Fibrin Induction of Tissue Plasminogen Activator Expression in Corneal Endothelial Cells In Vitro

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Purpose. Fibrin is deposited in the anterior chamber of the eye in response to injury and can damage corneal endothelial cells (CEC). Fibrin degradation is plasmin dependent and is regulated by the balance between plasminogen activators (PA), tissue-PA (t-PA), urokinase-PA (u-PA), and their inhibitors (PAI). Although several factors can modulate PA/PAI expression in cells, the effect of fibrin is inconclusive. We hypothesized that fibrin can regulate fibrinolysis in the anterior segment by modulating PA/PAI expression in CEC.

Methods. Bovine CEC (BCEC) were treated for 3 to 72 hours with in situ polymerized fibrin (2 mg/ml) ± 35S-methionine, cycloheximide, or actinomycin D. Polymerization was thrombin catalyzed, and control BCEC were incubated with or without thrombin or polymerization by-products. PA and PAI in conditioned medium, fibrin matrix, and cell fractions were analyzed by PA-specific zymographic and enzymatic assays.

Results. Fibrin treatment induced a dramatic (>20-fold) accumulation of extracellular, fibrin-bound PA. This activity was identified as t-PA by its Mw (70 kD) affinity for fibrin and sensitivity to inhibition by Erythrina. Induction of t-PA was not observed in control BCEC under any condition. Fibrin induction of t-PA was selective because the levels of u-PA (45 kD), PAI-1 (50 kD), or protein synthesis in general were unaffected. Fibrin induction of t-PA was not accompanied by changes in cellular t-PA levels and was dependent on both RNA and protein synthesis.

Conclusions. Fibrin selectively induces t-PA expression in CEC. Induced t-PA is released extracellularly and binds exclusively to the fibrin matrix. These findings suggest a role for fibrin and CEC in the regulation of fibrinolysis in the anterior segment of the eye. Invest Ophthalmol Vis Sci 1993;34:3207–3219.
from CEC and from trabecular meshwork endothelial cells\(^2\) seem likely. Despite the potential significance of PA/PAI production by ocular endothelial cells, little is known regarding the regulatory mechanisms involved in PA/PAI expression in these cells. In contrast, PA/PAI expression has been extensively studied in vascular endothelial cells and found to be modulated by several effector molecules, including hormones, growth factors, and cytokines.\(^{16-18,26-29}\) Although fibrin interacts with cells,\(^{29-31}\) affects cell function in some cells\(^{2,8,32-36}\) and is suggested to modulate PA or PAI production in vascular endothelial cells,\(^{31,37-40}\) a regulatory effect of fibrin on the PA/PAI system has not been conclusively demonstrated in any cell type.

We hypothesized that fibrin can modulate the PA/PAI balance in CEC and thus control local fibrinolytic activities within the anterior segment. We investigated this hypothesis using bovine CEC (BCEC) co-cultured with highly purified fibrin formed under conditions that simulate extravascular fibrinogenesis (i.e., in situ polymerization, physiologic concentrations of fibrinogen). Our results show that fibrin induces a 2- to 20-fold increase in extracellular PA activity that is entirely fibrin bound and that selectively results from increased BCEC expression of t-PA.

**METHODS**

**Animal Tissues**

The animal tissues used in this study (bovine plasma and eyes) were obtained from a local abattoir at the time of slaughter. The methods used to secure such tissues were humane and complied with the considerations described in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Purification of Bovine Fibrinogen**

Bovine fibrinogen was routinely purified by a chromatographic procedure developed previously in our laboratory.\(^{41}\) Briefly, fibrinogen was extracted from citrated bovine plasma by graded ethanol precipitation (2% to 12% ethanol in 55 mM citrate, pH 7.0, \(-3°C\)), washed (11% ethanol/citrate buffer, pH 6.5), resuspended (55 mM citrate, pH 6.5), and sequentially chromatographed on lysine sepharose and gelatin agarose affinity resins (Sigma, St. Louis, MO) to remove plasminogen, plasmin, plasminogen activators, and fibronectin, respectively. Affinity-purified fibrinogen was precipitated (final 11% ethanol/55 mM citrate, pH 6.5), resuspended (200 mM phosphate buffer, pH 6.5), and further purified by ion exchange chromatography on DEAE disks (Cuno, Meriden, CT). Purified fibrinogen was sterile-filtered (0.45 \(\mu\)m, Millex-HA filter, Millipore, Bedford, MA) and stored at 4°C in 55 mM citrate buffer to prevent spontaneous oxidation. Fibrinogen concentration was determined using \(A_{280} = 1.5\) for a 0.1% solution. The integrity and purity of fibrinogen preparations was routinely assessed by nonreducing and reducing SDS-PAGE. Fibrinogen purity with respect to plasmin or plasminogen was confirmed by the inability of fibrin clots to lyse spontaneously when polymerized in the absence or presence of plasminogen activators, respectively. Purity with respect to plasminogen activators was confirmed by PA-specific zymographic and enzymatic analysis.

