Fibrin Induction of Thrombospondin in Corneal Endothelial Cells In Vitro

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**Purpose.** Fibrin deposition in the anterior chamber of the eye occurs in response to injury or inflammation and can permanently damage the corneal endothelium. Fibrin functions as a mediator of inflammation and wound healing by affecting cell morphology and function in a variety of cells, including corneal endothelial cells. We hypothesized that fibrin can directly induce corneal endothelial cells to express injury-related proteins (eg, thrombospondin [TSP]) necessary for corneal repair processes.

**Methods.** Bovine corneal endothelial cells (BCECs) were pulse- or continuously labeled with 35S-methionine in the presence or absence of in situ polymerized fibrin (2 mg/ml). BCECs were harvested after 3–48 hr, and 35S-labeled proteins were analyzed by SDS-PAGE, autoradiography, and immunochemical techniques.

**Results.** Fibrin selectively induced BCECs to express a high molecular weight (MW) protein that was present extracellularly in conditioned medium and fibrin matrix. This induction represented a 3–5 fold increase relative to nonfibrin-treated BCECs, was not accompanied by corresponding changes in 35S-labeled intracellular proteins, and was evident at early (3 hr) or late times (24 hr) post-fibrin treatment. The induced protein had an apparent MW of 180 kD (reduced) and >420 kD (nonreduced), consistent with the characteristics of TSP. A polyclonal antibody to human TSP recognized the reduced form (180 kD) on Western blots and the native form (>420 kD) in immunoprecipitation studies.

that accompanies all of these complications is the formation and deposition of fibrin. Fibrin is a three-dimensional protein matrix formed in response to injury in intravascular and extravascular compartments. Fibrin deposition in the anterior chamber of the eye is considered detrimental, because fibrin itself may injure CECs.\textsuperscript{4,5} In vivo, fibrin adherence to the corneal endothelium is observed during ocular inflammation and is accompanied by morphologic retraction of CECs.\textsuperscript{6} CEC retraction is mimicked in vitro by fibrin treatment of corneas in organ culture.\textsuperscript{7} Fibrin-induced retraction also occurs in vascular endothelial cells\textsuperscript{8,9} and is thought to represent an initial stage in endothelial cell migration\textsuperscript{10} or reflect a cellular component of fibrinolysis.\textsuperscript{11,12} Therefore, fibrin-induced retraction appears to correlate with changes in cell function.

In addition to its role as protective sealant and temporary matrix, fibrin directly mediates several aspects of wound healing, including coagulation, fibrinolysis, chemotaxis, inflammation, angiogenesis and cell migration.\textsuperscript{13-16} Despite the multiple regulatory roles of fibrin, the direct effect of fibrin on cell function in general, and protein expression in particular, has not been fully addressed in any cell type. We hypothesized that fibrin can directly affect cell function and induce CECs to express injury-related proteins needed for wound healing.

In this report, we describe the effect of fibrin on protein synthesis in cultured bovine CECs (BCECs) and demonstrate that fibrin induces BCECs to express thrombospordin (TSP). TSP is an extracellular adhesive protein that mediates cell-cell and cell-matrix interactions. TSP functions in processes that require cell migration or tissue remodeling, including developmental processes, tumorigenesis, and wound healing. Our findings are relevant for understanding injury-repair mechanisms in the cornea in particular and the role of TSP in wound healing in general.

MATERIALS AND METHODS

Purification of Bovine Fibrinogen

To avoid artifactual results arising from contaminants in commercial fibrinogen preparations, bovine fibrinogen was purified to apparent homogeneity by a chromatographic procedure developed previously in our laboratory.\textsuperscript{17} Briefly, fibrinogen was extracted from citrated bovine plasma by graded ethanol precipitation (2-12%, -3°C, 55 mmol/l citrate, pH 7.0), followed by sequential chromatography on lysine Sepharose, gelatin agarose affinity resins (Sigma, St. Louis, MO), and ion-exchange chromatography on DEAE disks (Cuno, Meriden, CT). Purified fibrinogen was sterile-filtered (0.45 μm Millex-HA filters; Millipore, Bedford, MA) and stored as a stock solution (40–50 mg/ml in 55 mmol/l citrate buffer, pH 7) at 4°C. Fibrinogen concentration was determined using \( A_{280} = 1.5 \) for a 0.1% solution, and purity was assessed by SDS-PAGE.

