Patterns of Endothelial Microfilament Distribution in the Rabbit Aorta In Situ

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The available data on F-actin microfilament distribution in vascular endothelial cells in vivo is limited. In this study, the appearance and distribution of endothelial cell microfilaments in the rabbit thoracic aorta, the abdominal aorta and its major arterial branch points, and the aortic bifurcation were examined. Perfusion fixed rabbit aortas were stained in situ for F-actin by infusing rhodamine phalloidin via a peristaltic pump into the aortas at a slow flow rate. This new technique resulted in excellent visualization of branch points and allowed for a precise description of the actin microfilament bundles in endothelial cells along flow dividers. In the thoracic and abdominal aorta, away from branch ostia, actin microfilaments were localized in two regions of the endothelial cells, as a prominent band that completely outlined the cell periphery, and also as short central stress fibers. The central stress fibers were more frequent and prominent in cells of the abdominal aorta. At branch sites and at the aortic bifurcation, long, thick microfilament bundles were present in endothelial cells extending from the tip of the flow divider to a few millimeters along the branch arteries, the aorta, and the iliac arteries. Peripheral actin, however, no longer completely surrounded the cells. The thick bundles were not prominent in endothelial cells located adjacent to the proximal tip of branches or at the iliac arteries opposite the flow divider. This study shows that endothelial cell F-actin microfilament distribution in vivo is well defined along the aortic-arterial system. The prominent central microfilament bundles and the reduced peripheral microfilaments seen at localized regions may reflect an adaptive response to elevated shear stress at these sites. (Circulation Research 1989;64:21-31)

Since the endothelial lining of the vascular system is constantly subjected to shear stresses arising from blood flow, many studies have focused on the effect of fluid shear stresses on endothelial cell structure and function as it relates to the initiation of atherosclerosis. Although it is known that atherosclerotic lesions tend to localize at regions exhibiting large variations in shear stress, namely arterial branch sites, the role of shear stress in the development of atherosclerosis is unknown. Both high and low fluctuating shears can induce changes in endothelium that may be related to atherogenesis.

Shear stress alters the shape and orientation of endothelial cells and affects the repair of an experimentally wounded endothelial cell layer both in vitro and in vivo. Studies characterizing the F-actin microfilaments in vitro and in vivo have suggested that the F-actin microfilaments are important in the repair of the endothelium and in the maintenance of endothelial integrity.

The purpose of our study was to characterize the distribution of microfilaments in vascular endothelial cells at regions in the arterial tree which are subject to different shear stress levels. To carry out our study, we designed a new method to localize F-actin in situ and to observe the endothelial cells without stripping them from the vessel wall. The latter feature is critical because "Hautchen" stripping techniques yield poor preservation of endothelium at branch sites, which are of critical interest in the current study.

Materials and Methods

Preparation of Tissue for Fluorescence Microscopy

Aortic and arterial tissue specimens were obtained from 23 normal adult male New Zealand White rabbits weighing 2.5–3.5 kg. Prior to sacrifice, 1,000 units of heparin sodium (Organon Canada Inc, Toronto, Canada) was infused into the animals via an ear vein catheter. The animals were killed 1
minute later by infusing 1 ml of euthanasia solution, 200 mg/ml N-[2-(m-methoxyphenyl)-2-ethylbutyl-(1)]-γ-hydroxybutyramide, 50 mg/ml 4,4'-methylenebis-(cyclohexyltrimethylammonium iodide), and 5 mg/ml tetracaine hydrochloride (T-61, Hoechst Canada, Inc, Montreal, Canada), into the same ear vein catheter.

A cannula was introduced into the ascending aorta and advanced to the mid-descending thoracic aorta. A small catheter connected to a water column was introduced into the left femoral artery to monitor the perfusion pressure. After a brief flush with 60 ml of phosphate buffered saline (PBS), the aortas were perfusion fixed at a pressure of 100 mm Hg, the approximate systolic pressure in rabbits, for 15 minutes with 3% paraformaldehyde in 0.1 M phosphate buffer, 1 mM MgCl2, 0.1 mM CaCl2 (pH 7.4). A 15-minute PBS wash at 100 mm Hg followed, and then the arterial system was permeabilized by perfusion with Triton X-100 (Sigma Chemical, St. Louis, Missouri). In some instances, permeabilization with Triton X-100 was omitted.

