To the Graduate Council:

I am submitting herewith a thesis written by Kevin Wayne Belew entitled "Evaluation of the Morphological and Cytoskeletal Reorganization of Retrovirally Transduced Human Umbilical Vein Endothelial Cells in Response to Steady, Laminar Flow." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Engineering Science.

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We have read this thesis
and recommend its acceptance:

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Accepted for the Council:

Associate Vice Chancellor and Dean of
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Evaluation of the Morphological and Cytoskeletal Reorganization of Retrovirally Transduced Human Umbilical Vein Endothelial Cells in Response to Steady, Laminar Flow

A Thesis
Presented for the
Master of Science
Degree
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Kevin Wayne Belew
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Dedication

This thesis is dedicated to my parents, Wayne and Sheila Belew,

for their love, patience, and support;

and to my wife, Melissa, and sons, Trent and Luke,

who are my inspiration.

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Abstract

The role of the vascular endothelial cells in maintaining vascular wall integrity and a thrombus-free flow surface has been well documented. Extending this knowledge to the field of vascular grafting has the potential to improve the patency of synthetic vascular grafts, especially small inner diameter grafts. Research into seeding synthetic vascular grafts has shown promise in animal models, while limited human trials have been disappointing. Genetic alteration of endothelial cells in order to enhance their fibrinolytic action has emerged as a plausible improvement for endothelial cell seeded grafts; however, preliminary *in vitro* and *in vivo* experiments have suggested that genetically altered endothelial cells may be impaired in their ability to adhere to synthetic graft surfaces. The cause of this decreased adherence may be a consequence of integrin alteration secondary to retroviral gene transduction. The aim of the present study was to examine the effect of this integrin loss after retroviral gene transduction on human umbilical vein endothelial cell cytoskeletal development following fluid imposed shear stress. Genetically modified endothelial cells used in this study were transduced with the LN retroviral vector, conferring neomycin resistance to the cells. Naive human umbilical vein endothelial cells served as controls. The cells were seeded onto fibronectin coated glass slides and subjected to steady laminar flow for 66 hours with 25 dynes/cm^2 shear stress. A recirculating flow system and a parallel plate chamber provided the desired flow properties while maintaining a viable cell environment and facilitating collection of data. Through video microscopy, the response of endothelial cells within randomly

chosen fields were quantified by analyzing the digitized images at specified time points. The images were used to obtain two parameters related to endothelial cell morphology, the cell shape index and angle of orientation. Following the 66 hour flow period, the cells were immunofluorescently stained to allow inspection of the cytoskeletal protein, F-actin, and a focal adhesion protein, vinculin. The results of the experiment indicated a statistically significant difference in cell morphology between the initial and final time points when the same cell lines were compared as well as for transduced versus naive cell lines. Since the measures were different at the initial time point, the difference of the measures at the final time point may not be indicative of a difference in the ability of the cells to respond to the shear stress. Changes in shape index and angle followed a similar trend in both the naive and transduced endothelial cells for the duration of the experiment. Immunofluorescent staining of the cytoskeleton revealed no qualitative difference in the amount or pattern of the stress fibers. Vinculin staining was unsuccessful in allowing inspection of vinculin association with the actin fibers of the cytoskeleton.

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NOMENCLATURE

A	cell area
α	integrin subunit
β	integrin subunit
cm^2	area unit, square centimeter
g	gram
h	channel height
l	liter
ml	milliliter
mV	millivolt
P	cell perimeter
pH	degree of acidity or alkalinity
Q	volumetric flow rate
SI	cell shape index
τ_{wall}	wall shear stress
θ	angle of orientation
μ	viscosity of flow media
μg	microgram
μl	microliter
μm	micrometer (micron)
U	units

W	channel width
y	vertical position
$\frac{dp}{dx}$	differential change in pressure along the direction of flow

Abbreviations

ACE	angiotensin converting enzyme
CO_2	carbon dioxide
DMEM	Dulbecco's Modified Eagle Media
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediaminetetraacetic acid
EILDV	Glu-Ile-Leu-Asp-Val sequence
EGM	Endothelial Growth Media
ePTFE	expanded polytetrafluoroethylene
ERK1/2	extracellular signal-regulated kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HBSS	HEPES Buffered Saline Solution
hEGF	human Endothelial Growth Factor
HUVEC	human umbilical vein endothelial cells
LTR	long terminal repeat
MANOVA	multi-variant analysis of variance

neoR	neomycin resistance gene
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PGI_2	prostacyclin
PTFE	polytetrafluoroethylene
RGD	Arg-Gly-Asp sequence
RNA	ribonucleic acid
ssRNA	single stranded ribonucleic acid
tPA	tissue-type plasminogen activator
TNS	Trypsin Neutralizing Solution
TTV	thrombotic threshold velocity
VEGF	vascular endothelial growth factor
vWF	von Willebrand Factor

CHAPTER 1

INTRODUCTION

Vascular grafting is a common procedure in the treatment of severely diseased or traumatized blood vessels. Approximately 100,000 people with lower limb vascular disease undergo re-constructive surgery with either autologous blood vessels or synthetic vascular grafts annually (Burkel, 1988). Synthetic grafts have been moderately successful in replacing large diameter blood vessels (> 6 mm ID); however, small diameter synthetic grafts (≤ 6 mm ID) have repeatedly exhibited poor results (Hufnagel, 1983). In a multi-institutional randomized study by Veith et al. (1986) of infrainguinal arterial reconstructions, autologous vein grafts exhibited patency rates of 76% after 48 months while prosthetic (expanded polytetrafluoroethylene) graft patency rates were significantly lower at 54%. The use of autologous tissue for vascular grafts is restricted by the limited availability of vessels (Wesolowski, 1962). Therein lies the impetus for research into improving the synthetic grafts of today or developing new concepts for grafting materials of tomorrow.

In an attempt to resolve two major contributors to small diameter graft failure, thrombosis and anastomotic pseudointimal hyperplasia (Greisler, 1991), researchers are striving to incorporate an endothelial lining into the prosthetic graft (Zilla et al., 1987). The interest in this area of research has been spurred by heightened awareness of endothelial cell biology and by the potential use of genetically recombinant endothelial

cells to enhance the fibrinolytic and antiproliferative properties of synthetic grafts.

Over the past quarter of a century, an increased understanding of the active role that the endothelial lining plays in vascular function and the mechanisms by which endothelial cells sense and respond to stimuli has been realized. Aside from serving as a semi-permeable barrier between blood and underlying tissue that governs molecular transport, endothelial cells participate in regulation of several homeostatic processes including control of vascular tone, growth of smooth muscle cells and fibroblasts, angiogenesis, coagulation, and fibrinolysis (Palade, 1988). In addition, research has shown that vascular endothelial cell function and morphology are dependent upon the physiological environment to which the cells are exposed (Gimbrone and Bevilacqua, 1987). In the normal quiescent state, endothelial cells act to enhance the nonthrombogenic properties of the vessel wall, but when injured or altered the endothelial cell function tends toward coagulation (Libby and Birinyi, 1987).

Many researchers are investigating the pathways by which endothelial cells sense and respond to the mechanical and biochemical forces present in their environment. Studies have shown that the association of integrins, cell surface adhesion receptors, with the cell cytoskeleton are vital in many mechano-chemical pathways (Wechezak et al., 1989; Morita et al., 1993; Girard and Nerem, 1995; Burridge and Chrzanowska-Wodnicka, 1996; Malek and Izumo, 1996; Blum et al., 1997; Irigoyen et al., 1997). An increased understanding of endothelial cell biology and advances in the field of genetics have spurred research interest into the potential use of gene therapy as an enhancement to the endothelialization of synthetic vascular grafts.

Gene therapy is an exciting research field that has potential use in the treatment of rare genetic disorders, such as hemophilia, and more common disorders including cardiovascular diseases and cancer (Lyon and Gorner, 1995). The aim of the treatment may be to correct a genetic defect or to induce the production of desired therapeutic or marker proteins (Callow, 1990). Many researchers have sought to implement vascular gene therapy in the management of two vascular pathologies that plague vascular grafts: anastomotic pseudointimal hyperplasia and thrombosis (Flugelman, 1995). Because of the wealth of information available about endothelial cell function and improved culturing techniques, the endothelial cell is an attractive target for delivery of the therapeutic proteins (Callow, 1990). Several investigators have successfully modified endothelial cells to express various proteins (Dichek et al., 1989; Zweibel et al., 1989; Callow, 1990; Flugelman, 1995; Dunn et al., 1996; Sackman et al., 1995).

Capitalizing upon the positive aspects of endothelialized vascular grafts hinges upon the ability to completely endothelialize the lumen of the graft (Graham et al., 1991; Truskey and Reichert, 1996). While promising results have been shown with endothelial seeding in the canine model (Burkel et al., 1982; Koveker et al., 1986; Budd et al., 1991; Pasic et al., 1995), limited human trials have been disappointing in attaining a completely endothelialized layer (Walker et al., 1987). Recent studies using genetically recombinant endothelial cells *in vitro* and in the canine model have shown poorer adherence to the graft material for recombinant endothelial cells (Huber et al., 1995; Sackman et al., 1995; Dunn et al., 1996). A possible factor for this apparent decrease in adherence has been suggested by Sackman et al. (1997). Their investigation found that retroviral transduction

of both human umbilical vein endothelial cells and canine jugular vein endothelial cells with vectors containing phosphotransferase genes resulted in altered expression of the β_1 and β_3 integrin subunits as evidenced by the absence or truncation of these subunits. They hypothesized that the altered integrin subunits of the transduced cells may be a result of overexpression of the phosphotransferase gene.

Adherence to a substrate by an endothelial cell subjected to flow is believed to depend upon the cell's ability to elongate and align in the direction of the flow (Dewey et al., 1981; Nerem et al., 1981; Levesque and Nerem, 1985). The mechanism by which endothelial cells sense and respond to the flow stimulus is not fully understood at the molecular level. However, rearrangement of the cytoskeleton, and in particular the formation of bundles of F-actin called stress fibers, has been shown to be an important factor in adhesion (Franke et al., 1984; Wechezak et al., 1989). Integrins providing a link between the substrate and the cytoskeleton. Since alteration or deletion of integrin subunits has been shown to prevent cell binding to the extracellular matrix and the cytoskeleton, Sackman et al. (1997) further reasoned that the truncated or absent β subunits may play a role in the decreased adherence of retrovirally transduced endothelial cells.

The aim of the present study was to test the hypothesis that the recombinant endothelial cells were impaired in their ability to rearrange their cytoskeleton in response to fluid imposed shear stress. Naive and retrovirally transduced endothelial cells were seeded onto fibronectin coated glass slides and subjected to a shear stress of 25 dynes/cm^2 for 66 hours using a parallel plate flow system. In order to quantitatively compare the cell

shape change and reorientation, the cell shape index and angle with respect to flow were determined using a public domain image analysis program. The reorganization of the actin cytoskeleton and association of a focal adhesion protein, vinculin, were observed using immunofluorescent staining and visualization techniques.

CHAPTER 2

BACKGROUND

The Vascular System

The human vascular system consists of specialized tissues involved in the transport or circulation of blood throughout the body. The blood vessels involved in the circulation of blood can be divided into three broad categories: arteries, capillaries, and veins. These general divisions are characterized by functional differences. Arteries carry blood away from the heart and progressively become smaller as they branch out to all the tissues in the body. Arterioles are the smallest of the arteries and empty into a bed of capillaries. Capillaries serve as the middle ground between arteries and veins and provide an effective place for exchange of nutrients and oxygen in the blood for waste produced by the surrounding tissue. The deoxygenated blood begins its return to the heart through venules, tiny veins. The venules unite to form increasingly larger veins that eventually return the blood to the heart. Certain structural differences allow efficient function of each type of vessel. (Fung and Liu, 1993)

Walls of arteries and veins can be divided into three regions: the intima, the tunica media, and the adventitia as shown in Figure 2.1. The intima is the innermost layer of a blood vessel. This luminal surface contains endothelial cells which adhere to a basal lamina, a specialized mat of extracellular matrix proteins such as fibronectin, collagen,

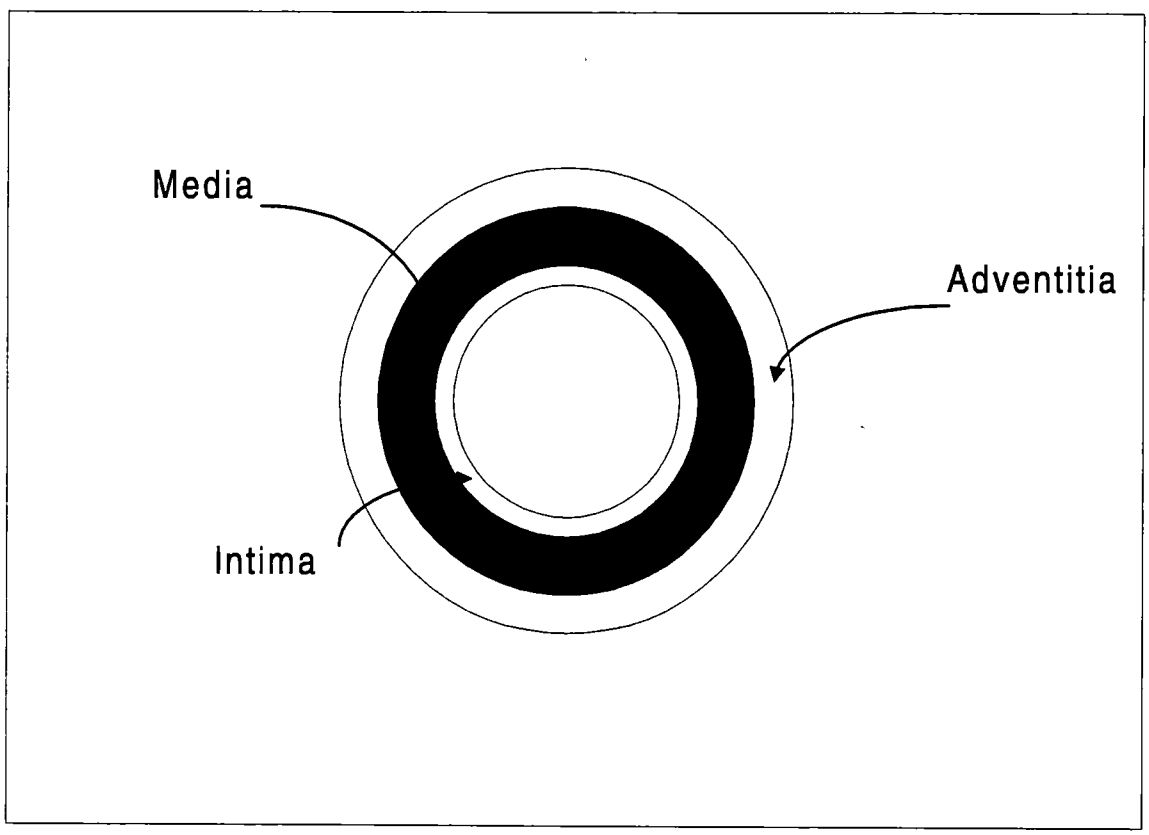

Figure 2.1. Three Layers of Typical Artery or Vein Wall

smooth muscle cells, elastic fibrils, and collagenous bundles. The tunica media is characterized by a layer of smooth muscle cells, elastic sheets, a network of elastic fibrils, and bundles of collagenous tissue. The adventitia is the outermost layer of a blood vessel. It primarily consists of collagen fibers and ground substances, but may also contain vasa vasorum, nerves, fibroblasts, and macrophages. Capillaries are composed simply of endothelial cells and a basal lamina. (Fung and Liu 1993)

The three types of blood vessels can be identified by the differences in the composition of their three regions. These variations optimally support different functions for each type of vessel. Arteries in general have thicker walls and contain more smooth muscle cells than veins of the same inner diameter. These characteristics allow arteries to withstand the relatively higher pressures of arterial blood flow and efficiently pump blood throughout the body as evidenced by palpable pulses at the extremities such as wrists and ankles. Capillaries with their small diameters and thin walls allow rapid diffusion of molecules between the blood and the surrounding tissues. Veins, suited for low pressure driven flow, are generally more collagenous and have less elastin with a relatively thick adventitia. In addition, veins contain one way valves which allow unidirectional flow of the blood toward the heart, but prevent counterproductive backflow. (Fung and Liu, 1993)

The Cytoskeleton and Integrins

Of particular interest in the present study are the cell cytoskeleton and cell surface receptor proteins known as integrins. These structures found in endothelial cells are

common to many eukaryotic cells. Therefore, an overview of the basic structure of these important cell components is necessary.

