Corneal Activation of Prothrombin to Form Thrombin, Independent of Vascular Injury

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Purpose. Two major functions of thrombin observed in the cornea are activation of thrombin-sensitive, proteinase-activated receptors and cleavage of fibrinogen to fibrin. The purpose of this study was to determine whether the normal human cornea itself is competent to convert prothrombin to thrombin and synthesizes the mRNA for the proteins required.

Methods. Human corneas were processed for immunolocalization studies or separated into epithelial, stromal, and endothelial layers for proteins and RNA isolation. The protein extracts were used for Western blots, prothrombin time, and activated partial thromboplastin time assays and fibrinopeptide A generation tests. RNA was used for RT-PCR. Apoptosis of cultured human corneal cells was induced with sodium nitroprusside or camptothecin and activation of prothrombin tested.

Results. Prothrombin and its mRNA were present in all three layers of human donor cornea. It was found to be associated with the cells and the extracellular matrix at similar levels across the cornea. With corneal stromal extracts, activation of either the intrinsic or extrinsic coagulation pathways resulted in thrombin activation and fibrin formation with fibrinopeptide A release. Detection of key components of the coagulation cascades confirmed noninjured human corneas contain factors required for prothrombin activation. In addition, mRNAs for representative factors and inhibitors were detected by RT-PCR and confirmed by sequencing. Apoptotic corneal stromal cells provide a surface for prothrombin activation.

Conclusions. These studies suggest that the normal avascular human cornea contains and synthesizes the components required for thrombin generation and that this process does not depend on a breech in the limbal vascular endothelium. (*Invest Ophthalmol Vis Sci.* 2007;48:134–143) DOI:10.1167/iovs.06-0339

Thrombin, the final activated protease in the coagulation pathway, not only is involved in cleavage of fibrinogen to fibrin but also in cleavage of other molecules in the coagulation pathway (Fig. 1), activation of matrix metalloproteinases, and initiation of signaling pathways through the cleavage of the N-terminal peptide of protease-activated receptors (PARs).[1] Cleavage of fibrinogen to fibrin occurs after both corneal scrape wounds and those involving both the epithelial and stromal layers.[2,3] On injury of the avascular cornea, fibrin is deposited on the wounded surface and within the extracellular matrix of the stroma.[4] Although fibrin may not play a critical role in wound healing, failure to remove fibrin results in corneal opacity, persistent inflammatory response, scarring, and neovascularization.[3,5]

Cleavage and activation of PARs by thrombin represents an unusual function of proteases. PARs are receptors in which the N-terminal peptide of the receptor contains a cryptic autoligand that is exposed on proteolytic cleavage.[6,7] These seven membrane-spanning receptors control signaling in cells through activation of coupled small G proteins. Thrombin directly cleaves PAR-1, -3, and -4 with 100- to 1000-fold higher affinity than other proteases and is thought to be the major activator of these receptors. PAR cleavage activates signal transduction pathways that control multiple functions in a cell-dependent manner.

PARs are present on cells of normal corneas.[7–11] PAR-1 has been detected in cells of all three layers of the cornea. Corneal epithelial cells also synthesize and express surface PAR-2 and -4 (Ansel JC et al. *IOVS* 2004;45:ARVO E-Abstract 3497; Strande JL et al. *IOVS* 2005;46:ARVO E-Abstract 2803).[8] In corneal endothelial cells, thrombin induces myosin light chain phosphorylation through cleavage and activation of PAR-1. In the epithelial cells, thrombin and the PAR-1 agonist peptide increase the synthesis of IL-6, IL-8, TNFα, and matrix metalloproteinase.[8,9]

Although it has been assumed that thrombin is released from the vascular system immediately before PAR cleavage, PAR responses are observed under conditions that do not involve a breech in the vascular system.[7,8,12] Of note, PAR1 signaling induced by low levels of thrombin (40 pM) increases endothelial barrier protection against leakage of vessels.[13] The mechanism of thrombin generation by the avascular cornea for activation of PARs is not known.

Activation of prothrombin in the vascular system is now considered to be initiated by the extrinsic coagulation system when factor VII from blood binds to tissue factor and phosphatidylserine, which are present on the surface of activated cells at the injury site (Fig. 1).[14] Formation of this complex leads to the activation of factor X, which in turn converts prothrombin to thrombin on activated platelets. Fibrinogen is cleaved by thrombin, the only enzyme known specifically to cleave fibrinogen to form fibrin.[15] Once low levels of thrombin are generated in the initiation phase of clotting, tissue factor pathway inhibitor (TFPI) inhibits the extrinsic pathway. In the propagation phase of clotting, factor X is activated by factor IXa generated either in the initiation phase by active factor VIIa or by factor XIa activated by thrombin in a feedback mechanism.[14,16] Factor Xa then activates additional prothrombin molecules to thrombin. Both of these mechanisms involve a cascade system of protease activation.

In the cornea, it is possible that prothrombin activation does not depend on factors leaking from the limbal or conjunctival vessels even in wound-healing conditions, because convective flux across the cornea is very small.[17,18] We hypothesized that prothrombin can be activated to thrombin by a

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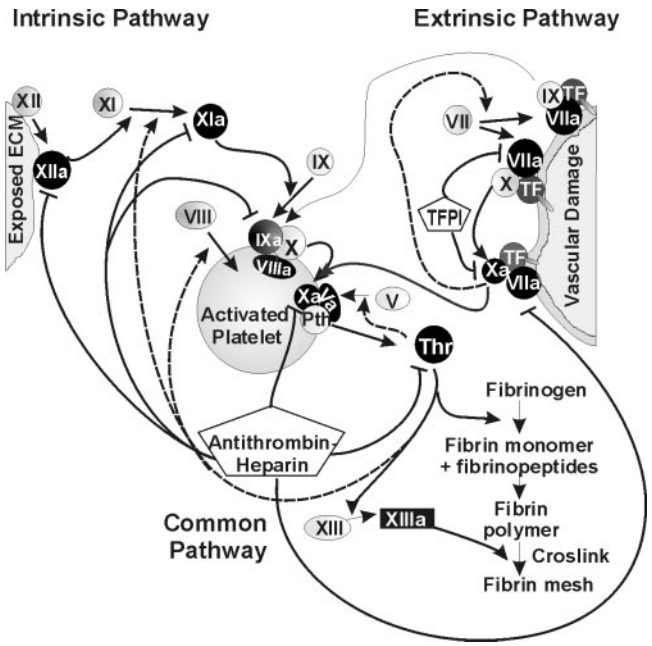

FIGURE 1. Vascular coagulation pathways lead to the activation of prothrombin and fibrin formation. The extrinsic and the intrinsic pathways initiate coagulation and both contain a common final pathway. Exposure of tissue factor on cells at the site of vascular injury initiates the extrinsic pathway. Binding of factor XII to exposed extracellular matrix molecules initiates the intrinsic pathway. Factors are given as Roman numerals with an "a" at the end signifying the active form. F, Factor; TF, tissue factor; Pth, prothrombin; Thr, thrombin; TFPI, tissue factor pathway inhibitor. *Circles*, protease factors; *ovals*, high-molecular-weight cofactors; *pentagons*, inhibitors; *rectangles*, cross-linking enzyme; *dashed lines*: feedback activation by thrombin or factor Xa.

mechanism that involves components present in the normal cornea and does not involve an increase in vascular permeability. We show that the human cornea contains and synthesizes prothrombin, factors required for cleavage of prothrombin to form thrombin, and factors that control this process. Activation of either the extrinsic or intrinsic coagulation cascade in human corneal stromal extracts results in the conversion of prothrombin to thrombin.

