The Effect of Thrombin on Actin Filament and Vinculin of Corneal Endothelial Cells

Taiji Sakamoto,* Yutaka Nakashima,* and Katsuo Sueishi*

Purpose. The authors examined the effect of thrombin on confluent bovine corneal endothelial (BCE) cells in vitro, especially on cellular integrity and on the redistribution of F-actin and vinculin.

Methods. Immunofluorescent stainings against F-actin and vinculin were carried out on confluent BCE cells, and the effect of thrombin was evaluated.

Results. F-actin was distributed at the cytoplasmic peripheries of confluent BCE cells, forming dense peripheral bands (DPB), whereas vinculin was linearly located at the cell borders. Enzymatically active thrombin caused the loss of DPB and an increase of central micronlament bundles, associating the dissociation of vinculin-cell plaques and the formation of intercellular gaps. However, enzymatically inactive thrombin did not induce such changes. The thrombin effect was reversible and occurred in a concentration-dependent manner. The pre-incubation of BCE cells with disrupting agents of microtubules, such as colchicine and demecolcine, or voltage-dependent Ca2+ channel blockers did not affect these thrombin-induced changes, whereas forskolin and energy blockers such as oligomycin AB and C and antimycin A inhibited these changes.

Conclusions. Enzymatic-active thrombin affects the arrangement of the cytoskeletal structure of BCE cells and cell-substratum interaction and plays an important role in the re-integrity or repair processes of the monolayer of BCE cells. Invest Ophthalmol Vis Sci. 1993; 34:438-446.
The present study was undertaken to determine the effect of thrombin on confluent BCE cells in vitro, especially on cellular integrity and on the redistribution of F-actin and vinculin.

**MATERIALS AND METHODS**

**Reagents**

Bovine alpha-thrombin was purified by cationic ion exchange chromatography from commercially available thrombin (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) as previously described. The final preparation had a specific activity of about 3000 NIH U/mg of protein. Diisopropylfluorophosphate (DIP; Sigma, St. Louis, Mo)-treated thrombin, an enzymatic inactive thrombin, was prepared by reacting thrombin with 10 mmol/l DIP at pH 7.2 for 1 hr at 37°C. Then, DIP-thrombin was dialyzed against phosphate-buffered saline (PBS), pH 7.4. Thrombin activity was assayed by a chromogenic method using S-2238 (Kabi Pharmacia, Uppsala, Sweden). The osmolarity of the medium that contained 5 U/ml of thrombin used in this study was 301 mosm/kg.

Cytochalasin B as a microfilament disrupting agent, colchicine and demecolcine (Colcemid) as microtubule disrupting agents, antimycin A (Streptomyces hitazawaiensis) and oligomycin AB and C as energy blockers, and verapamil-hydrochloride and nifedipine as voltage-dependent Ca2+ channel blockers were purchased from Sigma. Trypsin was from Mochida Pharmaceutical Co. Ltd. Cytochalasin B was dissolved in dimethyl sulfoxide (DMSO), colchicine and Colcemid in PBS, antimycin A, and oligomycin AB and C in absolute ethanol. Verapamil-hydrochloride was dissolved in 10% (volume/volume) ethanol in DMSO. These reagents were stored at −20°C until they were used. Each drug was diluted with culture medium to the final concentration indicated later.

**Cell Culture**

BCE cells were isolated and cultured by a method previously described. BCE cells were cultured on 21 cm² Falcon plastic culture dishes (Beckton Dickinson Labware, Oxnard, CA) in Dulbecco’s modified Eagle medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) that contained 10% fetal calf serum (FCS; Flow Laboratories, North Ryde, Australia), 100 U/ml penicillin, and 100 μg/ml streptomycin. BCE cells of the fifth to tenth passages were used for the following studies. In the authors’ opinion, methods for securing animal tissue were humane, included proper consent and approval, and complied with the ARVO Resolution on the Use of Animals in Research.

For immunofluorescent studies, cells were plated at 5 × 10⁴ cells per well onto sterile glass coverslips (Nunc, Naperville, IL). The cultures were fed every three days and allowed to grow to confluency. In the subsequent studies, the BCE cells were used 3 to 5 days after confluence.

