Lateral Zone of Cell-Cell Adhesion as the Major Fluid Shear Stress–Related Signal Transduction Site

Yumiko Kano, Kazuo Katoh, Keigi Fujiwara

Abstract—It has been proposed previously that actin filaments and cell adhesion sites are involved in mechanosignal transduction. In this study, we present certain morphological evidence that supports this hypothesis. The 3D disposition of actin filaments and phosphotyrosine-containing proteins in endothelial cells in situ was analyzed by using confocal microscopy and image reconstruction techniques. Surgical coarctations were made in guinea pig aortas, and the same 3D studies were conducted on such areas 1 week later. Stress fibers (SFs) were present at both basal and apical regions of endothelial cells regardless of coarctation, and several phosphotyrosine-containing proteins were associated with SF ends. Apical SFs had one end attached to the apical cell membrane and the other attached to either the basal membrane or the lateral cell border. Within the coarctation area, the actin filament–containing and vinculin-containing structures became prominent, especially at the apical and the lateral regions. Substantially higher levels of anti-phosphotyrosine and anti-Src staining were detected in the constricted area, particularly at the cell-cell apposition, whereas the anti–focal adhesion kinase, anti–CT10-related kinase, anti–platelet endothelial cell adhesion molecule-l, anti-vinculin, and phalloidin staining intensities increased only slightly after coarctation. We propose that apical SFs directly transmit the mechanical force of flow from the cell apex to the lateral and/or basal SF anchoring sites and that the SF ends associated with signaling molecules are sites of signal transduction. Our results support the idea that the cell apposition area is the major fluid shear stress–dependent mechanosignal transduction site in endothelial cells. (Circ Res. 2000;86:425-433.)

Key Words: endothelium ■ mechanotransduction ■ stress fibers ■ phosphotyrosine ■ coarctation

Numerous studies have indicated that endothelial cells (ECs) have some mechanism for responding to fluid flow.1 When cultured ECs are exposed to the flow of culture medium for a short period of time, a number of so-called early flow responses occur, including NO release,2 G-protein activation,2–4 Ca2+ mobilization,5–7 protein kinase C activation,8–11 increased levels of tyrosine phosphorylation,1,12 platelet endothelial cell adhesion molecule-l (PECAM-l) tyrosine phosphorylation,13,14 mitogen-activated protein kinase activation,11,12 and directed cell migration.15,16 These events are followed by later responses that become detectable typically after several hours of flow application, including changes in various gene expressions,1 changes in cell shape,17–22 and cytoskeletal reorganization.1,23–27 It is not certain whether and how any of the early types of responses relate to any of the later events. It is also uncertain whether these flow-induced responses are expressed by in situ ECs that are constantly exposed to blood flow. Although these observations demonstrate the ability of ECs to respond to fluid flow, the mechanism for flow sensing and signal transduction remains largely unknown. Fluid shear stress is the frictional force that acts on the apical cell surface. If the cell surface is easily deformable, the force will mostly be dissipated by deformation and may not be able to elicit flow responses in cells. Thus, shear stress–dependent signal transduction is expected to begin at sites where cell surface deformation is less likely to occur. One possible place is the apical stress fiber (SF)–plasma membrane attachment site (the apical plaque),28 although there is no evidence that the apical plaque is a site for mechanosignal transduction. There are other rigid structures associated with the plasma membrane, such as the cell-substrate adhesion site (focal adhesion) and the cell-cell adhesion site. Bundled actin filaments are associated with all of these sites, and via the actin bundle network, the force of shear stress acting on the apical plaque may be transmitted to the other fixed parts of the cell, where activation of specific signaling molecules may occur. This scheme suggests the possibility that some flow-dependent signaling events are initiated at the focal adhesion site, which is not directly exposed to flow. Indeed, in cultured cells, flow-dependent and cell adhesion–dependent mitogen-activated protein kinase activation has been reported,11,12 and there are apical SFs that appear to span the distance between the apical and the basal cell surfaces.28 This scheme also predicts signal transduction at the cell-cell attachment site. This laboratory has shown that PECAM-1, a cell-cell adhesion molecule, is tyrosine-phosphorylated when ECs are exposed to flow.13,14 This PECAM-1 phosphorylation could...
be triggered by the apical SF system that links the apical plaque and the cell adhesion site. Indeed, we have shown that PECAM-1 tyrosine phosphorylation does not occur in ECs treated with cytochalasin D. Whether apical SFs terminate on the cell-cell adhesion site requires investigation.