Cell Cytoskeleton

The cytoskeleton is an intracellular network of fibrous material constituting a dynamic framework providing mechanical strength and intracellular organization. It is composed of three types of fibers. Microfilaments, microtubules, and intermediate filaments are most often present within the cytoskeleton. These fibers are connected to each other and other cell structures by various proteins. The components of the cytoskeleton are in a state of constant assembly and disassembly facilitating shape changes and migration of the cell. (Prescott, 1988)

Changes in cell shape are caused by reorganization of the cytoskeleton. Stimuli for cytoskeleton reorganization include both external and internal forces. Cell division and differentiation are examples of internal signals, while mechanical stress is an example of an external stimulus. Certain substances such as phalloidin, cytochalasin, and colchicine possess the ability to prevent the assembly or disassembly of the fibrous components of the cytoskeleton and have been utilized to study the cytoskeleton. (Prescott, 1988)

Microfilaments

Microfilaments are important components of the cytoskeleton in that they are involved in the movement of both muscle and nonmuscle cells. They are composed of long filaments of the protein molecule actin and typically arranged with other

microfilaments in parallel bundles. These bundles are often very prominent within cells and have been termed stress fibers. Stress fibers are connected to integral transmembrane proteins called integrins. Integrins are in turn directly connected to adhesion proteins in the extracellular matrix. (Prescott, 1988)

Integrin Structure and Function

Adhesion of cells to proteins in the extracellular matrix provides signals for a number of cellular activities including motility, gene expression, and division. Major participants in the adhesion of cells to the basal lamina are adhesion receptors found on the cell surface known as integrins. Integrins are a class of cell surface proteins that participate in both cell-cell and cell-matrix adhesion. They are heterodimers consisting of noncovalently joined α and β subunits. Figure 2.2 illustrates three distinct regions of integrin structure: the cytoplasmic domain, the transmembrane segment, and the extracellular domain. Currently twenty-two different integrins, which differ in their respective combinations of the known fourteen α and eight β subunits, have been discovered along with many alternatively spliced variants of each integrin. (Hynes, 1992)

Integrins participate in adhesion by binding to specific polypeptide sequences found within a potential ligand. Binding of the protein or ligand occurs within the extracellular domain of an integrin. The ability to bind to more than one specific protein has been exhibited by some integrins. An extreme example of ligand diversity is the $\alpha_v\beta_3$ integrin which can serve as a receptor to vitronectin, fibronectin, fibrinogen, von Willebrand factor, thrombospondin, and osteopontin (Johansson et al., 1997). It is also

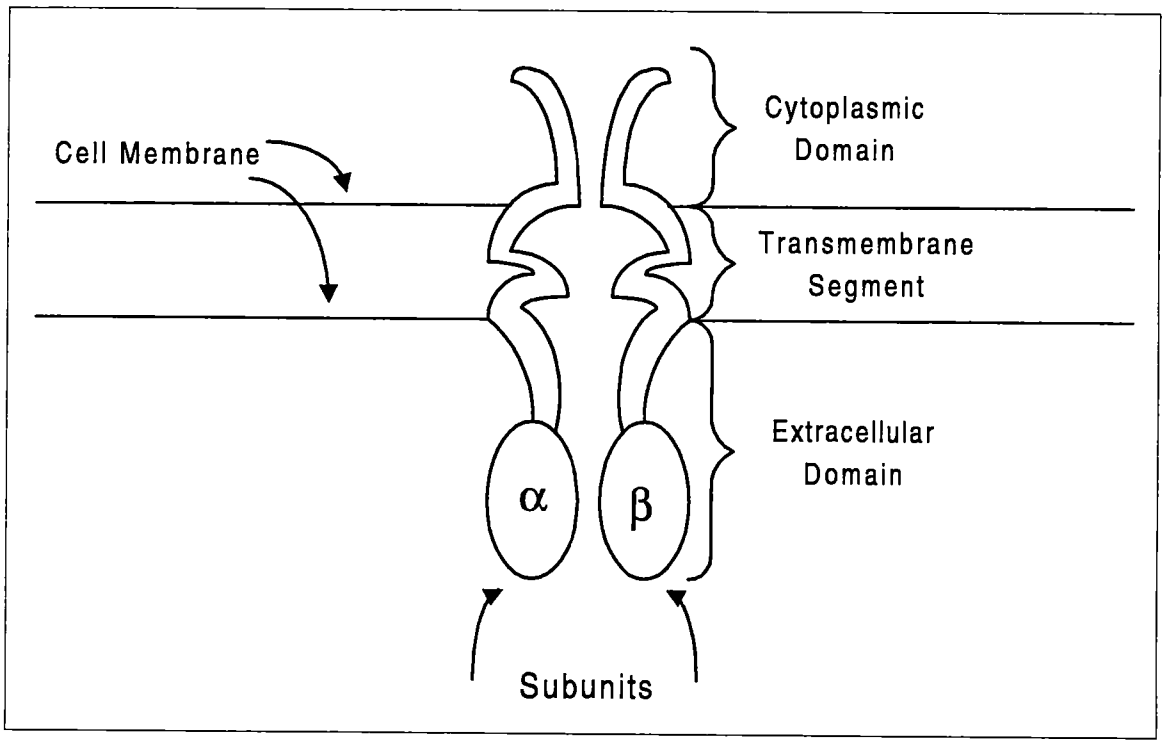

Figure 2.2. Integrin Structure

noteworthy that a particular protein may be bound by more than one integrin. For example, fibronectin is vital to many cell functions as evidenced by the fact it is recognized by ten different integrins (Hynes, 1992). Two of these fibronectin-associated integrins are the $\alpha_5\beta_1$ and the $\alpha_4\beta_1$ integrins. The main binding region on fibronectin for the $\alpha_5\beta_1$ integrin, whose only known ligand is fibronectin, is the widely recurring binding motif, the Arg-Gly-Asp (RGD) sequence. Also able to bind fibronectin and vascular cell adhesion molecule-1, the $\alpha_4\beta_1$ integrin interacts with the Glu-Ile-Leu-Asp-Val (EILDV) sequence of fibronectin (Johansson et al., 1997).

Because cells often need to detach from the extracellular matrix, as during migration or division, integrins exist in active and inactive binding states (Hynes, 1992). Energy dependent reactions occurring at the cytoplasmic tails of integrins cause conformational changes in the extracellular domain. These conformational changes are thought to activate and deactivate integrins by preventing the extracellular domain of the integrin from physically interacting with the main binding sequence of the ligand (Johansson et al., 1997).

Focal Adhesions

A focal adhesion or focal contact is a site of tight adhesion of the plasma membrane to the extracellular matrix that provides a physical connection between the actin cytoskeleton and the extracellular matrix. More than just being sites of anchorage, focal adhesions are involved in the transduction of signals that relate to growth control. A focal adhesion is composed of aggregated, ligand-bound integrins associated with

bundles of cytoskeletal actin, or stress fibers, through an intricate network of proteins termed a focal adhesion plaque. Proteins commonly found in focal adhesion plaques are α-actinin, filamin, vinculin, talin, tensin, zyxin, radixin, fimbrin, profilin, focal adhesion kinase, and paxillin. Proteins present in the focal adhesion plaque provide either structurally important connections of proteins and/or signals related to many vital cell functions. Vinculin is a structurally abundant protein in focal adhesions and is known to associate with or bind actin. (Burridge and Chrzanowska-Wodnicka, 1996)

Present research indicates that the first step in the assembly of a focal contact is activation of the integrin. This activation has been induced experimentally and can be caused by extracellular or intracellular signals. The second step is ligand binding by a number of integrins. Binding of the integrin with a ligand induces a conformational change of the integrin's cytoplasmic domain. This change allows the β subunit to interact with either talin or α-actinin, proteins found in focal adhesion plaques. Talin and α-actinin possess the ability to bind β subunits and actin, thereby, providing a structural link between the integrin and the actin cytoskeleton of the cell. Aggregation of the bound integrins and components of the focal adhesion plaque follow. The aggregation of the integrins activates focal adhesion kinase that results in the assembly of a multi-component signaling complex that is associated with the focal adhesion. An important regulator of focal adhesion assembly is the protein Rho, hypothesized to stimulate focal adhesion assembly through the bundling of stress fibers which aggregates the ligand-bound integrins. (Burridge and Chrzanowska-Wodnicka, 1996)

The Vascular Endothelial Cell

Endothelial cells line the lumen of the entire human vascular system forming a continuous monolayer of cells in constant contact with the blood. They actively participate in many vital physiological functions ranging from thrombosis to the control of vascular tone. In tissue culture, endothelial cells are characterized by contact inhibited growth, staining for Factor VIII or von Willebrand Factor (vWF), detection of angiotensin converting enzyme (ACE), presence of Weibel Palade bodies, and prostacyclin (PGI_2) production. The ability to isolate and efficiently culture vascular endothelial cells has opened the door to a staggering amount of research dealing with this once overlooked but extremely vital component of the vascular system.

Endothelial Cell Function

The past quarter century has seen the realization of the active role that the endothelial lining plays in vascular function. As regulators of macromolecular transport between the blood and the surrounding tissue, endothelial cells are important in the supply of nutrients to all tissues within the body and in the removal of waste from these same tissues. Endothelial cells also secrete components of the underlying basal lamina, such as collagen IV, fibronectin, and other proteoglycans (Palade, 1988). By altering the synthesis of proteins and metabolism of various substances found in the plasma, the endothelial cell is involved in many homeostatic events. Endothelial cells have demonstrated the ability to regulate vascular tone by production of both vasodilators, such as prostacyclin, nitric oxide, believed to be the major endothelium-derived relaxing factor

(EDRF), and vasoconstrictors, such as endothelin (Ando and Kamiya, 1993). Endothelial cells also produce and uniquely respond to vascular endothelial growth factor (VEGF) which has been implicated in angiogenesis (Herman, 1987).

Of particular interest to researchers involved in improving the patency of synthetic vascular grafts are the roles which endothelial cells assume in preventing thrombosis and controlling smooth muscle cell growth. Under normal physiological conditions, the endothelial lining is a non-proliferating monolayer of cells that provides an anti-coagulant surface for blood flow. Heparin sulfates secreted by endothelial cells are important in maintaining an anticoagulant surface (Pearson, 1987). Prostacyclin and EDRF, both vasodilators, also confer anti-platelet aggregating properties (Ando and Kamiya, 1993). The endothelial cell is also intimately involved in the anticoagulatory protein C/protein S pathway. Thrombomodulin, expressed on the surface of endothelial cells, binds to and inactivates thrombin, an important protein in the formation of fibrin. The thrombin/thrombomodulin complex then activates protein C. In turn, the activated protein C binds to protein S found on the surface of endothelial cells and inactivates the clotting factors VIIIa and Va (Esmon, 1988). Secretion of tissue-type plasminogen activator, tPA, a fibrinolytic protein, from endothelial cells has also been observed (Moscatelli et al., 1984).

When activated by mechanical injury or by inflammation, endothelial cells promote coagulation and thrombosis as a means of hemostasis. Under these conditions, the endothelial cell surface has been shown to be an important site for the activation of factors involved in the clotting cascade, such as Factor X. Endothelial cells also

synthesize clotting factor V/Va and vWF, an adhesion molecule for platelets (Stern et al., 1988). Endothelial cells have also demonstrated the ability to secrete interleukin 1 (IL-1), an immune response mediator, that decreases fibrinolytic activity (Curriden et al., 1988).

Evidence further suggests that endothelial cells are involved in the inhibition and stimulation of smooth muscle cell proliferation. Heparin-sulfates secreted by endothelial cells, which play a role in anti-coagulation, also act to inhibit smooth muscle cell proliferation. In contrast, activated endothelial cells synthesize and secrete a platelet derived growth factor (PDGF), that stimulates proliferation of the underlying smooth muscle cells (Castellot et al., 1984).

Effects of Shear Stress on Endothelial Cells

Endothelial cells line the interior of all blood vessels in the body and actively participate in the regulation of blood flow through the vessels. Over the past two decades, fluid imposed shear stress has been shown to be an important modulator of endothelial cell morphology and function (Ando and Kamiya, 1993). Table 2.1 briefly outlines the setup for past shear stress experiments to be covered in the proceeding discussion. With the exception of Kim et al. (1989) and Nerem et al. (1981), all the experiments shown in Table 2.1 were conducted *in vitro* and utilized various flow systems to subject the cells to varied levels and durations of shear stress.