**Cells**

Bovine eyes from young animals (1 to 4 years of age) were obtained from a local abattoir at the time of slaughter and transported to the laboratory in ice-cold Ringer’s solution. BCEC were isolated from the posterior surface of the cornea by an established procedure.\(^{42}\) BCEC were grown to confluence in 25 cm\(^2\) plastic culture flasks (Falcon Labware, Oxnard, CA) in 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). Cultures were incubated at 37°C in a humidified atmosphere of 95% air 5% CO\(_2\), and the medium was changed three times per week. Cells were passaged at 1:3 split ratios after disaggregation with 0.05% trypsin/0.02% EDTA (Gibco, Grand Island, NY). BCEC were used 2 to 4 days after confluence and at the second to twenty-third passages.

**BCEC-Fibrin Co-cultures**

BCEC-fibrin co-cultures were prepared by thrombin-catalyzed in situ polymerization of purified bovine fibrinogen. Briefly, BCEC monolayers were washed twice with Heps buffered saline (20 mM, pH 7.2 to 7.4) and once with serum-free MEM. Fibrin was polymerized in situ by adding equal volumes of fibrinogen (4 mg/ml) and thrombin (0.088 U/ml) solutions directly to BCEC flasks. Working solutions were freshly prepared by diluting stock solutions of purified bovine fibrinogen (40 to 50 mg/ml in 55 mM citrate buffer, pH 7) and bovine thrombin (260 U/ml) in serum-free MEM. Bovine thrombin free of clotting factors, plasminogen, or plasmin (T-6634, Sigma, St. Louis, MO) was used, and purity was confirmed by SDS-PAGE analysis on 5% to 25% gradient gels (Phast Gel System, Pharmacia-LKB, Piscataway, NJ). Fibrinogen polymerization was complete within 15 minutes at 37°C and produced a three-dimensional gel composed of fibrin and entrapped culture medium that was loosely adherent to the BCEC. Flasks were handled carefully before harvest to avoid disrupting BCEC-fibrin contacts. Control BCEC received the following: serum-free MEM only; serum-free MEM supplemented with thrombin (0.044 U/ml) and carrier BSA (2 mg/ml); or polymerization by-products. BSA (2-10 mg/ml) did
not affect the ability of thrombin to catalyze fibrinogen polymerization. Polymerization by-products were prepared by polymerizing 2 mg/ml fibrinogen with 0.044 U/ml thrombin in the absence of cells and centrifuging the fibrin gel to obtain the unconditioned MEM phase (unconditioned clot liquid).

Inhibition of Protein and RNA Synthesis
BCEC were treated with cycloheximide (1 to 4 mg/ml) or actinomycin-D (0.4 to 1 mg/ml) to inhibit protein and RNA synthesis, respectively. Treatment with 1× inhibitor followed 15 minutes of preincubation in 2× concentrated inhibitor. Inhibitors (Sigma) were prepared as stock solutions (1 mg/ml), stored at −20°C, and diluted in serum-free MEM immediately before use. Inhibitors were used at concentrations that were compatible with the maintenance of cell viability, as confirmed by trypan blue exclusion, and that enabled BCEC to recover normal morphology within 4 hours upon transfer to treatment-free, inhibitor-free, serum-containing MEM.

Harvest of BCEC-Fibrin Co-cultures
BCEC were harvested at 5, 6, 18 or 24, 48, and 72 hours. The conditioned fibrin gel from fibrin-treated BCEC was removed by gently tapping the culture flask to loosen fibrin adherent to BCEC. The loosened gel was carefully slid from the cell monolayer by tilting the culture flask on its side, and then it was removed by inverting the flask and pouring. BCEC monolayers remained intact as evidenced by phase-contrast microscopy. The fibrin gel was centrifuged (15 minutes, 10,000g) to separate conditioned medium (CMf) from conditioned fibrin (cFibrin). Conditioned medium from control cultures (CMw) was centrifuged in parallel. The cFibrin was washed extensively in saline before analysis by SDS-PAGE or subfractionation by urea extraction.

