EFFECT OF BIO-MIMETIC MATRIX MOLECULES ON DIFFERENTIATION OF ENDOTHELIAL CELLS GROWN *IN VITRO* ON BIOMATERIALS FOR TISSUE ENGINEERING

A Thesis Presented

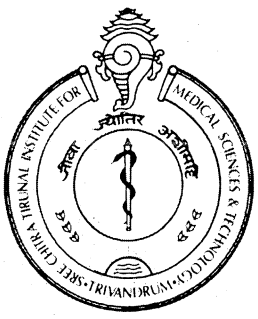
By

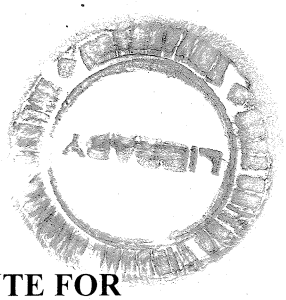
Krishna Prasad C

To

The Division of Thrombosis Research Unit

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy of





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April 2006

DECLARATION

I, Krishna Prasad C, hereby declare that I had personally carried out the work depicted in the thesis entitled **"EFFECT OF BIO-MIMETIC MATRIX MOLECULES ON DIFFERENTIATION OF ENDOTHELIAL CELLS GROWN IN *VITRO* ON BIOMATERIALS FOR TISSUE ENGINEERING"** under the direct supervision of Dr. Lissy K Krishnan, Scientist F & Leader, Thrombosis Research Unit, Biomedical Technology wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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This is to certify that Mr. Krishna Prasad C., in the division of Thrombosis Research Unit of this institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technolgy, Thiruvananthapuram. The work relating to his thesis entitled "EFFECT OF BIO-MIMETIC MATRIX MOLECULES ON DIFFERENTIATION OF ENDOTHELIAL CELLS GROWN *IN VITRO* ON BIOMATERIALS FOR TISSUE ENGINEERING" was carried out under my direct supervision.

27/04/06
Dr. Lissy K Krishnan

The Thesis

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ABBREVIATIONS

ACD	Acid Citrate Dextrose
AcLDL	Acetylated Low Density Lipoprotein
ADP	Adenosine Di Phosphate
AOE	Angina on Exertion
ASMI	Antero Septal Myocardial Infarction
ATP	Adenosine Tri Phosphate
BAEC	Bovine Aortic Endothelial Cell
bFGF	basic Fibroblast Growth Factor
CABG	Coronary Artery Bypass Grafting
CAD	Coronary Artery Disease
CC	Composite Coated
CCPS	Composite Coated Poly Styrene
COPD	Chronic Obstructive Pulmonary Disease
CPM	Count per Minute
DAB	Diamino Benzidine
Dacron	Polyethylene Terephthalate
DiI	1,1-dioctadecyl-3,3,3,3-tetramethyl Indocarbocyanine perchlorate
DLC	Diamond Like carbon
DLC_Ti	DLC coated Ti
DM	Diabetes Mellitus
DMSO	Di Methyl Sulfoxide
DOE FC II	DOE FC II: Dyspnea on Exertion Functional Class II
DVD	Double Vessel Disease
EC	Endothelial Cell
ECGF	Endothelial Cell Growth Factor
ECM	Extra Cellular Matrix
EDTA	Ethylene Diamine Tetra Acetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay

eNOS	endothelial Nitric Oxide Synthase
ePTFE	expanded PTFE
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FCPS	Fibrin Coated Poly Styrene
FDP	Fibrin Degradation Products
FITC	Fluorescein Iso Thio Cyanate
FN	Fibronectin
GAG	Glycosaminoglycan
GCPS	Gelatin Coated Poly Styrene
GF	Growth factor
HBSS	Hank's balanced Salt Solution
HGF	Hepatocyte Growth Factor
HRP	Horseradish Peroxidase
HSVEC	Human Saphenous Vein Endothelial Cell
HTN	Hypertension
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM-1	Inter Cellular Adhesion Molecule-1
IGF	Insulin like Growth Factor
IHD	Ischemic Heart Disease
IL	Interleukin
LDL	Low Density Lipoprotein
LM	Light Microscope
LMCA	Left major coronary artery
Ln	Laminin
MOD	Multi organ disease
NBCS	New Born Calf Serum
NO	Nitric Oxide
PAGE	Poly Acrylamide Gel Electrophoresis
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PDGF	Platelet Derived Growth Factor
PGI2	Prostaglandin I2
PND	Paroxysmal Nocturnal Dyspnea
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PS	Poly Styrene
PTFE	Poly Tetra Fluoro Ethylene
RGD	Arginine-Glycine-Aspargine
SDS	Sodium Dodycyl Sulphate
SEM	Scanning Electrom Microscope
SMC	Smooth Muscle Cell
SS	Stainless Steel
TCA	Tri Chloro Acetic acid
TCPS	Tissue Culture Poly Styrene
TF	Tissue Factor
TGF	Transforming Growth Factor
Ti	Titanium
TIA	Transient Ischemic Attack
tPA	tissue type Plasminogen Activator
TVD	Triple Vessel Disease
TX	Thromboxane
UHMWPE	Ultra High Molecular Weight Poly Ethylene
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor
WB	Whole Blood

NOTATIONS

γ	Gamma
λ	Lambda
~	Approximately
±	Plus or Minus
Ci	Curie
Ct	Threshold Cycle
h	Hours
IU	International Units
kDa	Kilo Daltons
M	Moles/Molar
mM	Milli Moles
mm	Micro Meter
ng	Nano Gram
pg	Pico Gram
RT	Room Temperature
w/v	Weight by Volume
α	Alpha
β	Beta
Δ	Delta
µ	Micro
µg	Micro Gram
µl	Micro Liter

Synopsis

Diseases of blood vessels, account for a significant number of deaths in various parts of the world. The vasculature has been redefined as a vital organ that can regulate its own tone and structure through numerous cellular mechanisms. The endothelium plays the role of gatekeeper in this process, sensing and responding to various stimuli.

Endothelial cells (EC) occupy a strategically important location between circulating blood and tissues and have the ability to respond to changes in its physical, chemical, and humoral environment by the production of a host of biologically active substances. The normal endothelium modulates the tone of underlying vascular smooth muscle, maintains a non adhesive luminal surface, and mediates hemostasis, cellular proliferation, and inflammatory and immune mechanisms in the vascular wall. The endothelium also secretes a myriad of powerful biochemical mediators that regulate blood cell trafficking, cell growth, and vascular remodeling.

