The effect of thrombin on release of plasminogen activators (PAs) was studied using cultivated endothelial cells of the bovine cornea. Species of PAs released into the conditioned medium were determined by fibrin autography and immunological analyses. Chromogenic peptide (S-2251) microassay was used for a quantitative estimation of the PA activity in conditioned medium and enzyme-linked immunosorbent assay (ELISA) for tissue plasminogen activator concentration. Fibrin autography revealed that cultured bovine corneal endothelial cells released into the conditioned medium tissue plasminogen activator (t-PA) and urokinase type plasminogen activator (u-PA). Addition of increasing concentrations (0.1 to 10.0 U/ml) of thrombin to the confluent cultures led to a dose-dependent increase in the rate of release of t-PA, while there was no significant increase in the release of u-PA. About a 2-fold increase in the t-PA concentration occurred when 10.0 U/ml thrombin was to the confluent cultures for 24 hr. Thrombin induced an increase in the release of t-PA, in a time-dependent manner. The addition of cycloheximide or actinomycin D to the thrombin-treated cultures resulted in a reduction of t-PA levels in the media. These findings indicate that the enhancing effect of thrombin is due to an increase in t-PA production, via protein synthesis. Thrombin inactivated with diisopropylfluorophosphate (DFP) did not induce an increase in t-PA levels. A 100-fold excess of DFP-treated thrombin did not inhibit the thrombin-induced increase. These findings indicate that binding ability and the effect of t-PA release depend on the enzymatically active site of thrombin. Invest Ophthalmol Vis Sci 30:1576-1583, 1989

With certain pathologies, such as chronic uveitis or postoperative inflammation, plasma proteins enter the eye following a breakdown of the blood–aqueous barrier and there is an increase in the concentration of proteins in the aqueous humor. The proteinaceous exudate is clinically visible. The presence of fibrin within the exudate was demonstrated by immunofluorescence and it was considered to be a major component of the exudate. Fibrin formed in the anterior chamber of the eye induces various undesirable effects. In addition to inhibiting proper vision, adhesion of a fibrin clot to the endothelium can cause cell damage, cell death and a permanent denudation. Central reactions in the fibrinolytic system relate to activation of plasminogen to plasmin by plasminogen activators (PAs). The fibrinolytic system has received increasing attention with regard to the pathophysiologic roles in various ocular structures. We investigated the release of PAs from cultured bovine corneal endothelial cells, in an attempt to clarify the fibrinolysis properties of the corneal endothelium and its protective activity against fibrin. To study mechanisms by which the fibrinolysis process of the corneal endothelium is accelerated, we focused on the clotting factor thrombin and examined its effect on the release of PAs.

Materials and Methods

Materials

Human tissue PA (t-PA) was purified from the conditioned medium of melanoma cell line (Bowes). Urokinase type PA (u-PA) of a molecular weight (M.W.) of 55,000 was a kind gift from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). Antisera against human t-PA and u-PA were raised in rabbits. The IgG fractions were isolated by ammonium sulfate precipitation and protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) column chromatography. The rabbit anti-human t-PA IgG inhibited human t-PA activity (50% inhibitory IgG concentration for 100 mU/ml of human t-PA: 0.03 μg/ml) but did not inhibit human u-PA activity, while the rabbit anti-human u-PA IgG inhibited human u-PA activity (50% inhibitory IgG concentration for 100 mU/ml of human u-PA: 0.6 μg/ml) but did not inhibit human t-PA activity. Human PA in-
Inhibitor 1 (PAI-1) was purified from the conditioned medium of HT-1080 fibrosarcoma cell line. Goat anti-human PAI-1 IgG and monoclonal mouse anti-human PAI-2 IgG were purchased from Biopool AB (Umeå, Sweden).

Bovine alpha-thrombin was purified by cationic ion exchange chromatography from commercially available thrombin (Mochida Pharmaceutical Co., Ltd.). The final preparation had a specific activity of about 2500 U/mg of protein. Diisopropylfluorophosphate (DFP)-treated thrombin was prepared by reacting thrombin with 10 mM DFP at pH 7.2 for 1 hr at 37°C after which DFP-thrombin was dialyzed against phosphate-buffered saline, pH 7.4 (PBS).

