 100% for all treatments. As shown in Figure 3, more than 90% of the thrombin-induced VEGF protein production was abolished by CAPE, Nac (25 mM), and DPI (92%, 100%, and 93%). Nac inhibited thrombin-induced VEGF by 92% and 87% at 25 and 10 mM, respectively, whereas Nac at 1 mM resulted in only slight inhibition (Fig. 3B). The stimulated VEGF expression was also highly sensitive to Ro318220, U0126, SB202190, Ly294002, genistein, and Sp600125, with inhibition rates of 88%, 80%, 80%, 75%, 73%, and 70%, respectively. By contrast, thrombin-induced VEGF production was resistant to AG490. To compare the signaling pathways induced by thrombin with those stimulated by TGF-β2, results of similar experiments were obtained (data not shown).

Additive Increase in Thrombin-Induced Human RPE VEGF Production by TNF-α and Human Monocyte Coculture

The study of thrombin in combination with other proangiogenic stimuli is also important for a full understanding of the

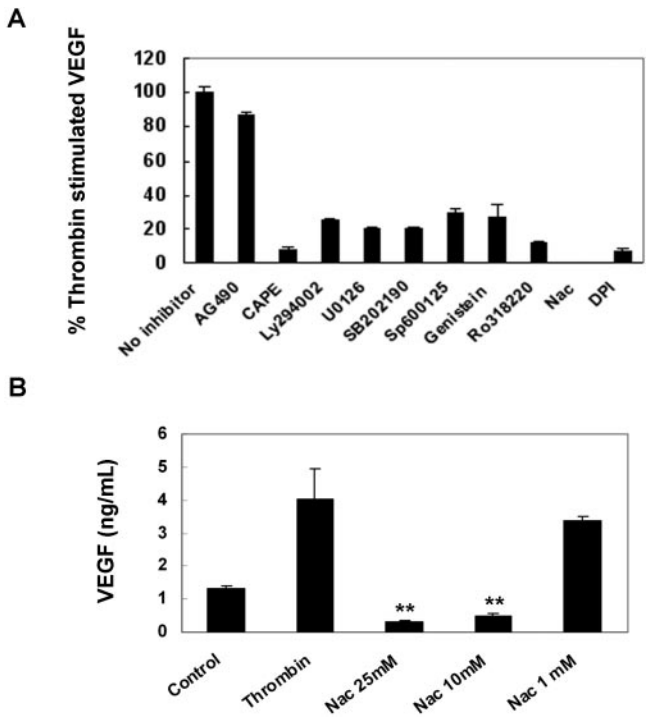

FIGURE 3. Inhibition of thrombin-stimulated human RPE VEGF production by inhibitors specific for various signaling pathways (**A**) and Nac dose response (**B**). RPE cells were pretreated with AG490 (50 μM), CAPE (25 μg/mL), Ly294002 (100 μM), U0126 (20 μM), SB202190 (30 μM), Sp600125 (20 μM), genistein (25 μg/mL), Ro318220 (10 μM), Nac (25, 10, and 1 mM), or DPI (25 μM) and then costimulated with thrombin (10 U/mL) for 24 hours. Thrombin-stimulated RPE cells were the positive control. Results are expressed as the mean ± SEM ($n = 3$). $^{**}P < 0.001$ versus the corresponding control.

role of thrombin in vivo. TNF-α has been shown to induce human RPE VEGF.[32] We have shown that thrombin-induced TNF-α is responsible for human RPE/monocyte coculture-induced chemokine expression.[24] These interactions led us to examine induction of RPE VEGF secretion by thrombin plus TNF-α and by thrombin plus coculture with monocytes. Incubation of human RPE cells with TNF-α (20 ng/mL) induced VEGF protein secretion above basal levels (Fig. 4). Compared with induction by TNF-α and by thrombin alone (0.48 $\pm$ 0.04 and 1.96 $\pm$ 0.07 ng/mL, respectively), combined stimulation with both agents resulted in enhanced VEGF secretion very close to the sum of VEGF induced by the two factors alone (2.71 vs. 2.44 ng/mL), suggesting that TNF-α and thrombin are additive in stimulating VEGF production in RPE cells.

We then tested the effect of thrombin on monocyte-induced RPE VEGF secretion. Similar additive induction was observed when monocytes were overlaid onto human PRE cells in the presence of thrombin. Monocytes cocultured with PRE cells for 24 hours induced VEGF secretion (3.28 $\pm$ 0.27 ng/mL) more than that obtained with 10 U/mL thrombin (2.74 $\pm$ 0.1 ng/mL). The combined induction by monocyte coculture with thrombin was approximately equal to the sum of VEGF induced by each of these stimulants alone (4.58 vs. 4.46 ng/mL).

