We report the first topographical data of the surface of living endothelial cells at sub-light-microscopic resolution, measurements essential for a detailed understanding of force distribution in the endothelium subjected to flow. Atomic force microscopy was used to observe the surface topography of living endothelial cells in confluent monolayers maintained in static culture or subjected to unidirectional shear stress in laminar flow (12 dyne/cm² for 24 hours). The surface of polygonal unsheared cells was smooth, with mean excursion of surface undulation between peak height (over the nucleus) and minima (at intercellular junctions) of 3.4 ± 0.7 μm (mean ± SD); the mean height to length ratio was 0.11 ± 0.02. In cells that were aligned in the direction of flow after a 24-hour exposure to laminar shear stress, height differentials were significantly reduced (mean, 1.8 ± 0.5 μm), and the mean height to length ratio was 0.045 ± 0.009. Calculation of maximum shear stress and maximum gradient of shear stress (στ/σz, where τ is shear stress at the cell surface) at constant flow velocity revealed substantial streamlining of aligned cells that reduced στ/σz by more than 50% at a nominal shear stress of 10 dyne/cm². Aligned cells exhibited ridges extending in the direction of flow that represented imprints of submembranous F-actin stress-fiber bundles mechanically coupled to the cell membrane. The surface ridges, ~50 nm in height and 200 to 1000 nm in width, were particularly prominent in the periphery of the aligned cells. These observations (1) represent the first measurement of endothelial surface topography in living cells, (2) demonstrate significant changes in surface topography as a result of exposure to hemodynamic forces, probably as a result of submembranous cytoskeletal reorganization, and (3) facilitate computation of detailed cell-surface force distribution. (Circ Res. 1994;74:163-171.)

Key Words • shear stress • atomic force microscopy • endothelial cells • morphology • cytoskeleton • flow • cell surface

The cellular responses to fluid forces are complex; they depend on the magnitude of shear stress as well as temporal and spatial gradients in shear stress. Flow separation due to arterial curvature or bifurcation gives rise to spatial gradients in vivo. Similar flow patterns reproduced in vitro have been shown to influence cell morphology and proliferation. However, these are gradients in average wall shear stress that vary smoothly over several cell lengths. In contrast, the waviness of the surface due to the presence of endothelial cells introduces gradients in shear stress on a subcellular scale. This was recognized in a recent theoretical study of flow over a sinusoidally undulating surface simulating the endothelium that demonstrated variations in shear stress over the cell surface that were strongly dependent on the model geometry. The shear stress distribution depended on the aspect ratio (length of the cell in the direction of flow divided by the width) and the height to length ratio of the cell. The model indicated that shape changes predicted to occur in response to shear stress (including elongation and alignment with flow direction) tend to reduce the maximum stress and the stress gradients experienced by the cell. However, while the two-dimensional shape changes, characterized by the aspect ratio or by the shape index, are well documented, neither cell height profiles nor surface topography have been accessible in living cells.

The state of stress in endothelial cells resulting from the application of fluid flow depends on the exact loading conditions (distribution of shear stress at the cell surface), the geometry of the cell membrane, and...
the structural properties of the cytoplasm underlying the membrane. Fung and Liu\(^\text{19}\) performed a simple analysis of the mechanics of the endothelium loaded by shear stress. The tensile stress in the membrane was related to its contour and the mechanical properties of the cytoplasm. However, they did not consider explicitly the effect of discrete cytoskeletal elements interacting with the membrane. Endothelial cells both in vivo and in vitro respond to shear stress by reorganization of F-actin into thick bundles aligned with flow.\(^9,18,20,21\) Information on the nature of the mechanical coupling of this structure to the apical membrane is needed to fully evaluate the model of Fung and Liu and/or to modify it. The cytoskeletal reorganization in response to flow is associated with an increase in cell stiffness as measured by a pipette aspiration technique.\(^21\) Furthermore, a recent report by Wang et al.\(^25\) demonstrated mechanical coupling of the apical membrane of unsheared endothelial cells to the cytoskeleton in which deformation at the surface was resisted primarily by actin but also, to a lesser degree, by other cytoskeletal elements. Together, these findings suggest that close interactions between the cytoskeleton and the cell membrane play a key role in cell shape rearrangement during flow.