MATERIALS AND METHODS

Corneal Tissues

Whole human eyes or anterior segments of eyes were obtained from the Lions Eye Bank of Wisconsin and from The National Disease Research Interchange within 24 to 48 hours of death. The corneas were dissected by cutting two mm from the limbus with a trephine, for all experiments except the immunohistochemistry experiments in which the anterior portion of the eye was used. For some experiments, the corneas were cut using a trephine to separate central and peripheral portions. Whole, central, and peripheral corneal sections were then further dissected into individual layers. The epithelial layer was removed using a gill knife, which retained Bowman's layer as part of the stromal layer. The endothelial layer was removed by tear striping, which removed both Descemet's membrane and the monolayer of endothelial cells. Each experiment was performed by using three to eight donor samples. Contamination between cells of different layers was ruled out by RT-PCR with specific primers in different exons (Table 1) for the genes for keratocan for stromal cells, keratin 3 for epithelial cells, and keratin 12 for epithelial and endothelial cells[19–21] (data not shown).

The experiments adhered to the tenets of the Declaration of Helsinki (1989) of the World Medical Association. The Human Resource Committee at the Medical College of Wisconsin approved the use of donor corneas for the experiments.

Immunolocalization of Prothrombin

Deparaffinized, paraformaldehyde fixed sections (10 μm) were treated with H_2O_2 to inactivate endogenous peroxidases. The sections were incubated with a mouse anti-human prothrombin monoclonal antibody (HematoLogics, Inc., Seattle, WA) and a rabbit anti-mouse secondary antibody (Bio-Rad, Hercules, CA), followed by the reagents in a kit (R. T. U. Vectastain Universal Quik Kit and Hematoxylin QS; Vector Laboratories, Burlingame, CA). Negative controls were incubated with preimmune IgG (BD PharMingen, San Diego, CA) matching the isotype of the primary antibody.

Reverse-Transcription Polymerase Chain Reaction

The epithelial, stromal or endothelial layer of the cornea from a given donor was placed in extraction reagent (TriReagent; Sigma-Aldrich, St. Louis, MO) and subjected to lysing beads (Lysing Matrix D Beads; Q-Biogene, Carlsbad, CA) in a cell disruptor (Scientific Industries, Inc., Bohemia, NY). Total RNA was extracted using the manufacturer's

TABLE 1. Primers Used for the Recognition of Coagulation Cascade Components

Protein	Location	Sequence
FII	Forward 835–858 (Exon 7)	5′-TGAGGAGGGCGTGTGGTGCTATGT-3′
	Reverse 1090–1112 (Exon 9)	5′-CGATGTAGGATTCCAGGAGCTCT-3′
FV	Forward 371–392 (Exon 3)	5′-GCTGAAGTCGGAGACATCATAA-3′
	Reverse 1120–1142 (Exon 7)	5′-TCTTCATGTGCCGCCTCTGCTCA-3′
FX	Forward 328–349 (Exon 4)	5′-TAAAGACGGCCTCGGGGAATAC-3′
	Reverse 832–854 (Exon 7)	5′-AGTGGGCTGCCGTTAGGATGTAG-3′
TF	Forward 399–420 (Exon 3)	5′-CGACGAGATTGTGAAGGATGTG-3′
	Reverse 787–808 (Exon 5)	5′-TTCGGGAGGGAATCACTGCTTG-3′
TFPI	Forward 45–66 (Exon 1)	5′-ATGCCTGCTGCTTAATCTTGCC-3′
	Reverse 578–599 (Exon 5)	5′-GGAGTCAGGGAGTTATTCACAG-3′
AT	Forward 550–571 (Exon 3)	5′-CTTACCTTCAATGAGACCTACC-3′
	Reverse 1146–1169 (Exon 5/6)	5′-CCTTCTGCAACAATACCTGGGAGT-3′
γ-Glutamyl carboxylase	Forward 133–154 (Exon 2)	5′-CCGAATAGGGAAACTCTTGGGT-3′
	Reverse 1052–1073 (Exon 8)	5′-TATACACACAGGAAACACTGGG-3′
Keratin 3	Forward 1350–1371 (Exon 6)	5′-GCGGGCAGAGATCGAGGGTGTC-3′
	Reverse 1787–1697 (Exon 9)	5′-CTGCTGCCGCCGCCAAATCCAC-3′
Keratin 12	Forward 82–102 (Exon 1)	5′-CGGCTCTCCTCGCAGAGTGTG-3′
	Reverse 980–1000 (Exon 4)	5′-CCCCGCTCTTTTCAATGAACC-3′
Keratocan	Forward 1234–1256 (Exon 2)	5′-GAATATGCCTCCAAGATTACCAG-3′
	Reverse 1534–1512 (Exon 3)	5′-GCTGGGACATATTACAGAGACAT-3′

standard extraction protocol (Sigma-Aldrich) for the epithelial and endothelial layers and the high proteoglycan content protocol for the stromal layer.

RT was performed on total RNA from the three corneal layers and human liver (Invitrogen, Carlsbad, CA) using random hexamers. PCR was performed using primers for selected coagulation factors (Invitrogen) designed so the PCR products would span one or more introns (Table 1). All the no-RT and no-cDNA control analyses were negative. No genomic DNA products were observed. The Protein and Nucleic Acid Facility of the Medical College of Wisconsin sequenced the RT-PCR products from at least two different donors for each factor (PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA).

Protein Extraction and Determination of Protein Concentrations

The epithelial, stromal, or endothelial layer or the whole cornea from a given donor was placed in either 100 mM Tris buffer (pH 7.6), containing 154 mM NaCl plus either a protease inhibitor cocktail (2 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM EDTA, 1 mM iodoacetamide, and 5 μg/mL leupeptin; Sigma-Aldrich), to prevent premature activation of the coagulation zymogens and degradation of coagulation-related proteins. In some extractions, citrate buffer (21.4 mM sodium citrate dihydrate in PBS [pH 7.6]) was used to chelate calcium and prevent activation of the coagulation cascades. The proteins were released from the layers with the cell disruptor and lysing beads described for the RT-PCR experiments. After centrifugation, protein levels in the supernatant fractions were determined with the protein dye reagent (Bio-Rad) and were used for normalization.

Western Blot Analysis and Quantification of Prothrombin

The proteins in the corneal extracts and standards (HematoLogics, Inc.) were separated by SDS-PAGE under reducing conditions and transblotted to nitrocellulose membranes (Bio-Rad). The specific proteins were reacted with rabbit polyclonal antibodies to antithrombin and mouse monoclonal antibodies to prothrombin; tissue factor; factors X, XI, and XII (HematoLogics, Inc.); and Gla residues (γ-carboxyglutamate; American Diagnostica, Greenwich, CT). The secondary antibodies were horseradish peroxidase–conjugated goat anti-mouse IgG or sheep anti-rabbit IgG (Bio-Rad; and Kirkegaard & Perry Laboratories, Gaithersburg, MD). The enhanced chemiluminescence (ECL) reagents and autoradiograph film (Hyperfilm ECL; GE Healthcare, Piscataway, NJ) were used to visualize the bands.

