Activated Blood Coagulation Factor X (FXa) Induces Angiogenic Growth Factor Expression in Human Retinal Pigment Epithelial Cells

Margrit Hollborn,1 Leon Koben,1,2 Cornelia Werschnik,3 Lore Tietz,3 Peter Wiedemann,1 and Andreas Bringmann1

PURPOSE. To determine the transcriptional regulation of the blood coagulation factor X (FX) in cultured human retinal pigment epithelial (RPE) cells, and whether the effects of FXa on the chemotaxis and expression of angiogenic growth factors are mediated by autocrine growth factor signaling.

METHODS. Alterations in gene expression and secretion of growth factors were determined by real-time RT-PCR and ELISA, respectively. Cellular proliferation and chemotaxis were investigated with a bromodeoxyuridine immunoassay and a Boyden chamber assay, respectively.

RESULTS. The gene expression of FX in RPE cells was increased by hypoxia and prostaglandin E2, and decreased by blood serum, FXa, thrombin, transforming growth factor beta (TGF-β1), and platelet-derived growth factor (PDGF). The serum-induced downregulation of FX was mediated by thrombin and TGF-β1 signaling. FXa induced chemotaxis of RPE cells via activation of the p38 mitogen-activated protein kinase signal transduction pathway. FXa also induced expression of vascular endothelial growth factor (VEGF), heparin-binding epidermal growth factor–like growth factor (HB-EGF), and basic fibroblast growth factor (bFGF), as well as release of VEGF bFGF and TGF-β1 from RPE cells. The stimulatory effects of FXa on the expression of growth factors and secretion of VEGF were prevented by inhibition of the TGF-β activin receptor-like kinase, but not by the thrombin inhibitor hirudin. FXa induced phosphorylation of ERK1/2, p38, and Akt proteins.

CONCLUSIONS. FXa induces chemotaxis of RPE cells, as well as expression and release of angiogenic growth factors from RPE cells, including VEGF. The effects of FXa on the expression and secretion of VEGF are mediated by autocrine/paracrine TGF-β1 signaling. (Invest Ophthalmol Vis Sci. 2012;53:5930-5939) DOI:10.1167/iovs.11-9214

From the 1Department of Ophthalmology and Eye Hospital, University of Leipzig, Leipzig, Germany; the 2Helios Klinikum Aue, Aue, Germany; and the 3Helios Klinikum Schwerin, Schwerin, Germany.

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Corresponding author: Margrit Hollborn, Department of Ophthalmology and Eye Hospital, University of Leipzig, Liebigstrasse 10-14, D-04105 Leipzig, Germany; hollbm@medizin.uni-leipzig.de.
PAR-3, and PAR-4 are cleaved by thrombin, whereas the activated factor X (FXa) can activate (in addition to other receptors) PAR-1 and/or PAR-2. It has been shown that RPE cells express PAR-1 and PAR-3, but not PAR-2 and PAR-4. Until now, it is not known whether further coagulation factors in addition to thrombin have cellular effects in the RPE, and whether the effects are dependent on the action of thrombin. Therefore, we determined whether FXa induces responses in cultured human RPE cells, which may be implicated in the development of choroidal neovascularization and subretinal edema in situ (i.e., RPE cell migration and proliferation) and expression of angiogenic factors including VEGF.

**Materials and Methods**

**Materials**

The following agents and substances (with their suppliers) were used in this study: human recombinant bFGF, heparin-binding epidermal growth factor–like growth factor (HB-EGF), PDGF-BB, tumor necrosis factor (TNF)-α, TGF-β1, and VEGF-A165 (R&D Systems, Wiesbaden, Germany); interleukin (IL)-1β (Relatech, Braunschweig, Germany); human recombinant matrix metalloproteinase (MMP)9 (purified active form), FXa, Akt inhibitor, AG1296, BAPTA/AM, G60976, U73122, SU1498, PD98059, SP600125, and LY294002 (Calbiochem, Bad Soden, Germany); AG1478 (Alexis, Grünberg, Germany); SB203580 (Tocris, Ellisville, MO); SP600125, and LY294002 (Calbiochem, Bad Soden, Germany); AG1478 (Alexis, Grünberg, Germany); SB203580 (Tocris, Ellisville, MO); SB600125, and LY294002 (Calbiochem, Bad Soden, Germany); SU1498, SP600125, SB203580, and PD173074 (Pfizer, Karlsruhe, Germany); rivaroxaban (BAY59-7939) (Bayer Pharma AG, Berlin, Germany); SB431542, α-thrombin, hirudin, and all other agents used were from Sigma-Aldrich (Taufkirchen, Germany), unless otherwise indicated. At the concentrations used, the test substances had no effects on cell viability as determined by trypan blue staining (data not shown). The cultures were pretreated with blocking substances for 30 minutes before the test substances were added.