**Stimulation of Confluent BCE Cells With Thrombin or DIP-Thrombin**

To stimulate BCE cells, the confluent cultures were washed three times with DMEM and incubated at 37°C or 4°C in DMEM containing 0.35% bovine serum albumin (BSA) and thrombin or DIP-thrombin, at the indicated concentrations. The incubation time varied from 15 min to 24 hr. The cells were observed with a phase contrast microscope (Nikon, Tokyo, Japan). The BCE cells incubated with DMEM that contained 0.35% BSA were employed as a control.

**Recovery from Thrombin Exposure**

After the BCE cells were pretreated with thrombin (2 U/ml for 60 min), the culture medium containing thrombin was removed and BCE cells were washed and incubated with the following media for 2 hr: DMEM with 10% FCS, DMEM with 0.35% BSA, or DMEM alone.

**Pretreatment With Several Agents Before Thrombin Exposure**

BCE cells were pretreated with the following agents: cytochalasin B (0.05 or 0.005 ng/ml for 30 min), colchicine (0.1 μmol/l or 0.5 μmol/l for 5 hr), demecolcine (10 nM or 50 nM for 5 hr), verapamil (10 μmol/l for 30 min), nifedipine (10 μmol/l for 30 min), oligomycin (20 μg/ml for 40 min), antimycin A (50 μg/ml for 40 min), and forskolin (10 μg/l or 100 μg/l for 20 min). Just after the preincubation, the cells were washed three times with DMEM containing 0.35% BSA and were exposed to the thrombin (2 U/ml) in DMEM for 60 min at 37°C.

**Stimulation of Confluent BCE Cells With Other Drugs**

Confluent BCE cells also were incubated with DMEM that contained the following drugs. Trypsin, one of the serine proteases, was used in various concentrations (0.1–5 U/ml; Mochida Pharmacological Co.) and incubated for 30 min to 4 hr, or phorbol 12-myristate 13-acetate (1–10 ng/ml; Sigma) was incubated for 30 min to 4 hr. The reagents used for this study have been reported to affect the rearrangement of the actin complex in endothelial and other cells in vitro. The concentrations applied to BCE cells were chosen based on these experiments.

**Distribution of Vinculin and F-actin**

The distribution of vinculin and F-actin in BCE cells was examined by the following double immunofluorescence labeling method.
BCE cells cultured on a slide were washed three times with PBS and fixed with cold acetone for 20 min. After air drying, the cells were incubated with PBS containing 1% BSA and 0.1% saponin (Sigma) for 30 min. They then were reacted with mouse monoclonal antibody against vinculin (dilution 1:100; BioMarkor, Rehovot, Israel) and rhodamine-phalloidin (dilution 1:200; Sigma) for 1 hr. After washing three times with PBS, they were incubated with a fluoresceinated secondary antibody for 1 hr to visualize vinculin distribution. The incubations with primary and secondary antibodies were carried out at 4°C. The cells were mounted in 50% glycerol/50% PBS and examined with a fluorescent microscope equipped with phase contrast and epifluorescence optics (Zeiss, Oberkochen, Germany). As a negative control, normal mouse serum (diluted 1:1000 in PBS) was used instead of the primary antibody or rhodamine-phalloidin.

RESULTS

Effect of Thrombin on BCE Cells

Confluent BCE cells cultured in DMEM that contained 0.35% BSA showed a polygonal shape in cobbled-

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933396/)

**FIGURE 1.** Photomicrographs of confluent BCE cells labeled for F-actin with rhodamine-phalloidin (A) and vinculin with anti-vinculin monoclonal antibody (B). F-actin is observed at the periphery of the cells, forming a dense peripheral band (arrows), and vinculin is located at the cell-cell contact areas. (Original magnification X1025.)

