Micropipette Aspiration of Cultured Bovine Aortic Endothelial Cells Exposed to Shear Stress

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The mechanical properties of cultured bovine aortic endothelial cells exposed to a fluid-imposed shear stress were studied using the micropipette technique. The cells, which were attached to a Thermanox plastic substrate, were exposed to a specific steady shear stress of either 10, 30, or 85 dynes/cm² and for a duration ranging from 0.5 to 24 hours. Morphological changes in shape and orientation were observed, and following each experiment, the mechanical properties were measured using the micropipette aspiration technique applied to cells detached from the substrate. Fluorescent microscopy was carried out to observe cytoskeletal F-actin filaments stained with rhodamine phalloidin. During exposure to shear, the en face shape of the endothelial cells on the substrate became more elongated and their long axis became oriented to the direction of flow. There was also an alteration in the F-actin filaments. These changes were dependent on both the level of shear stress and the duration of exposure. After detachment, the cells exposed to shear maintained their deformed shape. This is in contrast to cells in a static, no-flow environment which became spherical in shape upon detachment. Cells exposed to shear stress demonstrated a mechanical stiffness significantly greater than that of control cells, which was dependent on both the level of shear stress and the duration of exposure. Furthermore, it appears that the influence of shear stress on endothelial cell mechanical stiffness may be related to alterations in cytoskeletal structure. (Arteriosclerosis 7:276-286, May/June 1987)

Although there is considerable indirect evidence that hemodynamic forces are a factor in the process of atherogenesis, the detailed mechanisms are still poorly understood and the precise role is uncertain. The accumulation of lipids within the intima is believed to be an important factor in the early stages of atherosclerosis, and the process of transendothelial macromolecule transport, in particular shear stress, received early attention. More recently, hemodynamic forces have been found to affect the shape and orientation of endothelial cells studied both in vivo and in vitro. Furthermore, the cytoskeleton (in particular stress fibers), which is an important structure in the support of the membrane and in the maintenance of cell shape, also appears to differ in regions of differing shear stress.

It thus appears that when endothelial cells are exposed to a fluid-imposed shear stress the process of adaptation or response involves not only cell orientation and elongation, but also a change in the supporting, internal structure. Furthermore, this could be reflected in the mechanical properties of the endothelial cells, and any change in these properties could be important to the deformation a cell undergoes as part of the adaptation process. For this reason, it was believed that a measurement of the mechanical properties of an endothelial cell would be instructive. Although such properties would appear to be an important correlate of cell structure and function, to date there are no data available on the mechanical properties of the endothelial cell.

Several experimental methods for examining the mechanical properties of a cell have been proposed. These include: 1) osmotic swelling in a hypotonic solution; 2) compression between two flat plates; 3) aspiration in a micropipette; 4) deflection of the surface by a rigid spherical particle; 5) fluid shear on a cell tethered to a flat plate at one point; and 6) forced flow through polycarbonate sieves. Although these methods have been mainly applied to red blood cells, other cell types have also been considered.

In this investigation, the mechanical properties of cultured bovine endothelial cells exposed to a fluid-imposed shear stress and then detached were studied using the micropipette technique. These data demonstrate an important effect of shear stress on the mechanical properties of the endothelial cell. It is these measurements which are presented here, and their interpretation is discussed in the context of cell cytoskeletal structure.

Methods

Materials

Bovine aortic endothelial cells cultured in our laboratory on Thermanox plastic coverslips were used. Fully confluent cultured endothelial cell populations from the 7th to 9th
generation were studied. The age or passage time of the cultures varied from 2 to 9 days. The cells were exposed to a steady shear stress using a parallel plate flow chamber. The description of the flow chamber and the analysis of the laminar fluid flow conditions have been reported previously.8