Nerem et al. (1981) studied the shape and orientation of endothelial cells in the aortic intercostal ostia and found their morphology to be an indicator of the pattern of blood flow. Many investigators have confirmed, both *in vitro* and *in vivo*, that

Table 2.1. Fluid Imposed Shear Stress Experiments

Authors	Cell Type	Substrate	Shear Level (dynes/cm^2)	Duration (hrs)	Notes
Davies et al. (J. Clin. Inv. 1994)	BAEC (Bovine Aortic Endothelial Cells), confluent	glass and gelatin coated glass coverslips	10	24-48	Cells aligned @24-48 hrs. Coating retarded rearrangement
Davies et al. (Proc. Nat. Acad. Sci.1986)	BAEC, confluent	glass coverslips	Utilized both laminar (8-15) and turbulent shear stress (15)	24	Cells exposed to laminar rearranged within 24 hrs. Turbulent flow induced cell retraction, rounding and loss
Davies et al. (J. Clin. Inv. 1984)	BAEC, confluent	glass coverslips	Utilized steady (1-15), periodic square wave (0-13), and oscillating (3-13)	up to 24	Found rate of pinocytotsis greatly influenced by alterations in fluid shear stress.
Dewey (J. Biomech. Eng. 1984)	BAEC, confluent	glass coverslips	8	72	Cells not significantly realigned until 48 hrs.
Dewey et al. (J. Biomech. Eng. 1981)	BAEC, post and subconfluent	glass coverslips	5&8	24 and 48	Observed shape change @48 hrs
Franke et al. (Nature 1984)	HUVEC (Human Umbilical Vein Endothelial Cells), confluent	glass coverslips precoated with a layer Bovine Corneal Endothelial Cell Extracellular Matrix	2	4	Stress fibers induced upon exposure time of 3 hrs as evidenced by intensity and quantity after fluorescent staining
Girard and Nerem (J. Cell. Phys. 1995)	BAEC, confluent	slides cut from tissue culture dishes or mylar sheets	30	48	Vinculin and $\alpha_v\beta_3$ integrins localized at upstream of cell. Increase in surface level of $\alpha_5\beta_1$.
Hu and Chien (J. Hist. Cyt. 1997)	HUVEC, confluent	glass slides precoated w/ 2% gelatin	20	0.5	Determined that shear stress increases the amount and alters the distribution of protein kinase C. Double staining also revealed association of actin with protein kinase C in static and sheared cells.
Kim et al. (Arterioscl. 1989)	RAEC, (Rabbit Aortic Endothelial Cells)	Not Applicable	Not Applicable	2 weeks	Experimentally altered shear stress within aorta via a coarctation. Cells stained in situ and revealed that cells in high shear area elongated in direction of flow and exhibited actin reorganization.

Table 2.1 (continued)

Authors	Cell Type	Substrate	Shear Level (dynes/cm^2)	Duration (hrs)	Notes
Levesque and Nerem (J. Biomech. Eng. 1985)	BAEC, confluent	Thermanox plastic coverslips	10, 30, 85	24	Cells orient with direction of flow, strong correlation between degree of alignment and elongation, and higher stress leads to more elongation.
Malek and Izumo (J. Cell Sci. 1996)	BAEC, confluent	Not Available	20	24	Explored mechanism by which cells respond to shear. Concluded tyrosine kinase, intracellular calcium, and intact microtubule network to be vital to response.
Morita et al. (J. Clin. Inv. 1993)	PAEC, confluent	tissue culture dish	5	24	Study concluded that rearrangement of actin cytoskeleton mediates shear stress induced changes in endothelin-1 gene expression.
Nerem et al. (J. Biomech. Eng. 1981)	RAEC	Not Applicable	Not Applicable	Not Applicable	Found that aortic endothelial cells aligned with the predicted pattern of blood flow.
Ookawa et al. (Front. Med. Biol. Eng. 1993)	PAEC (Porcine Aortic Endothelial Cells), confluent	glass coverslips	20	0-24	Observed stress fiber @3hrs. Cell elongation @6hrs. Clearly observed cell elongation and orientation @24hrs.
Remuzzi et al. (Biorheology 1984)	BAEC	glass coverslips	8	72	Quantified relaxation after exposure to 72 hrs of flow.
Sato et al. (J. Biomech. Engr., 1996)	PAEC, confluent	glass coverslips	20	24	Elongated cells were determined to be stiffer after detachment from substrate.
Sato and Ohshima (Biorheology, 1994)	PAEC	Glass and ECM coated coverslips	20	24	Cells on coated slides were more elongated in static culture and retarded orientation.

Table 2.1 (continued)

Authors	Cell Type	Substrate	Shear Level (dynes/cm^2)	Duration (hrs)	Notes
Takahashi and Berk (J. Clin. Inv. 1996)	HUVEC (confluent)	bacteriological plastic coated w/ human fibronectin	12	5, 10, 20, 90 min after only 10 min adhesion	Suggest that adhesion and shear stress activates ERK1/2 partially independent of cytoskeletal integrity
Thoumine et al. (Exp. Cell Res.1995)	BAEC (confluent)	Plastic tissue culture and rat tail tendon type I collagen	30	24	Findings suggest that cytoskeleton is important in the transduction of forces that cause elongation.
Wechezak et al. (J. Cell. Phys. 1989)	BAEC (subconfluent, in order to examine response of single cell))	glass coverslips	93	2	Observed qualitatively the change in shape and cytoskeletal components.
Zhao et al. (Artscl. Thrmb. Vas. Biol. 1995)	BAEC (confluent)	Sylgard tubes coated with human fibronectin	shear stress of 6 hoop stretch 6.9%	24	Concluded that circumferential stretch synergistically aided cell response.

endothelial cells exposed to higher shear stresses are more elongated and more oriented with the direction of flow (Dewey et al., 1981; Levesque and Nerem, 1985; Kim et al., 1989). The degree and rate of this change in morphology was shown to be magnitude and time dependent with respect to the shear stress (Dewey et al., 1981). Remuzzi et al. (1984) found that the altered structure of the endothelial cell will revert to that seen under static conditions when removed from exposure to flow. Zhao et al. (1995) concluded that circumferential stretch in conjunction with fluid shear stress also induces cell morphology and cytoskeletal rearrangement.

Along with the cell shape change, the cytoskeleton of cells exposed to shear is rearranged in a manner consistent with the cell shape change. Franke et al. (1984) reported the induction of stress fibers in endothelial cells through exposure to controlled levels of shear stress. *In vivo* modulation of shear stress also induced bundling in the actin microfilament system (Kim et al., 1989). Ookawa et al. (1993) studied the time course of the cytoskeletal rearrangement and found that it precedes the change in cell shape and orientation. Analysis of the relationship between the fields of force within a cell and the experimental alterations in the cytoskeleton suggest that the cytoskeleton is vital to the transduction of forces that cause the elongation in endothelial cells (Thoumine et al., 1995).

Wechezak et al. (1989) suggested that rearrangement of the actin cytoskeleton and its associated focal adhesion plaques is essential for cell adhesion under shear stress. A study by Davies et al. (1994) revealed rapid remodeling of focal adhesions in response to

shear stress. Girard and Nerem (1995) studied the response of focal adhesion associated proteins during cytoskeletal reorganization during exposure to shear stress. They found that vinculin, a protein found in focal adhesion plaques, and $\alpha_v\beta_3$ integrins were localized at the upstream end of the cells, the focal adhesion plaque protein talin was evenly distributed, and that the surface levels of $\alpha_5\beta_1$ integrins increased. In addition, a change in the mechanical properties of endothelial cells previously exposed to shear has been reported (Sato et al., 1996). They used the micropipette technique to measure the viscoelastic properties of porcine endothelial cells exposed to shear stress and concluded that the elongated cells were stiffer. Anchorage to a protein substrate, such as extracellular matrix coated slides, was found to retard endothelial shape change and reorientation when exposed to fluid imposed shear stress (Sato and Ohshima, 1994).

The biological function of endothelial cells has been shown to be altered by shear stress as well. Among the many cell functions observed by various authors to be affected are migration, proliferation, protein synthesis and secretion, membrane permeability, and endothelial-neutrophil interaction (Ando and Kamiya, 1993). An early study by Davies (1984) concluded shear stress increases the rate of pinocytocis in endothelial cells. Hu and Chien (1997) reported that shear stress affected the distribution of protein kinase C, an important molecule in the signaling of various physiological processes.

Investigation into the mechanisms by which endothelial cells sense shear stress and respond by changes in morphology, orientation, and function have implicated integrin-mediated signal events. Integrins are a logical choice for the site of signal

mediation because they provide a structural link between the actin cytoskeleton and extracellular matrix. Takahashi and Berk (1996) reported that shear stress stimulates focal adhesion kinase tyrosine phosphorylation, an important event in focal adhesion assembly, and that β_1 activation stimulates extracellular signal-regulated kinase (ERK1/2) which is known to regulate gene expression via protein phosphorylation. Cytoskeletal reorganization has recently been shown to activate various signaling pathways which modulate cell function by regulating expression of genes. Morita et al. (1993) concluded that disruption of the actin cytoskeleton mediates shear stress-induced endothelin-1 gene expression. A study by Irigoyen et al. (1997) found that cytoskeletal rearrangement, induced by pharmacological agents, activated the urokinase-type plasminogen activator gene via the ras/extracellular signal-regulated kinase signaling pathway.

In conclusion, endothelial cells are involved in the regulation of many important physiological functions. Research has shown that endothelial cells are responsive to fluid forces. Although the mechanisms by which the endothelial cells respond to the fluid imposed stimuli are not fully understood, research indicates that the perturbation of the cell morphology and orientation are controlled by the reorganization of the cell cytoskeleton. Structurally associated with the cell cytoskeleton via focal adhesion plaques and vital to cell signal transduction are cell surface protein receptors known as integrins. Due to their important position within the chain of signal transduction, the role of integrin mediated adhesion in the modulation of endothelial cell function and structure due to mechanical forces is worthy of further investigation.

Vascular Grafting in the Treatment of Vascular Disease

History

The timeline of vascular grafting is marked by bold and promising experimentation followed by results that are encouraging but not altogether satisfactory. Thus, the search for the ideal vascular graft continues. The first attempts at bridging vascular defects or traumas were carried out using autologous vein grafts. In fact, autologous arteries or veins are still the grafting material of choice for small diameter grafts where their anticoagulant nature is most critical. However, the limited supply of acceptable autologous tissue restricts its usage. For larger diameter grafts (> 6 mm ID) satisfactory results have been attained with several prosthetic materials. The smaller diameter graft has had moderate success only with autologous tissue, therefore the need for an acceptable synthetic alternative is crucial to the health and well-being of many. (Wright, 1983)

Materials

The materials currently available for vascular grafting are many. Three materials are widely used: autologous vein or artery, Dacron, and expanded polytetrafluoroethylene (ePTFE). The autologous tissue grafts are superior to the Dacron and ePTFE with respect to tissue reaction, thrombogenicity, and compliance (Bosher, 1983). Wagner et al. (1994) concluded that biologic vascular grafts were preferred for arterial reconstruction due to their greater resistance to bacterial infection, lipid uptake, and anticoagulant nature. The

location and limited availability of donor grafts severely limits the use of autologous grafts, especially in the case of very large blood vessels such as the aorta and in relatively long grafts with small inner diameters as in the extremities (Greisler, 1991). The potential advantage of being able to supply a large number of grafts in a wide range of sizes with the synthetic grafts is negated by their poor performance *in vivo*. Veith et al. (1986) reported on the significantly better patency rates for autologous saphenous vein grafts over ePTFE grafts during a six year study.

Dacron and ePTFE are used more widely than other synthetic materials due to their biocompatability and ease of handling (Hufnagel, 1983). Dacron is a textile graft, meaning that it is constructed of knitted or woven fine strands of polyester fashioned into tubes. The tightness of the knit or weave controls the porosity of the graft. Expanded PTFE is composed of solid nodes of PTFE interconnected by fibrils of PTFE. The mean fibril distance, defined by the distance between nodes, is an index of the porosity of the material. Porosity of the graft is an important physical characteristic that affects the method of implantation, graft healing, and thrombogenicity. Although grafts with high porosity must be preclotted to prevent excessive blood loss upon implantation (Greisler, 1991), they allow better tissue ingrowth than grafts with low porosity which do not require preclotting (Wesolowski, 1962). Current research by Nagae et al. (1995) and Martakos and Karwoski (1995) examined the effects of varying graft porosity throughout the wall thickness of the graft in order to control the need for preclotting while allowing tissue incorporation into the graft.

Graft Healing

Muller and Dasbach (1994) examined 28 vascular prostheses made of Dacron or ePTFE, placed in the high-pressure arterial system of human patients to characterize their tissue incorporation during healing. They concluded that graft incorporation is typified by three phases: the early phase, the organization phase, and the late phase; and closely resembles inert, aseptic foreign body incorporation.

The early phase is defined as the time from implantation until about 2 weeks. At the end of the early phase, the graft lumen is covered by a thin fibrinous layer, which is typically more well developed at the anastomoses and has become loosely anchored to the surrounding tissue. Shortly after implantation, a perivascular haematoma is formed and the cellular reactions and protein adsorption on the inner surface start at the onset of blood flow through the prosthesis. After only one hour of blood flow, deposits of fibrin, platelets, protein and erythrocytes are present within the prosthetic graft gaps. Many components of the extracellular matrix are observed after four days and continue to become deposited rapidly. The initially formed perivascular haemotoma is replaced by loose granulation tissue after 1-2 weeks. This provides some degree of vascular graft attachment to the surrounding tissue. (Muller and Dasbach, 1994)

The organization phase, spanning from approximately 2 weeks to about 6 months, leads to a firmly anchored prosthesis with a confluent capillary bed, spotty fibrin accretions on the lumen, and tongue-shaped endothelialization only near the anastomoses. The connective tissue network has penetrated the prosthetic mesh after 2-3 weeks and by

3-4 weeks the organization of the extravascular part of the prosthesis is complete. Along with invasion of the connective tissue, neovascularization is evidenced early on by small capillary precursors. Neovascularization continues throughout this phase as the myoendothelial cells of the endogenous arteries begin to proliferate over the junction of the artery and prosthesis. It is of particular importance that endothelialization does not proceed to the inner portions of the graft at any point in time. Extracellular matrix deposition continues to increase and fibroblasts become markedly more numerous. (Muller and Dasbach, 1994)

The late phase, also referred to as the scarring phase, occurs 6 months post-operatively and is characterized by a firmly attached prosthesis with a fibrously lined inner capsule and is plagued by chronic inflammation, recurring thrombosis, and intimal hyperplasia. Islets of endothelial cells may also be visible within the middle portion of the graft and are attributed to capillaries which have infiltrated the graft, but proliferation from the anastomoses has not proceeded. (Muller and Dasbach, 1994)

Complications

The final fate for any failing graft, barring mechanical failure of the graft material or surgical technical errors, is occlusion due to thrombosis (Burkel, 1988). Less frequent causes of failure include aneurysmal dilatation and infection. The distribution for the cause of this final occlusion is generally considered to be bimodal with respect to time. Early occlusions are attributed to the inherent thrombogenicity of a prosthetic graft or

technical error, while late failure is normally due to anastomic pseudointimal hyperplasia which results in reduced graft diameter and subsequent thrombosis (Greisler, 1991).

Thrombosis is a normal hemostatic event within blood vessels that protects the body from excessive blood loss by plugging the vessel with a fibrin-platelet plug (Collins, 1983). Despite its innate usefulness, uninhibited thrombosis within a graft leads to a significant number of early occlusions attributed to either surface thrombogenicity or technical failure (Greisler, 1991). The physical properties of the surface, such as texture, electronegativity, surface tension, and hydrophobicity, affect the types of proteins that are adsorbed and thereby influence many important processes including thrombosis, platelet deposition, and cellular regeneration (Greisler, 1991). Incomplete endothelialization, low blood flow, technical errors, and patient hypercoaguability may also augment the prosthetic surface thrombogenicity (Collins, 1983). Below a particular minimal flow rate specific to each type of material, termed the thrombotic threshold velocity (TTV), platelets and procoagulants found in the plasma are activated by contact with the prosthetic surface (Sauvage, 1983). Turbulence caused by any number of sources such as kinking of the graft, suture lines, or progressing atherosclerotic disease also enhances thrombogenicity by increasing the dwell time of platelets and procoagulants with the graft surface and thereby activating them (Sauvage, 1983).