Cell Fractionation
BCEC monolayers (~5 × 10^6 cells/T25 flask) were fractionated using a differential detergent extraction protocol previously shown to yield distinct cell fractions enriched in cytosolic, membrane/organelle, and cytoskeletal/matrix proteins. Briefly, BCEC were rinsed twice with ice-cold saline and incubated on ice (5 to 10 minutes, gentle agitation) with 1 ml of digitonin buffer (0.015% digitonin, 10 mM Pipes, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2, 5 mM EDTA, 1.2 mM PMSF). The extract, consisting of digitonin-released cytoplasmic proteins, was removed, and the cell ghosts were further extracted with 1 ml of Triton X-100 buffer (0.5% Triton X-100, 10 mM Pipes, pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2, 5 mM EDTA, 1.2 mM PMSF, on ice, 30 minutes, gentle agitation). The Triton extract containing membranes and organellar contents was removed, and the insoluble residue—comprised of residual cytoskeletal, nuclear matrix, and extracellular matrix proteins—was solubilized in ×2 SDS-PAGE sample buffer ± 2-mercaptoethanol. Aliquots of digitonin and Triton extracts were stored at −70°C in their native state or at −20°C in ×5 SDS-PAGE sample buffer ± DTT.

SDS-PAGE
Conditioned medium, cFibrin, and cell fractions were analyzed by 10% SDS-PAGE. Samples were prepared in Laemmli sample buffer ± reducing agent, volume-normalized and electrophoresed (5 µl/lane to 25 µl/lane) at 20 mA in parallel with Mw standards (Sigma). SDS-PAGE gels were either processed for zymography or autoradiography, or stained and dried.

Zymography
Fibrin zymography was performed by a modification of the method of Loskutoff et al. Briefly, proteins were resolved by 10% SDS-PAGE under nonreducing conditions, washed twice (2.5% Triton X-100, 250 ml/gel, 45 minutes) to remove SDS, and overlaid on an agarose indicator gel previously poured on GelBond (FMC BioProducts, Rockland, ME). Indicator gels contained 1% SeaKem agarose (FMC BioProducts, Rockland, ME), 0.8% fibrinogen (plasminogen-rich, Organon Teknika, Holland) and 0.9 U/ml thrombin. Gel overlays were incubated at 37°C in a humidified atmosphere for 18 to 20 hours, after which the agarose indicator gel was stained (0.1% amido black, 70% methanol, 10% acetic acid), destained (70% methanol, 10% acetic acid), air dried, and photographed using back lighting. To verify the equality of protein loading, replicate gels containing reduced samples were electrophoresed in parallel and silver stained. Reverse fibrin zymography was performed as described for fibrin zymography, except that the indicator gel also contained 0.8 U/ml u-PA (American Diagnostica, Greenwich, CT).

Zymography in the presence of PA-specific antibodies was conducted by incorporating goat anti-human u-PA (Calbiochem San Diego, CA) or t-PA (American Diagnostica) directly into the indicator gel at 5 µg/ml to 20 µg/ml. Zymography in the presence of PA-specific inhibitors was conducted by incorporating the u-PA-specific inhibitor amiloride (Sigma), or the t-PA-specific inhibitor Erythrina (American Diagnostica), directly into the agarose indicator gel at final concentrations of 10^4 M and 10^5 M, respectively. Human u-PA and t-PA standards (American Diagnostica) were zymographed in parallel with samples (not shown).

Urea Extraction of cFibrin
cFibrin was extracted at 25°C for 60 minutes with 1 ml of urea-containing buffer (50 mM Tris-HCl, pH 7.4,
150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 5 M urea). Urea extracts (FBe) were separated from the insoluble fibrin residue (FBr) by centrifugation (8 minutes, 10,000g). The efficiency of urea extraction was monitored zymographically, and PA in urea extracts was quantified by enzymatic assay.

**PA-Specific Enzymatic Assay**

PA activity was quantified by enzymatic assay using the chromogenic substrate Spectrozyme. All reagents (Spectrozyme, glu-plasminogen, DESAFIB) and human u-PA and t-PA standards (American Diagnostica) were reconstituted in assay buffer (50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.01% Tween-80). Assays were conducted in triplicate in microtiter plates in a final volume of 125 μl. PA standards were assayed in the presence and absence of urea or detergent buffers to verify that buffer components did not affect enzymatic activity. Urea extracts of fibrin were diluted 1:50 with assay buffer, and digitonin and Triton cellular extracts were assayed neat. At these dilutions, linear time versus absorbance graphs were obtained for both samples and PA standards. Samples assayed in the absence of glu-plasminogen were inactive. For inhibitor studies, samples were assayed in the presence of 10^{-4} M amiloride or 10^{-5} M Erythrina because these concentrations completely inhibited u-PA or t-PA standards, respectively. PA antigen could not be quantified because bovine-specific antibodies able to detect PA at the levels commonly produced by cells in culture are unavailable, and commercially available ELISA to human PA (American Diagnostica) did not cross-react in our system.