Cultured endothelial cells on a coverslip with a diameter of 13 mm were positioned in the central part of the flow chamber. This flow chamber had a flow section which was 220 µm in height, 17 mm in width, and 50 mm in length. The chamber, a reservoir, and a circulation circuit were filled with culture medium (modified Dulbecco medium (MDM), containing 25 mmol/l Hepes buffer and 20% fetal bovine serum and antibiotics). The endothelial cells in the chamber were exposed to a specific shear stress condition defined by the dimensions of the flow chamber and the pressure drop across the chamber. The pressure drop was measured and controlled by adjusting the height of the upstream reservoir. A roller pump was used to return the outflow from the chamber to this feeding reservoir. The MDM in the reservoir was kept at a constant temperature of 37 ± 0.5°C. A gas mixture of 95% air and 5% CO2 was provided to the MDM in the reservoir. In the 25 experiments reported here, the exposure time of cells to shear stress was 0.5, 1, 2, 4, 12, or 24 hours and the shear stress imposed on the cell population was either 10, 30, or 85 dynes/cm². Photographs of the cells were obtained at the end of each flow experiment in order to observe changes in en face cell shape and orientation. These microphotographs of endothelial cells were analyzed quantitatively using a Zeiss Videoplan image analyzer (Zeiss Incorporated, New York, New York). The following morphological parameters were determined: area, perimeter, length, width, angle of orientation, and shape index. Only one parameter is presented in this report, the shape index, which is defined as $4\pi A/P^2$ where $A$ is the en face surface area and $P$ is the cell perimeter.26

Microscopy

Fluorescent microscopy was carried out: 1) on intact confluent monolayers grown on Thermanox coverslips under static (i.e., no flow) conditions and after exposure to shear stress and 2) on completely detached cells from monolayers using either trypsin or a mechanical method. The following steps were carried out at room temperature.24 Actin filaments were stained with rhodamine phalloidin (Molecular Probes, Incorporated, Eugene, Oregon) which is specific for F-actin.27 All cell samples were briefly rinsed with phosphate-buffered saline (PBS) and then fixed in 4% formaldehyde in PBS for 5 minutes. For detached cells, each step was followed by a brief centrifugation. After fixation, cell samples were rinsed in PBS and were incubated in 0.1% Triton (Sigma Chemical Company, St. Louis, Missouri) for 5 minutes. Then the cells were rinsed and incubated with 3.3 x 10⁻¹¹ mol of rhodamine phalloidin in 200 µl PBS for 20 minutes for intact monolayers and partly detached cells and for 5 minutes for detached cells. Cells were rinsed and mounted over glycerol/PBS (1:1) and were observed with a Nikon photomicroscope equipped with epifluorescence optics. Photgraphs were taken on Kodak TRI-X films at 400 ASA. The films were processed using the standard procedure recommended by the film manufacturer.

Figure 1. Schematic diagram of experimental setup used for micropipette technique. The left part of the diagram shows the pressure reservoirs and pressure measuring device. The center shows the micropipette and cell suspension on the microscopy stage. The top right shows the data acquisition system.
**Micropipette Experiments**

After exposure of cells to a known shear stress and for a specified length of time, the endothelial cells were detached from their substrate by scratching the coverslip with a sterile wood stick. The sheet of cell aggregates was detached further by aspiration through a 1 ml pipette. Before measurement, endothelial cell suspensions were stirred for approximately 15 seconds on a test-tube stirrer. As will be seen, detached cells that had not been exposed to shear stress were spherical in appearance, while those that had been exposed to shear stress were elongated in shape. The prepared cells were studied at room temperature and as soon as possible after detachment, usually within 6 hours. Cell shape and the measured mechanical properties did not show any significant change with time during this period.

Micropipettes with an internal diameter ranging from 2.0 to 3.4 μm (2.7 ± 0.5 μm), were prepared from glass tubes (ID = 0.7 mm, OD = 1.5 mm) with the use of a micropipette puller (Model 700D, David Kopf Instruments, Tujunga, California). The micropipette was filled through a 0.2 μm membrane filter with the same MDL used for the cell culture by use of a 1 ml syringe. This fluid-filled micro-