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933396/)

**FIGURE 2.** Phase contrast micrographs of confluent BCE cells. (A) Before thrombin exposure. (B) After incubation with 2 U/ml of thrombin for 30 min. BCE cells became elongated and formed intercellular gaps after thrombin exposure. (Original magnification X410.)

stone appearance without any apparent intercellular gap formation. F-actin filaments were distributed along the edge of the cells, forming dense peripheral bundles (DPB) (Fig. 1A). Vinculins were observed mainly at the margin of the cells, especially along the cell boundaries (Fig. 1B). BCE cells incubated with 0.1, 1, 2, or 8 U/ml of thrombin (1 U/ml is equivalent to about 9 × 10^-9 mol/l) elongated and formed intercellular gaps within 30 min (Figs. 2A, B). These changes were noted with every concentration of thrombin and occurred rapidly and intensely as the concentration of thrombin increased. Microfilaments located at the cellular peripheries as DPB decreased. With the change in cell shape, central microfilament bundles began to appear and became aligned with the long axis of the cells (Figs. 3A, C). The vinculin patches lost their linear distribution at the peripheral part of the cells and were redistributed in a scattered pattern (Figs. 3B, D). The formation of intercellular gaps began to appear focally after 30 min and became more prominent after 2 hr. In addition, these changes occurred rapidly and prominently as the concentration of thrombin was increased. Thrombin exposure at 4°C...
FIGURE 3. Thrombin effect on the distributions of F-actin (A, C) and vinculin (B, D). BCE cells were labeled for F-actin with rhodamine-phalloidin (A, C) or vinculin with anti-vinculin monoclonal antibody (B, D) by double fluorescence labelling method. BCE cells were incubated with 2 U/ml of thrombin for 30 min (A, B) or 4 U/ml of thrombin for 60 min (C, D). Dense peripheral bands (DPB) disappeared at every concentration. Such changes occurred more rapidly at the higher concentration. BCE cells became more elongated and intercellular gaps are more prominent at the higher concentration. (Original magnification ×1025.)

did not influence the cell morphology of BCE cells or the distribution of F-actin and vinculin.

Effect of DIP-Thrombin on BCE Cells

The exposure of BCE cells to DIP-thrombin (90 nM, 900 nM, 9000 nM for 60 min) had no effect on the distribution of F-actin and vinculin plaque, compared with exposure to enzymatically active thrombin. This suggests that these changes were thrombin active-site dependent. To determine whether the high-affinity active-site-independent thrombin receptor was involved in this change, thrombin was added to the cultures in the presence of excess amounts of DIP-thrombin. However, the simultaneous incubation with thrombin and an excess amount of DIP-thrombin, including up to 100 times in molar ratio, had the same effect on BCE cells as incubation with thrombin alone (Table 1).

Recovery from Thrombin Exposure

Beginning 1 hr after the exchange of culture medium containing thrombin to medium containing 10% FCS, the intercellular gaps between BCE cells began to disappear and return to their original hexagonal shape. F-actin was redistributed at the peripheral portion of the cytoplasm, and vinculin plaques were linearly arranged at the border of the BCE cells (Figs. 4A, B). However, BCE cells did not recover their original hexa-

### Table 1. Effect of Thrombin or DIP-Thrombin on the Distribution of F-actin and Vinculin of BCE

<table>
<thead>
<tr>
<th>Thrombin</th>
<th>DIP-Thrombin</th>
<th>Rearrangement of F-actin and Vinculin</th>
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<tbody>
<tr>
<td>0</td>
<td>90 × 10⁻⁹ mol/l</td>
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<tr>
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<tr>
<td>10 U/ml*</td>
<td>900 × 10⁻⁹ mol/l</td>
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* 10 U/ml: 90 × 10⁻⁹ mol/l.

FIGURE 4. Restoration of F-actin (A) and vinculin (B) redistribution after thrombin exposure. BCE cells were incubated with the medium containing 5% fetal calf serum for 2 hr after exposure to 3 U/ml of thrombin for 30 min. F-actin was rearranged at the periphery of the cell as forming DPB, and vinculin was located at the cell border again. (Original magnification X270.)

agonal shapes even after 2 hr, when the incubation medium was changed to a medium containing BSA alone or serum-free medium.