Detailed measurements of cell surface geometry are required for complete analysis of the fluid forces acting on the cell surface and the mechanical responses of the cell to such forces. Until the advent of the atomic force microscope (AFM),\(^23\) quantitative characterization of surface topography at this scale has been inaccessible. The AFM, which raster-scans a sample past a sharp stylus tracking the surface undulations, can image samples in a fluid environment allowing living cells to be kept in a physiological medium. AFM has been used to image hard surfaces with atomic resolution,\(^24\) biologic molecules bound to a surface,\(^25-29\) and both fixed and living cells.\(^30-33\) In the present study, we describe the use of the AFM to measure the surface contours of living endothelial cells.

Materials and Methods
Cell Culture and Shear Stress
Bovine aortic endothelial cells were isolated and cultured using standard procedures\(^34\) in complete medium (Dulbecco's modified Eagle's medium [DMEM], high glucose, containing 10 mmol/L HEPES, 2 mmol/mL glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated calf serum, all obtained from GIBCO Laboratories, Grand Island, NY). Cells in passages 20 through 24 were removed from standard tissue culture flasks by treatment with 0.1% trypsin, suspended in complete medium, and centrifuged at 220g for 6 minutes. Cells were resuspended in complete medium and placed in 100-μL drops (10\(^6\) cells per microliter) on 12-mm-diameter coverslips (Belco Biotechnology, Vineland, NJ) and then incubated at 37°C while the cells attached to the glass (30 minutes), at which time excess complete medium was added. Cells were then grown to confluence before application of shear stress or observation in the AFM.

Shear stress was applied to confluent endothelial cells using a cone-and-plate device introduced by Bussolari et al.\(^19\) and modified by DePaola.\(^36\) A unidirectional shear stress of 12 dyn/cm\(^2\) (82 rpm, complete medium) was applied in laminar flow for 24 hours. Coverslips were then quickly removed from the base plate, immersed in Dulbecco's phosphate-buffered saline (PBS containing [mmol/L] CaCl\(_2\), 0.9; KCl, 2.7; KH\(_2\)PO\(_4\), 1.2; MgCl\(_2\), 0.5; NaCl, 138.0; and Na\(_2\)HPO\(_4\), 8.1; Gibco), and transferred to the AFM chamber, where imaging was performed at room temperature with the cells bathed in PBS. Coverslips of unsheared control cells maintained in the incubator were handled identically.

Atomic Force Microscopy
An AFM (Nanoscope III, Digital Equipment, Santa Barbara, Calif.) was used in an open fluid cell configuration.\(^31\) A triangular silicon nitride (Si3N4) cantilever (110-μm-long, 24-μm-wide arms; spring constant = 0.38 N/m; Digital Equipment) was held fixed while the integral tip (Si3N4) interacted with the cell surface as the cantilever was scanned past. The deflection of the cantilever due to the force of interaction with the sample was detected by measuring the motion of a laser beam reflected off the gold-coated cantilever with a split photodiode. The signal from the photodiode was used to control the vertical position of the sample such that the cantilever deflection remained nearly constant. The three-dimensional contour of the sample is constructed by recording the position of the sample as it is scanned past the stylus using the J-head piezoelectric positioner with a maximum scan size of 143 μm (Digital Equipment). The vertical travel of the J-head was 5.50 μm; the height data was sampled with 16-bit resolution corresponding to a height resolution of 0.1 nm. Simultaneously, the small but finite cantilever deflection, the error signal, was recorded. This error-mode imaging detects edges and very small surface features.\(^31-33\)

The nearly constant cantilever deflection controlled by a feedback circuit determines the force of interaction between the tip and the sample. To test the sensitivity of the contour measurement to imaging force, the same portion of a cell was imaged at a range of imaging forces. Forces greater than 10 nN appeared to compress the cell, reducing the height and flattening the image. When the force was reduced to <1 nN, the stylus became disengaged with the surface. At imaging forces of 1 to 10 nN, engagement was maintained without significant change in the measured contour.