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**Figure 2.** Photomicrographs of rhodamine phalloidin-stained endothelial monolayers grown on Thermofax. **A.** Control conditions. **B.** After exposure to a shear stress level of 85 dynes/cm² for one-half hour.
pipette was connected to a pressure control line and was fixed to the stage of the microscope (Model BHA-P, Olympus, Lake Success, New York). A schematic diagram of the experimental system used is shown in Figure 1. The pressure control system was modeled after that of Chien et al. This system is composed of three different reservoirs. One is a damping chamber which is connected to the micropipette and partially filled with water. This chamber will be used in future dynamic tests to examine viscoelastic properties. The line between the micropipette and the chamber was filled with MDM. The other two reservoirs are adjustable, and a difference between these two heights can produce a negative pressure at the tip of the micropipette. One of these reservoirs was open to the atmosphere and can be controlled with an accuracy of better than 10 μm by the use of a micrometer. A part of the line between the micropipette and the chamber was connected to a pressure transducer (Model P23Db, Gould, Incorporated, Cleveland, Ohio), and the pressure level of the micropipette system was recorded on a multichannel recorder (Gould). The transducer was calibrated with a water manometer before each experiment.

The endothelial cell suspension was loaded into the cell chamber (2 mm in height and 10 mm in width) which was

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Figure 2 continued. C. After exposure for 4 hours. D. After exposure for 12 hours. Bar = 50 μm. Arrow indicates the flow direction.
held by a manipulator. The suspended cells were observed through a long working distance objective lens (×20) under the microscope as shown in Figure 2. The tip of the micropipette was made to approach the surface of a spherical endothelial cell by manipulating both the cell chamber and the stage of the microscope. When a negative pressure, Δp, was applied to the tip of the micropipette, the cell was drawn toward and into the pipette. To determine the zero pressure level, the height of the reservoir was changed by a slight adjustment of the micrometer so as to just maintain the cell in its initial position. First, a negative pressure of about 2 mm H2O was set by using the micrometer, and then a portion of the endothelial cell was aspirated into the micropipette.

In preliminary experiments, the aspirated part of the cell continued to deform after the application of pressure, but an almost steady state was observed to occur within 8–10 minutes. Therefore, in our experiments the negative pressure was maintained for 10 minutes at each setting and then increased in a stepwise fashion several times, and a photograph of the aspirated portion of the cell was taken at each setting by a camera connected to the microscope. In

Figure 3. Photomicrographs of rhodamine phalloidin-stained endothelial monolayers grown on Thermoflux and exposed for 24 hours to fluid shear stresses of (A) 30 and (B) 85 dynes/cm². Bar = 50 μm. Arrow indicates flow direction.
this way, the aspirated length (L) of the membrane in the micropipette was measured at several different negative pressures during the loading process. After the aspirated length of the cell membrane had attained a length twice the radius of the micropipette, the negative pressure was decreased and the cell was unloaded.

In a few experiments, the recovery of the deformed portion of the cell membrane was measured during this unloading process. When the pressure was returned to zero, it was determined whether the aspirated portion of the cell had fully recovered. If the cell remained deformed at zero pressure, then the data were discarded. The image of the cell shape and the deformation process was also observed by a TV camera (Model ITC-47, Ikegami Tsushinki Company, Limited, Maywood, New Jersey) and was recorded on a video tape recorder (Model NV-8050, Panasonic Industrial Company, Secaucus, New Jersey). After each experiment, the aspirated length of the cell was measured on a TV monitor screen by replaying the recorded tape or from photographs.

The data in the form of L versus \( \Delta p \) represents, in effect, a stress-strain relationship. Measurements of L versus \( \Delta p \) have been carried out previously by other investigators for both red cells and white cells. The data are presented here in the form of a mechanical stiffness parameter, \( K = \frac{R \times \Delta p}{L/R} \), where \( R \times \Delta p \) is the tension being exerted and \( L/R \) is the non-dimensional strain imposed on the cell (L is the aspirated length in the micropipette and R is the micropipette radius). With the use of a Mine II microcomputer, linear regression analysis was performed on individual experiments using a least-squares curve fit. In all experiments, it was possible to assess linearity over the full range of pressures.

**Results**

The changes in en face endothelial cell shape due to exposure to shear stress for a specified time have been shown previously. At the control condition of time zero, the cell shape is polygonal, and after exposure to shear stresses, the cells elongate and become oriented in the direction of flow.