Pretreatment With Agents Before Thrombin Exposure

Pretreatment of BCE cells with colchicine, Colcemid, verapamil, or nifedipine did not influence the effect of thrombin on cell morphology and distribution of F-actin or vinculin (Fig. 5). In contrast, the pretreatment of BCE cells with oligomycin AB or antimycin A inhibited the morphologic changes induced by thrombin. After preincubation with a high dose of forskolin (100 \( \mu \)g/ml), thrombin did not induce a redistribution of actin filament and vinculin. However, with a low dose of forskolin (10 \( \mu \)g/ml), a partial thrombin effect was noticed. We could not examine the influence of cytochalasin B on the thrombin effect, because the treatment of BCE cells with cytochalasin B alone changed the distribution of F-actin and vinculin.

Effect of Other Drugs

In incubation with trypsin, BCE cells began to round up and separate from each other within 30 min, so that prominent intercellular gaps were observed between the cells. After 3 or 4 hr, some cells began to float up in the medium. The DPB of cells were partly observed, but vinculin plaques mostly disappeared (Figs. 6A, B). In incubation with phorbol ester 12-myristate 13-acetate, neither an intercellular gap nor cell floating was apparent within 2 hr, but F-actin was diffusely distributed throughout the cells—thus, the DPB disappeared. Vinculin plaques also became obscure (Figs. 6C, D).

DISCUSSION

The mechanism that regulates the monolayer organization of BCE cells is important in light of such factors as corneal endothelial permeability, repair process, and maintenance of corneal transparency. Actin filament is a cytoskeleton that participates in the preservation of cell shapes, cell-substratum adhesion, and cell motility. Vinculin is involved in the attachment of actin filament bundles to the plasma membrane.

The present study demonstrates that enzymatically active thrombin changes the shape of confluent BCE cells and induces a formation of intercellular gaps and an irregular distribution of F-actin and vinculin. Those changes were more rapid and prompt as the concentration of thrombin was increased. F-actin of confluent BCE cells was located in the peripheral cytoplasm, forming DPB, and vinculin was linearly arranged at the margin of the cells. After exposure to thrombin, DPB and the lining vinculin plaques were markedly reduced, and central microfilaments and patchily distributed vinculin appeared. In addition, these morphologic changes of BCE cells were specific for enzymatically active thrombin and were different from the changes caused by trypsin or phorbol ester. Thus, trypsin might have a dissociating effect on the cell-substrate matrix interaction that is stronger than cytoskeleton rearrangement. Recently, McDermott et al reported that thrombin affected the morphology of human corneal endothelium. The concentration of thrombin used in their study (100–1000 U/ml) was much higher than that of our study, and their thrombin contained various other constituents, compared with our highly purified alpha-thrombin.

Wong and Gottlieb reported that DPB and peripheral vinculin plaques were important to the ability of vascular endothelial cells to maintain close cell-cell contact and restrict cell migration. They also showed that DPB and vinculin plaques disappeared during cell migration. Therefore, we speculate that the disappearance of DPB and vinculin plaques in BCE cells by
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Figure 5. Photomicrographs of confluent cultured BCE cells labeled for F-actin (A, C) and vinculin (B, D). BCE cells were treated with 10 ng/ml of nifedipine for 30 min (A, B). BCE cells were incubated with 5 U/ml thrombin for 1 hr after the pretreatment of nifedipine (C, D). F-actin and vinculin distributions were not changed by nifedipine treatment alone, but were markedly changed by thrombin, even after the pretreatment of nifedipine (Original magnification X270.)

The cellular shape and microfilamentous distribution of BCE cells were affected by enzymatically active thrombin, but not by DIP-thrombin. Moreover, the simultaneous incubation of BCE cells with high doses of DIP-thrombin did not inhibit the effect of active thrombin. These thrombin-induced changes also were temperature dependent. The present findings suggest that the effect of thrombin on BCE cells was mediated via the specific receptors for enzymatically active thrombin. Isaac et al reported that the corneal endothelium has binding sites to enzymatically active thrombin.19 Our present findings are very similar to those reported by Isaac for BCE cells, and the present morphologic changes of BCE cells seemed to be mediated by thrombin-binding sites or receptors. However, it still is possible that thrombin directly acted on BCE cells in addition to the receptor-mediated mechanism. Further studies about thrombin receptors of BCE cells thus will be necessary.

