CHAPTER TWO

Physiologic Stress-Mediated Signaling in the Endothelium

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Abstract

Although the vasculature was once thought to be a passive conduit for blood, it is now known that the endothelium is responsible for healthy vascular homeostasis and the progression of many cardiovascular-related diseases. Because the endothelium lines blood vessels, it is subjected to the mechanical forces due to of blood flow. It is now well established that endothelial cells transduce these mechanical signals into chemical signals that are evident in the

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mechanoregulation of a number of signal transduction pathways and endothelial cell phenotype. Despite the significant volume of work in the field of endothelial cell mechanotransduction, the exact mechanism by which mechanical forces are sensed and transduced into chemical signals is not yet well established. In this chapter, we focus on the specific role of fluid shear stress, the frictional drag force caused by blood flow, and cyclic stretch caused by the pumping action of the heart, in regulating vascular homeostasis and vascular signaling. The regulation of flow-mediated signaling in the endothelium is typically studied with well-characterized *in vitro* flow and stretch devices. Here, we examine various platforms used to analyze flow-mediated and stretch-mediated signals and describe the method for the implementation of these techniques.

1. Introduction

The endothelial cell layer of the vasculature directly contacts the circulating blood, providing a dynamic barrier to the surrounding tissue. Because of its unique position in the body, it is exposed to three primary mechanical forces caused by blood flow: pressure caused by the hydrostatic force within the vessel, hoop stresses caused by the balance between the cell–cell contacts and vasomotion of the vessel, and the shear stresses caused by the friction of the blood flow against the vessel wall. In addition, the endothelium modulates a number of biologic processes within the vessel wall, including active regulation of vascular tone and blood pressure through the production of nitric oxide, control over the coagulation cascade and fibrinolytic processes through the production of prothrombotic and antithrombotic factors, regulation of vascular remodeling through the production of growth factors and vasoactive substances, and control over the inflammatory response by providing a platform by which leukocytes can home to inflamed tissue. As such, the endothelium mediates the progression of several pathologic conditions, including chronic inflammation, wound healing, and the development of various cardiovascular diseases including atherosclerosis.

In healthy blood vessels, endothelial cells are typically quiescent, presenting an antithrombotic nonadhesive surface to the passing blood (Garin and Berk, 2006; Pearson, 2000). However, on activation by cytokines, such as tumor necrosis factor (TNF-α) or interleukin-1β (IL-1β), or other stimuli, endothelial cells upregulate a number of genes, including E-selectin, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, monocyte chemoattractant protein-1 (MCP-1), interleukin-1 and 8, plasminogen activator inhibitor-1 (PAI-1), and tissue factor (TF) (Cybulsky and Gimbrone, 1991; Diamond *et al.*, 1989; Malek *et al.*, 1999). (Fig. 2.1) In general, endothelial cell turnover increases, and

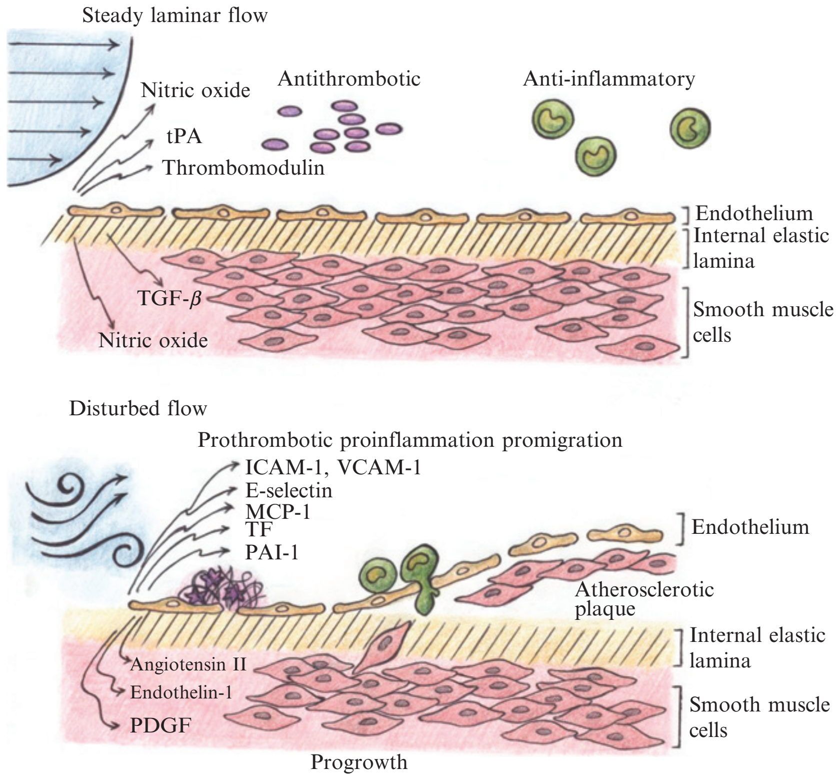

Figure 2.1 Flow-mediated effects on atherosclerotic plaque formation. Steady laminar flow promotes endothelial cell survival and is antithrombotic and antiinflammatory. Disturbed flow elicits the opposite response, stimulating endothelial apoptosis, vascular smooth muscle growth, thrombosis, and monocyte infiltration, resulting in a proatherogenic phenotype. Image drawn by Ms. Becky Zhao.

the production of antithrombotic mediators decreases. Endothelial cell activation is required for a number of adaptive processes, including clotting and leukocyte adhesion during wound healing (Hajjar and Deora, 2000). However, chronic endothelial cell activation is linked to chronic inflammatory diseases, including atherosclerosis (Davignon and Ganz, 2004).