This signal transduction involving actin filaments predicts a certain SF organization in the cell. More specifically, it predicts the presence of strategically placed SF–plasma membrane attachment sites on the apical, lateral, and basal parts of ECs and the association of signaling molecules with the lateral and basal sites. Previously, we have provided evidence for the presence of apical SFs and plaques in ECs in situ. In the present study, by using guinea pig aortas and confocal microscopy together with image reconstruction techniques, we show the 3D organization of actin filament–containing structures in ECs in situ. In the present study, by using guinea pig aortas and confocal microscopy together with image reconstruction techniques, we show the 3D organization of actin filament–containing structures in ECs in situ.

Figure 1. Stereo pairs of in situ ECs in a control guinea pig aorta doubly stained by phalloidin (A) and anti-phosphotyrosine (PY-20) (B). A, 3D distribution of actin filaments. Thick SFs are observed at the base, and thin SFs are observed at the apical portion. Some apical SFs run into the actin circumferential ring (arrowheads), and some attach to the basal part of cells (arrows). B, PY-20 staining associated with the plasma membrane, especially the lateral portion. Anti-phosphotyrosine staining without corresponding actin filament staining was rarely observed.

Figure 2. Stereo pairs of in situ ECs in the coarctation zone doubly stained with phalloidin (A and C) and anti-vinculin (B). A and C, Compared with Figure 1A, the actin cytoskeleton is more developed. Arrows indicate apical SFs connecting to the basal portions of ECs. At high magnification (C), an apical plaque is shown by an arrowhead. B, Vinculin localization is shown. Bright and large staining spots are observed at both the cell apex and base. Bar=20 μm.

The flow rate (as well as fluid shear stress) in the vessel is increased by making a region of coarctation, SF expression is increased, especially the apical type. Also increased in the coarctation area is the expression of Src and tyrosine-phosphorylated proteins, especially in the cell-cell overlap area. These results are consistent with the idea that the lateral cell apposition zone is a major fluid flow–related mechanosignal transduction site.

Materials and Methods

En Face Aortic Sample Preparation

Guinea pig abdominal aortas were used. A small silicon tube was placed over some of the aortas for a week to analyze the effect of increased fluid shear stress on various morphological parameters. Aortas were fixed, longitudinally cut, and spread flat to expose the endothelium.
Microscopy and 3D Image Reconstruction

En face aortic samples were stained with various antibodies and observed under a laser scanning microscope. To make 3D images, serial optical sections were made and, with the use of computer software, reconstructed into stereo images. Each 3D image is presented as a pair of sized-matched appropriately mounted micrographs that should be viewed through a special stereo viewer.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Actin Filament Organization

Whole-mount preparations of guinea pig abdominal aortas were stained with fluorescein-labeled phalloidin, and 3D images were reconstructed from confocal serial optical sections (Figure 1A). Each EC was delineated by a ring of strong staining near the lumenal surface, presumably representing actin filaments of the adherens junction. However, there were also actin rings near the base of the cell. Whether adherens junctions are present near the base of ECs requires investigation. SFs were generally aligned in the direction of blood flow. Thick SFs were present at the base, and some of them were quite short. Thin SFs were found in the apical portion of the cell. These apical SFs tended to span the whole length of the cell and appeared to run into the actin ring of the apical location (Figure 1A, arrowheads). Some SFs ran vertically, connecting the apical and the basal surfaces (Figure 1A, arrows).

