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I am submitting herewith a thesis written by Carey E. Romoser entitled "Static Adhesion and Cell Proliferation of Endothelial Cells on Vacuum Ultraviolet Surface Modified Expanded Polytetrafluoroethylene." 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.

Judy L. Cezeaux, Ph.D., Major Professor

We have read this thesis
and recommend its acceptance:

Roberto S. Benson, Ph.D.

John H. Forrester, Ph.D.

Jill E. Sackman, D.V.M., Ph.D.

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

STATIC ADHESION AND CELL PROLIFERATION OF ENDOTHELIAL CELLS ON VACUUM ULTRAVIOLET SURFACE MODIFIED EXPANDED POLYTETRAFLUOROETHYLENE

A Thesis
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Carey Edward Romoser
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DEDICATION

This thesis is dedicated to my grandmothers

Thelma King Romoser

and

Winnie Ruscoe Montgomery

both of whom died during its writing.

Grandmother, your encouragement never ended, even when words could no longer be voiced. And, Nanny, you always said a degree from the University of Tennessee was really something special.

One day we'll be together again.

I miss you both.

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ABSTRACT

Small diameter (≤ 6 mm ID) expanded polytetrafluoroethylene (ePTFE) vascular grafts, used as vessel replacements in patients without usable saphenous veins, have a cumulative patency rate of 38% after 5 years (Veith et al., 1986). Thrombus formation and anastomotic hyperplasia, an increase in luminal smooth muscle cells at the graft-host boundary which leads to progressive narrowing and eventual occlusion of the vessel, are primarily responsible for synthetic graft failure. In an effort to curtail these processes and, hence, increase patency rates of synthetic grafts, investigators have seeded grafts with endothelial cells prior to implantation. This technique has been moderately successful for the endothelialization of synthetic grafts in animal models, but not in humans thus far (Zilla et al., 1987; Wigod and Kitzman, 1993; Herring et al., 1994).

The carbon-fluorine bonds within ePTFE have high bond energy (486 kJ/mol) and low polarizability (Yamada et al., 1996), resulting in a hydrophobic surface. Studies have shown that endothelial cells (EC) exhibit poor adhesion to hydrophobic materials (Dekker et al., 1992); rather, EC adhere well on moderately water-wettable polymers (Van Wachem et al., 1985). Several methods of surface modification have been developed aimed at making polymers more hydrophilic. The purpose of this study is to evaluate the effect of vacuum UV (VUV) modification of ePTFE on endothelial cell adhesion and proliferation. VUV modification of ePTFE has shown increased initial adhesion of fibrin glue (Szymankiewicz et al., 1997), providing some reason to believe VUV modification will help EC adhesion.

Pieces of ePTFE graft material were exposed to 10 W, 20 W, or 40 W VUV radiation for 10, 20, or 40 minutes using an ultraviolet excimer lamp. After modification, the graft pieces were autoclaved and cut into pledgets, half of which were coated with fibronectin (20 μg/ml). Static cell adhesion was measured by seeding tritiated thymidine-(^{3}H-thy-) labeled human umbilical vein endothelial cells (HUVEC) onto the modified and unmodified pledgets for 60 minutes at 37°C. Following a wash, pledget radioactivity was measured using β-scintillation counting. For the cell proliferation experiments, pledgets were seeded with unlabeled HUVEC which were allowed to adhere to the graft material for 18 hours at 37°C. The cells were then exposed to ^{3}H-thy (1 μCi/ml) for 52 hours. Again, incorporation of ^{3}H-thy was measured using β-scintillation counting.

VUV modification had no significant effect on EC adhesion or proliferation on ePTFE vascular graft material for the power levels and exposure times examined. In the presence of fibronectin, cell proliferation on ePTFE exposed to 20 and 40 W for 40 and 10 minutes, respectively, appeared to be improved as compared to proliferation on unmodified ePTFE, but these results were not statistically significant. Analysis of variance showed significant improvement in adhesion and proliferation in the presence of fibronectin. Statistical significance was assumed to be at $P < 0.05$. Scanning electron microscopy demonstrated that cyanoacrylate used to secure the graft pledgets for the adhesion and proliferation experiments may have invalidated the results by altering the graft surface characteristics.

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LIST OF ABBREVIATIONS

ACE	angiotensin-converting enzyme
ADP	adenosine diphosphate
ANOVA	analysis of variance
APD	ablative photo-decomposition
ASV	autologous saphenous vein
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophoshate
C-C	carbon-carbon
C-F	carbon-fluorine
cGMP	cyclic guanine monophosphate
DMSO	dimethyl sulfoxide
DPM	decays per minute
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGM	endothelial cell growth medium
ePTFE	expanded polytetrafluoroethylene
Fn	fibronectin
HBSS	Hank's balanced salt solution
^{3}H-thy	tritiated thymidine

HUVEC	human umbilical vein endothelial cell
ID	inner diameter
MS	mass spectrometry
MVEC	microvascular endothelial cell
MW	molecular weight
NO	nitric oxide
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PGI_2	prostacyclin
PET	polyethylene terephthalate
PTFE	polytetrafluoroethylene
SEM	scanning electron microscopy
SMC	smooth muscle cell
TNS	trypsin neutralizing solution
tPA	tissue type plasminogen activator
TxA_2	thromboxane A_2
UV	ultraviolet
VSMC	vascular smooth muscle cell
VUV	vacuum ultraviolet
vWF	von Willebrand's factor
XPS	X-ray photoelectron spectroscopy

CHAPTER 1

Introduction

Myocardial infarctions, stroke, and amputations due to lower extemity athero-occlusive disease are major causes of mortality today (Wilson et al., 1989). Each year, 150,000 people have lower limb vessel replacements because of peripheral athero-occlusive disease related conditions in this country alone (Gupta et al., 1988). Reconstruction of diseased portions of the vasculature necessitates the availability of suitable conduits for vessel replacement. Such alternatives have included autologous saphenous vein (ASV) (Veith et al., 1986) and synthetic vascular grafts (Herring et al., 1978; Graham et al., 1980; Stanley et al., 1982; Clagett et al., 1984; Allen et al., 1984; Veith et al., 1986; Shepard et al., 1986; Hollier et al., 1986; Douville et al., 1987; Zilla et al., 1987; Herring et al., 1987; Herring et al., 1994). ASV as the conduit of choice for reconstructive surgery of small and medium-sized arteries has been widely acknowledged (Stanley et al., 1993). This biocompatible, compliant, endothelial cell-lined biologic vascular graft is preferred because of its inherent ability to actively control hemostasis and thrombosis, a characteristic attributed in the last two decades to the presence of a confluent, viable endothelial cell monolayer completely covering the lumen of the vessel (Kinley and Marble, 1980; Shireman and Pearce, 1996). In cases where the saphenous veins have already been used, or in patients whose ASV are not suitable, as in the 5 to 10 percent of cases where they are too small for vascular reconstructions (Stanley et al.,

1993), synthetic vascular grafts are used. Although several polymers have been utilized, including polyethylene terephthalate (PET) (Herring et al., 1978; Graham et al., 1980; Stanley et al., 1982; Clagett et al., 1984; Allen et al., 1984; Shepard et al., 1986; Hollier et al., 1986) and polyurethane, polytetrafluoroethylene (PTFE) is widely used for the manufacturing of vascular graft prostheses because of the polymer's biocompatibility, chemical inertness, and tensile properties (Douville et al., 1987; Zilla et al., 1987; Herring et al., 1987; Herring et al., 1994). Expanded PTFE was first used as an arterial conduit in patients in 1976 (Veith et al., 1986). Small diameter ($\leq$ 6 mm ID) ePTFE vascular grafts, used as lower-limb vessel replacements, unfortunately have a failure rate of 46% after 4 years (Veith et al., 1986). Synthetic vascular grafts with smaller inner diameters have consistently exhibited lower patency than larger synthetic grafts and vein grafts for a given interval of time (Zilla et al., 1994; Herring et al., 1994). Thrombus formation and intimal hyperplasia are major contributing factors in graft failure. Intimal hyperplasia is an increase in smooth muscle cells (SMC) in the lumen of the vessel, predominantly at the anastomoses, progressively narrowing the flow area through the graft. The presence of an endothelial cell layer plays the doyene role in maintaining vascular function and controlling thrombosis and intimal hyperplasia. Endothelialization of vascular prostheses may occur by 3 routes: proximally and distally from the adjacent native artery across the anastomoses and onto the graft lumen, capillary endothelial cell (EC) ingrowth through the interstices of the graft matrix to the intimal surface, and EC deposition or "fall out" from circulating blood (Clowes et al., 1985; Clowes et al., 1986; Asahara et al., 1997). Studies have shown that synthetic grafts never fully endothelialize when implanted in

humans, even after several years (Sentissi et al., 1986; Herring et al., 1994). It has been hypothesized that EC seeding of small-diameter synthetic vascular grafts prior to implantation may facilitate the development of a functional and confluent monolayer and subsequently improve patency rates. Endothelial cell seeding has resulted in EC monolayer formation, reduced platelet aggregation and SMC proliferation, and improved overall graft performance in *in vitro* studies (Sentissi et al., 1986; Pratt et al., 1988; Schneider et al., 1988; Greisler et al., 1989; Vohra et al., 1992; Zilla et al., 1994) and in animal studies *in vivo* (Herring et al., 1978; Graham et al., 1980; Stanley et al., 1982; Allen et al., 1984; Clagett et al., 1984; Shepard et al., 1986; Hollier et al., 1986; Douville et al., 1987). Zilla et al. (1994) reported that lower-extremity bypass grafts in humans remained patent for 32 months in 85% of seeded ePTFE grafts and only 55% of unseeded grafts. In another human *in vivo* study, Herring et al. (1987) documented patency at one year to be 82% for seeded ePTFE grafts compared to 31% for unseeded grafts. These results appear encouraging. Clinical trials in humans, however, have been inconsistent due to low seeding densities (Zilla et al., 1987; Herring et al., 1994) and incomplete graft coverage (Wigod and Kitzman, 1993). Common to all attempts of ePTFE endothelialization is the graft material's low affinity for EC adhesion and proliferation. Investigators have tried to improve EC adhesion to synthetic grafts by coating the graft surface with various protein substrates containing matrix ligands found in native vessels. Fibronectin (Fn), a large macromolecule found in plasma and in the subendothelium of the vascular wall, when used as a synthetic graft substrate, improves EC attachment and adhesion (Kesler et al., 1986; Hasson et al., 1986; Greisler et al., 1990; Wigod and

Kitzman, 1993). On ePTFE, Budd et al. (1990) reported significant increases in EC adhesion with increasing Fn concentration up to 20 μg/ml. While biocompatible and chemically unreactive, ePTFE has a low wettability making it very difficult for biological components to adhere and spread. Expanded polytetrafluoroethylene's strong carbon-fluorine (C-F) bonds lend to this hydrophobicity (Yamada et al., 1996). Several surface modification techniques have been examined to alter the chemistry and composition of a thin layer of the surface making it more wettable and, perhaps, more suitable for EC adhesion, while keeping the overall mechanical properties of the graft material uncompromised. Gaseous plasma modification (Youxian et al., 1991; Dekker et al., 1992; Sipehia et al., 1993; Sipehia et al., 1996; Yamada et al., 1996; Badey et al., 1996) and photochemical modification (Zhang et al., 1994; Esrom et al., 1995; Kang et al., 1996; Szymankiewicz et al., 1997) are means of modifying the surface of the graft material in an effort to increase its hydrophilicity. Gas plasma modification is a common method of surface modification whereby the polymer surface is exposed to a concentrated environment of highly reactive radicals that react with the C-F bonds, which are inert in conventional reactions. Changes in surface chemistry, including the substitution of fluorine (Youxian et al., 1991; Yamada et al., 1996; Badey et al., 1996), incorporation of nitrogen- and oxygen-containing groups (Dekker et al., 1992; Sipehia et al., 1993), and the formation of C-C double bonds (Youxian et al., 1991) or crosslinks have been reported to increase the wettability of ePTFE, confirmed by a significant decrease in water contact angles (Youxian et al., 1991; Dekker et al., 1992; Yamada et al., 1996; Badey et al., 1996). Several researchers have discussed that exposure to vacuum

ultraviolet (VUV) light using incoherent excimer lamps may have the effect of increasing the hydrophilicity of several polymers including ePTFE (Zhang et al., 1994; Esrom et al., 1995; Kang et al., 1996). Limited research has been conducted involving *in vitro* biological evaluation of EC attachment to synthetic vascular grafts whose surfaces have been VUV modified; however biological evaluation has been conducted after surface treatment using gaseous plasma modification (Dekker et al., 1992; Sipehia et al., 1993). Furthermore, no studies have been published to date which report the effects of VUV excimer lamp surface modification of ePTFE vascular grafts on EC adhesion and proliferation.

The research discussed in this thesis addresses the effect of VUV modification on EC adhesion to and proliferation on ePTFE vascular grafts in a static environment. This study examines the effects of UV radiation surface treatment at 10 to 40 W for up to 40 minutes on human umbilical vein endothelial cell (HUVEC) adhesion and proliferation to fibronectin-coated ePTFE.

Preliminary work was performed in the Vascular Research Lab, supervised by Dr. Jill Sackman, Department of Surgery, University of Tennessee Medical Center, Knoxville. VUV modification was performed by Mr. Chris Buck in the Laser Processing Laboratory at the University of Tennessee under the supervision of Dr. Roberto Benson, Department of Materials Science and Engineering. The adhesion and proliferation experiments were also performed at the University of Tennessee Medical Center under the supervision of Dr. Judy Cezeaux, Department of Mechanical and Aerospace Engineering and Engineering Science. Follow-up experimentation was carried out in the

Cellular Biomechanics Lab at the University of Tennessee in Knoxville. Samples for scanning electron microscopy (SEM) were prepared by Mr. Dick Williams, Division of Biology, University of Tennessee, Knoxville.

CHAPTER 2

Background

The Vasculature

The normal blood vessel must be viewed not only as a conduit but also as an organ containing endothelial and smooth muscle cells that can respond to physical and chemical stimuli in the blood. Vascular wall cells can communicate among themselves, express factors that can regulate cell proliferation and coagulation, and participate in local inflammatory reactions. (Clowes and Kohler, 1993)

Vascular Structure

Arteries are divided into three morphologic categories: large elastic arteries, medium-sized muscular arteries, and small arteries. Each type of artery is comprised of three layers or tunics, called the intima, media, and adventitia. The intima is composed of endothelium and the subendothelial extracellular matrix. The media is bound by the internal and external elastic laminae and contains smooth muscle cells embedded in a matrix of collagen, elastin, and proteoglycans. The adventitia lies outside the external elastic lamina and is composed of loose connective tissue, fibroblasts, capillaries, leukocytes, small nerve fibers, and vasa vasorum. These three categories of arteries differ in the role each tunic plays in the overall composition of the artery. Veins are comprised

of the same three layers; however, they tend to be larger and more thin-walled than arteries. (Clowes and Kohler, 1993)

Vascular Function

The primary function of the vasculature is to act as nonthrombogenic conduits for blood (Clowes and Kohler, 1993). Maintenance of endothelial cell function is a prerequisite for proper vasculature function. When a viable endothelial cell layer is not present, the vascular wall exhibits several different phenomena, including release of various proteins, platelet deposition, and SMC proliferation. Before discussing vascular reactions to the absence of endothelium or to disrupted endothelium, an understanding of platelet and SMC function is needed.

Platelets are discoid-shaped, anuclear cells having an average life span in the circulatory system of 8 to 12 days (Humphrey et al., 1993). They are released as cytoplasmic fragments of megakaryocytes within bone marrow. Platelets have surface receptors for thrombin, serotonin, fibrinogen, fibronectin, ADP, epinephrine, collagen, casopressin, thromboxane A_2 (TxA_2), platelet activating factor, factor X, and factor V (Humphrey et al., 1993). Platelets contain three types of storage granules: dense granules, which contain serotonin, ADP, ATP, and calcium; alpha granules, which themselves contain high-molecular-weight kininogen (HMWK), fibrinogen, fibronectin, factor V, von Willebrand's factor, and other coagulation factors, as well as platelet-derived growth factor (PDGF), platelet factor 4, β-thromboglobulin, thrombospondin, and other proteins; and lysosomes, which contain proteases, glycosidases, and acid hydrolases

(Humphrey et al., 1993). Following vascular injury, platelets adhere to the injured vessel wall. The initial stimulus may be exposed collagen fibrils. Platelets will not adhere to injury sites unless sufficient and functionally normal von Willebrand's factor is present within the subendothelium. Following adhesion, collagen, ADP, thrombin, and epinephrine stimulate platelets to release their granule contents. (Humphrey et al., 1993)

Arterial wall thickening is a prominant feature of most vascular pathologic processes, including atherosclerosis. This wall thickening could be due to intimal or medial thickening. In each case, it has been found that the proliferation of smooth muscle cells and the accumulation of extracellular matrix are important components. To understand smooth muscle growth, several models have been studied. To expand on one such model may provide sufficient information for a general knowledge of the phenomenon of arterial wall thickening. When an artery is injured and denuded of its endothelium, platelets immediately begin to adhere to the wall ECM. The platelets then spread and degranulate, or rupture, releasing their contents. The products released from the platelets include several growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor β, and an epidermal growth factor-like protein (Clowes and Kohler, 1993). One to 2 days later, the onset of medial smooth muscle proliferation and migration of these cells across the injured internal elastic lamina to form a neointima occurs. The reaction-to-injury hypothesis suggests that products released from platelets accumulate in the artery wall and stimulate SMC growth (Clowes and Kohler, 1993). Recent work suggests, however, that proteins released from platelets may be more important in stimulating migration than cell proliferation because the wall

thickens only after smooth muscle cells migrate from the media and proliferate in the intima (Clowes and Kohler, 1993). The intimal mass is further increased by the accumulation of extracellular matrix synthesized by the smooth muscle cells. Several observations have been made concerning the wall thickening process. First, the surface of the injured artery accumulates a layer of platelets. Second, intimal thickening does not develop without medial damage. The endothelium, therefore, might play a role in suppressing smooth muscle growth and migration from the media to the intima. Thirdly, endothelium can inhibit smooth muscle cell proliferation. The quiesent state of smooth muscle cells in normal arteries of adult animals might be actively maintained. This is in contrast to the idea that changes in the state of SMC are primarily due to the presence of growth factors. Fourthly, cells of the vascular wall seem to "speak" to each other and regulate the function of one another. Cell-cell communication, or "crosstalk", may happen two ways, either by direct contact through intercellular junctions or by means of molecules secreted into extracellular space and acting in a paracrine fashion. (Clowes and Kohler, 1993)

Arterial thrombosis occurs in regions of disturbed flow and in vessels with endothelial injury. Thrombosis is important for this work because when using synthetic vascular grafts, flow is inevitably disturbed and the endothelium is initially incomplete, if not altered, and these materials are highly thrombogenic. In regions of high shear stress, endothelial cells may be denuded, allowing platelet adherence and aggregation to occur on the exposed subendothelium. Following platelet adhesion and aggregation, coagulation is activated, leading to the formation of a mature thrombus, or blood clot.