**35S-Labeling of BCEC Proteins**

BCEC were labeled in serum-free MEM containing 70 μCi/ml 35S-met/35S-cys (Tran 35S-label, ICN Biochemicals, Costa Mesa, CA) in the presence or absence of fibrinogen (2 mg/ml) for various times. Cultures were harvested and fractionated as described above. Proteins in each fraction were resolved by 10% SDS-PAGE, silver-stained, and autoradiographed (−70°C, X-Omat diagnostic film, Eastman Kodak, Rochester, NY).

**RESULTS**

**Purity of Bovine Fibrinogen**

Fibrinogen was purified from bovine plasma by a method previously demonstrated to remove contaminating fibronectin, plasmin, plasminogen, PA, and complement components. The integrity and purity of final fibrinogen preparations was assessed by SDS-PAGE, and contaminating proteins were not evident (Fig. 1). A high Mw band at approximately 340 kD characteristic of the intact fibrinogen molecule was routinely observed upon analysis of nonreduced fibrinogen, indicating that subunit interactions were intact (Fig. 1, lanes 1 to 3). Analysis under reducing conditions demonstrated bands between 50 to 60 kD, corresponding to the a, b, and g chains of fibrinogen (Fig. 1, lanes 5 to 9). Clotting was greater than 95% at a thrombin:fibrinogen ratio of 0.022 U/mg. Clots aseptically maintained at 37°C for several months did not spontaneously lyse, indicating the absence of plasminogen (plasmin) contamination. When fibrinogen was polymerized in the presence of u-PA or t-PA, the resulting fibrin gel did not lyse after a 48-hour incubation period at 37°C, indicating the absence of plasminogen contamination. Purified fibrinogen was devoid of PA activity, as verified by zymographic (not shown) and enzymatic (see below) analysis. Thus, the fibrinogen used for fibrin treatment of BCEC was intact and of high purity.

![SDS-PAGE analysis of purified bovine fibrinogen](image-url)
**Fibrin-Induced BCEC Retraction**

The morphology of BCEC cultures treated \( \pm 2 \, \text{mg/ml} \) fibrin for 24 hours was examined by phase-contrast microscopy (Fig. 2). In contrast to control BCEC in which a cobblestone-like morphology typical of corneal endothelial cells was maintained (Fig. 2a), the monolayer in fibrin-treated cultures appeared disorganized, BCEC were elongated and spindle shaped, and intercellular spaces were evident (Fig. 2b). This “retraction” phenomenon was not produced in control BCEC incubated in serum-free MEM ± thrombin/BSA or polymerization by-products (unconditioned clot liquid). Retraction in fibrin-treated cultures was neither evident at 3 hours nor augmented by further incubation of cultures for 48 or 72 hours (not shown). Retraction was induced by fibrin treatment regardless of BCEC passage number or time postconfluence.

**PA and PAI in Conditioned Medium**

To determine if fibrin treatment stimulated PA or PAI release from BCEC, conditioned medium (CM) from control (CMc) and fibrin-treated (CMf) cultures was analyzed by fibrin zymography and reverse fibrin zymography, respectively (Fig. 3). Zymography demonstrated a predominant lytic band at 45 kD (Fig. 3a). This PA activity was quenched when amiloride or a polyclonal anti-human u-PA antibody were incorporated into the agarose indicator gel, but not by the presence of erythrina or a polyclonal anti-human t-PA antibody (not shown). Thus, the predominant PA released to conditioned medium by unstimulated BCEC was u-PA (45 kD), the levels of which were not affected by fibrin treatment (Fig. 3a).

Analysis of conditioned medium by reverse fibrin zymography revealed a darkly staining band (~50 kD) indicative of PAI, just above the lytic u-PA zone (Fig. 3b). Western blot analysis using polyclonal anti-human PAI-1 antibody supported the identity of this band as PAI-1 (not shown).

It should be noted that a lytic band at approximately 70 kD consistent with t-PA was sometimes observed in conditioned medium from control BCEC (Fig. 3a, lanes 1, 5, 7, 9 and Fig. 4b, lane 7). Additionally, lytic zones at higher Mws (110 kD), presumably representing PA-PAI complexes, were frequently present in conditioned medium from both control and fibrin-treated cultures (Fig. 3a, lanes 1, 4, 5, 7, 9, 10 and Fig. 4b, lane 7). The inconsistent presence of these additional bands did not correlate with differences in BCEC passage number or isolation but appeared to reflect differences in the degree of postconfluence. Despite the presence of t-PA and PA-PAI complexes, the predominant PA/PAI found in BCEC conditioned medium were u-PA and PAI-1, and their overall levels were not remarkably affected by fibrin treatment (Fig. 3).

