Endothelial Monolayer Integrity

Perturbation of F-Actin Filaments and the Dense Peripheral Band-Vinculin Network

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The role of the actin microfilaments in maintaining the integrity of the monolayer and activating endothelial repair processes is not well understood. This study was designed to characterize the prominent changes in F-actin distribution in endothelial cells that are associated with shape changes in the cells after perturbation of a confluent monolayer. F-actin was localized by using rhodamine phalloidin and fluorescence microscopy. The dense peripheral band (DPB) and vinculin cell-cell junctions were co-localized by using double fluorescence and immunofluorescence microscopy. Thrombin and 12-o-tetradecanoyl-myristyl-13-acetate (TPA) caused loss of the DPB and an increase in the central microfilament bundles, while agents that caused rounding of the cells (including plasmin, trypsin, and chymotrypsin) did not cause loss of the DPB although large gaps were formed between cells. The thrombin and TPA effects were rapid and reversible and were associated with an accompanying loss of vinculin cell-cell plaques. The mechanisms of the effects were not studied. It was postulated that thrombin and TPA were activating endothelial repair processes.

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Since the pathogenesis of atherosclerosis involves several initial events, one of which is the disruption of the physical integrity of the endothelial cell monolayer, we carried out studies on the role of the cell actin microfilaments in maintaining this integrity. It is well known that confluent aortic endothelial cells undergo shape changes both in vivo and in vitro in response to a variety of stimuli. These shape changes are modulated by the actin microfilaments; in some cases, these changes reflect cellular injury and in others, adaptation to injury. Vinculin containing adherens type junctions are associated with the dense peripheral band of actin microfilaments (DPB), which is present in vitro in confluent endothelial monolayers and in vivo in the cobblestone arranged cells present in low shear areas. It was hypothesized that the DPB of each individual cell was linked via the junctions to form a network of microfilaments in the monolayer, which maintained the physical integrity of the monolayer.

The aim of this study was to investigate the effect on actin filaments of substances that are known to disrupt the confluent endothelial monolayer. The reorganization of actin filaments was studied by using rhodamine phalloidin to localize F-actin. The compounds tested included some that produced an elongated shape change, such as thrombin and the tumor promotor, 12-o-tetradecanoyl-myristyl-13-acetate (TPA), and some that caused rounding of the cells, such as plasmin, trypsin, and chymotrypsin. The DPB-vinculin network was studied in thrombin disrupted monolayers by using double fluorescence and immunofluorescence localization of F-actin with either myosin or vinculin. Part of this work has been previously presented in abstract form.

Methods

Cell Culture

Primary porcine thoracic aortic endothelial cultures were obtained by the collagenase dispersion method and were grown in Medium 199 in Earle’s salts with 25 mM HEPES 0.3 mg/ml L-glutamine supplemented with 5% fetal bovine serum (FBS) (GIBCO, Grand Island, NY) as previously described in detail.

For immunofluorescence studies, cells were plated at 1 x 10^4 cells per dish onto 22 mm^2 sterile glass coverslips (Coming Glass, Coming, NY) in 35 mm Falcon tissue culture dishes (Benton, Dickinson, Oxford, CA). The cultures were fed twice weekly and allowed to grow to confluence, at which point they were fed for the last time and used 3 days later. All experiments were repeated at least four times across different cell, serum, and reagent lots.

Incubation of Test Compounds with Confluent Cultures of Endothelial Cells

Highly purified thrombin (3080 NIH units/mg protein) free of any blood clotting factors, and purified plasmin (4.2 units/mg), chymotrypsin (52 units/mg), and trypsin (9200 units/mg) in lyophilized form were obtained from Sigma Chemical, St. Louis, MO. In addition, highly purified α-thrombin and PPACK-thrombin were obtained from Dr. Frederick Ofosu, McMaster University, Hamilton,
were coded by another person in the laboratory and observed by one of the investigators (Michael Wong). The code was broken once the slides were assessed. Photographic recordings were made with Kodak Tri-Pan and Ilford HP-5 film and processed to an ASA rating of 400 and 1600, respectively. The film was developed with Microdol-X (Kodak) at 1:3 dilution for Tri-X Pan and Microphen (Ilford) at full strength for HP5 film.

**Results**

**Effect of Thrombin and TPA on Cell Monolayers and Actin Filaments**

Controls incubated with 0.35% albumin in Medium 199 did not show any cellular shape change or redistribution of microfilament bundles when compared to our standard culture conditions of Medium 199 supplemented with 5% FBS (Figure 1A).