Damage to EC initiates molecular changes, and a chain of events is set onto motion that leads to the proliferation and migration of SMC into the lumen, resulting in obstructive arterial lesions. Endothelial dysfunction involves an imbalance between vasoactive substances such that perturbations in the regulation of tone, hemostasis, and vessel structure result in the development of cardiovascular diseases, such as hypertension, atherosclerosis, and ischemia.

Surgical replacement of the affected blood vessel with autologous vessels or with artificial polymeric material is considered to be the most effective treatment method currently. When artificial vascular grafts are used, lack of endothelial cells on its lumen leads to major problems such as graft occlusion due to thrombosis and smooth muscle cell proliferation. To improve the patency of vascular grafts used for bypass surgeries, various approaches are being attempted: 1, *in vitro* seeding of endothelial cells on non degradable polymeric conduits; 2, tissue engineering using biodegradable scaffold; and 3, tissue engineering using multiple layers of vascular cell sheets grown on scaffold.

The fundamental assumption of this approach is that EC seeded on a prosthetic surface or scaffold would continue to exhibit the same salutary properties as do cells that line the normal blood vessel *in vivo*. However, we now understand that many functions of human vascular ECs are dynamic rather than fixed. Therefore, in all these approaches it is important to examine if the seeded cells retain their desired functions rather than just to achieve a viable lining of EC in the lumen of the construct. We must also consider which program of EC functions these cells will express.

The development of techniques for the isolation and reproducible culture of EC has facilitated structural and functional studies of EC. Several investigators have demonstrated that cell deterioration is prominent during serial passaging. Therefore, it is likely that the artificial environment affects the cell phenotype in an unpredictable manner and seeding of EC to produce functional vascular construct is rather challenging.

Extra cellular matrix (ECM) and growth factors are essential for cell proliferation and survival, rendering cells apoptotic in the absence of matrix and the surrounding matrix actively participates in modulating cell function. It is the continuous cross-talk between cells and the surrounding matrix environment that leads to the formation of patterns, the development of form (morphogenesis), and the acquisition and maintenance of differentiated phenotype. Therefore, to maintain cells for long time in tissue culture, it is important to use suitable matrix coated dishes so that they remain viable and functional.

Selection of an ideal matrix to support the growth of EC, without any adverse effects on the phenotype, forms an important aspect that determines success of tissue engineered constructs. This study was initiated with a hypothesis that a bio-mimetic matrix composition might improve EC growth and expression of proteins to maintain the normal physiology of endothelial cells.

Fibrin, an extracellular matrix component, is occupied in hemostasis, wound healing, and cancer. Fibrin is formed at the sites of tissue injury and provides a temporary matrix to support initial response of EC for tissue repair. Other than acting as a scaffold for cell attachment, it holds other extracellular components that are major integrin receptors such as fibronectin and thrombospondin that anchor the cells to the injured tissue apart from growth

factors that generate signals for optimal cell proliferation, survival and expression of appropriate functional proteins. In the light of this information this study was carried out to evaluate the effectiveness of a composite of fibrin, fibronectin, gelatin and vascular endothelial growth factor (VEGF). The goal of this study was to establish that gelatin which is a commonly used matrix for endothelial culture deteriorates cell properties due to lack of appropriate signal and to demonstrate that a composite matrix that is bio mimetic to the vessel matrix will improve the cell signaling for endothelial cell attachment spreading survival and expression of functional proteins required for maintaining the non thombogenic nature of the cells.

The objectives were achieved by systematic experiments that were carried out in three phases.

1. **Matrix standardization using human umbilical vein EC (HUVECs)**

1.i. Standardization of a matrix composition and the coating technique onto polystyrene culture plates to attain optimum EC adhesion, spreading and proliferation.

1.ii. Comparison of the composite matrix with conventional gelatin coating by evaluating the effect of passage progression on the cell behavior in terms of spreading and actin organization, proliferation and apoptosis, using HUVECs.

1.iii. Comparison of the function of EC monolayer by analysis of the mRNA expression and quantification of two pro-thrombotic and two anti-thrombotic molecules, using reverse transcriptase-polymerase chain reaction (RT-PCR) and biochemical assays.

2. **Human saphenous vein EC (HSVEC) growth and behavior on Composite Matrix**

2.i. Establish that HSVEC isolated from a small piece (~2cm) of saphenous vein from cardiovascular disease (CAD) patient can be grown on *in vitro* on standardized matrix to achieve functional autologous cells for therapeutic application;

2.ii. Evaluate expression and synthesis of functional molecules by HSVEC in monolayer culture to determine the balance of prothrombotic/antithrombotic factors owing to dysfunctional nature of EC in CVD patients.

2.iii. Establish that the matrix influences the phenotype of HSVEC grown *in vitro.*

2.iv. Demonstrate that the composite matrix regulates gene expression to maintain a balance between pro-thrombotic and anti-thrombotic proteins.

3. Establish effectiveness of standardized matrix for EC seeding on biomaterials

3.i. To standardize the composite matrix coating on surface of different biomaterials used to fabricate cardiovascular implants.

3.ii. To compare the composite matrix coated devices with bare devices, in terms of EC adhesion, spreading, proliferation, and survival.

3.iii. To compare the expression of functional molecules that determine prothrombotic and antithrombotic phenotype of EC grown on bare and composite coated surface.

3.iv. To compare the thrombogenicity of EC grown on bare devices against EC grown on composite matrix using in vitro blood compatibility studies.

This dissertation is divided into different chapters such as; Introduction, Materials & Methods, Results I, II &III, and Summary.

Chapter I: Introduction

The importance on EC monolayer in maintaining the vascular homeostasis is elaborated and the problems arising out of dysfunction of EC is reviewed. The problems associated with lack of endothelialization of cardiovascular implant devices are reviewed and the concept of *in vitro* tissue engineering is discussed. Hurdles in the isolation and culture of EC and requirement of coating of polystyrene dishes as a prerequisite for *in vitro* EC culture is reviewed. Role of cell-matrix interaction in modulating the cell phenotype and the need for an ideal matrix for the *in vitro* culture is highlighted. The importance of maintaining normal

phenotype of EC in *in vitro* culture is discussed and the current problem is stated by highlighting possible advantages of fibrin as a matrix. Finally the hypothesis and objectives are stated and relevant literature is reviewed.