Weakly fluorescent structures were lost during 3D image processing of confocal optical serial sections. Thus, structures consisting of a small number of actin filaments are not represented in the reconstructed images. It is known that in situ SF expression is high in the region of increased fluid shear stress, and indeed, in the guinea pig aorta, the actin cytoskeletal architecture was more exaggerated near bifurcations (data not shown). We had attempted to take advantage of this exaggerated actin filament organization for our analyses, but it was difficult to obtain flat whole-mount preparations of branching areas. Thus, to create an area with increased fluid shear stress that could be easily made into a whole-mount preparation, we made a coarctation in the straight region of the abdominal aorta. A stereo pair of the constricted area showed ECs with increased staining with rhodamine-labeled phalloidin. Apical SFs terminating on the circumferential actin ring were clearly demonstrated. Also clearly seen were the SFs that ran between the apical and the basal surfaces (Figure 2A, arrows). Stereo pairs showed that the actin cytoskeleton was membrane-associated, forming a cagelike structure underneath the plasma membrane and surrounding the entire body of cytoplasm. Increased shear stress upregulated the expression of apical SFs. Figure 2C, a more magnified stereo pair, shows the centrally located...
apical plaque (arrowhead) \(^{28}\) and SFs running between the apical plaque and the base of the cell (arrows). These studies demonstrate that apical SFs are connected to either the cell-cell adhesion site or the basal plasma membrane.

**Vinculin**

Anti-vinculin staining associated with both basal and apical plaques of in situ ECs has been shown.\(^{28,29}\) Stereo pairs of normal aortic ECs showed basal anti-vinculin spots but not the apical spots, presumably because of their low level of labeling (Figure 3). The extent of cell delineation by anti-vinculin was minimal. On the other hand, the level of anti-vinculin labeling in the coarctation area was considerably higher, and each staining spot in Figure 2B was significantly larger than the spots shown in Figure 3. The lateral and the apical vinculin spots also became noticeably prominent, indicating that heightened shear stress increased the size of vinculin-containing structures.

**FAK and Other Focal Adhesion-Associated Proteins**

Focal adhesion kinase (FAK) association with focal adhesions but not with apical plaques was reported in cultured cells.\(^{28}\) In ECs in vivo, this kinase was localized to discrete spots at the base of cells both in control (Figure 4A) and constricted (Figure 4B) vessels. They are presumably focal adhesions that are arranged linearly along the direction of blood flow. Under increased shear stress, the number and size of anti-FAK spots increased, suggesting that ECs reinforced their adhesion to the basement membrane by making more and larger focal adhesions.

CT10-related kinase (Crk), an adapter protein for both paxillin and pp130, also binds to FAK. Anti-Crk stained the basal portion of ECs, most notably focal adhesions (Figure 4C). Paxillin and pp130 were also immunolocalized to focal adhesions (data not shown). Our data indicate colocalization of FAK, Crk, paxillin, and pp130 in the focal adhesion of ECs in situ.

**Phosphotyrosine**

Because protein tyrosine phosphorylation occurs during intracellular signaling, we investigated phosphotyrosine levels in ECs in the constricted region (Figure 5A). There was a clear demarcation in anti-phosphotyrosine staining at the edge of coarctation. Although immunostaining in the low fluid shear stress area was low (Figure 5A, right), the area of increased shear stress exhibited a high level of staining (Figure 5A, left), which was closely associated with the lateral plasma membrane (Figure 5B). The similar sharp staining demarcation was observed at the proximal edge of coarctations. The lateral staining did not always form a sharp line, which might be expected if the adherens junction were labeled (Figure 5B). Instead, sheetlike staining patterns were observed, suggesting that the lateral cell membrane overlap area was labeled. A side view of ECs clearly showed staining along the entire cell-cell apposition (Figure 5C, arrows). Dotty staining was associated with the base of the cell and the apical cell membrane (Figure 5C, arrowheads).

A stereo pair of anti-phosphotyrosine–stained endothelium showed immunoreactivity being spread throughout the lateral plasma membrane overlap (Figure 1B). The specimen was also stained for actin filaments and showed colocalization of phosphotyrosine with actin filaments (Figure 1A). Although spotty staining corresponding to focal adhesions and apical...