Anastomotic pseudointimal hyperplasia is the overgrowth of subendothelial cells, in particular smooth muscle cells, at the ends of the graft. If severe enough, the consequent reduction in graft inner diameter has dire consequences because of the

associated reduction in flow velocity which may eventually lead to occlusion due to excessive fibrin build-up (Burkel, 1988). The reason for this hyperplastic response is not fully understood, but several contributing factors have been suggested. Under certain perturbed conditions, endothelial cells are known to secrete platelet derived growth factor (PDGF) and other substances that stimulate smooth muscle cell proliferation (Fox and DiCorleto, 1987). The spatial correlation of the overgrown tissue and the pannus-ingrowth of endothelial cells points toward the notion that the release of mitogens by endothelial cells may be an important factor in intimal hyperplasia (Fox and DiCorleto, 1987). Hemodynamic factors such as shear stress and flow velocity have also been implicated. Using a completely endothelialized graft in the baboon model, Kraiss et al. (1991) found that elevated levels of shear stress within the physiological range inhibited smooth muscle cell proliferation and neointimal thickening. Compliance mismatch and conformational stress have also been suggested as agents of the hyperplastic response at the anastomoses of grafts (Pomposelli et al., 1984).

Current Research

Attempts to resolve the problems associated with synthetic grafts, especially thrombosis and pseudointimal hyperplasia, have led to research in a variety of areas. In order to enhance the tissue ingrowth and endothelialization of synthetic grafts, investigators have sought to modify the microstructure of existing graft materials. Nagae et al. (1995) reported on the effect of a graft which had varying porosity. The highly

porous, 60 or 90 μm inner surface was designed for maximal tissue ingrowth, while the low porosity, 20 μm outer surface prevented leakage of fluid. They concluded that the composite porosity graft enhanced neointima formation while maintaining imperviousness. Martakos and Karwoski (1995) reversed the design of this composite graft with a highly porous outer layer, 60 μm, and a low porosity inner layer, 20 μm. Moreover, tapered through-pore channels were added to provide greater access from the adventitia to the lumen for cellular proliferation and tissue ingrowth. This study concluded that the design also enhanced cellularity and tissue ingrowth.

Modification of the inner surface of PTFE grafts with ammonia plasma was studied by Sipehia et al. (1996). They found that the plasma treated grafts enhanced the growth of human umbilical and saphenous vein endothelial cells. Researchers have also coated the inner surface with various proteins that enhance endothelialization and or reduce the thrombogenicty of the graft. A recent *in vitro* study by Weatherford et al. (1996) found that vascular endothelial growth factor combined with a fibrin based glue containing high concentrations of heparin promoted human aortic endothelial cell proliferation and inhibited smooth muscle cell proliferation.

Construction of various hybrid vascular grafts, such as the graft reported by Miwa et al. (1993), attempted to improve graft patency by incorporating vascular tissue *in vitro*. Investigators have also attempted to construct biodegradable grafts that provide a temporary scaffold for the growth of a new functional blood vessel (Van Der Lei and Wildevuur, 1989). Another area of intense research has been in the seeding of small

diameter prosthetic grafts with autogenous endothelial cells (Zilla et al., 1987). These investigations combined with the innovative field of gene therapy have defined a new direction for research into improvement of vascular grafting (Callow, 1990).

Endothelialized Vascular Grafts

The conceptualization of seeding vascular grafts with endothelial cells was born out of the knowledge gained about endothelial cell function in various vascular processes, in particular thrombosis and growth of surrounding tissues. Many researchers have investigated the positive aspects of implanting a graft with an intact endothelial lining (Zilla et al., 1987). It has been shown by various authors that a complete endothelial lining may be obtained in several animal models within a matter of weeks and that the patency rates of these grafts are improved (Burkel et al., 1982; Koveker et al., 1986; Budd et al., 1991; Graham et al., 1991; Pasic et al., 1995; Pasic et al., 1996). Burkel et al. (1982) found that autologous seeded Dacron grafts within the canine model formed a confluent endothelial layer quicker than unseeded grafts, decreased the thrombogenicity of the graft, and significantly reduced the neointima thickness in seeded grafts. The effect of seeding endothelial cells on ePTFE grafts in the canine model was reported by Budd et al. (1991) to improve patency and reduce early graft thrombogenicity as quantified by uptake of Indium radio-labeled platelets. The importance of attaining a complete endothelial monolayer in reducing intimal hyperplasia was stressed by Graham et al. (1991) who reported that in the canine model the extent of luminal coverage was

inversely proportional to the amount of distal anastomotic intimal hyperplasia. Blood vessel hemodynamics have also been shown to play a role in neointimal hyperplasia. Kraiss et al. (1991) found that high shear stresses within physiologic range reduced neointimal thickening in endothelialized baboon grafts. Also within the baboon model, increased blood flow has been shown to inhibit neointimal hyperplasia (Kohler et al., 1991).

Limited trials have been completed with human subjects and have not yielded the same encouraging results as in animal models (Fasol et al., 1987; Herring et al., 1994). A study in Vienna (Fasol et al., 1987) found no statistically significant advantage in favor of endothelial seeded ePTFE grafts placed in either the distal femoro-popliteal or femoro-crural positions. A multi-institutional randomized study by Herring et al. (1994) found the patency rate of seeded ePTFE grafts in the femoro-popliteal position to be only 38% after 30 months while 92% of autologous veins remained patent.

Gene Therapy

Gene therapy is emerging as an innovative approach to the treatment of cardiovascular diseases. By inserting genes into the living cells of patients, researchers are able to induce the cell to produce therapeutic or marker proteins. One of the earliest applications of gene therapy involved studies using gene therapy to mark cells, making it feasible to identify these cells and assess the efficiency of the prescribed treatment (Lyon and Gorner, 1995). Two well-known marker genes have been inserted into endothelial

cells. The lacZ gene encodes for a marker protein, beta galactosidase. Beta galactosidase is found natively in bacteria and causes the cell in which it is produced to become stained with a characteristic color when exposed to X-gal (Callow, 1990). By transducing cells with the lacZ gene, the cells which have been transduced can be identified visually by staining them with X-gal. The neomycin resistance (neoR) gene has also been inserted and infers resistance to neomycin, an antibiotic, or its analog G418 (Callow, 1990). Because this resistance allows researchers to select with a high degree of certainty only those cells which have been successfully transduced, the neoR gene is called a selectable marker gene (Callow, 1990). Successful integration and expression of potentially therapeutic substances for vascular grafts such as tPA (Dichek et al., 1989; Flugelman et al., 1995; Dunn et al., 1996; Sackman et al., 1997), nitric oxide synthase (Von Der Leyen et al., 1995), and VEGF (Van Belle et al., 1997) have been reported using various methods of gene delivery.

Methods of Gene Delivery

In a review by Baek and March (1998), four distinct gene delivery systems were outlined including plasmid DNA (deoxyribonucleic acid) gene transfer, adenoviral vectors, anti-sense oligonucleotide fragments, and integrating retroviral vectors. Typical concerns in the gene delivery systems include selectively targeting the cell of interest, stable expression of the gene, and transcription of the therapeutic gene leading to its expression within the cell (Hodgson, 1996).

Plasmid DNA gene transfer may be naked or rely upon cationic liposomal carriers to deliver the DNA to the cell interior. A major advantage of this system is the largely unlimited gene capacity of the plasmid DNA giving researchers a vehicle for large genetic sequences. Disadvantages of this system include its inability to integrate into the genome and inefficient transduction. While this method is typically inefficient, recent innovations in the composition of the lipid carrier have improved the transduction efficiency. Other improvements made upon this system include incorporation of ligands and viral proteins that enhance target cell binding and endosomal lysis. (Baek and March, 1998)

Adenoviral vectors have the ability to infect both nondividing and dividing cells; hence, they exhibit the highest transduction frequency. It is this same inherent infectiousness that elicits immune and inflammatory responses from the host due to target cell expression of viral proteins. Another disadvantage for this system is its inability to integrate into the host genome. This makes the gene expression transient, peaking at one week and limited to around two to four weeks. (Baek and March, 1998)

Oligonucleotide fragment anti-sense therapy has also been utilized to block the production of proteins. This system is different from previously discussed delivery systems because the oligonucleotide fragments delivered to the cell do not code for a therapeutic protein. Instead, they consist of genetic sequences which alter the expression of endogenous genes by binding to complementary sense RNA. (Baek and March, 1998)

A fourth gene delivery system uses replication incompetent retroviruses to integrate therapeutic genes into the host genome (Blau and Springer, 1995). These

vectors are constructed by deleting the region of the retroviral genome necessary for replication and replacing it with the desired marker or therapeutic gene. The advantages of using retroviral vectors are associated with the characteristics of retroviruses that make them efficient pathogens. The major advantage is stable integration into the host genome potentially providing long term gene expression, a high level of expression caused by powerful promoters present in the retroviral genome, and a flexible genome allowing changes in the virus infectability. One disadvantage to this technique is that the cells must be dividing in order to integrate the retroviral ribonucleic acid (RNA) into their genome. This limits the use of retroviral transduction in that it cannot be used in most adult tissues which are usually quiescent, meaning efficient transduction must take place *ex vivo*. A second disadvantage is that they can only carry genes of limited size. Lastly, the integration of the gene into host genome has the potential for disrupting normal cellular function or activating proto-oncogenes despite the fact that integration is random. (Hodgson, 1996)

Retrovirus Biology

Retroviruses are structurally simplistic organisms, schematically shown in Figure 2.3, consisting of genetic material in the form of two copies of single stranded ribonucleic acid (ssRNA) and essential viral enzymes, such as retroviral integrase and reverse transcriptase, contained within a capsid and enclosed by a protective coat called the envelope. Within the envelope are embedded transmembrane receptors that target

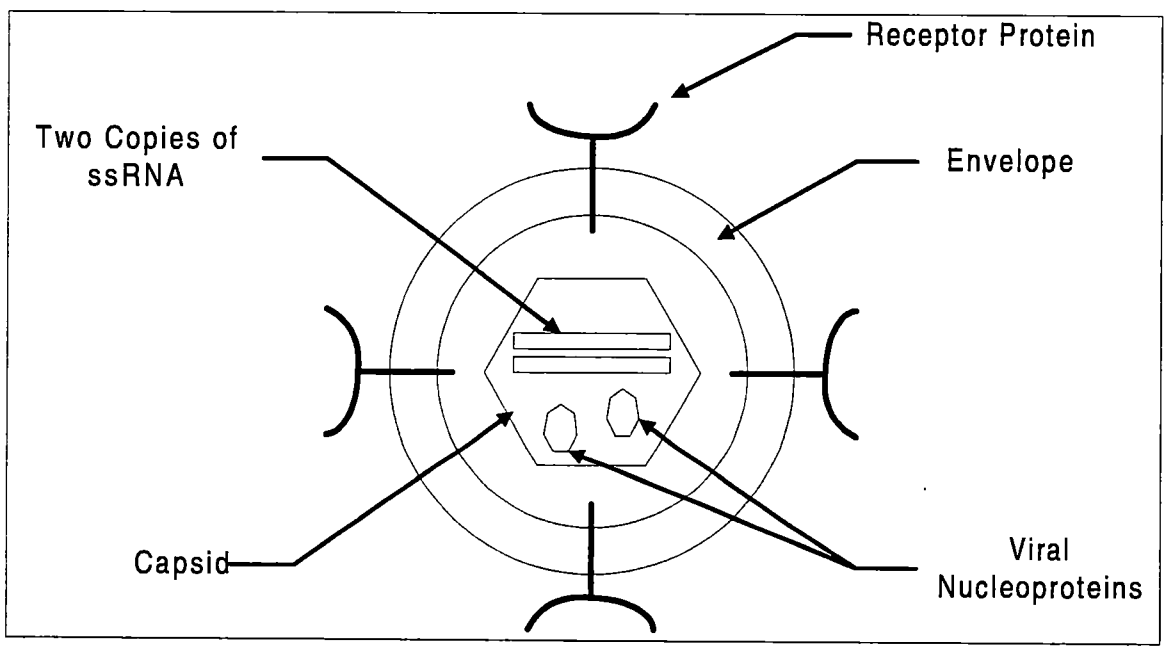

Figure 2.3. Retroviral Structure

proteins on the host cell giving the retrovirus its host specificity. Shown in Figure 2.4 is a schematic of retroviral ssRNA exhibiting the various regions important to the retroviral life cycle. (Hodgson, 1996)

Endocytosis requires recognition of host cell receptor proteins by the retrovirus. After entering the host cell, the viral coat must be dissolved to allow the viral genetic material to become exposed to the cytoplasm. Subsequent to uncoating, circular or linear double stranded DNA copies of the viral ssRNA are catalyzed by the retroviral reverse transciptase. For most retroviruses, excluding the human immunodeficiency viruses, integration into the host cell genome requires a round of cell division which breaks down the cell's nuclear membrane and allows the retroviral DNA to contact the host cell chromatin. The site of integration is usually random with respect to the host cell genome, but it has been shown that transcriptionally active sites are favored. After integration, transcription of the retroviral genome is possible and is promoted by sequences of the retroviral genome found in the long terminal repeat (LTR) regions. To form a new replication competent retrovirus, new genomic ssRNA and all of the structural proteins, termed "gag", "pol", or "env" proteins, are required. The region of the retroviral genome termed gag encodes for the nucleocapsid proteins. The pol region encodes the enzymes within the capsid. The env region encodes the envelope receptor proteins. Once all of the necessary components are present they are transported to the cell plasma membrane and are released through the process of budding, which forms the mature, replication competent virion. (Hodgson, 1996)

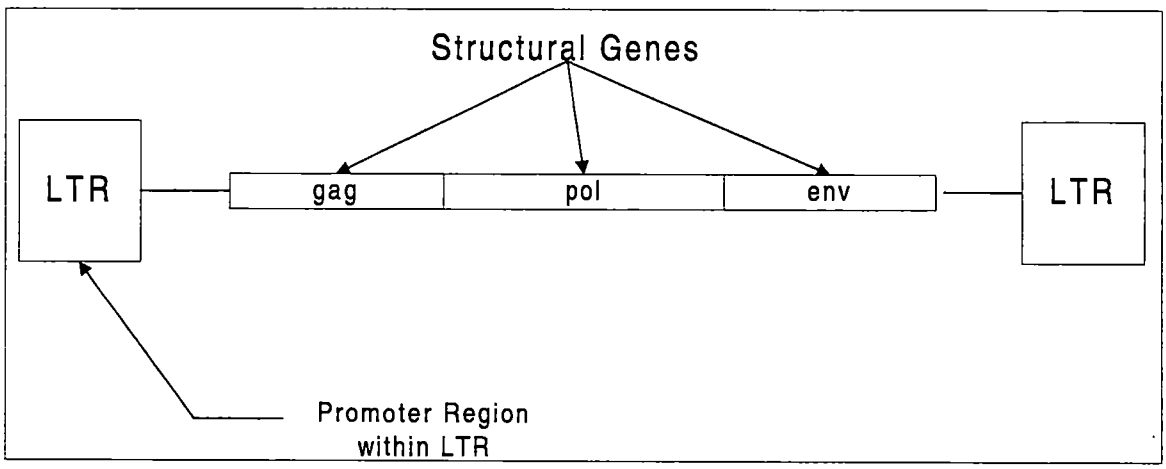

Figure 2.4. Schematic of Retroviral RNA

Retroviral Vectors

Retroviral vectors consist of retroviral RNA that has been genetically altered so that the structural genes have been deleted and replaced by an exogenous gene of interest. The LTR region and other vital regions must be conserved in order facilitate integration and expression of the vector in the host genome. The retroviral vector is replication incompetent because it is unable to produce the necessary proteins to package its genetic material. (Hodgson, 1996)

Because they cannot replicate the structural proteins, the infectious recombinant retroviruses that contain the retroviral vectors must be produced with packaging cells. A tissue cell line is transfected by DNA plasmids encoding for the retroviral structural genes. This cell line is now termed a packaging cell line because it produces the necessary retroviral structural genes to package a retroviral vector. The packaging cell line is then supertransfected by plasmids containing the recombinant retroviral vector. The cell line has now become a producer cell line and is capable of producing infectious yet replication incompetent retroviral vectors. Varying methods for producing retroviral vectors have been developed in order to reduce the risk of recombination events that would result in the regeneration of replication competent viruses within the packaging cell lines. (Hodgson, 1996)

Vascular Gene Therapy

Research involving recombinant gene expression in endothelialized vascular grafts has yielded limited success. The ability to efficiently transduce endothelial cells in tissue culture has been attained through the use of retroviral vectors carrying selectable marker genes, such as the neoR gene (Callow, 1990). However, seeding these cells onto vascular prostheses has elucidated a possible flaw in the implementation of recombinant endothelial cell seeding of vascular grafts.