To investigate the sensitivity of the technique to image small surface features, we intentionally produced small blebs by treating the cells with a low concentration (0.1 μg/mL of cytochalasin D. Within 10 minutes, small protrusions with dimensions 100 to 200 nm in width and 50 to 100 nm in height appeared (not shown). These features arose before any gross morphological changes occurred and were too small for an accurate size measurement to be made by light microscopy. The blebs were expected to be mechanically weak and roughly spherical in shape; therefore, the dimensions measured by AFM probably reflect some deformation due to the imaging force. The ability to image the small fragile structure without disrupting the membrane suggested that similarly sized features on the cell surface would be readily detected by AFM.

Fluorescence Staining
Cells were fixed with 4% formaldehyde in PBS for 20 minutes at 37°C. After three washes with PBS, the cells were permeabilized by 0.1% Triton X-100 in PBS for 3 minutes at room temperature and washed twice with PBS and then three times with 50 mmol/L ammonium chloride, pH 7.3, for 5 minutes each wash. Filamentous actin (F-actin) was stained by addition of N-(1-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin (NBD-phallacidin, Molecular Probes, Inc, Eugene, Ore.) at a dilution in PBS of 1:100 of a 3-mmol/L stock solution for 20 minutes at room temperature, followed by three final washes in PBS. After infusion of glycerol and PBS (1:1) containing 2 mg/ml p-phenylene diamine into the capillary tubes, cells were viewed using a Leitz fluorescence microscope equipped with epifluorescence using narrow band-pass excitation and emission filters for fluorescein and rhodamine (Omega Optical Co, Burlington, Vt.).
Results

Bovine aortic endothelial cells grown to confluence in the absence of flow were polygonal with smooth rounded surfaces (Fig 1). The boundaries between cells were clearly evident. Occasionally, small protrusions (50 to 100 nm in height, 100 to 200 nm in diameter) were observed. Cells were imaged for 1 to 3 hours without significant changes in cell morphology. Confluent monolayers exposed to 12 dyne/cm² steady shear stress for 24 hours became elongated and aligned with the direction of shear stress (Fig 2). The cell surfaces were more irregular, and boundaries between cells were less pronounced. In some cells, longitudinal ridges aligned in the flow direction (50 to 100 nm in height, 200 to 500 nm in width) were observed.

Cell height data were analyzed by examining sections taken through the highest point on the cell in directions parallel and perpendicular to the flow direction. Because the unsheared cells were randomly oriented, sections were taken in the $x$ and $y$ directions of the laboratory frame corresponding to the scan axes. The possibility of error due to scan-line artifacts was reduced by (1) avoiding sectioning through visible streaks in the image and (2) taking sections at several orientations on a few cells to check for any systematic bias introduced by taking sections in the scan direction. Furthermore, the line-to-line variations in height (making scan lines visible in Figs 1 and 2) are very small compared with the cell heights being measured. The cell-surface height differential was the average of the difference in height between the maximum, corresponding to the nuclear region, and each of the two minima at the cell boundaries for each section. The height to length ratio was the cell-surface height differential, as defined above, divided by the length of the longitudinal section (horizontal distance between cell boundaries). In addition, the aspect ratio (the ratio of the lengths of the two orthogonal sections) was obtained. These data for sheared and unsheared cells are summarized in Table 1.

Sections of a typical unsheared cell show a smoothly curving contour (Fig 3). The characteristic profile was independent of the orientation of the section (Fig 3, sections $xx'$ and $yy'$). Furthermore, the average aspect ratio was not significantly different from 1.0, indicating no preferred orientation or elongation of the unsheared cells. The average height differential was 3.4 $\mu$m, and the height to length ratio was 0.11. Changing the scan direction had no influence on the measured dimensions.