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**Figure 5.** Phosphotyrosine localization in the coarctation zone. A, Low-magnification micrograph showing the downstream edge of a coarctation (arrows). In the coarctation area (left), PY-20 staining is significantly increased. Coarctations formed folds in the vessel wall in the unconstricted area, and the top of folds showed increased labeling. B, Confocal image of the coarctation zone optically sectioned at the middle level of ECs. Staining is at the cell-cell junction. C, Side view of the endothelium. A confocal image obtained from a folded area shows strong staining at the cell-cell border (arrows) and weak linear staining along the apical plasma membrane (arrowheads). Bar=20 μm.
plaques was present, SFs were not labeled. These results suggest that the cell-cell border is an active site of signaling events and that increased shear stress upregulates signaling activities.

**Src**

Src family proteins are tyrosine-phosphorylated at the time of signal transduction and are tyrosine kinases. Localization of Src was investigated by using 2 antibodies with different specificity: SRC-2 (recognizing the Src family kinases) and N-16 (recognizing only c-Src). Cryosections of the aortic wall stained with SRC-2 showed labeling of the cell border and some basal spots, presumably focal adhesions (Figure 6A). The cell apex was also labeled in a dotted manner (Figure 6A). Cryosections stained with N-16 exhibited similar staining patterns, but anti-c-Src images were better defined than SRC-2 images (Figure 6B). Like anti-phosphotyrosine staining, the anti-Src signal was spread along the entire length of cell-cell apposition. When these 2 antibodies were treated with appropriate synthetic Src peptides and then used to stain specimens, no labeling was observed (data not shown).

A confocal optical section at the mid level of ECs showed dotty staining by SRC-2 at the cell-cell border (Figure 6C). N-16 gave a similar but better defined staining pattern (Figure 6D). An en face preparation stained doubly with anti-vinculin and SRC-2 was optically sectioned at the base of cells, where anti-vinculin stained focal adhesions. Figure 6E is a merged image of anti-vinculin (red) and anti-Src (green) signals and shows colocalization (yellow) of the 2 antigens at focal adhesions. A double-labeled confocal side view is similarly illustrated in Figure 6F, demonstrating localization of Src kinases at focal adhesions. This micrograph also demonstrates the localization of Src kinases at apical plaques (arrows).

Src expression was investigated in the coarctation zone. A low-magnification photograph showed increased anti-Src staining in the area of coarctation (Figure 7A). The border between low and high levels of Src expression was sharp and corresponded well with the edge of coarctation (Figure 7B). Increased staining occurred at all localization sites, including the general cytoplasm, but staining was particularly strong at the cell border (Figure 7C). The localization of Src kinases was remarkably similar to the distribution of phosphotyrosine-containing proteins.

**Csk**

C-terminal Src kinase (Csk) is a nonreceptor-type tyrosine kinase that phosphorylates a tyrosine residue in the C-terminal region of Src family kinases and inactivates them.
Strong anti-Csk staining was present at the cell-cell apposition (Figure 8). This staining was broad, inasmuch as it was seen with anti-Src kinases and anti-phosphotyrosine.

Discussion
The response of ECs to fluid flow has mostly been studied by using in vitro systems. Although this approach has provided information regarding the basic property of this cellular response, it is commonly believed that in vitro data may not totally reflect the situation in vivo. Therefore, it is of paramount importance that ECs in situ be closely studied. Understandably, however, performing experiments on cells in vivo is difficult, and available data on in situ ECs are severely limited. In the present study, using guinea pig abdominal aortic ECs in situ, we obtained certain morphological information pertinent to mechanosignal transduction by these cells. Although these immunolocalization studies do not provide definitive solutions to the mechanism of flow-induced signaling by ECs, we suggest that they provide insights into the way these cells respond to flow.