Thromboses are especially prone to develop in atherosclerotic vessels with altered endothelium and reduced flow. Endothelium that is disrupted may not express the anticoagulant agents needed for the clotting pathway, thus resulting in thrombus formation. Thrombus size is limited by arterial shear force, but an occlusive thrombosis will develop if the flow is sufficiently compromised. (Humphrey et al., 1993)

Atherosclerosis begins with an endothelial injury to the arterial wall, which could be secondary to hyperlipidemia and increased shear stress in hypertension. The earliest events in the development of atherosclerotic lesions involve recruitment of blood-borne monocytes into the subendothelial space. Some of these monocytes convert into ester-laden foam cells after engulfing cholesterol. The accumulation of foam cells in this space distorts the overlying single layer of endothelial cells. This distortion produces microseparations between the endothelial cells. It is here where platelets adhere and release PDGF and TxA_2. PDGF is essential for the division and growth of smooth muscle cells, which migrate into the subendothelial space, some converting into foam cells as well. As the accumulation of cells and lipid proceed, some SMC die and release their contents into the artery wall. This process continues to expand from the subintimal space toward the adventitia. When expansion in an adventitial direction is no longer possible the process impinges upon the lumen, leading to the obstruction of flow. (Fogelman et al., 1993)

Several hemodynamic factors play a role in the localization of plaque that causes atherosclerosis. It was once thought that high shear stress potentiated plaque formation by producing endothelial injury and disruption, thereby exposing the underlying artery

wall to circulating platelets and lipids (Ross and Harker, 1976; Ross, 1986). This point of view was strengthened by results of aorta constriction studies in animals showing that increased levels of wall shear stress, induced by vessel constriction, produced endothelial abnormalities (Zarins, 1993). Fry et al. (1968) increased wall shear stresses in the canine aorta to about 400 dynes/cm^2 (normal level is 15 to 20 dynes/cm^2) and found that the high shear stresses results in endothelial damage. Contradictorily, it is now suggested that it is low or oscillating shear stress that retards the mass transport of atherogenic substances away from the wall, resulting in increased intimal accumulation of lipids and EC damage (Caro et al., 1971). This debate was resolved by more comprehensive studies on the effects of high shear stress (Zarins et al., 1981; Reidy, 1985) and low shear stress on plaque formation. High shear stress studies showed that endothelial abnormalities were due to experimental problems and that neither high nor low shear stress causes endothelial damage (Reidy, 1985). Quantitative flow modeling of the human carotid bifurcation (Zarins et al., 1983) allowed researchers to monitor the effects of low and high shear stresses in the same region, revealing that it is the low shear regions, not the high shear regions, that form intimal plaques. Low shear stress may also interfere with exchange of essential substances at the endothelial surface. These substances are important in arterial wall nutrition and in the maintenance of proper endothelial metabolic function. Another hemodynamic factor in atherosclerosis is areas of flow separation, which exhibit low flow velocity and low shear stress. Particles in a region of flow separation have an increased residence time and would have greater opportunity to interact with the vessel wall. Additional factors in atherosclerosis, from a hemodynamic

standpoint, include oscillation of flow, turbulence, and hypertension (Zarins, 1993). Under normal, physiologic resting conditions, blood flow in the human body in laminar. However, at peak systole in the aorta, turbulent bursts have been observed, and turbulence can be more significant during exercise and in diseased vessels (Nerem, 1993). Hence, turbulence can exist *in vivo* and seems to play an atherogenic role in diseased vessels.

Endothelial Cell Function

In the past ten years, the scientific community has come to appreciate the complex interaction between endothelial cells and many of the circulatory system's regulatory mechanisms. Such mechanisms include thrombosis, fibrinolysis, vasoconstriction and vasodilation, and smooth muscle cell migration and proliferation.

The role of endothelial cells in the regulation of hemostasis is extensive. Endothelium possesses the innate ability to shift from its normal anticoagulant state into a procoagulant state. This shift is controlled by inflammation, trauma, and cytokines such as tumor necrosis factor-α (Shireman and Pearce, 1996). Several opposing factors work together to maintain hemostasis, the process by which bleeding from injured tissue is controlled. Hemostasis requires the interaction of the blood vessel wall, platelets, and coagulation and fibrinolytic proteins (Humphrey et al., 1993). Control of hemostasis is important to this work because thrombus formation frequently results in the occlusion of many synthetic vascular grafts (Clowes et al., 1985; Clowes et al., 1986; Poole-Warren et al., 1996). Many of the substances involved in hemostasis are synthesized by the

endothelium. In their normal quiescent state, endothelial cells are actively antithrombogenic (Humphrey et al., 1993).

Several proteins secreted by or expressed on EC are also involved with the promotion of coagulation and platelet adhesion. Platelet-activating factor stimulates platelet aggregation, while von Willebrand's factor (vWF) is the major platelet adhesion protein, found in the subendothelium and extracellular matrix. Fibronectin and collagen in the subendothelial cell matrix are also platelet adhesion proteins. Following vascular injury, tissue factor, or thromboplastin, when bound to factor VII, activates factor X initiating the clotting cascade either directly via prothrombinase, factor Xa, cofactor Va, and anionic phospholipid; or indirectly via factor IX, factor VIIIa, and phospholipid. Once to this point by either intrinsic or extrinsic pathways, prothrombin is cleaved to form thrombin, which enters the surrounding plasma by diffusion. Thrombin then cleaves fibrinogen to produce fibrin strands, the matrix of clots (Shireman and Pearce, 1996).

Several other proteins secreted by or expressed on EC are involved with the inhibition of coagulation. Tissue factor pathway inhibitor is the most important of these and participates in a negative feedback mechanism in the clotting pathway from factor X to thrombin. Tissue factor pathway inhibitor forms a complex with factor Xa, which combines with the factor VIIa-tissue factor complex. This inactivates this complex and thus keeps factor X from being activated which ultimately inhibits coagulation for as long as tissue factor pathway inhibitor and factor Xa are bound together (Shireman and Pearce, 1996). The protein C- protein S pathway also inhibits coagulation by participating in

another negative feedback mechanism. When thrombin, the product of the clotting pathway, binds to a receptor known as thrombomodulin on the EC membrane, protein C becomes activated and binds to protein S at the EC membrane which inactivates factors Va and VIIIa. As a reminder, without factors Va and VIIIa, thrombin cannot be produced. When thrombin and thrombomodulin bind together, thrombin's specificity changes such that protein C is activated faster than unbound thrombin (Shireman and Pearce, 1996). Heparan sulfate is a component of glycocalix which makes the surface of endothelium nonthrombogenic. There are five types of heparans and all are synthesized by EC. Thus, the presence of heparan sulfates, as well as other anticoagulants, largely contribute to the nonthrombogenicity of EC. Heparin acts as a cofactor for antithrombin III, which inactivates thrombin (Clowes and Kohler, 1993). Fibrinolysis, the digestion of the fibrin produced via the clotting pathway, is initiated at the EC surface. Tissue type plasminogen activator (tPA) and urokinase, both synthesized by endothelium, are two plasminogen activators that bind to fibrinogen and remain bound to fibrin (Clowes and Kohler, 1993), taking plasminogen that is bound to the endothelium and converting it into plasmin. Plasmin digests fibrin-rich thrombus. Tissue type plasminogen activator and urokinase are both inhibited by plasminogen activator inhibitors type 1 and 2, which are both synthesized by EC as well (Shireman and Pearce, 1996).

Endothelial cells form a confluent monolayer on the lumen of blood vessels. Because of their close proximity to vascular smooth muscle cells (VSMC) they play an integral role in the regulation of vasomotor tone by secreting or expressing vasodilators and vasoconstrictors agents. Vasodilatation is induced by prostacyclin (PGI_2), which

binds to adenylate cyclase and causes the production of cAMP within platelets, thus blocking platelet activation (Clowes and Kohler, 1993). Prostacyclin also prevents the aggregation of platelets on the endothelial cell surface. Thrombin, ADP, ATP, kallikrein, and histamine (Clowes and Kohler, 1993) stimulate EC production of PGI_2. The second mechanism of vasodilatation involves endothelium-derived relaxing factor (nitric oxide or NO-containing compound). NO is synthesized in the EC and diffuses into the VSMC where it elicits the formation of cGMP by activating guanylate cyclase. Cyclic GMP decreases intracellular calcium and causes VSMC relaxation. Thirdly, endothelium-derived hyperpolarizing factor changes the membrane potential of VSMC (Shireman and Pearce, 1996). Vasoconstriction occurs in several ways to counteract vasodilatation, as should be expected. EC synthesizes endothelin-1, which binds to endothelin receptor found on VSMC. These receptors cause vasoconstriction. A second vasoconstricting agent, angiotensin-II, allows constriction by catalyzing a pathway of reactions as well. Angiotensinogen becomes angiotensin I in the presence of renin. Angiotensin-converting enzyme (ACE) converts angiotensin I into angiotensin II which induces vasoconstriction by increasing permeability (Shireman and Pearce, 1996). TxA_2 also causes vasoconstriction. TxA_2 and PGI_2 are both arachidonic acid metabolites with opposing effects derived from EC membranes (Fogelman et al., 1993).

Endothelial cells have recently been found to have a part in the early stages of atherosclerosis, which many times develops into vessel occlusion. One of their points of involvement lies in the control of certain adhesion molecules during inflammation. Vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1

(ICAM-1) expressed on EC bind circulating monocytes. Once these monocytes have attached, they migrate through the ECM and form macrophages and foam cells. (Shireman and Pearce, 1996)

Endothelial cell adhesion depends upon both cell-cell adhesion and cell-matrix adhesion (Morgan, 1996), and different classes of integrins are involved with each. Integrins are adhesion receptors, present on endothelial cell membranes, which are responsible for binding extracellular proteins. They are composed of α and β transmembrane glycoprotein subunits. For example, the $\alpha_5\beta_1$ receptor binds to fibronectin. When an integrin binds to an extracellular protein, an intracellular protein complex forms, which links the integrin to the actin filaments in the cell cytoplasm. (Alberts et al., 1994)

Much of what has been discussed deals with the function of endothelial cells as pluripotential cells whose structure and function changes to accommodate the environment in which it finds itself. Endothelium is not a passive, non-thrombogenic barrier between blood and the vascular wall, but rather a dynamic one that is influenced by the hemodynamics of circulation. The endothelium recognizes its mechanical environment and adjusts its structure and function (Nerem, 1993). This behavior has been shown through *in vitro* (Dewey et al., 1981) and *in vivo* (Nerem, 1993) studies. *In vivo* experiments have shown that endothelial cells on the arterial wall orient themselves such that they are elongated in the direction of flow (Nerem, 1993). In addition, when the flow pattern changes, the cells reorient themselves as a function of the level of wall shear stress in the new flow environment. Further, cell culture studies have shown that when

an endothelial cell monolayer is exposed to laminar, steady state flow cells orient themselves along the streamlines and cell proliferation decreases. However, under turbulent conditions the cobblestone-shape appearance is retained and cell proliferation is increased (Nerem, 1993). Arterial diameter changes with wall shear stress. It is believed that the endothelium monitors shear stress levels and causes diameter changes in an effort to maintain a constant wall shear stress (Kamiya and Togawa, 1980; Langille and O'Donnell, 1986; Zarins et al., 1987). Endothelial responses to flow and the associated shear stress also result in changes to cytoskeletal localization, cell mechanical stiffness, protein synthesis and secretion, endocytosis, and intracellular signaling (Nerem, 1993). The endothelium serves as an important mediator of effects between blood and the underlying vessel wall.

Vascular Grafts

Autologous Grafts

Now that a general knowledge base of the physiological and pathological processes occurring in the vascular wall has been provided, attention can be focused on the idea of vessel replacement using what has been learned in regard to the injury response of the arterial wall. Much progress has been made in the repair of injured vessels by use of angioplasty, stents, and vascular reconstruction. However, when such attempts are futile, as in instances of severe damage or disease, segments of the arterial tree need to be replaced. The optimal substitute for a diseased artery is the arterial

autograft. Availability of suitable arteries is limited. The most commonly used peripheral arterial autograft has been the internal iliac artery, used primarily for renal reconstruction (Stoney and Wylie, 1980). One of the most important advantages of using autologous artery relates to maintenance of the vessel's intrinsic blood supply during the early implantation period. If the vasa vasorum, the system of vessels supplying the vasculature with blood and nutrients, are undisturbed the vessel will keep its own blood supply intact as it is transplanted (Stanley et al., 1993).

Autogenous saphenous vein as the conduit of choice for reconstructive surgery of small and medium-sized arteries, and its use in the lower extremity as a reversed conduit or *in situ* graft remains the standard by which other biologic and synthetic prostheses are judged (Stanley et al., 1993). Several vein graft alterations take place following arterial implantation. The intima of the vein graft begins to thicken. One form of this thickening is a proliferative response encompassing an overgrowth of cellular elements within the subendothelial tissue. Another is the result of fibrin layering on the graft surface, and appears to be the result of successive films of fibrin clot becoming organized in areas of unusually low or turbulent flow. These two specific forms of intimal thickening are referred together as dysplastic lesions. They may produce focal stenoses, as well as diffuse narrowings of the entire conduit. Although some degree of intimal thickening is inevitable, the extent and rapidity with which it progresses is variable. In cases of coronary artery reconstructions, this form of intimal proliferation has been alleged to account for 15 to 30 percent of occluded grafts during the first postoperative year (Stanley et al., 1993). The dominant pathologic feature in these lesions appears related to the

presence of myofibroblasts, or myointimacytes, which are modified smooth muscle cells. They have a highly secretory phase that may be initiated by luminal surface mitogens. One such mitogen is PDGF released as platelets adhere to the deendothelialized intima. The secretory phase of myofibroblasts may also be initiated due to local hemodynamic alterations, to circumferential distention and altered tangential stresses, or to rapid or low velocity blood flow (Stanley et al., 1993). In general, the venous circulation is resistant to arteriosclerosis. Veins used as arterial substitutes do not maintain the same immunity to this disease.

Synthetic Grafts

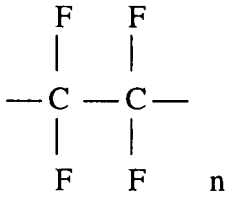
Dacron (polyethylene terephthalate) and Teflon (expanded polytetrafluoro-ethylene) fabric grafts are the two most used synthetic grafts clinically because these materials are essentially unchanged in tensile strength even after long periods of implantation. Teflon is slightly less reactive than Dacron. Because these grafts are initially foreign bodies introduced to the body, the healing of these fabric prostheses often is a major indication of the body's acceptance of the graft implant. Early failure of small ID grafts is due to thrombosis. Shortly after implantation, platelets and a fibrin layer form at the blood-surface interface -- usually $\leq$ 1 mm thick. This does not lead to thrombosis and graft occlusion in high flow, larger caliber prostheses; however, in small ones, especially in low-flow environments, the fibrin layer invariably increases in thickness and often contributes to occlusion of fabric prostheses less than 5 mm in diameter. The next stage of healing involves the fibrous tissue encapsulation and

organization of the graft's inner lining. The inner fibrous layer may arise from cells originating from the severed ends of the of host artery, from multipotential cells that are deposited from the bloodstream, or from the surrounding external tissues that grow through the interstices of the prosthesis to its inner surface. Ingrowth from the host vessel will never completely cover prostheses more than 4 cm in length. Therefore, the most important mechanism by which the fibrous layer is formed is the ingrowth of fibroblasts through the interstices of the graft. Depending of the porosity of the material, capillaries in the form of a "vasa vasorum" traverse graft interstices to nourish the inner lining. One complication with synthetic vascular grafts is that the materials soon become encased and ingrown by fibrous tissue, causing the loss of any preexisting elasticity. This loss and the resulting compliance mismatch have been implicated as a cause of endothelial injury and anastomotic intimal hyperplasia. (Stanley et al., 1993)

Expanded Polytetrafluoroethylene as a Biomaterial

A biomaterial is a biologically inert substance designed for implantation within or incorporation with a living system (Park, 1990). The most widely used biomaterial for vessel replacement is a fluorocarbon polymer known as expanded polytetrafluoro-ethylene. Pertinant information concerning its manufacture and its mechanical and chemical properties as they relate to vascular graft vessel replacement should be provided in order to understand the behavior of biological entities in contact with such a synthetic material.