In contrast, the longitudinal section of an elongated cell after exposure to flow shows a flatter profile with a
bulge in the region of the nucleus (Fig 4, section aa'). The average height differential of elongated cells (aspect ratio >2.0) was 1.8 μm. The longitudinal height to length ratio, 0.045, was less than half the control (no shear) value. The transverse section through the nuclear region was narrower than sections of unsheared cells and had a triangular shape (Fig 4, section bb'). Toward the periphery of the cell, upstream or downstream from the nucleus, longitudinal ridges were evident in section (Fig 4, section cc').

Error-mode imaging allowed small surface features to be observed by acting as an edge detector. The surface of unsheared control cells appeared smooth when observed in this imaging mode (Fig 5). In contrast, the surface of sheared cells displayed the imprint of an extensive fibrous network (Fig 6) that was continuous with the longitudinal ridges noted above and was aligned with the direction of shear stress. The larger surface imprints suggested the dimension of underlying bundles to be ≈0.5 to 1.0 μm in width. By reducing the scan size, the AFM resolved finer detail; impressions of fibers <200 nm in width were noted (Fig 7). Sheared cells stained with rhodamine-labeled phallacidin displayed a similar organization of intracellular F-actin stress fibers (Fig 8A). In contrast, F-actin in unsheared cells was located in prominent peripheral bands as well as in sparse randomly oriented fibers throughout the cell (Fig 8B). These images, however, give no spatial resolution in the out-of-plane dimension.

**Discussion**

Cell-surface imaging by atomic force microscopy has provided the first quantitative three-dimensional contour information needed to pursue analyses of endothelial mechanics at the cellular and subcellular scales. Data on cell-surface geometry are essential for determining microscopic flow characteristics and, therefore, topographical shear stress distribution. In addition, membrane contour and the structure of the supporting cytoskeleton are important determinants of the mechanical response of the cell to the applied loading conditions.

The three-dimensional contour of the apical surface of the endothelium governs the distribution of shear stresses acting on the cells for a given macroscopic applied flow. In analytical and numerical studies, Satcher et al. showed that two geometrical parameters, the aspect ratio (q) and the height to length ratio, determine the maximum shear stress and shear stress gradient developed for flow over an idealized sinusoidally undulating surface. Our values for the height to length ratio indicate that both sheared and unsheared cells fall within the regime for which their analytic linearized solution to the governing equations is valid:

![Image](http://circres.ahajournals.org/doi/abs/10.1161/01.RES.74.1.166)
Fig 4. Sections of cell aligned by flow. The gray-scale display of the height data for aligned cells (left) shows the position of orthogonal sections taken in the longitudinal direction (section aa'), parallel to flow, and the transverse direction (sections bb' and cc'), perpendicular to flow. The height scales for the section profiles (right) are half of that used in Fig 3. The longitudinal profile (section aa') is flat with a bulge at the nucleus. Transverse sections through the nuclei of aligned cells had the characteristic shape of section bb'. In section cc', the longitudinal ridges, not readily visible in the height image, appear as small bumps in the section profile.

(1) \[ \tau = \mu \sigma + 2 \pi \mu \sigma \frac{2 + q^2}{\sqrt{1 + q^2}} \frac{\eta}{\lambda} \cos \left(\frac{2 \pi x}{\lambda_x}\right) \cos \left(\frac{2 \pi y}{\lambda_y}\right) \]

where \( \tau \) is shear stress at the cell surface; \( \mu \) is fluid viscosity; \( \sigma \) is shear rate; \( q \) is the aspect ratio (length in the flow direction divided by the width transverse to flow) of the cell; \( \eta \) is the amplitude of the wavy surface (half of the cell height); \( \lambda_x \) is the cell length in the flow direction; and \( \lambda_y \) is the cell length transverse to the flow direction.