Monolayers incubated with 2 U/ml or 4 U/ml of thrombin began to lose their polygonal shape and became more elongated within 30 minutes. This occurred without the formation of detectable intercellular spaces. Observation of the F-actin cytoskeleton revealed some disorganization of the DPB (Figures 2A and 3A). There was a decrease in the density of microfilaments making up the DPB, and many short microfilaments were not present in this area. As cell elongation continued, centrally located microfilament bundles began to reorient and become aligned with the long axis of the cell. After 1 hour, the DPB was completely disrupted, with only an occasional microfilament bundle present at the cell periphery. The centrally located microfilament bundles became the most prominent F-actin structure within these cells. In addition, the number of these bundles increased when compared to cells incubated in thrombin for only 30 minutes. By 2 hours, the cells were maximally elongated (Figures 2C, 3C, and 5A) and did not significantly change shape with longer incubations in thrombin. Intercellular gaps were not apparent at this or later time points. The cells appeared to attain their maximum microfilament reorientation change within 2 hours of incubation. Cells incubated in 8 U/ml or 16 U/ml of thrombin (Figures 4C and 4D) appeared more elongated and attained their maximum cell shape change more rapidly than did their counterparts in 2 U/ml or 4 U/ml of thrombin. There was little difference between 2 and 4 U/ml of thrombin (Figures 4A and 4B). Cultures incubated with PPACK-thrombin at similar concentrations did not show shape change or reorientation of microfilaments (Figure 1F).

In cultures incubated with TPA, endothelial cells began to elongate; by 30 to 60 minutes, the monolayers appeared morphologically similar to those treated with thrombin. F-actin localization studies confirmed this observation. Incubation with TPA resulted in the disruption of the DPB, which occurred as the cells underwent a transition in shape from polygonal to elongated (Figure 5C). Elongated cells appeared to possess more stress fibers than did cells in control cultures. Washout of TPA-treated cultures gave rise to a rapid restitution of the DPB, which occurred as the cells resumed their polygonal shape and the stress fiber distribution returned to that of the resting state (Figure 5D).
DMSO controls and incubation with phorbol and with 4α-phorbol 12,13-didecanate did not show any effect on cell shape or on microfilament bundle distribution (Figures 1C, 1D, and 1E).

**Effect of Plasmin, Trypsin, and Chymotrypsin on Cell Monolayers and Actin Filaments**

Within 15 minutes of incubation, cells in plasmin, trypsin (not shown), and chymotrypsin began to round up and separate from each other so that by 30 minutes, prominent gaps were seen between the cells. This cell rounding progressed, and after 1 to 2 hours some endothelial cells had lifted off the dish and were floating in the medium. Use of these enzymes at 2 U/ml resulted in the loss of few cells from the monolayer after 2 hours. In contrast, a concentration of 5 U/ml gave rise to large denuded areas in the confluent monolayer. After 4 hours, the vast majority of cells had come off the plate in both concentrations.

Washout of the test substances after 2 hours of incubation by using 5% FBS in Medium 199 resulted in the prompt spreading of the cells and prevented further cell loss from the monolayer. Visualization of these cultures 2 hours after washout showed the presence of a continuous cell monolayer with only occasional intercellular gaps still present (Figures 5F and 5H).

Localization of F-actin revealed that, as the cells rounded up, centrally located stress fibers gradually disappeared (Figures 5E and 5G). The DPB, however, remained present and was very prominent in the attached round cells after 2 hours of incubation (Figures 5E and 5G). Immediately after washout, the DPB appeared to

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**Figure 1.** Photomicrographs of control confluent endothelial cell monolayers labeled for F-actin with rhodamine phalloidin. Cells were incubated for 2 hours. A. Standard culture conditions with 5% fetal bovine serum. B. 0.35% albumin in Medium 199. C. 0.35% albumin in Medium 199 with DMSO. D. Phorbol. E. 4α-phorbol 12,13-didecanate (3.2 × 10⁻⁷ M). F. PPACK-thrombin (4 U/ml). (Arrow indicates dense peripheral band, e.g., A, C). Bar=20 μm.
within the focal actin densities (Figures 3I and 3J). A short, thin microfilament bundles, which extended from the cells underwent the shape transition from elongated to rounded and showed disruption of the DPB. Thin microfilament bundles were no longer present between the DPB and the cell periphery. Vinculin plaques began to disappear from the cell-cell interface and this occurred concomitant with the fragmentation of the DPB (Figure 2B). As this was occurring, myosin disappeared from the cell periphery as the microfilaments making up the DPB become less prominent (Figures 3B and 3D). No change in the myosin and vinculin normally associated with central microfilament bundles was apparent. By 2 hours, there was a general paucity of vinculin along the cell margins and the vinculin plaques were still found in the elongated cells coinciding with the ends of the central stress fibers (Figure 2D).

