THE DYNAMIC RESPONSE OF VASCULAR ENDOTHELium TO FLUID SHEAR STRESS IN VITRO

by

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JUN 23 1983
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Submitted to the Department of Mechanical Engineering on May 17, 1983 in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

ABSTRACT

The vascular endothelial lining in vivo is constantly exposed to hemodynamic shear stress resulting from normal or altered patterns of blood flow. There is considerable evidence that fluid forces can influence several aspects of endothelial cell biology and thus may play a role in the pathogenesis of atherosclerosis and hypertensive vascular disease.

A theoretical model of the fluid flow at the level of the cell-fluid microenvironment has been developed. The endothelial surface is modeled as a rigid surface with waves of distinct wavelength parallel and perpendicular to the mean vessel flow. A small perturbation technique yields the distribution of wall shear stress and pressure. The magnitudes of the force perturbations are estimated, based on measurements of cultured bovine aortic endothelium.

An in vitro apparatus has been developed to assess the dynamic response of endothelial cultures to controlled levels of fluid shear stress. The flow system is similar in operation to commercial cone-and-plate viscometers and is capable of producing physiological levels of laminar and turbulent shear stress. The experimental parameters studied include: cell shape and orientation, mitotic activity, motility (wound repair), and cytoskeletal organization. The alteration of endothelial morphology is examined with particular regard to the interaction of the cells with the local flow field.

Title: Professor
ACKNOWLEDGEMENTS

The completion of this work was possible through the cooperation of many individuals and I will attempt to recognize them here.

To my advisor, C. Forbes Dewey, Jr., I owe a particular debt. His valuable counsel and skill as a designer and experimentalist were extremely valuable to me throughout my tenure as a student. I thank him for the many times he lent assistance and, more importantly, for recognizing the times when I only thought I needed help.

The present form of the apparatus and the design of the biological experiments resulted from the cooperation of Drs. Michael A. Gimbrone, Jr. and Peter F. Davies of the Vascular Pathophysiology Laboratory at the Brigham and Women's Hospital and the Harvard Medical School. As well as providing expertise in endothelial cell biology and the laboratory facilities necessary for the experiments reported here, they were actively involved in the engineering interpretation of the results. Some of the experiments described in Chapter V have been published [Dewey et al. (1981), Bussolari et al. (1982)] and appear there in summary form. I thank Sheila Cruise and Ethel Gordon of the Vascular Pathophysiology Laboratory for their technical assistance in the performance of these experiments and their suggestions for refinements in the apparatus design. Drs. Gimbrone and Davies were responsible for the protocol of
the growth, orientation, and endocytosis experiments. Glenn White of the Department of Anatomy, Harvard Medical School provided the cytoskeletal organization data. Dr. Helmut Rennke of the Pathology Department, Harvard Medical School, assisted me with the cell topography measurements reported in Chapter III.

The help of Dick Fenner and Andrea Remuzzi was extremely valuable in the design and construction of the shear stress apparatus. I appreciate the assistance of Professors Marten Landahl, Harvey Greenspan, and Sheldon Weinbaum who served on my thesis committee. They provided guidance in the selection of the analytical model of the fluid flow presented in Chapter IV.

My friends in the Fluids Lab have made my stay there a pleasant one and I take fond memories of them with me. The love and support of my wife, Marilee has made this effort much easier and I thank her for the sacrifices that she has made.

Finally, I am indebted to the Whitaker Health Sciences Fund, the NRSA Grant Program, and the National Heart, Lung, and Blood Institute who provided financial support for this work.
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</tr>
<tr>
<td>A_x</td>
<td>magnitude of shear stress perturbation in x-direction.</td>
</tr>
<tr>
<td>A_z</td>
<td>magnitude of shear stress perturbation in z-direction.</td>
</tr>
<tr>
<td>A_p</td>
<td>magnitude of pressure perturbation.</td>
</tr>
<tr>
<td>i</td>
<td>square root of -1.</td>
</tr>
<tr>
<td>k</td>
<td>square root of ([2 + 2^2]).</td>
</tr>
<tr>
<td>P</td>
<td>fluid pressure.</td>
</tr>
<tr>
<td>P*</td>
<td>dimensionless fluid pressure.</td>
</tr>
<tr>
<td>(\hat{P})</td>
<td>pressure perturbation.</td>
</tr>
<tr>
<td>q</td>
<td>axis ratio of endothelial cell.</td>
</tr>
<tr>
<td>R</td>
<td>dimensionless parameter of cone-plate flow.</td>
</tr>
<tr>
<td>Re</td>
<td>Reynolds Number.</td>
</tr>
<tr>
<td>r</td>
<td>radial distance from apex of cone.</td>
</tr>
<tr>
<td>U</td>
<td>linear shear flow in x-direction.</td>
</tr>
<tr>
<td>u</td>
<td>fluid velocity in x-direction (in radial direction for cone-plate flow).</td>
</tr>
<tr>
<td>(\hat{U})</td>
<td>velocity perturbation in x-direction.</td>
</tr>
<tr>
<td>(\hat{V})</td>
<td>fluid velocity vector.</td>
</tr>
<tr>
<td>v</td>
<td>fluid velocity in the y-direction.</td>
</tr>
<tr>
<td>(\hat{V})</td>
<td>velocity perturbation in y-direction.</td>
</tr>
<tr>
<td>w</td>
<td>fluid velocity in the z-direction.</td>
</tr>
<tr>
<td>(\hat{W})</td>
<td>velocity perturbation in z-direction.</td>
</tr>
<tr>
<td>x</td>
<td>horizontal coordinate.</td>
</tr>
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</table>
y  vertical coordinate.
z  horizontal coordinate.

**GREEK**

\( \alpha \)  cone angle or wavenumber in x-direction.
\( \beta \)  wavenumber in z-direction.
\( \eta \)  coordinate surface of wavy-wall.
\( \hat{\eta} \)  amplitude of wall waviness.
\( \lambda_x \)  wavelength in x-direction.
\( \lambda_y \)  wavelength in z-direction.
\( \mu \)  fluid viscosity.
\( \nu \)  fluid kinematic viscosity.
\( \rho \)  fluid density.
\( \sigma \)  velocity gradient of shear flow.
\( \tau_{w} \)  mean wall shear stress.
\( \tau^* \)  dimensionless shear stress.
\( \tau_{yx} \)  shear stress in x-direction.
\( \tau_{yx}^* \)  dimensionless shear stress in x-direction.
\( \tau_{yz} \)  shear stress in z-direction.
\( \tau_{yz}^* \)  dimensionless shear stress in z-direction.
\( \psi \)  transformed variable used in Appendix A.
\( \omega \)  angular velocity of cone.
CHAPTER I
INTRODUCTION

Endothelial Structure and Function

The vascular endothelium forms a selectively permeable lining that covers the luminal surfaces of healthy mammalian arteries. The cells that make up this lining form a single-cell thick layer (monolayer) in which each cell is in intimate contact through "tight junctions" with its neighbor. Though smooth muscle cells and elastic lamellae give the arterial wall the mechanical properties necessary to its role as an active pressure vessel, the endothelium, as the component in intimate contact with the flowing blood, must provide a blood compatible surface. The geometry of the vascular endothelium is well suited as an exchange membrane for nutrients with a thickness on the order of a few microns and a total surface area, in humans, of several hundred square meters [Gimbrone (1981)]. It is highly resistant to platelet aggregation or the formation of thrombi on its surface. Being a metabolically active tissue, the endothelium is capable, through biochemical synthesis, of influencing the behavior of other cells within the vessel wall.

The vascular endothelial cell monolayer appears to be the primary resistance to molecular transport between the interior of the vessel wall and the flowing blood [Caro et al. (1978)]. Recent theories have implicated endothelial change or dysfunction as the precursor to atherosclerotic lesion
formation [Ross et al. (1976), Fry (1976)]. Though endothelial damage can be caused by a variety of stimuli, the location of atherosclerotic plaques within regions of sharp vessel curvature and branching constitutes evidence that fluid forces may play a role in the initiation of the disease [Caro et al. (1969), Fry (1976)]. The correlation between arterial locations exhibiting focal lesions and regions of large variations in fluid shear stress has produced much speculation regarding the relationship between this fluid force and atherogenesis.

The vascular endothelial lining in vivo is constantly exposed to hemodynamic shear stress resulting from normal and altered patterns of blood flow. There is considerable evidence that hemodynamic forces can influence several aspects of endothelial cell biology that are critical to normal vessel wall function. Endothelial integrity, biosynthetic activity, and regrowth following injury are among the processes that have been shown to be affected by fluid shear stress.

Numerous studies have attempted to clarify the relationship between shear stress and arterial integrity and those most relevant to the scope of this work are summarized in Table 1. The principal parameter of these investigations, the fluid shear stress, $\tau_w$, is defined as the product of the fluid viscosity, $\mu$, and the average velocity gradient at the vessel wall, ($\partial v/\partial y$), based on pipe or channel flow models:

$$\tau = \mu \frac{\partial v}{\partial y}$$
The actual coupling between hemodynamic shear stress and the vascular endothelium occurs in the region immediately adjacent to the individual cells. The flow in this cell-fluid microenvironment has a characteristic length scale of the order of the cell vertical dimensions, (~1 micron). Although the global wall shear stress defined above provides a measure of the average force on an endothelial cell, the distribution of stress on a typical cell as well as the details of the flow past the cell have not yet been modeled. Endothelial shape, motility, endocytosis, myototic activity, and cytoskeletal structure are examples of cell function that may be strongly linked to the distribution of fluid forces on the cell surface.

In Vivo Observations of Endothelial Shape

Because of the possible role of fluid forces in the pathogenesis of arterial disease, much attention has been focused on the morphology of the vascular endothelium in vivo. When preparations are made of arteries such that the endothelial cell shapes are visible [Silkworth et al. (1975), Langille et al. (1981)], it is observed that the cells are elongated in shape and align themselves in what appears to be the average flow direction. The degree of elongation of the cells seems also to correspond to estimates of the magnitude of the local average fluid shear stress on the arterial wall. The endothelium in regions where the average shear stress is estimated to be high have a very elongated shape with a
length-to-width ratio of approximately 5. The major axis of these cells is aligned with the direction of average fluid shear stress in a very consistent and organized pattern [Reidy et al. (1978)]. Disruption of this cell alignment is observed in areas where flow separation or turbulence is expected to take place [Gutstein et al. (1973), Bjorkerud et al. (1972)]. In regions of supposed stagnation, the cells exhibit a more random polygonal shape with significantly less elongation. Some researchers have inferred the magnitude and direction of the blood flow in the arteries from observations of endothelial shape and orientation in complex vessel geometries such as those found near flow dividers and branching vessels [Nerem et al. (1981)].

It seems clear that the flow of blood in the major arteries and, in particular, the tractive force of that flow on the endothelium has an effect on gross cell morphology. The relationship between that force and cell shape as well as the mechanical coupling between the cells and the fluid in their immediate vicinity is the subject of part of this thesis. Because the flow in arteries is pulsatile and the arteries themselves are of complex geometry, it is quite difficult to form a sufficiently detailed model to examine quantitatively the relationship between the local flow and endothelial cell structure and function.

The Significance of Transport Phenomena

The exchange of macromolecules between the blood and the
vessel wall takes place, in part, through a mechanism involving the bulk transport of plasma by endocytic vesicles within the endothelium. This process appears to be the dominant factor in transport rather than simple fluid convection or molecular diffusion [Weinbaum et al. (1976)]. Mechanical or biochemical effects that compromise the continuity of the endothelial layer or affect the permeability of the cell junctions may produce large increases in macromolecular transport [Pfeffer et al. (1981)].

Consolidation theory [Kenyon (1979), Blackshear et al. (1980)] predicts the presence of large hydraulic gradients across the vascular endothelium due to pulsatile arterial flow. Increases in the magnitude of pressure pulsations downstream of arterial restrictions have been thought to locally increase the permeability of the artery wall [Newman et al. (1977)]. The effects of fluid shear stress on the formation of endocytic vesicles or on the permeability of intracellular gaps may have dramatic effects on the overall transport of large molecules to the interior of the vessel wall.