The concentration of prothrombin in seven stromal extracts was determined using Western blot analysis for quantification of the 72-kDa prothrombin form to assure activation intermediates and degradation products were not measured. Corneal samples at several dilutions were placed on the same blot as the prothrombin standard curve. The corneal prothrombin concentrations were within the linear portion of the prothrombin standard curve (Hematologics, Inc.) on the same blot.

Induction of Apoptosis of Corneal Stromal Cells

Immortalized human corneal stromal cells, obtained from Mitchell Watsky, University of Tennessee College of Medicine (Memphis),[22] were cultured in DMEM plus 5% FBS (Hyclone, Logan, UT), 10 μg/mL ciprofloxacin (Bayer, Leverkusen, Germany) and 0.1% Mito plus serum extender; and 5% CO_2 in a humidified atmosphere at 37°C. The cells were treated with 6 μM camptothecin (Sigma-Aldrich), 3 mM sodium nitroprusside (Sigma-Aldrich) or PBS for 5 hours. EDTA was used to release the cells which were then used for the thrombin generation assay. An aliquot of the cells was reacted with FITC-annexin V (Sigma-Aldrich) which binds to cell surface–exposed phosphatidyl serine and is indicative of apoptosis.[23] Total cells and annexin V–positive apoptotic cells were counted.

Thrombin Generation Assay

The protocol of Flynn et al.[24] was used to determine thrombin generation in the presence of apoptotic, normal immortalized corneal stromal cells or PBS. Factor X in platelet-poor ancrod (Sigma-Aldrich) defibrinated plasma was activated to factor Xa by Russell's viper venom (Sigma-Aldrich). Thrombin generation was assayed with a chromogenic substrate (S-2238; DiaPharma, West Chester, OH). The experiments were repeated three times with different batches of cells.

Prothrombin Time and Activated Partial Thromboplastin Time Kinetic Assays

The prothrombin time (PT) and activated partial thromboplastin time (aPTT) assays were based on the microassays reported by Jagadeeswaran and Sheehan.[25] Briefly, supernatant fractions from citrate buffer extracts of corneal stroma, plasma (Norm-Trol 1; Helena Laboratories, Beaumont, TX) or PBS were placed in 96-well plates (Nalge Nunc Int., Rochester, NY) and incubated in PBS containing 1 mg/mL fibrinogen (Calbiochem, La Jolla, CA). For the PT assay 0.25% thromboplastin (a kind gift from Emo Cimeth; Helena Laboratories) was added to provide tissue factor, a required membrane-bound protein that is not extracted into aqueous buffer, and to provide rabbit brain phospholipids to generate a surface for the assembly of the components of the pathway. For the aPTT assay 5% ellagic acid in a suspension of rabbit brain extracted phospholipids (aPTT-SA; Helena Laboratories) was added to activate factor XII through a conformational change and to provide the surface for the activation of the pathway. To determine whether the corneal extracts contained heparin activated inhibitors such as antithrombin, 1 U/mL heparin (Elkins-Sinn Inc., Cherry Hill, NJ) was added to some reactions to activate the inhibitors and inhibit coagulation. Coagulation was initiated by the addition of 10 mM $CaCl_2$ and mixed, and the absorbance at 405 nm was read initially at 7 minutes and then at 3-minute intervals. The experiments were performed with three to nine samples from different individual donors. Each sample from an individual donor was used in at least two different assays.

Fibrinopeptide a Competitive Enzyme-Linked Immunosorbent Assay Test

The presence of fibrinopeptide A (FPA) in the products of the PT and aPTT assays was determined (Zymutest FPA Kit; DiaPharma). In this assay, fibrinogen in the sample was removed by bentonite absorption. The sample FPA was reacted with rabbit anti-FPA. Noncomplexed antibody was measured by an ELISA with FPA bound to a plate, horseradish peroxidase–conjugated goat anti-rabbit IgG, 3,3′,5,5′ tetramethyl benzidine, and hydrogen peroxidase. The FPA concentrations in the experimental samples were within the linear range of the provided FPA standard curve. In some reactions, heparin was added to activate protease inhibitors such as antithrombin and inhibit coagulation. The experiment was repeated three times with three to seven samples per assay point.

Statistical Analysis

Data was analyzed for overall differences by one-way ANOVA, and individual comparisons were analyzed with the Tukey test (SigmaStat; SPSS, Chicago, IL).

Results

Prothrombin and Its mRNA in the Three Layers of the Cornea

Extracts of the three layers of the cornea contained an immunoreactive protein at 72 kDa, which comigrated with the prothrombin standard on Western blot analysis (Fig. 2A). The concentration of prothrombin in the stroma was 325 ± 35 ng/mg total protein (n = 7). Immunolocalization studies revealed that this molecule was distributed throughout the entire cornea and was associated with epithelial, stromal, and endothelial cells and stromal extracellular matrix (Figs. 2B–D). In the epithelial layer, prothrombin was more concentrated in the basal cells immediately above Bowman's layer than in the more