Chronic endothelial cell activation and increased incidence of atherosclerotic lesion formation is often detected in branch points or bifurcations within the vasculature (Gimbrone *et al.*, 2000; Malek *et al.*, 1999; World *et al.*, 2006). Notably, the blood flow patterns, fluid shear stresses, and stretch patterns imposed on the endothelium are also strikingly different in these regions than those measured in the straight portions of the vessel (Chien, 2007). The nature and magnitude of blood flow, shear, and vessel wall stretch is largely determined by the shape and structure of the blood vessel and the cardiac cycle. The shear stresses found in most major human

arteries have been found to be 2 to 20 dyn/cm^2, with localized increases to 30 to 100 dyn/cm^2 near branches and areas of sharp wall curvature (Dewey *et al.*, 1981). Typically, in the straight portions of a blood vessel, the flow is laminar. In these regions, endothelial cells align and elongate parallel to the direction of flow (Fig. 2.2). This realignment corresponds with a streamlining of the cell that reduces resistance to flow and is speculated to mediate the subsequent signaling response. In contrast, flow within abrupt curvatures, such as occurs at bifurcations, is typically disturbed, exhibiting flow reversal, separation, and low velocity. As a result, the endothelial cells do not reorient like those located in the straight portions of the vessel. Because the cells do not align with the flow, their topology exposes them to greater shear stress gradients across the length of the cell, and these areas are also more prone to atherosclerosis (Barbee *et al.*, 1994). For instance, within the carotid bifurcation, where atherosclerosis often develops, the flow separates, disrupting the laminar profile and producing disturbed streamlines (Ku *et al.*, 1985). The lateral wall experiences areas of flow reversal and recirculation varying with

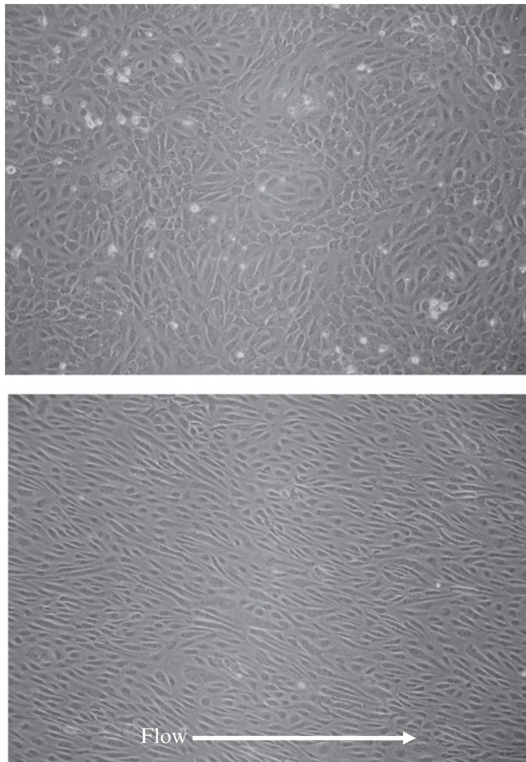

Figure 2.2 Phase image of HUVECs before and after 24 h of flow at 20 dyn/cm^2 with a cone and plate flow device as described in the text. Image taken at 10×.

the cardiac cycle, resulting in a time-averaged shear stress close to zero. Because areas subjected to laminar shear stresses are generally free from plaque formation and lesions correlate with areas of disturbed flow, it is believed that laminar shear stresses impose an atheroprotective force on the vasculature and help maintain healthy vascular homeostasis (Berk *et al*., 2002; Traub and Berk, 1998). A similar general theme based on endothelial cell response to stretch has been advanced by Chien (Chien, 2007). However, the mechanism by which laminar flow and unidirectional stretch are detected and translated into an atheroprotective force by the endothelium remains unclear.

Significant evidence exists that laminar flow at physiologic shear stresses inhibits platelet aggregation and can enhance endothelial cell survival by preventing apoptosis (Dimmeler *et al*., 1996; Garin *et al*., 2007; Yoshizumi *et al*., 2003). Physiologic levels of cyclic stretch also seem to provide beneficial effects on endothelial barrier function (Fujiwara, 2003). In addition, significant evidence exists that disturbed flow that exerts time-averaged low shear stresses on the endothelium induces endothelial expression of proapoptotic, proinflammatory, and procoagulant genes (Berk, 2008; Davies, 2007; Garcia-Cardena and Gimbrone, 2006). However, the upstream events that initiate the signaling cascade in response to shear stress are still unclear.

Because many questions regarding how endothelial cells mechanotranduce fluid shear stress and stretch into intracellular responses, the atheroprotective effects of these forces and the subsequent flow-mediated and stretch-mediated signaling continue to be an active area of research. Although there have been significant advances in this field with animal models, *in vivo* work presents a number of challenges. In addition to the difficulties associated with isolating the effects of the mechanical signals caused by blood flow from the chemical humoral effectors, it is also difficult to visualize various force fields within the vasculature and to relate those stresses to specific endothelial cell phenotype in real time. Therefore, *in vitro* methods have been designed to mimic *in vivo* forces for the studies of flow-mediated and stretch-mediated signaling in the endothelium. Here, we will discuss several methods used to subject cells to flow, and we will also describe methods we have used to stretch cells to compare these mechanosignaling responses to the response elicited by flow.

2. Parallel Plate Flow System

The parallel plate flow chamber is one of the most commonly used platforms for subjecting monolayers of endothelial cells to uniform laminar shear stresses. Its advantages include the following (Kandlikar *et al*., 2005):

- The fluid shear stress is relatively uniform within the chamber.
- It can be used on a temperature-controlled microscope stage, allowing for real-time visualization of the cells and/or flow patterns within the chamber.
- It is capable of applying a wide range of flow rates and fluid shear stresses.
- It allows for continuous sampling of the conditioned medium.
- It can be scaled down to micron-sized channels, with smaller volumes to conserve the use of rare or expensive molecules.

2.1. Design of a parallel plate flow chamber

Most parallel plate flow chambers contain several common components, depicted in Fig. 2.3. Typically, a reusable plastic upper surface is used to sandwich a gasket onto a tissue culture dish or glass plate such as a micro-slide. The gasket contains a rectangular cutout, creating the side walls of the flow domain. The upper surface contains ports at either end of the channel for the inlet and outlet of flow. Some devices also use a vacuum line to keep the apparatus pressed together without the use of anchors or clips. This helps to ensure that the height of the flow path created by the opening in the