Synergistic Effect of Thrombin Plus TGF-β2 on Stimulating RPE VEGF Expression

Because TGF-β2 is the predominant form of TGF-β found in ocular tissues,[33] we next focused on the effect of TGF-β2 on VEGF expression induced by thrombin, to determine whether TGF-β2 alone or in combination with thrombin alters VEGF mRNA and protein production in human RPE cells. We also performed experiments to determine whether thrombin induces TGF β2 production in hRPE cells and found that it does not do so (data not shown).

Incubation of RPE cells with TGF-β2 (10 ng/mL) for 24 hours potently induced VEGF secretion. The level of induction by TGF-β2 was approximately four times that stimulated by 10

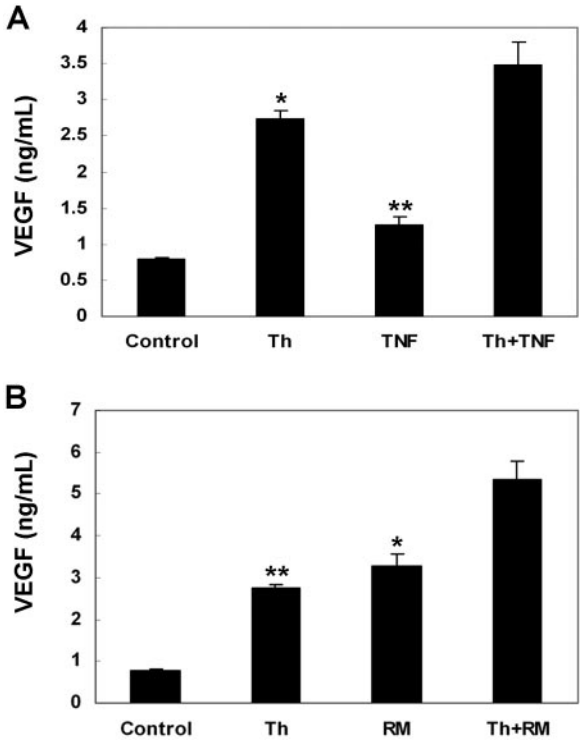

FIGURE 4. Effects of TNF-α and human RPE/monocyte coculture on thrombin-stimulated RPE VEGF secretion. The RPE cells were stimulated by thrombin (10 U/mL; Th) alone and in combination with TNF-α (20 ng/mL) (**A**) and cocultured with human monocytes (ratio, 1:1.5; RM; **B**) for 24 hours. Unstimulated RPE cells were the control. The conditioned media were assayed for VEGF by ELISA. The results are expressed as the mean $\pm$ SEM ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$ versus the control.