Using this solution, we can calculate the maximum shear stress (\( \tau_{\text{max}} \)) and the maximum gradient in shear stress \( \left( \frac{\partial \tau}{\partial x} \right)_{\text{max}} \) in terms of the average wall shear stress \( \left( \tau_{\text{avg}} \right) \) and the geometric parameters measured by AFM: height (H), length in the flow direction (L), and aspect ratio (q):

(2) \[ \tau_{\text{max}} = \left(1 + \pi \frac{2 + q^2}{\sqrt{1 + q^2}} \frac{H}{L} \right) \tau_{\text{avg}} \]

(3) \[ \left( \frac{\partial \tau}{\partial x} \right)_{\text{max}} = 2 \pi \frac{2 + q^2}{\sqrt{1 + q^2}} \frac{H}{L^2} \tau_{\text{avg}} \]

Although the real endothelial surface deviates from the ideal surface used for analysis, this solution allows us, as a first approximation, to evaluate the changes in hemodynamic stresses induced by the morphological responses to flow for living cells. Results in dimensionless form are summarized in Table 1. Results of a sample calculation using an average wall shear stress of 10 dyne/cm² and the average values of H, L, and q are given.

Fig 5. Error-mode image of unsheared endothelial monolayer. Unsheared control cells exhibited smooth surfaces. For low-frequency undulations (cell scale), the error signal is proportional to the slope of the surface in the scan direction (light grays indicate a positive slope, and dark grays indicate a negative slope), creating a shaded appearance. Regions where the signal is off scale (o) appear black and are not indicative of a loss of contact between cells. Field size is 143×143 \( \mu \text{m}^2 \).
Stress concentrations of 80% for the non-aligned cells and 40% for aligned cells were substantially higher than the values (34% and 20%, respectively) reported by Satcher et al.\textsuperscript{17} for their model geometries. The values for gradients of shear stress are at least an order of magnitude greater than values obtained in models of macroscopically disturbed flow.\textsuperscript{14} The utility of the analytic solution is that the dependence of the shear stresses on geometric parameters can be evaluated directly. For example, if the morphological changes in endothelial cells in response to flow represent an adaptation designed to reduce shear stress magnitudes and gradients, then it would appear that the decrease in cell height is the more important effect, because an increase in aspect ratio (with a constant height) increases the stress values. The actual shear stress distribution will be sensitive to shape,\textsuperscript{38} and full numerical simulation using the real cell geometries will be required to make more detailed comparisons between the stress fields acting on cells with different surface morphologies.

Significant changes in the actin filament network of endothelial cells in response to flow have been observed using fluorescent labeling techniques on fixed cells. Unexpectedly, we observed in the living cells a prominent fibrous structure manifested as surface ridges and organized in parallel arrays aligned with flow. The size range of the “fibers” (up to 1 \( \mu \)m) is consistent with the

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**FIG 6.** Error-mode image of aligned cells. Sheared cells display an extensive fibrous network (small arrows) aligned with the flow direction (large arrow). Error mode acts as an edge detector allowing sensitive detection of small surface protrusions. Regions where the signal is off scale (o) appear black and are not indicative of a loss of contact between cells. Field size is 143\( \times \)143 \( \mu \)m\(^2\).

**FIG 7.** Cytoskeletal detail. Error-mode image of a small area (7.9\( \times \)7.9 \( \mu \)m\(^2\)) gives high resolution of cytoskeletal fibers aligned in the flow direction (large arrow). Bundles of fibers (arrowhead) 0.5 to 1.0 \( \mu \)m in width are the same as the large fibers shown in Fig 6. At this resolution, thin fibers (small arrows) \(~\)100 nm in width were detected.
size of bundles of F-actin determined from electron micrographs.16,18 Because AFM images the surface of the cell, these data are strong evidence that the enhanced cytoskeletal structures found in sheared cells are closely associated with the apical membrane. These observations are consistent with the micropipette aspiration data of Sato et al,23 who measured the stiffness of endothelial cells after exposure to steady shear stress for 2 to 24 hours. The time course of the increasing stiffness correlates with that of the reorganization of actin filaments.18 The cell membrane alone is unlikely to account for the stiffening response. Rather, as noted by Sato et al, the major contribution to the mechanical properties of the cell surface is likely to be cortical cytoskeleton that is also aspirated into the pipette. Our detection of cortical stress fibers so closely associated with the membrane implies a tight mechanical coupling providing a structural basis for their results.