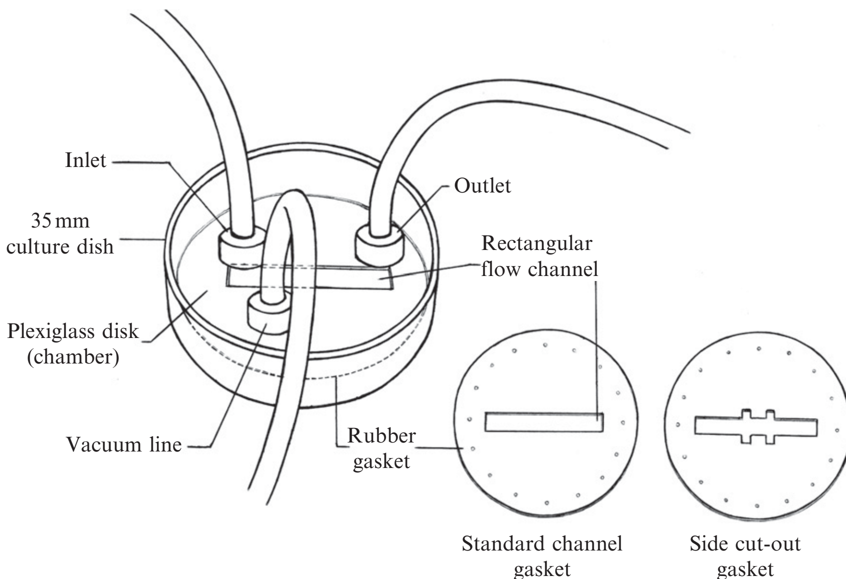

Figure 2.3 Schematic of a typical parallel plate flow chamber. The chamber and gasket are designed to fit within a standard 35-mm tissue culture dish. Insets indicate gasket designs for uniform laminar flow and disturbed flow containing spatial gradients of shear. Image drawn by Ms. Becky Zhao.

Table 2.1 Commercially available parallel plate flow chambers with relevant dimensions

Model	Manufacturer	Channel height (μm)
Circular flow chamber	Glycotech Corporation	125–150
ECIS flow system	Applied Biophysics	1000
Stovall flow cell	Stovall Life Science, Inc.	1000
Flow chamber system	Oligene	300–500
Adhesion flow chamber	Immunogenics, Inc.	250
Vacucell	C&L Instruments, Inc.	250

gasket is uniform. Alternately, the chamber may be secured by screws set at the perimeter, which must be tightened equally to ensure that the flow path is level.

Many parallel plate flow systems are commercially available with varying channel dimensions, dictating a range of fluid shear stresses. Several commercially available systems are listed in Table 2.1 (Kandlikar *et al.*, 2005), with their manufacturer and reported range of channel dimensions. Most commercially available flow chambers are designed to fit in commonly used 100-mm or 35-mm tissue culture–treated dishes, and many of these devices contain an optically transparent flow path, allowing for direct visualization of cell behavior under flow.

2.2. Calculation of the imposed fluid shear stress in a parallel plate flow chamber

Given a flow channel, where the length and width of the channel is much greater than the height, the flow profile can be approximated as flow between infinite parallel plates, often referred to as plane Poiseuille flow. For a Newtonian fluid, the shear stress at the surface of the plate is:

$$\tau = \frac{6Q\mu}{wh^2} \tag{2.1}$$

where Q is the flow rate, μ is the fluid viscosity, w is the width of the channel, and h is the height of the channel (i.e., the thickness of the gasket). To achieve a specific shear stress, the corresponding flow rate should be used. Flow rate can be controlled in several ways. For short-term studies of flow-mediated response, syringe pumps in withdraw-mode can pull media from a reservoir through the channel. However, depending on the capacity of the syringe and the number of syringes connected in parallel, the maximum duration for the application of flow ranges from 1 to 3 h. To apply flow for longer times, a flow loop is needed, where the culture medium is

moved by a peristaltic pump. However, peristaltic pumps do create some pulsation within the flow. There are several methods for minimizing this pulsation: (1) install a commercially available pulse dampener in the loop; (2) use a peristaltic pump with a larger number of rollers (>8 to 10) to smooth the flow; and (3) add two reservoirs into the flow loop, up and downstream of the flow chamber. The flow through the chamber is pressure driven on the basis of the height of the upper reservoir, and media is returned to the upper reservoir from the lower reservoir through a peristaltic pump (Go *et al.*, 1999).

2.3. Protocol for subjecting endothelial cells to laminar fluid shear stress with a parallel-plate flow chamber

2.3.1. Materials

- Glycotech Flow Chamber (cat #. 31-001): The flow chamber kit includes gaskets containing well-characterized channels of defined width, height, and length and quantitative characterization of the flow rates necessary to achieve a given wall shear stress.
- Peristaltic pump (Rainin, Cat #7103-058) or
- Syringe Pump with 60-ml syringes, depending on the length of experimentation.
- Silastic or Tygon Tubing (1/16 in ID).
- Corning 35-mm tissue culture dishes.
- Human umbilical vein endothelial cells (HUVEC) (available through Cascade Biologics or can be isolated from a primary source).
- HUVEC Media: 500 ml DMEM (Cascade Biologics) 10 ml LSGS (Cascade Biologics), 5% FBS (Gibco).
- Vacuum line.

2.3.2. Methods

Plate cells on 35-mm dishes in culture media several days before the experiment and allow the cells to grow to confluence. *Note*: if cells are grown significantly past confluence, cells may release from the dish in cell sheets upon the initiation of flow.

If the experiments do not involve any biochemistry and instead only require observation and immunocytochemistry, cell can be plated over the entire dish.

If the desired end experiments involve biochemistry (e.g., Western blotting of cell lysates after flow), then cells should be plated only within the flow channel. To position cells within the flow channel, a template is cut from a sheet of silicon (Scientific Instrument Services, #S11) to match the gasket and channel dimensions, and the silicon is pressed to seal to the culture dish. Cells are cultured within the silicon template, and the template is removed before the flow chamber is assembled.

Sterilize the chamber and gasket by rinsing in 200 proof ethanol and leaving under UV light for >20 min. Lay the gasket out onto a dry Kim wipe and seal the gasket to the chamber by carefully aligning the holes in the chamber with the flow channel in the gasket and pressing down. Push air bubbles out from beneath the gasket by smoothing.

2.3.3. Assembling the tubing and pump

The experiment is performed at 37° and 5% CO_2/95% air atmosphere. To prevent rusting and malfunction of the peristaltic pump, the pump remains outside of the incubator, and tubing is run in and out of the side port of the incubator. It is important to keep the flow path located outside of the incubator as short as possible to minimize the cooling of the media. Likewise, this apparatus can be assembled and placed on a microscope for real-time visualization, given that the microscope is equipped with a temperature/gas controlled incubator.