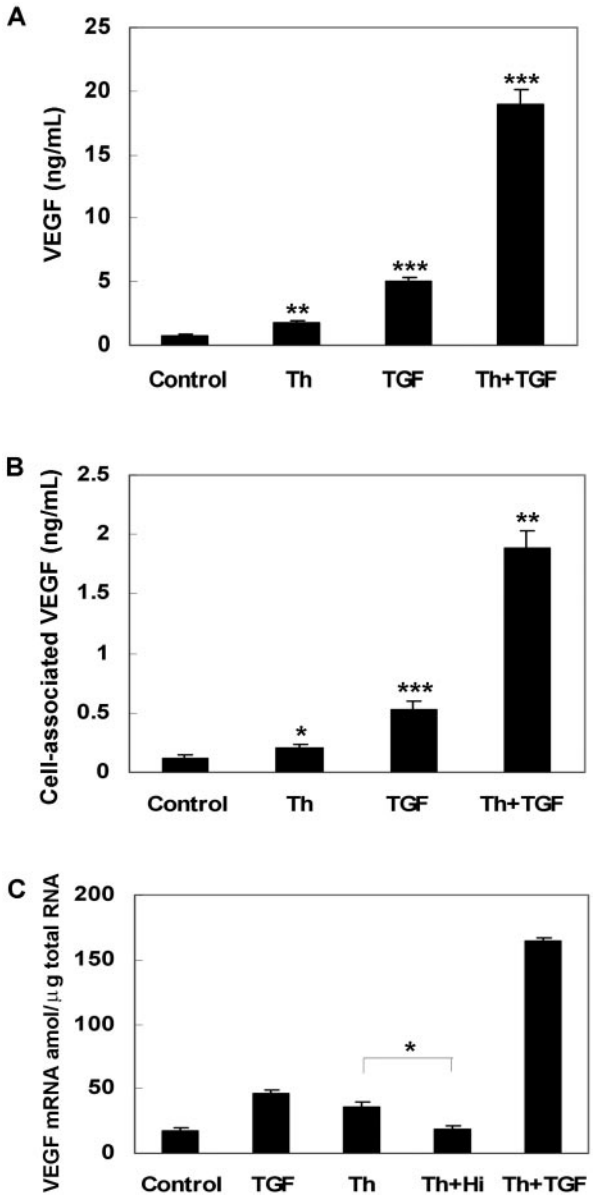

FIGURE 5. Effects of TGF-β2 on thrombin-stimulated human RPE VEGF protein secretion (**A**), cell-associated VEGF protein production (**B**), and VEGF mRNA expression (**C**). RPE cells were stimulated with thrombin (10 U/mL; Th) alone or in combination with TGF-β2 (10 ng/mL; TGF) for 6 (**C**) or 24 (**A**, **B**) hours. The media overlaying the cells were then collected, and the cells were lysed for VEGF determination by ELISA. For assessing VEGF mRNA expression in RPE cells, total RNA was extracted from RPE cells, and VEGF mRNA levels were determined with an mRNA colorimetric quantification kit. Unstimulated RPE cells and RPE cells incubated with hirudin (Hi) were the controls. (**A**) **Th versus the control, $P < 0.01$; ***TGF versus the control, $P < 0.001$; ***Th+TGF versus the sum of Th and TGF alone, $P < 0.001$. (**B**) *Th versus the control, $P < 0.05$; ***TGF versus the control, $P < 0.001$; **Th+TGF versus the sum of Th and TGF alone, $P < 0.01$. (**C**) *T+Hi versus Th alone, $P < 0.05$.

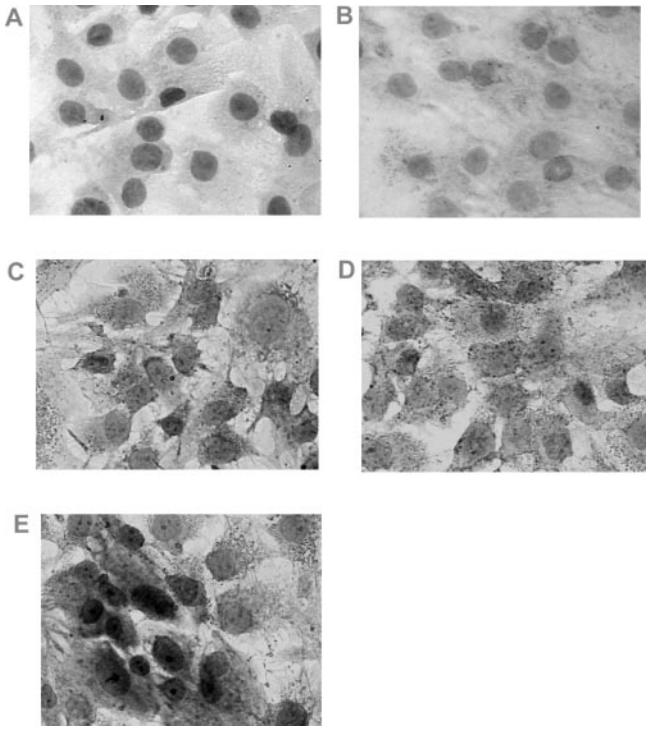

FIGURE 6. Immunohistochemical analysis of VEGF protein expression in cultured human RPE cells. RPE cells were exposed to thrombin, TGF-β2, or thrombin+TGF-β2 for 16 hours. (**A**) Cells stained with normal rabbit serum served as the nonspecific control. Untreated RPE cells (**B**) and cells treated with thrombin (**C**), TGF-β2 (**D**), or thrombin+TGF-β2 (**E**) were stained with rabbit anti-human VEGF antibody and the red chromogen, AEC.