**FIGURE 2. Effect of fibrin on BCEC morphology.** BCEC were incubated in the absence (a) or presence (b) of in situ polymerized fibrin (2 mg/ml) for 24 hours. Retraction of BCEC was induced by fibrin treatment irrespective of BCEC passage number or starting morphology, but it was not induced in control BCEC under any condition (serum-free MEM ± thrombin-BSA or unconditioned clot liquid). Phase-contrast microscopy (bar, 20 mm).

**PA and PAI in cFibrin: Induction of t-PA**

Because t-PA can bind fibrin, the fibrin matrix was next examined to determine if the seeming lack of effect of fibrin on extracellular PA/PAI levels was caused by their sequestration into the fibrin matrix.
Zymographic analysis of cFibrin revealed that significant PA activity was fibrin-bound. This activity was predominantly represented by a 70 kD (t-PA) species, but lesser amounts of 110 kD (PA-PAI) and 45 kD (u-PA) species were also present (Fig. 4a). The 70 kD lytic zone was completely quenched by incorporation of crithmum, but not amiloride, into the agarose indicator gel, consistent with its identity as t-PA (not shown). In contrast, the 70 kD PA was only partially inhibited by incorporation in the indicator gel of a polyclonal anti-human t-PA antibody, consistent with the lack of cross-reactivity observed between human and bovine t-PA in ELISA assays. When cFibrin was analyzed by reverse zymography, PAI-1 (50 kD) was not detected.

The relative abundance of the 70 kD PA in cFibrin was similar with 2 mg/ml fibrin (Fig. 4a, lane 1) and with 1 mg/ml (Fig. 4a, lanes 2, 3) but increased with
time in culture (Fig. 4a; 72 hours, lane 5 versus 24 hours, lane 4). This apparent dose independence was supported by subsequent dose-response experiments in which the magnitude of 70 kD PA induction was similar for BCEC treated with 0.125, 0.5, and 2 mg/ml fibrin for 24 hours (not shown). With respect to the time dependence of 70 kD PA induction by fibrin (2 mg/ml), activity was not evident at 3 to 6 hours (not shown), was dramatic/maximal by 18 or 24 hours (Fig. 4a, lane 4), remained constant between 24 and 48 hours (Fig. 4a, lanes 1,2), and increased further at 72 hours (Fig. 4a lane 5). Thus, 70 kD PA induction by fibrin required a threshold level of fibrin, proceeded after an initial lag-phase of >6 hours, and occurred in bursts at approximately 24 hours and 72 hours.

The 70 kD PA was tightly bound to the fibrin matrix and was not released or inactivated when cFibrin was washed extensively with PBS ± 1% to 2% Triton X-100 or water, even after several hours at room temperature (not shown). Although the 70 kD PA was released by SDS, the presence of SDS precluded further analysis by enzymatic assay. Therefore, a urea extraction protocol was devised to release the 70 kD PA from cFibrin for subsequent enzymatic assay because urea effectively dissociates interacting proteins, and protein denaturation by urea is reversible upon dilution. As demonstrated by zymography (Fig. 4b), the 70 kD PA was efficiently extracted from cFibrin using a 5M urea-containing lysis buffer (Fig. 4b, lanes 3, 6, 9).

To quantify and confirm the identity of the fibrin-induced 70 kD PA, urea extracts of cFibrin were diluted 1:50 and analyzed by enzymatic assay in the presence or absence of PA-specific inhibitors (Fig. 5). Urea buffer alone at this dilution did not affect the activity of u-PA or t-PA standards, which, however, were completely inhibited by amiloride (10^-4 M) or erythrina (10^-5 M), respectively (Fig. 5a). PA activity in urea extracts of cFibrin (Fig. 5b) was not affected by amiloride (FBe/A) but was completely inhibited by erythrina (FBe/E), confirming its identity as t-PA. Induction of t-PA was fibrin-specific because it was not induced by thrombin alone or polymerization by-products.

Thus, fibrin induced a 70 kD PA, which was localized extracellularly and tightly bound to the fibrin matrix. This activity was identified as t-PA by Mw, affinity for fibrin, and sensitivity to erythrina. Fibrin induction of t-PA was time dependent after a lag phase of >6 hours and occurred in bursts evident at 18 to 24 hours and at 72 hours. Induction by 24 hours was similar for fibrin doses between 0.125-2.0 mg/ml, suggesting a dose-independent, threshold response.