Chapter II: Materials and methods

The methods for isolation, culture and characterization of HUVEC and HSVEC are described. The analysis methods include; actin identification by specific phalloidin binding for EC attachment and spreading, immunostaining of vinculin for focal adhesion organization, PCNA staining for proliferation, Annexin V staining for apoptosis, von Willebrand factor (vWF) and Acetylated Low Density Lipoprotein (AcLDL) uptake for cell characterization. Analysis of immuno-stained cells was done using fluorescent microscope and/or laser scanning confocal microscope. Live/Apoptotic/Dead cells were quantified using flowcytometric analysis. For phenotypic expression molecules analyzed were; vWF – a prothrombotic molecule, nitric oxide (NO)- an antithombotic and vasodilator molecule, tissue plasminogen activator (t-PA) – a fibrinolytic factor, and plasminogen activator inhibitor (PAI)- an antifibrinolytic factor. Analysis was done by reverse transcriptase polymerase chain reaction (RT-PCR) for detection of m-RNA expression. The mRNA expression by HSVECs and EC grown on biomaterials were quantified by Real Time PCR. ELISA was performed to detect quantitative variation in release of functional proteins into the culture medium. NO release by ECs was detected using a commercial kit. Shear stress resistance of the EC monolayer was studied by perfusion of the seeded biomaterials. To examine how the phenotype of ECs grown on the biomaterials reflects on the *in vitro* blood compatibility of the devices, tests were selected from the list recommended in ISO10993 part4. These tests are standardized and validated in the laboratory and is accredited against quality system ISO/IEC 17025 by COFRAC (France). The thrombogenicity of EC grown materials was assessed by coagulation studies, qualitative platelet adhesion using scanning electron microscopy and quantitative assay using radiolabeled platelets (radioscintigraphy).

Results and discussion are divided into 3 chapters, Chapters III – V for convenience.

Chapter III: Matrix standardization using HUVEC

The objective of this part of the study was to compare the growth of HUVEC on conventional gelatin coated polystyrene (GCPS), and composite coated polystyrene (CCPS). Actin-specific staining has illustrated distinction of adhesion and spreading on GCPS, CCPS and in addition fibrin coated polystyrene (FCPS). Because cell spreading was not satisfactory on FCPS, further studies were not carried out using FCPS. Proliferation rate on GCPS decreased from 4th passage number, but on CCPS doubling time remained steady until 10th passage. It took about 85 days for EC to form confluent monolayer in repeated 15 passages on GCPS, whereas on CCPS in 50 days 15 passages of monolayer are formed. Percentage of apoptotic cells in GCPS varied from 30 % to 60 % between 9th to 15th passages, respectively, however, in CCPS it was only from 5 % to 15 %. The m-RNA expression and release of pro-thrombotic vWF and PAI-1 on GCPS increased passage by passage from 6^{th}on wards; however, detectable levels of these are observed only at a later passage in EC grown on CCPS. The fibrinolytic factor t-PA is expressed only at low levels in EC grown on GCPS and CCPS at earlier passages. But in later passages t-PA expression remained steady on CCPS it was not detectable in GCPS. The cells on GCPS expressed optimal level of eNOS mRNA up to 6th passage, which is found to decrease with passage number, whereas the protein expression is found to be almost steady until 15th passage in the case of cells grown on CCPS.

Chapter IV: HSVEC growth and behavior on Composite Matrix

In this chapter, results from isolation of EC from the saphenous vein of cardiovascular patients undergoing coronary bypass surgery, and the subsequent culture and characterization are described. These cells are probably dysfunctional as evidenced by high mRNA expression for both vWF and PAI-1 in early passage unlike in the case of HUVEC. In the case of tPA, the mRNA expression is not detectable until 6th passage in both GCPS and CCPS, by 9th passage expression improved and remained steady until 12th passage on CCPS, whereas, there was no detectable expression until the 12th passage when grown on GCPS. The eNOS mRNA expression was negligible in initial passages in EC grown on both matrices but the cells grown on CCPS increased the expression from 6th passage onwards

but not in the case of GCPS. An interesting observation was that the EC from one of the subjects behaved more or less similar to that of HUVEC, when it was sub cultured through several passages on CCPS and GCPS. The pro thrombotic factors vWF and PAI-1 remained stable on CCPS whereas on GCPS it was over expressed as the passage number progressed. Measurement of t-PA and eNOS through several passages was also similar to that of HUVEC in this subject. It indicates that HSVEC of this subject was not dysfunctional in terms thrombogenicity. Whereas the HSVEC from all other subjects were more pro thrombotic indicated by amplified vWF & PAI m-RNA expression and synthesis, and less anti thrombotic pointed out by reduced t-PA and eNOS m-RNA expression and synthesis by cells in early culture passage. Quantification of the synthesis and release of these molecules correlated well with the mRNA expression pattern.

Chapter V: EC seeding on Biomaterials: Tissue engineering of cardiovascular devices

This chapter gives results of EC seeding on biomaterials that are currently used for cardiovascular devices such as Polyethylene terephthalate (PET, Trade name: Dacron), titanium (Ti), diamond-like carbon (DLC)-coated Ti, Stainless steel (SS) and ultra high molecular weight polyethylene (UHMWPE). The bare materials that do not support EC adhesion and spreading are coated effectively with composite to obtain confluent monolayer on them *in vitro*. It is demonstrated that the EC do not survive on bare materials unless cells spread and form focal contacts with intermediate matrix proteins. The EC grown on composite coated Dacron is found to resist shear stress of the order of ~20 dynes/cm^2. It is demonstrated that EC seeded bare materials surfaces are prothrombotic, whereas EC seeding on surfaces pre-coated with bio mimetic molecules facilitated normal EC phenotype and reduced the risk of platelet adhesion and activation of blood coagulation. The data suggests that this approach of coating the designed composition of bio mimetic adhesive proteins and growth factors is suitable for cardiovascular tissue engineering using various base material or scaffold.

Chapter VI: Summary and Conclusions

The use of gelatin adsorbed surfaces for endothelial cell growth in tissue culture is found to cause cell transformation more prominently on serial passage.

The actin filament assembly and formation of focal adhesion on composite are able to regulate cell signaling for proliferation, survival and differentiation.