U/mL thrombin (4.18 vs. 0.94 ng/mL; $P < 0.001$; Fig. 5A). The same concentration of TGF-β2 markedly synergized thrombin-induced VEGF protein secretion. The amount of VEGF jointly induced by TGF-β2 plus thrombin was approximately 3.5-fold of the level anticipated if both factors were simply additive in stimulating VEGF secretion (18.1 vs. 5.12 ng/mL; $P < 0.001$). To further characterize this synergistic effect, we lysed the treated cells and subjected them to ELISA to determine cell-associated VEGF. In unstimulated RPE cells, VEGF was barely detectable (0.11 $\pm$ 0.03 ng/mL). Upon stimulation by thrombin (10 U/mL) for 24 hours, intracellular VEGF was enhanced, albeit at a very low level (0.1 ng/mL), whereas in TGF-β2 (10 ng/mL)-treated RPE cells, the induced VEGF protein was approximately four times higher than that caused by thrombin stimulation (0.42 vs. 0.1 ng/mL). Consistent with the synergistic effect of RPE VEGF secretion observed in the conditioned media, RPE cells exposed to TGF-β2 and thrombin contained VEGF at levels 3.4-fold more than that expected simply from an additive effect (1.78 vs. 0.52 ng/mL; $P < 0.01$).

To illustrate synergistic increases in VEGF mRNA production by coadministration of thrombin and TGF-β2, we examined VEGF mRNA by colorimetric quantification of PCR products. As seen in Figure 5C, combined stimulation by thrombin and TGF-β2 (146.5 $\pm$ 2.5 attamole/μg total RNA) resulted in a threefold increase in VEGF mRNA over the sum of the VEGF mRNA induced by TGF-β2 and thrombin separately (29.6 $\pm$ 1.4 and 19.0 $\pm$ 1.9 attamole/μg total RNA, respectively; $P < 0.001$). This synergistic increase (threefold) at the mRNA level was close to the protein increases (3.5-fold) found by ELISA, suggesting that the synergistic effect at the protein level is due to increases in both VEGF mRNA expression and translation. Thrombin-induced VEGF mRNA synthesis was also shown to be sensitive to inhibition of thrombin enzymatic activity by hirudin, consistent with the ELISA (Fig. 1) and RT-PCR findings (Fig. 2).

In agreement with the ELISA data depicted in Figures 5A and 5B, immunocytochemical staining of intracellular RPE VEGF protein by anti-VEGF antibodies showed similar qualitative trends (Fig. 6). Human RPE cells stimulated by thrombin plus TGF-β2 (Fig. 6E) showed more intense immunoreactivity when compared with cells stimulated by thrombin and TGF-β2 alone (Figs. 6C, 6D). By contrast, RPE cells treated with preimmune serum (Fig. 6A) were completely negative and unstimulated quiescent cells showed only weak immunostaining for VEGF (Fig. 6B).

Thrombin is a potent mediator of calcium mobilization in endothelial cells. This effect has been suggested to be the major metabolic link between thrombin and TNF-α in synergistically stimulating the expression of tissue factor (thromboplastin; factor III) in endothelial cells.[34] Thus, the role that thrombin-stimulated calcium mobilization plays in VEGF induction is of interest. To investigate this possibility, we pretreated human RPE cells with BAPTA/AM, a cell-permeable calcium chelator for 1 hour before challenge with TGF-β2, thrombin, or thrombin plus TGF-β2. ELISA data showed that BAPTA/AM did not significantly inhibit TGF-β2-induced VEGF secretion (Fig. 7). In contrast, more than 65% of thrombin-induced VEGF expression was blocked by BAPTA/AM ($P < 0.01$). VEGF stimulation due to thrombin plus TGF-β2 was only reduced by 20%. Although this inhibition was not statistically significant, the actual reduction of VEGF protein (2.1 ng/mL) induced by thrombin combined with TGF-β2 was approximately five times more than the amount of VEGF inhibited by BAPTA/AM when RPE cells were stimulated by thrombin alone (0.44 ng/mL). These data suggest that thrombin-mediated calcium mobilization, at least in part, accounts for the synergistic increases in VEGF expression caused by thrombin plus TGF-β2.