Review of Previous Experiments

The classic experiment of Fry (1968) demonstrated the existence of a critical shear stress (~400 dynes/cm²) at or beyond which vascular endothelium sustains substantial damage (cytoplasmic swelling, cell deformation, and erosion) after short exposure (~1 hour).

Flaherty (1972) performed an in vivo experiment using dogs
nuclei in the direction of the mean blood flow. A segment of aorta was excised and resewn in place in such a way that the endothelial nuclei were oriented 90° to the mean flow. After 7-8 weeks, the nuclei were observed to be once again aligned with the mean flow.

Carew (1971) used a wedge-shaped flow chamber to expose excised dog aortic tissue to spatially-varying fluid shear stress in vitro. The shear stress in this experiment varied from 150 to 1100 dynes/cm² during a time exposure of approximately 1 to 2 hours. The endothelial cell population density was observed to decrease at the locations of higher shear stress and the permeability of Evans blue labeled albumin was found to be a function of the applied shear stress. It was observed that at a shear stress of approximately 600 dynes/cm², 50% of the cell population exhibited abnormal morphology.

A series of in vitro experiments was performed by Krueger (1971) using cultured human endothelium. The cells were cultured on glass in a parallel plate flow chamber. The effects of low level fluid shear stress (10⁻³ to 10⁻¹ dynes/cm²) were observed for exposure times of up to 24 hours. The cultured specimens exhibited a tendency to become detached from the substrate, resulting in a marked decrease in cell population density. Shorter term (~1 hour) exposures were performed at shear stress levels of 1-10 dynes/cm². In both types of experiments, no cell deformation was observed nor was any mitosis apparent, although the cells seemed to become detached at a greater rate by the higher magnitudes of shear
stress.

The experiments described above and summarized in Table 1 demonstrate the existence of an endothelial cell response to fluid shear stress. There remain, however, questions as to the significance of this response with respect to the pathogenesis of vascular disease. The average physiological fluid shear stress experienced by the arterial endothelium is from 2 to 40 dynes/cm² in the resting human. Peak physiological levels may reach 100 dynes/cm² [Ling et al. (1968)]. Because these values are below those at which dramatic endothelial damage is evident (300 - 400 dynes/cm²), it would appear that cell dysfunction, if it exists, is of a more subtle nature. Certainly the time scale of atherosclerotic plaque formation would indicate a more gradual mechanism. This is not to say that the more subtle effects of fluid shear stress may not be detectible; rather, it suggests that experiments designed to elucidate them must be of substantial duration and performed under extremely well-characterized conditions.

Organization of the Thesis

An in vitro apparatus has been developed to study the effects of fluid shear stress on cultured vascular endothelium under controlled conditions [Bussolari et al. (1982)]. A review of the design and construction of the apparatus is presented in Chapter II. The device is similar in geometry and operation to commercial cone-and-plate viscometers. The apparatus is capable of producing laminar shear stress of .01
to 100 dynes/cm² and turbulent shear stress of 2 to 200 dynes/cm². It is designed to maintain a cell culture environment for long-term experiments (greater than 7 days). Its purpose is to provide the experimental environment necessary to assess the alteration of endothelial morphology by fluid shear stress with particular regard to the interaction of the cells with the local flow field.

A theoretical model of the distribution of fluid forces has been developed at the level of the cell-fluid microenvironment. This model is based on the solution of the equations of motion of a viscous fluid with appropriate boundary conditions defined by the geometry of the endothelial surface and the imposed velocity gradient of the vessel flow. The flow close to the cell is dominated by viscous forces (Reynolds Number << 1) and the imposed upper boundary condition appears as a quasi-steady linear shear flow. A geometrical model of the endothelial surface is postulated in the theoretical analysis. It consists of a rigid surface with waves of distinct wavelength in the x and z directions that represent the shape of individual cells (Figure 1). A small perturbation technique is employed to obtain the distribution of pressure and wall shear stress over the surface. The spatial variation of these fluid forces on individual cells will be expressed in terms of appropriate surface shape parameters.

The characteristic dimensions of the endothelial cell monolayer have been determined through microscopic examination
and measurement of cultured vascular endothelium in vitro. The measurement procedures and results are presented in Chapter III. Quantitative comparisons have been made between these measurements and similar data obtained in vivo by other researchers. Such a comparison is extremely valuable to the application of experimental results obtained with the in vitro system.

Chapter IV contains an outline of the calculations of the flow in the cell-fluid microenvironment and estimates of the distribution of fluid forces on individual cells. The details of the mathematics involved are presented in Appendix A.

A description of in vitro experiments performed with the shear stress apparatus appears in Chapter V. The experimental parameters studied with the apparatus include cell shape and orientation, growth to confluence, motility (wound repair), fluid endocytosis, and cytoskeletal modification. The effects of steady as well as time-varying shear stress as in pulsatile flow were examined.

The results of the in vitro experiments and their relationship to the fluid mechanics of the microenvironment are discussed in Chapter VI. There, the measurements presented in Chapter III will be used to estimate the distribution of shear stress and pressure on endothelial cultures in vitro. The results will be compared to measurements made in situ by other researchers.
Principles of Cone-Plate Flow

In order to study the effects of fluid shear stress on endothelial cell structure and function, an in vitro system was developed that utilizes the geometry of a cone-plate viscometer (Figure 2). Shear stress is produced in the fluid contained between a stationary plate and a rotating cone. Whereas commercial cone-plate viscometers produce laminar, purely azimuthal flow, the device described here is designed to produce a wide range of shear stress encompassing both laminar and turbulent flow.

The flow induced by the rotating cone has been well characterized [Sdougos et al. (1983)] and found to have three regimes defined by the dimensionless parameter:

\[
\tilde{R} = \frac{r^2 \omega \alpha^2}{12v}
\]

where \( r \) is the radial distance from the apex of the cone, \( \omega \) the angular velocity of the cone, \( \alpha \) the cone angle in radians, and \( v \) the kinematic viscosity (viscosity/density) of the fluid (Figure 3). The parameter \( \tilde{R} \) is analogous to the Reynolds Number in that it measures the ratio of centrifugal to viscous forces acting on the moving fluid.

If \( \tilde{R} \ll 1 \), the centrifugal forces are insignificantly small and the fluid velocity is purely azimuthal \( [u(y,r)=0] \) with a
linear gradient in the vertical direction. Using the boundary conditions of no slip at the cone and plate surfaces, the azimuthal velocity, $v$, is

$$ v = \frac{W}{\alpha} $$

The fluid shear stress $\tau_w$ is, therefore, constant over the plate surface:

$$ \tau_w = \mu \frac{\partial v}{\partial y} = \frac{\mu W}{\alpha} $$

where $\mu$ is the viscosity of the fluid.

As $\tilde{R}$ becomes of order unity or larger, the fluid adjacent to the rotating cone experiences a centrifugal force that causes a flow in the radial direction. There is a corresponding centripetal flow in the fluid close to the stationary plate.

This radial, secondary flow $u(y,r)$ (Figure 3) causes the fluid streamlines to deviate from the purely azimuthal, concentric circles observed when $\tilde{R} \ll 1$. The shear stress at the plate is changed in magnitude and direction due to the inward flux of fluid with higher azimuthal velocity. For values of $\tilde{R} > 1$, the flow shows a tendency to become turbulent as observed in flow visualization experiments [Sdougos et al. (1983)].

It should be emphasized that $\tilde{R}$ is a local parameter that describes the type of flow at a given radial distance from the cone apex with all other parameters [$v$, $\alpha$, $W$] held
constant. Thus it is possible, as one progresses radially from
the cone apex, to encounter purely azimuthal flow, laminar
secondary flow, and, ultimately, turbulent flow in the same
apparatus. This is an important consideration in the radial
placement of specimens for shear stress experiments.

A semi-empirical expression for the shear stress on the
plate surface for all flow regimes is given by the
dimensionless equation:

\[
\tau^* = 1 + 2.58 \left( \frac{\tilde{R}^{3/2}}{3.5 + \tilde{R}} \right) - 0.86 \left( \frac{\tilde{R}^{5/2}}{(3.5 + \tilde{R})^2} \right)
\]

where

\[
\tau^* = \frac{\tau_w}{(\mu w / \alpha)}
\]

This expression was used as an aid in determining the geometry
of the experimental apparatus. The parameter \( \tau^* \) measures the
departure of the surface shear stress from the simple primary
flow for which \( R < 1 \) and \( \tau^* = 1 \). The form of the expression for \( \tau^* \)
is motivated by theoretical considerations, but the numerical
factors appearing in it are chosen to conform with experimental
data.

Details of Construction

A schematic of the important elements of the experimental
apparatus is shown in Figure 4 and a photograph of the actual
device is given in Figure 5. A separate box contains circuitry
to control the temperature of the apparatus and the rotational speed of the cone. A Plexiglas culture chamber provides a sterile environment for the portion of the device that is in direct contact with the cells and the culture medium. The cone and plate are fabricated of type 304 stainless steel, a material compatible with the cultures, and the plate is mounted on a Nikon microscope stage so that it may be raised and lowered. The plate, which contains the endothelial specimens and the culture medium, is raised until contact is made with the apex of the cone for a shear stress test. Lowering of the plate provides access to the test specimens for removal.

Accurate positioning of the plate is accomplished by measuring the electrical resistance between the cone and the plate. Contact of the apex of the cone with the plate is seen as a drop in that electrical resistance. The microscope stage may then be locked, holding the plate in the correct position for application of shear stress. This system has been found to yield a repeatability of positioning the cone apex and plate of ±0.01 mm.

The cone is supported by precision stainless steel ball bearings and is driven by a variable speed Bodine d.c. motor. Long-term variations in cone rotational speed are less than 1% with this system.

The temperature to which the endothelial specimens are exposed is maintained at 37.0°C ± 0.3°C by means of resistance heaters affixed to the underside of the plate and the inside wall of the culture chamber. The pH of the culture medium is
kept constant by maintaining a 5 vol % CO₂, 95 vol % O₂ gas mixture within the chamber. The filtered and humidified gas mixture is supplied to the chamber at the rate of 1 standard cubic foot per hour.

The plate is designed to accept 12 test specimens, each 12 mm in diameter. The specimens are commercial Belko #1 glass coverslips 0.15 mm thick onto which the vascular endothelium has been cultured. The coverslips fit into precisely machined recesses in the plate surface and are held in place by a vacuum applied to the underside of each coverslip. Figure 6 depicts a typical coverslip location in cross section. A thin film of nontoxic silicone vacuum grease is applied to the underside of each coverslip. After the coverslip has been positioned, the O-ring sealed plunger is drawn down and locked into place, producing a partial vacuum beneath the coverslip. The vacuum grease prevents leakage of medium into the space beneath the specimen. The discontinuity between the coverslip and the plate surface is less than 0.03 mm and the insertion and removal of the coverslips is accomplished without damage to the cells themselves.

The cone outer radius is 6 in. and the specimens are located along two concentric circles at 1/3 and 3/4 of the cone outer radius from the cone apex. The eight specimens located in the outer circle are sufficiently distant from the outer radius to avoid the effects of the flow at the cone edge.

For very small values of the parameter $\tilde{R}$, all 12 specimens will experience equal shear stress. For higher values of $\tilde{R}$,
the outer eight specimens will experience larger shear stress than the four inner specimens. If judicious choices are made of the various parameters \( [\alpha, r, v, \omega] \), it is possible to provide a high degree of standardization between experiments as well as perform simultaneous experiments at two values of shear stress in the same apparatus.