Assemble the flow path by running the tubing through the flow pump and placing the upstream end of the tubing into a 15-ml conical tube containing cell culture media. Prime the pump, filling the tubing with media. Place a second tube between the outlet port of the chamber and the media-containing conical tube.

Attach the vacuum line to the vacuum port of the chamber.
Aspirate the media from the 35-mm dish containing the cells.
To prevent a bubble from forming within the flow path, the upstream tubing should be attached to the chamber and flow should be started, allowing the port in the upper surface to overflow before the chamber is sealed to the 35-mm dish.
Once the flow is started and the media have exited the inlet port, turn the chamber upside down and invert the 35-mm dish onto the chamber. Once assembled, the vacuum will hold the chamber together and the entire assembly can be turned right side up.
Continue flow for desired time. We have successfully run this system continuously for up to 48 h.
At the completion of the experiment, the chamber can be disassembled by stopping the flow, turning off the vacuum, and lifting the chamber from the 35-mm dish. The cells can then either be fixed for visualization or lysed for biochemical assaying with standard methods.

2.4. Disturbed flow chamber

Because regions of disturbed flow correlate well with regions prone to atherosclerotic lesions, it is equally important to understand endothelial cell behavior in areas containing shear stress gradients. A number of investigators have proposed methods to create these gradients, including uniquely shaped flow paths within the parallel plate design (Usami *et al.*, 1993),

introduction of an obstacle into the flow path (Tardy *et al.*, 1997), and use of a backward facing step (Skilbeck *et al.*, 2001). In our own laboratory, we have modified the parallel plate flow chamber to create sharp spatial gradients within the flow path. The chamber contains the standard flow path within the gasket, with rectangular cutouts within the sides of the channel (see Fig. 2.3). The cutouts create shear gradients and vortices within the flow path, dependent on flow rate (Fig. 2.4). This chamber, unlike the backward facing step, allows the decoupling of the effects of spatial shear stress gradients from those caused by flow reattachment at low flow rates, and its flow profile is easy to visualize. Because the design is based on the conventional parallel plate flow chamber, it permits the direct microscopic observation of the cells within the flow path.

The chamber is made using customized rectangular hole punch on a standard parallel plate flow chamber gasket. The flow loop is assembled in the same way the standard parallel plate is assembled. The flow profile is characterized as described below.

2.5. Experimental characterization and visualization of the flow field

2.5.1. Materials

- Parallel plate flow chamber assembly as described previously.
- Fluorescent tracer beads, 2 μm (Invitrogen).

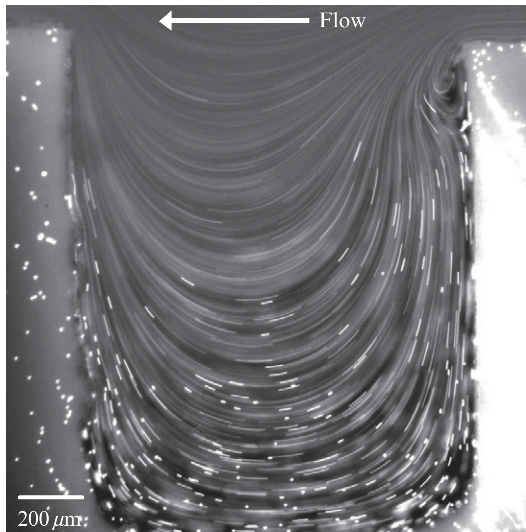

Figure 2.4 Streamlines of tracer beads flowing in the side cutout flow chamber, created as described in the text. Note the formation of a stable flow vortex at the inlet of the cutout.

- Cell culture medium.
- Inverted fluorescent microscope with CCD camera.

2.5.2. Methods

Sonicate beads to disperse aggregates.
Create a dilute suspension of tracer beads in cell culture medium: 10 μl of bead suspension into 20 ml of deionized water.
Assemble the flow chamber apparatus as described previously.
Pump the bead solution through the chamber at known flow rates.

After allowing the flow to equilibrate (>5 sec), collect fluorescent images at known exposure times. The exposure time is chosen on the basis of the flow rate to achieve clear streaks of the beads through the flow path. In the gasket shown, we use exposure times ranging from 0.2 to 1.0 sec for flow rates ranging from 0.1 to 9.0 ml/min.

The streaks created by the tracer beads outline the flow profile. See Fig. 2.4 for an example.

3. Cone and Plate Flow System

The cone and plate flow system works almost identically to a cone and plate viscometer. Actually, many laboratories, including our own, have adapted cone and plate viscometers to subject uniform shear stress distribution within the fluid environment of a cell culture dish. A number of systems are commercially available, including those from Wells-Brookfield, Thermo-Scientific, and Research Equipment Ltd., which can be adapted for use in cell culture. The cone and plate system consists of a cone with a well-defined angle that can rotate about its center axis. It is placed into a tissue culture dish and rotated, such that at sufficiently small Reynolds numbers, it produces a stable laminar flow.

The cone and plate system offers a number of advantages (Papadaki and McIntire, 1999), including the following:

- It produces a relatively stable flow such that small changes in the dimensions do not have significant effects on the wall shear stresses.
- The system requires very little medium because it requires a fixed volume, without a flow loop.
- The fluid flow is not dependent on a hydrostatic pressure gradient.
- Compared with the parallel plate chamber, there is a larger area of cell coverage, increasing the area for sampling and lysate recovery.
- The plates can be tissue culture dishes that are easily replaceable and are excellent for cell growth.

The cone and plate flow chamber does have some disadvantages. It is not easily adaptable for use on a microscope stage and real-time observation, and it does not allow for continuous sampling of the media. However, for studies of flow-mediated signaling, these disadvantages have not limited our experiments. An additional disadvantage of most commercially available viscometers is that they are typically open to the environment, permitting media evaporation. This prevents long-term studies of cell response to flow. We have addressed this problem by implementing a device that uses the cone and plate design but also covers the dish to prevent media loss.