Huber et al. (1995) studied, *in vitro* and *in vivo*, the effect of retroviral transduction upon the proliferation and adherence of canine endothelial cells on ePTFE grafts. They concluded that neither adherence nor proliferation of the endothelial cells *in vitro* was affected by the transduction. However, *in vivo* adherence of the transduced endothelial cells was significantly lower than that of naive endothelial cells for grafts harvested 1 hour after implantation. They concluded that the lower rates of surface endothelialization by transduced endothelial cells *in vivo* may result from decreased adherence after exposure to hemodynamic forces. An *in vitro* study utilizing a parallel plate flow chamber by Sackman et al. (1996) concluded that neoR gene transduction using the LN retroviral vector significantly altered the adhesive properties of canine endothelial cells. In the study, it was found that cells transduced with the neoR gene and exposed to a shear rate of 90 dynes/cm^2 detached from fibronectin coated slides at a much higher rate than either naive or mock transduced cells. Dunn et al. (1996) studied the effects of seeding endothelial cells expressing recombinant tPA onto Dacron grafts placed

in sheep. They concluded that tPA production also significantly reduced endothelial cell adherence. A study by Sackman et al. (1997) found that retroviral transduction altered the integrin expression of recombinant endothelial cells. In the study, human umbilical vein and canine jugular vein endothelial cells were either mock transduced with empty viral particles or with one of two recombinant gene vectors encoding human tPA or hygromycin resistance. Evaluation for the expression of both α and β integrin subunits revealed that the β_1 and β_3 subunits, but not the α subunits, integrins of the recombinant gene endothelial cells were not expressed in their complete form, possibly due to over-expression of the phosphotransferase selectable marker gene. Because a functional β subunit is critical for integrin function and signal transduction as discussed in a previous section, these findings may offer a plausible explanation for the apparent decreased adherence of recombinant endothelial cells.

Statement of Hypothesis

Based upon prior indications of decreased adherence of retrovirally transduced endothelial cells (Sackman et al., 1995; Huber et al., 1995; Dunn et al., 1996; Sackman et al., 1996) and recent research that indicated the presence of altered β subunits in retrovirally transduced endothelial cells (Sackman, 1997), it was hypothesized that the altered integrins are responsible for the decreased adherence of the recombinant endothelial cells. In order to test this hypothesis, naive and retrovirally transduced HUVEC were exposed to steady, laminar flow to induce the well documented (Table 2.1)

change in morphology common to naive endothelial cells. HUVEC that have been transduced with the neomycin resistance gene, neoR gene, were used as the recombinant cell line due to prior research that linked the presence of the neoR gene to decreased adherence of endothelial cells (Sackman, 1996). A parallel plate chamber was used to expose the HUVEC to steady, laminar flow due to the well defined characteristics of flow through parallel plate chambers. The flow rate was chosen so that the HUVEC were subjected to a shear stress of 25 dynes/cm^2, consistent with the mean wall shear stress in the carotid artery. The duration of flow exposure was sufficient to reorient the cells in the direction of flow based upon previously documented studies. The cells were seeded onto fibronectin coated slides based upon the findings of a previous study which showed a decreased adherence of the retrovirally transduced cells seeded onto fibronectin coated slides (Sackman et al., 1996). The comparison of cell morphology was based upon the analysis of the amount of cell elongation and orientation along the direction of flow. These two parameters have previously been studied using the cell shape index and the angle of orientation of the major axis of the cell with respect to the flow (Levesque and Nerem, 1985). Additionally, the stress fiber arrangement was observed for each experiment via immunofluorescent staining for F-actin. Two experiments for each cell line, naive and transduced, were conducted to provide an adequate sample size.

CHAPTER 3

MATERIALS AND METHODS

Experimental Procedure

Human umbilical vein endothelial cells (HUVEC) were seeded onto fibronectin coated glass slides and incubated for 24 hours at 37°C in a fully humidified environment. The slides were then placed in a parallel plate flow chamber and subjected to fluid imposed steady shear stress of 25 dynes/cm^2 for 66 hours. A randomly chosen field of cells was taped using video microscopy at several time points over the 66 hour flow exposure time. The cell shape index and angle of orientation with respect to the direction of flow were calculated for the various time points sampled. Image NIH 1.60, a public domain image analysis program created at the National Institutes of Health and available on the Internet, was used to obtain the data necessary to calculate the aforementioned measures of cell morphology. Following exposure to flow, the cells were immunofluorescently stained for F-actin to observe the stress fiber arrangement using fluorescent microscopy. Two trials for both naive and retrovirally transduced HUVEC were completed and the data combined for statistical analysis.

Cell Culture

Both naive and retrovirally transduced HUVEC were produced by Dr. Jill Sackman. They had been stored in a liquid nitrogen dewar. The transduced HUVEC were produced by supernatant transduction of HUVEC obtained commercially from American Type Culture Collection (Rockville, MD, Cat.# 1730-CRL). The LN retroviral vector used contains the genetic sequence for a bacterial protein, neomycin phosphotransferase, which confers resistance to the cytotoxins neomycin and the neomycin analog, geneticin or G418. The genetic sequence is known as the neoR gene and is termed a selectable marker gene because it allows researchers to preferentially select and grow only those cells that have been successfully transduced by culturing them in media containing G418. The neoR gene was used because past research had implicated expression of the neoR gene in the alteration of endothelial cell integrins (Sackman et al., 1997). The LNc11 producer line used by Dr. Sackman to produce the transduced cells was a gift from Dr. Don Kohn (Children's Hospital, Los Angeles, CA).

Immediately following retrieval from the dewar, the cells were rapidly thawed in a 37°C water bath and transferred to a T-75 Falcon tissue culture flask. Both transduced and naive endothelial cells were cultured in 10 ml of Endothelial Growth Media (EGM) from Clonetics Corp. (San Diego, CA, Cat.# CC-3024) which was supplemented with 10 ng/ml of human Endothelial Growth Factor (hEGF), 2% Fetal Bovine Serum (FBS), 1.0 μg/ml hydrocortisone, gentamycin, and amphotericin-B. Incubation of the slides and flasks at 37°C took place in a humidified, water-jacketed incubator (Forma Scientific,

Inc., Marietta, OH, Model 3546) that provided an atmosphere of 5% CO_2 and 95% air. A subculturing reagent pack (Clonetics Corp., Cat.# 5034) provided HEPES Buffered Saline Solution (HBSS), trypsin/ethylenediaminetetraacetic acid (EDTA), and trypsin neutralizing solution (TNS) to be used in subculturing the cell cultures. To harvest cells, the culture flasks were washed with 5 ml of HBSS in order to rid the cell surfaces of FBS that would inactivate the trypsin. Trypsin/EDTA, 5 ml at room temperature, was used to detach the cells from the flask bottom. Cell detachment was verified on an inverted microscope and halted with 5 ml of TNS. The cell suspension was then centrifuged at approximately 350 x g for 5 minutes to pellet the cells. The cell pellet was then resuspended with 1 ml of EGM. This cell suspension could then be transferred to a 75 cm^2 flask and covered with 10 ml EGM or used in seeding a fibronectin coated slide.

Fibronectin Coating of Glass Slides

Glass slides approximately $4\frac{5}{8}$" in diameter were first washed thoroughly with detergent, then rinsed with tap water followed by distilled water. The slides were subsequently soaked in methanol for 30 minutes and rinsed again in distilled water and allowed to air dry. The slides were then coated with an extracellular matrix protein, lyophilized human plasma fibronectin (Life Technologies, Inc., Grand Island, NY, Cat.# 33016-015) at a coverage of 1 $\mu g/cm^2$. The value of the coverage used was consistent with previous experiments related to the present research (Zhao et al., 1995). The fibronectin was equilibrated to room temperature and reconstituted with 1 ml of

sterile water. A final dilution of 20 μg/ml was obtained by adding 49 ml of Hank's Balanced Salt Solution (Worthington Biochemical Corp., Freehold, NJ). In order to contain the fibronectin within a specified region, a small plastic ring, with an approximate area of 6.16 cm^2, was placed in the center of a slide and its outline traced with a marker on the opposite side. With the ring still in place, the necessary volume of diluted fibronectin solution, about 0.3 ml, was added to the slide and incubated at 37°C for 90 minutes. The ring was removed and the excess fluid gently washed away two times with sterile water. After allowing to air dry, the coated slide was placed in a glass petri dish, wrapped with Parafilm® (American National Can Co., Greenwich, CT), and stored in the refrigerator until used.

Cell Seeding

Fibronectin coated slides were seeded with a high density of endothelial cells, $5x10^4$ cells/cm^2, and incubated for 24 hours prior to exposure to flow in order to achieve a confluent monolayer of cells in a short period of time. As per the subculturing protocol, the cells were detached from the flask. The resulting cell suspension was transferred to a centrifuge tube and centrifuged at 350 x g for 4 minutes. The supernatant was then aspirated and the pellet of cells was resuspended with 1 ml of EGM. To seed the cells on the slides at the specified density, the number of cells in the cell suspension was counted using a hemacytometer. A 1:40 dilution of 25 μl of the cell suspension was made by addition of 500 μl of 0.4% Trypan blue (Sigma Chemical Co., St. Louis, MO, Cat.# T-

1854) and 475 µl of PBS, Phosphate Buffered Saline (Life Technologies, Inc., Cat.# 21600-010). The Trypan blue stained the viable cells a distinctive violet color. This enabled estimation of the viable cells per unit volume when a small volume of solution was placed on a hemacytometer (Baxter Scientific Products, McGraw Park, IL, Cat # B3178-1) and the number of stained cells counted. In this manner, the necessary volume of the cell suspension to be placed on the glass slide was determined, which was typically around 100 µl. Guided by the marker outline, another plastic ring was placed on the fibronectin coated region. Within this plastic ring, the necessary volume of the cell suspension was added to the fibronectin coated region. Addition of 1 ml of EGM allowed sufficient coverage and spreading of the cells across the entire fibronectin coated region. The seeded slide was incubated at 37°C for 24 hours before exposure to flow.

Flow System and Parallel Plate Flow Chamber

The cells were subjected to shear stress within a parallel plate flow chamber. Parallel plate devices have been successfully used by other researchers to apply known shear stresses to the cells. Levesque and Nerem (1985) cited three advantages to the use of the parallel plate. First, the flow for this channel geometry is well defined and has been verified in their device by flow visualization studies. Secondly, high shear stresses which more closely approximate estimated physiologic values can be obtained. Lastly, the device can be made small enough to fit within a microscope stage allowing continuous monitoring of the cell response.

The parallel plate device, shown in Figure 3.1, consisted of four components: a threaded polycarbonate top, a cell seeded glass slide, an O-ring, and a threaded aluminum collar. The rectangular flow channel was milled into the bottom face of the polycarbonate top to a depth of approximately 285 μm, a width of 5.65 cm, and a length of 6.8 cm. Two pressure ports, 3.4 cm apart, drilled into the top allowed measurement of the pressure drop across a section of the plate. Apparatus assembly involved screwing the top into the collar sandwiching the glass slide. The O-ring insured a tight seal between the slide and top. The assembled parallel plate apparatus allowed application of a well defined flow to the cells.

The flow system shown in Figure 3.2 was designed to deliver steady flow to the parallel plate chamber, maintain a viable environment for the cells throughout each 66 hour experiment, and to accommodate collection of images of the cells during flow exposure for subsequent analysis. The flow circuit consisted of an elevated reservoir, the parallel plate chamber, a collection reservoir, a pump, and various types of tubing shown in Figure 3.2.