Optimum staining of the endothelium with minimal background staining was obtained when a solution of 0.2% (vol/vol) Triton X-100 was slowly infused with a syringe so that it took approximately 20–30 seconds to deliver 30 ml of the solution. This was followed by a 10-minute PBS wash at 100 mm Hg pressure. The tissue was then stained to localize both F-actin and the nucleus using the fluorescent dyes rhodamine phalloidin (Molecular Probes, Eugene, Oregon) and bisBenzimide (Hoechst No. 33258, Sigma), respectively.

Stock solutions of both reagents were diluted together in one flask with PBS to obtain final concentrations of 24.75 nM rhodamine phalloidin and 5 μg/ml bisBenzimide in a total volume of 100 ml. This solution was infused into the rabbits via a peristaltic pump (LKB, Bromma, Sweden) at a flow rate of 4.3 ml/min. This was followed by a 15-minute PBS wash at 100 mm Hg pressure. Then the aorta from the midthoracic region to the iliac arteries including the major arterial branches was gently excised. Tissue segments 5–6 mm in length were obtained from the following areas: the thoracic aorta; the coeliac, superior mesenteric, right and left renal arteries, and their aortic ostia; the abdominal aorta; the aortic bifurcation; and the common iliac arteries. The tissue segments from the thoracic and abdominal aorta were opened lengthwise along the dorsal side. This allowed for viewing of the ventral endothelial surface, which is free of branches. The tissue segments containing branch points and bifurcations were opened in various ways to allow for optimal viewing of the regions of interest. The tissue segments were mounted in 50% glycerol in PBS, and orientation with respect to the direction of blood flow was noted. The tissue samples were examined with a Zeiss Photomicroscope III equipped with epifluorescence optics. Zeiss filter sets 47714 and 487702 were used for rhodamine phalloidin and Hoechst 33258, respectively.

To show that the stained cells were indeed endothelial cells, samples were examined following balloon denudation of the abdominal aorta by the method of Baumgartner and Studer.16

Preparation of Tissue for Transmission Electron Microscopy

An additional four rabbits were killed and prepared for fixation in the same manner as in the fluorescence studies. The aortas were perfusion fixed at 100 mm Hg for 15 minutes with 2.5% glutaraldehyde-2% paraformaldehyde in 0.1 M Sorenson's phosphate buffer, 1 mM MgCl2, 0.1 M CaCl2 (pH 7.4). The thoracic aorta, abdominal aorta, aortic bifurcation, and iliac arteries were excised, trimmed of adventitia, opened by the same procedure as in the fluorescent studies, pinned down on dental wax, and flooded with 2.5% glutaraldehyde in 0.1 M phosphate buffer to reduce the amount of curling. Tissue segments 1×2 mm were then further fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 3 hours at 4°C, rinsed in 0.1 M phosphate buffer overnight, postfixed in 1% OsO4 in 0.1 M phosphate buffer for 2 hours at room temperature, rinsed twice with acetate buffer for 15 minutes each, and stained with 1% uranyl acetate overnight at 4°C. The blocks were washed with acetate twice for 15 minutes each, dehydrated with a series of graded concentrations of acetone, and then embedded in Epon Araldite. Thin sections were stained with uranyl acetate and lead citrate and examined on a Philips EM 400.

Morphometry

Photomicrographs of rhodamine phalloidin stained endothelial cells from the thoracic and abdominal aorta were analyzed from five rabbits quantitatively using Apple computer software and a graphics tablet. Three endothelial cell parameters were measured: area, length, and width. Length was defined as the distance of the longest cell axis. Width was defined as the maximal width perpendicular to the long axis of the cell. For each rabbit, one field on the ventral surface of both the midthoracic and midabdominal aorta was photographed. Ten cells were randomly selected in each field with a point hit method, for morphometric analysis. Thus, 10 thoracic aortic cells and 10 abdominal aortic cells were analyzed in each of the five rabbits. Differences in cell dimensions were assessed by paired t test. The level of statistical significance was p < 0.05.