F-actin microfilament bundles in confluent monolayers grown on a Thermox substrate are shown in Figure 2A for static conditions and in Figures 2B, 2C, 2D, 3A, and 3B for shear stress exposures. Examples of F-actin microfilaments after a short exposure time of one-half hour for a shear stress level of 85 dynes/cm² is presented in Figure 2B. With such a short exposure time, a change in cellular morphology was not observed with phase contrast optics. However, it was observed that the microfilament system

![Figure 4](http://atvb.ahajournals.org/) Example of cellular deformation after detachment for (A) control conditions, and after exposure for 24 hours to shear stress levels of (B) 10, (C) 30, and (D) 85 dynes/cm². Bar = 10 \( \mu \)m.
was affected. First, the dense peripheral band mostly disappeared and an increase in stress fibers was seen. The stress fibers seemed to have a more organized appearance.

Figures 2C and 2D show F-actin in response to a fluid shear stress level of 85 dynes/cm² for 4- and 12-hour exposures, respectively. The 4-hour exposure (Figure 2C) shows an increased number of stress fibers aligned with the flow field. It was also apparent that there is a widening of intercellular spaces with few connecting microfilaments.

With the longer exposure time of 12 hours, the stress fibers were mostly aligned with the direction of flow, but a few intercellular spaces were still present.

Figures 3A and 3B show F-actin microfilaments in cells after a 24-hour exposure to shear stress levels of 30 and 85 dynes/cm², respectively. In both cases, the stress fibers were aligned with the direction of flow. For the two lower shear stress levels (i.e., 10 and 30 dynes/cm²) intercellular spaces were often seen with short microfilaments that seem to connect adjacent cells together. For the highest

Figure 5. Photomicrographs of rhodamine phalloidin-stained endothelial cells after exposure to 85 dynes/cm² for 24 hours during the process of being detached from their substrate using trypsin (A), and in a completely detached cell using the mechanical method (B). Bar = 50 μm.
shear stress level, intercellular spaces were no longer visible and there was a higher concentration of microfilament bundles at the cell periphery.

Examples of cell deformation for endothelial cells which have been exposed to different shear stresses are shown in the series of photomicrographs in Figure 4. In contrast to control cells, the shapes of the cells exposed to shear stress are quite different. As noted earlier, cells exposed to high shear stress do not become spherical in shape when detached, but retain a more elongated shape. Furthermore, the degree of elongation appears to depend on the level of the shear stress to which the cell has been exposed. Figure 5A shows cells detached using trypsin from a monolayer exposed to a shear stress of 85 dynes/cm² for 24 hours. As may be seen, there is little alteration in cellular morphology occurring during detachment and a well organized F-actin microfilament system was preserved. In a completely detached endothelial cell obtained with the mechanical method (Figure 5B), the F-actin microfilament system was not disrupted. In addition, a narrowing of the cell seemed to occur. The cells exposed to high shear stress were stiffer than cells exposed to a low shear or to a static, no-flow condition. This is evidenced by the fact that a larger suction pressure was needed to deform the cell to the same extent. An example of this is shown in Figure 6 where a typical loading curve is shown for cells exposed to shear stresses of 10, 30, and 85 dynes/cm², respectively, as well as for a cell from a control, no-flow condition.

As may be seen from Figure 6, the relationship between the pressure difference and the aspirated length is approximately linear during the loading process. The slope of such a relationship is used to define the stiffness parameter, K. Values of the mechanical stiffness parameter, K = R x Δp/(L/R), obtained from cells exposed to shear stress for different exposure times are shown in Figure 7. The results for 0.5 to 1.5 hours of exposure are presented in Table 1. These values are not presented in Figure 7 because only the most elongated cells were measured and these may not be a good representation of the total population. It should be noted that the cells exposed to a shear stress of 85 dynes/cm² for only a few hours had a value of the stiffness parameter almost four times higher than that for control conditions. This is in contrast to a shear stress of 30 dynes/cm², where cells exposed for approximately 4 hours had a relatively low value of the mechanical stiffness parameter. However, with longer exposure times, the mechanical stiffness increased to a level approximately three times higher than that for control cells. This time-history of the mechanical stiffness parameter, K, during the response of endothelial cells to shear stress is illustrated in Figure 7. Data for cells exposed to a shear stress of 10 dynes/cm² are also included. These cells show a similar
trend to those at 30 dynes/cm², having a lower value of the mechanical stiffness parameter for times of 4 hours or less, but evidencing an increase for longer times. After 24 hours of exposure to shear stress, the increase in the stiffness parameter was statistically significant as compared to control values for all three shear stress levels employed (p < 0.05, two-tailed Student’s t test).