3.1. Design of a cone and plate chamber

We have adopted use of a cone and plate design first described by Jo and colleagues (Boo *et al.*, 2002; Jo *et al.*, 2006). The cone is designed to fit into either a 35-mm or 100-cm tissue culture dish (Fig. 2.5). Unlike a typical cone and plate viscometer and many other commonly used cone and plate devices, this design includes an upper lid that rests on the top of the dish, minimizing evaporation of the cell culture media and permitting long-term flow conditions. This lid also supports the free rotation of the cone on an

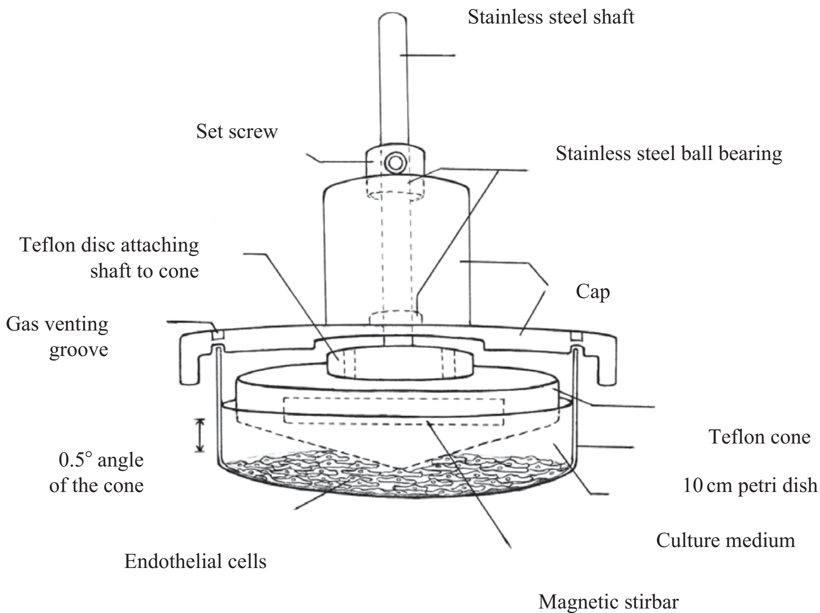

Figure 2.5 Schematic of a cone and plate design, initially described by Jo *et al.* (2006). The device is machined from Teflon and stainless steel, as described in the text. Image drawn by Ms. Becky Zhao.

axle secured by ball bearings and a setscrew. The setscrew controls the height of the cone from this dish. The cone is machined from Teflon to contain a 0.5-degree angle from its peak to its edge and is free from defects and machining marks so at to not affect the flow field. Teflon does not typically bind proteins and additives from the media, and it does not interfere with cell viability. To rotate the cone, a magnetic stir bar is embedded within the Teflon that can be turned with a laboratory magnetic stirrer. The entire system can be assembled in a temperature and CO_2 controlled chamber.

3.2. Calculation of the imposed fluid shear stress in a cone and plate flow chamber

The shear stress profile is uniform across the plate of a cone and plate flow chamber. This uniformity in shear stress is due to the increase of the gap height from the center of the cone countered by the linear increase in fluid velocity as a function of the radius. Because both the gap height and fluid velocity vary linearly with the radius, the shear stress is uniform throughout the flow field (Dewey *et al.*, 1981).

The shear stress imposed on the surface of the plate is calculated on the basis of the rotational speed of the cone:

$$\tau_w = \frac{\omega\mu}{\theta}, \qquad (2.2)$$

where ω is the rotation speed, μ is the fluid viscosity, and θ is the angle of the cone. The rotation of the cone in our system is determined by the speed set on the stir plate.

3.3. Protocol for subjecting endothelial cell to laminar fluid shear stress with a cone and plate flow chamber

3.3.1. Materials

- Human umbilical vein endothelial cells (HUVEC) are available through VEC Technologies (Rensselaer, NY) or can be isolated from a primary source.
- HUVEC media: 500 ml DMEM (Cascade Biologics) 10 ml LSGS (Cascade Biologics). 5% FBS (Gibco) carriage return.
- Cone and plate apparatus: needs to be machined to the preceding specifications. We have used the machine shop at the University of Alabama at Birmingham.
- Stir plate (Wheaton Microstir).

- 100-mm or 60-mm tissue culture–treated dishes: Size is chosen on the basis of the size of the cone.

3.3.2. Methods

Plate HUVEC on the tissue culture–treated dishes and allowed to grow to confluence.
Adjust the height of the cone by placing the cone in a dry tissue culture dish containing a #1 coverslip placed at its center. Align the coverslip with the peak of the cone and adjust the setscrew such that the peak of the cone rests on the coverslip
Add fresh media to the dish. The volume of media depends on the volume of the cone. Once the cone is added to the dish, the media should exceed the height of the cone, but should not come as high as the top edge of the dish. If media overflows once the cone is placed on the dish, the dish should be removed, and enough media should be removed to prevent the media from moving up the wall and overflowing. If the media are pulled to the top of the dish edge, capillary action will continue to draw media out of the dish causing the dish to dry out.
Position the cone into the dish, being careful not to trap air bubbles beneath the cone.
Place the cone and plate on the stir plate and initiate flow by turning on the stirplate to the desired speed.

4. Cyclic Stretching of the Endothelium

In addition to fluid shear stress, endothelial cells are exposed to cyclic stretch caused by the pumping action of the heart. For large human arteries, the vessel wall expands circumferentially by 5 to 12% under the normal physiologic condition (Birukov *et al.*, 2003; Nagel *et al.*, 1999). Effects of mechanical stretch on endothelial cells and other cell types have been studied with various types of cell stretch apparatuses. These *in vitro* studies have shown that stretch activates specific signaling pathways, causing cells to exhibit stretch-dependent phenotypes (Shi *et al.*, 2007).

To exert mechanical stretch onto cultured cells, several commercial devices are available, including those from Flexcell International and B-Bridge. Several different models of cell stretch devices are designed for different experimental purposes. In addition, investigators have developed their own apparatuses to meet the needs of specific studies (Arold *et al.*, 2007; Joung *et al.*, 2006; Lee *et al.*, 1996; Liu *et al.*, 2006; Takemasa *et al.*, 1997; Tschumperlin and Margulies, 1998; Yost *et al.*, 2000). Although these designs vary in their specific details, they all implement the same basic

technique: cells are grown on thin, stretchable, silicon membranes and mechanically stretched. For cells to be stretched, it is important that they must be firmly anchored to the membrane so that there is no slip between the cells and the membrane and also cells on the membrane should be stretched to the same extent as the membrane. To ensure firm cell attachment, silicon membranes are typically coated with an extracellular matrix protein such as collagen or fibronectin.