A summary of the operating characteristics of the shear stress apparatus is given in Figure 7. Cone angles of 0.5°, 1°, and 3° are employed at rotational speeds of up to 100 rpm producing shear stress from 0.01 to 200 dynes/cm² for fluid viscosities of \( 7.6 \times 10^{-2} \) to \( 3.04 \times 10^{-2} \) cm/sec. The four-fold change in culture medium viscosity may be obtained by addition of up to 7 wt % 80,000 molecular weight dextran. Dextran concentrations of up to 10 wt % in culture medium were found, by experiment, to be compatible with the growth of endothelial cell cultures [Bussolari (1980)]. For each cone angle, \( \alpha \), the variation in fluid viscosity, \( v \), cone rotation speed, \( \omega \), and specimen location, \( r \), permits operation in any region of \( \tilde{R} \) and \( \tilde{\tau}_w \) defined in Figure 7. Thus, for a given \( \tilde{\tau}_w \), the apparatus may be chosen to achieve laminar (\( \tilde{R} < 1 \)) or turbulent (\( \tilde{R} > 1 \)) flow conditions.

The preparation and handling of the endothelial cell specimens, up to the moment they are placed in the apparatus and from the time they are removed, follow standard cell culture techniques (see Chapter V). Chemical assay of the medium is simplified by the small fluid volume required in the cone-plate geometry (15-60 ml, depending on the cone angle).
With the motor detached, the entire Plexiglas chamber and its contents may be subjected to ethylene oxide sterilization.

The present form of the apparatus arose, in part, from a careful study of the problems encountered by previous methods of assaying cell response to fluid flow. An in\textit{ vitro} approach was chosen to avoid the deleterious effects associated with the surgical intervention of in\textit{ vivo} methods [Fry (1968), Greenhill et al. (1981)]. Cultured cells are free of these preparatory artifacts while maintaining the characteristics and morphology of the in\textit{ vivo} cell [Gimbrone (1976)]. The cone-plate system permits a wider dynamic range of shear stresses than other geometries (channel, fluid jet, concentric cylinders) [Haudenschild et al. (1980), Rosen et al. (1974), Thibault et al. (1980)] and requires only a small fluid volume. The present apparatus does not accommodate optical microscopy during shear stress application, but the ease with which the specimens are removed and replaced permits microscopic examination at selected points in time during an experiment. If desired, the cells may be fixed in\textit{ situ} under shear by infusing fixative into the fluid volume.

An important attribute of the present design is the availability of 12 replicate samples. The large number of specimens increases the reliability of all quantitative measurements, as well as allowing increased flexibility in choosing experimental protocols (e.g., the comparative response of two different cell types under identical conditions).
Biological Compatibility of the Apparatus

An experiment was performed to determine whether cells placed in the sterile apparatus under no-flow conditions might show altered growth kinetics compared to cells grown in standard culture dishes. Bovine aortic endothelial cells (strain 11-BAEC) were replicate plated on glass coverslips placed in the bottoms of Costar-24 culture wells \(4 \times 10^4\) cells/well) in growth medium supplemented with 10% fetal calf serum. After 48 hours, some coverslips were transferred to fresh plastic culture wells and others inserted into the baseplate of the apparatus. The baseplate was placed in the Plexiglas test chamber and raised until contact was made with the apex of the cone. The cone was not rotated during the experiment. The number of viable cells was determined on duplicate samples at daily intervals thereafter. As the population density increases to approximately \(1.8 \times 10^5\) cells per square centimeter, the endothelial cells normally form a continuous (confluent) monolayer in which further cell division and growth is inhibited by contact with neighboring cells. As seen in Figure 8, population doubling times and final (confluent) population densities were essentially the same in both the incubated cells and those in the apparatus under no-flow conditions. This verifies the fundamental biological compatibility (i.e., non-toxicity) of the apparatus.

Comparisons of the growth rate and stable population density were made between cultures subjected to fluid shear stress in the cone-plate apparatus and control cultures in
static medium. The growth behavior of sparsely seeded endothelial cell cultures continuously exposed to 1 dyne/cm² shear stress was essentially identical to that of replicate-plated cultures maintained under standard petri dish conditions (Figure 9). When cultures were plated at two different subconfluent densities (≈ 2.5 and 5.0 x 10⁴ cells/cm²) and then subjected to 5 dynes/cm² shear stress, cell growth proceeded at the same rate in both groups and the final cell densities attained were comparable to those of static control cultures (Figure 10). Post confluent monolayers exposed to 1 and 5 dynes/cm² maintained a stable cell density (approximately 1.5 x 10⁴ cells/cm²) for up to 1 week. Thus, constant exposure to moderate physiological levels of shear stress does not appear to alter the basic growth kinetics or population behavior of vascular endothelial cells in vitro.

The apparatus has been specifically designed to expose cultured endothelium to a controlled hemodynamic environment. Although this in vitro system does not, at the present time, attempt to reproduce precisely the flow and milieu experienced by vascular endothelium in vivo, it has been used to observe significant changes in endothelial cell shape and orientation, wound regeneration, fluid endocytosis, and platelet interaction upon exposure to shear stress of 1-15 dynes/cm² (see Chapter V). During these and other experiments, healthy endothelial cultures have been maintained in the apparatus for periods of up to 14 days.
CHAPTER III

ENDOTHELIAL CELL SURFACE TOPOGRAPHY

In order to estimate the external forces that are imposed on a cell layer by the flow, it is necessary to construct a model of its surface topography. This is only possible through actual measurement of the cells themselves in three dimensions. Here, the surface topography in vitro will be described for the specific substrate and culture conditions of the experimental apparatus described in Chapter II.

Measurement Techniques

The dimensions of a cell layer can be measured in several ways. Because the surface topography is of primary importance in this analysis, two methods were investigated: en face scanning electron microscopy (SEM); and light microscopic examination of cross-sections of the endothelial monolayer.

The scanning electron micrograph provides an image of the surface of the cell layer. The cells themselves must be fixed and coated with a metallic substance to provide the electron scattering necessary to produce the image. Alternatively, unfixed cells may be cast in a plastic medium in such a way as to produce a negative impression of the cell surface in the casting medium. The plastic cast may then be coated for SEM, thus eliminating the necessity for fixing the cells. The SEM has the capacity to produce stereoscopic micrographs of the specimen from which depth and topographical information can be
deduced. The potential disadvantage of any technique involving fixation of the cell specimens is the possibility of distortion of the cell surface topography. In general, the fixation procedure is designed to cross-link the proteins or lipids in the membrane and internal structures of the cells, causing them to become rigid and fixed in their geometry as well as impervious to decay. Once the structures have been fixed, the water in the cells must be removed without causing collapse of the surrounding material. This can be accomplished by air drying or critical point drying (CPD) in which the water is removed by replacing it with carbon dioxide at its critical pressure and temperature. Of the two techniques for removing the intracellular water, CPD is considered to be least likely to produce large changes in the shape of the cell.

In order to determine the suitability of SEM for the determination of cell surface topography, a series of preparations were made of cultured endothelium grown on the same glass coverslips used in the shear stress apparatus but maintained under standard petri dish conditions (no flow). The object of these experiments was to determine if enough resolution of the surface topography was possible to permit accurate measurements, and if the preparation procedure produced significant distortion of the cell.

Three techniques were utilized to prepare the cells for the SEM. The first involved fixation with gluteraldehyde followed by CPD and metallic coating. In the second procedure, air drying followed the gluteraldehyde fixation. Both
procedures were performed with the cells still on the coverslips. The third technique involved casting the cells in a plastic (Batson's medium). This casting approach has been used previously in the visualization of arterial endothelial shape in situ [Levesque et al. (1977)]. Both fresh and gluteraldehyde fixed cells were covered by a thick layer of Batson's medium which was allowed to harden. The cured plastic, containing an imprint or negative image of the cell surface, was then peeled away from the coverslip. Any cellular material that clung to the plastic was dissolved away by an acid solution. The plastic was then coated with gold-palladium to prepare it for the SEM. The purpose of the Batson's medium preparation was to attempt to produce an image of unfixed cells. This method is, potentially, the most reliable in replicating the surface topography because no prior chemical treatment of the cells was performed.

Scanning electron micrographs of cultured endothelial monolayers prepared by each of the three preparation procedures are presented in Figure 11. None of the specimens were exposed to fluid shear stress prior to fixation. It is clear from the photomicrographs of the fixed cells, taken at an oblique angle to the cell surface, that the cells appear quite flat and exhibit no real large scale topography. The nuclei are visible as slightly raised areas and the cytoplasm contains many large peri-nuclear vacuoles. The presence of these vacuoles may be an artifact of the culture itself. In fact, phase contrast microscopy of cultured endothelium does reveal the presence of
these vacuoles in living cells [Davies, P.F., personal communication]. However, the lack of gross surface topography indicates that the fixation techniques may have caused a collapse of the cells and thus did not produce an accurate representation of the cell surface topography. This hypothesis is supported by the photomicrograph of the unfixed cell surface cast in Batson's medium (Figure 11). The large peri-nuclear vacuoles are visible as faint impressions in the cast, suggesting that they are perhaps representative of the morphology in culture. The cast appears also to have a more prominent three-dimensional structure than either of the fixed specimens, though the amplitude of the surface waviness due to the cells is still quite small compared to their lateral dimensions.

Because of the lack of prominent surface topography, due either to fixation artifact or actual endothelial morphology, the use of SEM could not provide accurate measurements of the cell surface topography. Hence, alternate methods were investigated as described below.

**En Face Phase Contrast Microscopy**

Measurements of phase contrast photomicrographs of living cell cultures, such as those of Figures 17 and 18, are useful in determining the lateral dimensions of individual endothelial cells in culture. Cell length, width, shape and orientation have been routinely catalogued through the use of digital image processing techniques, [Milich (1982)]. The distribution of
cell eccentricity (the ratio of cell length/width) and orientation for cultures exposed to fluid shear stress in vitro are presented in Chapter V. Average values of cell length and width found through such measurements are presented in Table 2 for cells exposed to shear stress as well as static controls. The remaining cell surface parameter, the cell height, was measured by examining the cross sections as described below.

Thick Sectioning of Cultured Endothelium

The measurement of cross-sections of the endothelial specimens eliminates the need for stereoscopic photomicroscopy of the surface and instead provides a direct indication of cell thickness. In this technique, the cells are fixed in gluteraldehyde and the coverslip with the cells on it is imbedded in a plastic (Epon). The plastic is removed from the coverslip by immersion in liquid nitrogen so that the differences in coefficient of thermal expansion of the glass and the plastic causes the two to separate. The Epon, containing the cells, is cut into sections 1 micron thick on a microtome and the sections are stained and examined under the light microscope. Through optical measurement of these sections, the shape and height of the cell surface can be determined.

There are difficulties associated with attempting to totally reconstruct the cell surface topography from a series of two dimensional sections, but, when combined with the data taken from the en face phase contrast photomicrographs,
sufficient information can be extracted to permit a useful model to be made. Figure 12 shows the appearance of the stained sections of endothelial cultures that have been either subjected to 8 dynes/cm² for 72 hours or incubated under static conditions. The cross section of Figure 12(b) was taken perpendicular to the direction of flow. The similarity between the cross sectional shape of the cells exposed to shear stress and those incubated under static conditions is striking in view of the dramatic shape change the shear-exposed specimens exhibited in en face phase contrast micrographs (see Figure 18). The curvature and shape of the surface of the two specimens is not substantially different. An important parameter, the vertical dimension of the cell at its maximum height, was measured and averaged over 25 individual cells in both the static and shear-exposed specimens. Because the thickness of the sections is comparable with the dimensions of the endothelial cell nucleus, only those sections that contained cell nuclei were measured in order to ensure that the maximum height of the cell was apparent. The results were combined with the en face measurements and are summarized in Table 2. It will be demonstrated in Chapter V that the cell density (number of cells/unit area) does not change with exposure to shear stress. The cells, therefore, undergo shape change such that their length and width are altered, but not cell area. This, coupled with the observation of constant cell height, indicates that cell volume must not be substantially changed.
The three characteristic dimensions of the static and shear-exposed cells: length, width, and cell height given in Table 2 are used in Chapter VI to calculate the fluid forces (pressure and shear stress) to which the endothelial surface is exposed. There, the mathematical model of the flow over a three-dimensional wavy surface, presented in Chapter IV, will be employed to estimate the relationship between these forces and endothelial cell shape.
CHAPTER IV

MODEL OF THE CELL-FLUID MICROENVIRONMENT

Endothelial Surface Model

In the previous chapter, the endothelial surface was examined in vitro using scanning electron and light microscopy. Using these data and the theoretical model to be developed in this Chapter, it is possible to estimate the character of the fluid flow in the cell-fluid microenvironment and calculate the distribution of force applied to the endothelial surface by the fluid. An appropriate geometrical model for the cell surface topography must provide the necessary flexibility and detail to provide useful information about the flow. At the same time, the model must be simple enough to be analytically tractable. A model geometry that meets both requirements is described below.