Results

Thoracic and Abdominal Aortae

In both the thoracic and abdominal aortae, away from any arterial branch sites, the endothelial cells were aligned parallel to the long axis of the vessel. The endothelial cells of the abdominal aorta were
Aortic Bifurcation

The bifurcation of the abdominal aorta showed several characteristics. Immediately downstream from the flow divider there were long, thick stress fibers that were frequently wavy in appearance (Figures 2A, 2C, and 3A). Many of these were shown to be in the central portion of the cell in tissue double stained to show microfilaments and nuclei (Figure 4). Rhodamine phalloidin staining did not completely delineate any of the cell boundaries in this region. It is possible, however, that some of the long, thick microfilament bundles in this region are located at the cell periphery and represent partial staining of the boundaries (Figures 2A, 2C, and 3A). The stress fibers occasionally showed a nonuniform periodic staining pattern for F-actin. Furthermore, downstream from the flow divider along the adjacent wall of the iliac arteries, the stress fibers decreased in thickness and length, and the F-actin microfilaments delineating cell boundaries became discernible with increasing distance along these walls (Figure 3). Cells with circumferential F-actin microfilaments appeared only at distances of more than 3.5 mm from the flow divider (Figures 3B and 3C). The opposite lateral wall showed F-actin distribution similar to that observed in the thoracic and abdominal aortas (Figure 2B).

Branch Sites

At the arterial branch sites, the region of the flow divider showed F-actin microfilament patterns similar to those found around the flow divider of the bifurcation. In the coeliac and superior mesenteric arteries, cells with circumferential F-actin microfilaments and less prominent stress fibers first appeared at about 3 mm from the flow divider. In the renal arteries, cells with circumferential microfilaments and decreased stress fibers were present only at distances beyond 6–8 mm from the flow divider. Along the aortic side of the branch sites, a return to circumferential microfilaments occurred at less than 2 mm from the flow divider for the coeliac and superior mesenteric branch points and at about 3 mm for renal branch sites. On the lip of the flow divider, rounded cells exhibited a prominent peripheral band of microfilaments outlining the cells. Adjacent cells showed prominent stress fibers (Figure 5).

Endothelial cells on the walls opposite the flow divider showed F-actin distribution similar to that observed in the thoracic and abdominal aortas, that is, they had a circumferential bundle of F-actin microfilaments at cell boundaries and often had short, thin stress fibers.

In all experiments, fluorescent staining was poor without prior permeabilization of the aorta. In control experiments, in which the animals had the endothelium from the abdominal aorta removed by balloon catheter, the staining patterns observed in the normal nondenuded aortas was not seen. Instead, rhodamine phalloidin stained smooth muscle cells oriented orthogonally to the direction of blood flow were seen (not shown).

Transmission Electron Microscopy

Transmission electron microscopy of endothelial cells in the region next to the aortic flow divider showed very large stress fibers in the abluminal portion of the cells (Figure 6). The stress fibers were focally associated with the basal plasma membrane (Figure 6A). Occasionally, stress fibers were associated with both the basal and lateral plasma membranes. On the opposite wall, the endothelial cells did not display these large stress fibers. Similarly, these large stress fibers were not seen in the thoracic and abdominal aorta away from branches, which is consistent with our fluorescent microscopy findings.

Discussion

A new method, developed to localize endothelial cell F-actin in situ with rhodamine phalloidin without removal of the endothelium, has allowed us to carry out a detailed study of the F-actin distribution at the aortic bifurcation and major aortic branch sites. Our data demonstrates that in response to

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**Table 1.** Length, Width, and Area of Endothelial Cells in the Thoracic and Abdominal Aorta

<table>
<thead>
<tr>
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<th>Length (μm)</th>
<th>Width (μm)</th>
<th>Area (μm²)</th>
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<tbody>
<tr>
<td>Thoracic aorta</td>
<td>48.5±1.6</td>
<td>8.52±0.76</td>
<td>262±21</td>
</tr>
<tr>
<td>Abdominal aorta</td>
<td>55.6±0.6*</td>
<td>6.82±0.27</td>
<td>249±9</td>
</tr>
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Lengths, widths, and areas of 10 cells from the thoracic aorta of each of five animals were averaged. Data presented are the means of these five values plus the between-animal SEM. Identical analyses were performed with abdominal aortic endothelial cells. *Significantly different from thoracic aortic value (paired t test, p<0.05, n=5 rabbits/group).

significantly longer than those of the thoracic aorta, but there was no difference in the individual endothelial cell areas between the two regions (Table 1). In both locations a prominent bundle of microfilaments was present at the periphery of the cell. These microfilaments were continuous around the entire cell (Figures 1A and 1B). In addition, the endothelial cells in the thoracic aorta displayed fewer stress fibers than those of the abdominal aorta. The latter showed many short stress fibers (Figure 1B) that were only approximately aligned with the long axis of the cells. Deviations of up to a 30° angle were frequently observed. The stress fibers were not preferentially localized to certain intracellular sites; rather, they were distributed throughout the cells.
local environmental factors, alterations in both central and peripheral F-actin occur. These organizational patterns are far more profound than previously reported.