The following polyclonal antibodies were used: a neutralizing pan-specific rabbit anti-TGFβ-directed against human TGFβ1, porcine TGFβ1 and β2, and amphibian TGFβ5 (20 μg/mL; R&D Systems), rabbit anti-human extracellular signal-regulated kinases 1 and 2 (ERK1/2; p44/p42; 1:1000; New England Biolabs, Frankfurt/M., Germany), a rabbit anti-phosphorylated ERK1/2 (1:1000; New England Biolabs), a rabbit anti-human p38 mitogen-activated protein kinase (p38 MAPK; 1:1000; New England Biolabs), a rabbit anti-human phosphorylated p38 MAPK (1:750; New England Biolabs), a rabbit anti-human Akt (1:1000; New England Biolabs), a rabbit anti-human phosphorylated Akt (1:1000; New England Biolabs), a rabbit anti-MAPK kinase (MKK)-3 (1:1000; Cell Signaling Technology, Beverly, MA), a rabbit anti-phosphorylated MKK-3/6 (1:750; Cell Signaling Technology), a rabbit anti-GAPDH (1:2000; New England Biolabs), and an anti-rabbit IgG conjugated with alkaline phosphatase (1:2000; Chemicon, Hoffenheim, Germany).

**Cell Culture**

The use of human material was approved by the Ethics Committee of the University of Leipzig, and was performed according to the Declaration of Helsinki. Human RPE cells were obtained from several donors within 48 hours of death, and were prepared and cultured as described in the Supplementary Material (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9214/-/DCSupplemental). Cells were cultured under normoxic (95% air, 5% CO2) and hypoxic conditions (1% O2, 5% CO2, 94% N2), respectively. DNA Synthesis Rate and Chemotaxis

The proliferation rate was determined by measurement of the DNA synthesis rate using a bromodeoxyuridine immunoperoxidase, as described in the Supplementary Material (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9214/-/DCSupplemental). Chemotaxis was determined with a modified Boyden chamber assay described in the Supplementary Material (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9214/-/DCSupplemental).

**RT-PCR and Real-Time PCR**

The preparation of the total RNA from cultured cells, and the RT-PCR and real-time PCR analysis, were conducted using standard methods described in the Supplementary Material (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9214/-/DCSupplemental). PCR was carried out using primer pairs described in Supplementary Table S1 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9214/-/DCSupplemental). The mRNA expression was normalized to the levels of beta-actin mRNA (ACTB).

**ELISA**

The cells were cultured at 3 × 10^5 cells per well in 96-well plates (100 μL culture medium per well). At a confluency of approximately 80%, the cells were cultured in serum-free medium for 16 hours. Subsequently, the culture medium was changed, and the cells were stimulated with the test substances, in the absence and presence of blocking substances. The supernatants were collected after 6 and 24 hours, and the levels of TGFβ1, VEGF-A165, bFGF, and HB-EGF, respectively, in the cultured media (200 μL) were determined by ELISA (R&D Systems).

**Western Blotting**

The cells were seeded at 1 × 10^5 cells per well in 6-well plates in 1.5 mL complete medium, and were allowed to grow up to a confluency of approximately 80%. After growth arrest for 16 hours, the cells were treated with test substances for different time periods. Then, the medium was removed, the cells were washed twice with prechilled phosphate-buffered saline (pH 7.4; Invitrogen, Paisley, UK), and the monolayer was scraped into 150 μL lysis buffer (Mammalian Cell Lysis-1 Kit; Sigma-Aldrich). The total cell lysates were centrifuged at 10,000g for 10 minutes, and the supernatants were analyzed by immunoblot. Equal amounts of protein (30 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis. Immunoblots were probed with primary and secondary antibodies, and immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. In addition to near-confluent cultures, confluent cultures were used, as indicated.

**Statistics**

The migration rate and the levels of growth factors in the cultured media are expressed as percentage of untreated control (100%). For each test, at least three independent experiments were carried out in triplicate. Data are expressed as means ± SEM; statistical significance (Mann-Whitney U test and Kruskal-Wallis test followed by Dunn’s comparison for multiple groups) was accepted at P < 0.05.