One of the key findings is that the dysfunctional EC isolated from cardiovascular patients express more prothrombotic molecules and less antithrombotic molecules in most of the cases and are regenerated to express a normal anti-thrombotic phenotype when they were grown on the composite coated matrix in serial passages. Initially it was anticipated that HSVEC may not grow and express normal physiology *in vitro*. Therefore, it was proposed that HSVEC may be grown on ECM deposited on growing EC on the fibrin composite matrix. The finding is that normalization of HSVEC phenotype has taken place on composite matrix and this has far reaching significance in the field of vascular biology suggesting that adherence of the endothelium to the matrix is a dynamic interaction allowing endothelium to change and adapt to various physiological and pathological states.

Finally, since the matrix favors the maintenance of normal phenotypic expression, the composite matrix can be used for the tissue engineering of cardiovascular devices to reduce thrombogenicity of implants. It has been demonstrated in this study that though bare materials give wrong signal to the EC seeded on the surface and turn them prothrombotic if these surfaces are pre-coated with bio mimetic molecules they help to maintain normal EC phenotype and reduce the risk of platelet adhesion and activation of blood coagulation. The dysfunctional EC isolated from patients' autologous vein can be grown on small diameter vascular graft coated with the composite matrix for coronary artery bypass grafting.

The emerging field of tissue engineering is focusing on the *in-vitro* generation of functional organ replacements using living endothelial cells and other vascular cells for which non degradable or biodegradable scaffold base materials are used. Since the data from this study suggest that the approach of coating the designed composition of bio mimetic adhesive proteins and growth factors is suitable for cardiovascular tissue engineering using any material of choice as base material or scaffold, future work should focus on the coating of biodegradable polymeric scaffolds to achieve completely biological cardiovascular conduits.

Chapter I

INTRODUCTION

I.1. SUMMARY

Blood vessels are the life-giving conduits that connect out tissues and organs to the heart and to each other. Diseases of the blood vessels, principally of the small and medium caliber muscular arteries, account for the majority of deaths in various parts of the world annually. The high incidence of atherosclerosis, in which formation of fatty plaques leads to clogging of the arteries, and related diseases means that diseased muscular arteries are surgically replaced frequently. Recent insights into the pathogenesis of vascular disease have opened up a new frontier that has implications for future therapies. The vasculature has been redefined as a vital organ that can regulate its own tone and structure via numerous cellular mechanisms. The endothelium plays the role of gatekeeper in this process, sensing and responding to stimuli and activating various vasoactive systems that function as mediators.

Key discoveries in the past two decades have revealed that the vascular endothelium is an important regulatory organ that is involved in maintaining cardiovascular homeostasis in health and contributes significantly to the patho-mechanism of several cardiovascular diseases. The endothelium is much more than a gate keeper for blood and blood-borne components. Its complexity is underscored by the numerous functions it performs in coordination with the rennin-angiotensin, coagulation, fibrinolytic, complement and immune system of

the body. When their integrity is maintained, endothelial cells exert anticoagulant properties via the synthesis of thrombomodulin, tissue factor (TF) pathway inhibitor and tissue-type plasminogen activator (t-PA); it regulates the transport of nutrients, leukocytes and many other substances by regulation of membrane protein expression; it participate in the modulation of vascular tone and plays a major role in the control of blood pressure by synthesis and release of NO and PGI_2; and it forms new vessels in response to angiogenic stimuli. Local production of NO by endothelial cells is a major mechanism regulating blood flow and NO also participates in other far-ranging physiologic functions including regulation of proliferation of smooth muscle cells, platelet aggregation and immune response (McIntire and Eskin 1984). On activation or damage, endothelial cells quickly manifests prothrombotic properties by the release of proteins like multimeric von Willebrand factor (vWF), which promotes platelet adhesion and aggregation, and plasminogen activator inhibitor-1, a member of the serpin family; it secretes mediators of vasoconstriction like endothelin; and up-regulates receptors involved in coagulation, complement activation and leukocyte migration. In addition, TF expression by endothelium leads to initiation of the extrinsic blood coagulation pathway (Verstraete, 1995). Another important feature of endothelial cells is their ability to direct cells of the immune system to specific sites in the body. Constitutively expressed or cytokine-inducible cellular adhesion molecules [e.g., E-selectin and intercellular adhesion molecule-1 (ICAM-1)] and soluble factors such as chemoattractants, cytokines, and chemokines act in concert to recruit the immune cells to lymphoid organs or inflammatory sites (Carlos and Harlan, 1994). Last, endothelial cells are actively involved in vascular remodeling during, for example, ovulation, wound healing, tumor growth, and diabetic retinopathy. Although complex in regulation and sometimes difficult to functionally analyze *in vitro*, as well as during disease progression, data have become available that link (parts of) these endothelial cell functions to various steps in the angiogenic cascade.

Native atherosclerosis and the accelerated arteriopathies that follow therapy share a common initial pathological event, loss of EC integrity and function. Damage to EC removes endothelial compounds, and a chain of events is set onto motion that leads to the proliferation and migration of SMC into the lumen, resulting in obstructive arterial lesions. Endothelial dysfunction involves an

imbalance between vasoactive substances such that perturbations in the regulation of tone, hemostasis, and vessel structure result in the development of cardiovascular diseases such as hypertension, atherosclerosis and heart failure.

Modern medical practice routinely violates the delicately balanced homeostasis that involves the interaction of the endothelium with soluble and cellular components of blood and results in a gradual exposure of blood elements to a nonendothelial, "nonself" surface. Artificial vascular grafts, stents and heart-valve sewing cuffs are examples of devices that remain in the body permanently and thus require a surface that will retain hemocompatibility throughout its service life. A "blood compatible" material would perfectly substitute for the endothelium so that soluble and cellular blood components would remain entirely naïve of its foreign presence. Although biomaterial science has advanced sufficiently in the past 50 years, true biocompatibility of artificial surfaces remains a relatively distant and elusive goal. As on today, implantation of mechanical valves and stents necessitates lifelong anticoagulation that is associated with the possibility of hemorrhage, the risk of thromboembolic events, and predisposition to lifelong risks of infection (Vongpatanasin et al 1996; Kuntze et al 1998). Surface modification has been attempted by several researchers to provide blood compatibility to the devices; however, the diverse array of medical devices demands a corresponding range of hemocompatible surface properties. To successfully modify the variety of devices requiring blood-compatible surfaces, many different methods need to be developed and tested (Ito et al 1992; Skarja and Brash 1997). Endothelial cells (EC) line the internal walls of blood vessels, and as such prevent platelet attachment and blood coagulation (Becker et al 2000), and surfaces covered with these cells is the best option for implant devices.