DISCUSSION

VEGF protein exists as a homodimer of four alternatively spliced variants of a single gene.[35] Upon binding to its tyrosine kinase receptors (Flt-1/VEGF-R1 and KDR/VEGF-R2), VEGF trig-

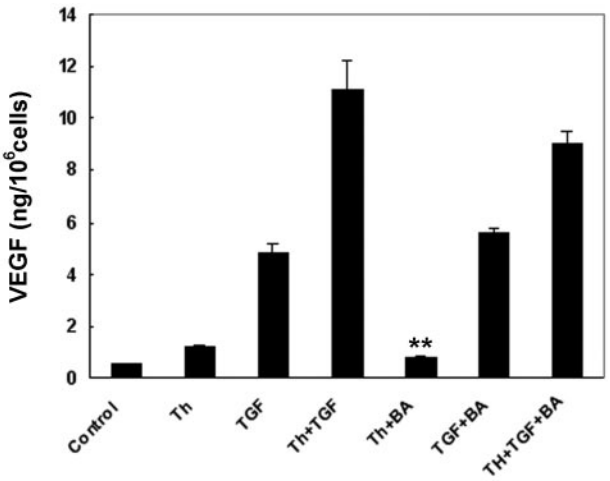

FIGURE 7. Effects of intracellular calcium chelation on VEGF expression induced by thrombin and TGFβ-2. RPE cells were stimulated after preincubation with 5 μM BAPTA for 1 hour, then with thrombin (10 U/mL), TGFβ-2 (10 ng/mL) or both for 24 hours. The conditioned media were assayed for VEGF by ELISA. The data are expressed as the mean $\pm$ SEM ($n = 3$). Unstimulated RPE cells were the control. BA, BAPTA; TGF, TGF-β2; Th, thrombin. **Th versus Th+BA, $P < 001$.

gers a cascade of downstream signaling events, which leads to physiological and pathologic angiogenesis.[36] Although VEGF is expressed in various cell types, expression of KDR and Flt-1 is primarily restricted to vascular endothelial cells.[37]

There is ample immunohistochemical, molecular, genetic, and pharmacologic evidence that VEGF is one of the most important factors leading to ocular neovascularization.[38,39] For example, intraocular VEGF levels are elevated in patients with PDR, and may reach 15 to 30 times higher than those present in nondiabetic control patients. VEGF is a proangiogenic mitogen that also promotes vascular permeability. It is implicated as a cause of blood–retinal barrier breakdown within the neurosensory retina in diabetic retinopathy. In addition, RPE-derived VEGF may also contribute to dysfunction in the neurosensory and outer blood–retinal barriers and may induce choroidal neovascularization. In experimental animals, injection of adenoassociated virus-VEGF into the subretinal space of rats causes extensive subretinal neovascularization, degeneration of photoreceptors, and proliferation of RPE cells. In addition, silencing VEGF by small interfering (si)RNA effectively inhibits choroidal neovascularization in a mouse model.

In contrast, overexpression of VEGFs and their receptors increases microvascular permeability and angiogenesis of the outer retina in AMD.[40] VEGF protein is known to be expressed in human RPE cells of surgically excised choroidal neovascular membranes in AMD and other ocular diseases.[32] It is induced by various stimuli, including hypoxia, increased levels of reactive oxygen species, vasopressor hormones (angiotensin II and vasopressin, advanced glycation end products), cytokines (TGF-β, IL-1), and growth factors such as basic fibroblast growth factor (bFGF). The induction of VEGF is thought to be the consequence of a local inflammatory response.[41] In this study, we demonstrated for the first time that thrombin is a potent stimulus of VEGF expression by RPE cells. This action is mediated by thrombin receptor PAR-1, not PAR-3. The thrombin-specific action was evidenced by inhibition with hirudin, a thrombin antagonist, and by mimicking the stimulation with a PAR-1 specific agonist. These results suggest that thrombin is a potential angiogenic factor in RPE-mediated neovascularization and that RPE-derived VEGF may initiate its own production by enhancing thrombin accumulation at sites of blood–retinal barrier breakdown.

The angiogenic action of thrombin has been demonstrated both in vivo and in vitro.[42,43] Many thrombin-mediated cellular effects on endothelial cells may contribute to angiogenesis. It has been reported that thrombin causes time- and dose-dependent decreases in endothelial attachment to basement membrane components, permits endothelial cell migration, and increases DNA synthesis in endothelial cells, thus promoting endothelial cell proliferation.[15] Thrombin also upregulates expression of integrin $\alpha v\beta 3$, a marker for angiogenic endothelial cells, and activates matrix metalloproteinase 2.[15] Furthermore, our laboratory and others have shown that thrombin stimulates proangiogenic IL-8 and MCP-1 secretion by RPE cells.[24,44]