**RESULTS**

**Transcriptional Regulation of FX**

It has been shown that RPE cells express factors of the extrinsic coagulation pathway including factor X (FX). To determine the transcriptional regulation of FX, we stimulated RPE cell cultures with different agents. As shown in Figure 1A, chemical hypoxia induced by the treatment of the cultures with CoCl2 resulted in a significant (P < 0.05) increase in the gene expression of FX. No alterations in the gene expression of FX were observed in the presence of various other divalent cations (CaCl2, NiCl2, MgCl2, and CdCl2, respectively, each 150
μM; data not shown). The gene expression of FX was also increased under hypoxic (1% O₂) conditions (Fig. 1A). Stimulation of the cultures with serum (10%), TGF-β1, and PDGF decreased the expression of FX (Fig. 1A). The expression of FX was also decreased in the presence of activated factor X (FXa) and thrombin (Fig. 1A). Whereas the inflammatory factors IL-1β and TNFα had no effects, prostaglandin E₂ (PGE₂) increased the expression of FX (Fig. 1A).

Serum constituents, which induce the downregulation of FX (Fig. 1A), may include prothrombin, which can be activated at the surface of RPE cells, as well as TGF-β. To determine the factors present in serum that downregulate the expression of FX, we tested the thrombin inhibitor hirudin and the blocker of the TGF-β activin receptor-like kinase, SB431542. As shown in Figure 1B, coadministration of both blockers fully abrogated the serum-induced downregulation of FX, suggesting that thrombin and TGF-β are serum-derived factors that induce downregulation of FX.

**FXa-Induced Tissue Factor Expression**

In situ, blood coagulation in areas of tissue damage and hemorrhage is initiated by the exposure of tissue factor. In various cell systems, the expression of the tissue factor was found to be increased by FXa. We found that treatment of the cultures with FXa induced a transient increase in the gene expression of tissue factor in RPE cells (Fig. 1C).

**FXa-Induced Chemotaxis**

Addition of FXa to the culture media did not result in alteration of the RPE cell proliferation (Fig. 2A). However, FXa stimulated dose-dependently the chemotaxis of RPE cells (Fig. 2B). FXa-induced chemotaxis was prevented in the presence of the competitive FXa inhibitor rivaroxaban (Fig. 2C). The motogenic effect of thrombin in RPE cells was previously shown to be mediated by autocrine/paracrine transactivation of growth factor receptors. To determine whether the FXa-induced chemotaxis is also mediated by receptor transactivation, we tested selective inhibitors of the PDGF, epidermal growth factor (EGF), and FGF receptor tyrosine kinases, and of the TGF-β activin receptor-like kinase. As shown in Figure 2C, FXa-induced chemotaxis was not abrogated in the presence of these inhibitors, suggesting that the motogenic effect of FXa was not mediated by transactivation of growth factor receptors. The motogenic effect of FXa was also not mediated by thrombin signaling, as indicated by the lack of inhibition by the thrombin inhibitor hirudin (Fig. 2C). Thrombin and FXa exerted nonadditive effects on the chemotaxis (not shown).

To determine the intracellular signaling pathways implicated in FXa-induced chemotaxis, we tested pharmacologic blockers of key intracellular signal transduction molecules. As shown in Figure 2C, the FXa-induced chemotaxis was abrogated in the presence of a selective inhibitor of p38 MAPK activation, SB203580. Inhibitors of ERK1/2 activation, the c-Jun N-terminal kinase (JNK), and of Akt, which is a key molecule of the phosphatidylinositol-3 kinase (PI3K)-Akt signaling pathway, did not reduce FXa-induced chemotaxis (Fig. 2C). The data suggest that the FXa-induced chemotaxis is mediated by activation of the p38 MAPK signal transduction pathway. The data are in agreement with previous studies that showed that activation of p38 MAPK is critical in ligand-induced chemotaxis of RPE cells. The stimulatory effect of PD98059 on the chemotaxis under control conditions (Fig. 2C) is presumably caused by ERK1/2-mediated disinhibition of p38 MAPK, as previously shown in various cell systems.