The endothelial surface is approximated as the three-dimensional coordinate surface described by the equation

\[ y = \hat{n} \equiv \hat{n} \cos(\alpha x) \cos(\beta z) \]

This is a surface with waviness in the x and z directions, each with a distinct wavenumber, \( \alpha \) and \( \beta \) (see Figure 1). The amplitude, \( \hat{n} \), is the third parameter that defines the surface topography. By independent variation of the wavenumbers and the amplitude, a large number of cell surface geometries may be simulated. Figures 13 and 14 are illustrations of this
geometry and coordinate system. In Figure 13, the wavenumbers $\alpha$ and $\beta$ have been chosen to be equal and the amplitude equal to approximately $1/4$ of the wavelength. The resulting regular and symmetric pattern is similar to that found in endothelial cells cultured under static conditions. It should be noted that actual cell geometries as observed by the methods of Chapter III have relative vertical dimensions much smaller that those depicted in Figures 13 and 14. This exaggeration of surface topography in the figures serves to illustrate the geometry rather than actual cell shape. In Figure 14, the wavenumber $\alpha$ has been decreased by a factor of 4 (thus increasing the wavelength in the $x$-direction by the same factor) while $\beta$ and $\eta$ have remained constant. The resulting elongated shapes are suggestive of the spindle-shaped endothelium observed in vitro and in vivo (see Chapter V).

Equation 7 may be expanded as follows:

$$y = \frac{\eta}{2} \left[ \cos(\alpha x + \beta z) + \cos(\alpha x - \beta z) \right]$$

and the desired geometry expressed as the superposition of two (or more) wavy surfaces of the form

$$y = \frac{\eta}{2} \cos(\alpha x + \beta z)$$

where $\alpha$, $\beta$, and $\eta$ are chosen appropriately. The wavenumbers $\alpha$ and $\beta$ are related to the length and width of the cells as follows:
\[ \alpha = \frac{2\pi}{\lambda x} \]
\[ \beta = \frac{2\pi}{\lambda z} \]

where \( \lambda_x \) is the length of the cell in the x-direction and \( \lambda_z \) is the length in the z-direction. A length-to-width ratio may then be defined as:

\[ q = \frac{\lambda x}{\lambda y} = \frac{\beta}{\alpha} \]

For convenience in solving the equations of motion of the fluid, the complex form

\[ y = \eta \ e^{i(\alpha x + \beta z)} \]

the real part of which is equal to Eq. 9 is used.

**Fluid Mechanics of the Microenvironment**

The flow adjacent to the endothelial cell layer has a Reynolds Number (the ratio of inertial forces to viscous forces) that can be estimated using the parameters illustrated in Figure 15. The endothelium experiences a shear stress that is generated by the velocity profile of the blood (or, in vitro, the culture medium) flowing in the vessel. The length scale of the flow in the cell-fluid microenvironment is extremely small compared to the diameter of any major artery.
Therefore, the influence of the bulk flow of blood in the artery upon the endothelial layer will be modeled as a quasi-steady linear shear flow. This assumption is justified by the extremely small vertical dimensions of the cell layer [\( \hat{\eta} \approx 1\mu m \)] and the ability of the viscous flow to readjust rapidly to changes in the upper boundary condition over such distances through momentum transfer. The Reynolds Number can, therefore, be calculated by the following expression:

\[
Re = \frac{\sigma \hat{\eta}^2}{\nu}
\]

where \( \sigma \) is the shear rate, \( \hat{\eta} \) is the characteristic height of the cell and \( \nu \) is the kinematic viscosity of the blood. Typical physiological values of these parameters are

\[
\sigma \approx 10^3 - 10^4 \text{ sec}^{-1}
\]

\[
\hat{\eta} \approx 10^{-5} \text{ cm}
\]

\[
\nu \approx 10^{-2} \text{ cm}^2/\text{sec}
\]

The Reynolds Number, therefore, is quite small, being on the order of \( 10^{-2} \) to \( 10^{-3} \). The small value of \( Re \) is indicative of the dominance of viscous forces in the flow adjacent to the endothelium. It is the assumption of very small (essentially zero) Reynolds Number that is key to the analysis that follows. The equations of motion of a Newtonian fluid with
negligible inertia are:

\[ \nabla^2 \mathbf{v} = \frac{1}{\mu} \nabla p \]

\[ \nabla \cdot \mathbf{v} = 0 \]

where \( \mathbf{v} \) is the fluid velocity vector, \( \mu \) is the fluid viscosity and \( p \) is the pressure.

The effect of the sinusoidal boundary is introduced as a velocity perturbation of the following form:

\[ \mathbf{v} = \mathbf{v}(y) e^{i(\alpha x + \beta z)} \]

A similar form is adopted for the pressure perturbation. Each velocity and pressure perturbation is composed of a function of the vertical coordinate, \( y \), multiplied by a fixed function of \( \alpha \) and \( \beta \). This function introduces a sinusoidal modulation in the \( x \) and \( z \) directions that will be made to match that of the cell surface model of Eq. 7.

The boundary condition at \( y=\infty \) is a linear shear flow in the \( x \) direction:

\[ \left. u \right|_{y \to \infty} = \sigma y \]

where \( \sigma \) is the shear rate.

The boundary conditions at the cell surface are zero velocity:
The boundary \( \eta \) is defined in a way that facilitates superposition of multiple solutions in order to arrive at a general solution for the boundary of Eq. 7. If the amplitude of the sinusoidal boundary is small and the wavelength is large, the boundary conditions at \( y=\eta \) can be linearized. The details of this solution are given in Appendix A and the results presented here for the endothelial surface model of Eq. 7.

**Calculation of Fluid Forces on the Endothelial Model**

The shear stress and pressure force on the model endothelial surface are reproduced here from Eqs. A-59 to A-61 of Appendix A (for \( y=0 \)):

\[
\tau_{yx} = \mu \sigma \left[ \frac{2\alpha^2 + \beta^2}{\sqrt{\alpha^2 + \beta^2}} \hat{\eta} \cos(\alpha x) \cos(\beta z) + 1 \right] \quad 23
\]

\[
\tau_{yz} = \mu \sigma \left[ - \frac{\beta \alpha}{\sqrt{\alpha^2 + \beta^2}} \hat{\eta} \sin(\alpha x) \sin(\beta z) \right] \quad 24
\]

\[
P = \mu \sigma \left[ - 2\alpha \hat{\eta} \sin(\alpha x) \cos(\beta z) \right] \quad 25
\]

Here, \( \tau_{yx} \) is the surface shear stress in the \( x \)-direction and \( \tau_{yz} \) is the surface shear stress in the \( z \)-direction.

The term \( \mu \sigma \) is the mean wall shear stress imposed by the flow far (i.e. many times the cell height) from the endothelial
surface. When normalized by this average shear, the non-dimensional forms, denoted by a superscript \( \ast \), of the shear stress and pressure become:

\[
\tau_{yx}^\ast = \tau_{yx} \mu_\sigma \quad 26
\]

\[
\tau_{yz}^\ast = \tau_{yx} \mu_\sigma \quad 27
\]

\[
p^\ast = p \mu_\sigma \quad 28
\]

The resulting equations describing the pressure and shear stress are:

\[
\tau_{yx}^\ast = 2\pi \frac{2 + q^2}{\sqrt{1 + q^2}} \frac{\hat{n}}{\lambda_x} \cos(\alpha x) \cos(\beta z) + 1 \quad 29
\]

\[
\tau_{yz}^\ast = -2\pi \frac{q}{\sqrt{1 + q^2}} \frac{\hat{n}}{\lambda_x} \sin(\alpha x) \sin(\beta z) \quad 30
\]

\[
p^\ast = -4\pi \frac{\hat{n}}{\lambda_x} \sin(\alpha x) \cos(\beta z) \quad 31
\]

The above expressions for the pressure and shear stress are each proportional to the ratio of the cell height, \( \hat{n} \), to its length in the \( x \)-direction, \( \lambda_x \).

**Effect of Cell Shape on the Distribution of Fluid Forces**

The surface shear stress in the \( x \)-direction (Eq. 29) consists of the sum of the average stress imposed by the vessel
flow (normalized to 1) and a spatially varying stress perturbation due to the cell shape. The magnitude of the shear stress perturbation depends on $q$ and $\hat{\eta}/\lambda_x$. As $\hat{\eta}/\lambda_x$ increases, the longitudinal shear stress perturbation increases linearly. This is expected because $\hat{\eta}/\lambda_x$ is a direct measure of the prominence of the cell height in the $x$-direction.

Less intuitive is the behavior of the term involving the length-to-width ratio, $q$. A graph of this term is shown in Figure 16. For values of $q$ less than approximately 2, the quadratic nature of the term is apparent. However, as $q$ becomes greater than 2, the function rapidly approaches the asymptote, $q$, shown as the dashed line in Figure 16. In general, as the cell length-to-width ratio becomes larger ($q$ increases), $\hat{\eta}/\lambda_x$ should tend to decrease. This indicates a competing effect of the two cell shape parameters on the magnitude of the shear stress perturbation in the $x$-direction. Since the term involving $q$ asymptotes quickly to $q$, the magnitude of the shear stress will be proportional to the product of $q$ and $\hat{\eta}/\lambda_x$.

$$|\tau_{yx}^*| \sim q \frac{\hat{\eta}}{\lambda_x}$$  

The phase of the perturbation is identical to that of the cell shape (see Eq. 7): the shear stress perturbation is at a maximum at the highest point on the cell surface.

A cell surface with no waviness subjected to a linear
shear flow in the x-direction will experience no shear stress perpendicular to the direction of flow. However, the presence of surface waviness introduces a lateral shear stress perturbation (Eq. 30). As with $\tau^*_{y-x}$, the magnitude of this perturbation is linear with $\hat{\eta}/\lambda_x$. The dependence of $\tau^*_{y-z}$ on $q$ is markedly different, however, as shown in Figure 16. For values of $q$ greater than 2, the variation of magnitude with $q$ disappears. Therefore, as the cell elongates to axis ratios greater than 2, the lateral shear stress depends only on $\hat{\eta}/\lambda_x$. The relationship between the phase of the surface waviness and $\tau^*_{y-z}$ is quite different from that of $\tau^*_{y-x}$. The lateral shear stress is 90° out of phase with the surface waviness, $\tau^*_{y-z}$ being maximum or minimum at the points of maximum surface slope. It is also asymmetric with respect to the cell, tending to force the cell boundaries away from the longitudinal axis of the cell on the proximal surface and toward that axis on the distal surface.

The pressure perturbation shows no dependence at all on the length-to-width ratio, $q$, but is linear with $\hat{\eta}/\lambda_x$. It is asymmetric with respect to the cell longitudinal axis, tending to increase the pressure on the proximal side and reduce it on the distal side. This pressure perturbation is in addition to the pressure imposed on the cell layer by the vessel flow (i.e. the systolic blood pressure).