Temperature and pH were maintained via an air stream incubator and CO_2 addition to a sodium bicarbonate buffering system. A HEPA-VENT™ In-Line Filter Device (Whatman Inc., New Jersey, Cat.# 6723-5000) placed between the collection reservoir and the CO_2 tank removed 99.97% of particles greater than 0.3 μm from the incoming CO_2. Electrodes positioned within the collection reservoir allowed measurement of the temperature and pH of the flow media. To vent the collection

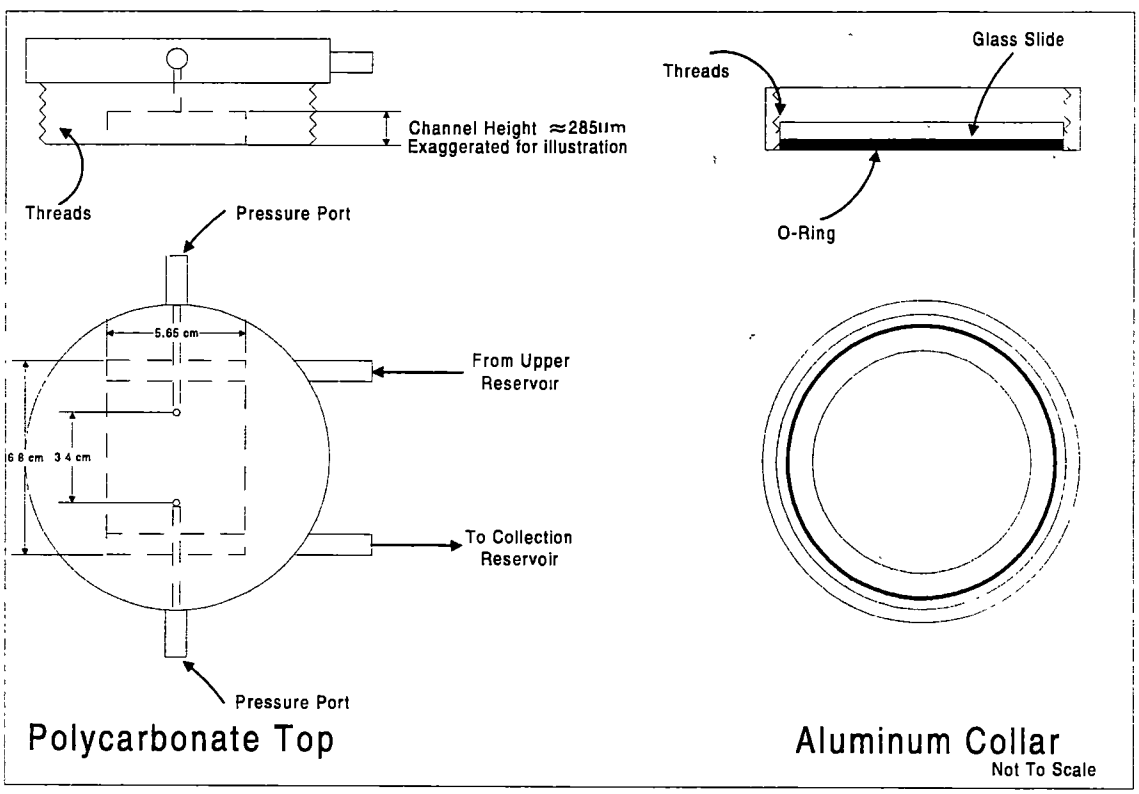

Figure 3.1. Parallel Plate Flow Chamber

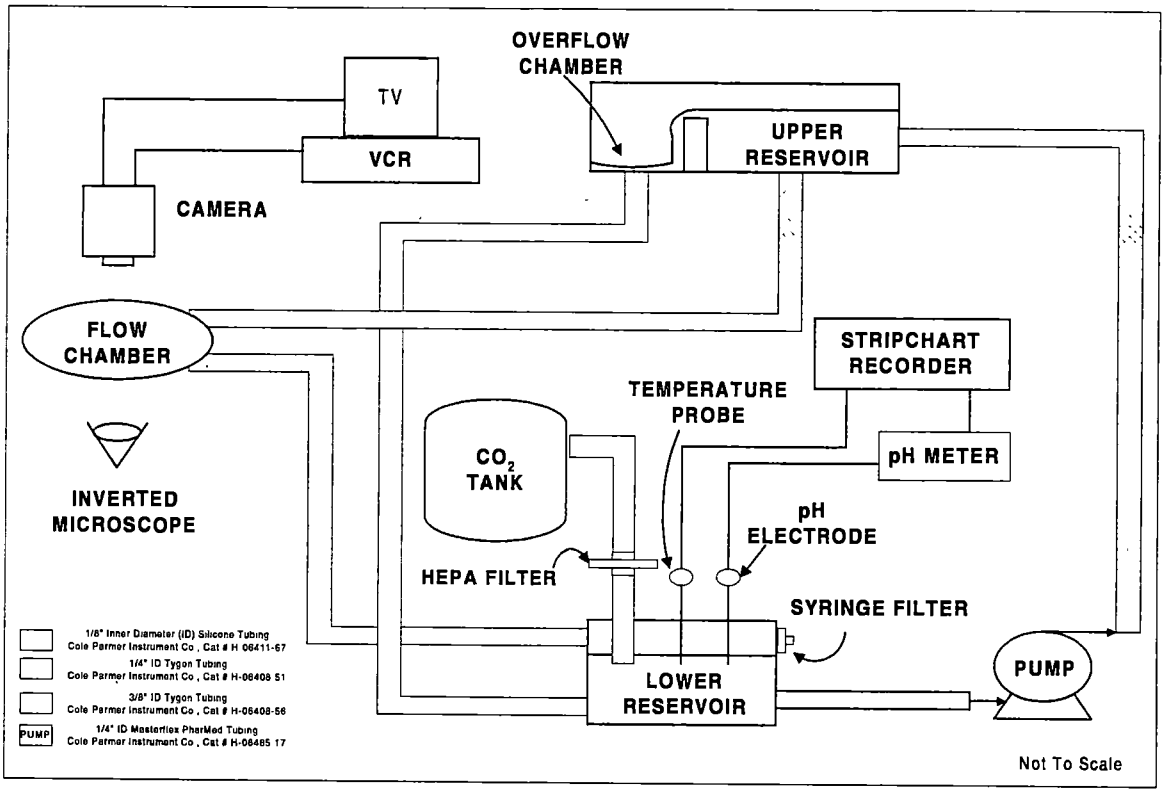

Figure 3.2. Flow System Schematic

reservoir and maintain the integrity of the closed flow system, an Acrodisc® CR PTFE syringe filter (Gelman Sciences, Michigan, Cat.# 4225), with a pore size of 0.2 μm, was placed in the side of the collection reservoir. An inverted microscope permitted observation of the cells on slide, while a camera, television monitor, and VCR permitted taping of the cells during each experiment.

The flow media drained from an upper reservoir, Figure 3.3, to the parallel plate apparatus. The constant height of flow media in the upper reservoir ensured steady flow to the parallel plate. The perfusate consisted of Dulbecco's Modified Eagle Media (DMEM) from Life Technologies, Inc. (Cat.# 12100-046) containing 4,500 mg/L of D-glucose and L-glutamine and supplemented with 3.75 g/L of sodium bicarbonate (Life Technologies, Inc., Cat.# 25080-094), 2% fetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Pen-Strep Liquid, Life Technologies, Inc., Cat.# 15140-122). Prior to addition of the fetal calf serum and penicillin-streptomycin, the DMEM was sterilized by membrane filtration. Outflow from the parallel plate chamber and the overflow chamber drained into the collection reservoir shown in Figure 3.4. The flow media was returned to the upper reservoir via a Masterflex® pump controller (Cole-Parmer Instrument Co., Illinois, Cat.# 7553-50), equipped with an Easy-Load® pump head.

In order to provide a viable environment for the cells during the experiment, the temperature and pH of the flow media were maintained as close to physiological values as possible, 37°C and 7.4 respectively. To maintain the flow media temperature, the flow

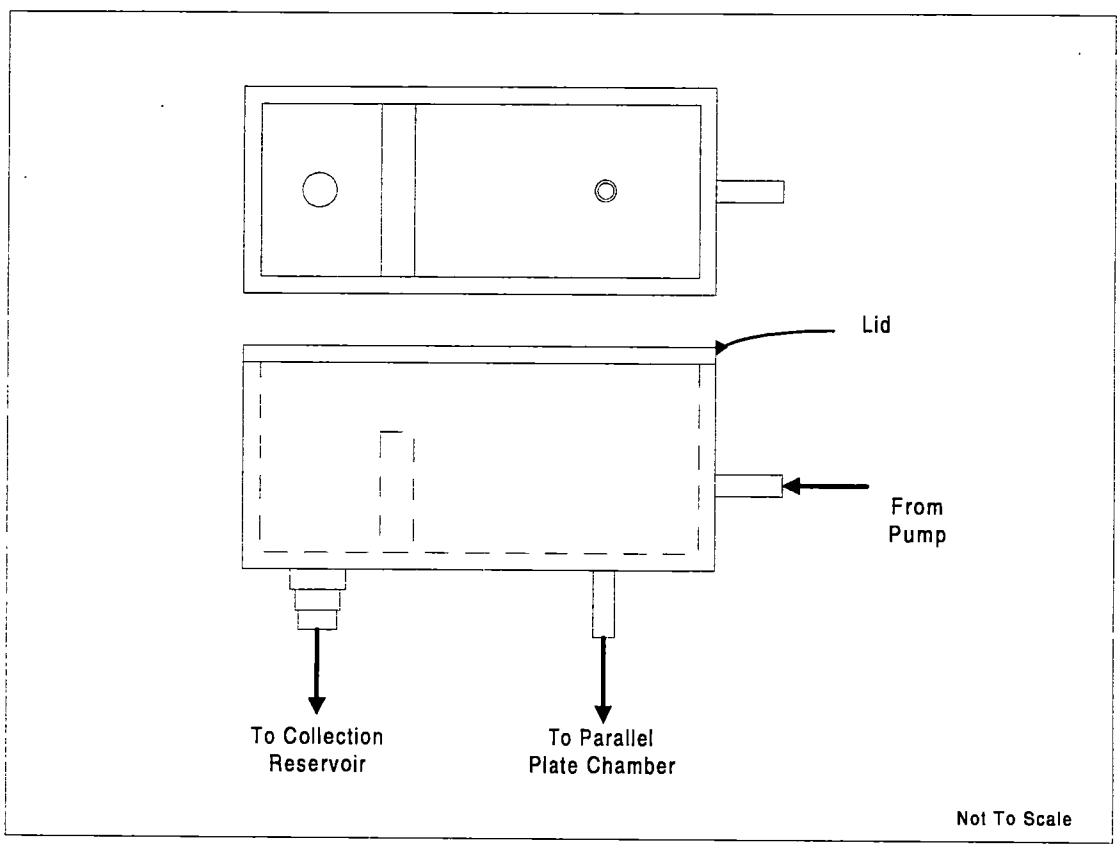

Figure 3.3. Upper Reservoir

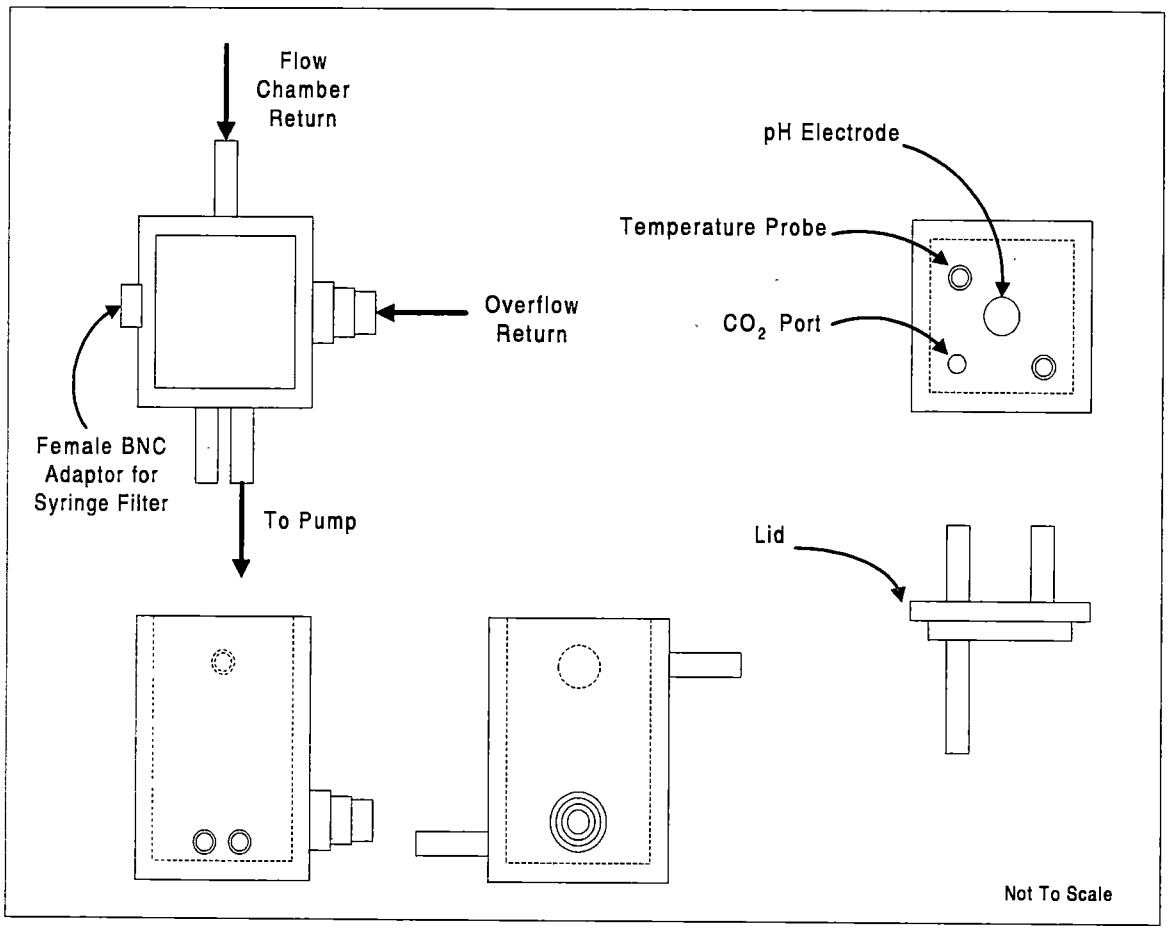

Figure 3.4. Collection Reservoir

system was contained within an enclosure made primarily of Foamular® insulation board (Owens Corning) and heated by an air stream incubator (Nicholson Precision Instruments, Gaithersburg, MD, Model ASI400). To allow access to the flow system, a plastic front with openings was attached to the enclosure. In order to maintain the pH of the perfusate, a flowmeter (Cole-Parmer Instrument, Co., Cat.# E-32013-15) was used to control the rate of CO_2 addition to the media in the collection reservoir. The temperature and pH were monitored in the collection reservoir by an Omega temperature electrode (Omega Engineering, Inc., Stamford, CT, Cat.# 871A) and a combination electrode (Cole-Parmer Instrument Co., Cat.# 5992-20) and Chemcadet® pH/mV meter (Cole-Parmer Instrument Co., Cat.# 5986-60). The temperature and pH values for the entire 66 hour period were recorded on a stripchart recorder (Linseis, Princeton, NJ, Model L6514B). All autoclavable components of the flow system were autoclaved at 250°F and 15 psi for 20 minutes. The other components were either soaked in alcohol or wiped down thoroughly with an alcohol soaked tissue.

Determination of Shear Stress

Modeling the flow over the cells within the parallel plate chamber as flow between infinite parallel plates, as shown in Figure 3.5, is validated by the relatively small channel height, 285 μm, to channel width and length, 5.65 cm and 6.8 cm respectively. Assuming that the flow media is a Newtonian fluid, the shear stress imposed upon the top and bottom walls of the parallel plate is directly proportional to the

shear rate at the walls as shown in Equation 3.1. By determining the velocity profile and the necessary physical properties of the fluid, calculation of the wall shear stress is straightforward.

$$\tau_{wall} = -\mu \frac{du}{dy}\bigg|_{y=\pm\frac{h}{2}} \tag{3.1}$$

τ_{wall} is the wall shear stress, μ represents the viscosity of flow media, u is defined as the flow media velocity, y and h are the vertical direction and channel height respectively as shown in Figure 3.5.