**Regulation of Growth Factor Expression by FXa**

To determine whether FXa regulates the expression of growth factors that are known to be implicated in neovascularization in situ, we carried out real-time RT-PCR analysis. As shown in Figure 3, FXa had no effects on the gene expression of TGF-β1, hepatocyte growth factor (HGF), PDGF-A, and PDGF-B. However, FXa induced time-dependent upregulation of HB-EGF, bFGF, and VEGF-A in RPE cells (Fig. 5). FXa-induced upregulation of HB-EGF (Fig. 4A), bFGF (Fig. 4B), and VEGF...
was abrogated in the presence of the inhibitor of the TGF-β activin receptor-like kinase, SB431542. This suggests that autocrine/paracrine TGF-β signaling is involved in mediating the effect of FXa on the gene expression of growth factors. It has been shown that thrombin stimulates the gene expression of HB-EGF, bFGF, and VEGF in RPE cells. Coadministration of FXa and thrombin resulted in a nonadditive increase in HB-EGF expression (Fig. 4A). On the other hand, the transcriptional activation of bFGF (Fig. 4B) and VEGF (Fig. 4C) was blocked when FXa and thrombin were coadministered.

Regulation of Growth Factor Secretion by FXa

To determine whether FXa induces secretion of growth factors from RPE cells, we measured the levels of TGF-β1, VEGF-A165, bFGF, and HB-EGF proteins in the cultured media by ELISA. As shown in Figure 5A, FXa induced secretion of TGF-β, VEGF, and bFGF; whereas it had no effect on the secretion of HB-EGF. Hypoxia is a major factor that induces secretion of VEGF from retinal cells. Triamcinolone acetonide inhibits the expression and secretion of VEGF from RPE cells. Triamcinolone also inhibited the FXa-induced secretion of VEGF (Fig. 5B). FXa-induced secretion of VEGF was further prevented by pharmacologic blockers of ERK1/2, p38 MAPK activation, SB203580 (10 μM), and the inhibitor of p38 MAPK activation, SB203580 (10 μM), respectively. The following agents did not inhibit the FXa-induced chemotaxis: the inhibitor of the PDGF receptor tyrosine kinase, tyrphostin AG1296 (10 μM), the inhibitor of the EGF receptor tyrosine kinase, AG1478 (600 nM), the inhibitor of the FGF receptor tyrosine kinase, PD173074 (500 nM), the blocker of the TGF-β activin receptor-like kinase, SB431542 (10 μM), the thrombin inhibitor, hirudin (20 U/mL), the MEK inhibitor, PD98059 (20 μM), the JNK inhibitor, SP600125 (10 μM), and Akt inhibitor (Akt-I; 30 μM). Vehicle control was made with DMSO (1%). As positive control, the effect of PDGF (10 ng/mL) was determined. Significant blocking effect: **P < 0.01. Significant difference versus control (100%): *P < 0.05; **P < 0.01.
MAPK, JNK, and PI3K activation (Fig. 5D). These data are consistent with previous studies that showed that the ligand-induced expression and secretion of VEGF in RPE cells depends on activation of various intracellular signal transduction pathways.33,39

We found that the FXa-induced secretion of VEGF depends on autocrine TGF-β1 signaling (Fig. 5D). Both FXa (Fig. 5A) and thrombin (Fig. 5E) induced release of TGF-β1 from RPE cells. Coadministration of FXa and thrombin increased the release of TGF-β1 to a similar extent as that of each factor alone (Fig. 5E). Active TGF-β1 is known to be released by cleavage from the latent TGF-β1 complex, which is linked to the extracellular matrix.40 Cleavage of active TGF-β1 may be mediated by different proteinases including MMPs and thrombin.40,41 To determine whether the FXa-induced release of TGF-β1 depends on the generation of thrombin at the surface of RPE cells, we tested the thrombin inhibitor, hirudin. Hirudin did not inhibit the FXa-induced release of TGF-β1 (Fig. 5E), whereas the FXa inhibitor rivaroxaban abrogated the FXa-induced release of TGF-β1 (Fig. 5E). The data suggest that the effect of FXa is independent of thrombin formation. Furthermore, the broad-spectrum metalloproteinase inhibitor, 1,10-phenanthroline (10 μM), did not inhibit the FXa-induced release of TGF-β1 from RPE cells (not shown). The proteinase involved in FXa-induced release of TGF-β1 remains to be determined in future experiments.

**Figure 3.** Effect of FXa on the gene expression of growth factors in human RPE cells. The mRNA levels were determined by real-time RT-PCR after stimulation of the cells with FXa (1 U/mL) for 2, 6, and 24 hours, respectively. Means ± SEM of five independent experiments using cells from different donors. Significant difference versus control: *P < 0.05; **P < 0.01.