Endothelial cells have been an important focus of research in the past two decades. The development of techniques for the isolation and reproducible culture of EC has facilitated structural and functional studies of EC. However, as several investigators have demonstrated, the up regulation of many endothelial genes *in vitro* indicates that care needs to be exercised when extrapolating from cell culture systems to *in vivo* situations. The cultured EC should maintain their differentiated functions to a sufficient degree to allow the *in vitro* dissection of

many mechanisms by which the endothelium resists thrombus formation in the normal vessel, as well as many other important aspects of endothelial biology.

Understanding the importance of endothelium in maintaining a thrombus free luminal surface, *in vitro* seeding of EC is under evaluation in various laboratories. The fundamental assumption of this approach is that ECs seeded on a prosthetic surface would continue to exhibit the same salutary properties as do cells that line the normal blood vessel *in vivo*. However, we now understand that many functions of human vascular ECs are dynamic rather than fixed. These new findings indicate that, it is important to examine that the seeded cells retain their desired properties. It may not be sufficient to achieve a viable lining of EC on a prosthetic material. We must also consider which program of EC functions these cells will express.

It has been well known that in long term cultures and repeated splitting to obtain large number of cells, endothelial cells lose the potential to regenerate as the passage number progresses; however, related phenotypic alteration is not so well studied. Extra cellular matrix is essential for endothelial cell survival and proliferation response to growth factors, rendering cells apoptotic in the absence of matrix. Also, it has now become equally clear that the surrounding matrix actively participates in modulating endothelial function and those EC products, in turn, can regulate matrix content and structure. It is the continuous cross-talk between cells and the surrounding matrix environment that leads to the formation of patterns, the development of form (morphogenesis), and the acquisition and maintenance of differentiated phenotype. Therefore, to expand the cell number in tissue culture, it is important to use suitable matrix coated dishes so that they remain viable and functional. As much is it important to have a matrix with optimum composition to get EC attachment, spreading, proliferation and differentiation on biomaterial surface so that the modification is effective to prevent device thrombosis.

Therefore, a systematic study is required to understand the influence of matrix components to normalize differentiation of EC in culture so that the most suitable matrix composition may be used for vascular tissue engineering technique that involve endothelial cell culture.

I.2. REVIEW OF LITERATURE

I.2.1. Culture of Endothelial Cells

Endothelial cells normally have a low rate of proliferation in the adult with a life span of 100–10,000 days (Hobson, B., and Denekamp, J., 1984), but the endothelium retains its capacity for proliferation, which occurs physiologically in the corpus luteum and uterus and also during wound healing. The relative lack of success in maintaining human endothelial cell cultures so that they may grow vigorously for long periods of time (and with a high mitotic index) when passaged at low density could be explained by the use of cells derived from the umbilical vein. This vascular territory could be pre programmed to senesce rapidly after birth because of its unique function as a prenatal link between the mother and the foetus. Alternatively, the difficulty in culturing these cells could result from inadequate conditions under which they are maintained *in vitro*.

The single most important breakthrough for EC culture, and still probably the most used technique, was published by Jaffe and coworkers in 1973. Since then several modifications and improvements have been published (Gimbrone et al. 1974, Maciag et al, 1981), with the fundamental principle remaining unchanged, i.e. the incubation of the vessel, ideally clamped at both ends, with a collagenase containing solution, and the subsequent plating and cultivation in a protein-coated tissue culture flask, the cells being fed with a medium enriched by relatively large amounts of serum and growth factors. It should be remembered that ECs in culture are something very different than ECs in natural environment. Compared to many, if not most other cell types, it is harder to mimic *in vitro* the natural circumstances under which ECs operate. This must be remembered when interpretations of EC functions are made, and the *in vivo* correlate remains the only true measure of the predictive value of *in vitro* findings.

One of the major hurdles in the culture of ECs was its failure of satisfactory attachment and growth on un-coated tissue culture flasks. It is generally accepted that some matrix component pre coated on the tissue culture treated plastic is beneficial. Most often, as also originally described by Jaffe et al, denatured bovine collagen, i.e. gelatin, can be used. The role of fibronectin as a promoter of cell migration and tight anchorage of ECs to many biomaterials has

been described extensively (Kesler et al 1986a; Vohra et al 1990). Therefore, polystyrene culture dishes are frequently pre coated with fibronectin (Fn). Other adhesion substances like collagen, laminin (Ln), vitronectin (Vn) and extracellular matrix (ECM) are also used extensively.

As the coated matrix controls the EC adhesion, spreading and proliferation, the selection of matrix for the coating is very important. It is the interaction between the cell and the matrix and the subsequent organization of focal adhesion and signaling, which have the ultimate say on the cell behavior. It has been well known that during serial passaging, endothelial cells lose the potential to regenerate as the passage number progresses (Sato et al. 1993; Yang et al. 1999; Wagner et al. 2001; Zhang et al. 2002). Maier et al (1990) reports rapid loss of endothelial characteristics in HUVEC when cultured beyond fourth passage on gelatin coated tissue culture plates. This has been shown to be the result of production of interleukin (IL)-1α by the HUVEC itself. Administration of antisense IL-1α oligomers blocks senescence (Maier et al. 1990).

When the adhesion of ECs on different matrices were compared by Relou et al. (1998), it was found that human umbilical vein EC adhere well to and stretch well with plastic coated with fibronectin, collagen, and gelatin in contrast to non-coated plastic. The spontaneous proliferation of EC was enhanced in fibronectin, collagen and gelatin-coated wells as compared to non-coated wells. A markedly up-regulated proliferation was measured on fibronectin and collagen while EC on gelatin-coated plastic only showed moderate bFGF-induced proliferation. Balcells and Edelman (2002) examined the adhesion and the biochemical secretion of bovine aortic endothelial cells (BAEC) on tissue culture polystyrene (TCPS) discs coated with fibronectin (Fn), laminin (Ln), or gelatin. The three coating proteins nonspecifically promote sub-confluent and post-confluent endothelial cell production of total protein up to 2.5-fold of the reference value. Total soluble glycosaminoglycan (GAG) production slightly increased with the different coatings only at low cell density. In contrast, Ln and Fn, not gelatin, drastically enhanced post-confluent BAEC production of prostaglandin (PGI_2). However, antibody-blockage of the α_5 integrin, constituent of the Fn receptor in BAEC, appeared to inhibit the up regulation of PGI_2 production observed on Fn-coated surfaces. The results indicate that the cell adhesion mediators used as

coating agents dictate cell biological production as well as adhesion and proliferation (Balcells and Edelman, 2002).