Bovine Corneal Endothelial Cells

Bovine eyes were obtained from a local slaughterhouse, and BCECs were isolated as described.\textsuperscript{18} BCECs were grown to confluence on plastic culture flasks (Falcon Labware, Oxnard, CA) in minimal essential medium (MEM; Gibco, Grand Island, NY) supplemented with 15% calf bovine serum (HyClone, Logan, UT), 0.5 mg/ml fungizone (Gibco, Grand Island, NY), and 20 mg/ml gentamycin sulfate (Elkins-Sinn, Cherry Hill, NJ). The medium was changed three times a week and cells were passaged at 1:3 split ratios after disaggregation with 0.05% trypsin/0.02% EDTA (Gibco, Grand Island, NY). BCECs were used at 5th to 23rd passage and 3–4 days post-confluent.

Coculture of BCECs with Fibrin

To simulate in vivo conditions, BVEC-fibrin cocultures were prepared in situ by polymerizing physiologic amounts of fibrinogen with low concentrations of thrombin directly in the presence of BCECs. Briefly, post-confluent BCECs were washed twice with HEPES-buffered saline (20 mmol/l, pH 7.2–7.4) and once with serum-free MEM. In situ fibrin clots were formed by adding equal volumes of fibrinogen and thrombin (Sigma, St. Louis, MO) solutions in serum-free MEM directly to BVEC flasks to achieve final conditions of 2 mg/ml fibrin and 0.044 U/ml thrombin. Working solutions of fibrinogen and thrombin were freshly prepared from stock solutions of purified bovine fibrinogen (40–50 mg/ml in 55 mmol/l citrate buffer, pH 7) and bovine thrombin (260 U/ml) by dilution in serum-free MEM. The bovine thrombin used was essentially free of clotting factors, plasminogen, or plasmin (T6634; Sigma, St. Louis, MO). The purity of the thrombin was confirmed by SDS-PAGE. Polymerization was complete within 15 min at 37°C and produced a three-dimensional gel of polymerized fibrin and entrapped culture medium. Flasks were handled carefully to avoid disrupting interactions between fibrin and BCECs. Control BCECs received the following: (1) serum-free MEM; (2) serum-free MEM supplemented with thrombin (0.044 U/ml) and bovine serum albumin (BSA; 2 mg/ml); (3) unconditioned clot liquid, obtained from the liquid MEM phase of fibrin clots formed in vitro in the absence of cells; and (4) lipopolysaccharide (LPS) 10 or 100 μg/ml.

Harvest of Control and Fibrin-Treated BCECs

Control BCECs and BVEC-fibrin cocultures were harvested after 3–48 hr. Flasks containing BVEC-fibrin
Fibrin, Thrombospondin, and Corneal Endothelial Cells

To determine if fibrin treatment affected protein synthesis. The MEM-fibrin gel was centrifuged (15 min, 10,000 x g) to separate conditioned MEM (CM+) from conditioned fibrin (cfibrin). Conditioned MEM from control cultures (CM−) was centrifuged in parallel. The cfibrin was washed extensively in saline and solubilized in SDS-PAGE sample buffer.

35S-Labeling of BCEC Proteins

To determine if fibrin treatment affected protein synthesis in BCECs, BCEC cultures were pulse- or continuously labeled with 35S-methionine in the presence or absence of fibrin (2 mg/ml). Labeling was performed using serum-free RPMI-1640 medium that contained one fourth the normal methionine concentration (3.75 mg/ml) and 70 µCi/ml 35S-met/35S-cys (Tran 35S-label; ICN Biochemicals, Costa Mesa, CA). At harvest, conditioned medium or fibrin gel was removed and fractionated as described. The remaining cell monolayer was washed twice with ice-cold phosphate buffered saline (PBS) and lysed with 2× SDS-PAGE sample buffer. Proteins in extracellular and intracellular compartments (CM, cfibrin, cell) were analyzed by SDS-PAGE and autoradiography. Continuous-labeling was conducted by adding 35S-amino acid to BCEC cultures at time zero and incubating cultures for 3, 24, or 48 hr. Pulse-labeling was performed for 3 hr and was initiated after a pre-incubation period of 3 or 24 hr with or without fibrin. At the time pulse-labeling was begun, the conditioned medium or fibrin gel from the pre-incubation period was removed and replaced with fresh medium or fibrin plus radioactive isotope.