Fung and Liu19 have presented an elegant model for the mechanics of the endothelium in which the assumptions of a tension-field theory are used to simplify the analysis, thereby providing valuable insights. The close association of an aligned fibrous structure with the apical membrane noted here suggests that the membrane mechanical properties are derived from the composite structure of the membrane and the underlying cytoskeleton. One would expect this structure to be capable of bearing a much larger tensile stress in the direction of the aligned fibers (the flow direction) than in the transverse direction. This is consistent with the assumption of a uniaxial tension in the flow direction used in the model of Fung and Liu. However, cells aligned by flow were imaged under no-flow conditions. Therefore, the inherent tension generated by the contractile activity of the actin stress fibers appears to be sufficient to produce the cell profile predicted for the case of a uniaxially stressed membrane stretched over a deformable nucleus.19 Surface contour data on endothelial cells obtainable by AFM should permit testing of other aspects of their model.

Previously reported imaging techniques have not provided quantitative high-resolution topographical data of living cells. Nomarski and Hoffman modulation optics provide only a qualitative low-resolution impression of

**TABLE 2. Maximum Shear Stress and Shear Stress Gradient**

<table>
<thead>
<tr>
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<th>Maximum Shear Stress, dyne/cm²</th>
<th>Maximum Gradient, dyne/cm²</th>
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<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>18140</td>
</tr>
<tr>
<td>Sheared</td>
<td>14</td>
<td>8220</td>
</tr>
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For an average wall shear stress of 10 dyne/cm² applied to cells with either the sheared morphology or the unsheared control morphology, the values of maximum shear stress and maximum shear stress gradient were calculated using the parameters in Table 1.
surface contours of live cells by creating a shading effect. Scanning electron microscopy is subject to artifacts of fixation and dehydration of the sample. Transmission electron microscopy of a vertical section gives a high-resolution quantitative height profile, but for only a single section and only of fixed dehydrated cells. Contours imaged this way fall into the range of the profiles measured by AFM, but with transmission electron microscopy, there is uncertainty about where the section is taken (ie, not necessarily through the point of maximum height).

The two-dimensional information obtained by AFM is consistent with the findings of others for light-microscopic measurements (phase contrast and fluorescence). The aspect ratios are similar to those previously reported, although longer exposure to shear stress has been shown to produce higher aspect ratios in the range of 3 to 4. The organization of the fibrous structure detected by AFM is similar to the actin cytoskeleton revealed by immunofluorescence, although AFM was able to detect fibers that were smaller than could be accurately measured by light microscopy. Electron micrographs have shown actin bundles of various thicknesses up to 1 μm for the largest stress fibers terminating at focal adhesions. Our data are consistent with the electron microscopic data as well as AFM images of actin fibers in other cells.

AFM has allowed imaging of the surface morphology of living endothelial cells for the first time. The technique provides high resolution in the measurement of cell topography and requires no fixation and drying of the specimen. We have taken measures to ensure (1) that the cells were not significantly deformed by the imaging force and (2) the validity of detecting small projections from the cell surface at the imaging forces used. Furthermore, the images of unsheared cells were stable for up to 3 hours after removal from the incubator, indicating that the interactions with the probe did not significantly disturb the cell during this time period. It has been previously reported that at 3 to 4 hours in static culture at 37°C, aligned endothelial cells begin to revert rapidly back to an unaligned configuration. We did not observe this response during 4 hours of imaging; however, at room temperature, this response could be expected to be delayed somewhat. An incidental finding was that the sheared cells appeared to be much more resistant to imaging forces (producing stable images up to 4 hours after removal from the cone-and-plate device) because of the structural support afforded by the enhanced cytoskeleton, an observation consistent with the retention of shape by unfixed sheared cells noted in the membrane deformation experiments of Sato et al.

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Shear stress-induced reorganization of the surface topography of living endothelial cells imaged by atomic force microscopy.

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