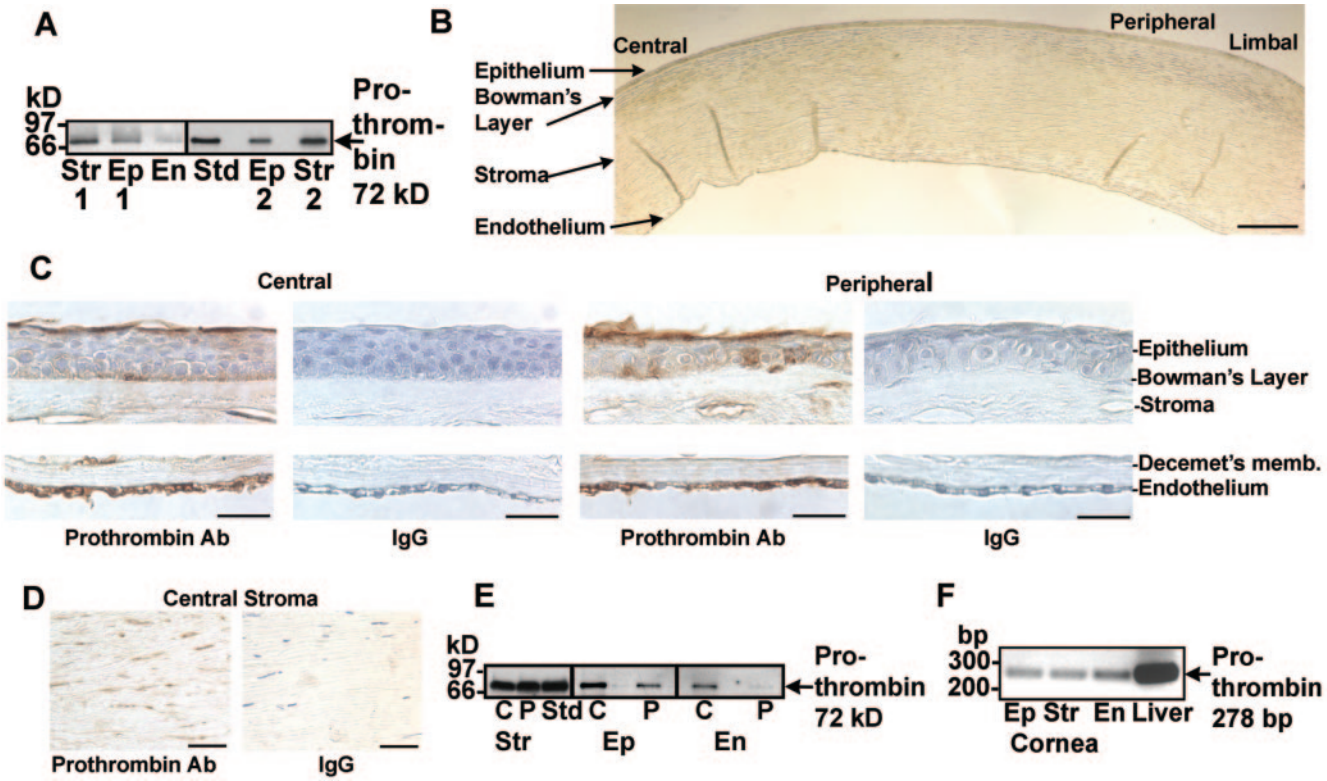

FIGURE 2. Prothrombin and its mRNA are present in the three layers of the human cornea. (**A**) Extracts of epithelial (Ep), stromal (Str 1 and 2), and endothelial (En) layers of human corneas and purified human prothrombin (Std) were separated by SDS-PAGE, transblotted, and detected by using mouse monoclonal antibodies to human prothrombin. The stromal and epithelial samples 1 and 2 are from two different donors. (**B**–**D**) Distribution of prothrombin from the central to the limbal areas of the human cornea. A mouse monoclonal antibody to prothrombin and a horseradish peroxidase–conjugated secondary antibody were used to localize prothrombin, and the section was counterstained with hematoxylin. (**B**) Photomerge of three photographs across half of the cornea. Controls using isotype matched IgG showed only the counterstain (data not shown). (**C**, **D**) Localization of prothrombin in the central and peripheral cornea (**C**) and central stroma (**D**) with controls using preimmune isotype-matched IgG to that used for the specific antibody. (**E**) Western blot analysis of extracts from the central *lane C* and peripheral *lane P* portion of the three corneal layers normalized for protein content. (**F**) RT-PCR of total RNA from the corneal epithelial, stromal and endothelial layers and liver (control) using specific primers within different exons for prothrombin. Samples were normalized for total RNA. The control containing no cDNA was negative (not shown). Equal amounts of total RNA were used. The PCR products were sequenced to confirm their identity. All figures are representative of experiments with three to five donors. Bar: (**B**) 1 mm; (**C**, **D**) 100 μm.

anterior wing cells (Fig. 2C). Prothrombin was also associated with the flattened squamous surface cells. This prothrombin could be derived from either the cells or from the tear film. Similar prothrombin localization patterns were observed for corneal sections from five individuals.

If the limbal blood vessels that surround the cornea were a major source of prothrombin, it would be expected that significantly higher levels of prothrombin would be observed in the peripheral cornea in a manner similar to that observed for albumin.[17,26] No major differences were noted by immunohistochemistry between the amounts of prothrombin present in the central versus the peripheral cornea close to the limbus (Figs. 2B, 2C). Neither were there major differences in the amounts of prothrombin in extracts of the central stroma (Fig 2E, lane C) versus the extracts of the peripheral stroma (Fig 2E, lane P). In the three corneas in which peripheral and central corneal extracts were made, the amount of prothrombin in the peripheral epithelial and endothelial layers was always less than in the central epithelial and endothelial layers; however, the ratio varied from one cornea to another. These differences were probably too small to be observed by immunohistochemistry. Similar levels or lower levels of prothrombin in the peripheral corneal versus the central cornea suggest that blood protein leakage from the limbal vessels is not a major source of prothrombin in the normal cornea.

To determine whether corneal cells synthesize prothrombin mRNA, RT-PCR with specific primers for prothrombin located in different exons was performed (Table 1, Fig. 2F). A product of the expected size (278 bp) for prothrombin was amplified from total RNA extracts of the epithelial, stromal, and endothelial layers. The identity of the products as prothrombin cDNA was confirmed by sequencing. Thus, the three layers of the cornea express prothrombin mRNA, which indicates the cells of the cornea synthesize prothrombin, and an external source of prothrombin is not needed to generate thrombin in the cornea.

Components of the Prothrombinase Complex in the Layers of the Cornea

In vascular coagulation, prothrombin is activated by the prothrombinase complex composed of prothrombin, the activating enzyme factor Xa, and the high-molecular-weight activated cofactor factor Va and is assembled on phospholipids, including phosphatidylserine and phosphatidylcholine of activated platelets or apoptotic cells (Fig. 3A).[14,24] In the extracts of all three layers of the cornea, the 47-kDa heavy chain (HC) of the mature zymogen form of factor X was detected by a monoclonal antibody on Western blots generated from samples separated under reducing conditions (Fig. 3B). This immunoreac-