SDS-PAGE

To detect and characterize BCEC proteins induced by fibrin, 35S-labeled BCEC proteins in CM, cfibrin, and the cell fraction were analyzed by SDS-PAGE and autoradiography. SDS-PAGE was performed on 10% polyacrylamide gels.19 Samples were prepared in Laemmli sample buffer with or without reducing agent,20 were volume-normalized (5–25 µl/lane), and were electrophoresed at 20 mA. SDS-PAGE protein standards were from Sigma. Gels were silver-stained to verify uniformity21 and dried, and autoradiography was performed (24 h, -70°C, X-Omat diagnostic film; Eastman Kodak, Rochester, NY). Molecular weight (MW) approximations for high MW proteins (not shown) were determined using 7.5% polyacrylamide gels (linear range 50–200 kD) and high marker proteins from Sigma (myosin 205 kD, β-galactosidase 116 kD, phosphorylase b 97 kD, BSA 66 kD, egg albumin 45 kD, carbonic anhydrase 29 kD).

Western Blot Analysis

To determine the identity of a 180 kD extracellular protein induced by fibrin treatment of BCECs, 35S-labeled proteins in CM and cfibrin were analyzed by Western blotting.22 Briefly, proteins were resolved by 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose (140 volt hours). To improve transfer of high-MW protein, transfer buffer (25 mmol/l Tris-HCl, pH 8.3, 192 mmol/l glycine, 20% methanol) also contained 0.05% sodium dodecyl sulfate. After transfer, gels were silver-stained and the nitrocellulose was stained with Ponceau S (Sigma Chemical Co., St. Louis, MO) to verify transfer efficiency and uniform protein binding, respectively. Blots were air dried and blocked for 3 hr with 5% casein in PBS that contained 0.05% Tween-20. Blots were reacted with primary antibody (1:1000, 18 hr) using a rabbit polyclonal to human thrombospondin (Gift of Dr. G. J. Raugi, Department of Biochemistry and Medicine, University of Washington, Seattle, WA) or a rabbit polyclonal to bovine fibronectin (Sigma), followed by reaction with secondary antibody (1:1000, 2 hr) using an alkaline phosphatase conjugated anti-rabbit IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN)). Immunoreactivity was visualized using the BCIP/NBT phosphatase system (Kirkegaard and Perry Labs. Inc., Gaithersburg, MD).

Immunoprecipitation

Immunoprecipitation studies were performed to determine if the fibrin-induced protein was recognized in its native state by the anti-TSP polyclonal antibody. Aliquots of conditioned medium from control and fibrin-treated BCECs were mixed 1:1 with lysis buffer (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 10 mmol/l EDTA, 0.5% Triton X-100) and pre-absorbed for 4 hr with protein-A Sepharose CL-4B (Sigma) pre-equilibrated (3 hr) with lysis buffer. Pre-absorbed supernatants were incubated with antisem to human TSP, normal rabbit serum (NRS) or antisem to bovine fibrogen (prepared in our laboratory). Immune complexes were precipitated with fresh protein-A Sepharose pre-equilibrated in lysis buffer, washed (3X, lysis buffer), and resuspended in SDS-PAGE sample buffer with or without reducing agent. Radiolabeled proteins in bound and unbound protein-A Sepharose fractions were analyzed by SDS-PAGE and autoradiography.

The animal tissues used in this study (bovine eyes and plasma) were obtained from a local abattoir at the time of slaughter. In our opinion, the methods used to secure such tissue were humane, included proper consent and approval, and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
RESULTS

Purity of Bovine Fibrinogen

Fibrinogen was purified from bovine plasma by a method previously demonstrated to remove contaminating fibronectin, plasmin, plasminogen, plasminogen activators, and complement components. Analysis by SDS-PAGE demonstrated a single band at approximately 340 kD under nonreducing conditions, consistent with the intact fibrinogen molecule, and three bands at 50–60 kD under reducing conditions, representative of the α, β, and γ fibrinogen subunits (not shown). Thus, the fibrinogen used for fibrin-treatment of BCECs was undegraded and of high purity.