**Figure 4.** Regulation of the FXa-induced gene expression of HB-EGF (A), bFGF (B), and VEGF (C). The mRNA levels were determined by real-time RT-PCR after stimulation of the cells with FXa (1 U/mL) for 2 (A) and 6 hours (B, C), respectively. The FXa-induced upregulation of the three growth factors was blocked in the presence of the inhibitor of the TGF-β activin receptor-like kinase, SB431542 (10 μM). Thrombin (10 U/mL) induced expression of HB-EGF (A), bFGF (B), and VEGF (C). The transcriptional upregulation of bFGF (B) and VEGF (C), but not of HB-EGF (A), was blocked when FXa and thrombin were coadministered. Means ± SEM of four to five independent experiments using cells from different donors. Significant difference versus control: *P < 0.05; **P < 0.01. Significant difference versus FXa: +P < 0.05; ++P < 0.01.
FXa-induced secretion of TGF-β1 was dependent on intracellular calcium signaling and activation of protein kinase C, as indicated by the inhibitory effects of the cell-permeable calcium chelator, bis-(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxyethyl ester (BAPTA/AM), and the inhibitor of the calcium-dependent isoforms of protein kinase C, Go6976, respectively (Fig. 5E). On the other hand, the phospholipase C inhibitor U73122 had no significant effect on the FXa-induced secretion of TGF-β1 (Fig. 5E).

Activation of Intracellular Signal Transduction Molecules

We found that the FXa-induced secretion of VEGF depends on activation of various intracellular signal transduction pathways (Fig. 5D). We used Western blot analysis to determine whether FXa activates key molecules of intracellular signal transduction cascades. FXa induced dose- (Fig. 6A) and time-dependently (Fig. 6B) phosphorylation of ERK1/2, p38, and Akt proteins in RPE cells. FXa-induced increase in the phosphorylation level of the three proteins was observed in both nonconfluent and confluent RPE cell cultures (not shown). FXa also induced phosphorylation of the activators of p38 MAPK, MKK-3/6 (not shown).

FXa-induced phosphorylation of p38 was prevented in the presence of the cell-permeable calcium chelator, BAPTA/AM (100 μM), and the inhibitor of protein kinase C, Go6976 (1 μM), respectively; but not in the presence of the phospholipase C inhibitor, U73122 (10 μM). Vehicle control was made with DMSO (1%). The data are expressed as percentage of untreated control (100%). Means ± SEM of four to ten independent experiments using cells from different donors. Significant difference versus control: *P < 0.05; **P < 0.01. Significant difference versus FXa: *P < 0.05; **P < 0.01; ***P < 0.005.
decreased the FXa-induced phosphorylation of Akt (Fig. 6C). Interestingly, chelation of calcium by BAPTA/AM and inhibition of phospholipase C increased the phosphorylation level of p38 under control conditions (Fig. 6C), suggesting that calcium ions inhibit activation of p38 in RPE cells. On the other hand, BAPTA/AM and U73122 did not increase the phosphorylation level of Akt (Fig. 6C).

We found that FXa induces release of growth factors from RPE cells (Fig. 5A). This suggest that FXa-induced phosphorylation of intracellular signaling proteins may be (at least in part) mediated by transactivation of growth factor receptors. However, whereas VEGF and TGF-β1 induced phosphorylation of ERK1/2, they did not induce phosphorylation of p38 and Akt (Supplementary Fig. S1, http://www iovs org/lookup/ suppl doi:10.1167/ iovs.11-9214/-/ DC supplemental). Furthermore, the FXa-induced phosphorylation of p38 and ERK1/2 was not blocked in the presence of inhibitors of KDR/flk-1 (SU1498) and the TGF-β activin receptor-like kinase (SB431542), respectively (Supplementary Fig. S2, http://www iovs org/lookup/ suppl doi:10.1167/ iovs.11-9214/-/ DC supplemental). Further investigations are required to determine whether growth factor action contributes to FXa-induced activation of intracellular signaling molecules.