Calculations of the magnitudes of the pressure and shear stress perturbations are possible given the actual dimensions of the endothelial cell monolayer. In Chapter VI, estimates of
the distribution of surface shear stress and pressure are made using \textit{in vitro} and \textit{in situ} measurements.
CHAPTER V
SHEAR STRESS EXPERIMENTS

Endothelial Cell Culture Methods

Methods for the isolation and culture of vascular endothelial cells are described in detail in the literature [Gimbrone (1976)]. Primary cultures are obtained from segments of yearling calf thoracic aortas that are clamped in situ and excised into sterile containers at a local slaughterhouse. The endothelium is harvested from the aortas by perfusing them with a collagenase solution that dissolves the matrix by which the cells are attached to the vessel wall. Individual cells become suspended in the perfusate and are plated at a density of 1-2 x $10^4$ cells/cm$^2$ in Dulbecco's-modified-Eagle's medium (DME) supplemented with 20% fetal calf serum. After growth to confluence (stable density, 1-2 x $10^5$ cells/cm$^2$; 6-7 day time course), cells can be harvested and plated on 12mm glass coverslips for experimentation. In all the experiments reported here, a single strain of bovine aortic endothelial cells (II-BAEC), that is free of smooth muscle cell contamination was utilized.

Endothelial cell cultures that are subjected to fluid shear stress in the cone-and-plate apparatus are always compared with control specimens. The controls are from the same set of replicate cultures and are plated on identical coverslips and incubated under static conditions in a volume of medium comparable to that in the shear stress apparatus.
Endothelial Growth in the Presence of Shear

As presented in Chapter II, the basic growth kinetics and stable population density of cultured endothelial monolayers are not affected by exposure to moderate levels of fluid shear stress (5 dynes/cm² or less).

In order to determine the level of cell replication (mitosis) induced by exposure to fluid shear stress, experiments were performed in which the incorporation of ^3H^H-thymidine (^3H-TdR) into the DNA of the endothelial nuclei was measured. Positive radioactive labeling of a cell nucleus indicates that the cell has entered the cell division cycle. Confluent 11-BAEC monolayers were exposed to fluid shear stress of 1 or 8 dynes/cm² for two hours, removed from the apparatus, and incubated with ^3H-TdR for 22 hours under static conditions. The number of labeled cell nuclei were essentially equivalent to control specimens that were maintained in static culture. This indicates that exposure to these levels of shear stress does not cause confluent monolayers to enter the cell division cycle. This is in contrast to the results of Stoker (1973) who observed increased cell replication in culture of Balb/C-3T3 (mouse fibroblast) cell monolayers exposed to a variable shear stress of approximately 0.1 dynes/cm². The results of the ^3H-TdR labeling assays are important in the interpretation of the fluid endocytosis experiments presented below.
Shear Effects on Cell Shape and Orientation

In the growth experiments described above, the endothelial monolayers were examined for signs that the cells had become elongated and oriented in the direction of the imposed shear stress as has been observed in vivo (Silkworth et al. (1975), Reidy (1978), Cornhill et al. (1978), and Langille et al. (1981)). When the cultures were continuously exposed to 1 or 5 dynes/cm², for up to 8 days, their appearance did not vary significantly from that of the static controls. A slight tendency for cell alignment with the flow was observed in subconfluent specimens exposed to 5 dynes/cm².

When confluent monolayers were exposed to 8 dynes/cm², dramatic morphological changes developed. After up to 24 hours exposure to shear, the appearance of the monolayer was essentially that of the static control (Figure 17). However, 48 hours of shear exposure produced cell elongation and orientation in the direction of flow (Figure 18). Continued exposure to shear stress beyond 48 hours produced no further shape changes. Figure 19(a) and (b) illustrate quantitative measurements of the degree of cell shape change observed. The measurements were obtained by calculating an ellipse that represented the best fit to the individual cell shape [Milich (1982)]. The cell eccentricity is the ratio of the major to minor axes of this best fit ellipse and the orientation angle is measured between the major axis and the direction of flow. The aligned cells exhibit an eccentricity of approximately 3.5 and show a definite alignment with the direction of flow.
whereas static controls have an eccentricity close to 1.
Similar experiments were performed using cultures of smooth
muscle and 3T3 cells. Neither smooth muscle nor 3T3 cultures
exhibited any tendency to become elongated or align with the
flow after 72 hours of exposure to 8 dynes/cm² shear stress.

Endothelial monolayers aligned by exposure to 8 dynes/cm²
for 72 hours were exposed to the same magnitude of shear stress
at 90° to the original direction by rotating the glass coverslip
in its location in the apparatus. The endothelium tended to
become realigned with the new direction of flow. In all cases
of cell elongation and alignment with the direction of shear
stress, the cultures showed a tendency to resume the polygonal
shape and random orientation typical of static controls within
approximately 24 hours after removal from the apparatus. Time
lapse cinematography was used to study this relaxation process.
This technique [White et al. (1982)] produces a series of still
photomicrographs on 16mm film. The resulting motion picture,
when viewed at normal speed, demonstrates the movement of the
endothelial monolayer at 400 times normal speed. Figure 20
contains three frames of the time lapse film taken at intervals
of 1, 8, and 15 hours post-shear. The progression from
ellipsoidal cells, aligned with the direction of flow to
resumption of a random, polygonal shape is evident. The motion
picture is quite dramatic in its depiction of short time scale
cell movement. Individual cells were seen to move several
times their own diameter during reorientation periods of
approximately 10-20 minutes. Groups of cells exhibited gross
movement in concert, punctuated by periods of non-movement. Occasionally, a cell would undergo surface spiculation and rapid (~1 minute) cytoplasmic oscillations while remaining attached to a single site. The frequency and amplitude of the cell movement gradually decreased as the monolayer assumed the appearance of the static controls.

Regeneration of Monolayer Under Shear

In preliminary experiments, confluent endothelial monolayers were given a standardized linear defect ("wound") by scratching the cultures with a sharp instrument. The specimens were then exposed to 8 dynes/cm$^2$ to determine the effects of shear exposure on cell migration and proliferation. Figure 21(a) shows a culture in which the direction of shear stress is parallel to the wound. Cells migrating into the denuded area are strongly oriented with the flow, the degree of orientation decreasing with distance from the edge of the migrating layer. In Figure 21(b), the wound is oriented perpendicular to the flow and cells appear to migrate much more rapidly in the direction of flow and less rapidly when they must travel upstream.

Shear Effects on Fluid Endocytosis

The uptake of a tracer molecule, horseradish peroxidase, (HRP) was measured in a variety of experiments as a measure of the degree of fluid endocytosis by the endothelial monolayer. As seen in Figure 22, there is a significant increase in the
appearance of intracellular HRP in cultures exposed to a steady shear stress of 1 or 8 dynes/cm² over static controls.

In a second series of experiments, the effect of accommodation to shear stress on fluid endocytosis was investigated. Endothelial monolayers were exposed to 8 dynes/cm² shear or static conditions for 48 hours then incubated with HRP for 2 hours under shear or static conditions. As shown in Figure 23, the specimens that were accommodated either to no flow or 8 dynes/cm² exhibited equal rates of endocytosis when they were incubated with HRP at the conditions under which they had become accommodated. However, if the shear stress was changed, either by subjecting no-flow accommodated cells to 8 dynes/cm² or cells accommodated to 8 dynes/cm² to no-flow conditions, the rate of fluid endocytosis was almost doubled.

The effect of a periodic variation in shear stress upon fluid endocytosis was assessed in an experiment during which specimens were alternately exposed to steady shear stresses of 1 and 8 dynes/cm². The shear stress level was changed every 15 minutes. The rate of fluid endocytosis was found to be almost twice that of static controls. Similar experiments, in which the shear stress level was changed every 5 minutes did not produce a significant increase in the rate of fluid endocytosis over static controls.

Finally, the response of the cultures to high frequency oscillating shear stress was investigated. The cone axis of
of rotation, producing a slight "wobble" in the cone rotation. This produced a sinusoidal oscillation in the shear stress of frequency 1 Hz whose magnitude varied from 3 to 13 dynes/cm$^2$, with an average shear stress of 8 dynes/cm$^2$. In experiments covering more than 30 combinations of shear stress and time of sampling subsequent to addition of HRP, no observable change in fluid endocytosis was found. The results of these experiments involving periodic application of shear stress are summarized in Figure 24.

Shear Effects on Cytoskeletal Organization

The effects of shear stress on endothelial microfilaments (stress fibers) in vitro and their distribution in normal as well as hypertensive rats has been investigated by White (1983). The in vitro studies were carried out with the shear stress apparatus and the results are summarized here. Endothelial specimens were exposed to 8 dynes/cm$^2$ shear stress for 72 hours and upon removal from the apparatus, were stained with antibodies specific to actin and myosin, the two major components of cytoskeletal stress fibers, and the appearance of the cells compared to static controls. The resulting photomicrographs are shown in Figure 25. There is a marked difference between the static controls on the left and the shear-exposed specimens on the right. The stress fibers in the cultures exposed to shear stress appear larger, more numerous, and organized in the direction of flow. This is in contrast to
the case of anti-actin, a diffuse cytoplasmic staining, that is less prominent in the specimens exposed to shear stress.

The experiments described above demonstrate significant changes in endothelial structure and function upon exposure to physiological levels of fluid shear stress. A discussion of the experimental results and their relationship to the flow in the cell-fluid microenvironment will be given in the following Chapter.
CHAPTER VI

DISCUSSION

Variation of Fluid Forces with Cell Shape

The data obtained through measurement of the cultured endothelial monolayers presented in Chapter III may be utilized to estimate the magnitude and distribution of fluid forces on individual cells. The theory developed in Chapter IV expresses the perturbation in fluid shear stress and pressure as a function of two cell shape parameters: the ratio of length to width, \( q \), and the ratio of height to length, \( \eta/\lambda \). The results of Chapter IV can be summarized below:

\[
\tau_{yx} = \tau_w [A_x \cos(\alpha x) \cos(\beta z) + 1]
\]

\[
\tau_{yz} = \tau_w [-A_z \sin(\alpha x) \sin(\beta z)]
\]

\[
P = \tau_w [-A_p \sin(\alpha x) \cos(\beta z)]
\]

where the terms \( A_x \), \( A_z \), and \( A_p \) have been introduced to represent the magnitude of the shear stress and pressure perturbations. The term \( \tau_w \) in these equations represents the mean wall shear stress imposed on the endothelial surface by the vessel flow. The sinusoidal terms are spatial fluctuations in shear stress and pressure induced by the topography of the cell layer. The functional dependence of \( A_x \), \( A_z \), and \( A_p \) on the cell shape parameters is given in Chapter IV.
Table 3 summarizes the calculation of the cell shape parameters and the perturbation magnitudes for the in vitro endothelial specimens. It is immediately apparent that while the cell height did not change under flow, there were significant changes in cell length and width. The monolayers exposed to shear stress exhibited a 3-fold increase in length and a slight decrease in width. The shear stress and pressure perturbation magnitudes are quite significant, representing spatial fluctuations of greater than 30% of the mean wall shear stress in endothelium that have not become elongated in the direction of flow. The specimens that have been exposed to 8 dynes/cm$^2$ shear stress for 72 hours exhibit a surface topography such that the force perturbations are significantly less than the static controls. It should be noted that because the average area of the endothelium does not appear to change during the elongation process, the total force exerted on a cell (as calculated by integrating the shear and pressure over the cell surface) will not change. However, an endothelial monolayer that exhibits the random, polygonal morphology characteristic of static cultures will experience a significantly greater spatial variation of fluid forces than one that has accommodated to flow by cell elongation in the direction of the mean flow.

Nerem et al. (1981) used a vascular casting technique to measure the shape of rabbit endothelium in situ in the vicinity of the aortic-intercostal artery flow divider. Table 4 presents estimates of the magnitude of the spatial variation in
shear stress and pressure over the cells from three areas in which the shear stress is expected to be successively greater: 50-150 \( \mu \text{M} \) upstream of the flow divider, just proximal to the flow divider and just distal to the flow divider. For purposes of the calculations, a cell height of 1 \( \mu \text{M} \) was assumed as this was not reported in the measurements. As was demonstrated in Chapter IV, the magnitudes of the force perturbations are linear with cell height so that the relative changes with cell shape will remain the same, provided that the cell height remains the same. The assumption of constant cell height is based on the in vitro observations of Chapter III. The cells in areas of estimated elevated shear stress exhibit morphology that reduces the spatial variation of stress on their surfaces as calculated by the theory. Clearly, careful in situ measurements of the cell height must be made before the theory can be systematically applied to in vivo work, but these preliminary results are consistent with the in vitro observations of cell shape under shear and the calculations of the theory.