Various methods have been employed to visualize the microfilaments in vascular endothelial cells. Hautchen procedures require prior removal of the endothelium before fluorescent staining.\textsuperscript{14,17} Whole-
FIGURE 2. Rhodamine phalloidin staining of endothelial cells at the region of the aortic bifurcation. A: The region of the flow divider displays very long, thick stress fibers. B: The lateral wall at the entry to the common iliac artery is characterized by endothelial cells with a prominent circumferential band of F-actin microfilaments and short, thin stress fibers. C: Very striking stress fibers, similar to those at the flow divider, are present in the medial wall of the common iliac artery very close to the flow divider. Line drawing indicates location of A, B, and C. The direction of blood flow is indicated by an arrow on the line drawing. Bar, 10 μm.
Rhodamine phalloidin staining of endothelial cells along the medial wall of the common iliac artery showing a decrease in stress fibers with increasing distance from the flow divider. A: 1.8 mm; B: 3.6 mm; C: 5.8 mm. Line drawing indicates location of A, B, and C, and direction of blood flow. Bar, 10 μm.
FIGURE 4. Double staining of endothelial cells for (A) F-actin with rhodamine phalloidin and (B) nucleus with Hoechst 33258 at aortic bifurcation. Rhodamine phalloidin staining shows very striking microfilament bundles. The nucleus superimposed on rhodamine phalloidin staining shows that many of the microfilament bundles are in the periluminal region; that is, they occupy a central location in the endothelial cells (arrow). Direction of blood flow is from top to bottom. Bar, 10 μm.
mount preparations, which leave the endothelium intact but in which fluorescent staining is implemented after the vessel is excised and cut into segments, have also been used. Our method of infusing the fluorescent stain into the intact, unopened aorta and then preparing whole-mount specimens offers several advantages. First, staining the aorta before cutting tissue for whole mounts offers a reduced background because the stain infiltrates into the aorta only from the luminal side. Second, all of the permeabilization, staining, and rinsing is done in the unopened vessel; thus, excised segments can be mounted immediately without having to be handled excessively. Third, we avoid artifacts that may be created by removal of the endothelium from the vessel wall.

The most striking observations of the current study are the profound changes in microfilament distribution near flow dividers of aortic ostia. A complete or partial loss of peripheral F-actin microfilaments was coupled with the formation of very long and thick stress fibers in cells on both the branch and aortic side of the flow dividers.

The altered distribution of F-actin near flow dividers is probably a response to local blood flow conditions. High velocity flows impact on the dividers and generate much elevated shears on adjacent, downstream vessel walls. Redistribution of F-actin from the cell periphery to central stress fibers may enhance cell substrate adhesion under these high shears. The close association of stress fibers with the abluminal surface of the cells, seen by transmission electron microscopy, is certainly consistent with a role in substrate adhesion. Enhanced substrate adhesion may, however, be at the expense of controls afforded by peripheral F-actin, since peripheral F-actin has been implicated in the maintenance of cell-cell adhesion, in the stabilization of the endothelial monolayer, and, more recently, in the maintenance and regulation of the endothelial permeability barrier. Frequently the stress fibers exhibited a wavy, or perhaps, corkscrew appearance. This may be a

FIGURE 5. Rhodamine phalloidin staining of endothelial cells on the lip of the flow divider of the superior mesenteric artery. Note the rounded cells on the flow divider exhibiting a continuous peripheral band of microfilaments (arrow). Note stress fibers of adjacent cells just distal to the lip of the flow divider (arrowhead). Flow into the superior mesenteric artery is from top to bottom. Bar, 10 μm.
FIGURE 6. Transmission electron micrographs of endothelial cells at the medial wall of the common iliac artery very close to the aortic bifurcation. Stress fibers are present in the abluminal portion of the cell (arrows). Note that stress fiber is associated with cell membrane cytoplasmic density (arrowhead). A: Transverse section; B: longitudinal section. L, lumen. Bar, 0.1 μm.

Passive structural characteristic of very large F-actin microfilament bundles, or it may be a manifestation of the tension generating mechanism of contractile proteins. Since this wavy appearance was present in only some of the bundles, it is unlikely that it reflects an artifact of tissue shrinkage, although this cannot be ruled out.