Thrombin exposure occurs to allow each cell to move less hindered by its neighbors.

The thrombin-induced morphologic changes were reversible, and this reversibility occurred in the presence of FBS. Thus suggests that some serum proteins may be indispensable or helpful in restoring the original shape of BCE cells. We examined several constituents of serum, such as serum albumin, vitronectin, fibronectin, and hyaluronate (data not shown). But they did not have an apparent recovery effect on BCE cells. Other factors may recover the morphology of BCE cells. Serum protein may have accelerated BCE cells to internalize or neutralize thrombin.20

We incubated various agents before exposing BCE cells to thrombin to clarify the mechanisms involved in thrombin-induced changes. Colchicine and the colcemid did not prevent those changes. Although the microfilament system for maintaining the cell shape of fibroblasts has been reported to be partly controlled by a special microtubule-dependent mechanism,21 our results suggest that microtubules did not influence the thrombin-induced shape changes. The similar rearrangement of actin filament induced by
thrombin in vascular endothelial cells was reported by Galdal et al. Therefore, the effect of thrombin on BCE cell shape may have resulted from depolymerization of actin filament, as those authors speculated.

We could not elucidate the mechanism by which forskolin caused such an inhibitory effect on BCE cells against thrombin-induced change. However, some explanations can be derived based on the findings for vascular endothelial cells. The regulation of intracellular Ca\(^{2+}\) concentration in vascular endothelial cells and corneal endothelium has been reported to be very important in maintaining the cytoskeleton and cell shape. Thrombin is known to induce the release of Ca\(^{2+}\) from intracellular stores and substantial Ca\(^{2+}\) influx across the plasma membrane. Also, the increase of cytosolic Ca\(^{2+}\) is the initial event in the thrombin-induced shape changes of vascular endothelial cells. It has been established that thrombin stimulates the rapid hydrolysis of phosphatidyl inositol 4,5-biphosphate and the accumulation of inositol 1,4,5-triphosphate, which acts as a second messenger within the cell to stimulate Ca\(^{2+}\) release from the endoplasmic reticulum.

The agents that increase intracellular cAMP, including forskolin, have been reported to decrease phosphatidyl inositol biphosphate hydrolysis and consequently reduce the cytosolic Ca\(^{2+}\) concentration. Indeed, in vascular endothelial cells, forskolin effectively inhibits a thrombin-evoked cytosolic Ca\(^{2+}\) increase. The same events may have occurred in BCE cells, and thrombin-induced shape change was inhibited by forskolin in the present study. In addition, our results imply that voltage-dependent Ca\(^{2+}\) channel blocked by nifedipine and verapamil does not play a major role in thrombin-induced shape changes. On the other hand, oligomycin and antimycin are mitochondrial energy blockers that inhibit adenosine triphosphate (ATP) production. ATP is known to be an important factor in the contraction of the actomyosin system. Therefore, we can say that thrombin-induced shape change is an energy-dependent reaction, but is independent of the microtubular system and...
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Voltage-dependent Ca\textsuperscript{2+} channels and is inhibited by an increase of cAMP.

Thrombin, however, may have a negative effect on maintaining the transparency of the normal cornea, because thrombin induces gap formation between BCE cells, followed by the increased permeability of the endothelium and corneal stromal opacity. To confirm the pathologic effect of thrombin on corneal endothelial cells, it is necessary to evaluate the concentrations of enzymatically active thrombin in the anterior chamber, with relation to the thrombin inhibitors such as the complex of anti-thrombin III and heparin.

In conclusion, when corneal endothelium is impaired or denuded, wound repair can be achieved by a sequence that involves the changing shape, spreading, migration, and proliferation of corneal endothelial cells. Therefore, it is reasonable to say that enzymatically active thrombin that is produced after injury and that induces degeneration and denudation of corneal endothelium plays an important role in enhancing the healing processes of the corneal endothelium by mobilizing the adjoining endothelial cells.

Key Words
actin, calcium, corneal endothelium, thrombin, vinculin.

References