**Magnitude of t-PA Induction by Fibrin:** >20-fold

The magnitude of t-PA induction by fibrin was derived by comparison of PA activity in cFibrin to PA activity in fibrin

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**FIGURE 5.** (a) Inhibition of u-PA and t-PA standards by PA-specific inhibitors. PA standards (0.1-1.0 U/ml) were analyzed by enzymatic assay using the chromogenic substrate spectrozyme ± the u-PA-specific inhibitor amiloride (10^-4 M) or the t-PA-specific inhibitor erythrina (10^-5 M). (b) Effect of PA-specific inhibitors on PA activity in cFibrin. PA activity in urea extracts of cFibrin (FBe) was assayed ± the u-PA-specific inhibitor amiloride (A, 10^-4 M) or the t-PA-specific inhibitor erythrina (E, 10^-5 M). Enzymatic analysis was performed on 1:50 dilutions of FBe. The PA activity in cFibrin was insensitive to amiloride (FBe/A) but was completely inhibited by erythrina (FBe/E), supporting its identity as t-PA. Data represent the mean ± SD of three independent experiments.
in conditioned medium and cell fraction (Fig. 6). Consistent with zymographic observations (Fig. 3), PA activity in the conditioned medium was not remarkably different for control versus fibrin-treated BCEC (Fig. 6, CMc versus CMf). Similarly, cellular PA also were not dramatically affected by fibrin treatment (Fig. 6, CELLCc versus CELLCf). However, consistent with zymographic analysis of cFibrin (Fig. 4), PA activity in urea extracts of cFibrin was dramatic (Fig. 6, FBe) and represented at least a 20-fold increase in total PA activity for fibrin-treated BCEC relative to controls.

Effect of Fibrin on Protein Synthesis and Intracellular PA

BCEC-Fibrin co-cultures were pulse-labeled or continuously 35S-labeled for both short and long periods (not shown) to determine the effect of fibrin on protein synthesis in general. Although differences in isotope-incorporation were evident for short versus long labeling periods (not shown), a generalized upregulation of total protein synthesis was not observed for fibrin-treated BCEC relative to controls for any given labeling protocol (Fig. 7). However, a 180-kD radiolabeled protein subsequently identified as thrombospondin was specifically induced by fibrin treatment. To determine if fibrin affected the levels of cell-associated PA or PAI, the fractions described in Figure 7 were analyzed by zymography and reverse zymography (not shown). These studies demonstrated the presence of t-PA and u-PA to varying degrees in all cell fractions and a selective partitioning of PA-PAI complexes in the matrix fraction. However, consistent with the results of activity assays (Fig. 6), differences in PA/PAI levels between control and fibrin-treated BCEC were not found for any cellular fraction.

FIGURE 7. Effect of fibrin on protein synthesis in BCEC. BCEC were continuously labeled with 35S-amino acid for 48 hours in the absence (c) or presence (f) of fibrin (2 mg/ml). Radiolabeled proteins in conditioned medium (CM), cell fractions (Cyto, MO, MAT), and urea extracts of cFibrin (FBe) were resolved by 10% SDS-PAGE and analyzed by autoradiography. Detergent fractionation enriched for select populations of radiolabeled proteins and fibrin treatment did not stimulate overall protein synthesis in general. A fibrin-specific induction of a high Mw radiolabeled band (~180 kD) was observed, however, and was subsequently identified as thrombospondin. Similar results were obtained with other continuous-labeling or pulse-labeling protocols. Data are representative of at least four independent experiments. Cyto = cytosolic, digitonin extract; MO = membrane/organelle, Triton extract; MAT = cytoskeletal/extracellular matrix, detergent insoluble proteins; Sw = saline wash of cFibrin; FBr = cFibrin residue after urea extraction.
were incubated in the presence or absence of cycloheximide or actinomycin D to inhibit protein and RNA synthesis, respectively. Cycloheximide treatment decreased the PA levels in both extracellular and cellular compartments, as demonstrated by zymography (Fig. 8) and resulted in a 69 ± 13.2 % (n = 6) decrease in fibrin-bound PA activity. Actinomycin D treatment caused a similar depletion of PA in both cellular and extracellular compartments (not shown).

DISCUSSION

Fibrin seals injured tissue and provides a matrix for cell migration during wound healing. Fibrin also exhibits regulatory capabilities and—through interactions with thrombin, factor X, t-PA, plasminogen, and antiplasmin—plays a pivotal role in modulating the balance between fibrinogenesis and fibrinolysis. In addition, fibrin or its degradation products can affect cell function, including cell migration and chemotaxis, in some cells, and the presence of fibrin in tissues correlates with the induction of angiogenesis, collagen deposition, and the development of granulomas or scar tissue. Fibrin degradation is plasmin dependent and is regulated by the balance between PA and PAI. Although CEC produce t-PA, t-PA, and PAI-1 and fibrin affects CEC morphology, our study is the first study to investigate the effect of fibrin on PA/PAl expression in CEC.