Here we describe a protocol for stretching a confluent culture of bovine aortic endothelial cells (BAECs) with a uniaxial cell stretch apparatus (Model NS-500) obtained from K. K. Scholar Tec (Osaka, Japan). The apparatus was designed by Naruse *et al.* (1998). Cells are grown in a stretch chamber (30 × 30 × 10 mm) made of silicon. (Fig. 2.6). The bottom of this chamber is a 100-μm-thick silicon membrane on which cells are plated. The chamber is mounted onto a mechanical stretch apparatus that is controlled by a computer to stretch the entire chamber by 5 to 25% at 1 to 60 cycles/sec. Experiments are performed in a tissue culture incubator (humidified and 5% CO_2 atmosphere). With this setup, cells can be kept under a cyclic stretch condition for several days.

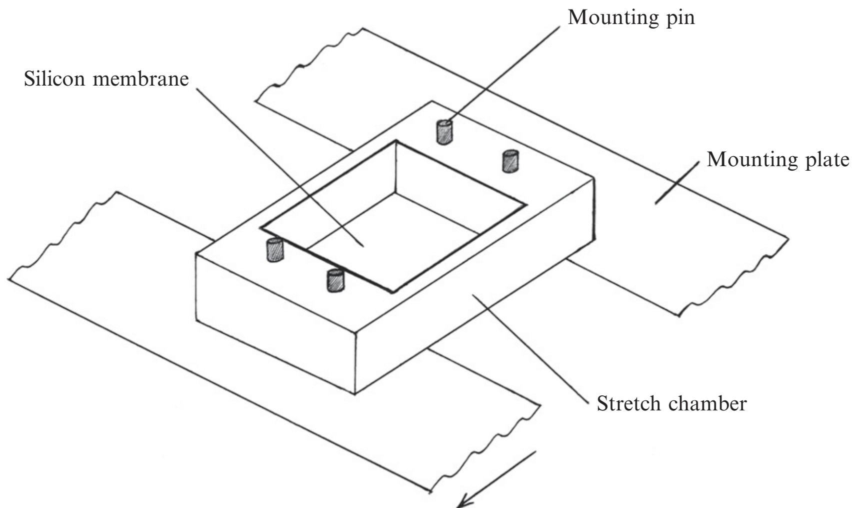

Figure 2.6 Schematic of a cell stretch chamber mounted on the metal plates of a stretch apparatus (Model NS-500). The frame of the chamber is made of 10-mm-thick silicon with a thin silicon membrane glued at the edges to create the bottom of the chamber. Cells are plated on this membrane. The chamber has four pin holes used to secure it to the mounting plates of the stretch apparatus. Initially, the mounting plates are separated such that when a chamber is mounted, it is at its rest length. One of the plates is fixed in position, whereas the other plate is connected to a motor and moved back and forth (arrow), cyclically stretching the chamber. Each mounting plate has 12 pins so that up to six chambers can be stretched simultaneously. Image drawn by Ms. Becky Zhao.

4.1. Protocol for subjecting endothelial cells to cyclic stress

4.1.1. Materials

- Rat Tail Type I Collagen (BD Biosciences).
- 0.02 *N* Acetic acid.
- Stretch apparatus and chambers as described previously.
- 10-cm Petri dishes.

4.1.2. Methods

- Each stretch chamber is autoclaved, air-dried, and placed in a sterile 10-cm petri dish in a laminar flow hood.
- Coat the bottom of the stretch chambers with rat tail type I collagen by adding 2 ml of 0.1 mg/ml of collagen in 0.02 *N* acetic acid to each chamber. Allow the solution to evaporate completely in the hood overnight.
- Wash chambers thoroughly with sterile phosphate-buffered saline (PBS: 137 m*M* NaCl, 8 m*M* Na_2HPO_4, 2.7 m*M* KCl, 1.6 m*M* KH_2PO_4, pH 7.4) to remove the acetic acid and sterilize them under UV light for at least 20 min.
- Plate BAECs, between passages 6 and 10, at the desired cell density in 2 ml of media. In our experiments, we plate 4 to 6×10^5 cells in 2 ml of DMEM supplemented with 10% FBS, 10 m*M* HEPES, and penicillin (50 IU/ml)/streptomycin (50 μg/ml), and allow them to form a tight monolayer over 2 to 3 days of culture. Other types of endothelial cells may require different culture media and plating conditions, which should be determined by each investigator.
- Set stretch conditions through the control box positioned outside of the tissue culture incubator. It provides various preset levels of amplitude (5, 6, 8, 10, 12, 15, 20, and 25% uniaxial stretch) and frequency (1, 5, 10, 20, 30, 40, 50, and 60 stretches/min). These values are for the cell stretch apparatus (Model NS-500). Other models may provide different sets of conditions. Our model is capable of stretching six chambers at a time, so that six stretch samples under the same condition or samples from different time points may be obtained.
- The duration of stretch is controlled through the main switch of the apparatus. It can be as short as a second or as long as days. We have grown cells under cyclic stretch for several days. Because cyclic stretch at high amplitude and frequency will cause the medium to splash, the culture medium should be periodically replenished.
- Control cells should be plated in stretch chambers as described previously and mounted in the stretch apparatus without stretching for the same length of time as experimental conditions. To account for the vibrations caused by the stretch apparatus, a second control is placed in a petri dish and on the lid of the stretch apparatus.

NOTES: When cells are cyclically stretched, they also experience shear stress caused by movements of culture medium. To avoid this problem, we have used a single sustained stretch (Chiu *et al.*, 2007). We operate the stretch machine manually such that the chamber is gently stretched to a desired length and kept at that length for the duration of experiments. A single sustained stretch is only feasible for short-term experiments, because it is anticipated that cells may adjust their adhesion points in time. When this ''reattachment'' takes place, cells would no longer be in a stretched state.

Stretched endothelial cultures may be analyzed for signaling molecule and enzyme activities, protein modifications, morphologic changes, gene and protein expression, and secretion of bioactive substances into the media. Here we describe the protocol for morphologic studies of cells, specifically immunocytochemistry, and harvesting cells for immunoblotting studies.