One would expect that studies done with cell populations maintained in inadequate tissue culture conditions which give rise to senescent populations would lead to misleading conclusions as to what the cells are really capable of doing. The elucidation of the functions and mechanisms through which endothelial cells achieve these functions can only be achieved by first establishing the natural requirements for survival and proliferation of this cell type. These requirements are best reflected by long-term maintenance of endothelial cells at low density as well as by high mitotic index of the cultures. Such cell lines should provide an adequate model to study the metabolic function of the vascular endothelium.

I.2.2. Cell-Matrix Interactions in Physiology and Pathology

Adhesive interactions between endothelial cells and the structural and superficially bound proteins that make up the extra cellular matrix are critical to maintaining the properties of the endothelium. Vascular research over the past 20 years has highlighted the importance of the endothelial lining in maintaining vascular function. However, it has now become equally clear that the surrounding matrix actively participates in modulating endothelial function and those EC products, in turn, can regulate matrix content and structure. It is the continuous cross-talk between cells and the surrounding matrix environment that leads to the formation of patterns, the development of form (morphogenesis), and the acquisition and maintenance of differentiated phenotype.

Endothelial cells regulate the export of secretion products and the expression of membrane receptors to react to changes in stimuli from the external environment or subendothelium (Defilippi et al. 1993; Dejana et al.1993). Most of these regulatory functions of ECs are mediated by adhesive interactions between membrane receptors and soluble factors or immobilized molecules in the extra cellular matrix.

Extracellular matrix regulates many physiological and pathological processes (Robert et al. 1990; Carey, 1991; Haralson, 1993; Adams and Watt, 1993). Alterations of these endothelial-matrix interactions have been implicated

as important steps in a variety of pathologic conditions, including wound healing, tumor metastasis and angiogenesis (Folkman, 1985; Lafrenie et al. 1992; Luscinskas and Lawler, 1994; Raaf and Raaf, 1994). Atherogenesis and restenosis are two disease processes relevant to coronary artery vascular biology that are directly related to extracellular matrix collagen synthesis and degradation, primarily because the hallmark of the disease is mechanical obstruction of coronary blood flow secondary to medial and intimal hyperplasia.

The importance of a functionally intact endothelium in atherosclerosis is firmly established (Schwartz et al. 1993). Less well characterized, however, is the role of the endothelium in extra cellular matrix metabolism, despite the observation that the extra cellular matrix comprises the bulk of the media in health and the bulk of the abnormal neointima during disease. In the pathological condition of atherosclerosis, characteristic extracellular matrix changes accompany development of the atherosclerotic lesion (Keeley and Johnson, 1985; Barnes, 1985; Stary, 1990; Park et al. 1990; Katsuda et al. 1992; Rekhter et al. 1993), and these changes significantly impact the course of the disease (Wight, 1980; Schwartz et al. 1992). The role of extracellular matrix in atherosclerosis is increasingly recognized (Madri et al. 1991; Yamamoto et al. 1993; Rekhter et al. 1993), and atherosclerotic calcification represents an extreme case of matrix alteration. Vascular cells are known to synthesize their own complex extracellular matrix, and this synthesis is affected by the pre-existing matrix as well as the state of differentiation of the cell (Streuli and Bissel, 1990). Numerous changes in the extracellular matrix occur in atherosclerosis, and alterations in the composition of artery wall matrix contributes to atherogenesis (Wight, 1980; Schwartz et al. 1992; Park et al. 1990; Stary, 1990; Katsuda et al. 1992; Rekhter et al. 1993). In addition to direct effects of matrix molecules on the cells, matrix-bound growth factors may also modulate calcification. Extracellular matrix specifically binds cytokines and growth factors, affecting both their availability and biological activity (Flaumenhaft and Rifkin, 1992). One growth factor that may be particularly important in vascular calcification is transforming growth factor-β1 (TGF-β1). TGF- β1 is bound by a variety of matrix molecules, including fibronectin (Fava and McClure, 1987; Mooradium et al. 1989), and expression of the TGF-β1 gene is regulated by extra cellular matrix (Streuli et al. 1993). TGF-β1 is thought to be a major contributor to

the sclerosis and fibrosis seen in atherosclerosis, as well as the end-stage fibrosis of parenchymal organs such as the liver, lungs, and kidneys (Border and Ruoslahti, 1992). It has been proposed that many of the biological effects of TGF-β1 are mediated by its ability to regulate the synthesis of extracellular matrix, and TGF-β1 is known to greatly increase the production of collagen I and fibronectin (Ignotz and Massague, 1988).

As receptors for the extracellular matrix, integrins mediate a variety of signals that regulate several important events, including differentiation (Luscinskas and Lawler, 1994). Several diseases as well as normal physiological processes are known to be modulated by changes in the extracellular matrix. Further research on the interplay between vascular cells, their extracellular matrix, and cellular integrins may lead to new therapeutic approaches to treat atherosclerotic calcification. Additionally, the same integrin interactions on the basal surface of the cell which maintain endothelial function are also regulatory components of thrombosis and thrombolysis at the apical surface (Hawiger, 1994). Thus, endothelial cell-matrix interactions, and in particular integrin mediated interactions, are key components of vascular regulation.

I.2.3. ECM effects on Cell Adhesion and Migration

The vascular extra cellular matrix contains a heterogenous mixture of structural and adhesive proteins; principal among them are laminins, collagens, elastin, fibronectin, vitronectin, and a variety of proteoglycans and glycosaminoglycans. Although a number of endothelial surface molecules can participate in adhesion to these matrix components (Dejana, 1993), the principal endothelial adhesion molecule belongs to the integrin super family (Luscinskas and Lawler, 1994). Treatment of endothelial monolayers with peptides that mimic or block integrin activity leads to disruption of the monolayer, increased trans-endothelial permeability, proliferation and migration of cells, and even cell death (Pierschbacher et al. 1981; Chen and Hawiger, 1991; Meredith et al. 1993; Brooks et al. 1994a). These underscore the importance of integrin mediated cell-matrix interactions in maintaining vessel integrity and regulating endothelial function.