The effect of fibrin on the PA/PAl system in CEC is investigated here using a bovine cell culture model system in which physiologic concentrations (2 mg/ml) of highly purified fibrinogen are polymerized in situ. This system has two major advantages. First, the use of highly purified fibrinogen enables polymerization to be rapidly achieved using low concentrations of thrombin (0.022 U/mg), thereby avoiding complications arising from fibrinogen-associated impurities or thrombin-specific effects. The latter is especially relevant because thrombin alone can induce t-PA expression in vascular and corneal endothelial cells. However, the concentrations required for a twofold induction (5 to 10 U/ml) are >100-fold those used (0.044 U/ml) to catalyze polymerization in our system, and, as control studies verify, t-PA is not induced in BCEC treated with 0.044 U/ml thrombin.

Second, in situ polymerization simulates in vivo conditions and permits fibrin–cell interactions such as may be required to evoke cellular responses. To discern between fibrin-specific responses and effects due to by-products of the polymerization process (i.e., unpolymerized or modified fibrinogen, soluble fibrin, free thrombin, soluble thrombin–fibrin complexes, and fibrinopeptides A and B), the liquid phase of fibrin gels polymerized in vitro in the absence of cells (unconditioned clot liquid) is included as a control treatment. Our results demonstrate that fibrin treatment induces BCEC retraction and concomitant t-PA expression. These responses are fibrin-specific because they are not induced by any control treatment.

Fibrin and Retraction

Our finding that fibrin induces BCEC retraction in cell culture extends previous observations of fibrin-associated CEC retraction in vivo during ocular inflammation and in vitro during fibrin treatment of whole corneas. Retraction represents a morphologic change from a hexagonal to a spindlelike shape and was first described in vascular endothelial cells. Fibrin-induced retraction is suggested to represent an initial stage in endothelial cell migration involving t-PA release. Our present results support a temporal correlation between CEC retraction and t-PA induction because both phenomena attain peak responses within a similar time frame between 6 and 24 hours.

Retraction is fibrin-specific because it is not induced in control cultures under any condition (serum-free medium alone, thrombin/BSA, or unconditioned clot liquid). Whether retraction and t-PA induction are primary responses to fibrin (possibly mediated by RGD-dependent interactions between fibrin and integrins) or secondary responses (perhaps mediated in an autocrine fashion consequent to BCEC elaboration of cytokines or adhesion proteins) requires further study. For example, it is possible that retraction arises consequent to the disruption of focal adhesion contacts between BCEC and the extracellular matrix. Such a mechanism is likely in view of our recent finding that fibrin treatment of BCEC induces an early and sustained increase in thrombospondin expression.
Thrombospondin is an extracellular matrix/adhesion protein that can mediate cell attachment and disrupt focal adhesion in vascular endothelial cell cultures.

**PA and Corneal Endothelial Cells**

Our findings are consistent with previous studies in showing that u-PA and PAI-1 are the predominant PA/PAI species present in the conditioned medium of unstimulated BCEC and corroborate reports that BCEC synthesize t-PA. However, t-PA production by unstimulated BCEC probably is not characteristic of the quiescent state because we have found that BCEC produce significant quantities of t-PA during subconfluent proliferative stages, and this synthesis may persist through the confluence-postconfluence interface (authors’ unpublished observations, 1991–1992). Thus, just as the time required to attain confluence varies for early versus late passage BCEC (2 to 15 days), so too does the postconfluence time to quiescence (hours versus days). Although this suggests that the absence of t-PA production in unstimulated cells might serve as a marker for quiescence, quiescence is not required for fibrin to induce t-PA. Fibrin induces t-PA expression in BCEC regardless of passage number, time postconfluence, or the presence of basal t-PA production.