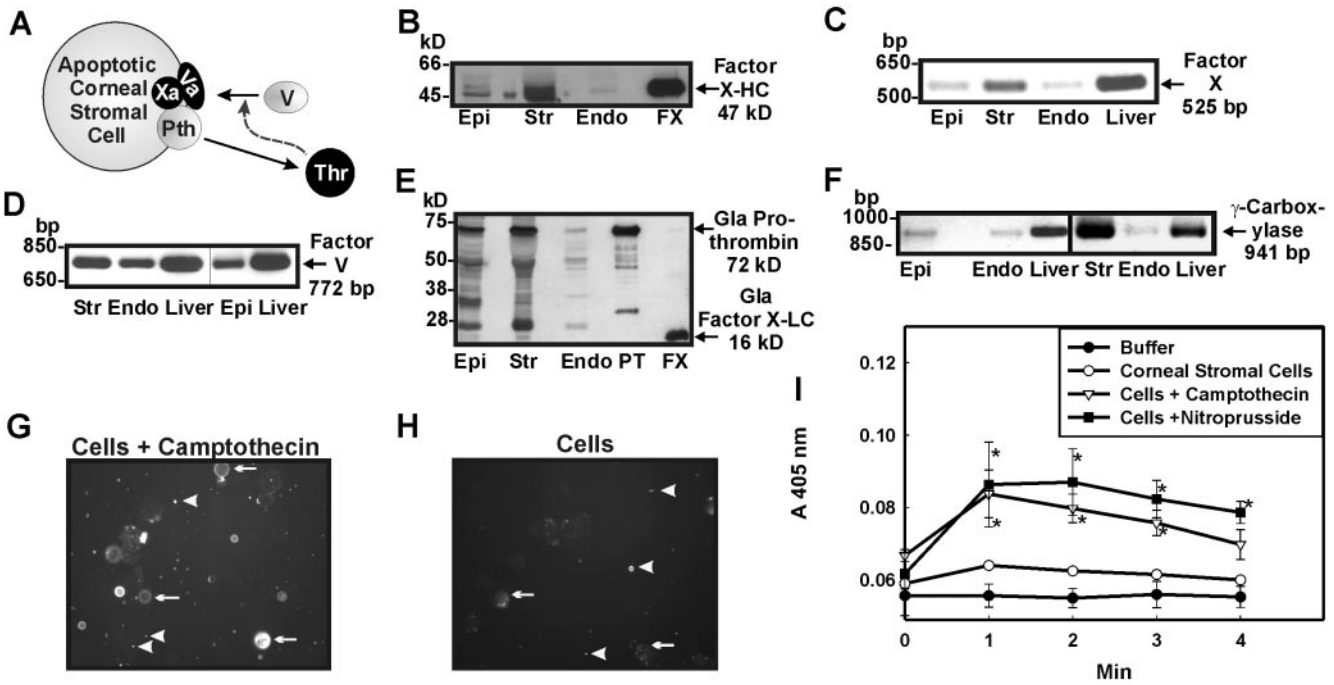

FIGURE 3. Prothrombinase complex components, factors X and V and prothrombin, are present in human corneal extracts and apoptotic corneal stromal cells can serve as a surface for prothrombin activation. (**A**) Diagram of the prothrombinase complex on an apoptotic cell. Factor Xa (Xa) and prothrombin (Pth) interact with the activated high-molecular-weight cofactor factor Va (Va) and respond to phosphatidylserine on the apoptotic corneal cell surface through N-terminal Gla residues (glutamic acid residues with additional carboxyl groups on the γ carbon). Factor Xa cleaves prothrombin forming thrombin (Thr), which can feedback and activate factor V. (**B**, **E**) Western blot analysis of human corneal epithelial (Epi), stromal (Str), and endothelial (Endo) extracts and standard proteins isolated from plasma (FX, Factor X, Pth, prothrombin) using mouse monoclonal antibodies to human factor X (**A**) and to γ-carboxylated glutamic acid residues (Gla) (**E**). Similar results were shown for three donors. HC, heavy chain; LC, light chain. (**C**, **D**, **F**) RT-PCR of total RNA from the three layers of the cornea and liver (control) with specific primers to sequences located in different exons of factor X (**C**), factor V (**D**), and the vitamin K–dependent γ-carboxylase (**F**). The control containing no cDNA was negative (not shown). All PCR products were sequenced to confirm their identity. Similar results were shown with three different donors. (**G**–**I**) Stimulation of the conversion of prothrombin to thrombin by apoptotic corneal stromal fibroblasts. Apoptosis was induced by treatment of human corneal stromal fibroblasts with 3 mM sodium nitroprusside or 6 μM camptothecin for 5 hours FITC-annexin V staining of phosphatidyl serine on the surface of the cells (*arrows*) and apoptotic bodies (*arrowheads*) was greater on the treated cells (**G**) than on the nontreated cells (**H**). Equal numbers of cells were placed on the slides in (**G**) and (**H**). Factor X in platelet-poor ancrod defibrinated plasma was activated by Russell's viper venom and was added to the apoptotic or control cells or buffer alone. Thrombin generation was measured using the thrombin-specific substrate S-2238 (**I**). The values were corrected by subtraction of the background activity of the substrate without added prothrombin. Points are the averages of results in three experiments. $^*P < 0.01$ relative to buffer only.

tive band from the corneal extracts migrates to the same extent as the heavy chain of mature factor X standard isolated from human plasma (Figs. 3B, FX). In addition, the mRNAs for factors X and V were present in extracts from the three layers of the cornea as detected by RT-PCR and confirmed by sequencing (Figs. 3C, 3D), which suggests that the corneal cells express not only prothrombin, but also factors V and X of the prothrombinase complex.

In the prothrombinase complex, prothrombin and its activating enzyme factor Xa are localized on the surface of activated platelets by binding directly to membrane-bound factor Va and to phosphatidylserine through N-terminal γ-carboxylated glutamic acid residues (Gla) associated with calcium ions (Fig. 3A).[27] Gla residues were detected on numerous corneal proteins including prothrombin and the N-terminal light chain (LC) of the mature zymogen form of factor X using a Gla-specific monoclonal antibody (Fig. 3E). The mRNA for the modifying enzyme, vitamin K–dependent γ-glutamylcarboxylase was detected in the cells of the three layers by RT-PCR and confirmed by sequencing (Fig. 3F). These results imply that vitamin K–dependent γ-glutamylcarboxylase may posttranslationally modify corneal prothrombin and factor X, as well as, other proteins.

Because platelets are not found in normal or wounded avascular corneas,[28] apoptotic stromal and epithelial cells found at the site of injury or observed during the normal turnover of cells could serve as a surface on which the prothrombinase complex forms.[29] Apoptotic cells, like activated platelets, expose on their outer surface phosphatidylserine, the phospholipid required for interaction with the Gla residues on coagulation factors. To test whether human corneal stromal cells undergoing apoptosis can support thrombin activation, immortalized corneal stromal cells were exposed to camptothecin or sodium nitroprusside to induce apoptosis. Treated cells and generated cell bodies reacted with the phosphatidylserine binding protein, annexin V, which was labeled with FITC (Fig. 3G). In the untreated stromal fibroblasts, FITC-annexin V staining was observed for some cells and cell bodies indicating the presence of a low level of apoptotic cells (Fig. 3H). This low level probably reflects the normal level of apoptosis of the cultured stromal cells. In the presence of these apoptotic cells, factor X activated with Russell viper venom increased the activation of prothrombin to form thrombin as measured using the thrombin-specific substrate S-2238 (Fig. 3I). Much less prothrombin activation was measured in the presence of untreated corneal cells than that observed for the camptothecin- and nitroprusside-treated cells. More prothrombin activation was observed in the presence of untreated cells than that for prothrombin alone (Fig 3I, Cells versus Buffer). This low level of prothrombin activation was probably due to the apoptotic

cells in the nonstimulated control cells (Fig. 3H), which suggests that apoptotic corneal stromal cells, like other apoptotic cells,[24,30] can serve as a surface for formation of the prothrombinase complex.

Formation of Fibrin by Activation of the Extrinsic Pathway in Corneal Stromal Extracts

Either the extrinsic or the intrinsic coagulation pathway can activate factor X, which in turn converts prothrombin to thrombin (Fig. 1).[14,31] The PT assay, which detects the presence of factor VII and the prothrombinase components factors V and X and prothrombin, was used to determine the presence of the extrinsic pathway in corneal stromal extracts (Fig. 4A). On addition of thromboplastin (tissue factor and phospholipids) to aqueous extracts of human corneal stromas or plasma, a fibrin clot was formed (Figs. 4B, 4C) and fibrinopeptide A was cleaved from fibrinogen (Fig. 4D). The clot initiation time for this representative corneal extract was similar to that of 1:2000 diluted plasma with a long propagation phase (Fig. 4B versus 4C). Considering the ninefold dilution factor, the activity of the extrinsic pathway in the cornea given in Figure 4B was equivalent to a 1:220 dilution of plasma. For corneal stromas from nine different individuals, the average PT activity was equivalent to a 1:400 dilution of plasma with a range of 1:200 to 1:650. The variation between experiments, for the same samples was approximately 20%. These results support the presence of the extrinsic pathway; factors V, VII, and X; and prothrombin in the corneal stroma extracts.