One reason that PTFE has been so attractive as a biomaterial is because it can be modified to various structural forms (Florian et al., 1976). In its manufacture, however, PTFE cannot be injection molded or melt extruded because it has a very high melt viscosity; PTFE cannot be plasticized, either. PTFE is made from tetrafluoroethylene sintered under pressure to above 327°C with a peroxide catalyst in the presence of excess water for removal of heat. The monomer comprising the polymer is similar to that of polyethylene except that the hydrogen atoms are replaced by fluorine atoms (Park, 1990):



Expanded PTFE consists of bulky parallel nodes fixed at a certain internodal distance by transversely situated thin fibrils. By increasing the fibril length and, thus, the internodal distance, an increase in porosity can be achieved.

In terms of its mechanical properties, PTFE has a low modulus of elasticity (0.5 GPa), low tensile strength (17-28 MPa), very high density (2.15-2.2 g/ml), a low friction coefficient (0.1), and very low surface tension (18.5 ergs/cm^2). In addition, PTFE is highly crystalline (> 94%). From a chemical perspective, PTFE has an average MW of $5x10^5$ - $5x10^6$ g/mol. The C-F bond energy is very high at 486 kJ/mol (Park, 1990; Yamada et al., 1996).

As early as 1976, it was postulated that high-porosity ePTFE grafts with long fibril length and low density could serve as vascular conduits in small vessels for extended periods (Florian et al., 1976).

Mechanisms of Arterial Graft Healing and Failure

Mechanisms of arterial graft failure were investigated using 30 μm internodal distance PTFE arterial bypass grafts placed end-to-side in carotid and iliac regions of baboons. The specific types of connective tissue cells involved in the healing, or ultimately failing, process and definitive answers as to their origins and destinations were sought. It was found that within 24 hours after graft implantation, EC and VSMC begin migrating from the arterial end across the anastomoses towards and eventually into the graft. EC migrate and proliferate at the growing, or leading, edge in an effort to form a confluent monolayer, while SMC migrate beneath and along with the EC leading edge, migrating to the surface wherever EC is absent and returning to basal levels once an EC monolayer is established. In contrast to a healing injured artery, EC and SMC continue to proliferate at the anastomoses of synthetic grafts even after endothelium has formed a continuous sheet along the lumen. This behavior is perhaps explained by recurring endothelial injury at the anastomoses even though the endothelial cell monolayer appears to remain intact. Continued proliferation of these connective tissue cells is the cause of intimal thickening at ePTFE synthetic graft anastomosis in baboons and their eventual failure by occlusion (Clowes et al., 1985).

In a subsequent study, Clowes et al. (1986) found that while endothelial and VSMC migration in 30 μm PTFE was due to growth from the arterial ends, endothelial covering of higher porosity (60 μm) PTFE occurred by a completely different mechanism. Others had suggested that endothelium might be derived from capillaries growing through the graft matrix from the outside (Herring et al., 1978; Stanley et al., 1982; Herring et al., 1985). In accordance with these beliefs, Clowes et al. (1986) found that by using 60 μm PTFE that endothelium rapidly covered the luminal surface entirely in 2 weeks time. Endothelial migration and proliferation from the adjacent arterial ends seemed to be limited to about 2 cm. Substantial evidence indicates that the remaining majority of endothelium was derived from capillary endothelium via capillary ingrowth through the high porosity graft to the lumen. Evidence supporting this mechanism includes the presence of microvessels penetrating the graft wall. Once a confluent endothelium was established (2 weeks) SMC proliferation was detected beneath the endothelial surface. Intimal thickening seemed to be distributed evenly along the length of the graft at 1 and 3 months in the baboon in contrast to thickening at the anastomoses observed using 30 μm PTFE. It is suggested in conclusion that SMC proliferation persists even in the presence of a complete EC monolayer because either EC or SMC or both are producing mitogens, such as PDGF-like proteins (Clowes et al., 1986).

Endothelial Cell Seeding

The presence of an endothelial lining on the inner surface of the vasculature is known to be instrumental in controlling platelet deposition, smooth muscle cell migration and proliferation, SMC conversion to foam cells (Clowes and Kohler, 1993). Other pathological processes that may otherwise lead to intimal or medial thickening, thrombosis, arteriosclerosis and, ultimately, graft occlusion, may also be controlled by the presence of endothelium. One of the main reasons why native vessels are excellent thromboresistant surfaces is because of the presence of a confluent, viable monolayer of endothelial cells. Synthetic grafts do not have this important biological component. A synthetic vascular prosthesis completely lined by an intact endothelium should theoretically be protected from graft failure (Sentissi et al., 1986). Despite early hopes of spontaneous endothelialization, prosthetic vascular grafts implanted in humans lack a confluent endothelial lining even after several years (Sentissi et al., 1986; Wigod and Kitzman, 1993; Herring et al., 1994). Establishing an earlier confluent endothelium is believed to be essential in increasing the success rate of synthetic grafts as compared to autologous vein grafts. According to one study, 68% of vein graft remained patent as compared to 38% ePTFE grafts in small diameter reconstruction (Veith et al., 1986). Efforts at endothelialization have to this point included endothelial seeding, endothelial "sodding", and *in vitro* culturing of an endothelial cell monolayer on the prosthetic surface before implantation (Greisler et al., 1989). EC sodding holds a semantic implication (as in sowing grass, if you will) in that while EC seeding may involve

embedding cells within the matrix of the graft, EC "sodding" attempts to lay the cells on the graft surface. *In vitro* culturing of EC on synthetic vascular grafts prior to implantation is an effort aimed at achieving a complete endothelialized luminal surface before the graft is subjected to physiological flow-induced shear stress (Jarrell et al., 1986).

Seeding synthetic prostheses with autogenous endothelial cells has made possible the development of highly thromboresistant endothelialized surfaces in dogs (Herring et al., 1978; Graham et al., 1980; Sharefkin et al., 1982). This apparent success does not translate directly to clinical success because, just as the autogenous endothelial cells were seeded at the time of implantation in the canine models, a vascular prosthesis would have to be seeded with the relatively small numbers of cells that could be obtained from a short segment of a patient's vein. Thus, seeding density, which is a noted factor in the success of endothelial cell coverage, will be lower in the clinical application of these canine studies. Watkins et al. (1984) verified that harvested endothelial cells could grow to confluence from low densities *in vitro* under proper conditions. Hasson et al. (1986) postulated that failure of either spontaneous or low seeding density graft endothelialization is not because the seeded endothelial cells can not reproduce but rather is due to unfavorable graft surface conditions in which the cells are expected to grow to confluence.

Protein Substrates

In an effort to optimize the graft surface conditions in which endothelial cells are expected to adhere and grow, several different protein substrates have been tested (Greisler et al., 1989; Greisler et al., 1990). Greisler et al. (1990) coated 4 mm ID polyester grafts with fibronectin at a concentration of 10 μg/ml prior to endothelial cell seeding. Documentation of early methods of endothelial seeding directly onto synthetic grafts resulted in less than 20% adherence which then fell to 4% after exposure to 24 hours of a simulated flow environment (Greisler et al., 1990). They asserted that adhesion improves when prosthetic surfaces are pretreated with adhesive proteins, such as fibronectin, laminin, or collagen. Vohra et al. (1992) coated PTFE with a 50 μg/ml fibronectin solution. This concentration was chosen by virtue of earlier attachment studies they conducted on fibronectin coated ePTFE. To evaluate a collagen-fibronectin coating as a substrate for endothelial cell attachment, Sentissi et al. (1986) pretreated grafts with collagen and fibronectin. It was found that these substrates, used in combination, promoted both attachment and adherence of endothelial cells even under flow conditions (Sentissi et al., 1986). Kesler et al. (1986) conducted studies on endothelial cell attachment and adherence to polyester and ePTFE grafts coated with fibronectin at 100 μg/ml using untreated grafts as controls. After exposure to 15 dynes/cm^2 for 1 hour, they found significantly improved attachment to both materials with the fibronectin substratum (Kesler et al., 1986). Wigod and Kitzman (1993) seeded microvascular endothelial cells (MVEC) onto ePTFE treated 5 different ways. One group of grafts was coated with fibronectin, another with fibronectin after denucleation, a group

with tridodecylmethylammonium chloride (TDMAC) following denucleation, one more with TDMAC and fibronectin after denucleation, and a final group with TDMAC and a synthetic polymer with arginine-glycine-aspartic acid sequences following denucleation. The reason for conducting the study was to compare endothelial cell adhesion on synthetic vascular grafts coated with various adhesive substrates. Fibronectin is a cell attachment protein found in the extracellular matrix. TDMAC is a cationic surfactant that has been used to bond heparin or antibiotics to biomaterials. Arginine-glycine-aspartic acid (RGD) is an amino acid sequence responsible for the adhesive properties of many glycoproteins including fibronectin (Wigod and Kitzman, 1993). Attachment of fibronectin or RGD onto the graft surface may enhance cell adhesion. Denucleating a material refers to the removal of gas nuclei from the interstices of the material which increases the available surface area and may lessen a material's thrombogenicity. Results indicated that TDMAC alone or with fibronectin increases adhesion of human microvascular endothelial cells to denucleated ePTFE. These findings are important because they show that these substances when present on the surface of a graft may increase EC adhesion.

Substrates, such as the ones already mentioned, need to be used to support cell growth at low densities. Glycoproteins, such as fibronectin and laminin, as well as collagenous substrates, are highlighted because of their reputation of promoting cell attachment and growth on culture surfaces. Hasson et al. (1986) investigated the effects of matrix proteins on adult human vascular endothelial cell attachment by using a NaOH lysis technique. Using tritiated thymidine as a marker, they found that fibronectin coated

polystyrene (tissue culture plastic) was the favored surface with 63% attachment as compared to 18% attachment on untreated polystyrene after 60 minutes. Their results imply that endothelial cell attachment and growth is facilitated to the greatest extent by fibronectin when compared to laminin and collagen on tissue culture plastic. Many experiments have been conducted to arrive at an optimal concentration of fibronectin substrate for surface coating of synthetic vascular grafts. Different concentrations have been used: 10 μg/ml (Greisler et al., 1989), 40 μg/ml (Wigod and Kitzman, 1993), 50 μg/ml (Vohra et al., 1992), 100 μg/ml (Kesler et al., 1986), 1000 μg/ml (Sentissi et al., 1986), and 10 $\mu g/cm^2$ (Hasson et al., 1986; Greisler et al., 1990). Budd et al. (1990) documents work in which the effect of various fibronectin concentrations on PTFE seeded with HUVEC was studied. Varying the concentration from 2 to 100 μg/ml, they found no significant increase in attachment at concentrations higher than 20 μg/ml. Therefore, a fibronectin concentration of 20 μg/ml may be the most efficient in terms of cell attachment and cost.

In vitro Culturing of Seeded Grafts

Thrombus formation and platelet deposition play respected roles in graft failure. Endothelial cell seeding, while praised for the potential to decrease long-term platelet accumulation, actually provides no protection against platelet deposition and thrombotic occlusion in the early post-implantation period because seeding involves deposition of a relatively small number of harvested EC at the time of graft placement. Seeding of grafts in humans may not be as adequate at covering the graft's luminal surface as seeding of

grafts in animals because human vascular EC replicate and migrate at a slower rate (Schneider et al., 1988). Due to the problems associated with cell loss immediately after implantation, *in vitro* culturing of seeded grafts has been investigated as a process whereby pure populations of endothelial cells from a short segment of vein are isolated, cultured, and seeded on prostheses *in vitro* prior to implantation. Schneider et al. (1988), in order to establish a technique for forming a confluent endothelial cell monolayer on small-caliber prosthetic grafts before implantation, documented the possibility of producing a confluent EC monolayer on 4 mm ID ePTFE and Dacron (PET) after only 2 hours of *in vitro* perfusion (15 ml/min) when seeded at a density of $3.6x10^5$ cells/cm^2 graft. This study and others focused on the establishment of a confluent EC monolayer prior to graft implantation in an effort to contribute to the long-term success or patency of prosthetic vascular grafts. Another way to address the likelihood of long-term success is to improve cell retention following exposure to flow. Miyata et al. (1991) developed an *in vitro* pulsatile flow circuit to study the effects of two variables on cell retention: cell density at the time of seeding and postseeding incubation time. Grafts exposed to flow 90 minutes after seeding exhibited significantly less cell retention when compared to greater incubation times. Maximal retention of 92% at 24 hours after seeding was observed when cells were seeded at confluent density ($1x10^5$ cells/cm^2). Miyata's results indicate higher success in cell retention when seeded grafts experience delayed exposure to flow induced shear stress. In a clinical study monitored by Zilla et al. (1994), 33 patients received synthetic PTFE grafts confluently lined with cultured autologous endothelial cells through a two-stage seeding method and 16 patients received untreated PTFE

prostheses. Angiography and platelet adhesion studies over three years revealed 84.7% patency for endothelialized grafts and 55.4% for control grafts. Platelet deposition was also lower in the endothelialized group throughout the 3-year period. It should also be mentioned that cell adhesion and retention on vascular grafts has been enhanced *in vitro* by pre-conditioning the seeded endothelial cell monolayer with long-term shear stress (Ott and Ballermann, 1995).

Cell Adhesion Studies

In vitro Studies

In an effort to evaluate the adherence of endothelial cells under flow conditions and to evaluate the use of certain substrates to facilitate adherence to the graft surface, Sentissi et al. (1986) seeded 22 PTFE grafts with bovine aortic endothelial cells at a seeding density of $4x10^5$ cells/cm^2 and compared them at 2 weeks with 22 PTFE non-seeded control grafts. They found that 20 of 22 seeded grafts had a confluent monolayer. After labeling the seeded grafts with Indium-111 oxine, so that the cells that incorporated the indium could be counted later using a gamma counter, all grafts were exposed to nonpulsatile flow for 60 minutes at low and high rates of flow of 25 and 200 ml/min, respectively (Sentissi et al., 1986). Light microscopy, scanning electron microscopy, and radionuclide scans showed an insignificant loss of endothelial cells during exposure of flow, with 7 and 11 percent loss with low and high flow, respectively. Greisler et al. (1989) documented a study in which they seeded 4-mm ID polyester grafts with endothelial cells harvested from canine jugular veins at a density of $3.4x10^5$ cells/cm^2.

Grafts were cultured to achieve a confluent monolayer. After culture, grafts were exposed to flow in an *in vitro* perfusion circuit at either 90 or 195 ml/min for 120 minutes. It was found that 90% of the initial monolayer remained after the perfusion for both hemodynamic conditions. In a later study, Greisler et al. (1990) seeded circular patches of expanded PTFE with endothelial cells from canine external jugular veins. Patches were cultured to confluence, then exposed to shear stresses at 2.5 and 5.0 dynes/cm^2 using a cone and plate rotational-flow device. Endothelial cell retention did not differ between the two shear stresses, but both resulted in continued cell loss over time with 60% and 53% for low and high flows, respectively, at 1 hour, while static controls exhibited 92% and 95%, respectively. These results seem to contradict what was found in Greisler's earlier study, where 90% of the initial monolayer remained after exposure to flow at 90 and 195 ml/min. This may be due to the different labeling techniques used. Also aiming to compare the effects of shear stress of flow on endothelial cell monolayers, Vohra et al. (1992) seeded ePTFE and gelatin-impregnated Dacron graft segments with endothelial cells harvested from adult saphenous veins at a density above $1.4x10^5$ cells/cm^2. Two groups of seeded grafts were incubated, at 30 minutes and 90 minutes, and exposed to stresses at flow rates of 200 and 300 ml/min for 25 seconds. Cell retention was 55.4% and 56.5% on ePTFE and was 69.0% and 66.5% on Dacron for the two flows, respectively. Therefore, this study shows implies that seeded grafts may perform better regarding cell retention by culturing *in vitro* for a sufficient period of time prior to implantation.

In vivo Studies

Adhesion studies involving the seeding of endothelial cells onto synthetic vascular grafts and their subsequent behavior *in vivo* have been conducted in dogs (Herring et al., 1978; Graham et al., 1980; Stanley et al., 1982; Clagett et al., 1984; Allen et al., 1984; Douville et al., 1987), baboons (Shepard et al., 1986), pigs (Hollier et al., 1986), and humans (Zilla et al., 1987; Herring et al., 1987; Herring et al., 1994). These studies have utilized both Dacron and ePTFE graft material.

As early as 1978, Herring et al. (1978) harvested endothelial cells from autogenous vein at the time of implantation and seeded them on an arterial prosthesis. They found better cellular organization in seeded grafts and thromboresistance similar to normal endothelium. Their results implied that seeding led to an endothelium that was more similar to native endothelium than to the pseudointima found on unseeded grafts. Graham et al. (1980) found that seeded grafts after 4 weeks had 80% endothelial coverage while unseeded grafts exhibited limited pannus in-growth in 6 mm ID Dacron. Clagett et al. (1984) documented that platelet survival time and platelet serotonin levels were approaching normal at 8 and 12 weeks, respectively, on seeded grafts but were reduced in non-seeded controls. These two parameters were studied to determine a time course for development of thromboresistance and to test the ability of EC seeded prostheses to produce prostacyclin, which is produced by endothelium. Additionally, thrombus, composed of platelets, white cells, and fibrin, covered only 6% of seeded graft luminal surface area, compared to 27% of unseeded grafts. Graft patency studies of seeded vs. unseeded grafts *in vivo* in dogs using both Dacron and ePTFE have shown that seeded

grafts offer improved patency in small-diameter grafts. Dacron grafts were patent in 73% of seeded grafts and 27% in unseeded grafts at 16 weeks (Stanley et al., 1982), while at 7 months, a different study reported patency in 96% of seeded grafts and 29% of unseeded grafts (Allen et al., 1984). At 1 year, Douville et al. (1987) documents patency in 58.3% of seeded ePTFE grafts compared to 27.8% of grafts that were not seeded.

Seeded Dacron in baboons (Shepard et al., 1986), however, showed no significant difference in patency when compared to unseeded 4 mm Dacron at 5 weeks, although morphology revealed that seeding accelerated cellular organization of neointima and promoted the formation of an endothelial lining. Thrombogenicity was also found to be less in seeded grafts than in controls.