In the present study, we also investigated the signaling pathways leading to enhanced VEGF gene expression by thrombin and TGF-β2. The mechanisms underlying regulation of VEGF gene expression and protein production have been the subject of intensive investigation.[31,45] Expression of VEGF is controlled at both transcriptional and posttranscriptional (mRNA stabilization) levels via complicated signaling pathways[31,45–47] that lead transcription factors, including HIF-1, Smads, Ap-1, Ap-2, Sp-1, and NF-κB, to bind the VEGF promoter at specific, functional DNA-binding sequences.[46–48] VEGF expression is further tuned by stabilization of VEGF mRNA[14,49,50] and through crosstalk between different pathways.[51] In this study, we used a pharmacological approach, demonstrating that the stimulation of VEGF expression by thrombin was mediated by multiple signaling pathways post-PAR-1, including the MAPK/ERK, p38, JNK MAPK pathways and the PI3K and NF-κB pathways, as well as by activation of PTK, PKC, and ROS. Those signaling pathways are also shared by TGF-β2 in stimulating VEGF expression in RPE cells, although thrombin-induced VEGF expression in the RPE was more sensitive to inhibition by Sp600125 than that induced by TGF-β2 (data not shown), suggesting that the JNK pathway is more important for thrombin induction than for TGF-β2 induction of RPE VEGF.

Depending on cell types and stimuli, all these signaling molecules have been reported in VEGF gene expression.[31,45,52] Although STAT3, the downstream target of Jak2, is required for transcriptional control of VEGF gene expression and blockade of Jak2 by AG490 abrogates the induced VEGF production in many cell types,[53] the present studies do not support an involvement of the Jak2 pathway in thrombin- and TGF-β2-induced VEGF gene expression in RPE cells.

Upregulation of VEGF has been observed in various cell types by thrombin,[13,14,54] TNF-α,[44] TGF-β2,[55] and monocyte contact.[56] As demonstrated in the present study, thrombin, monocyte cell-to-cell contact, TGF-β2, and TNF-α all independently stimulate human RPE to produce VEGF. Our results with the latter two proangiogenic factors were consistent with those in previous reports.[44,55] In addition to these angiogenic effects by thrombin alone, thrombin may amplify its effects through interplays with other proangiogenic agents such as TNF-α and TGF-β. For example, thrombin works in synergy with TNF-α, causing large increases in endothelial permeability,[57,58] and simultaneous treatment of HepG2 cells with thrombin and TGF-β synergistically enhances the levels of plasminogen activator inhibitor type-1.[59] As shown in this study, stimulation of human RPE VEGF by thrombin and TGF-β2 appears to be mediated by highly overlapped signaling pathways. However, the mechanism underlying the synergistic effect of thrombin and TGF-β2 in stimulating RPE VEGF is not fully understood. The synergy between thrombin and TGF-β2 is likely to result from the combination of the signaling pathways unique to each stimulus. The Smad pathway by TGF-β[51] and the Ca^{+2} pathway by thrombin[60] are likely candidates for the synergistic action of the two factors. Consistent with this hypothesis is our finding that 36% of thrombin-induced and 19% of thrombin+TGF-β2–induced, but not TGF-β2-induced, RPE VEGF stimulation was sensitive to the calcium chelator BAPTA. The remaining BAPTA-insensitive synergy between the two factors remains to be investigated. The TGF-β2-specific Smad pathway is likely to be the major contributor.

Our findings of the independent and interactive upregulation of VEGF production by thrombin, TNF-α, TGF-β2, and monocytes in hRPE cells are likely to be pathologically relevant. Thrombin is generated in areas of hemorrhage and in diseased retinal tissue.[61,62] Thrombin has been thought to be a potent stimulus for inflammation, which aggravates disease processes.[21,63] The role of thrombin in ocular neovascularization is reminiscent of its role in angiogenesis and tumor progression.[64] On the one hand, thrombin induces VEGF expression in tumor cells. On the other hand, it upregulates thrombin receptors (KDR and Flt-1) on endothelial cells. The dual role of thrombin results in a synergistic activation of angiogenesis. Likewise, it is possible that plasma-derived thrombin potentiates other proangiogenic cytokines such as TGF-β2 and TNF-α, as well as mononuclear phagocytes to stimulate RPE VEGF secretion. The secreted VEGF then binds to VEGF receptors in endothelial cells that are upregulated by thrombin, exacerbating ocular angiogenesis. Further studies of the interplay among those proangiogenic factors in ocular neovascularization under diseased conditions will provide more insight into the important role of these factors in PDR, PVR, and AMD, and suggest

potential approaches for pharmacological interventions. One such intervention may be the specific inhibition of PAR-1-mediated thrombin induction of RPE VEGF.

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