To determine whether heparin-activated serpin type inhibitors are present in the stromal extracts, heparin was added to the PT assay (Figs. 4B–4D). Heparin stimulated inhibition of clot formation (Figs. 4B, 4C) and fibrinogen cleavage (Fig. 4D) in the presence of corneal stromal extracts and in plasma. Antithrombin, a serpin type inhibitor, is the major heparin

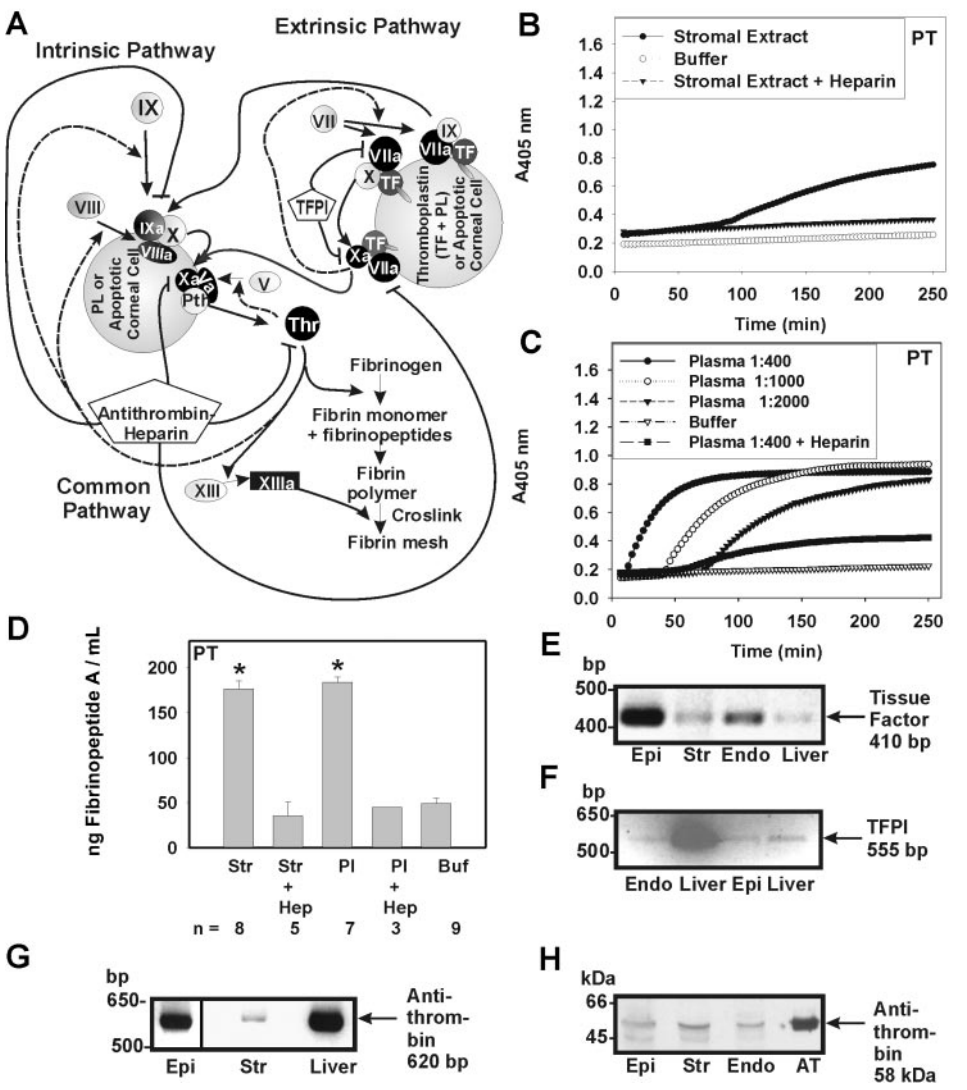

FIGURE 4. Initiation of the extrinsic coagulation pathways in human corneal stromal extracts results in fibrin formation and release of fibrinopeptide A. (**A**) Diagram of the extrinsic pathway modified to represent the conditions of the PT assay where the added thromboplastin preparation provides tissue factor (TF) and phospholipids (PL). In the cornea, apoptotic corneal cells probably provide TF and the PL surface. Activation of all factors may occur on the same apoptotic cell. Two cells are shown for simplicity. Factors are given as Roman numerals with an "a" at the end signifying the active form. TF, tissue factor; Pth, prothrombin; Thr, thrombin; TFPI, tissue factor pathway inhibitor. *Circles*; protease factors; *ovals*, high molecular weight cofactors; *pentagons*, inhibitors; *rectangles*, cross-linking enzyme; *dashed lines*, feed back activation by thrombin or factor Xa. (**B–D**) Comparison of the levels of the extrinsic pathway in corneal extracts and plasma using the PT test. Rabbit brain thromboplastin was first mixed with corneal stromal extracts (**B**) or diluted plasma (**C**), and then calcium chloride was added to initiate the extrinsic coagulation pathway. Heparin was added to some samples to measure the presence of heparin-activated coagulation inhibitors. Turbidity was measured every 3 minutes at A_{405} with the first point at 7 minutes. Similar results were observed in nine individual samples. (**D**) Total fibrinopeptide A release in the presence and absence of heparin. Total fibrinopeptide A released during the PT assay shown in (**B**) for corneal extracts and in (**C**) for diluted plasma was measured by the competitive enzyme-linked immunosorbent assay. The no-thromboplastin control results were the same as the buffer control (data not shown). Error bars, SD. Str, stromal extract; Pl, plasma; Hep, heparin; *n*, number of samples. $^*P < 0.001$ relative to sample+heparin and to buffer only. (**E**) Western blot of the heparin-activated inhibitor antithrombin in extracts of the three corneal layers and plasma-derived antithrombin, using monoclonal antibodies. Similar results were observed in three different samples. Epi, epithelial; Str, stromal; Endo, endothelial; AT, antithrombin. (**F–H**) RT-PCR of total RNA from the three layers of the cornea and liver (control) using specific primers to sequences located in different exons of antithrombin (**F**), tissue factor (**G**), and tissue factor pathway inhibitor (TFPI) (**H**). The control containing no cDNA was negative (not shown). All PCR products were sequenced to confirm their identity. Similar results were shown with three different donors.

activated inhibitor that inhibits thrombin and factors VIIa, IXa, Xa, XIa, and XIIa. Antithrombin was detected in the corneal epithelial, stromal, and endothelial extracts on Western blots (Fig. 4E). The mRNA for antithrombin was detected in the cells of epithelial and stromal layers of the cornea by RT-PCR and confirmed by sequencing of the cDNA (Fig. 4F). The mRNA for antithrombin was not detected in total RNA from the endothelial layer, possibly due to low levels. Thus, heparin-activated coagulation inhibitors, such as antithrombin, may control the conversion of prothrombin to thrombin and the activity of thrombin in the cornea.

In these experiments, tissue factor as thromboplastin was added to the aqueous corneal stromal extracts. As expected, tissue factor, an integral membrane protein required for the extrinsic pathway was not detected on Western blots of these aqueous extracts (data not shown). However, the mRNA for tissue factor was present not only in the stromal cells but also in the epithelial and endothelial cells, as determined by RT-PCR and confirmed by sequencing of the cDNAs (Fig. 4G). This indicates that the cells of the cornea could synthesize tissue factor.