**DISCUSSION**

It has been suggested that activated blood coagulation factors act synergistically with angiogenic factors such as VEGF in promoting choroidal neovascularization and breakdown of the outer blood–retinal barrier constituted by the RPE. RPE cells were shown to express factors of the extrinsic coagulation pathway and, thus, may provide a procoagulant surface for the formation of thrombin from serum-derived prothrombin (Fig. 7). Thrombin exerts various effects on RPE cells including upregulation of VEGF (Fig. 7) and formation of intercellular gaps in RPE monolayers. In the present study, we show that (in addition to thrombin) the activated blood coagulation factor X (FXa) also has cellular effects. FXa

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**FIGURE 6.** FXa induces phosphorylation of ERK1/2, p38, and Akt in RPE cells. (A) Dose dependence of the FXa effect. The cultures were stimulated with the factors for 15 minutes. (B) Time dependence of the effects of FXa (1 U/mL) on the phosphorylation of ERK1/2, p38, and Akt. (C) Effects of the inhibitor of p38 MAPK activation, SB203580 (10 μM), the phospholipase C inhibitor U73122 (10 μM), the cell-permeable calcium chelator BAPTA/AM (100 μM), and the FXa inhibitor rivaroxaban (Rivaro; 30 μM) on the FXa (1 U/mL)-induced phosphorylation of p38 and Akt. The cultures were stimulated with the factors for 15 minutes. Blocking agents were preincubated for 30 minutes. In (A) and (C), PDGF (10 ng/mL) was used as a positive control. Amounts of total proteins are shown above; amounts of phosphorylated proteins are shown below. Similar results were obtained in three independent experiments using cells from different donors.
induces chemotaxis of RPE cells and stimulates the expression and release of angiogenic growth factors from the cells (Fig. 7). Because the effects of FXa are not prevented by the thrombin inhibitor hirudin (Figs. 2, 5D), FXa acts independently from the formation of thrombin in RPE cells.

We found that the stimulatory effects of FXa on the expression of growth factors (Figs. 4A–C) and secretion of VEGF (Fig. 5D) depend on autocrine/paracrine TGF-β signaling; that is, FXa induces release of TGF-β1 (Fig. 5A), which activates the TGF-β receptor-like kinase. TGF-β is a well-known inducer of VEGF in RPE cells. It has been shown previously that (in addition to FXa) thrombin also induces TGF-β-dependent secretion of VEGF from RPE cells. Both thrombin and FXa stimulate the release (Fig. 5E) but not the expression of TGF-β (Fig. 3). The FXa-induced release of TGF-β1 from RPE cells was dependent on intracellular calcium signaling and activation of protein kinase C (Fig. 5E). It is conceivable but remains to be proven that calcium-dependent activation of protein kinase C may activate a (presently unknown) proteinase at the surface of the cells, which cleaves TGF-β1 from the latent TGF-β1 complex. Accordingly, these events are apparently independent of gene expression regulation of TGF-β.

FXa-induced secretion of TGF-β1, VEGF, and bFGF, but not of HB-EGF, from RPE cells (Fig. 5A). The failure of FXa-induced secretion of HB-EGF may be explained by the absence of MMP activation, which is required to shed HB-EGF from its membrane-bound precursor. This assumption is in agreement with the finding that the broad-spectrum metalloproteinase inhibitor 1,10-phenanthroline did not inhibit the FXa-induced release of TGF-β1. A similar failure of HB-EGF secretion despite transcriptional upregulation of HB-EGF was found in the case of thrombin. However, under in situ conditions when matrix metalloproteinases are activated by other factors, FXa-induced increase in HB-EGF expression may contribute to the HB-EGF–induced stimulation of RPE cell migration and increase in tissue VEGF level.

The present data indicate that FXa induces chemotaxis and upregulation of angiogenic factors such as VEGF and bFGF in
cultured RPE cells. Whether cellular effects of FXa play a pathogenic role in choroidal neovascularization in situ remain to be determined in future in vivo experiments. Further pathogenic factors such as hypoxia and inflammatory factors contribute to the pathologic process in situ. The additive effect of chemical hypoxia and FXa on the secretion of VEGF (Fig. 5B) may suggest that ischemia-hypoxia and hemorrhage additively induce VEGF-mediated edema and angiogenesis; this assumption may explain in part the clinical observation that the presence of hemorrhages at sites of vascular leakage is associated with greater reduction in retinal function.43 Because FXa is a factor generated before thrombin in the coagulation pathway and required for the generation of thrombin from prothrombin (Fig. 7),15 inhibition of FXa might have a greater impact in suppression of coagulation-induced angiogenesis than inhibition of thrombin.

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