Plasminogen was prepared from human plasma by affinity column chromatography on lysine-Sepharose (Pharmacia Fine Chemicals). Horseradish peroxidase (HRP), bovine serum albumin (BSA), DFP, hirudin, cycloheximide and actinomycin D were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Adult bovine eyes were purchased from a local abattoir, transported on ice, and the excisions of corneas were performed within 1 hr of death. Corneal endothelial cells were isolated from the cornea according to Stocker et al and Gospodarowicz et al and were cultured on 21 cm² Falcon plastic culture dishes (Becton Dickinson Labware, Oxnard, CA) in Dulbecco’s modified Eagle medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS, Flow Laboratories, North Ryde, Australia), 100 U/ml penicillin, and 100 µg/ml streptomycin. Bovine corneal endothelial cells (BCE cells) of fifth to tenth passages were used for the following studies.

Stimulation of BCE Cells

Stimulation of the BCE cells was performed by washing confluent cultures three times with DMEM and incubation at 37°C in 1.5 ml DMEM containing 1% FBS and thrombin or DFP-thrombin, at the indicated concentrations. The conditioned medium was centrifuged at 15,000 g to remove cell debris. Tween 80 was added to make the final concentration of 0.01%, and the preparation was then kept at −75°C until use.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Fibrin Autography and Western Immunoblotting

According to Laemmli, SDS polyacrylamide slab gels were prepared using a separating gel of 10% acrylamide (Nakarai Chemicals, Ltd., Kyoto, Japan) and a stacking gel of 4% acrylamide. We used the molecular weight standards (Combithek, Boehringer Mannheim GmbH Biochemica, Mannheim, FRG) were α-(39,000), β-(155,000), and β'-subunit (165,000) of RNA-polymerase, bovine serum albumin (68,000) and trypsin inhibitor (21,500).

Fibrin autography was performed according to Levin and Loskutoff. The indicator gel was composed of 1% agarose containing 0.1% fibrin and 7 µg/ml of plasminogen, and was stained with Coomassie Brilliant Blue (Nakarai Chemical, Ltd.). To determine the immunological nature of the PAs, 5 µg/ml of anti-t-PA IgG or 500 µg/ml of anti-u-PA IgG was added to the fibrin agarose gel.

Western immunoblotting was performed as follows. Serum-free conditioned medium was precipitated with 24% trichloroacetic acid and 20-fold condensed samples was electrophoresed in SDS-PAGE. Purified human PAI-1, t-PA or u-PA was also submitted to SDS-PAGE as control. Electrophoresed proteins in the gels were transferred to nitrocellulose papers according to the method of Towbin et al. All subsequent incubations were carried out at room temperature and washing was done in PBS containing 0.05% Triton X-100. The nitrocellulose sheets were first washed and remaining accessible sites blocked with 1% BSA in PBS. Primary incubation with goat anti-human PAI-1 IgG, monoclonal mouse anti-human PAI-2 IgG and rabbit anti-human t-PA or u-PA IgG was carried out for 1 hr. Sheets were washed and incubated with HRP-conjugated rabbit anti-goat IgG, rabbit anti-mouse IgG and goat anti-rabbit IgG for 30 min. After washing the papers were visualized with 4-chloro-l-naphthol and 0.01% hydrogen peroxide.

Preparation of Monospecific Anti-Human t-PA IgG Conjugated with HRP

Monospecific anti-t-PA IgG was prepared by passing the IgG fraction of rabbit antiserum through a column of Sepharose 4B coupled with purified t-PA. The absorbed IgG was eluted with 0.1 M glycine-HCl, pH 2.5. To each eluate fraction (1 ml) was added 50 µl of 1 M Tris-HCl, pH 9.0. The fractions containing the IgG were pooled and dialyzed against 0.01 M carbonate buffer, pH 9.5. The monospecific IgG was conjugated with HRP by the peridate method.