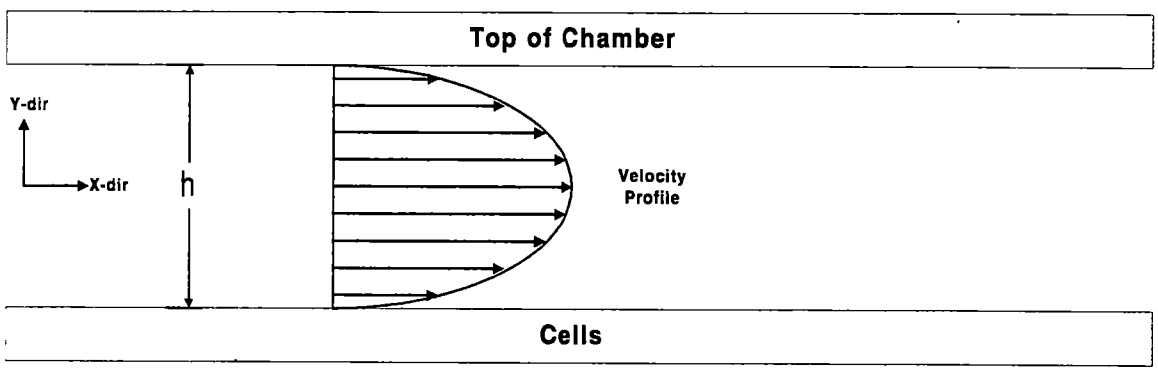

Figure 3.5. Perfusate Velocity Profile

Knowledge of both the density and the viscosity of the perfusate are necessary for the calculation of the shear stress. Density was previously determined to be 1.006 g/ml by weighing a known volume of flow media pre-warmed to 37° C. A falling ball type viscometer (Gilmont® Instruments, Barrington, IL, Cat.# GV-2100) with a glass ball was

used to determine the viscosity. The average of three trials yielded a viscosity of 7.65 x 10^{-4} Poise at 37°C.

Two assumptions are made in the determination of the velocity profile of the flow media through the parallel plate chamber. The assumption of steady flow through the parallel plate is justified due to the overflow chamber in the upper reservoir which maintains a constant fluid height and therefore a constant flow rate. It is also assumed that there were no appreciable body forces in the direction of flow.

Applying the aforementioned assumptions to the continuity equation and the Navier-Stokes equations, yields the parabolic velocity profile of Equation 3.2 characterizing steady, laminar flow between infinite parallel plates. A graphical representation of the velocity profile was shown in Figure 3.5.

$$u = \frac{h^2}{2\mu}\left(\frac{dp}{dx}\right)\left[\left(\frac{y^2}{h^2}\right) - \frac{1}{4}\right] \tag{3.2}$$

Due to the assumptions made about the flow, the pressure gradient along the direction of flow, $\frac{dp}{dx}$, is constant.

Substituting the solution for the velocity profile into Equation 3.1 yields the wall shear stress as a function of the pressure drop and channel height. However, the flow rate through the parallel plate is more accurately obtainable during the experiments. Relating the pressure drop in terms of the flow rate and substituting into Equation 3.1 yields the

shear stress in terms of readily obtained flow parameters, Equation 3.3. The parameters Q and W represent the volumetric flow rate and the channel width respectively.

$$\tau_{wall} = \frac{6Q\mu}{Wh^2} \tag{3.3}$$

Two important parameters concerning the nature of fluid flow are the Reynolds number and the entrance length. The Reynolds number is a dimensionless parameter that characterizes a flow as either laminar or turbulent. The Reynolds number equation from White (1991) is given in Equation 3.4 below

$$\mathrm{Re} = \frac{\rho UD}{\mu} \tag{3.4}$$

where ρ and μ are the fluid density and viscosity respectively, U is the average velocity, and D is the hydraulic diameter. Flow characterized by Reynolds numbers less than 1500 are generally considered laminar, meaning the flow streamlines do not cross (White, 1991). For Reynolds numbers above 1500, the flow is considered unstable or turbulent, meaning that the flow fluctuates randomly (White, 1991). Hence, the exact solution obtained for the velocity profile, Equation 3.2, is only valid for laminar flow. Given the geometry of the parallel plate, the density and viscosity of the fluid, and the desired shear stress, the Reynolds number for the perfusate flow is estimated to be 120, which is much less than the turbulent transition number of 1500.

When flow enters a duct, there is an entrance effect where wall friction causes viscous layers to develop and grow with increasing distance in the direction of flow until these layers meet and the flow is said to be fully developed (White, 1991). The entrance

length is an estimation of the distance from the flow inlet to the point of transition into fully developed flow (White, 1991). The entrance length for the parallel plate geometry and flow conditions is determined based upon Equation 3.5 below for arbitrarily shaped ducts (White, 1991)

$$\frac{x_L}{D} \cong 0.05\,\mathrm{Re} + 0.5 \tag{3.5}$$

where x_L is the entrance length and Re is the Reynolds number as computed by Equation 3.4. The entrance length is estimated to be 4 mm for the present experiments. Therefore, fully developed flow over the cell seeded region is assumed, which means that the velocity profile over the area of interest does not vary.

The accuracy to which the shear stress is computed depends upon the combined accuracy of the four terms on the right side of the Equation 3.3. Two of the four terms on the right hand side of the equation, the channel height and width, are known quantities that do not vary. The viscosity is a fluid property that varies with temperature; therefore, confidence in its value during an experiment depends heavily on maintaining the same temperature at which the viscosity was determined. The flow rate is determined by collecting the outflow in the collection reservoir over a twenty second period. The process of setting up the flow circuit, warming the flow system up to 37° C, and calculating the shear stress for various upper reservoir heights determined the required height to achieve the desired level of shear stress.

Data Collection

The assembled parallel plate chamber was placed on the stage of the Zeiss Axiovert 35 microscope prior to the introduction of flow and a field of cells was randomly chosen. The accompanying time lapse VCR (Hitachi, Model TLC 1800) was used to tape 60 second segments of the chosen field at 0, 3, 12, 24, 36, 48, and 66 hours following the onset of perfusion. The resulting videotaped images were digitized using Image NIH 1.60. To facilitate analysis, the borders and shape of 80-100 cells on each image were enhanced. The images were then ready for analysis within Image NIH 1.60. Figure 3.6 contains representative images of the three steps involved in analyzing the image.

For each time point, the cell shape index (SI) and the angle of orientation (θ) were computed for the outlined cells within an image. The area, perimeter, and angle of each cell were computed by Image NIH 1.60 and the SI was calculated via a spreadsheet program. The SI compared the ratio of an individual cell's area to the square of its perimeter with the same ratio for a circle. The equation for SI is

$$SI = \frac{4\pi A}{P^2} \tag{3.5}$$

where the variable A represents the cell area and P is the perimeter of the cell.

The range of SI values were from 0 to 1 and corresponded to the two limiting cases of a line and a circle, respectively. As a measure of the roundness of cell shape, the cell SI indicated the degree to which the cell was elongated. In past research, endothelial

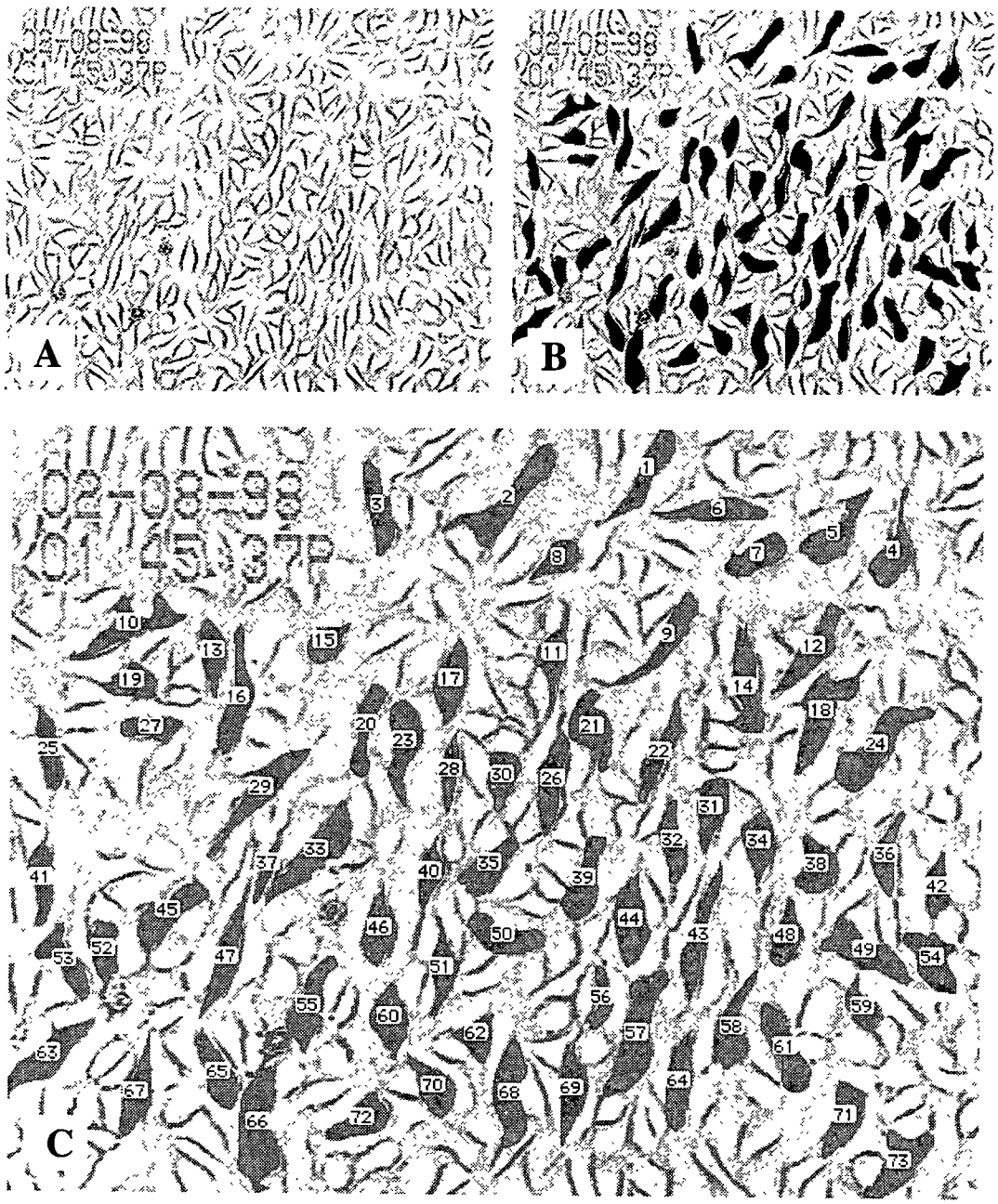

Figure 3.6. Analysis of Cell Images: A) Image Capture with NIH 1.60, B) Shape Enhancing, C) Data Generation within Image NIH

cells elongated when exposed to shear. Hence, the SI values for the flow exposed cells should decrease from an initial value relatively close to the upper limit of 1, depending upon the initial confluency of the slide. The angle of orientation was defined as the absolute value of the angle between the major axis of a cell and the direction of flow and ranged from 0° to 90°. Theoretically, the average θ for a randomly oriented field of cells would be 45°, while a field oriented with the direction of flow would be 0°. Because endothelial cells often exhibit localized fields of preferred orientation in static culture conditions, we did not expect θ for at the initial time to be exactly 45°, but rather closer to 45° than 0°.

Statistical Analysis

The data at each time point from the two trials of each cell type was combined and the mean and standard deviation of SI and θ were computed. Multi-variant analysis of variance (MANOVA) was conducted because SI and θ were not likely to be independent variables based upon prior research finding a high correlation between the elongation and alignment of endothelial cells exposed to shear stress (Levesque and Nerem, 1985). MANOVA determined that cell type and time were important factors. Comparisons of the mean values of SI and θ at the initial and final time points were made using the unpaired student t-test with a level of significance of $p < 0.05$.

Immunofluorescent Staining and Microscopy

In order to qualitatively analyze the ability of the transduced cells to rearrange their cytoskeleton in response to shear stress, a major component of the cytoskeleton, F-actin, and a focal adhesion protein associated with F-actin, vinculin, were viewed by fluorescent microscopy. The slides were double stained for F-actin and vinculin to investigate the association of vinculin with the F-actin after exposure to shear stress.

F-actin was stained by rhodamine-conjugated phalloidin (Sigma Chemical Co., Cat.# P5282). Phalloidin is a toxin that binds to the F-actin within the cell cytoskeleton. This property of phalloidin has been used to visualize the F-actin within cells by covalently conjugating the phalloidin to a dye that fluoresces red when excited by green light.

Monoclonal anti-vinculin (Sigma Chemical Co., Cat.# V-4505) was used in this study for the indirect immunocytochemical localization of vinculin. Indirect immunofluorescence utilizes two antibodies: a primary antibody specific for the protein of interest and a secondary antibody, carrying a chemically attached dye, specific for the primary antibody. This process reduces the chance of non-specific binding of the dye to the protein of interest and thereby increases the overall efficiency of the staining. Purified vinculin from chicken gizzard smooth muscle cell served as the immunogen in the production of monoclonal anti-vinculin. Harvested from a mouse immunized with the chicken vinculin, myeloma cells and splenocytes were fused to form a hybridoma from which the monoclonal anti-vinculin was derived. The fluorescent secondary antibody

used was Fluorescein Isothiocyanate (FITC)-conjugated AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, Cat.# 115-095-003). The goat anti-mouse IgG is an antibody specific for heavy and light protein chains found in mouse immunoglobulins. It was formed within a goat immunized with immunoglobulins from a mouse. The resulting antibody was then conjugated with the fluorophore, FITC, which fluoresces green when exposed to blue light.

After exposure to flow, the slide was removed from the parallel plate chamber and the cells gently rinsed in PBS prewarmed to 37°C. To contain the staining reagents within the cell populated area of the slide, a hydrophobic slide marker, PAP pen (BioGenex Laboratories, San Ramon, CA, Cat.# XT001-PP), was used to draw a ring around the cell region. The cells were then fixed in prewarmed (37°C) 3% paraformaldehyde for 20 minutes. After another gentle washing in prewarmed PBS, the cells were permeabalized by incubation at 37°C with 0.1% Triton-X in PBS for 3.5 minutes followed by another wash in prewarmed PBS. In order to reduce non-specific binding of the primary antibody, the cells are incubated with a goat protein block (BioGenex Laboratories, Cat.# HK112-9K) for 7 minutes. The excess serum was tapped off, not washed, prior to staining. The cells were double stained for vinculin and F-actin. Slides were first incubated with 150 µl of a 1:100 dilution of anti-vinculin in a common antibody diluent (BioGenex Laboratories, Cat.# HK156-5K) for 30 minutes at 37°C. Addition of the secondary antibody and rhodamine phalloidin followed with incubation of a mixture of FITC at a 1:100 dilution and rhodamine phalloidin at a 1:20 dilution for

20 minutes at room temperature. After the final incubation, the slide was washed in room temperature PBS and mounted in 50:50 glycerol/PBS.