Washout of thrombin after 2 hours of incubation (Figures 2E to 2J, and 5B) resulted in the rapid restitution of microfilaments at the cell periphery beginning after 15 minutes (Figure 2E) and progressing over the next 2 hours (Figures 3G and 3I) to return to the control state. As the cells underwent the shape transition from elongated to polygonal, vinculin plaques began to appear in areas where cells were closely appositioned to each other (Figures 2F, 2H, and 2J). These developing plaques were sometimes associated with short, thin microfilaments, which emanated from discrete F-actin rich densities within the DPB (arrows in Figure 2E). Myosin remained restricted to the microfilaments of the DPB and did not extend into the area in which intercellular vinculin plaques were found (Figures 3F, 3H, and 3J). In addition, myosin also appeared to be absent from the short, thin microfilament bundles, which extended from the focal actin densities (Figures 3l and 3J).

**Effect of Thrombin on Association of Vinculin and Myosin with Dense Peripheral Band of Microfilaments**

Within the first 60 minutes of thrombin incubation, the cells became elongated and showed disruption of the DPB. Thrombin microfilament bundles were no longer present between the DPB and the cell periphery. Vinculin plaques began to disappear from the cell-cell interface and this occurred concomitant with the fragmentation of the DPB (Figure 2B). As this was occurring, myosin disappeared from the cell periphery as the microfilaments making up the DPB became less prominent (Figures 3B and 3D). No change in the myosin and vinculin normally associated with central microfilament bundles was apparent. By 2 hours, there was a general paucity of vinculin along the cell margins and the vinculin plaques were still found in the elongated cells coinciding with the ends of the central stress fibers (Figure 2D).

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**Discussion**

Reversible perturbation of the confluent endothelial monolayer results in either rounding of the cells and persistence of the DPB or elongation of the cells with the loss of the DPB and its associated vinculin plaques. In the former, there are prominent gaps formed between the cells, while in the latter, the cells rearrange their shape, yet gaps are not present. The realignment of the cells in this manner maintains endothelial integrity while still allowing shape changes to occur.

Both TPA and thrombin cause a similar rapid reversible reorganization of the actin filaments, which is reminiscent of that occurring in migrating endothelial cells during re-endothelialization. In those cells, the marked reduction or loss of the peripheral actin microfilament bundles occurred in endothelial cells at the edge of a large denuding wound made in a confluent monolayer. In addition, we have recently reported that very precise cytoskeletal changes occurred during wound closure even during the process of limited endothelial cell migration. Loss of the DPB and enhancement of the central stress fibers, which are parallel to the long axis of the cell, are very important changes that favor cell migration. This was part of the initial events that occurred during re-endothelialization. It is well recognized that cell proliferation and secretion and modulation of the matrix are important factors in long-term regulation of repair. It is, however, intriguing to postulate that both thrombin and the tumor promoter, TPA, activate cytoskeletal changes that favor migration to promote re-endothelialization if it is necessary. TPA has been shown to induce angiogenesis in vitro. After injury to the confluent endothelium, covalent binding sites for thrombin rapidly appear. In addition, human umbilical vein endothelial cells treated with concentrations of thrombin similar to those used in our studies to produce rounding showed enhanced polymerization of actin and prominent microfilament bundles. Thus, it is possible that the presence of thrombin at the site of vascular injury may initially act to prime the cells for migration, an important repair process. The effect is reversible and thus the redistribution to a resting confluent monolayer pattern can be quickly re-established if migration is not needed. This characteristic DPB loss and central microfilament reorganization has also been observed in human endothelial cells treated with recombinant thrombin for 2 hours (Figures 2E to 2J, and 5B) resulted in the rapid restitution of microfilaments at the cell periphery beginning after 15 minutes (Figure 2E) and progressing over the next 2 hours (Figures 3G and 3I) to return to the control state. As the cells underwent the shape transition from elongated to polygonal, vinculin plaques began to appear in areas where cells were closely appositioned to each other (Figures 2F, 2H, and 2J). These developing plaques were sometimes associated with short, thin microfilaments, which emanated from discrete F-actin rich densities within the DPB (arrows in Figure 2E). Myosin remained restricted to the microfilaments of the DPB and did not extend into the area in which intercellular vinculin plaques were found (Figures 3F, 3H, and 3J). In addition, myosin also appeared to be absent from the short, thin microfilament bundles, which extended from the focal actin densities (Figures 3l and 3J).

**Figure 2.** Photomicrographs of cells doubly labeled for F-actin (A, C, E, G, and I) and vinculin (B, D, F, H, and J). Cells were incubated with 4 U/ml of thrombin and photographed at 30 minutes (A and B) and 2 hours (C and D), at which time thrombin was washed out. Cells were photographed at 15 minutes (E and F). 60 minutes (G and H), and 120 minutes (I and J) after washout. Note the loss of peripheral vinculin in cells that have lost their dense peripheral band (DPB) (small arrows in C and D). Within 15 minutes after the removal of thrombin, the DPB and vinculin plaques reappeared. At the early time points, the reconstitution of these vinculin areas appeared to be associated with F-actin-rich densities within the DPBs (arrows in E). After 2 hours, the vinculin distribution resembled that of the control. Bar=20 μm.