A major control mechanism of the extrinsic pathway is inhibition of factor VIIa and Xa by the Kunitz-type proteinase inhibitor, TFPI.[32] Because the required amount of this inhibitor is very low and may not be detectable by Western blot, we looked for the mRNA for this protein. The mRNA for this inhibitor was detected in the cells of the three layers of the cornea by RT-PCR (Fig. 4H). The sequences were confirmed for the cDNA samples.

Formation of Fibrin by Activation of the Intrinsic Pathway in Corneal Stromal Extracts

The aPTT assay, which detects the presence of factors VIII, IX, XI, and XII, as well as, the prothrombinase component factors V and X and prothrombin (Fig. 5A) was used to determine the presence of the components of the intrinsic pathway for prothrombin activation in corneal stromal extracts. Ellagic acid was added to initiate this pathway in the corneal extracts with the formation of fibrin (Figs. 5B, 5C) and the release of fibrinopeptide A (Fig. 5D). The kinetic curve for the corneal stromal extracts was similar to that for diluted plasma (Figs. 5B, 5C), with the activity being similar to that for a 1:200 dilution of plasma. Considering the ninefold dilution during tissue extraction, the activity of the intrinsic pathway in the assayed corneal stroma is approximately equal to a 1:22 dilution of plasma. For corneal stromal extracts from five different individuals, the corneal stromas had an average activity equal to a 1:50 dilution of plasma with a range of 1:20 to 1:100. These results suggest that the components of the intrinsic pathway are present in human corneal stroma. Heparin also inhibited the cleavage of fibrinogen with the release of fibrinopeptide A and the formation of fibrin clots in the corneal stromal extracts as well as plasma (Figs. 5B, 5D).

To confirm the presence of this pathway, two early components of this assay, factors XII and XI, were detected by Western blot analysis of extracts of the corneal stromal and epithelial layers (Figs. 5E, 5F). Factor XII was also noted in the endothelial layer (Fig 5E). Factor XI was not observed in the endothelial extracts, but the levels may be too low for detec-

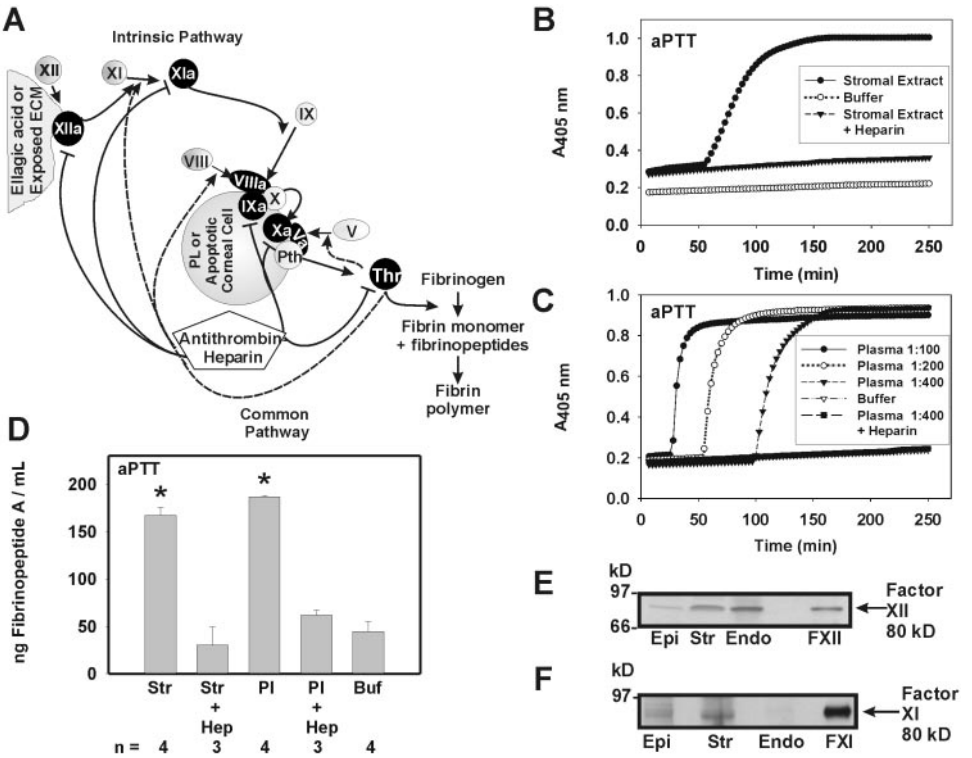

FIGURE 5. Initiation of the intrinsic coagulation pathways in human corneal stromal extracts results in fibrin formation and release of fibrinopeptide A. (**A**) Diagram of the intrinsic pathway modified to represent the conditions of aPTT, where ellagic acid substitutes for the wounded surface and activates factor XII. The added phospholipids (PL) replace activated platelets. In the cornea, the corneal extracellular matrix probably replaces the injured surface, and apoptotic corneal cells may provide the surface for factor activation. Factors are given as Roman numerals with an "a" at the end signifying the active form. PL, phospholipids, Pth, prothrombin, Thr, thrombin. *Circles*, protease factors; *ovals*, high-molecular-weight cofactors; *pentagons*, inhibitors; *rectangles*, cross-linking enzyme; *dashed lines*, feedback activation by thrombin. (**B–C**) For the aPPT assay, ellagic acid with phospholipids was first mixed with the corneal stromal extracts (**B**) or diluted plasma (**C**), and then calcium chloride was added to initiate the intrinsic coagulation pathway. Heparin was added to some samples to measure the presence of heparin-activated coagulation inhibitors. Turbidity was measured every 3 minutes at A_{405}, with the first point at 7 minutes. Similar results were observed in five individual samples. (**D**) Total fibrinopeptide A release in the presence and absence of heparin. Total fibrinopeptide A released during the aPTT assay of corneal extracts (**B**) and diluted plasma (**C**) in the presence and absence of heparin was measured by competitive enzyme-linked immunosorbent assay. The no-thromboplastin control results were the same as the buffer control (data not shown). Error bars, SD. Str, stromal extract; Pl, plasma; Hep, heparin; *n*, number of samples. $^*P < 0.001$ relative to sample+heparin and to buffer only. (**E**, **F**) Western blot analysis of factors XII (**E**) and XI (**F**) of the intrinsic coagulation pathway present in the extracts of the three layers of the cornea and plasma purified standards using monoclonal antibodies. Similar results were observed in three individual samples. Epi, epithelial; Str, stromal; Endo, endothelial; FXII, factor XI; and FXI, Factor XI.

tion. These results indicate the presence of the intrinsic pathway for prothrombin activation in normal human corneal stromal extracts, which is more active, relative to plasma, than the extrinsic pathway.

Discussion

Previously, it was assumed that thrombin release from the blood stream into the tissue was required immediately before PAR activation. This is the first study to demonstrate that components present in the cornea can activate prothrombin to form thrombin without the need for a breech in the vascular endothelial system to release components of the coagulation system. Extracts of the avascular cornea dissected inside the limbus generated activated thrombin as measured by fibrinopeptide cleavage and clot formation on initiation of either the extrinsic or the intrinsic coagulation pathways. This implies that all aqueous components required to activate prothrombin by these two pathways are present within the cornea without the requirement of recruitment of factors from the vascular system. In addition, this is the first study to show the synthesis of mRNAs for many of the components of the coagulation cascades and molecules that control this system in a nonhepatic tissue. These results support the hypothesis that the cornea has a vascular-independent mechanism, to convert prothrombin to thrombin.