4.1.2.1. Immunofluorescence

Remove a stretch chamber from the stretch apparatus and place it in a 10-cm petri dish so that the bottom of the chamber (i.e., the thin silicon membrane) is supported. Slightly tilt the dish and remove culture medium by aspiration. Wash the inside of the stretch chamber with warm (room temperature −37°) PBS. Fix cells for 5 to 10 min at room temperature by directly adding 3 ml of 3.7% formaldehyde in PBS and wash thoroughly with PBS. If permeabilization is required, treat the fixed cells with 0.1% Triton X-100 in PBS for 1 to 2 min at room temperature and wash with PBS. Cold methanol may be used to fix cells also. After fixation, one may treat the specimen with a blocking solution. When an aspirator is used, one must be careful not to touch the thin membrane, which is easily damaged.

Stain cells with primary and secondary antibodies following a routine immunofluorescence protocol specific for the antibodies used.

To observe immunofluorescently stained cells in the chamber, it is most convenient to use a water immersion objective lens, which can be dipped directly into a PBS-filled stretch chamber. It should also be possible to place a coverslip on the membrane and use a dry or oil immersion lens, although we have not used this approach.

Fixed cells in stretch chambers stained for immunofluorescence should be removed when microscopy is done. Add 2% SDS solution in deionized water to the chamber and scrape cells off with a rubber policeman. Store chambers in a clean beaker with tap water until further and thorough cleaning (see following).

4.1.2.2. Western blotting

Remove a stretch chamber from the stretch apparatus and place it in a 10-cm petri dish so that the bottom of the chamber (i.e., the thin silicon membrane) is supported. Place the dish on ice and by slightly tilting the dish, remove culture medium by aspiration.

Wash the inside of the stretch chamber with cold PBS and add 0.1 ml SDS-PAGE sample buffer. Gently scrape off cells with a rubber policeman and collect the sample into a small vial with a pipette.

The sample is ready for a routine SDS-PAGE, followed by immunoblotting.

With appropriate solutions, one may fractionate stretched cells for biochemical analyses or isolating mRNA.

Chambers may be reused by washing them thoroughly (see later).

4.1.2.3. Cleaning chambers for reuse

Used chambers should be kept in a clean beaker with tap water until further cleaning.

To begin cleaning, place used chambers in a beaker containing hot detergent and sonicate for 15 min.

Rinse thoroughly (>five times) with hot tap water and then with RO (reverse osmosis) water five times. Sonicate in RO water for 15 min and rinse five times with RO water.

Rinse thoroughly with deionized water, air-dry, and keep them in a clean box.

Before reusing the chambers, autoclave.

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REFERENCES

Arold, S. P., Wong, J. Y., and Suki, B. (2007). Design of a new stretching apparatus and the effects of cyclic strain and substratum on mouse lung epithelial-12 cells. *Ann. Biomed. Eng.* **35,** 1156–1164.

Barbee, K. A., Davies, P. F., and Lal, R. (1994). Shear stress-induced reorganization of the surface topography of living endothelial cells imaged by atomic force microscopy. *Circ. Res.* **74,** 163–171.

Berk, B. C. (2008). Atheroprotective signaling mechanisms activated by steady laminar flow in endothelial cells. *Circulation.* **117,** 1082–1089.

Berk, B. C., Min, W., Yan, C., Surapisitchat, J., Liu, Y., and Hoefen, R. (2002). Atheroprotective mechanisms activated by fluid shear stress in endothelial cells. *Drug News Perspect* **15,** 133–139.

Birukov, K. G., Jacobson, J. R., Flores, A. A., Ye, S. Q., Birukova, A. A., Verin, A. D., and Garcia, J. G. (2003). Magnitude-dependent regulation of pulmonary endothelial cell barrier function by cyclic stretch. *Am. J. Physiol. Lung Cell Mol. Physiol.* **285,** L785–L797.

Boo, Y. C., Sorescu, G., Boyd, N., Shiojima, I., Walsh, K., Du, J., and Jo, H. (2002). Shear stress stimulates phosphorylation of endothelial nitric-oxide synthase at Ser1179 by Akt-independent mechanisms: Role of protein kinase A. *J. Biol. Chem.* **277,** 3388–3396.

Chien, S. (2007). Mechanotransduction and endothelial cell homeostasis: The wisdom of the cell. *Am. J. Physiol. Heart Circ. Physiol.* **292,** H1209–H1224.

Chiu, Y., McBeath, E., and Fujiwara, K. (2007). Fyn- and stretch-dependent PECAM-1 tyrosine phosphorylation in detergent extracted endothelial cell model. *Mol. Biol. Cell* **18** (Supp), 425.

Cybulsky, M. I., and Gimbrone, M. A., Jr. (1991). Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* **251,** 788–791.

Davies, P. F. (2007). Endothelial mechanisms of flow-mediated athero-protection and susceptibility. *Circ. Res.* **101,** 10–12.

Davignon, J., and Ganz, P. (2004). Role of endothelial dysfunction in atherosclerosis. *Circulation* **109,** III27–III32.

Dewey, C. F., Jr., Bussolari, S. R., Gimbrone, M. A., Jr., and Davies, P. F. (1981). The dynamic response of vascular endothelial cells to fluid shear stress. *J. Biomech. Eng.* **103,** 177–185.

Diamond, S. L., Eskin, S. G., and McIntire, L. V. (1989). Fluid flow stimulates tissue plasminogen activator secretion by cultured human endothelial cells. *Science* **243,** 1483–1485.

Dimmeler, S., Haendeler, J., Rippmann, V., Nehls, M., and Zeiher, A. M. (1996). Shear stress inhibits apoptosis of human endothelial cells. *FEBS Lett.* **399,** 71–74.

Fujiwara, K. (2003). Mechanical stresses keep endothelial cells healthy: Beneficial effects of a physiological level of cyclic stretch on endothelial barrier function. *Am. J. Physiol. Lung Cell Mol. Physiol.* **285,** L782–L784.

Garcia-Cardena, G., and Gimbrone, M. A., Jr. (2006). Biomechanical modulation of endothelial phenotype: Implications for health and disease. *Handb. Exp. Pharmacol.* **176,** 79–95.