Discussion

Endothelial cells in vivo are aligned with the direction of flow and have an elongated tear drop shape. These phenomena (i.e., cell orientation and elongation) have also been reported by others for in vitro cultured endothelial cells exposed to fluid shear stress. Although much has been learned about the effect of fluid mechanics on endothelial elongation and orientation, little is known about other properties of endothelial cells. However, since a cell’s mechanical properties may be related to the process of its elongation and orientation, this investigation has focused on endothelial cell mechanical properties. In their preliminary studies, Dewey et al. did show that certain endothelial cell functions, including fluid endocytosis, cytoskeletal assembly, and nonthrombogenic surface properties, are sensitive to shear stress. Frangos et al. demonstrated enhanced PGI₂ production in endothelial cells exposed to shear stress. These results taken together suggest that fluid mechanical forces can directly influence endothelial cell structure and function.

The experiments presented here were conducted under laminar, steady flow which is in contrast to in vivo conditions where pulsatile flow exists. However, it is recognized that other types of flow (e.g., pulsatile, oscillatory, turbulent) may affect endothelial cell properties differently. The complexity of the flow fields would make it difficult to assess the effect of shear stress alone since other flow components may indeed interfere with endothelial cell biology. As a first step, it thus is appropriate to evaluate the effect of shear stress on endothelial cell mechanical properties using well defined steady flow conditions.

The results presented suggested that exposure to shear stress exerted such an alteration in the microfilament network that even after detachment, the elongation of the endothelial cell was preserved. This is in contrast to cells under a no-flow condition which, upon detachment, became spherical. In the latter case, there was some evidence that a submembranous cortical layer was formed. For this latter case, the analysis of the mechanical properties of these detached spherical endothelial cells was carried out using a membrane type of equation as originally proposed by Evans and Chien et al. In this investigation, however, the shear-exposed cells did not become spherical upon detachment. Thus, their behavior could not be analyzed using this previous approach. As a first step in the analysis of these data, a mechanical stiffness parameter, K, was used. This parameter represents the slope of the variation in the tension, R × ΔP, with the nondimensional length of aspiration, L/R. It is very similar to the approach used in previous erythrocyte, leukocyte, and platelet micropipette studies.

Along with the changes in endothelial cell shape, it has been observed that endothelial cells become stiffer in response to shear stress. The effect of shear stress on shape as a function of time was reported previously. The changes with time in cell mechanical properties appear to be due to certain structural changes in the cytoskeleton which are a response to shear and are discussed below. The relationship between endothelial cell shape and the mechanical stiffness parameter is demonstrated quantitatively in Figure 8 where a correlation between the en face cell shape index and the stiffness parameter is presented.

The increase in cell stiffness may parallel the reorganization of the cytoskeleton and, in particular, the microfilament system. In an independent study, we have investigated the importance of two components of the cytoskeleton, (e.g., microfilaments and microtubules) in relation to endothelial cell mechanical properties (unpublished results). Endothelial monolayers under no-flow conditions were incubated with cytochalasin B and colchicine to disrupt the microfilament or microtubular system respectively. We found that microfilaments contributed more to endothelial cell mechanical properties than microtubules. This suggests that, in the presence of shear stress, the microfilaments may make the greater contribution to endothelial cell mechanical properties.
Even exposure to shear stress for short times had a dramatic effect on the F-actin microfilament system. This was also true for the stiffness parameter of some cells (Table 1). With regard to the latter, it was felt that these results may not be representative of the actual shear stress effect for at least two reasons. First, it was the most deformed cells which were selected for measurement. Second, it was observed that most of the cells selected for short exposure times did not become spherical after detachment, but acquired a rather potato-type appearance with the formation of a cortical cytoskeletal layer. It is suspected that this cortical layer may have contributed to the increase in cell stiffness, which may be accounted for by the increase in stress fibers shown in Figure 2. This is consistent with our previous work on passage effect where we found that cells from older monolayers were stiffer, but also appeared to have a higher concentration of stress fibers. Franke et al. have shown that for human vascular endothelium in vitro, fluid shear stress acts directly on the stress fiber system without affecting cellular shape and orientation. They exposed cell monolayers to a shear stress level of 2 dynes/cm² for 3 hours and observed that the quantity and intensity of stress fibers had dramatically increased. They also observed, as shown here, that there was a preferential alignment, or direction, to the newly formed stress fibers.