The significance of the pressure perturbation calculated by the theory is a matter of question due to the large baseline values of vessel pressure due to the pulsatile flow. Peak systolic pressure in humans is, on the average, 120 mm Hg. This imposes a force of \( 1.6 \times 10^5 \) dynes/cm\(^2\) uniformly over the cell surface. Clearly, a pressure perturbation on the order of the wall shear stress is not a significant fraction of this total pressure. No attempt has been made in this work to
evaluate the effects of pressure forces on vascular endothelium by experiment. The regional distribution of atherosclerotic lesions observed in situ despite the relatively uniform distribution of pressure in the arterial tree suggests that factors such as shear stress may be more important in the pathogenesis of this disease.

Time vs Shear Effects on Cell Morphology

The dramatic difference in cell morphology between cultures subjected to 8 dynes/cm² and those exposed to 5 dynes/cm² suggest that changes in cell shape are quite sensitive to the magnitude of the applied shear stress. The time course of this phenomenon seems to suggest that there is a threshold stress at which elongation and alignment with the flow occurs precipitously. Cultures exposed to 8 dynes/cm², a level of shear stress that produces considerable shape change in 48 hours, show no obvious signs of alignment after 24 hours (Figure 17). If the alignment process were a simple first-order accommodation to an input force, significant shape change would be expected within 24 hour after exposure to shear stress in view of the dramatic changes observed at 48 hours. The design of the shear stress apparatus does not, at present, accommodate microscopic examination of the specimens during application of fluid shear stress. However, the time lapse cinematography of shear-exposed cultures relaxing to the randomly oriented, polygonal morphology after removal from shear provides insight to the mechanism of endothelial
monolayer movement. Because of the intimate contact between each cell and its neighbors, large changes in cell geometry may necessitate the coordination between groups of cells as seen in the film. Whether the coordinated movement observed during relaxation was a result of mechanical coupling of the cells or biochemical signaling is unknown.

Cultures that were confluent for longer periods of time prior to shear exposure showed less elongation and alignment with the flow than subconfluent cultures subjected to the same level of shear stress. It is possible that cell-cell contact or extracellular matrix secreted by the endothelium cause older, more densely-packed cultures to be more resistant to fluid forces.

**Culture Substrate Effects**

The in vitro experiments reported in the previous chapters were performed with endothelium cultured on sterile glass coverslips. The effects of substrate composition have not yet been systematically examined. The wound-healing experiments [Figure 21 (a) and (b)] have demonstrated significant effects of flow direction on cell migration into a denuded region. This is in contrast to the observations in situ of Buck (1979) who noted that the regeneration of a damaged section of rat aorta occurred at equal rates upstream and downstream. He hypothesized that the sub-endothelial structure of the vessel wall provides "contact guidance" for the migrating endothelium, enabling the cells to overcome the forces imposed by the
flowing blood by providing suitable anchorage sites. This same contact guidance may, in some respects, act as a template of cell shape, causing new cells to assume an elongated shape in the flow direction [Schwartz et al. (1978)]. Clearly, the anchorage sites available to the endothelium cultured on smooth glass are quite different from those in vivo. It is evident from the wound-healing photomicrographs that endothelium cultured on glass do not migrate as freely under physiological shear stress as they appear to in situ. The production of extracellular matrix in vitro may have significant effects on cell attachment. The shear stress apparatus design would lend itself well to the detailed examination of substrate-dependent phenomena.

The Endothelial Cytoskeleton

The presence of actin and myosin filaments in cultured endothelium has been well documented [White et al. (1982)]. The role of the cytoskeleton in endothelial cell function and the distribution of such stress fibers in situ have only recently been systematically examined [Wong et al. (1983)]. The words "stress fiber" and "cytoskeleton", though provocative in their imagery, refer to the appearance of these structures rather than their function, though it has certainly been postulated that they may provide the endothelial cell with structural integrity. The presence of actin and myosin support this structural hypothesis. Once thought to be an artifact of cell culture, microfilaments have been observed in
the vascular endothelium of rats [White (1983)]. There appears to be a positive correlation between the number of stress fibers found in the cells and their location in regions of elevated fluid shear stress. In addition, the endothelium of hypertensive rats exhibit larger numbers of stress fibers than normal rats.

The experiments performed with the shear stress apparatus demonstrate a significant change in cytoskeletal appearance at physiological levels of fluid shear stress. The stress fibers of static cultures seem to be randomly oriented and fewer in number than those of shear-exposed specimens. It is not known if the fibers depolymerize and repolymerize in the organized pattern when the cell is subjected to shear or if whole fibers re-orient as units. Experiments conducted by White (1983) demonstrate that the relaxation of endothelial shape after removal from the shear stress apparatus is not accompanied immediately by randomization of the stress fibers. Cells that are observed to have morphology similar to static cultures 24 hours after removal from shear still show the organized cytoskeleton of oriented specimens. Only after 48 hours do the stress fibers resume the random orientation of the static monolayer. This suggests that cytoskeletal structure is not a passive function of cell shape but perhaps is an actively constructed network that may well have a structural role.

The visualization of stress fibers is usually performed by fixing specimens and staining them with specific antibody stains to enhance the microfilament structure. This
necessitates killing the cells and prevents real-time or time-lapse photography such as that described in Chapter V. However, stress fibers may be seen with polarized light microscopy without fixing the cells and such techniques may prove valuable in studying the reorganization process. The investigation of the role of endothelial cytoskeleton involvement in cell structure and function is an important area of further research.

Fluid Endocytosis

One of the characteristics of atherogenesis is the accumulation of lipid material in focal regions of the arterial wall. It has been hypothesized that local fluid forces may alter the permeability of the vascular endothelium. A marked increase in wall permeability could result in lipid accumulation. The study of the effects of fluid shear stress on fluid endocytosis is, therefore, of particular interest. The ability to expose cultured endothelium to controlled shear stress in the apparatus provides an excellent experimental tool for a variety of such experiments.

In the first experiments, a dramatic increase in fluid endocytosis was observed in cultures exposed to steady shear stress of 1 or 8 dynes/cm² (Figure 22). Specimens exposed to 8 dynes/cm² exhibited an approximately 50% increase in the uptake of HRP within 1 hour after initiation of shear and a 100% increase after 2 hours when compared with static controls. The short time scale of these increases is remarkable when compared
with the 48 hour time course of the shape change process. Furthermore, when specimens were exposed to steady shear for periods in excess of 4-6 hours, their HRP uptake returned to the levels of static controls [Davies, P.F., personal communication]. This suggested a transient readjustment or accomodation to shear stress of relatively short duration. The experiments of Figure 23 were designed to elucidate this apparent accomodation effect by allowing cells to stabilize under shear or static conditions for 48 hours and then subject some of the specimens to a change in shear, either from 8 dynes/cm$^2$ to zero or vice-versa. The remaining specimens served as controls. In both cases where the conditions were changed, the rate of HRP accumulation increased almost two-fold.

The results of the $^3$H-thymidine labeling experiments demonstrated that confluent monolayers are not stimulated to enter the cell division cycle when exposed to shear stress. The cultures retained, therefore, the quiescence of contact inhibition and stable population. Increases in cell cycle were not responsible for the augmentation of fluid endocytosis observed.

Figure 24 contains a summary of several experiments performed to determine the threshold of shear stress necessary to trigger the increase in HRP uptake and to simulate pulsatile flow. The two-fold increase in uptake was observed for 1 and 2 hour exposures to 15 dynes/cm$^2$ as for 2 hours at 8 dynes/cm$^2$. Thus, a doubling of the shear stress did not seem to increase
the rate of fluid endocytosis. When specimens were exposed to periodic intervals of 8 dynes/cm², (of 15 min duration) separated by equal periods of no-flow, a significant increase in uptake was still noted. Decreasing the period of shear application to 5 minutes seemed to eliminate the effect. Finally, the sinusoidal application of physiological shear stress of frequency 1 Hz also produced no effect though the mean shear stress, if steady, is sufficient to produce a large increase in endocytosis.

The mechanism by which such changes in fluid endocytosis occur remains to be shown. It is possible that the imposition of tractive forces on the cell surface may affect the mechanical process of vesicle formation or the mobility or position of surface receptors, but this area certainly deserves further study. The time scale of the effect and an apparent frequency response suggest quite a different phenomenon than the gross changes observed in cell shape and cytoskeletal organization.
CHAPTER VII

CONCLUSION

The possible role of the vascular endothelium in the pathogenesis of arterial disease has prompted much study of the structure and function of this important tissue. In view of the patterns exhibited by atherosclerotic lesions, the fluid forces imposed by the blood on the arterial wall have been postulated as causing endothelial removal or dysfunction. Early research demonstrated the susceptibility of endothelium to damage by elevated fluid shear stress in vivo. The complex geometry of the arterial tree and the pulsatile nature of the flow within permits only approximate analysis of the local fluid dynamics, but such estimates indicate that peak physiological levels of fluid shear stress are well below the values found to cause gross cell damage. The long time course of lesion formation and growth would seem to favor more subtle alterations in endothelial structure and function as the result of fluid forces. There is a need, therefore, to design and perform controlled experiments that will assess the response of vascular endothelium to physiological flow.

To date, the fluid shear stress imposed on the endothelial cell monolayer has been considered to be the average wall shear stress produced by the blood flowing down the vessel. This picture ignores the coupling between the cells and the flowing blood that takes place in a region within a few cell dimensions of the endothelial surface. It is in this region, called the
cell-fluid microenvironment, that the local distribution of flow and stress is important. Receptor-mediated processes, the mechanical formation of membrane vesicles, the patency of tight junctions, and the organization of internal cell structures may all be influenced by local flow dynamics.

The model of the endothelial cell surface described in this Thesis was created to estimate the magnitude and phase of the spatial variation of fluid shear stress in terms of appropriate cell shape parameters. When the dimensions of endothelial monolayers measured in vitro were applied to this model, it predicted changes in local shear that are large fractions of the mean wall shear stress. The induction of a significant shear stress component perpendicular to the flow direction was also predicted. Cultured endothelial cells that have undergone shape change in response to fluid shear stress demonstrate significantly less spatial variation when their dimensions are applied to the model than those that have grown in no-flow conditions. Measurements of the en face endothelial geometry in situ by other researchers are consistent with this model. That is, the cells in regions of elevated fluid shear stress exhibit a shape that would reduce the spatial variation of shear stress on the cell surface. The systematic application of this model to in situ endothelium requires more topographical data than are presently available. The prediction of large spatial variation of fluid forces over surfaces with low amplitude waviness illustrates the need for more detailed study of the in vivo endothelial topography.
The development of the cone-and-plate shear stress apparatus has facilitated the routine assay of a number of cell functions under controlled in vitro conditions. It represents a significant improvement over previous flow systems in terms of its ability to produce a large dynamic range of physiological fluid shear stress and the capacity for long-term shear exposure. The apparatus will continue to be a valuable tool for the investigation of shear effects on endothelial cell biology.

Cultured endothelial monolayers demonstrate dramatic shape and cytoskeletal changes when exposed to physiological levels of fluid shear stress. These changes take place over a time scale of 48 to 72 hours and are reversible upon removal from shear. The effects of shear stress on cell motility point to possible substrate dependence on this important wound healing phenomena. The process of fluid endocytosis is significantly increased by steady shear stress after as little as 1 hour of exposure. The diminishing of this increase as exposure to shear increases to beyond 4 hours indicates a time-dependent accommodation to shear stress. The absence of any change in fluid endocytosis upon exposure to sinusoidally varying shear stress (1 Hz) of mean value 8 dynes/cm² constitutes evidence that the mechanism has a fundamental frequency response as a result of mechanical or biochemical time constants.