Actin Bundle Organization and Signal Transduction
A role of the cytoskeleton in mechanosignal transduction by ECs has been proposed. We have suggested that the actin cytoskeleton might transmit mechanical forces directly from one part of the cell to another. Such a scheme predicts a certain pattern of actin bundle disposition. In the present study, we have demonstrated the presence of SFs that run in the specific ways that have been predicted by our mechanosignal transduction hypothesis.

Of particular interest are the SFs located in the apical part of ECs. In cultured fibroblasts, one of the ends of the apical SF is attached to the apical plasma membrane via the apical plaque, and the other end appears to terminate at the basal plasma membrane. Likewise, one end of the apical SF in ECs in situ is attached to the apical plaque, whose molecular makeup is similar to that of the apical plaque of cultured cells. However, it was not clear where the other end of the apical SF was located. In the present study, we demonstrated that this end was attached to either the basal focal adhesion or the cell-cell apposition site. The apical SF attachment to the focal adhesion site is presumably mediated by the integrin system. The lateral termination sites were clearly above the base of the cell, indicating that they were not the cell-substrate attachment sites. This brings up a new set of questions regarding the structure and the molecular makeup of this lateral SF–plasma membrane binding site, which may
be different from the binding sites involved in the integrin-based anchoring of SFs to the membrane. Integrin is not concentrated at the cell-cell adhesion site. Cadherin and PECAM-I are the major transmembrane proteins at the lateral cell adhesion site, and both proteins have a β-catenin binding activity. Actin filament bundles running into the lateral portion of cultured cells has been reported, and β-catenin is localized to such sites. There is a circumferential ring of actin filaments associated with the lateral plasma membrane of ECs forming a monolayer. In many instances, apical SFs appeared to run into this structure, at the level of resolution of a light microscope. Whether these 2 actin filament bundles are physically linked is an intriguing question, which we are now investigating.

As we have outlined in the introductory section of the present study, fluid shear stress acts on the rigid part of cell. The apical plaque is such a part because it is anchored by the SF system. Thus, it is a candidate site for shear stress sensing and initial signal transduction. However, it is also possible that through the SF system, the force is transmitted to focal adhesions and cell-cell apposition sites, where appropriate signaling pathways can be activated. Thus, any individual region or combinations of these 3 regions are good candidates for fluid shear stress sensing and signal initiation sites. The present study has provided a structural basis for this hypothesis.

Localization of Signaling Molecules
Tyrosine residues of many proteins involved in signal transduction are phosphorylated at the time of their activation or inactivation. Thus, the level of tyrosine phosphorylation at a given area in a cell can be a reflection of local signal transduction activities. Anti-phosphotyrosine staining was detected at apical plaques, regions of cell-cell apposition, and focal adhesions. The focal adhesion has been identified as a site of signal transduction, and several tyrosine-phosphorylated polypeptides, such as FAK, vinculin, paxillin, Crk, pp130Cas, and Csk, have been localized. These proteins may be responsible for anti-phosphotyrosine staining of focal adhesions and apical plaques, but other proteins, perhaps specific for shear stress signal transduction, may also contribute to this staining.

Anti-phosphotyrosine staining was much stronger at the cell-cell contact site than at the other 2 locations, suggesting more active signaling events at the lateral cell overlap. Because increased shear stress significantly increased this staining, the region of cell-cell apposition may be the most active signal transduction site of shear stress. We have reported previously that when a confluent monolayer of cultured ECs is exposed to >5 dyne/cm² of shear stress or osmotic changes, PECAM-I is tyrosine-phosphorylated within 1 minute. PECA-I is an EC adhesion molecule localized at the membrane overlap region between neighboring cells. Thus, a portion of the phosphotyrosine localized at the cell-cell border may be in PECAM-I. Unlike vascular endothelial (VE)-cadherin localization, which appears as a line at the cell-cell overlap, anti-PECAM-I staining was seen as a belt. As shown in the present study, anti-phosphotyrosine staining was broad along the cell-cell border.