In vivo adhesion studies conducted in humans reported that synthetic prostheses seeding as a means to facilitate endothelialization of the graft's luminal surface was inferior to that documented in animal studies. Although Herring et al. (1987) found 93% and 85% patency in seeded and unseeded ePTFE grafts, respectively, at 3 months and 82% and 31% patency, respectively, at 1 year, Zilla et al. (1987) reported no significant differences between seeded and unseeded ePTFE grafts. Such a discrepancy in the behavior of autologous endothelial cells seeded onto ePTFE graft material by two teams in the same year could possibly be attributed to Zilla's extremely low seeding density of 3100 cells/cm^2 of graft. In a subsequent study, Herring et al. (1994) contradicted the 1987 study, demonstrating that seeded ePTFE grafts had lower patency rates than vein grafts and failed due to anastomic hyperplasia despite that initial attachment of endothelium was satisfactory.

Surface Modification

Failure of small diameter prostheses is due to thrombotic occlusion and intimal and anastomic hyperplasia (Dekker et al., 1992; Poole-Warren et al., 1996) developing because of a lack of a confluent EC monolayer on the luminal surface of the graft. Endothelial cell seeding as a means of producing a viable, confluent monolayer has been looked at extensively (Herring et al., 1978; Graham et al., 1980; Stanley et al., 1982; Clagett et al., 1984; Allen et al., 1984; Sentissi et al., 1986; Shepard et al., 1986; Hollier et al., 1986; Douville et al., 1987; Herring et al., 1987; Pratt et al., 1988; Schneider et al., 1988; Greisler et al., 1989; Vohra et al., 1992; Zilla et al., 1994); however, achieving a confluent EC monolayer that is shear stress resistant (in the physiologic range) on a synthetic vascular graft is extremely difficult. Endothelial cells do not adhere or spread well on hydrophobic materials such as ePTFE or polyethylene terephthalate (PET). Endothelial cells do adhere better to moderately water-wettable polymers (Dekker et al., 1992), hence, if the wettability of ePTFE could be improved, it is postulated that the ability of endothelial cells to form a confluent monolayer in a reasonable amount of time could be improved as well.

Several means of modifying the surface of the graft material with the purpose of making it more hydrophilic and, hence, more wettable, have been studied. Two such surface modification techniques are gas plasma modification and photochemical modification. A working knowledge of different tools used to quantify a surface modifi-

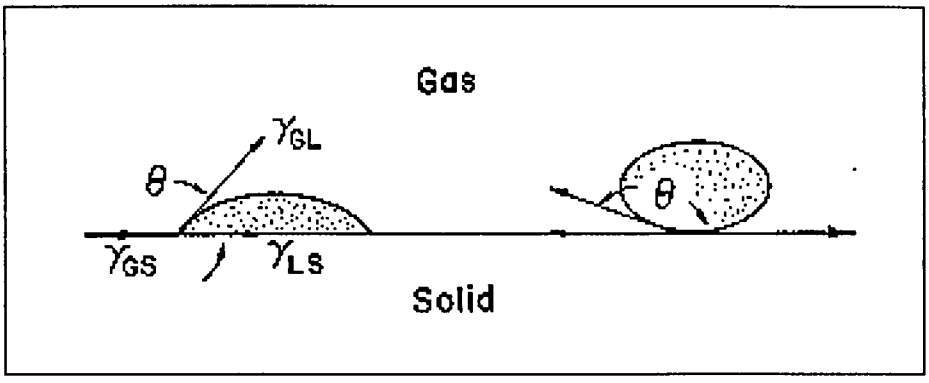


Figure 2.1. The Wettability of a Liquid on the Flat Surface of a Solid. Note the Contact Angle, θ.

cation must be gathered prior to an exploration of each technique. These methods include, but are not limited to, contact angle measurements, electron spectroscopy, and *in vitro* biological evaluation.

Contact angle measurements as a means of quantifying the wettability of a liquid on a flat surface of a solid are widely used (Youxian et al., 1991; Dekker et al., 1992; Yamada et al., 1996; Kang et al., 1996; Badey et al., 1996). When a drop of a liquid is placed on a solid flat surface, it will either spread or make a spherical bubble depending on the wettability of the surface material. The liquid is free to move until equilibrium is established but once force equilibrium is established, the surface tension among the solid, liquid, and gas phases in the solid plane should be zero. The contact angle, as shown in Figure 2.1, is the angle that the solid surface makes with the direction of surface tension between the liquid and gas phases and its measure is indicative of the wetting characteristics for a given solid and liquid. γ_{GL}, γ_{GS}, γ_{LS} denote the direction of surface

tension between the gas and liquid, gas and solid, and liquid and solid phases of matter, respectively. Partial wetting occurs with a contact angle ranging between 0° and 90°, while angles greater than 90° are interpreted as nonwetting surfaces. A contact angle of 0° indicates complete wetting of the surface (Park, 1990). The liquid used to measure contact angles is most reportedly water, which is a polar liquid. Methylene iodide, an apolar liquid, is also used for contact angle measurements (Youxian et al., 1991; Badey et al., 1996). There exists a variety of ways to measure contact angles. Dekker et al. (1992) used the captive bubble method; contact angle meters have also been utilized (Youxian et al., 1991; Yamada et al., 1996); and others have calculated the angles based on the height and base diameter found with a scaled telescope (Yamada et al., 1996; Badey et al., 1996). Both receding and advancing contact angles can be measured so that contact angle hysteresis can be evaluated which can provide significant information about the wettability of the surface. In short, a decrease in contact angle from one surface treatment to the next usually indicates that the surface has been made more wettable by the treatment.

Changes in chemical composition on a material's surface have been recognized by studying the surface using X-ray photoelectron spectroscopy (XPS) (Youxian et al., 1991; Dekker et al., 1992; Sipehia et al., 1993; Zhang et al., 1994; Yamada et al., 1996; Kang et al., 1996; Sipehia et al., 1996; Badey et al., 1996). XPS uses a photon source, either magnesium (Dekker et al., 1992; Yamada et al., 1996; Kang et al., 1996) or aluminum (Youxian et al., 1991; Badey et al., 1996), to identify the functional groups present, their relative abundance, and the surface concentration of various elements: carbon, nitrogen,

oxygen, and fluorine. When surface-treated samples are compared to untreated samples with respect to chemical composition, the effects of the treatment are readily realized.

In vitro biological experiments have been carried out (Dekker et al., 1992; Sipehia et al., 1993; Sipehia et al., 1996) on ePTFE surfaces modified by various methods and compared both qualitatively and quantitatively to unmodified ePTFE for endothelial cell adhesion and proliferation.

Gaseous Plasma Modification

Gaseous plasma treatment is a widely used surface modification technique whereby the polymer surface is placed in a plasma environment. Plasma contains radicals, along with electrons and ions, which are highly reactive, possessing the ability to initiate chemical reactions. Expanded polytetrafluoroethylene (ePTFE), a commonly used biomaterial praised for its chemical inertness and tensile properties, is also very water repelling. Its hydrophobicity is a result of the strong carbon-fluorine bonds comprising the material. These high-energy bonds within the polymer render a non-reactive surface with a low surface energy of 18.6 mJ/m^2 (Yamada et al., 1996). The interaction between the bonds within the polymer surface and the activated radicals of the plasma is desired to incur a beneficial change in surface chemistry, namely, the substitution of fluorine at the PTFE surface. The significance of gas plasma modification is that reactive species in the plasma may attack the C-F bonds, which are difficult to substitute by conventional chemical reactions (Youxian et al., 1991). It should be noted that since only a thin layer

of the polymer surface is modified with this technique, a few nanometers thick at the most, the mechanical properties for which PTFE is applauded are not compromised.

Plasma is usually generated at the electrodes of a plasma reactor at optimal electrode power and pressure, realized by diffusing a gas at the appropriate flowrate. Air (Youxian et al., 1991), ammonia (Sipehia et al., 1993; Sipehia et al., 1996; Badey et al., 1996), argon (Youxian et al., 1991; Kang et al., 1996), hydrogen (Yamada et al., 1996; Badey et al., 1996), nitrogen (Dekker et al., 1992), oxygen (Youxian et al., 1991; Dekker et al., 1992), and water vapor (Youxian et al., 1991) are several of the different gases that have been used in plasma treatments. Although noble in theory, gaseous plasma modification has proved all the nobler in practice, especially with regard to decreased hydrophobicity of PTFE surfaces. To examine the effects of gas plasma treatment on PTFE surface composition and adhesion, Dekker et al. (1992) observed increased wettability, abstraction of fluorine, and improved endothelial cell adhesion on plasma treated PTFE and ePTFE surfaces using oxygen and nitrogen plasmas. The improved EC adhesion documented was attributed to the increased hydrophilicity of the surface compared to untreated PTFE and ePTFE surfaces. The X-ray photoelectron spectra of carbon showed a peak shift which indicated a change in carbon bonding to some degree, which was confirmed by peaks in the oxygen and nitrogen regions showing that the PTFE incorporated oxygen- and nitrogen-containing groups. Increased wettability, inferred by the change in chemical composition, was verified by a large decrease in water contact angles as compared with those of untreated surfaces. Prior to this study, Youxian et al. (1991), using air, oxygen, argon, and water vapor plasmas, suggested a pathway by which

plasma treated PTFE surfaces are made more polar and given higher surface energies. Youxian et al. (1991) reported a significant decrease in both water and dimethyl iodide contact angles compared with untreated surfaces. Also, XPS revealed a decreased fluorine-to-carbon (F/C) ratio, an increased neutral carbon signal, and insignificant oxygen uptake. From these findings a specific pathway for plasma modification of PTFE has been suggested: breakage of C-F bonds by electron impact dissociation resulting in fluorine abstraction from surface followed by either chain scission, reaction with other radicals on polymer surface, or reaction with other species in plasma environment. Combination of carbon-centered radicals is preferred, which results in crosslinking if radicals are on adjacent chains and carbon double bonds if radicals are neighbors on the same chain. All other studies reviewed further verified that gaseous plasma modification resulted in polytetrafluoroethylene surfaces that were increasingly hydrophilic and, hence, more wettable (Sipehia et al., 1993; Yamada et al., 1996; Kang et al., 1996; Sipehia et al., 1996; Badey et al., 1996).

Photochemical Modification

Not only highly reactive radicals contained in generated gas plasma, but also photons can be used to improve the wettability of polymers by changing the morphology and chemical properties of the polymer surface (Zhang et al., 1994). The energy contained within the photons induces changes in bond chemistry in the exposed polymer. Intense radiation via pulsed excimer ultraviolet (UV) lasers and incoherent excimer UV lamps (Zhang et al., 1994; Esrom et al., 1995; Kang et al., 1996; Szymankiewicz et al., 1997)

have been used as a source of photons for surface modification in numerous applications and means by which photochemical modification takes place. Etching of polymers is also widely discussed which involves the removal of a thin layer of the polymer surface. The removal of thin layers of organic polymers with intense radiation is known as ablative photodecomposition (APD). Photochemical etching is useful in optics, microelectronics, etc., but also in determining the thickness of the modified surface layer. For example, Dekker et al. (1992) found that after etching a 1 nm layer from oxygen plasma-treated surfaces and analyzing with XPS, 20% of incorporated nitrogen and oxygen atoms were still present in the surface.

Photochemical surface modification and dry etching of polymethylmethacrylate (PMMA), polyimide (PI), polyethylene terephthalate (PET), and polytetrafluoroethylene (PTFE) is documented utilizing both excimer UV lasers (coherent particle beams) and excimer UV lamps (incoherent particle beams) and the mechanisms by which these surfaces are modified were disclosed based on SEM, mass spectrometry (MS), and XPS (Zhang et al., 1994). When using excimer lasers, the incident energy must exceed a certain threshold level, or fluence, for ablation to occur. This fluence is dependent on the polymer's absorption coefficient and, in turn, the wavelength of the UV radiation. Esrom et al. (1995) found this value to be 1.4 J/cm2 for PTFE. Because of the relatively small cross-section of the excimer laser beams, efficient treatment is limited to small surface areas ($< cm^2$). Etch rate saturation occurs with increasing laser intensity, but an etch depth as high as several micrometers can be achieved. The morphology of the etched region reveals that a significant thermal component is involved in the process (Zhang et

al., 1994). Irradiation of PTFE (undoped) surfaces using standard excimer lasers (193, 248, and 308 nm) results in poor surface treatment exhibited by cracks, bubbles, and disruptions. This is because PTFE absorbs weakly in the UV spectral region (170-300 nm) and hence, experiences a high thermal load (Esrom et al., 1995). Because the incorporation of dopants increases the absorbency of a material, expanded polytetrafluoroethylene (ePTFE) may undergo modification by excimer UV lasers more successfully.

Zhang et al. (1994) also noted several observations concerning the etching of polymers using incoherent excimer lamps. Contrary to the wavelength-dependent threshold fluence observed using lasers, no threshold was found using lamps. Etch depth was found to depend linearly on UV intensity; and the morphology of the etched region showed no indications of a thermal component as opposed to the strong indication observed in the laser experiments. The etching of polymers using excimer UV lamps seems to be photolytic in nature (Zhang et al., 1994). Therefore, given that surface modification and area-selective dry etching of polymers using excimer lasers is established, such surface modifications are also possible with novel incoherent excimer UV sources, and the absence of threshold fluences, area restrictions, and thermal components as well as the increased cost and maintenance of laser technology makes the use of lamps particularly desirable in certain applications and with certain materials, possibly including PTFE.

Examining the effects of UV irradiation via excimer lamps on PTFE in particular, it was found that at a wavelength of 222 nm and an intensity of 40 mW/cm^2 for 40 minutes

exposure time at 1 cm away and a background pressure of 100 mbar air, the surface exhibited a continuous microroughness, through a photooxidation process, with a mean peak-to-valley distance of about 500 nm. Microroughness is dependent on exposure time, VUV/UV intensity, and the distance between sample and excimer lamp (Esrom et al., 1995). Surface microroughness is an important parameter in obtaining good adhesion of electrolessly deposited metals (Zhang et al., 1994; Esrom et al., 1995). Its role in facilitating good adhesion of biological species such as endothelial cells and proteins is sought. Expanded PTFE graft material, exposed to VUV radiation for 30 minutes using an excimer lamp, showed increased initial adhesion of fibrin glue (composed of human fibrinogen and bovine α-thrombin) to the modified ePTFE surface (Szymankiewicz et al., 1997). This may suggest a certain improvement in adhesion of biological components to UV/VUV modified ePTFE.

Thus, surface modification of polymers is accomplished with gaseous plasma modification and photochemical modification. In an effort to provide a thorough summary, it should also be noted that these techniques have been performed on polymers in succession. Kang et al. (1996) subjected argon plasma-treated PTFE films to further surface modification with UV light and that the additional modification provided resistance to the gradual deterioration of the surface reactivity they observed after gas plasma treatment alone.

CHAPTER 3

Materials and Methods

Experimental Procedure

Naive human umbilical vein endothelial cells (HUVEC) were labeled with tritiated thymidine (^{3}H-thy) and seeded onto fibronectin-coated VUV surface modified expanded polytetrafluoroethylene (ePTFE) vascular graft pledgets and incubated for 1 hour to examine the effect of VUV modification on static endothelial cell (EC) adhesion. In order to elucidate the effect of VUV modification on EC proliferation, unlabeled HUVEC were allowed to adhere to ePTFE graft pledgets for a period of 18 hours at which time ^{3}H-thy was introduced and incubated for an additional 52 hours. At the end of each experiment, the graft pledgets were washed and analyzed by liquid scintillation counting. Seeding unmodified pledgets in a identical manner provided controls. The thymidine incorporated by cells on the modified ePTFE pledgets were compared to incorporation of thymidine by the cells on the unmodified pledgets to determine the percentage of attached cells after the pledgets were washed. Scanning electron microscopy (SEM) was done to study cell and graft morphology. Analysis of variance (ANOVA) were performed on both adhesion and proliferation data and statistical significance was assumed for $P < 0.05$. Detailed descriptions of the materials and methods are following.

Cell Culture

All cell culture was performed under aseptic conditions in a biological safety cabinet. Human umbilical vein endothelial cells (HUVEC) (Cat. # 1730-CRL, American Type Culture Collection, Rockville, MD) at passage 30 were thawed from liquid nitrogen by suspending them in a water bath at about 40°C. After about 30 seconds, the liquid vial contents were transferred to a T-75 tissue culture flask under sterile conditions. Ten ml of Endothelial Cell Growth Medium (EGM) (Cat. # CC-3024, Clonetics, Walkersville, MD) were added to the flask slowly in order to prevent osmotic shock. The flask was then labeled with cell-type, passage number, and date, and placed in an incubator at 37°C and 5% carbon dioxide. The following day, the flask was removed from the incubator and media in the flask was changed to remove the dimethyl sulfoxide (DMSO). The old media was aspirated from the flask and 10 ml EGM was added to the flask using a 10 ml pipette under sterile conditions. The flask was returned to the incubator. Cell growth was monitored by light microscope each day. Three days after thawing the HUVEC, when cells were nearly confluent, they were passaged. EGM was aspirated from the flask and 5 ml Hepes Buffered Saline Solution (Cat. # CC-5022, Clonetics, Walkersville, MD) was added to wash the cells. The saline solution was then aspirated and 5 ml Trypsin/EDTA (Cat. # CC-5012, Clonetics, Walkersville, MD) were added to cleave the cells from the polystyrene surface of the tissue culture flask. After 5 minutes, which was long enough for the cells to detach from the surface, 5 ml Trypsin Neutralizing Solution

(TNS) (Cat. # CC-5002, Clonetics, Walkersville, MD) were added to the flask to neutralize the trypsin/EDTA. Contents of the flask were pipetted into a 15 ml conical tube using a 10 ml pipette and centrifuged at 387 g for 4 minutes. Next, the supernatant was aspirated and the pellet was resuspended in 2 ml EGM. One ml was pipetted into each of two sterile T-75 flasks. Nine ml of EGM was added to each flask. Flasks were labeled and placed in the incubator.