Garin, G., Abe, J., Mohan, A., Lu, W., Yan, C., Newby, A. C., Rhaman, A., and Berk, B. C. (2007). Flow antagonizes TNF-alpha signaling in endothelial cells by inhibiting caspase-dependent PKC zeta processing. *Circ. Res.* **101,** 97–105.

Garin, G., and Berk, B. C. (2006). Flow-mediated signaling modulates endothelial cell phenotype. *Endothelium* **13,** 375–384.

Gimbrone, M. A., Jr., Topper, J. N., Nagel, T., Anderson, K. R., and Garcia-Cardena, G. (2000). Endothelial dysfunction, hemodynamic forces, and atherogenesis. *Ann. N. Y. Acad. Sci.* **902,** 230–239; discussion 239–240.

Go, Y. M., Park, H., Maland, M. C., and Jo, H. (1999). *In vitro* system to study role of blood flow on nitric oxide production and cell signaling in endothelial cells. *Methods Enzymol.* **301,** 513–522.

Hajjar, K. A., and Deora, A. (2000). New concepts in fibrinolysis and angiogenesis. *Curr. Atheroscler. Rep.* **2,** 417–421.

Jo, H., Song, H., and Mowbray, A. (2006). Role of NADPH oxidases in disturbed flow- and BMP4- induced inflammation and atherosclerosis. *Antioxid. Redox Signal* **8,** 1609–1619.

Joung, I. S., Iwamoto, M. N., Shiu, Y. T., and Quam, C. T. (2006). Cyclic strain modulates tubulogenesis of endothelial cells in a 3D tissue culture model. *Microvasc. Res.* **71,** 1–11.

Kandlikar, S., Garimella, S., Li, D., Colin, S., and King, MR. (2005). "Heat Transfer and Fluid Flow in Minichannels and Microchannels." New York: Elsevier Science, New York.

Ku, D. N., Giddens, D. P., Zarins, C. K., and Glagov, S. (1985). Pulsatile flow and atherosclerosis in the human carotid bifurcation. Positive correlation between plaque location and low oscillating shear stress. *Arteriosclerosis* **5,** 293–302.

Lee, A. A., Delhaas, T., Waldman, L. K., MacKenna, D. A., Villarreal, F. J., and McCulloch, A. D. (1996). An equibiaxial strain system for cultured cells. *Am. J. Physiol.* **271,** C1400–C1408.

Liu, J., Liu, T., Zheng, Y., Zhao, Z., Liu, Y., Cheng, H., Luo, S., and Chen, Y. (2006). Early responses of osteoblast-like cells to different mechanical signals through various signaling pathways. *Biochem. Biophys. Res. Commun.* **348,** 1167–1173.

Malek, A. M., Alper, S. L., and Izumo, S. (1999). Hemodynamic shear stress and its role in atherosclerosis. *JAMA* **282,** 2035–2042.

Nagel, T., Resnick, N., Dewey, C. F., Jr., and Gimbrone, M. A., Jr. (1999). Vascular endothelial cells respond to spatial gradients in fluid shear stress by enhanced activation of transcription factors. *Arterioscler. Thromb. Vasc. Biol.* **19,** 1825–1834.

Naruse, K., Yamada, T., and Sokabe, M. (1998). Involvement of SA channels in orienting response of cultured endothelial cells to cyclic stretch. *Am. J. Physiol.* **274,** H1532–H1538.

Papadaki, M., and McIntire, L. V. (1999). Quantitative measurement of shear-stress effects on endothelial cells. *In* "Tissue Engineering Methods and Protocols" (J. R. M. M. L. Yarmush, ed.), Humana Press, Totowa, NJ.

Pearson, J. D. (2000). Normal endothelial cell function. *Lupus* **9,** 183–188.

Shi, F., Chiu, Y. J., Cho, Y., Bullard, T. A., Sokabe, M., and Fujiwara, K. (2007). Down-regulation of ERK but not MEK phosphorylation in cultured endothelial cells by repeated changes in cyclic stretch. *Cardiovasc. Res.* **73,** 813–822.

Skilbeck, C., Westwood, S. M., Walker, P. G., David, T., and Nash, G. B. (2001). Population of the vessel wall by leukocytes binding to P-selectin in a model of disturbed arterial flow. *Arterioscler. Thromb. Vasc. Biol.* **21,** 1294–1300.

Takemasa, T., Sugimoto, K., and Yamashita, K. (1997). Amplitude-dependent stress fiber reorientation in early response to cyclic strain. *Exp. Cell Res.* **230,** 407–410.

Tardy, Y., Resnick, N., Nagel, T., Gimbrone, M. A., Jr., and Dewey, C. F., Jr. (1997). Shear stress gradients remodel endothelial monolayers *in vitro* via a cell proliferation-migration-loss cycle. *Arterioscler. Thromb. Vasc. Biol.* **17,** 3102–3106.

Traub, O., and Berk, B. C. (1998). Laminar shear stress: Mechanisms by which endothelial cells transduce an atheroprotective force. *Arterioscler. Thromb. Vasc. Biol.* **18,** 677–685.

Tschumperlin, D. J., and Margulies, S. S. (1998). Equibiaxial deformation-induced injury of alveolar epithelial cells *in vitro*. *Am. J. Physiol.* **275,** L1173–L1183.

Usami, S., Chen, H. H., Zhao, Y., Chien, S., and Skalak, R. (1993). Design and construction of a linear shear stress flow chamber. *Ann. Biomed. Eng.* **21,** 77–83.

World, C. J., Garin, G., and Berk, B. (2006). Vascular shear stress and activation of inflammatory genes. *Curr. Atheroscler. Rep.* **8,** 240–244.

Yoshizumi, M., Abe, J., Tsuchiya, K., Berk, B. C., and Tamaki, T. (2003). Stress and vascular responses: Atheroprotective effect of laminar fluid shear stress in endothelial cells: Possible role of mitogen-activated protein kinases. *J. Pharmacol. Sci.* **91,** 172–176.

Yost, M. J., Simpson, D., Wrona, K., Ridley, S., Ploehn, H. J., Borg, T. K., and Terracio, L. (2000). Design and construction of a uniaxial cell stretcher. *Am. J. Physiol. Heart Circ. Physiol.* **279,** H3124–H3130.