During angiogenesis, endothelial interactions with the matrix molecules and the type and conformation of the matrix play a crucial role in cell migration

and the development of blood vessels (Cockerill et al. 1995; Baldwin, 1996; Hanahan, 1997; Kumar et al. 1998). The intimate relationship between matrix adherence and endothelial structure would suggest that disruption of endothelial adhesion could have profound influence not only on cell structure but also on function. When bovine aortic ECs are plated on a collagen matrix, they migrate across the surface. Plating on fibronectin, however, inhibits cell migration (Madri et al. 1988a). On collagen substrates, actin filaments realign to form coarse filamentous networks within the EC, oriented in the direction of migration (Pratt et al. 1984). In contrast, on substrates such as fibronectin, which do not allow migration, endothelial actin filaments lack this unidirectional orientation (Madri et al. 1988a). Although evidence is still mostly circumstantial, it is hard to ignore the consistent co-localization of specific integrin distribution patterns to the migratory status of ECs (Madri et al. 1991; Shattil, 1995), suggesting that the alterations in cytoskeletal organization and integrin distribution are functionally linked. With the absence of well developed focal contacts, the cell motility is strongly inhibited (Lambugnani et al. 1990).

HUVECs migrate and arrange themselves in tubular structures when cultured for 12 hours on a matrix isolated from Engelbreth-Holm-Swarm (EHS) tumors (Kleinman et al. 1982). When the cells are cultured on collagen type I, however, tubular structures do not form in this period of time, but if they are grown for a week inside collagen gels, giving ECs time to deposit their on basement membrane; tubes do develop (Montesano et al. 1983; Madri et al. 1988b). The rapid tubulogenesis on EHS versus collagen I suggests that one or more components of the basement membrane plays an important role in the development of the capillary-like structures, a speculation that has been confirmed both in culture and *in vivo* (Sakamoto et al. 1991; Grant et al. 1992). Pre-incubation of the EC with antibodies to laminin prevents the formation of tubules *in vitro* and synthetic peptides containing the sequence SIKVAV, present in the A chain of laminin, induce endothelial cell adhesion and elongation and promote angiogenesis (Grant et al. 1992).

When endothelial cells are completely denied from adhesion to the substrate, they lose viability within 10 hours (Re et al. 1994). The best evidence for the mechanism of cell death in these cases has come from microscopic experiments that identified many hallmark attributes of apoptosis; namely nuclear

fragmentation, blebbing, and the formation of apoptotic bodies (Meredith et al. 1993; Re et al. 1994). The involvement of the apoptosis pathway has been confirmed by functional studies revealing that bcl-2 will prevent the death of the detached cells (Frisch and Francis, 1994). Although integrins are implicated, the loss of viability appears not to be exclusively an integrin-mediated phenomenon. When EC in suspension are incubated with RGD peptide coated microbeads, the cells bind the beads via integrin receptors. However, the cells remain in suspension and do not spread. Under these conditions, EC still become apoptotic (Re et al. 1994). These findings indicate that spreading and perhaps integrin-mediated cytoskeletal organization, are also intimately linked to viability.

I.2.4. ECM effects on Cell Proliferation

Interactions of matrix molecules with cells can also modulate the cellular response to growth factors and is critical for cell cycle progression. Adhesion to the extracellular matrix activates intracellular signal transduction pathways important in the regulation of cell growth (Howe et al., 1998), death (Frisch et al., 1997) and differentiation (Roskelley et al., 1995). Information present in the extra cellular matrix is transduced largely through integrin receptors which localize in focal adhesion structures at sites of cell-matrix contact (Jockusch et al., 1995; Burridge et al., 1988). At such sites, integrin receptors are linked to the actin microfilament system in a functional unit which 'senses' both mechanical and chemical changes in the matrix and transduces this information through effects on cellular architecture resulting in changes in gene expression and growth behavior (Chen et al., 1997; Maniotis et al., 1997; Ingber, 1993) and in activation of chemical mediators (Akiyama et al., 1994; Ginsberg et al., 1992; Guan et al., 1991). Indeed, a striking correlation exists between fibronectin matrix organization and the actin cytoskeleton, suggesting that the fibronectin matrix may be a potential modulator of actin organization (Roman et al., 1989; Singer, 1979; Ali et al., 1977; Hynes et al., 1978), functioning, thereby, to influence cell signaling and growth.

Cells which are anchorage-dependent for proliferation require both adhesion to the extracellular matrix and growth factor stimulation to progress through the cell cycle (Howe et al., 1998; Assoian and Zhu, 1997). Integrin and growth factor based signaling pathways both activate MAP kinases (Short et al.,

1998; Renshaw et al., 1997; Miyamoto et al., 1996) and regulate the synthesis and activities of cyclin D-cdk 4/6, cyclin A as well as cyclin E-cdk2 complexes (Zhu et al., 1996; Fang et al., 1996; Schulze et al., 1996; Guadagno et al., 1993; Bohmer et al., 1996). Higher order constraints, however, can modulate this growth response. The need for a particular cell shape and/or cytoskeletal organization appears superimposed upon the requirement for integrin ligation in order for cells to pass through the cell cycle (Chen et al., 1997; Hansen et al., 1994).

Ligation of integrins activates tyrosine kinases and small 3226 GTPases necessary for the reorganization of actin required for cell spreading (Clark et al., 1998; Nobes and Hall, 1995). Agonist-induced actin restructuring is apparently followed by the Rho-dependent activation of cell contractility, resulting in the formation of actin stress fibers, clustering of the $\alpha 5\beta 1$ integrin and the assembly of mature focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996). Therefore, changes in the actin skeleton as a consequence of integrin ligation results in the consolidation of structural as well as signaling molecules to focal adhesion complexes (Burridge et al., 1988; Yamada and Geiger, 1997).