**Fibrin and t-PA Binding**

As our results show, fibrin-induced t-PA is localized extracellularly and is tightly bound to the fibrin matrix. This localization is consistent with the high affinity of t-PA for fibrin, which enhances the catalytic rate of plasmin formation, protects t-PA from inactivation by PAI-1, and confines plasmin degradation to the domain of the fibrin clot. Our observation that t-PA can bind to the fibrin matrix several (>6) hours after polymerization contrasts with in vitro studies in which t-PA binding to fibrin is minimal to performed clots, but maximal if present coincident with polymerization. Because t-PA can bind to proteolytically generated carboxy-terminal lysines, postpolymerization binding of t-PA to fibrin may reflect limited proteolysis of the fibrin matrix by BCEC-derived proteinases and the generation of additional t-PA binding sites. Alternatively, because the structure of fibrin clots can be modified by fibrin-binding proteins such as fibronectin or thrombospondin, incorporation of such proteins into the fibrin matrix might alter the spatial organization of fibrin fibrils and enhance the accessibility of internal t-PA binding sites in fibrin. Interestingly, both fibronectin and thrombospondin are synthesized by BCEC and detectable in cFibrin.

**Fibrin and t-PA Induction**

Our investigation shows that fibrin dramatically induces t-PA expression in BCEC (>20-fold). This induction is fibrin-specific because t-PA is not induced under any control condition (serum-free MEM, thrombin/BSA, or unconditioned clot liquid) and selective because corresponding changes in the levels of u-PA, PAI-1, or protein synthesis in general do not occur. Although fibrin has previously been suggested to modulate t-PA release or production in vascular endothelial cells, two recent investigations into the effect of fibrin on the PA/PAI system in human umbilical vein endothelial cell (HUVEC) cultures yielded conflicting and inconclusive results.

In the first study by Kaplan et al., HUVEC were cultured with plasma as a source of fibrinogen, and calcium was added to activate thrombin and to catalyze in situ polymerization. In this system, t-PA induction resulted and was concluded to be fibrin-specific because t-PA was not induced when HUVEC were cultured in the presence of collagen gels or when polymerization was catalyzed by reptilase. Considering the heterogeneity of the system, it is possible that t-PA was induced by other calcium-sensitive plasma constituents acting alone or in synergy with fibrin.

In the second study by Fukao et al., HUVEC were incubated with a preformed clot prepared in vitro using high concentrations of thrombin (10 U/ml) and commercial fibrinogen (10 mg/ml). The preformed clot was washed extensively to remove excess thrombin and, upon incubation with the HUVEC, failed to induce t-PA but caused a moderate (~threefold) decrease in PAI-1 production. In our experience, fibrin clots formed in vitro may irreversibly collapse or condense when subjected to mechanical manipulations such as washing and may float when subsequently added to cell cultures. Collapsed or floating clots may not be able to engage in physiologic interactions with cells and hence may fail to evoke cellular responses. In addition, the fibrin matrix was not analyzed for PA activity and, had t-PA induction occurred, it could have gone undetected.

Despite their inconsistencies, these studies support an involvement of fibrin in modulating the PA/PAI system in vascular endothelial cells. Although our results agree with the study of Kaplan et al., a direct correlation cannot be inferred because vascular and corneal endothelial cells differ with respect to their embryologic origins (mesenchymal versus neural crest, respectively) and, hence, may differ metabolically. Thus, the question of fibrin induction of t-PA in vascular endothelial cells as well as other cell types requires further investigation.

**Regulation of t-PA Expression**

Our present results are consistent with de novo synthesis as the mechanism for fibrin induction of t-PA because induction follows a lag phase, occurs in bursts, is
not accompanied by depletion of t-PA in cellular pools, and requires both RNA and protein synthesis. Although the regulatory events involved in t-PA gene expression are poorly understood, they are likely to be complex because t-PA expression can be induced by a variety of diverse factors—e.g., vasoactive substances (thrombin), biogenic amines (histamine), differentiation inducing agents (retinoids, butyrate), growth factors (bFGF, EGF), glucocorticoids (dexamethasone), gonadotropins (FSH, LH), and tumor promoters (PMA). The magnitude of induction achieved by these agents also varies, ranging from 2.5-fold for induction of t-PA by thrombin in BCEC, to 20-fold for PMA induction of t-PA in vascular endothelial cells, to 50-fold for gonadotropin stimulation of t-PA synthesis in granulosa cells. The 20-fold induction of t-PA by fibrin reported here ranks fibrin as among the most potent inducers of t-PA and identifies fibrin-BCEC co-cultures as a good model system for studying t-PA gene regulation.

CONCLUSION

In summary, fibrin selectively induces t-PA expression in bovine corneal endothelial cells in culture. Newly synthesized t-PA is released to the culture medium, where it associates exclusively with the fibrin matrix and is protected from PAI-1 inhibition. The net effect of this induction on the PA/PAI balance is a minimal and is protected from PAI-1 inhibition. The net effect of expression are poorly understood, they are likely to where it associates exclusively with the fibrin matrix synthesis and cell function. Comp Biochem Physiol. 1988:90B:691-708.


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