Graft Modification

Eight-millimeter ID ePTFE graft material having an internodal distance of 30 μm (I.D. # 1196-16B,W.L. Gore & Associates, Inc., Flagstaff, AZ) was cut longitudinally and then cut into 9 segments, exposing an inner surface of the graft material of about 14 cm^2. The inner surfaces were irradiated with ultraviolet light using a vacuum excimer lamp (Figure 3.1). Graft modification was performed by Mr. Chris Buck, Materials Science

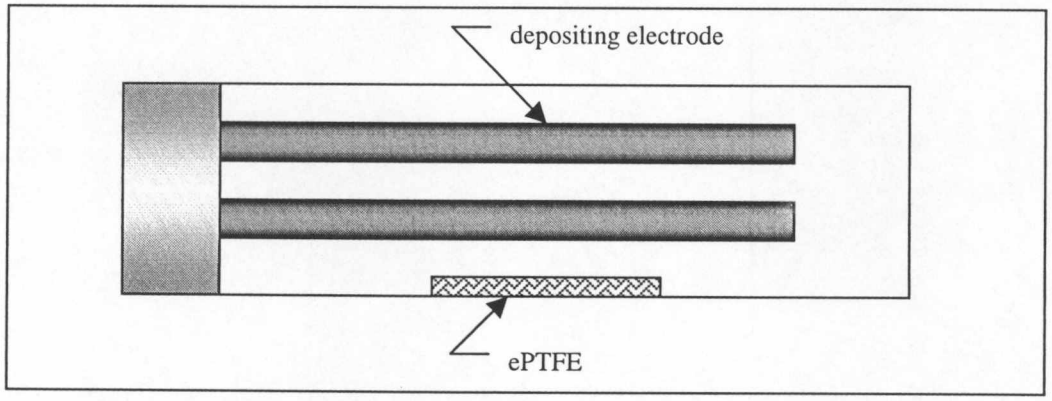


Figure 3.1. Diagram of Vacuum Excimer Lamp. Note electrodes and piece of ePTFE graft material.

and Engineering Department, University of Tennessee, Knoxville. Three graft pieces were exposed for 10, 20, and 40 minutes at 10 W, 3 more for 10, 20, and 40 minutes at 20 W, and 3 for 10, 20, and 40 minutes at 40 W. Irradiation was done at a frequency of 260 - 275 kHz and a pressure of 100 mbars in an argon gas environment. Each sample was placed in the vacuum chamber of the VUV excimer lamp at distance of approximately 3 cm from the depositing electrode. Both electrodes could be rotated with respect to the sample. Previous VUV modification work had shown that when the depositing electrode was positioned 120° from the sample, a balance was achieved between silica deposition and UV exposure.

Sterilization and Pledget Handling

Because the adhesion and proliferation experiments were conducted at a separate time, the modified ePTFE was divided into 2 sets prior to sterilization. The modified graft pieces were cut into two pieces and graft material that had not been irradiated was cut into 6 pieces in order to allow the availability of unmodified and modified graft material for both the adhesion and proliferation experiments. Each piece of ePTFE was packaged separately in sterilization packages and were sterilized by autoclaving at 120°C and a pressure of 15 psi for 20 minutes. Under sterile conditions, circular pledgets were punched from a sterilized control graft piece and from each of the modified graft pieces using a sterile hole punch and glued in a systematic arrangement in the central 32 wells of a sterile 96-well dish (Cat. # 25860, Corning Glass Works, Corning, NY) with sterile

Nexaband Quick Seal (Cat. # 92105, Veterinary Products Laboratory, Phoenix, AZ), a topical quick sealing tissue adhesive (formulated cyanoacrylate). These pledgets were glued in the dish luminal surface up.

Fibronectin Coating

One milligram of lyophilized human plasma fibronectin (Cat. # 40008, Becton Dickinson Labware, Bedford, MD) was removed from the -70°C freezer and allowed to come to room temperature in the sterile hood for approximately 1 hour. The fibronectin was reconstituted by adding 1 ml of sterile water at room temperature to the fibronectin vial using a 5 ml syringe. The fibronectin vial containing 1 ml of water was then gently agitated back and forth between palms to mix well and allowed to sit for 30 minutes at room temperature. The reconstituted fibronectin was added to 49 ml of HBSS, resulting in a fibronectin concentration of 20 μg/ml. Using a multi-channel pipetter, 100 μl of fibronectin solution was added to each of 16 wells containing ePTFE pledgets. To the other 16 wells containing pledgets, 100 μl of HBSS was added. To prevent evaporation from the dish, 100 μl of normal saline were added to the remaining 64 wells surrounding the 32 pledget-containing wells. The cover was then placed on the 96-well dish which was incubated at 37°C for 90 minutes. The fibronectin solution and HBSS were then aspirated and the pledgets were washed with HBSS.

Static Adhesion

Two days after the cells were passaged, the two T-75 flasks were removed from the incubator and placed in the sterile hood. Tritiated thymidine (Cat. # TRA120 B377, Amersham Lifescience, Arlington Heights, IL) was taken from the refrigerator and placed in the sterile hood. Isopropyl alcohol (70%) was used to clean the top of the ^{3}H-thy vial. A 1 ml syringe was used to retrieve 0.01 ml of ^{3}H-thy and inject into a 15 ml conical tube containing 10 ml EGM, yielding a concentration of 1 μCi/ml. The media in one T-75 flask was aspirated and the prepared EGM/ ^{3}H-thy solution was added. The flask was labeled with "Radioactive" tape, cell-type, passage number, date, and initials, and placed in the incubator. This process was repeated for the second T-75 flask.

Once cells had been labeled with ^{3}H-thy for 48 hours, they were removed from the incubator and placed in the hood. The media in the flask was aspirated into a 15 ml conical tube and disposed of as radioactive waste. The cells were trypsinized and centrifuged as described previously. The supernatant was aspirated and the pellet was resuspended in 1 ml of EGM. Next, 500 μl of Hank's Balanced Salt Solution (HBSS) (Cat. # 14175-079, GibcoBRL, Grand Island, NY), 475 μl of 0.4% Trypan Blue Dye (Cat. # T-8154, Sigma Cell Culture, St. Louis, MO), and 25 μl of cell suspension were added to a 2 ml tube, resulting in a 1:40 dilution. Trypan Blue Dye has a greater affinity for dead cells than for viable cells. Since dead cells readily take up the dye, counting the cells that remain clear gives the experimenter an accurate viability. The 2 ml tube was gently vortexed and approximately 12 μl were placed on each grid of a hemocytometer. The

cells were counted to determine the number of cells per milliliter. The volume for 8,000 cells was then determined. The cell suspension volume was increased to 5 ml by adding 4 ml of EGM. The volume for 8,000 cells was then determined by the equation:

$$y = (4x10^7)\ x$$

where x = number of cells per milliliter and y = the volume (µl) containing 8,000 cells. Approximately 80 µl of EGM were added to each of the 32 wells using a multi-channel pipetter. Next, the determined volume containing 8,000 cells was pipetted into each well. The dish was again covered and returned to the incubator for 60 minutes at 37°C. To allow for less intensive harvesting of pledgets, the static adhesion experiment was divided into three parts, corresponding to the seeding of ePTFE pledgets exposed to 10, 20, and 40 W VUV, respectively. Each part of this experiment was performed on a different day with its own control.

Cell Proliferation

The second set of modified and control ePTFE pledgets were sterilized and coated with fibronectin as previously described. Unlabeled HUVEC cultured as described previously were removed from the incubator and placed in the hood. The cells were then harvested and counted as discussed above. Approximately 80 µl of EGM were then added to each of 32 wells in each of three 96-well dishes containing ePTFE pledgets.

Next, the calculated volume, y, containing 8,000 cells was pipetted into each well. The three dishes were covered and incubated at 37°C for 18 hours to allow the cells to adhere to the ePTFE graft pledgets. Next, each dish was returned to the hood and the media in each of the 32 wells was aspirated and replaced by 100 µl of EGM containing 1 µCi/ml of ^{3}H-thy. The dishes were incubated for 52 hours at 37°C.

Liquid Scintillation Counting

For both the static adhesion and cell proliferation assays, liquid scintillation counting was utilized as a means of measuring radioactivity incorporated by the cells. Once experiments were completed, the dishes were returned to the hood and the contents of each well were pipetted into separate scintillation vials each containing 3 ml of Cytoscint scintillation fluid (Cat. # 882453, ICN Pharmaceuticals Inc., Costa Mesa, CA). Each well was washed with 100 µl of phosphate buffered saline (PBS) twice and added to the respective vials. Pledgets were transferred to separate scintillation vials containing 3 ml of Cytoscint using sterile forceps. All vials were uniformly mixed by shaking and were placed sequentially in liquid scintillation β-counter cartridges. All vials were then analyzed in a 1900 TR Tri-Carb Liquid Scintillation Analyzer (Packard, Meriden, CT), which output the decay per minute (DPM) exhibited by the radioactivity within the cells. The β-counter analyzed all vials for a time period of 1 minute.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed on pledgets that were seeded and allowed to proliferate for a period of 4 days. An additional 96-well dish was prepared at the time of the cell proliferation experiment which contained 2 pledgets from unmodified graft material and 2 pledgets from each modified graft piece. These pledgets were sterilized and fibronectin coated in the manner described and seeded with unlabeled HUVEC. After 18 hours of incubation at 37°C the media was aspirated and fresh EGM was added. The pledgets were incubated for 3 additional days, at which time the media in each well was again aspirated and replaced with 200 µl of a cacodylate buffer, made from cacodylic acid (Cat. # C-0125, Sigma Chemical Co., St. Louis, MO), with 2.5% glutaraldehyde (Cat. # 16310, Electron Microscopy Sciences, Ft. Washington, PA) and stored at 4°C. This solution fixed the cells to the ePTFE graft pledgets. After 24 hours, the cacodylate/glutaraldehyde solution was aspirated and replaced with 200 µl of cacodylate buffer. Recipes for these buffer solutions are included in the appendix. The dish was covered, labeled and stored at 4°C.

The samples were then dehydrated by successive ethanol treatments of increasing concentrations. After critical point drying in carbon dioxide to remove the ethanol, the samples were mounted, and coated with silver. Photographs were made using an electron beam microscope. A representative picture was sought but attention was also given to certain features that may have provided useful information. Mr. Dick Williams, Division

of Biology, University of Tennessee, Knoxville, performed all SEM preparation and supervised all photography.

Statistical Analysis

An analysis of variance (ANOVA) was performed on the adhesion and proliferation data. For the static adhesion experiment, average percent cell adhesion was determined and a 2-way analysis of variance for each power level was done with a full factorial design having fibronectin and time as factors. Data exhibited a normal distribution and, thus, did not need to be transformed. For the cell proliferation experiment, average thymidine incorporation values were normalized by dividing through by the control values. A 3-way analysis of variance was done with a full factorial design having fibronectin, time, and power level as factors. A box-cox transformation was performed on the proliferation data because the data did not exhibit a normal distribution. JMP, a statistical analysis software package, was used. Statistical significance was assumed to be at a value $P < 0.05$.

CHAPTER 4

Results

Static Adhesion

VUV modification had no effect on cell adhesion for all radiation intensities studied. Adhesion varied more with the day on which the experiment was conducted than with UV radiation power or exposure time. Figure 4.1 shows cell adhesion on uncoated or fibronectin-coated pledgets modified at a power level of 10 W and its uncoated or fibronectin-coated control. Similar graphs for the 20 W and 40 W experiments are shown in Figures 4.2 and 4.3, respectively. Percentages shown are the averages of four replicate samples. The error bars denote the standard error of the mean. Unmodified pledgets served as controls and exhibited 49, 27, and 37% adherence with fibronectin and 44, 37, and 29% adherence without fibronectin for 10, 20, and 40 W, respectively. The largest increase in adhesion of modified ePTFE over controls was 7% after 40 minutes at 40 W without fibronectin. Adhesion was significantly improved in the presence of fibronectin independent of VUV modification.

Cell Proliferation

VUV modification had no significant effect on cell proliferation whether or not Fn was present on the ePTFE surface. Figure 4.4 shows thymidine incorporation by HUVEC

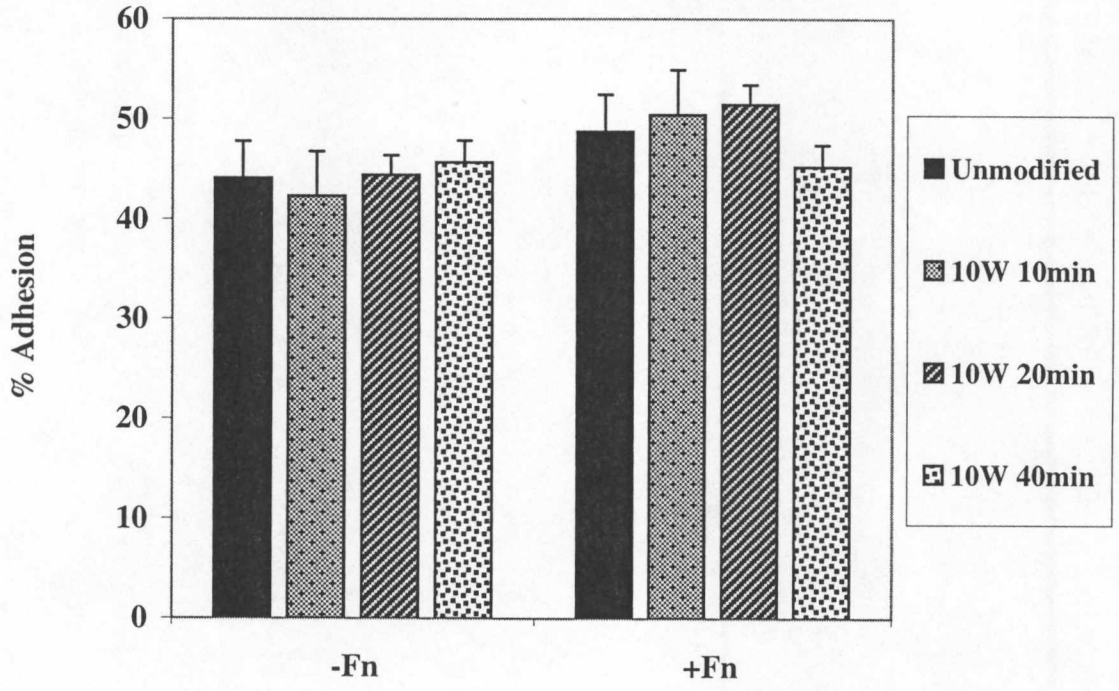


Figure 4.1. Cell Adhesion on 10 W VUV Modified ePTFE without Fibronectin (-Fn) and with Fibronectin (+Fn).

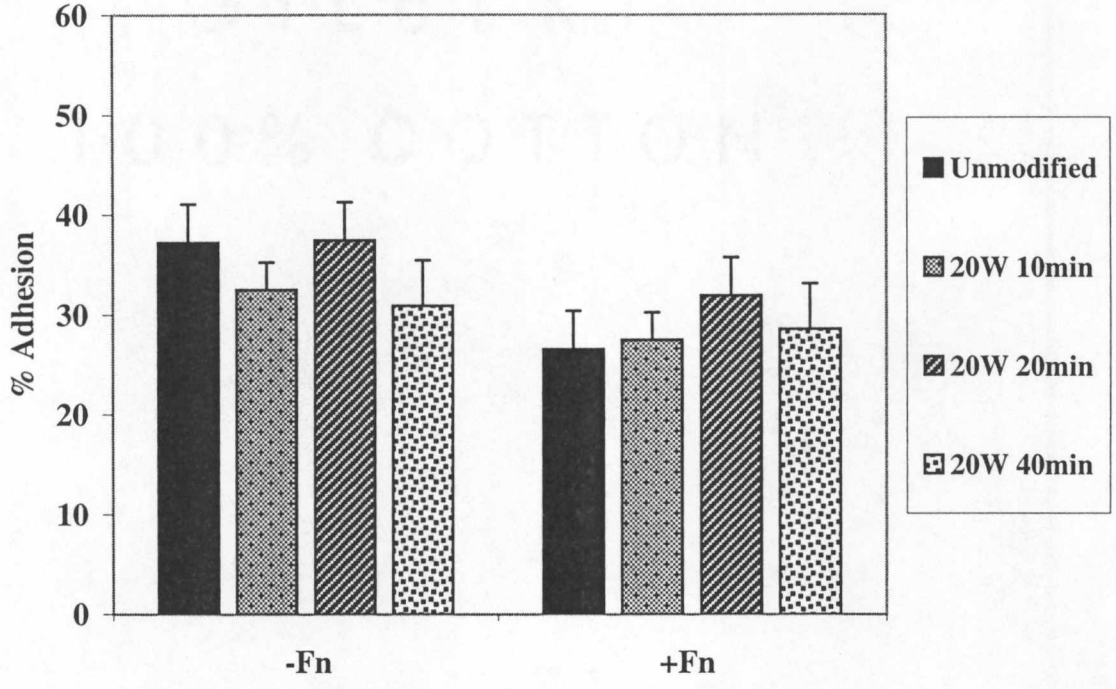


Figure 4.2. Cell Adhesion on 20 W VUV Modified ePTFE without Fibronectin (-Fn) and with Fibronectin (+Fn).