Src family kinases and Csk were also localized to the entire cell-cell overlap. Thus, PECAM-1, Src, and Csk, all of which are phosphotyrosine-containing molecules, are localized in a near-identical pattern to the cell-cell border. These results suggest that all or some of these molecules work together for mechanosignal transduction at the cell-cell border. Indeed, our in vitro study shows that e-Src is an effective PECAM-1 kinase. Immuno-localization also suggests that the activity of Src kinases is tightly controlled at the cell-cell border by Csk.

Effect of Coarctation
A coarctation was made in the straight region of the abdominal aorta to create a region of increased fluid shear stress that can be easily made into an en face specimen for microscopy. The same approach has been taken by other investigators. Precise fluid mechanical analyses on the blood flow pattern and assessment of the magnitude of the increase of fluid shear stress within the constricted region were not performed. Because we have used a straight region of the blood vessels, it is expected that the flow pattern within the coarctation is generally laminar (as is assumed for a nonbranching straight blood vessel) and that the level of fluid shear stress in the constricted zone is significantly heightened. The flow pattern in the region immediately distal to the coarctation is expected to be nonlaminar, and accordingly, the fluid shear stress level there is expected to be lower than that of the normal flow area. Although these local differences in fluid shear stress levels are likely, ECs are expected to be under higher shear stress in the constricted region than in the areas immediately proximal and distal to a coarctation. Thus, we expect that the expression of any shear stress-dependent events is heightened in the area of coarctation. The basic localization patterns of various proteins were the same between regions with and without a coarctation. However, the actin cytoskeleton and the structures labeled with anti-vinculin, anti-paxillin, and anti-FAK were enlarged in ECs in the coarctation area. Inasmuch as most of these latter structures are focal adhesions, our results suggest that cells under increased shear stress reenforce their adhesion to the substrate by making larger and possibly more numerous adhesion sites.

All these changes, however, were detectable only at a high magnification. When en face specimens stained with anti-FAK, anti-vinculin, anti-paxillin, or fluorescent phalloidin were observed at low magnifications, there was no fluorescence boundary between the low and the high shear stress areas. This indicates that the extent of increased expression of these proteins in the coarctation area is not incredibly high. However, in the specimens stained with anti-phosphotyrosine or anti-Src, a clear demarcation was detected between the low and the high shear stress regions, indicating that Src expression is substantially upregulated by shear stress and that the amount of proteins with phosphorylated tyrosines is also highly concentrated in the high shear stress area. Observations at high magnifications revealed that increased staining was mostly associated with the lateral cell overlap region. It is not clear at this time what types of signaling pathways are activated at the cell border, but they are presumably fluid shear stress–dependent signaling events perhaps involving PECAM-1, Src, and Csk. These data suggest the cell-cell
apposition to be a important site for mechanosignal transduction in ECs.

Many types of EC flow responses, especially the early ones, are transient. The present study indicates that although the ECs in the coarctation zone have been exposed to increased shear stress for 1 week, certain types of signaling events are still occurring. Consistent with this interpretation is our observation that both anti-Src and anti-phosphotyrosine heavily stain ECs at arterial branching, where the average fluid shear stress is always higher than the straight region of the same vessel. Some of the long-lasting responses are related to cell morphology, including cell-shape changes and SF alignment, and these responses are known to be reversible. To maintain the expression of a stimulus-dependent, reversible response, cells may need to continuously monitor the level of the stimulus and to generate a positive signal for the response. Thus, we suggest that the increased expression of SFs and focal adhesions and the elongated cell shape are maintained in ECs in the region of high shear stress because the fluid shear stress–dependent signal transduction machinery of these cells is continuously active and keeps generating positive signals. This may be the reason for the increased expression of phosphotyrosine in the zone of high shear stress. We propose that the expression and organization of the SF system, which includes the SF–plasma membrane attachment site and possibly of other morphological parameters in ECs in situ, are dynamically maintained by fluid shear stress–dependent signals transmitted continuously from the area of cell-cell adhesion.

Acknowledgments

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The lateral zone of cell-cell adhesion as the major fluid shear stress related signal transduction site.