The results obtained for F-actin microfilaments are generally consistent with the results reported by others, although others have not looked at the effect of both shear stress level and exposure time on stress fiber reorganization in as detailed a fashion. White et al. looked at the orientation of the endothelial cytoskeleton using cultured bovine aortic endothelial cells under both static, no-flow, and shear stress conditions in vitro. Cultured cells under static, no-flow conditions exhibited a random stress fiber orientation. However, after exposure to 8 dynes/cm² for 72 hours, these cells became aligned with the flow direction, and their cytoskeletal structure underwent a dramatic reorganization; stress fibers appeared much more prominent and the cells showed an orientation with the major cell axis and with the direction of flow. The organization of the extracellular matrix beneath the endothelial monolayer also appeared to be influenced by shear stress. Similar results were reported by Wachezak et al. for fibronectin and F-actin redistribution in cultured endothelial cells exposed to shear stress.

In addition to the above, we have demonstrated that the dense peripheral band is also influenced by shear stress. For static endothelial cell monolayers, it has been suggested that the dense peripheral band may be contractile and help maintain cell shape and yet at the same time be intimately associated with the ability of the cell to spread after wounding. This dual function is thought to reflect the demands of the endothelial cell environment. Our results are perhaps more compatible with the in vivo condition, and it would seem that endothelial cells in vivo must remain closely appositioned to each other in order to function as a selective barrier. Figure 3 shows that higher shear may contribute not only to an increase in stress fiber density, but also to a tightening of the monolayer. For example at the highest shear stress, individual cells are not distinguishable, whereas at the lower shear stresses, not only are cells distinguishable but also there are intercellular spaces devoid of stress fibers. This suggests a possible influence of stress on intercellular junctions.

The occurrence and distribution of stress fibers also has been examined in vivo using mouse and rat endothelial cells in perfusion-fixed large arteries and veins. As an example, in the lower thoracic aorta, the stress fibers were strongly aligned with the flow, while in the inferior vena cava there were very few stress fibers with no preferred orientation. Since the latter might be expected to have lower shear stresses than the former, these results also suggest that local hemodynamic factors may influence both the abundance and orientation of such contractile, cytoskeletal elements. The same results were obtained for vertebrate tissues by Wong et al. They concluded that the shearing force of blood may be a determinant of endothelial cell stress fiber elaboration and orientation.

The present results also may have implications for transendothelial transport, a subject which has been of interest in the study of the influence of wall shear stress on the blood-arterial wall transport of macromolecules. In these earlier experiments, the accumulation of materials in the wall was measured, but the precise pathway through the endothelium and into the intima was not known. More recently Davies et al. examined the effect of shear stress on endothelial cell pinocytosis using cultured bovine aortic endothelial cells. The intracellular horseradish peroxidase accumulation in confluent endothelial monolayers exposed to 0, 1, or 8 dynes/cm² shear stress was measured. These results demonstrated that the rate of fluid endocytosis was enhanced in response to a step-change in shear stress.

Although somewhat speculative, it might be expected that at an early stage in atherogenesis the cytoskeleton of endothelial cells could play an important role in determining the permeability of endothelium to macromolecules, e.g., via transendothelial and junctional transport. Thus, cells in high shear regions, in response to their mechanical stress environment, would have a more highly developed cytoskeletal structure which could inhibit both transendothelial and junctional macromolecule transport. The result would be that low shear regions, with their higher influx of macromolecules like LDL, would have a higher predilection for the initiation of lesions.

Finally, the present data and that of others suggest that the response of an endothelial cell to shear stress includes an alteration in both its structure and its mechanical properties. More detailed comparisons of structure and cell function between in vivo and cultured endothelial cells are needed to apply the results of in vitro observations of mechanical properties to the endothelial cells of living blood vessels.

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