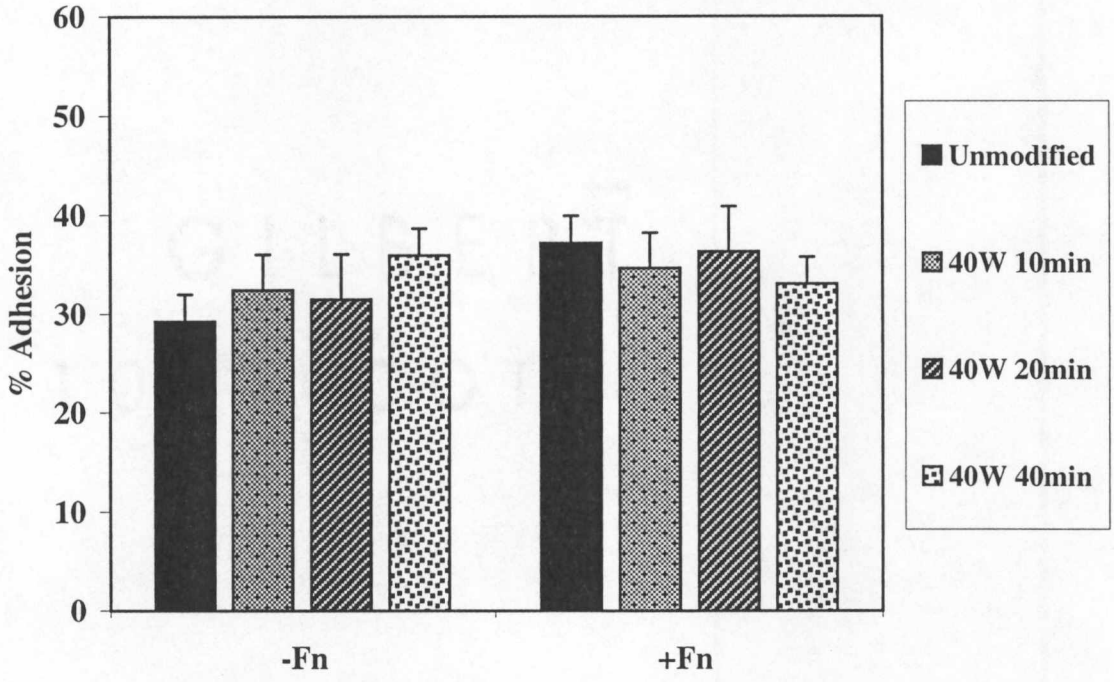


Figure 4.3. Cell Adhesion on 40 W VUV Modified ePTFE without Fibronectin (-Fn) and with Fibronectin (+Fn).

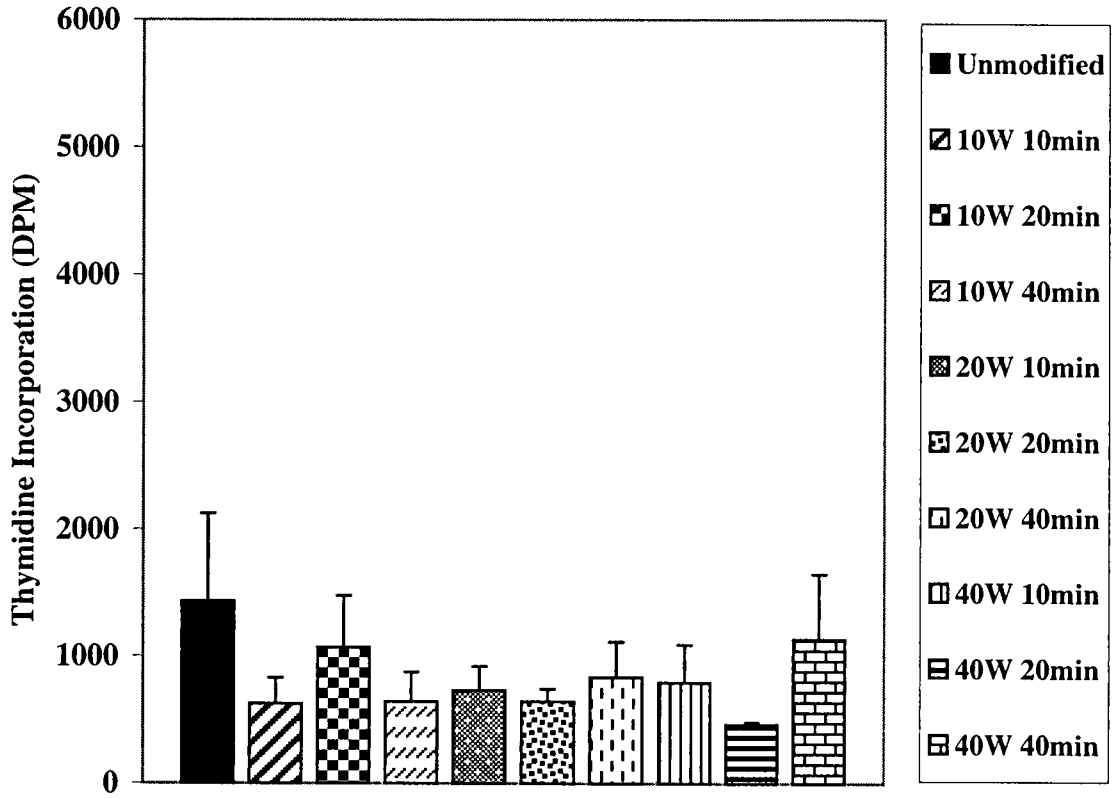


Figure 4.4. Average Thymidine Incorporation by HUVEC on Uncoated ePTFE.

on uncoated ePTFE modified at each power level and exposure time. Incorporation values are averages of four replicate samples (n=4).

For cell proliferation in the presence of Fn it appears that several modifications exhibited greater proliferation rates relative to unmodified pledgets, but these results were not statistically significant. Thymidine incorporation by HUVEC on fibronectin-coated ePTFE at each modification is shown in Figure 4.5. Expanded PTFE modified for 40 minutes at 20 watts and 10 minutes at 40 watts in the presence of Fn had incorporation values of 2712 and 3913 DPM, respectively, compared to 1193 DPM for unmodified ePTFE with Fn. The error bars denote standard error of the mean for the appropriate modification. Cell proliferation was also significantly improved when ePTFE was coated with fibronectin regardless of the extent of VUV modification.

Scanning Electron Microscopy

The surfaces of endothelial cell seeded ePTFE graft pledgets evaluated at 4 days by scanning electron microscopy demonstrated sparse endothelial cell coverage on all samples studied. Cells exhibited a spherical shape while adhered to both unmodified and VUV modified ePTFE graft material (Figures 4.6, 4.7, and 4.8). Endothelial cells seeded on ePTFE modified at 40 W for 40 minutes, however, appeared to be more discoid-shaped (Figure 4.9). Although cell proliferation results indicate improved proliferation on fibronectin-coated ePTFE graft material as compared to uncoated ePTFE, independent

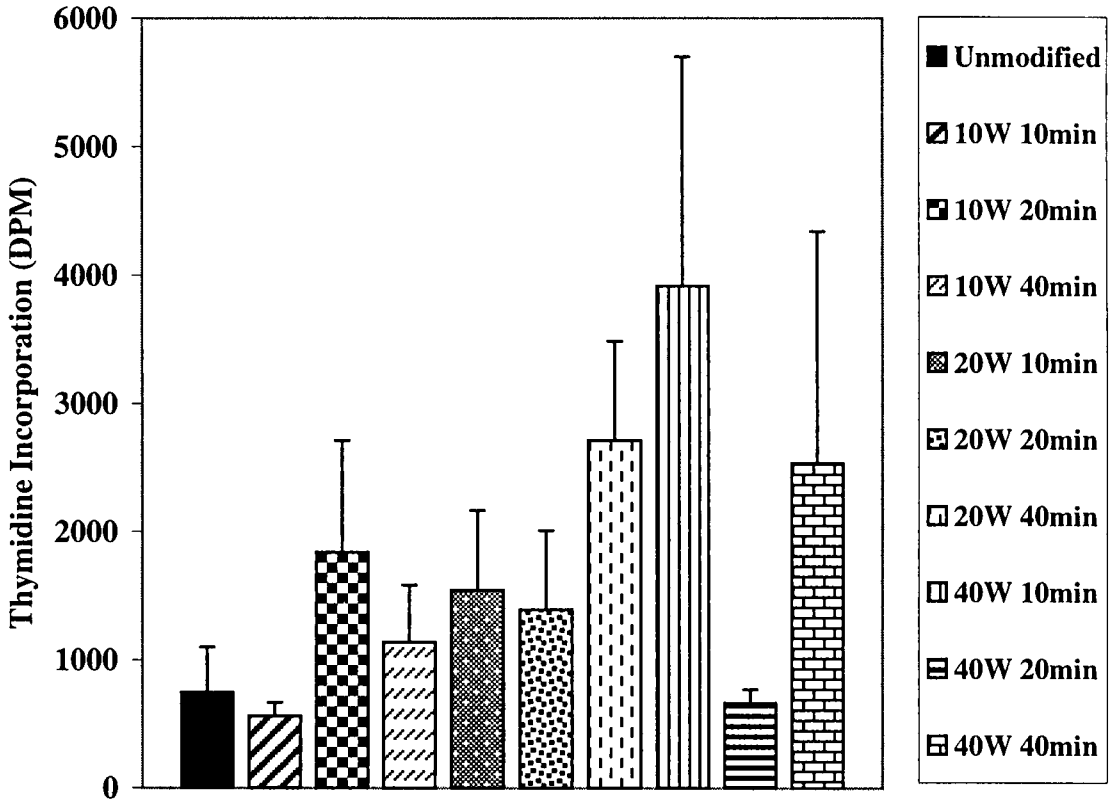


Figure 4.5. Average Thymidine Incorporation by HUVEC on ePTFE Coated with Fibronectin.

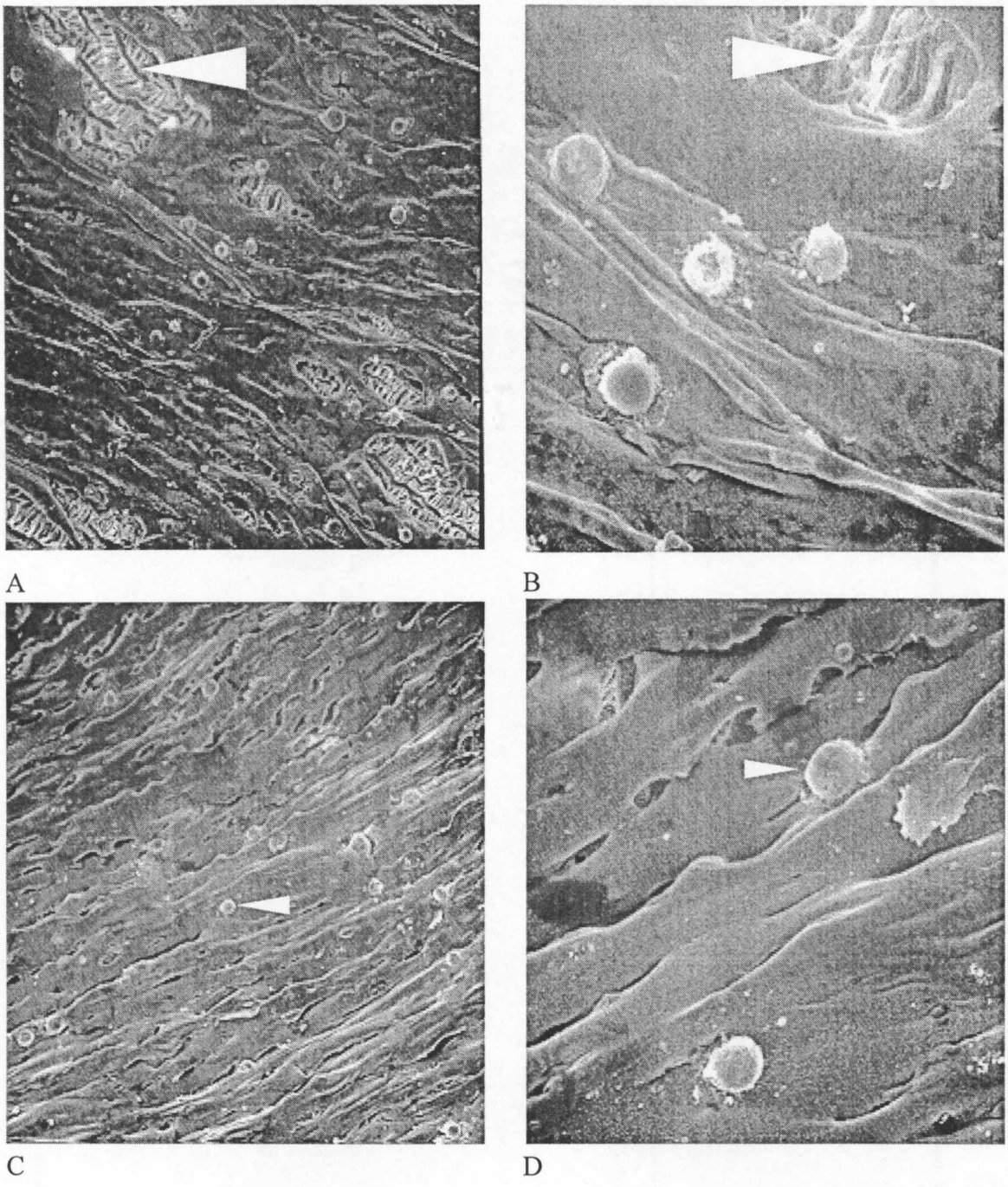


Figure 4.6. Scanning electron micrographs of unmodified ePTFE graft pledgets seeded with HUVEC at 4 days. A. without fibronectin, original magnification 250X; B. without fibronectin, original magnification 1000X; C. with fibronectin, original magnification 250X; D. with fibronectin, original magnification 1000X; large arrows-ePTFE, small arrows-cells.

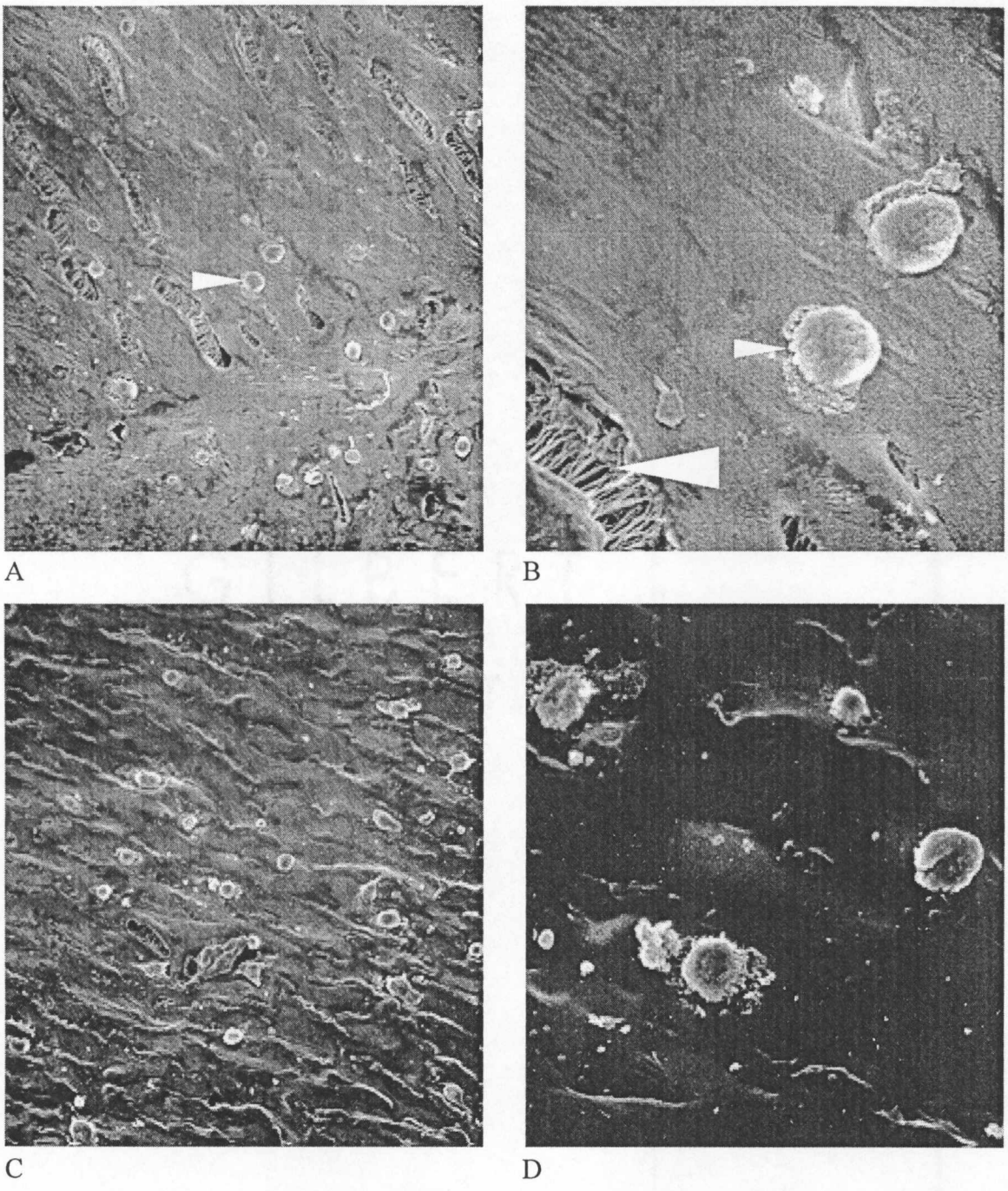


Figure 4.7. Scanning electron micrographs of ePTFE graft pledgets modified at 10 W for 10 minutes and seeded with HUVEC at 4 days. A. without fibronectin, original magnification 250X; B. without fibronectin, original magnification 1000X; C. with fibronectin, original magnification 250X; D. with fibronectin, original magnification 1000X; large arrow-fibrils, small arrows-cells.