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Selected Abbreviations and Acronyms: FAK; focal adhesion kinase.
Crk; CT10-related kinase. Csk; C-terminal Src kinase. Cas; v-Crk-associated tyrosine kinase substrate. PECAM-1; platelet endothelial cell adhesion molecule-1.
Materials and Methods

Preparation of blood vessels

Aortae were obtained from normal adult guinea pigs weighting 400-600 g. All animals were anesthetized with an overdose of sodium pentobarbital by an intraperitoneal injection. After perfusion with 0.85 % NaCl containing 200 U heparin sodium via left ventricle, abdominal aortae were excised and fixed with cold methanol (4 °C) for 5 minutes or 2 % paraformaldehyde using a microwave irradiation method as previously reported using a special microwave device (MI-77, Azumaya Ikakikai, Tokyo) ¹.

Antibodies and fluorescent reagents

Monoclonal anti-vinculin (Sigma), anti-Crk (Transduction Laboratories, Lexington, KY), anti-Csk (Transduction Laboratories), (Upstate Biotechnology, Lake Placoid, NY), anti-phosphotyrosine (clone PY-20; ICN Pharmaceuticals, Aurora, Ohio), anti-pp130CAS (Transduction Laboratories) and anti-paxillin (Zymed) were purchased. Polyclonal anti-Src (Santa Cruz Biotechnology, Santa Cruz, CA), anti-FAK (Takara Biomedicals, Shiga, Japan), fluorescein-labeled goat anti-rabbit IgG (Cappel, Durham, NC) and anti-mouse IgG (Cappel) were also purchased. Rhodamine- or fluorescein-labeled phalloidin were obtained from Sigma. Anti-PECAM was described previously ².

Immunofluorescence Procedures
Fixed aortae were rinsed with PBS and cut into small pieces. Segmented specimens were permeabilized with 0.5 \% Triton-X 100 in PBS for 5 minutes. After washing with PBS, they were treated with 10 \% normal goat serum for 20-60 minutes at room temperature and incubated with phalloidin or primary antibodies for 60-120 minutes that were appropriated diluted: anti-phosphotyrosine (1:200), anti-FAK (1:100), anti-Crk (1:50), anti-Csk (1:100), anti-Src (1:20-50), anti-vinculin (1:400), anti-paxillin (1:100), anti-ppl30CAS (1:50) and anti-
PECAM-1 (1:500). They were washed with PBS and incubated with secondary antibodies. Fluorescein-labeled goat anti-rabbit IgG (1:100 - 1:200) or anti-mouse IgG (1:100 - 1:200) were used as secondary antibodies. Some specimens were doubly stained with rhodamine-labeled phalloidin. Specimens were mounted in 90\% glycerol in PBS containing 2.5\% 1,4-diazabicyclo [2.2.2]-octane (DABCO, Aldrich) and were examined by a confocal laser scanning microscope (GB-200, Olympus) with a plan-apochromat X10 (N.A. 0.25), X20 (N.A. 0.6) or X63 (N.A. 1.4, oil) objective lens. Stereo pair images were reconstructed from 20-30 serial sections by using the NIH IMAGE software system. Fluorescent secondary antibodies alone did not stain cells.

Preparation of surgical coarctation

Animals were anesthetized with sodium pentobarbital (35 mg/ kg) by intraperitoneal injection. According to a modified method described by Gabriels et al. \textsuperscript{3}, the abdominal aorta was exposed and a silicon tube (inner diameter 1.0 mm; outer diameter 2.0 mm; 3.0 mm in length) was wrapped around it. The tube was positioned 4-5 millimeters above the common iliac artery branching site. This part of aorta is straight without branching. Two sutures were placed near each end of the tube to prevent
it from slipping or coming off. The peritoneum and the abdominal wall were sutured separately. Animals were fed normally for 7 days and sacrificed as described above. After 7 days from the operation, the blood vessel diameter was reduced to about 70% of the original size.

**Reference**