The Effect of Fibrin on BCEC Morphology

Control and fibrin-treated BCECs were examined by phase-contrast microscopy after incubation with or without fibrin (2 mg/ml) for 3, 24, or 48 hr. At 3 hr post-treatment, control and fibrin-treated BCECs appeared similar (not shown). At 24 hr, control BCECs still maintained a cobblestone-like morphology typical of endothelial cells (Fig. 1A), whereas significant retraction was prevalent in fibrin-treated BCECs (Fig. 1B). Retraction was characterized by an elongation of BCECs to a more spindle-like shape and was accompanied by significant disorganization of the cell monolayer. Retraction was induced regardless of BCEC passage number and was not induced in control cultures incubated with serum-free MEM, thrombin, unconditioned MEM from fibrin gels formed in the absence of cells, or LPS, as described in “Materials and Methods.” BCEC cultures at 48 hr were not significantly more retracted than at 24 hr.

The Effect of Fibrin on BCEC Protein Synthesis

SDS-PAGE and autoradiography of 35S-labeled BCEC proteins demonstrated the appearance of a high MW radiolabeled band that was present in fibrin-treated, but not control BCECs (Figs. 2A, B). The apparent MW of this protein was determined relative to high-MW marker proteins on 7.5% SDS-PAGE (not shown) and was found to be approximately 180 kD. This protein was present extracellularly in the conditioned medium (Figs. 2A, B, lane 2) and fibrin (Figs. 2A, B, lane 5). The radiolabeled 180 kD band was not detected by silver stain, indicating that it was present in low concentrations. The 180 kD protein was not released from the fibrin matrix when fibrin was washed extensively in saline with or without 2% Triton X-100, indicating that it was specifically associated. Low levels of the 180 kD protein sometimes were detected in CM— (Figs. 2A, B; lane 1), but not intracellularly in control or fibrin-treated BCECs (Figs. 2A, B; lanes 3, 4). The approximate fold-stimulation for fibrin induction of TSP was determined densitometrically from autoradiograms of conditioned medium from BCECs at passage 5, 16, 18, and 19, which were pulse- or continuously labeled for 3, 24, or 48 hr in the presence or absence of fibrin. The level of TSP induction under these conditions and from these passages ranged from 3–7 fold, with a mean and standard deviation of 4.2 ± 1.7, n = 6 (Fig. 2C). Fibrin induced the expression of the 180 kD band regardless of BCEC isolation or passage number (ie, passage 5, 8, 10, 16, 18, 19, 23).
extracellularly in conditioned medium and cFibrin (a). Molecular weight approximations were determined as described in “Materials and Methods.” Autoradiograms of the 35S-labeled BCEC proteins (b) were analyzed by densitometry (c). Relative to control, the 180 kD protein was induced at least 3-7 fold by fibrin (4.2 ± 1.7, n = 6). Data are from BCECs at passage 16. Comparable autoradiograms were obtained for analysis of samples from BCEC passages 5, 8, 10, 18, 19, and 23.

Characteristics of the Fibrin-Induced 180 kD Protein

The 180 kD fibrin-induced protein was detected in CM+ at early (Fig. 3; 0–3 hr, lane 6; 3–6 hr, lane 2) and late times (Fig. 3; 0–24 hr, lane 8; 24–27 hr, lane 6). This indicated that the 180 kD protein was rapidly induced by fibrin and that synthesis was sustained throughout fibrin treatment. Despite evidence for sustained synthesis, the 180 kD protein did not appear to accumulate with increased labeling time (Fig. 3; lane 6 versus 8), suggesting rapid turnover. Induction of this protein at 3 hr preceded the appearance of significant retraction (see above) and was blocked by cycloheximide and actinomycin D (not shown). The fibrin-induced protein comigrated with purified human TSP on SDS-PAGE and bound heparin agarose (not shown).

The 180 kD protein had a nonreduced MW >420 kD, as determined by its migration relative to fibronectin (FN) on nonreduced gels (Fig. 4). The 180 and >420 kD protein was not induced in control BCECs incubated with or without thrombin/BSA (Fig. 4). The radiolabeled band below FN in the nonreduced gel of thrombin-treated BCECs (Fig. 4) probably reflects nonspecific absorption of 35S-label by BSA contaminants and not a thrombin-induced protein. The MW characteristics of the fibrin-induced protein, its heparin- and fibrin-binding abilities, extracellular localization, and its rapid induction and turnover suggested the fibrin-induced protein was TSP.