The experiments performed with the apparatus have demonstrated three fundamental time constants: one associated with the shape change of the cells under shear stress; a second
controlling the activation of fluid endocytosis following shear change; and a third defining the maximum frequency of shear change at which fluid endocytosis will respond.

The present understanding of the effects of fluid forces on vascular endothelial structure and function has greatly improved since flow was first identified as a causal element in arterial disease. *In vitro* experimentation has demonstrated important changes in endothelium exposed to fluid shear stress. Improvements in the mechanical and biochemical modeling of the fluid-cell interaction may well form a more deterministic theory of the role of these forces in the pathogenesis of arterial disease.
## Table 1

### SOME PREVIOUS STUDIES OF FLOW EFFECTS ON ARTERIAL PROPERTIES

<table>
<thead>
<tr>
<th>Investigator</th>
<th>System</th>
<th>Shear Stress Range</th>
<th>Parameters Studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carew (1971)</td>
<td>Excised sections dog aorta, laminar flow channel</td>
<td>20-500 dynes/cm²</td>
<td>Arterial permeability, critical shear stress</td>
</tr>
<tr>
<td>Krueger et al. (1971)</td>
<td>Cultured MDBK cells laminar flow channel</td>
<td>10⁻³ - 10 dynes/cm²</td>
<td>Cell detachment and morphology</td>
</tr>
<tr>
<td>Mansfield (1979)</td>
<td>Cultured endothelium</td>
<td>400-600 dynes/cm²</td>
<td>Attachment to substrate</td>
</tr>
<tr>
<td>Fry (1968)</td>
<td>Dog aorta, plug inserted to enhance shear stress</td>
<td>100-600 dynes/cm²</td>
<td>Morphology; cell loss</td>
</tr>
<tr>
<td>Rosen et al. (1974)</td>
<td>Cultured bovine aortic endothelium, rectangular flow channel</td>
<td>2.8-6.2 dynes/cm²</td>
<td>Histamine synthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5 hours</td>
<td></td>
</tr>
<tr>
<td>DeForrest and Hollis (1978)</td>
<td>Rabbit aorta in situ, mechanical pump</td>
<td>22-109 dynes/cm²</td>
<td>Histamine synthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 hour</td>
<td></td>
</tr>
<tr>
<td>Mansfield (1979)</td>
<td>Calf aorta, tubular graft with cultured endothelium</td>
<td>Normal physiological flow</td>
<td>Resistance to thrombus formation, endothelial integrity, cell alignment</td>
</tr>
<tr>
<td>Schwartz et al. (1978)</td>
<td>Rat aorta, endothelium denuded with balloon catheter</td>
<td>Normal physiological flow</td>
<td>Arterial permeability by EBS. Patterns of endothelial migration and replication</td>
</tr>
</tbody>
</table>
**TABLE 2**

**MEASUREMENTS OF CULTURED ENDOTHELIUM**

**AVERAGE DIMENSIONS (MICRONS)**

<table>
<thead>
<tr>
<th>Static Controls</th>
<th>Length</th>
<th>Width</th>
<th>Height</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static Controls</td>
<td>30</td>
<td>30</td>
<td>1.5</td>
</tr>
<tr>
<td>8 dynes/cm²</td>
<td>90</td>
<td>25</td>
<td>1.5</td>
</tr>
<tr>
<td>For 72 hours</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 3

**ESTIMATES OF SURFACE SHEAR STRESS**

*IN VITRO* MEASUREMENTS APPLIED TO WAVY-WALL MODEL

<table>
<thead>
<tr>
<th></th>
<th>$8$</th>
<th>$\tilde{\eta}/\lambda$</th>
<th>$A_x$</th>
<th>$A_z$</th>
<th>$A_p$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>STATIC CONTROLS</strong></td>
<td>1.0</td>
<td>0.025</td>
<td>0.33</td>
<td>0.11</td>
<td>0.31</td>
</tr>
<tr>
<td><strong>8 DYNES/CM$^2$ FOR 72 HOURS</strong></td>
<td>3.5</td>
<td>0.0083</td>
<td>0.20</td>
<td>0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>LOCATION</td>
<td>$\bar{x}$</td>
<td>$\theta/\lambda_x$</td>
<td>$A_x$</td>
<td>$A_p$</td>
<td>$\rho$</td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------</td>
<td>---------------------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>PROXIMAL AORTA</td>
<td>5.0</td>
<td>0.19</td>
<td>0.63</td>
<td>0.12</td>
<td>0.24</td>
</tr>
<tr>
<td>PROXIMAL TO INTERCOSTAL FLOW DIVIDER</td>
<td>5.0</td>
<td>0.017</td>
<td>0.57</td>
<td>0.11</td>
<td>0.02</td>
</tr>
<tr>
<td>DISTAL TO INTERCOSTAL FLOW DIVIDER</td>
<td>5.0</td>
<td>0.0012</td>
<td>0.04</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Fig. 1  Wavy-wall model of endothelial cell surface showing distinct wavelengths in the x and z-directions. The upper boundary condition is a linear shear flow in the x-direction.

Fig. 2  Geometry of the cone-plate test apparatus showing location of typical test specimen.

Fig. 3  Definition of nomenclature used in cone-plate flow description.

Fig. 4  Line drawing of shear stress apparatus. For testing, the plate is raised until contact is made with the apex of the rotating cone.

Fig. 5  Photograph of shear stress apparatus. Temperature and motor speed controller is at right.

Fig. 6  Detail drawing of coverslip hold-down arrangement. After placement of the coverslip, the piston is drawn down, producing a partial vacuum beneath the coverslip that holds it in place.

Fig. 7  Operating range of the experimental apparatus. \((\tau_{wu})\) is the shear stress acting on the plate and
specimens of endothelium. $\bar{R}$ is evaluated at the specimen locations.

Fig. 8 Growth of endothelial cells under static conditions: control and apparatus specimens.

Fig. 9 Growth of endothelial cells subjected to 1 dyne/cm$^2$ fluid shear stress: control and apparatus specimens.

Fig. 10 Growth of endothelial cells subjected to 5 dynes/cm$^2$ fluid shear stress: control and apparatus specimens.

Fig. 11 Scanning electron micrographs of cultured endothelial monolayers prepared by: (a) Glutaraldehyde fixation and air drying, (b) Glutaraldehyde fixation and critical point drying, and (c) Unfixed, negative cast produced with Batson's medium. Bar on photomicrograph represents 10 microns.

Fig. 12 Photomicrograph of thick (1 micron) sections of cultured endothelial monolayers: (a) static controls, (b) exposed to 8 dynes/cm$^2$ for 72 hours.

Fig. 13 Illustration of the endothelial surface model. Here, (alpha) = (beta).
Fig. 14 Illustration of the endothelial surface model. Here, $(\alpha) = 4 \times (\beta)$.

Fig. 15 Illustration of parameters used to calculate the Reynolds Number of the flow adjacent to the endothelial cell monolayer.

Fig. 16 Dependence of shear stress perturbation magnitude on the cell eccentricity, $q$, found by the theory of Appendix A. Data points represent values of $q$ found in vitro with the shear stress apparatus and in situ in rats [Nerem et al. (1981)].

Fig. 17 Photomicrograph of endothelial specimens 24 hours from start of experiment: (a) static control, (b) exposed to 8 dynes/cm$^2$ (mag 100X).

Fig. 18 Photomicrograph of endothelial specimens 48 hours from start of experiment: (a) static control, (b) exposed to 8 dynes/cm$^2$ (mag 100X).

Fig. 19 Shape distribution of endothelial cultures subjected to 8 dynes/cm$^2$ shear stress for 72 hours: (a) eccentricity, (b) orientation angle.

Fig. 20 Three frames of time-lapse film of endothelial monolayer relaxing after cessation of fluid shear
stress (8 dynes/cm²): (a) 1 hour post shear, (b) 8 hours post shear, (c) 15 hours post shear.

Fig. 21  Regeneration of linear defect in confluent endothelial monolayer exposed to 8 dynes/cm² shear stress for 24 hours following "wounding" (arrow = direction of flow): (a) flow parallel to wound. Left portion of photograph illustrates an area of the monolayer at a distance from wound edge. (b) Flow perpendicular to wound. Denuded surface area between the two edges has been removed from the photograph for clarity.

Fig. 22  Shear effect on fluid endocytosis. Intracellular HRP accumulation was measured in confluent endothelial monolayers exposed to 0, 1, or 8 dynes/cm² shear stress for the indicated times, and the corresponding volumes of extracellular fluid uptake calculated (mean ± 1 SD, triplicate determination).

Fig. 23  Transient effect of shear stress on fluid endocytosis. Specimens were incubated for 2 hours with HRP after a 48 hour "pre-conditioning" with either 8 dynes/cm² shear stress or no-flow conditions.
Fig. 24  Summary of effects of periodic application of fluid shear stress on fluid endocytosis.

Fig. 25  Photomicrograph of endothelial monolayers stained with antibodies specific to actin and myosin. Frames to left are static controls. Frames to right are specimens exposed to 8 dynes/cm² for 72 hours.
\[ u = \sigma y \quad (y \to \infty) \]

\[ \lambda_x = \frac{2\pi}{\alpha} \]

\[ \lambda_z = \frac{2\pi}{\beta} \]
CONE-PLATE TEST APPARATUS

FIGURE 2
FIGURE 6
GLASS COVERSLOTS
11-BAEC, Sc-32
DME + 10% C.S.

NO FLOW

IN APPARATUS
IN PETRI DISH

PETRI DISH
APPARATUS OR PETRI DISH

CELL DENSITY PER CM² (X10⁵)

0 0.5 1.0 1.5 2.0
DAYS

0 2 4 6 8 10

FIGURE 8
SHEAR EFFECT: CELL GROWTH

11-BAEC (SPARSE)

CELLS X 10^4 / COVERSILP

\[\begin{align*}
\Delta & \quad 1 \text{ DYNE/CM}^2 \\
\square & \quad \text{STATIC CONTROL}
\end{align*}\]

FIGURE 9
SHEAR EFFECT: GROWTH
II-BAEC (SUBCONFLUENT)

CELLS X 10^4 / COVERSIP

HOURS

STATIC CONTROLS

5 DYNES/CM²

FIGURE 10
\[ u = \sigma y \quad (y \to \infty) \]

\[ \eta = \hat{\eta} \cos(\alpha x) \cos(\beta z) \]
\[ u = \sigma y \]
\[ (y \to \infty) \]

\[ \eta = \hat{\eta} \cos(\alpha x) \cos(\beta z) \]
\[ u = \sigma y \]

\[ R_e = \frac{\sigma \hat{\eta}^2}{\nu} \]

**ENDOTHELIAL CELL**

\[ \hat{\eta} \sim 1 \mu M \]

**FIGURE 15**
\[ \text{IN VITRO (8 \frac{\text{Dynes}}{\text{CM}^2}, 48 \text{ hr})} \]

\[ \text{IN SITU [NEREM et al (1981)]} \]

\[ \frac{2 + q^2}{\sqrt{1+q^2}} \]

\[ \frac{q}{\sqrt{1+q^2}} \]

FIGURE 16
SHEAR EFFECT FLUID ENDOCYTOSIS
II-BAEC (CONFLUENT)
HRP (Type VI)

FIGURE 22
SHEAR STRESS: TRANSIENT EFFECT

FLUID ENDOCYTOSIS
(nl/mg protein/2hr)

\[
\begin{align*}
20.6 \pm 1.9 & \quad (SD) \\
39.1 \pm 9.2 \\
21.1 \pm 7.2 \\
35.2 \pm 7.3
\end{align*}
\]

0 48 50 hours

\[\text{STATIC} \quad \text{8 dynes/cm}^2\]

FIGURE 23
Time course of shear

Rate of fluid endocytosis (% of static controls)