The slides were immediately placed under the microscope equipped with epifluorescent cubes and pictures were taken at various magnifications with a Minolta X-700 SLR camera (Norcross, GA) using T-MAX 400 black and white Kodak print film (Eastman Kodak Company, Rochester, NY) shot at 1600 ASA. The epifluorescent cubes contained a filter set composed of an excitation filter and a barrier filter. The excitation filter, positioned between the microscope light source and stage, passed a specific wavelength of light on to the cells. The fluorescent dye on the stained cells emitted light of a different wavelength than that passed by the excitation filter. The barrier filter in the epifluorescent cube allowed only that wavelength of light at which the dye fluoresces to pass to the objective lens. A fluorescein cube (Zeiss, Cat.# 487909) and a rhodamine cube (Zeiss, Cat.# 487915) were used to view the staining patterns of the respective dyes. The fluorescein cube excitation filter passed blue light, a wavelength range of 450-490 nm, and the barrier filter passed green light, wavelength of 520. The excitation filter in the rhodamine cube permitted green light, wavelength of 546 nm, to pass, while the barrier filter passed red light, wavelength of 590 nm.

CHAPTER 4

RESULTS

Image Analysis

Images of a randomly chosen field of cells were obtained through video microscopy at 0, 3, 12, 24, 36, 48, and 66 hours following initiation of perfusion. Two trials each were completed for the naive and transduced HUVEC. The images were digitized, enhanced to better differentiate cell borders, and subsequently analyzed to yield the necessary morphometric data. From the morphometric data, the cell shape index and angle of orientation were computed for each cell. The combined sample size at each time point for both naive and transduced cells was between 150 and 180 cells. Figure 4.1 and Figure 4.2 represent time histories of the mean shape index and mean angle of orientation for both naive and transduced HUVEC. The error bars are the standard error of the mean for each time point. It is apparent from the figures that both the naive and transduced cells exhibited some degree of elongation and reorientation after 66 hours of exposure to flow. Furthermore, the changes in SI and θ follow similar trends for both cell types.

The mean values of shape index and angle for each cell type before the onset of flow were compared to the values for the same cell type at 66 hours. This was done to determine the level of significance of the change in cell shape or angle across time for a

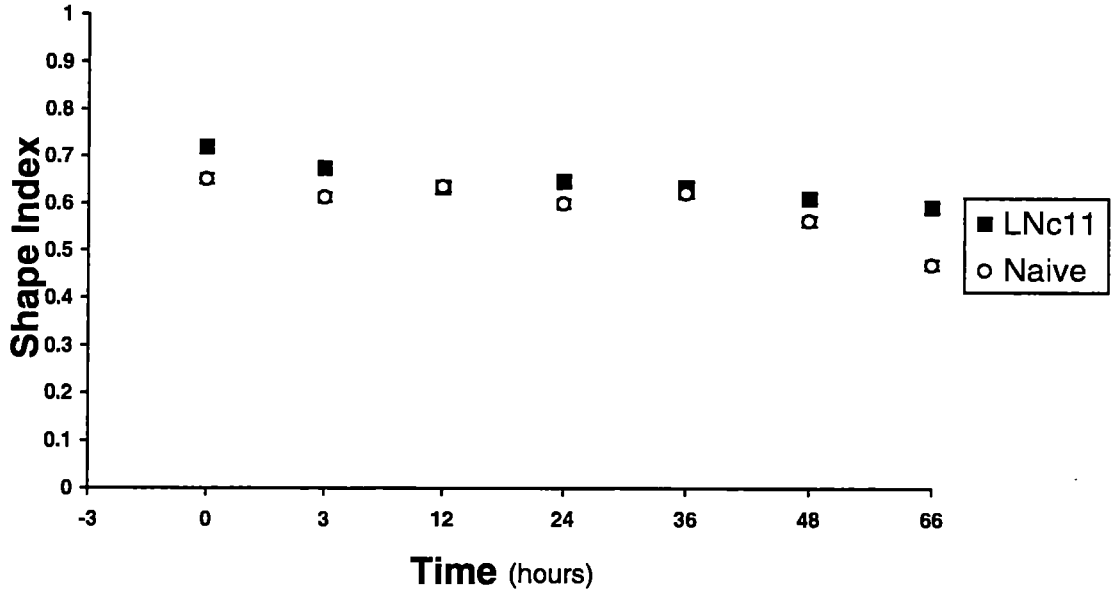

Figure 4.1. Endothelial Cell Elongation

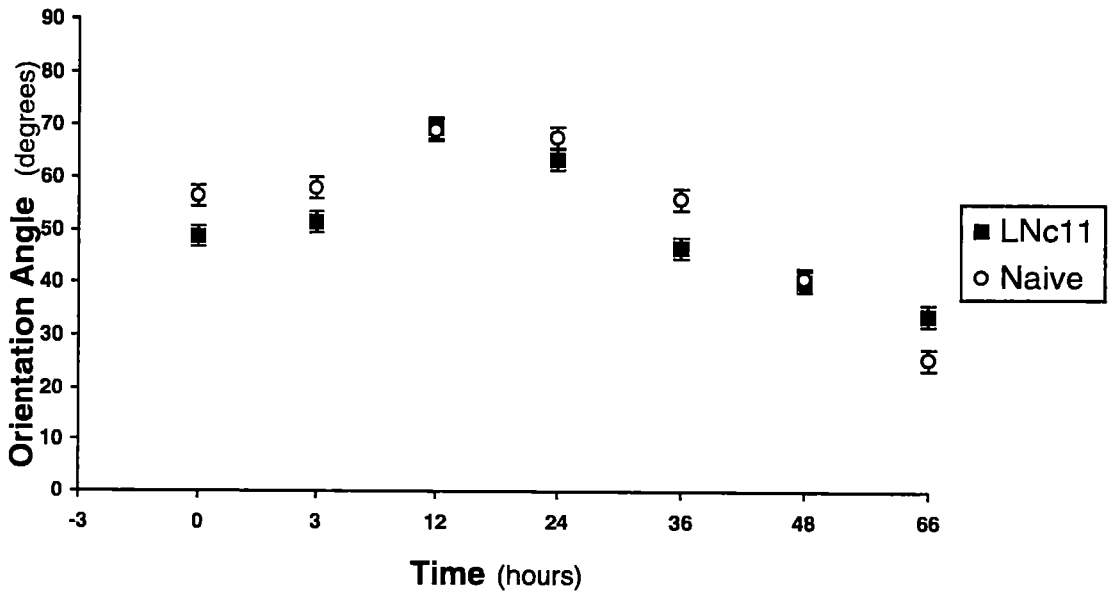

Figure 4.2. Endothelial Cell Orientation

particular cell type, either naive or transduced cells. Additionally, the data at 0 and 66 hours for each cell type was compared to the corresponding data of the other cell type. This comparison checked for statistically significant differences in cell shape or angle between naive and transduced cells at the same point in time. The comparisons concluded two things. First, the shape index and angle of orientation for both naive and transduced cells changed significantly ($p < 0.05$) during the course of the experiment. Secondly, the shape index and angle of orientation of the naive and transduced cells differed significantly ($p < 0.05$) from each other at the initial and final time points.

Immunofluorescent Staining

Immunofluorescent stains of the slides were successful in revealing the F-actin cytoskeleton of the flow exposed cells. However, the vinculin staining was unsuccessful for both naive and retroviral cells. Figure 4.3 contains a representative stain for the naive HUVEC and Figure 4.4 shows the staining pattern of the LNc11 transduced HUVEC. Figure 4.3 and Figure 4.4 were taken upon completion of the experiment, 66 hours, with 200x and 300x magnification respectively. The direction of flow in the photographs was vertical, from the top to the bottom of the photograph. Visible within both photographs are numerous stress fibers. However, no noticeable differences in either the number or thickness of the stress fibers were seen between the naive and transduced cells.

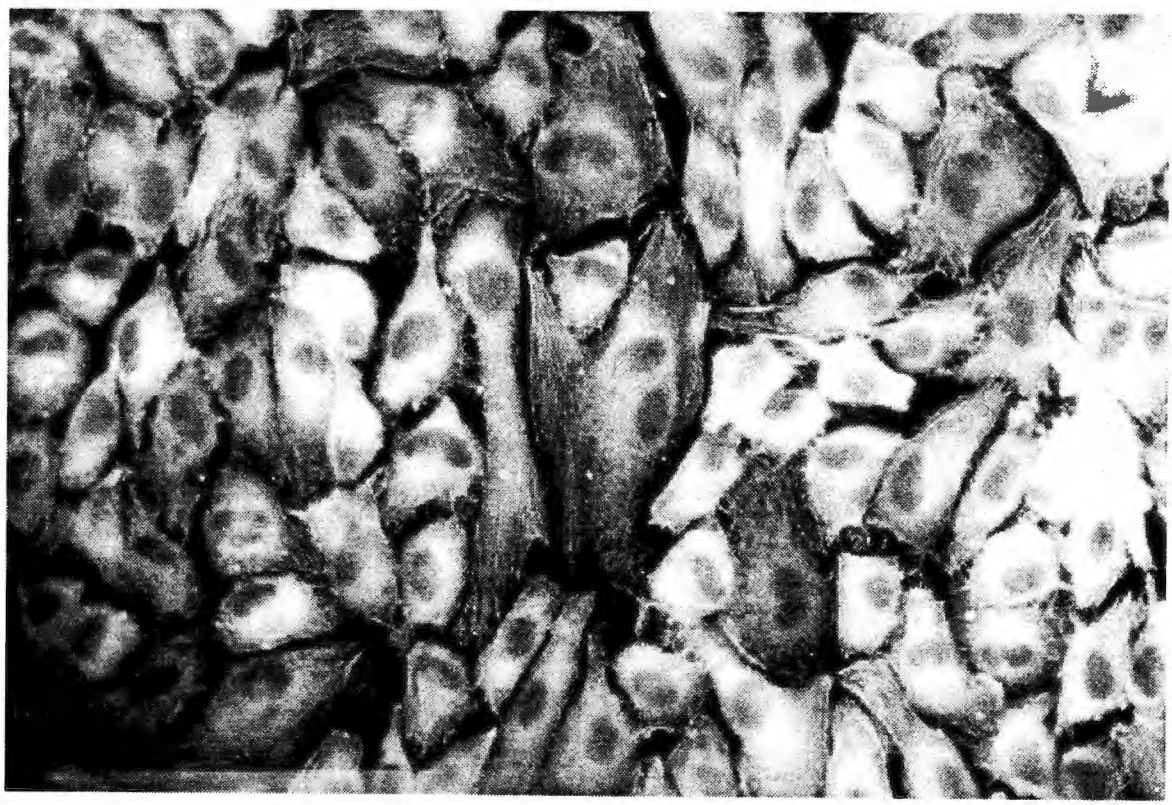

Figure 4.3. Naive Cell Staining Pattern after 66 Hours of Flow

Figure 4.4. Transduced Cell Staining Pattern after 66 Hours of Flow

CHAPTER 5

DISCUSSION

The use of genetically modified endothelial cells to reduce thrombus formation and intimal hyperplasia in small ID vascular grafts depends upon the ability of the transduced cells to adhere to their substrate. Unfortunately, *in vivo* and *in vitro* studies suggest that the modified endothelial cells are impaired in their ability to adhere to synthetic materials (Dunn et al., 1996; Sackman et al., 1995). A recent study by Sackman et al. (1997) found that the β_1 and β_3 subunits of transduced endothelial cell integrins were not present in their complete form, possibly hindering the integrin's ability to bind to extracellular adhesive proteins.

Because integrins are associated with the cell cytoplasm and are known to be vital in the signaling of various cell processes, it was hypothesized that the altered β integrins found in the retrovirally transduced endothelial cells (Sackman et al., 1997) may adversely affect the recombinant endothelial cells' ability to adhere to a substrate and also hinder the rearrangement of the cell cytoskeleton in response to fluid imposed shear stress. The cytoskeletal rearrangement of naive endothelial cells in response to flow is well documented, as evidenced in Table 2.1, and is believed to be key for continued adherence. In order to evaluate the aforementioned hypothesis, naive and retrovirally transduced cells plated onto fibronectin coated slides were subjected to a shear stress of

25 dynes/cm^2 for a duration of 66 hours. Because expression of the neomycin phosphotransferase gene may be one factor in the decreased endothelial cell adhesion in a previous study (Sackman et al., 1996), cells transduced by the neoR gene with the LNc11 vector were used in the present study. Based upon the findings of Sackman et al. (1996) that neoR transduced endothelial cells adhered more poorly to a fibronectin substrate than naive endothelial cells, the cells used in this study were seeded onto fibronectin coated slides similar to the original study. A randomly chosen field of cells for each experiment was videotaped at regular intervals in order to obtain data pertaining to the cell shape and amount of alignment with the direction of flow. In addition, each slide of cells was subsequently immunofluorescently stained for the cytoskeletal protein F-actin and the focal adhesion protein vinculin.

These experiments indicate a statistically significant difference between naive and transduced HUVEC with respect to the cell shape and angle of orientation at the beginning and end of the experiments. However, a comparison between naive and transduced cells in terms of the percent change in shape or angle could not be made because the data was unpaired, i.e. the percent change of particular cells could not be calculated because the procedure did not involve tracking the same cells across time. Additionally, the transduced cells did exhibit the ability to elongate and reorient in the direction of flow. The experiments also revealed that the changes in transduced and naive cell shape and orientation followed similar trends during the course of the experiment. Furthermore, the cytoskeletal staining did not reveal noticeable differences in either the

number or thickness of stress fibers. The vinculin staining was unsuccessful and did not yield any information concerning the association of vinculin with the F-actin fibers.

In a previous study by Sackman et al. (1996), transduced cells were able to adhere to fibronectin coated slides while subjected to a shear stress of 20 dynes/cm^2. This implies that the fibronectin-associated integrin may still be partially functional. During the same study at a higher shear stress of 90 dynes/cm^2, the neoR transduced cells were impaired in their ability to adhere (Sackman et al., 1996). Perhaps a higher shear stress would exaggerate the effect, if any, that the altered integrin may have upon the cell shape, angle with respect to flow, and/or stress fiber formation.

The formation of stress fibers in response to *in vitro* fluid shear stress is well documented, Table 2.1. While the F-actin staining revealed no qualitative difference among naive and transduced cells in stress fiber formation, the present experiment was unable to successfully stain either the naive or transduced cells for vinculin. Therefore, the effect of retroviral transduction upon the association of focal adhesion plaque proteins with the altered integrin subunit of transduced cells remains to be documented.

CHAPTER 6

CONCLUSIONS

The aim of the presented work was to observe the morphological change and reorganization of the cytoskeleton within recombinant endothelial cells exposed to flow. The present research was successful in implementing a procedure for the study of endothelial cells subjected to fluid imposed shear stress over an extended time period, 66 hours. Utilizing video microscopy and image analysis computer software, data was obtained describing the cell shape and angle of orientation. Lastly, immunofluorescent staining revealed the F-actin cytoskeleton of the flow exposed cells.

Although the present study did not conclusively support or contradict the experimental hypothesis, additional studies using similar experimental protocols should prove more informative. Pairing the data, tracking the change in cell shape and angle of individual cells across time, would yield more reliable statistical evidence by allowing a comparison between naive and transduced cells with respect to the percent change in shape and angle. Because the rings used in coating the slides often leaked, a more reliable technique would increase confidence in the calculated fibronectin substrate coverage and integrity and possibly eliminate a variable introduced by incomplete or inadequate substrate coverage. Finally, successful staining of focal adhesion proteins,

such as vinculin, should provide evidence of any impaired interaction of the cell cytoskeleton with the altered integrins.

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