Identification of the 180 kD Protein as Thrombospondin

To confirm the identity of the fibrin-induced protein, 35S-labeled proteins in conditioned medium and cFibrin were analyzed by immunoblotting (Fig. 5) and immunoprecipitation (Fig. 6). Autoradiography of nitrocellulose blots showed that the 180 kD protein was effectively transferred and bound to nitrocellulose (Figs. 5A, C; lanes 2, 3). Western blot analysis demonstrated that a polyclonal antibody to human TSP recognized the reduced form (180 kD) of the fibrin-induced protein in CM+ (Fig. 5B; lane 2), and in cFibrin, although the latter did not photograph well (Fig. 5B; lane 3). The 180 kD protein was not detected when purified TSP was added in the presence of primary antibody (not shown). The anti-TSP polyclonal antibody also exhibited limited cross reactivity with FN (Fig. 5B; lanes 1–3), but did not react with other proteins in the conditioned medium (not shown). A polyclonal antibody to bovine TSP recognized the FN band only and did not cross react with the 180 kD TSP protein (Fig. 5D; lanes 1–3).

Immunoprecipitation studies were conducted to determine if the fibrin-induced protein was recognized by the anti-TSP polyclonal antibody in its native,
Fibrin deposition in the anterior chamber of the eye occurs as a result of trauma and disease and surgical and postoperative factors. Fibrin adherence to the corneal endothelium can damage CECs, promoting a loss of stromal detergescence and corneal transparency. We proposed that fibrin can directly affect cell function and induce CECs to express proteins involved in injury-repair processes. Our results indicate that fibrin induces CECs to express TSP, a multifunctional glycoprotein implicated in wound healing.

TSP was first identified in platelet α-granules and subsequently was determined to be a normal component of the extracellular matrix, present in the culture medium of a variety of cells. TSP is made up of three subunits of identical molecular weight linked together by disulfide bonds. These subunits migrate as a single band on reduced SDS-PAGE gels and, depending on the electrophoretic system, exhibit an apparent molecular weight of 140–190 kD. The molecular weight of the homotrimeric protein is estimated to be 420 kD by velocity sedimentation and 450 kD by nonreducing SDS-PAGE. These values are consistent with the electrophoretic profiles of the 180 kD fibrin-induced protein described here.

TSP, like other adhesive proteins, contains multiple domains and discrete binding regions (e.g., a heparin binding domain near the globular N-terminus, a collagen binding domain in the central rod-like region, and a calcium-sensitive, RGD-containing domain near the globular C-terminus). As a result, TSP engages in numerous bi- and trimolecular interactions and binds extracellular matrix proteins (collagen, fibrinogen, thrombin, urokinase- and tissue-plasminogen activator), and saccharides and membrane glycoproteins (sulfated glycolipids, heparin, histidine-rich glycoprotein, glycoprotein IV). TSP and FN interact with each other and share sequence homologies. Therefore, interactions between TSP and FN could explain the partial coprecipitation of these proteins in our immunoprecipitation studies. Alternatively, because of the polyclonal nature of the TSP antibody, communoprecipitation and cross-reactivity on Western blots could be explained by epitopic similarities.

TSP is expressed by numerous cell types, including CECs. Although the factors that regulate TSP expression are poorly defined, TSP production...
Fibrin, Thrombospondin, and Corneal Endothelial Cells

FIGURE 4. Analysis of the fibrin-induced protein by nonreducing and reducing SDS-PAGE. \(^{35}\)S-labeled proteins in conditioned medium from control (−) and fibrin-treated (+) BCECs were resolved by 10% SDS-PAGE with or without reducing agent. Shown is an a composite autoradiogram of samples resolved by nonreduced (left) or reduced (right) SDS-PAGE in which the polyacrylamide gels are aligned relative to FN. The 180 kD protein migrated below FN on reduced gels but above FN (420 kD) on nonreduced PAGE. Arrow indicates the position of the fibrin-induced protein. Samples were from BCECs at passage 19 and represent other passages.

varies developmentally and is rapidly induced by platelet-derived growth factor and basic fibroblastic growth factor.\(^{24,25}\) TSP exhibits a short half-life and quickly disappears from areas of cell contact,\(^{31}\) possibly because of receptor-mediated endocytosis.\(^{37}\) Rapid induction of TSP is consistent with the early synthesis of the 180 kD protein induced in our system by fibrin treatment of BCECs. A short half-life would explain its continuous synthesis, yet seemingly constant levels during the course of fibrin treatment.