Other investigators have noted spatial variations in endothelial microfilament distribution, but these are usually limited to changes in the number of stress fibers that are observed or the proportion of cells containing stress fibers. The findings that most resemble our results near branch ostia are those of Gabbiani et al., who described marked stress fiber expression immediately adjacent to flow dividers of rabbit intercostal ostia. Details of the actin distribution were not described, but the regions exhibiting stress fiber formation were highly localized, and their appearance was attributed to local shear. White and Fujiwara used in situ labeling of endo-
thelial myosin in rats and observed more stress fibers near branch sites, but these were seen both upstream and downstream of ostia. No loss of peripheral staining was observed. Wong et al. also reported increased stress fibers in regions where high hemodynamic forces are exerted. Finally, Herman et al. have used a sophisticated perfusion system to impose pulsatile hemodynamics on dog carotid arteries in vitro. They saw some increase in stress fiber formation at high flows. This and other aspects of their study are difficult to interpret, however, because of apparent high rates of cell loss from the luminal surface of the vessels. Cell densities were frequently decreased by 50–75% following challenges with various hemodynamic conditions. Thus, processes related to endothelial repair may have strongly influenced actin distribution.

Failure of these previous studies to report both the disruption of peripheral F-actin and the very large stress fibers we observed could reflect differences in methodology. However, this seems unlikely given that similar results were obtained at sites distant from branches. Species differences are also possible, or it may be that very careful examination of the flow divider region is necessary near small branches. We have shown that microfilament distribution characteristic of these regions is very localized, and therefore, may easily be missed, especially when Hauthen techniques are used to strip endothelium. This localization probably means that the most extreme hemodynamic conditions at ostia are involved.

We observed that stress fibers distributed through the central regions of the cell run parallel to each other. This finding confirms previous observations for quiescent endothelium exposed to flowing blood or medium. There was no preferential distribution of these stress fibers in the proximal half of the endothelial cells as was reported by White et al. Frequently, the alignment of these fibers was very well defined, but it did not always match cell orientation. Instead, the angle between stress fibers and major cell axis was up to 30°. Endothelial cells are oriented in the direction of time-averaged blood flow and stress fibers also align with flow in vitro. However, actin interacts with extracellular proteins, for example, fibronectin; thus, the organization of endothelial cell substratum may influence stress fiber orientation. Endothelial cells can also be aligned by the substratum, but this appears to happen only in the absence of flow.

We observed some graded variations in the presence of central stress fibers. First, we noticed that cells immediately over the flow divider contained stress fibers, but these were not as prominent as in adjacent cells. Furthermore, the cells were less elongated and peripheral actin was retained. At this site, flow velocities are normal to the surface and a small “stagnation zone” with relatively lower shear may be created. Flow deflected by the divider then travels parallel to the vessel surface and generates high shear in adjacent areas. We also noted that fewer central stress fibers and rounder cells characterized the thoracic versus the abdominal aorta. Cornhill et al. argued that rounder cells in the thoracic region may reflect lower, or less consistently aligned, shear stress. Our findings of fewer stress fibers in this region is consistent with the presumption of lower shear; however, direct assessments of shear have not yet been made.

The findings of this study suggest that high shears induce stress fiber formation and that the mechanism may involve a redistribution of F-actin from the cell periphery, where it may regulate cell-to-cell interactions and permeability, to the central cell regions, where stress fibers may enhance substratum adhesion. Other endothelial cell responses can elicit similar microfilament reorganization. Acute redistribution of F-actin from the cell periphery to stress fibers occurs in endothelial cells adjacent to zones of denudation during monolayer repair. A major difference between repair responses and adaptations to high shear is that the latter is permanent. Thus, it is surprising that cells do not appear to ultimately synthesize enough actin to replace lost contractile protein at the cell periphery. One possible explanation is that actin turnover proceeds at maximal rates under high shear. Central stress fiber formation might then take precedence over peripheral actin expression. Functions of peripheral actin may be sacrificed chronically or other cell junctional adaptations may compensate.

In any event, confirmation that shear stress causes actin redistribution awaits in vivo demonstration that experimentally elevated shears can elicit such actin redistribution in cells normally exposed to moderate shear levels.

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References


**KEY WORDS** • shear stress • microfilaments • endothelium
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