Enzyme-Linked Immunosorbent Assay (ELISA) for t-PA

The assay we used was a modification of that of Bergsdorf et al. In brief, the rabbit anti-human t-PA IgG (100 µl, 5 µg/ml) in 0.1 M NaHCO₃, was placed on a Costar 96-well microtiter plate (Data Packing Co., Cambridge, MA) and kept overnight at 4°C.
After coating this preparation with 1% BSA, samples or t-PA standards were added to each well and the preparation kept at 4°C overnight. HRP-conjugated monospecific rabbit anti-t-PA IgG (100 µl, 0.5 µg/ml) was put into each well and the preparation incubated for 3 hr at room temperature. Then, 100 µl citrate phosphate buffer (0.1 M, adjusted to pH 5.0 with 0.1 M citric acid) containing 0.4 mg/ml o-phenylenediamine and 0.01% H₂O₂ was added and the preparations incubated for 2 hr in the dark at 25°C. The enzymatic reaction was halted by adding 25 µl of 4.5 M H₂SO₄. The absorbance was measured at 492 nm using a multiscan spectrophotometer (Corona Co., Tokyo, Japan). Standard curve values in the range of 0.05 to 1.2 ng/ml gave a linear dose-response curve, as shown in Figure 1.

**Assay of PA Activity**

Fibrinolytic activity was estimated using a chromogenic assay. Assay solution was prepared by mixing 0.1 mg/ml Glu-Plasminogen and 3 mM plasmin substrate (S-2251, KabiVitrum, Stockholm, Sweden) in dilution buffer (0.05 M Tris-HCl, pH 7.3, 0.1 M NaCl, 0.01% NaN₃). To measure the total PA activity in each sample, an equal volume of the activator reagent containing plasminogen and S-2251 was added to 100 µl of the sample. The reaction was done with the addition of 2 µl (7 mg/ml) soluble fibrin. 47.5 U/ml of hirudin (Sigma) was added to the sample to inhibit the direct hydrolytic effect of thrombin against S-2251. This concentration of hirudin did not interfere protease activity of plasmin or PA in the sample. After incubation at 37°C for 3–4
Fig. 4. Immunoblotting with goat anti-human PAI-1 IgG of condensed serum-free conditioned medium of BCE cells (lanes 1 and 2) and purified human PAI-1 (lane 3). Samples were separated on SDS polyacrylamide gels and electrophoretically transferred to nitrocellulose papers. Lane 1 was loaded with 40 μl of 20-fold condensed serum-free conditioned medium (28 μg of protein/lane). Lane 2 was loaded with 80 μl of 20-fold serum-free condensed conditioned medium (56 μg of protein/lane). Lane 3 contained 100 ng of purified human PAI-1. The 48,000 and 124,000 bands showed specific binding to anti-human PAI-1 IgG. Lane 1: 124,000 band was recognized. Lane 2: 48,000 and 124,000 bands were recognized. The higher M.W. band was regarded as the reaction of aggregate. Lane 3: purified human PAI-1 from HT-1080 conditioned media showed an apparent M.W. of 48,000.

Fig. 5. Thrombin-mediated increase of t-PA release. Confluent cultures were incubated with 1% serum-containing medium plus thrombin at the concentrations indicated. After incubation for 24 hr, the t-PA was measured by ELISA. The values are mean ± SEM of four experiments with duplicate measurements in each. *P < 0.05, **P < 0.01: statistically significant differences from the control (no stimulus).

Fig. 6. Effect of increasing concentrations of thrombin on plasminogen activator release from BCE cells. Confluent BCE cells were incubated with 1% serum-containing medium plus indicated concentrations of thrombin. Plasminogen activator activities of total (●), t-PA (●), and u-PA (○) in the conditioned media were measured 24 hr later, as described in Materials and Methods. The values expressed the means of duplicate determinations from a representative example of four experiments.
solution, because the residual activity was completely neutralized by simultaneous addition of 500 μg/ml of anti-u-PA IgG.

Statistical Analyses

Statistical analyses were conducted by analysis of variance, t-test or Bonferroni test.

Results

Fibrin Autography of Conditioned Medium of Cultured BCE Cells

Fibrin autography revealed that cultured BCE cells released into the conditioned medium PAs with a M.W. of about 45,000, 72,000 or 124,000 (Fig. 3). The first one was u-PA because this PA activity was quenched by anti-u-PA IgG, but not by anti-t-PA IgG. The latter two were identified as t-PA-related activators because these PA activities were quenched by anti-t-PA IgG, but not by anti-u-PA IgG.