TSP regulates cell-cell and cell-matrix interactions.\(^{37}\) TSP synthesis is increased in response to in-

FIGURE 5. Autoradiography and Western blot analysis of the fibrin-induced protein. \(^{35}\)S-labeled proteins in cFibrin and conditioned medium from control (−) and fibrin-treated (+) BCECs were resolved by 10% SDS-PAGE under reducing conditions, electroblotted to nitrocellulose, and analyzed by autoradiography (a, c) and immunoblotting (b, d). Immunoblotting was performed using rabbit polyclonal antibodies to human TSP (b) or bovine FN (d). Immunocomplexes were detected using alkaline phosphatase-conjugated anti-rabbit IgG. The anti-TSP antibody recognized the 180 kD protein but also exhibited limited cross-reactivity with FN (b). The anti-FN antibody reacted only with FN (c). Data represent more than three independent experiments. Arrow denotes the position of the 180 kD band.
jugy, and TSP is present at sites of wound healing. TSP can influence the structure of fibrin clots, the efficiency of fibrinolysis, endothelial cell adhesion, and migration. In addition, TSP is chemotactic for neutrophils and is necessary for recovery from heat shock in vascular endothelial cells. Together, these capabilities suggest a role for TSP in injury/repair processes.

Our study supports a role for TSP in injury/repair and is consistent with the induction of TSP in BCECs by freeze-induced injury. Our results are the first to demonstrate the induction of TSP synthesis by fibrin. Our finding that a significant amount of newly synthesized TSP is fibrin-associated is consistent with TSP's ability to bind fibrin and its presence in vivo in blood clots. TSP incorporation into the fibrin matrix affects clot structure by promoting increased branching and the formation of numerous thin fibrils. These changes in fibrin structure may influence the efficiency of fibrinolysis, a plasmin-dependent process involved in wound healing. In addition to structural considerations, TSP may influence fibrinolysis by directly affecting the activity of plasmin or plasminogen activators.

A scenario consistent with the foregoing observations is that after corneal injury, fibrin deposition on the corneal endothelium induces the rapid expression of TSP by CECs that persists as long as fibrin is present. Newly synthesized TSP is secreted to the extracellular environment, where it partitions between the extracellular matrix, fibrin clot, and aqueous humor (ie, culture medium). TSP localized in the extracellular matrix may disrupt CEC interactions at focal adhesion sites, and thereby promote CEC “retraction” and migration. Retraction and fibrin-CEC interactions may induce the expression of other injury-repair proteins or biomolecules by activating second messenger systems in the CEC membrane. Consistent with this possibility is our recent finding that fibrin can induce the latent expression of tissue plasminogen activator in BCECs, the primary regulator of fibrinolysis. Because TSP is chemotactic, aqueous TSP could induce neutrophil recruitment into the anterior chamber. Because of fibrin-bound TSP, infiltrating polymorphonuclear leukocytes then might accumulate in the fibrin clot and be stimulated to release proteases involved in fibrin degradation. Thus, through effects of TSP on fibrin structure, the plasmin/plasminogen activator system, and neutrophil infiltration, TSP could influence fibrinolysis and wound healing in the anterior chamber of the eye.

In summary, fibrin, a multifunctional fibrous protein, induces BCECs to express TSP, a multifunctional matrix protein involved in injury-repair processes. The ability of fibrin and TSP to interact with each other and with cells, matrix proteins, and components of the fibrinolytic system, as well as the ability of both to affect endothelial cell migration and neutrophil...
Fibrin, Thrombospondin, and Corneal Endothelial Cells

chemotaxis, supports the conclusion that fibrin and TSP together are important mediators of inflammation and wound healing.

Key Words
corneal endothelial cells, fibrin, inflammation, thrombospondin, wound healing.

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