**Figure 3.** Photomicrographs of cells doubly labeled for F-actin (A, C, E, G, and I) and myosin (B, D, F, H, and J). Cells were incubated with 4 U/ml of thrombin and photographed at 30 minutes (A and B) and 2 hours (C and D) at which time thrombin was washed out. Cells were photographed at 15 minutes (E and F), 60 minutes (G and H), and 120 minutes (I and J) after washout. Two hours of thrombin incubation resulted in the disruption of the myosin, which is normally associated with the microfilament making up the dense peripheral band (DPB) (C and D). Within 15 minutes after washout, myosin reappeared at the cell periphery concomitant with the restitution of the DPB (large arrow in E and F). Note that myosin was not found in the area between the DPB of adjacent cells, even though F-actin was present (smaller arrows in F). After 2 hours, the microfilaments that made up the DPB were individually distinct (bracketed by the double arrows in I and J). Note also that myosin was not associated with the many short, thin microfilament bundles that transversed the area between DPBs (G to J). Note especially arrowhead pointing to intercellular space in J. Bar=20 μm.
binant tumor necrosis factor or immune (gamma) interferon, as well as in hyperoxic injury to endothelial cells. Thrombin modulates endothelial cell shape in the confluent monolayer. In our studies, it is noteworthy that cell loss from the monolayer does not take place. This is unlike reports using similar concentrations of thrombin, which showed major gap formation and cell loss from the monolayer upon incubation with thrombin. This may be species-related, since these investigators used human umbilical vein endothelium. It also appears that cell density is an important consideration since the thrombin-induced shape change is less pronounced as confluency is reached. Garcia et al. have reported evidence of a thrombin-induced increase in albumin permeability across an in vitro endothelial monolayer, which they attributed to a reversible change in endothelial cell shape with formation of intercellular gaps. Thrombin washout experiments revealed that intercellular vinculin plaques reappeared at the same time as the microfilaments of the DPB. No vinculin plaque formation in areas of the cell periphery devoid of DPBs was observed. The first vinculin plaques appeared after the washout of thrombin and were often associated with microfilaments emanating from areas of the DPB that contained F-actin rich densities. What makes these F-actin densities able to initiate cytoskeletal rearrangement is not known; however, it is possible that actin-binding proteins are involved. Myosin is not involved, as seen in our studies. These observations support the conclusion that the peripheral vinculin plaques rely primarily upon an intact DPB. Indeed, such a relationship between vinculin and F-actin has been supported by work from Schliwa et al. and Herman and Pledger which show that vinculin adhesion plaque formation is intimately associated with F-actin bundles. Schliwa et al. reported that the disruption of stress fibers by TPA preceded the loss of vinculin from cell-substratum focal adhesion sites, while in Balb/c-3T3 cells, the opposite was reported. In our study, we did not detect any time difference between the loss or reappearance of the DPB and vinculin in the cell-cell vinculin plaques. Although this work was not designed to study the mechanisms by which the agents act upon the DPB, several points can be made. The TPA effect was not seen when phorbol or the inactive analogue of TPA, esterolytically inactive thrombin did not show any effect on endothelial cell shape or microfilament distribu-

Figure 4. Photomicrographs of confluent endothelial cell monolayers labeled for F-actin with rhodamine phalloidin, which were incubated with: (A) 2 U/ml, (B) 4 U/ml, (C) 8 U/ml, and (D) 16 U/ml of thrombin in Medium 199 for 2 hours. Dense peripheral bands (DPBs) were disrupted at all these concentrations. Disruption occurred most rapidly at the higher concentrations. Central microfilament bundles became very prominent and were aligned with the long axis of the cells. Bar=20 μm.
Figure 5. Photomicrographs of confluent endothelial cell monolayers labeled for F-actin with rhodamine phalloidin, which were incubated with 2 U/ml thrombin (A and B), 0.2 μg/ml 12-0-tetradecanoyl-myristyl-13-acetate (TPA) (C and D), 2.0 U/ml chymotrypsin (E and F), and 2.0 U/ml plasmin (G and H) for 2 hours (A, C, E, and G). Then they were washed out and observed 2 hours later (B, D, F, and H). The effects of all compounds were reversible in that the dense peripheral bands (DPB) reappeared upon removal of the chemical. TPA caused DPB changes that mimicked those of thrombin. In contrast, the nonthrombin proteases tested resulted in cell rounding without any preferential effect on the DPB. Bar=10 μm.
matrix in modulating thrombin and TPA effects on the microfilaments requires future consideration.

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