Western Immunoblotting

Blots of condensed serum-free conditioned medium of BCE cells probed with goat anti-human PAI-1 IgG showed antibody binding sites with M.W. of 48,000 band and 124,000 band (Fig. 4). Rabbit anti-human t-PA IgG recognized two bands with M.W. of 72,000 and 124,000, but the reaction was rather weak, probably because of poor cross-reactivity of anti-human t-PA IgG for bovine t-PA, as shown in Figure 2. Monoclonal mouse anti-human PAI-2 IgG and rabbit anti-human u-PA IgG detected no immunostained band.

Effect of Thrombin on Release of PA from BCE Cells

The addition of increasing concentrations of thrombin (0.1 to 10.0 U/ml) to confluent cultures for 24 hr induced a dose-dependent increase in the level of t-PA in the conditioned medium (Figs. 5, 6). The t-PA level in the thrombin-free medium was 0.47 ng/ml in concentration and 0.28 U/ml in activity. The increase in t-PA levels was first detectable with 1.0 U/ml thrombin (0.64 ng/ml). A maximum level of t-PA (0.95 ng/ml) was observed with treatment with 10.0 U/ml thrombin. Thrombin at a level of 31.6 U/ml induced no additional increase in the t-PA levels. The level of u-PA activity was not significantly affected by the addition of thrombin. Fibrin autography also supported these findings (Fig. 7). The total PA activity in the conditioned medium was 0.41 U/ml. The maximum level of total PA activity was 1.0 U/ml at the stimulation with 3.16 U/ml thrombin. Thrombin at levels over 10.0 U/ml inhibited additional increases in PA activity.

Changes in the level of t-PA concentration were measured at various times during 24 hr after addition of 10.0 U/ml thrombin (Fig. 8). The release of t-PA during the first 15 min was not detectable, but during 1 hr the release of t-PA increased over the control values. The average rate of t-PA release increased to 0.10 ng/ml per hr between 1 and 3 hr and declined 0.05 ng/ml per hr between 3 to 6 hr. These findings indicated that the thrombin effect on t-PA release was present from an early time and had no apparent lag.
phase. Fibrin autography also supported the time dependency of the thrombin enhancement of t-PA release. The fibrinolytic zones of t-PA gradually increased with the incubation time and were wider than those in nontreated preparations at the respective times. The fibrinolytic zones of M.W. 124,000 band also increased with incubation (Fig. 9).

Effects of Cycloheximide and Actinomycin D

To determine whether the thrombin-mediated t-PA release required protein synthesis or mRNA synthesis, the cultures were treated with various concentrations of cycloheximide or actinomycin D for 24 hr after adding 10.0 U/ml thrombin, and the amount of t-PA in the conditioned medium was measured. Although small doses of cycloheximide had no effect, doses over 0.1 μg/ml decreased the level of t-PA in the conditioned medium. When 1.0 μg/ml cycloheximide was added, the final t-PA concentration was 43% of thrombin-treated cultures (Fig. 10A). Treatment with actinomycin D also lowered the t-PA levels (Fig. 10B).

Effect of DFP-Thrombin

Little increase in the level of t-PA was observed when the cells were treated with DFP-thrombin at various concentrations equivalent to 0.01 to 31.6 U/ml active thrombin (Fig. 11). This result indicated that the enhanced release of t-PA was thrombin active site-dependent. To determine whether the high-affinity active site-independent thrombin receptor was involved in the increase of t-PA release, thrombin was added to cultures in the presence of excess amounts of DFP-thrombin. Little thrombin enhancement of t-PA release was observed when the cells

---

**Fig. 10.** Effect of cycloheximide and actinomycin D on the thrombin-induced t-PA release from BCE cells. Confluent cultures were exposed to 10.0 U/ml thrombin and indicated concentrations of cycloheximide (A) or actinomycin D (B). At 24 hr, culture medium was removed and t-PA concentration was measured by ELISA. The values are mean ± SEM of four experiments with duplicate measurements in each. *P < 0.05, **P < 0.01: statistically significant differences from values measured in the presence of only 10 U/ml thrombin.