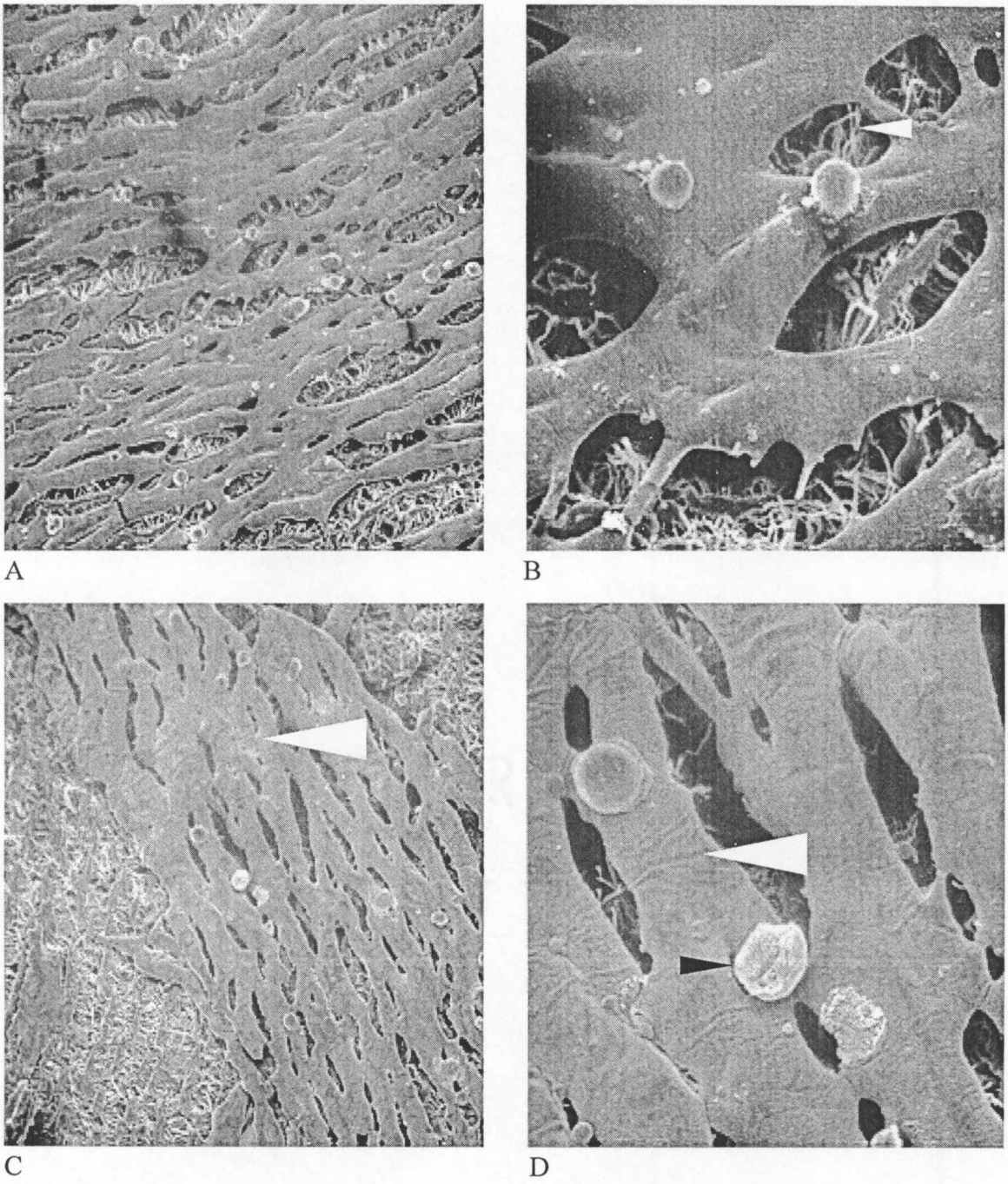


Figure 4.8. Scanning electron micrographs of ePTFE graft pledgets modified at 40 W for 10 minutes and seeded with HUVEC at 4 days. A. without fibronectin, original magnification 250X; B. without fibronectin, original magnification 1000X; C. with fibronectin, original magnification 250X; D. with fibronectin, original magnification 1000X; large arrows-unknown substance, small arrow-fibrils, solid arrow-cell.

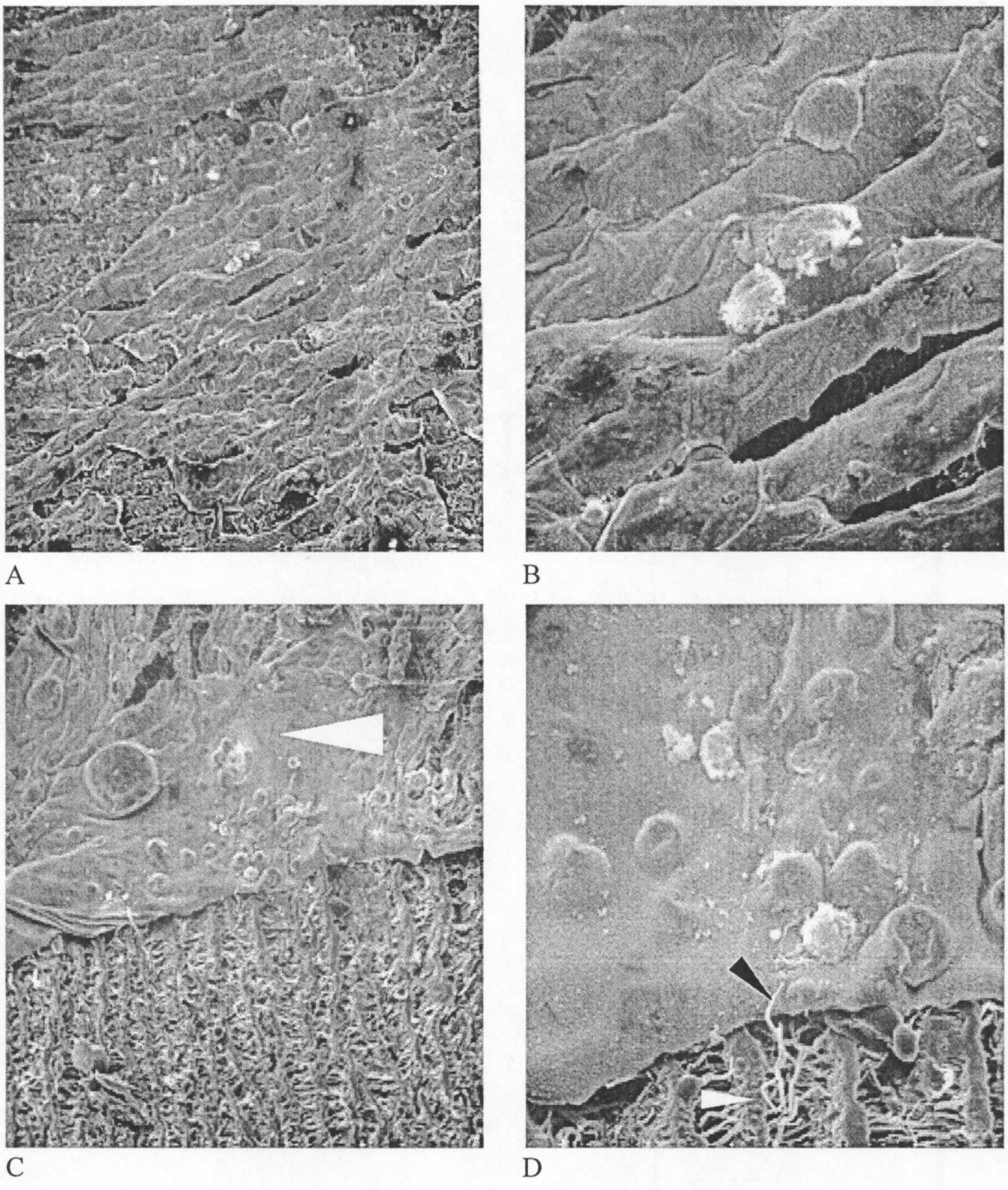


Figure 4.9. Scanning electron micrographs of ePTFE graft pledgets modified at 40 W for 40 minutes and seeded with HUVEC at 4 days. A. without fibronectin, original magnification 250X; B. without fibronectin, original magnification 1000X; C. with fibronectin, original magnification 250X; D. with fibronectin, original magnification 1000X; large arrow-unknown substance, small arrow-node, solid arrow-fibrils.

of VUV modification, this was not observed with scanning electron microscopy (Figures 4.6, 4.7, 4.8, and 4.9).

The morphology of the unmodified ePTFE graft material can be seen in several exposed areas (Figure 4.6 A). Note that the fibrils spanning the ePTFE nodes can be observed in several exposed areas on ePTFE modified at 10 W for 10 minutes (Figure 4.7 A, B). These fibrils are much more apparent in ePTFE modified at 40 W (Figures 4.8 and 4.9). Note the distinct change in graft morphology as compared to unmodified ePTFE. Many fibrils appear to be disconnected from their nodes (Figures 4.8 B and 4.9 D).

In addition to the ePTFE graft material and the adherent endothelial cells, another substance is obviously present and seems to cover much of the ePTFE. In Figures 4.6, 4.7, and 4.8, this substance is a fairly confluent layer having a wavy texture. In Figure 4.8, however, this third element has a networked texture and does not coincide with the nodal direction of the ePTFE graft material (Figure 4.8 C).

In an effort to determine the identity of the substance observed in the cell proliferation experiment scanning electron micrographs, unmodified and VUV modified ePTFE graft pledgets exposed to 4 different conditions for 4 days were evaluated by scanning electron microscopy. Graft pledgets incubated for 4 days in media containing no serum are shown in Figure 4.10. Note the presence of a substance layer covering areas of ePTFE (Figure 4.10 A). A similar layer was observed on fibronectin-coated ePTFE in media containing no serum (Figure 4.11). On uncoated and fibronectin-coated ePTFE incubated in media containing 2% serum, a layer of substance is present also, perhaps, to

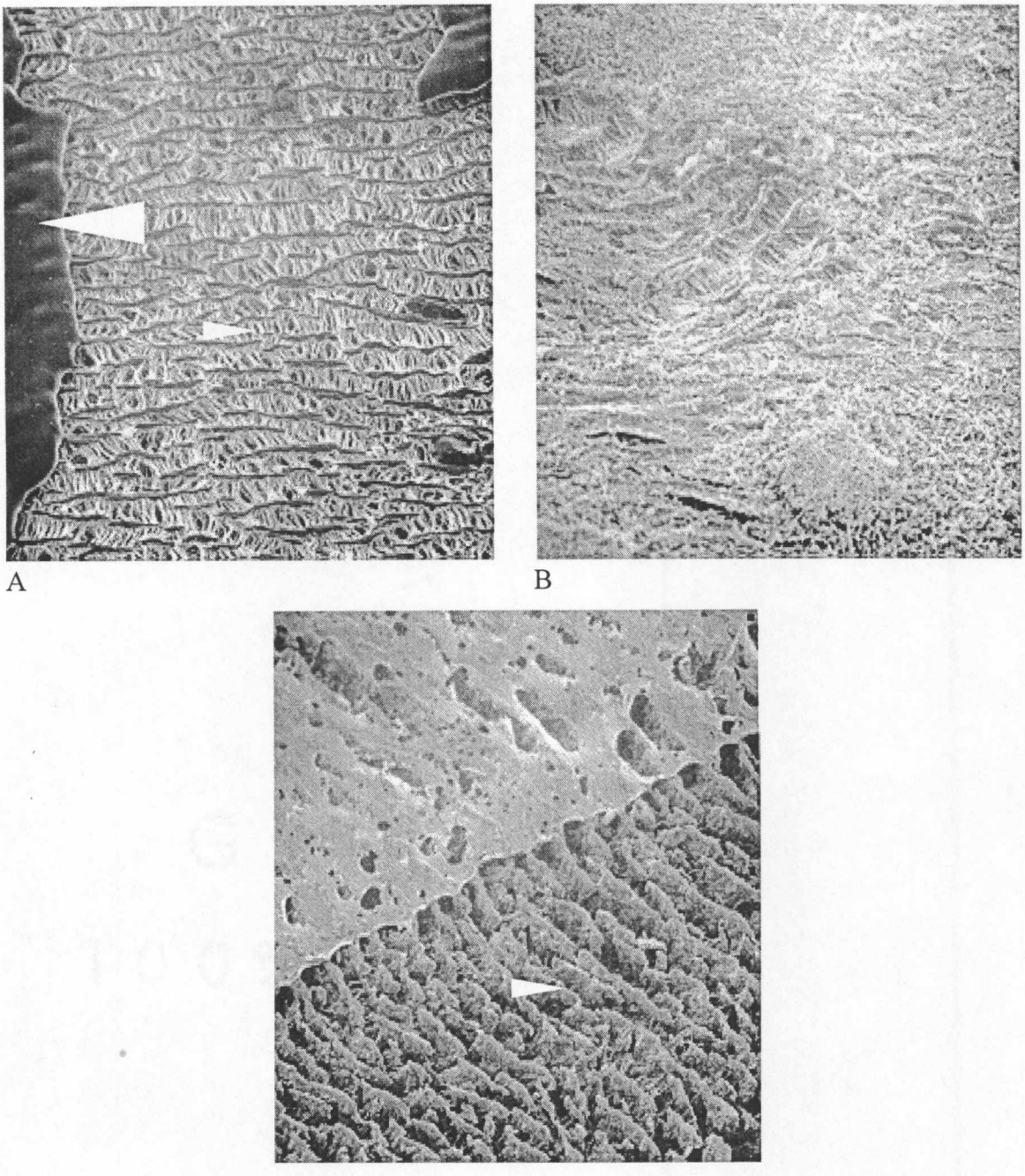


Figure 4.10. Scanning electron micrographs of uncoated ePTFE graft pledgets at 4 days incubation in media containing no serum. Original magnification 250X. A. unmodified; B. modified at 10 W for 10 minutes; C. modified at 40 W for 40 minutes; large arrow-unknown substance, small arrows-ePTFE.

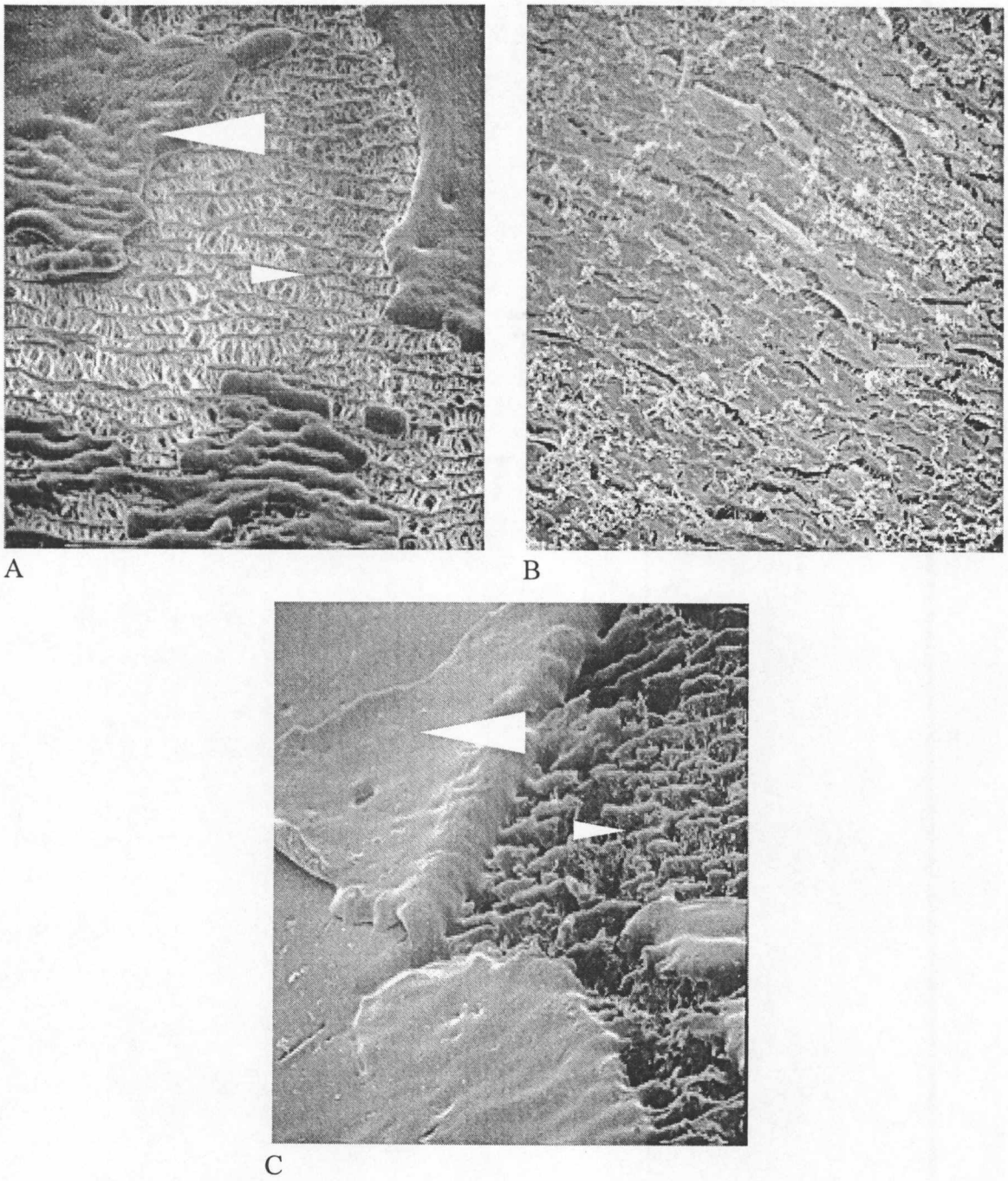


Figure 4.11. Scanning electron micrographs of fibronectin-coated ePTFE graft pledgets at 4 days incubation in media containing no serum. Original magnification 250X. A. unmodified; B. modified at 10 W for 10 minutes; C. modified at 40 W for 40 minutes; large arrows-unknown substance, small arrows-ePTFE.

an even greater extent (Figures 4.12 and 4.13). The wavy-textured layer seen in Figure 4.8 did not appear to be exhibited in figures 4.10, 4.11, 4.12, or 4.13.