- 15 DYNES/cm²

- 2 hr

increased 177%

- 15 DYNES/cm²

- 1 hr

increased 161%

- 8 DYNES/cm²

- 2 hr

increased 210%

- 15 min

- 8 DYNES/cm²

- 2 hr

increased 173%

- 5 min

- 8 DYNES/cm²

- 2 hr

no increase 97%

- 1 sec

- 13 DYNES/cm²

- 2 hr

100%

FIGURE 24
APPENDIX A

WAIVY-WALL FLOW SOLUTION

The equations of motion of a viscous fluid are solved here for the conditions of Reynolds Number zero (i.e. the inertial forces are insignificantly small). The assumption of zero Re is based upon the calculations of Chapter IV. For this case, the Navier Stokes Equations reduce to the following form:

\[ \nabla^2 V = \frac{1}{\mu} \nabla p \]

\[ \nabla \cdot V = 0 \]

where \( V \) is the fluid velocity, \( \mu \) is the fluid viscosity and \( p \) is the pressure. The coordinate system used in the analysis presented here is shown in Figure 1. The lower boundary condition is zero fluid velocity at the wall (no slip) and the upper boundary condition is a linear shear flow at \( y = \infty \). Expressing the equations a-1 and a-2 in terms of this coordinate system yields:

\[ \frac{\partial u}{\partial x} + \frac{\partial v}{\partial y} + \frac{\partial w}{\partial z} = 0 \]

\[ \frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2} + \frac{\partial^2 u}{\partial z^2} = \frac{1}{\mu} \frac{\partial p}{\partial x} \]
\[ \frac{\partial^2 v}{\partial x^2} + \frac{\partial^2 v}{\partial y^2} + \frac{\partial^2 v}{\partial z^2} = \frac{1}{\mu} \frac{\partial p}{\partial y} \]  
\[ \frac{\partial^2 w}{\partial x^2} + \frac{\partial^2 w}{\partial y^2} + \frac{\partial^2 w}{\partial z^2} = \frac{1}{\mu} \frac{\partial p}{\partial z} \]

The method of solution involves the assumption of a form for the velocity that is the sum of a linear shear flow and a small perturbation. The linear shear flow, \( U \), will simply be that defined by the upper boundary condition:

\[ U = \sigma y \]

where \( \sigma \) is the shear rate. The small perturbation will be constrained to vanish at \( y = \infty \), thus satisfying the upper boundary condition. The sum of the linear shear flow and the perturbation must be zero at the wall, in keeping with the lower boundary condition.

The wavy-wall can be expressed in analytical form by the equation

\[ y = \eta = \eta e^{i(\alpha x + \beta z)} \]

Only the real part of Eq. a-8 is taken to have physical signifigance. This defines a surface with waviness in both the \( x \) and \( z \) directions with amplitude \( \eta \) and wavenumber \( \alpha \) and \( \beta \). To facilitate manipulation of the equations, the symbol \( \eta \) will be used to indicate the surface, implying the right-hand side of equation a-8.
The form assumed for the velocities and pressure are given below:

\[ u = U + \hat{u}(y) e^{i(\alpha x + \beta z)} \]  \hspace{1cm} a-9

\[ v = \hat{v}(y) e^{i\alpha x + \beta z} \]  \hspace{1cm} a-10

\[ w = \hat{w}(y) e^{i(\alpha x + \beta z)} \]  \hspace{1cm} a-11

\[ p = \hat{p}(y) e^{i(\alpha x + \beta z)} \]  \hspace{1cm} a-12

The form of the pressure and velocity perturbations are the product of an unknown function of \( y \) alone and a periodic function of \( x \) and \( z \) that matches the form of the boundary \( \eta \).

The velocity in the \( x \)-direction, \( u \), is the sum of the linear shear flow (the upper boundary condition imposed in this problem) and the perturbation.

The boundary conditions on the velocities are given below:

\[ \hat{u}(\eta) + \sigma \eta = 0 \]  \hspace{1cm} a-13

\[ \hat{v}(\eta) = 0 \]  \hspace{1cm} a-14

\[ \hat{w}(\eta) = 0 \]  \hspace{1cm} a-15
The conditions of vanishing perturbation velocities at \( y = \infty \) are:

\[
\hat{u}(\infty) = \hat{w}(\infty) = \hat{v}(\infty) = 0 \quad a-16
\]

and

\[
\hat{u}'(\infty) = \hat{v}'(\infty) = \hat{w}'(\infty) = 0 \quad a-17
\]

where primes denote differentiation with respect to \( y \).

When the velocities and pressure given by equations \( a-9 \) through \( a-12 \) are substituted in equations \( a-3 \) through \( a-6 \), the following results.

\[
i\hat{u} + \hat{v}' + i\beta \hat{w} = 0 \quad a-18
\]

\[
\hat{u}'' - K^2 \hat{u} = \frac{1}{\mu} i\alpha \hat{P} \quad a-19
\]

\[
\hat{v}'' - K^2 \hat{v} = \frac{1}{\mu} i\alpha \hat{P}' \quad a-20
\]

\[
\hat{w}'' - K^2 \hat{w} = \frac{1}{\mu} i\beta \hat{P} \quad a-21
\]

where

\[
K^2 = \alpha^2 + \beta^2 \quad a-22
\]

Equations \( a-18 \) through \( a-21 \) can be combined to produce a single fourth-order differential equation in terms of yielding:
\[ \hat{v}'' - K^2 \hat{v} = \frac{1}{K^2} (\hat{v}''' - K^2 \hat{v}'') \]

**Linearization of the Boundary Conditions**

If the amplitude of the waviness \( \hat{\eta} \) is small and the wavelengths in both the \( x \) and \( z \) directions are long, the lower boundary condition can be linearized to the following form:

\[ \hat{u}(0) = -\sigma \hat{\eta} \]

\[ \hat{v}(0) = 0 \]

\[ \hat{w}(0) = 0 \]

The form of the continuity equation (equation a-18) provides a second constraint on \( \hat{v} \) at \( y = 0 \).

\[ \hat{v}'(0) = i \tan \sigma \]

**Transformation of Variables**

The following transformation of variables was used to solve equation a-23.
\[ \psi = (\hat{\psi}'' - K^2 \hat{\psi}) \]

The transformed form of equation a-23 is

\[ \psi'' - K^2 \psi = 0 \]

which has as a general solution

\[ \psi = A e^{K y} + B e^{-K y} \]

Substituting equation a-30 into equation a-28 and solving for \( \hat{\psi} \) yields

\[ \hat{\psi} = \left[ \frac{2AKy + 4CK^2 - A}{4K^2} \right] e^{K y} - \left[ \frac{2BKy - 4DK^2 + B}{4K^2} \right] e^{-K y} \]

where A, B, C, and D are arbitrary constants to be determined by the boundary conditions (equations a-25, a-16, a-17 and a-27). Application of equation a-25 results in the following:

\[ C - \frac{A}{4K^2} + D - \frac{B}{4K^2} = \]

The constraints on \( \hat{\psi} \) and its \( y \)-derivative at \( y = \infty \) produce:
\[ \hat{v}(\infty) = \lim_{y \to \infty} \left[ \frac{2AKy + 4CK^2 - A}{4K^2} e^{Ky} \right] \]  
\[ \hat{v}'(\infty) = \lim_{y \to \infty} \left[ \frac{-A + 2KAy + 4CK^2}{4K} e^{Ky} \right] \]

Equations $a-16$ and $a-17$ imply

\[ A = C = 0 \]  

Applying equation $a-27$ yields

\[ \hat{v}'(0) = \frac{A + 4CK^2}{4K} - \frac{B + 4DK^2}{4K} = i\tan\sigma \]  

Using equations $a-35$, $a-32$, and $a-36$, the constants $B$ and $D$ can be solved for:

\[ B = -2i\tan\sigma K \]  
\[ D = -i\tan\sigma/2K \]  

Therefore the expression for $\hat{v}$ that satisfies the boundary conditions is

\[ \hat{v}(y) = i\tan\sigma e^{-Ky} \]  

Using similar techniques, the functions $\hat{U}$ and $\hat{W}$ may be solved for, and the results of such calculations are given below:
\[ \hat{u}(y) = \frac{\alpha^2 \eta}{K} y e^{-Ky} - \eta e^{-Ky} \]  
\[ \hat{w}(y) = \left( \frac{\alpha \eta K}{\beta} - \frac{\alpha \eta}{BK} \right) y e^{-Ky} \]

**Final Solution for Velocity, Pressure and Shear Stress**

Using the solutions of equations a-39 through a-41, the final form of the velocities and pressure can be written as the real part of equations a-9 through a-12.

\[ u = \left[ \frac{\alpha \eta}{K} y - \eta \right] e^{-Ky} \cos(\alpha x + \beta z) + \sigma_y \]
\[ v = -\alpha \eta \gamma e^{-Ky} \sin(\alpha x + \beta z) \]
\[ w = \frac{\beta \alpha \eta}{K} y e^{-Ky} \sin(\alpha x + \beta z) \]
\[ p = -2\mu \eta \gamma e^{-Ky} \sin(\alpha x + \beta z) \]

From the above, the shear stress imposed by the fluid on the wavy surface may be calculated with the following formulae:

\[ \tau_{yx} = \mu \left( \frac{\partial u}{\partial y} + \frac{\partial v}{\partial x} \right) \]
\[ \tau_{yz} = \mu \left( \frac{\partial v}{\partial z} + \frac{\partial w}{\partial y} \right) \]

The direction of each of the two surface shear stresses is shown in Figure 1 and their values are given below:
\[ \tau_{yx} = \mu \sigma \left[ \frac{\alpha^2 + K^2 \eta}{K} - 2\alpha^2 \eta y \right] e^{Kx \cos(\alpha x + \beta z)} \] 

\[ \tau_{yz} = \mu \sigma \left[ \frac{\beta \alpha \eta}{K} - 2\beta \eta y \right] e^{-Kx \cos(\alpha x + \beta z)} \]  

At the cell surface (\( y = 0 \) in the linearized solution), the shear stresses are

\[ \tau_{yx} \big|_{y=0} = \mu \sigma + \mu \left( \alpha^2 + K^2 \eta \right) \] 

\[ \cos(\alpha x + \beta z) \]  

\[ \tau_{yz} \big|_{y=0} = \frac{\mu \beta \alpha \eta}{K} \cos(\alpha x + \beta z) \]  

**Superposition of Solutions**

In order to estimate the surface shear stress and pressure force on the model endothelial surface

\[ y = \eta \cos(\alpha x) \cos(\beta z) \]  

the superposition of two solutions of the form of equations a-42 through a-45 must be made. The boundary conditions will hold for a surface of the form

\[ y = \frac{\eta}{2} \left[ \cos(\alpha_1 x + \beta_1 z) + \cos(\alpha_2 x + \beta_2 z) \right] \]  

where
so that $\alpha$ and $\beta$ are as in equation a-52. The equations of motion are linear and the results of the superposition of two solutions yields the following results for the velocities, pressure, and shear stress:

$$u = \left[ \frac{\alpha^2 \eta \sigma}{K} y - \sigma \hat{\tau} \right] e^{-Ky} \cos(\alpha x) \cos(\beta z) + \sigma y$$  \hspace{1cm} a-56

$$v = -\alpha \eta \sigma y e^{-Ky} \sin(\alpha x) \cos(\beta z)$$  \hspace{1cm} a-57

$$w = \frac{-\beta \alpha \eta \sigma}{K} y e^{-Ky} \sin(\alpha x) \sin(\beta z)$$  \hspace{1cm} a-58

$$p = -2\mu \alpha \eta \sigma e^{-Ky} \sin(\alpha x) \cos(\beta z)$$  \hspace{1cm} a-59

$$\tau_{yx} = \mu \sigma \left[ \frac{\alpha^2 + K^2}{K} \eta - 2 \alpha \eta \hat{\tau} \right] e^{-Ky} \cos(\alpha x) \cos(\beta z) + \mu \sigma$$  \hspace{1cm} a-60

$$\tau_{yz} = \mu \sigma \left[ 2 \alpha \beta \eta - \frac{\beta \alpha \eta \hat{\tau}}{K} \right] e^{-Ky} \sin(\alpha x) \sin(\beta z)$$  \hspace{1cm} a-61
REFERENCES