The cornea probably serves as its own source of prothrombin based on its distribution in the cornea. Prothrombin is nearly evenly distributed across the cornea associated with the epithelial, stromal, and endothelial cells and the extracellular matrix of the stroma. The nearly uniform distribution pattern demonstrated for prothrombin is observed for other molecules synthesized by the cornea which are also present in plasma: the serine proteinase inhibitors, α1-proteinase inhibitor, α1-antichymotrypsin, and α2-antiplasmin and the high-molecular-weight inhibitor, α2-macroglobulin.[33–38] In contrast, serum albumin is nonuniformly distributed in the cornea. Serum albumin is found at much greater levels in the anterior peripheral cornea near the limbal vessels than in the central cornea.[17,26] These results suggest that prothrombin is mainly synthesized by the cornea and that little is derived from the vascular system.

It is not surprising that the avascular cornea uses the same cascade systems as the vascular system for the conversion of prothrombin to thrombin, because these systems have evolved multiple mechanisms to control this process. These mechanisms include the ability to activate specifically the zymogens of the pathway in the proper order through specificity of the activated proteases, localization of the reactions through formation of multicomponent complexes on activated cells, and the need for posttranslational γ-glutamylcarboxylation of the zymogens for binding to the activated cells, feedback activation, and formation of complexes between active proteases with tissue factor pathway inhibitor and heparin-activated inhibitors such as antithrombin.[39–41] Although it has not been addressed in this study, the cornea probably also contains the activated protein C pathway for inactivation of factors V and VIII. Thrombomodulin, which binds to thrombin forming a complex that activates protein C, is present and synthesized by the cornea.[42] The observed association of prothrombin with corneal epithelial, stromal and endothelial cells suggests that stimulated release of prothrombin may be an additional control mechanism for prothrombin activation.

Thrombin generation in a vascular independent mechanism is probably most important for thrombin cleavage of PARs. The concentration of prothrombin in the cornea was approximately one fiftieth that in plasma. The concentration was approximately 37 nM in the corneal stroma relative to 2 μM in plasma. These levels are more than adequate to generate sufficient thrombin for PAR activation. The concentration of thrombin necessary to activate PAR signaling is in the range of 20 to 5000 pM.[13,43]

The presence of prothrombin and the ability of corneal components to generate thrombin suggest that this enzyme may play various roles in normal and wounded corneas through activation of PAR receptors and activation of other molecules. Thrombin may regulate normal and abnormal corneal wound healing through regulation of the synthesis and/or release of growth factors, cytokines, chemokines, extracellular matrix molecules, proteases, and their inhibitors (Strande JL et al. *IOVS* 2005;46:ARVO E-Abstract 2803).[8,44–48] Thrombin activation of PAR-1 and/or -4 on corneal epithelial cells stimulates the synthesis and release of the inflammatory factors, IL-1α and -β, IL-6, IL-8, and TNFα (Strande JL et al. *IOVS* 2005;46:ARVO E-Abstract 2803).[8] Prostaglandin synthesis by corneal endothelial cells is regulated by thrombin.[49] Thrombin may influence the levels of corneal proteinases and their inhibitors through altering gene expression and/or release. In corneal epithelial cells, thrombin regulates the release and synthesis of plasminogen activators by corneal endothelial cells and matrix metalloproteinase (MMP)-1, -2, and -9 and tissue inhibitor of metalloproteinase (TIMP)-1 and -2 by corneal epithelial cells (Ansel JC et al. *IOVS* 2004;45:ARVO E-Abstract 3497).[50,51] Fibrosis in the corneal may also be stimulated by thrombin. Procollagen type I is increased by thrombin in fetal lung fibroblasts and vascular smooth muscle thrombin.[52,53] Cleavage of PAR-1 by thrombin can lead to fibrosis of the lung, kidney, and liver through induction of platelet-derived growth factor (PDGF) and connective tissue growth factor (CTGF) synthesis.[54–56]

Corneal thrombin may influence the function of nerves in the stroma and epithelial layers. Corneal wounding activates corneal nerves releasing neuropeptides, which stimulate inflammation and lead to hyperalgesia.[57] This process may be induced by corneal thrombin. In other tissues, this protease cleaves PAR-1 on sensory nerves and induces analgesia and release of neuropeptides, leading to neurogenic inflammation.[58,59]

Although many of the effects of thrombin on cells are mediated by the PARs, some effects may involve other molecules. Thrombin cleaves and activates membrane-bound MMPs which in turn activate MMP-2.[1] Other PAR-independent thrombin functions include stimulation of adhesion of macrophages and monocytes to extracellular matrix molecules; stimulation of angiogenesis, apoptosis; or cell survival, induction of chemotaxis, and induction of VEGF and MMP-9 release.

Other effects of thrombin in the cornea may not be mediated by its proteolytic activity. Enzymatically inactive thrombin can stimulate chemotaxis and growth of macrophages and monocytes.[1] Two thrombin peptides corresponding to residues 367-380 (TP367) and 50-530 (TP508) of thrombin can mediate some of thrombin's function. TP367 is chemotactic for monocytes and acts as a growth factor for monocytes, vascular endothelial cells, and fibroblasts.[60–63]

In wound-healing conditions, cornea synthesized prothrombin may also be involved in cleavage of fibrinogen for deposition of fibrin on the wounded surface and within the extracellular matrix of the stroma.[2,3] In the current work, cornea-generated thrombin cleaved fibrinogen to form fibrin. Because bleeding does not occur within the cornea, the slower coagulation rate observed for the stromal extracts relative to plasma may not present a problem. Although we have only been able to detect mRNA for the γ-chain of fibrinogen (Twining SS, unpublished data, 2005), the corneal cells, similar to lung cells, may synthesize the Aα and Bβ chains of fibrinogen on cytokine stimulation during wound healing.[64]

Prothrombin activation to thrombin is observed in tissues other than the cornea under noninjury conditions. Without injury, the tight junctions of the vascular endothelial cells would be expected to be intact, and the release of prothrombin into tissues unlikely within the time frame of a response. Examples of PAR-regulated processes that occur without injury include renal hemodynamics, ion transport across intestinal cells, normal contraction and relaxation of smooth muscle cells of the gut and airways, uterine contraction, calcium signaling in cells including neurons and astrocytes, and release of cytokines and chemokines by lung epithelial and fibroblast cells, microglial cells, and corneal epithelial, stromal, and endothelial cells.[7,9,65–69]

Before the current characterization of the corneal system, synthesis of components of the coagulation cascades was reported in some vascularized tissues. Prothrombin and its mRNA were found in the brain cortex, cerebella, hippocampus, muscle, and kidney.[70–73] The mRNA for a few other factors were detected in brain (factors VII and X),[74,75] in macrophages (factor X)[76] and in mesangial cells (factors V and VIII).[77] Thus, generation of thrombin for the activation of PARs in vascularized tissue may not require release of coagulation factors from the circulation.

In summary, the avascular cornea contains the components required for the conversion of prothrombin to thrombin by the two coagulation cascades, the intrinsic and extrinsic pathways. In addition, corneal cells produce mRNA for coagulation factors. Because of the lower level of coagulation factors in the cornea relative to the plasma, the major function of cornea-activated thrombin may be to activate the thrombin-sensitive PARs.

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