Unmodified ePTFE graft material not exposed to the Nexaband adhesive (cyanoacrylate) was examined by scanning electron microscopy and demonstrated a clean graft surface and the absence of the layer observed in previous samples.

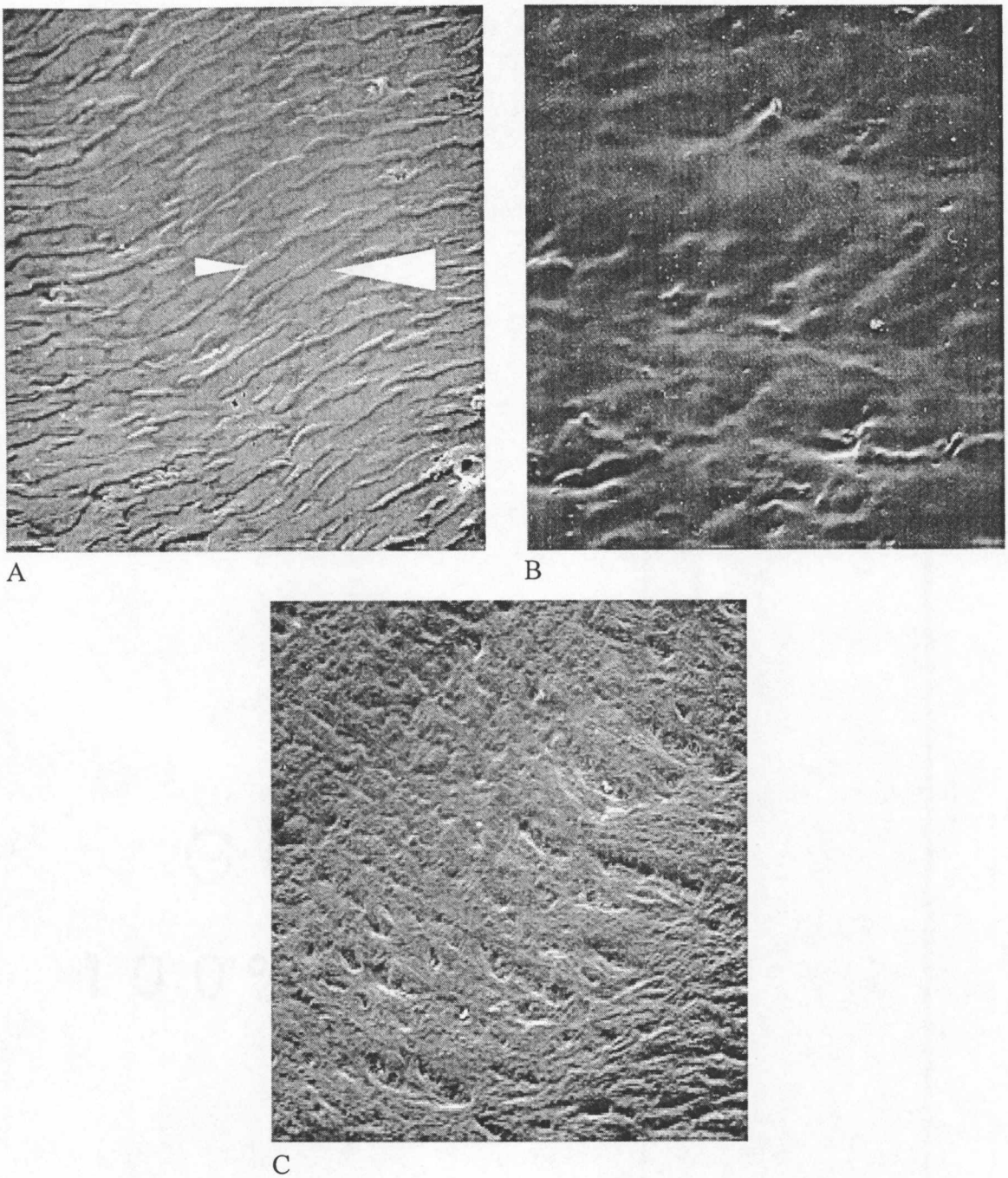


Figure 4.12. Scanning electron micrographs of uncoated ePTFE graft pledgets at 4 days incubation in media containing 2% calf serum. Original magnification 250X. A. unmodified; B. modified at 10 W for 10 minutes; C. modified at 40 W for 40 minutes; large arrow-unknown substance, small arrow-node.

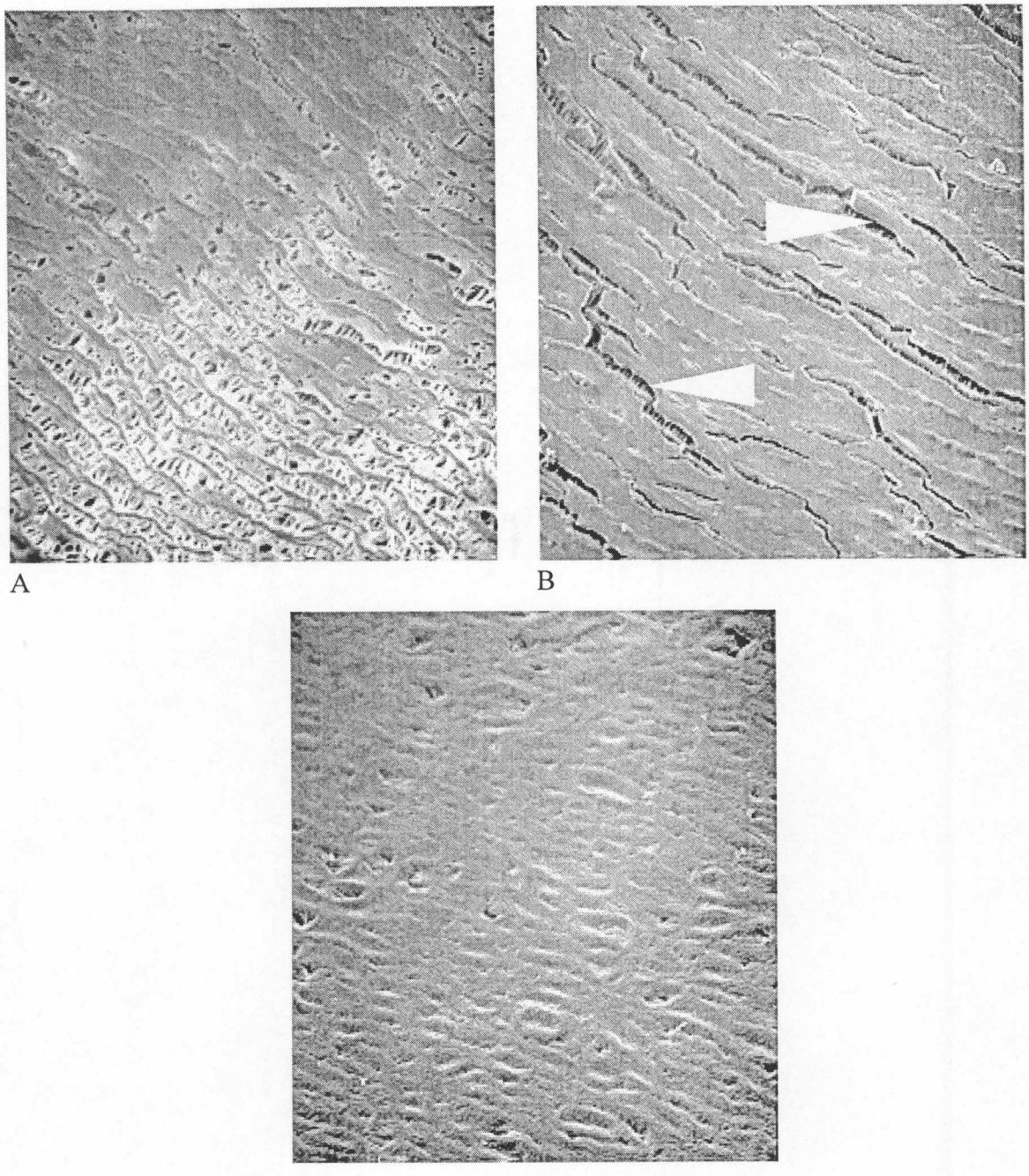


Figure 4.13. Scanning electron micrographs of fibronectin-coated ePTFE graft pledgets at 4 days incubation in media containing 2% calf serum. Original magnification 250X. A. unmodified; B. modified at 10 W for 10 minutes; C. modified at 40 W for 40 minutes; arrows-separations in unknown substance.

CHAPTER 5

Discussion

One of the greatest problems encountered in synthetic vascular graft research is the low wettability of the polymeric materials from which the grafts are manufactured. As previously discussed, the hydrophobicity of ePTFE is due to the low surface free energy and low polarizability of its C-F bonds. The different surface modification techniques described endeavor to substitute the fluorine atoms at the surface with functional groups that are less repelling than fluorine, thereby yielding a material that may better accommodate adhesion of biological components such as endothelial cells and the protein substrates used to facilitate adhesion and proliferation. Modification of any kind may be capable of altering the favorable mechanical and chemical properties that make ePTFE such a good biomaterial. However, because these modification techniques affect only a very thin layer of the surface, several nanometers maximum, the properties of the material as a whole remain uncompromised. With increasing radiation dose, VUV modification of fluoropolymers causes an increase in the surface free energy. This diminishes the strong water and oil repellency of PTFE in such a way that homogeneous incorporation of aqueous and organic liquids is possible (Lunkwitz et al., 1995).

Several investigators have reported increased adhesion of endothelial cells seeded onto PTFE films and ePTFE patches as a result of gaseous plasma surface modification (Dekker et al., 1992); however, improved adhesion and proliferation as a result of VUV

modification has not yet been documented. Despite the lack of information concerning the behavior of endothelial cells on VUV irradiated ePTFE, it has been reported that surface treatment of PTFE via UV excimer lamps renders more hydrophilic surfaces by changing the chemical composition at the polymer's surface (Zhang et al., 1994; Esrom et al., 1995; Kang et al., 1996). Although VUV modification has not yet been shown to improve EC adhesion or proliferation, VUV modification of ePTFE has been shown to significantly improve the initial adhesion of fibrin glue (Szymankiewicz et al., 1997). This is relevant because if VUV modification of ePTFE is found to increase subsequent adhesion of protein substrates such as fibrin glue or Fn, then it may also indirectly affect EC adhesion based on studies showing some protein substrates to facilitate EC adhesion and proliferation. Specifically, EC adhere better on Fn-coated ePTFE than on uncoated ePTFE (Kesler et al., 1986; Vohra et al., 1992).

In the present study, VUV surface modification of ePTFE had no effect on endothelial cell adhesion in the first 60 minutes following seeding for the parameters studied. There exists the possibility that a radiation intensity of 40 W is not significant enough to elicit a change in the way endothelial cells behave on ePTFE. Cell adhesion was improved with the Fn substrate at 20 μg/ml independent of VUV modification. This finding is supported by other studies confirming that Fn used as a substrate on ePTFE contributes to improved EC adhesion (Kesler et al., 1986; Budd et al., 1990; Vohra et al., 1992).

Pretreatment of ePTFE with Fn has been reported (Kesler et al., 1986; Budd et al., 1990; Vohra et al., 1992) to increase initial endothelial cell attachment.

Endothelialization is a slow process *in vivo* taking 6 weeks for ePTFE in the canine model (Budd et al., 1990) and even longer in humans (Schneider et al., 1988). Studies involving EC seeding have demonstrated that Fn facilitates EC proliferation on ePTFE vascular grafts (Greisler et al., 1990; Wigod and Kitzman, 1993). In the present study, VUV modification had no significant effect on cell proliferation for the parameters studied during the interval of 18-66 hours following EC seeding. Cell proliferation was improved by Fn independent of VUV modification.

Scanning electron microscopy evaluation of the cell proliferation experiment raised the question of the identity of a third component present on the surface of unmodified and VUV modified ePTFE. The first two components discussed were the endothelial cells and the ePTFE graft material. The third component, the substance present as a thick layer, may be the tissue adhesive (sterile cyanoacrylate) used to attach the ePTFE pledget to the tissue culture plastic in which the experiments were conducted. If this is, in fact, true, it must be reasoned that the cyanoacrylate seeped through the ePTFE graft matrix and remained on the surface of the pledget, possibly interfering with the graft-protein adhesion of the Fn-coating and endothelial cell seeding processes that followed.

It is not certain the effect that the cyanoacrylate had on the endothelial cell seeded ePTFE graft surface. Cyanide, a toxin containing the cyano functional group, has been found to block cell metabolism by interfering with ATP production, causing cell, tissue, organ, and system death. Studies completed in mice (Kreuter et al., 1995) and rabbits (Macsai et al., 1990) focused on the effects of cyanoacrylate injection into the brain.

Scanning electron microscopy revealed that injection of the adhesive produced a polymerized mass that remained throughout the course of the study (Macsai et al., 1990). An enlarging "cocoon" of fibrin and inflammatory cells surrounding the adhesive marked an active inflammatory response. As a result of the injected cyanoacrylate, the corneal endothelial cells became swollen for the first 14 days following injection (Macsai et al., 1990). Hence, cyanoacrylate has caused tissue reactions and adverse cell reactions eliciting foreign-body and inflammatory responses *in vivo*, and may have affected EC adhesion and proliferation in this study.

CHAPTER 6

Conclusions

The research reported herein includes the work necessary to study the effects of VUV surface modification of ePTFE vascular grafts on subsequent endothelial cell adhesion and proliferation. VUV modification was accomplished using a VUV excimer lamp at powers ranging from 10 to 40 watts and exposure times not exceeding 40 minutes. Following surface modification, *in vitro* biological evaluation consisting of static HUVEC adhesion and proliferation experiments were conducted. Cell adhesion was determined by labeling cells with ^{3}H-thy prior to graft seeding, while thymidine incorporation during the proliferation experiment was performed by introducing ^{3}H-thy to adhered HUVEC. For both experiments, radioactivity was measured using β-scintillation counting. The effects of the presence of fibronectin were included as a parameter in this study. Scanning electron microscopy was used to study cell morphology.

VUV modification had no significant effect on EC adhesion to or proliferation on ePTFE vascular graft material for the power levels and exposure times examined whether fibronectin was present or not. In the presence of fibronectin, cell proliferation on modified ePTFE appeared improved for certain parameters as compared to proliferation on unmodified ePTFE, but these results were not statistically significant. Cell adhesion and proliferation was significantly improved by the presence of fibronectin independent of VUV modification. Scanning electron microscopy revealed the presence of a thick

layer of unknown substance covering much of the graft surface, which was later hypothesized to be cyanoacrylate due to additional SEM examination on ePTFE not exposed to cyanoacrylate or serum protein. Limited differences were seen for cell morphology from one modified graft to another with respect to radiation intensity or exposure time. However, a distinct change in ePTFE morphology was observed with increasing radiation intensity.

Further studies of endothelial cell adhesion and proliferation on VUV modified ePTFE vascular grafts could render more encouraging results. A revised experimental method should be seriously considered before this work is repeated. A procedure of securing the ePTFE graft pledgets mechanically, as to eliminate the use of cyanoacrylate or any other substance which adds additional experimental variables to the study, should be mastered. Further examination should be undertaken to decipher the interactions of other proteins present on the ePTFE surface with fibronectin, including growth factors and serum proteins. Additional methods of quantitating increases in wettability via contact angle measurements and XPS may provide more definitive results as to the effects of VUV modification.

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APPENDIX

APPENDIX

Introduction

Several solutions needed for the cell adhesion and proliferation experiments and for subsequent fixation prior to SEM had to be prepared fresh at the time of usage. The recipes for these solutions are given in this appendix.

Buffer Solutions

Cacodylic Buffer

Recipe to make 500 ml of cacodylic buffer

1. Measure out 10.7 grams of cacodylic acid
2. Dilute to approx. 450 ml with distilled water
3. Bring pH to 7.23
4. Dilute to 500 ml with distilled water
5. Store at 5°C

Cacodylic Buffer with 2.5% Glutaraldehyde

Recipe to make 50 ml of cacodylic buffer with 2.5% glutaraldehyde

1. Add 2.5 ml of 50% glutaraldehyde solution to 50 ml of cacodylic buffer
2. Store at 5°C

Protein Substrate Solutions

Fibronectin Solution

Recipe to make 50 ml of fibronectin solution (20µg/ml)

1. Take 1 mg human plasma fibronectin from -70°C freezer and allow to thaw to room temp. for approx. 1 hour
2. Add 1 ml sterile water to Fn vial using 5 ml syringe
3. Rub vial back and forth in palms to mix well
4. Allow to sit for 30 minutes at room temperature
5. Add contents of vial to 49 ml of HBSS

VITA

Carey Edward Romoser was born in West Palm Beach, Florida on April 21, 1973. He attended public and private schools in the West Palm Beach area and in the Palm Beach County Public School System, where he graduated from Palm Beach Gardens Community High School in June, 1991. He entered the University of Tennessee, Knoxville, during August of 1991 where in May, 1995, he received the Bachelor of Science in Engineering Science with a concentration in Biomedical Engineering. He then entered graduate school in Engineering Science at the University of Tennessee, Knoxville in August of 1995, officially receiving the Master of Science degree in December, 1997. Carey is scheduled to begin work as an engineer associate for Pratt & Whitney Aircraft, a subsidiary of United Technologies Corp., in West Palm Beach, Florida, in December, 1997.