**Discussion**

The presence of PAs in BCE cells has been reported.19-21 Pandolfi and Lantz noted that the BCE
Table 1. Effect of DFP-thrombin and active dium plus DFP-thrombin, at the concentrations indicated. After incubation for 24 hr, the t-PA concentration was measured by ELISA. The values are mean ± SEM of four experiments with duplicate measurements in each. The values at any concentrations of DFP-thrombin were not significantly different from that at no DFP-thrombin (no stimulus). P < 0.05.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>t-PA level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No stimulant</td>
<td>100.0 ± 6.0</td>
</tr>
<tr>
<td>Thrombin (10 U/ml)</td>
<td>205.1 ± 9.0*</td>
</tr>
<tr>
<td>DFP-thrombin (100 U, 40 µg/ml) and thrombin (10 U/ml)</td>
<td>210.4 ± 10.2*</td>
</tr>
<tr>
<td>DFP-thrombin (1000 U, 400 µg/ml) and thrombin (10 U/ml)</td>
<td>208.3 ± 11.5*</td>
</tr>
</tbody>
</table>

Confluent cultures were treated with the indicated amounts of thrombin or DFP-thrombin for 24 hr. Average level of t-PA concentration in non-stimulated conditioned media was expressed as 100%. The values listed are mean ± SEM of three experiments represented as the percentage of average level of t-PA in nonstimulated conditioned media (no stimulant).

* P < 0.001: statistically significant differences from the t-PA level in nonstimulated conditioned media.

Fig. 11. Effect of DFP-thrombin on t-PA release from BCE cells. Confluent cultures were incubated with 1% serum-containing medium plus DFP-thrombin, at the concentrations indicated. After incubation for 24 hr, the t-PA concentration was measured by ELISA. The values are mean ± SEM of four experiments with duplicate measurements in each. The values at any concentrations of DFP-thrombin were not significantly different from that at no DFP-thrombin (no stimulus). P < 0.05.

We employed separate assays for t-PA and u-PA in the conditioned medium. A similar method was reported by other investigators.24 In our experiments, the fibrinolytic activity of the conditioned medium was completely quenched by simultaneously adding sufficient amounts of rabbit anti-human u-PA IgG and rabbit anti-human t-PA IgG. A large amount of rabbit anti-human u-PA IgG was necessary to completely quench bovine u-PA activity, probably due to the poor immunological cross-reactivity of rabbit anti-human u-PA IgG for bovine u-PA.

Other investigators reported that thrombin inhibited corneal endothelial cell-mediated fibrinolysis.19 However, according to our results, thrombin induced an increase in the release of t-PA, in a dose-dependent manner, and enhanced the fibrinolytic activity of BCE cells. This appears to be protective for corneal endothelial cells against fibrin clot formation, because t-PA is a fibrin-specific fibrinolytic agent. High levels of thrombin over than 10 U/ml did not induce the additional increase of t-PA release. This is probably due to the cytotoxic effect of thrombin at high concentrations, although morphological damage or decrease in cell number was not found.

Thrombin induced an increase in the release of t-PA in a time-dependent manner and this effect was observed from the early period and had no apparent lag phase. This result suggests that an initial release of stored t-PA is involved in thrombin-mediated t-PA release. However, the amount of t-PA release within 1
hr is very small compared with the total amount of t-PA released for 24 hr. So thrombin-mediated t-PA release is mainly considered to be due to t-PA synthesis, as shown in the cycloheximide and actinomycin D data.

Enzymatically inactive DFP-thrombin did not increase the release of t-PA, thereby indicating that the active site of thrombin is necessary for the thrombin-mediated t-PA release. This effect of thrombin does not appear to involve high-affinity, active site-independent thrombin binding sites on the BCE cells. Other workers showed that thrombin binds covalently to its receptor sites on the surface of corneal endothelial cells.

In conclusion, we obtained evidence that corneal endothelial cells release two kinds of PAs, t-PA and u-PA, and only the t-PA release is enhanced by treatment with thrombin. Thrombin may play an important role in the intraocular regulation not only of coagulation but also of fibrinolysis. However, to confirm the pathophysiological effects of thrombin, it is necessary to establish the concentrations of free thrombin actually functioning in vivo, with relation to the action of inhibitors of thrombin such as antithrombin III.

**Key words:** corneal endothelial cell, cultivation, thrombin, plasminogen activator, ELISA

Acknowledgments

We thank Professor H. Inomata for persistent suggestions and M. Ohara for comments on the manuscript.

**References**