The assembly of the focal adhesion is associated with the reorganization of substrate fibronectin into fibrils (Fay et al., 1996; Mattey and Garrod, 1984; Avnur and Geiger, 1981). Exogenous full-length fibronectin, as well as its 70 kDa amino terminal fragment, bind to adherent endothelial cell layers and co-localize with fibronectin fibers (Christopher et al., 1997). Such interactions between the amino-terminal region of fibronectin and the cell surface is the initial step in the assembly of exogenous fibronectin into extracellular matrix (Quade and McDonald, 1988; McKeown-Longo and Mosher, 1985) and is believed to represent one of the intermolecular homophilic binding events critical for fibronectin polymerization (Hocking et al., 1994, 1996; Morla and Ruoslahti, 1992).

Some of the ECM effects on the cell proliferation involve cooperation with growth factors. Cells which are anchorage dependent for proliferation require both adhesion to the extracellular matrix and growth factor stimulation to progress through the cell cycle (Howe et al. 1998; Assoian and Zhu, 1997). For example, basic fibroblast growth factor (bFGF), interleukins (IL-1, IL-2, IL-6), hepatocyte

growth factor (HGF), platelet derived growth factor-AA (PDGF-AA), and Transforming Growth factor-β (TGF-β) are found in association with ECM at high concentrations and are released at specific times for interactions with their receptors (Schuppan et al. 1994).

Increased matrix rigidity increases the level of phosphotyrosine in focal adhesions, while strengthening the linkages between integrins and the cytoskeleton (Choquet et al., 1997; Pelham and Wang, 1997). Changes in the mechanical properties of the matrix are expected to have profound effects on cell migration and proliferation. Reversible deformation of ECM, in fact, accompanies neutrophil migration (Mandeville et al., 1997). In addition, increased matrix rigidity as well as increased level of fibronectin matrix (Morla et al., 1994) is associated with decreased rates of cell migration. Also, changes in the matrix rigidity have been shown to affect cell cycle progression (Hansen et al., 1994).

I.2.5. Endothelial Integrin Binding and Organization of the ECM

The interaction between different vascular cells and the matrix is mediated by cell surface receptors, primarily integrins. These dynamic interactions allow cells to perceive, respond, and influence signals from the extracellular matrix and the flowing blood (Bissel et al. 1982; Hynes, 1992; Defilippi et al. 1993; Shattil, 1995). Endothelial cells express at least nine separate integrins. For matrix interactions, the β_1 and $\alpha_v\beta_3$ (Hynes, 1987) integrins appear to be most important.

Studies indicate that the β_1 integrins bind many components of the sub endothelial matrix, including collagen, laminin, fibronectin, thrombospondin, and perlecan and these integrins co-localized to the basal surface of endothelial cells in contact with the sub endothelial matrix (Dejana et al. 1987; Dejana et al. 1988). Endothelial cells also express the $\alpha_v\beta_3$ integrin receptor that can bind matrix as well as several plasma proteins, such as vitronectin, fibrinogen and thrombin (Luscinskas and Lawler, 1994). Under specific conditions these plasma proteins can become trapped within the sub endothelium and are important modulators of cell adherence.

The integrin mediated cell adherence to the sub endothelial matrix appears to be critical in maintaining endothelial integrity. RGD containing molecules that block specific integrin binding also disrupt endothelial monolayers

(Charo et al. 1987; Dejana et al. 1987). Immuno localization studies show that cells can reorient the distribution of their integrins in response to alterations in the matrix composition. When placed on a pure vitronectin substrate, $\alpha_v\beta_3$ integrins on the basal surface form clusters (Dejana et al. 1988). In contrast, the β_1 integrins remain dispersed. However, if fibronectin is used as the substrate, the β_1 but not β_3 integrins cluster (Dejana et al. 1988). It has also been shown that clustering of a single class of integrins could support subsequent formation of focal contacts and the local assembly of cytoskeletal proteins (Dejana et al. 1988).

Investigation of integrin expression in cells plated on fibrinogen revealed a more complex interaction with the matrix (Dejana et al. 1990). The binding of fibrinogen to the $\alpha_v\beta_3$ integrins subsequently induced synthesis and secretion of fibronectin. The secreted fibronectin mediated endothelial spreading in a manner similar to that seen on pure fibronectin substrates (Dejana et al. 1988). Treatment with monensin to inhibit fibronectin synthesis and secretion did not inhibit initial attachment to the fibrinogen substrate, but did inhibit spreading and the cells did not organize their cytoskeleton and adhesion structures (Dejana et al. 1988). It is clear from these studies that, there exists a strong correlation between the matrix composition, integrin receptor localization, stress fiber organization and the cell shape and spreading. For β_1 integrins particularly, matrix binding has been correlated with a reorganization of β_1 into focal area that match the distribution of stress fibers (Pratt et al. 1984; Leto et al. 1986). In addition, double labeling experiments co-localize the focal integrin accumulations with the distribution of cytoskeletal molecules such as F-actin, vinculin and talin which are known to be components of focal adhesion plaques (Dejana et al. 1988; Singer et al. 1988; Burridge et al. 1992). These studies imply that adherence to the matrix and cytoskeletal organization is intimately linked.

In addition to the function of integrins in mediating cell adhesion, integrins have been widely recognized as important molecules in the transduction of positional cues from the ECM to the intracellular signaling machinery. For example, integrin ligation is known to induce a wide range of intracellular signaling events, including the activation of Ras; MAP kinase; focal adhesion kinase (FAK); Src; Rac, Rho, and cdc42 GTPases; phosphatidylinositol-3-kinase (PI3-kinase); Abl; and integrin-linked kinase (Miyamoto et al. 1996; Aplin et al.

1998; Schwartz and Shattil, 2000; Ridley, 2000). Adapter proteins including CAS/Crk and Shc are important for coordinating intracellular signals during integrin-mediated cell migration (Klemke et al. 1998; Collins et al. 1999). In addition, integrin ligation increases intracellular pH and Ca^{2+} levels, inositol lipid synthesis, cyclin synthesis, and the expression of immediate-early genes (Schwartz et al. 1995). Interestingly, many of the signaling pathways and effectors, which are activated by integrin ligation, are also activated after growth factor stimulation. This suggests that integrin and growth factor mediated cellular responses may synergize and may function to coordinate biochemical responses in multiple cell types.

I.2.6. Integrin and Growth Factor Receptor Crosstalk

Recent work from a number of laboratories has demonstrated that cell adhesion receptors and growth factor receptors are important molecular determinants in providing specificity for signaling during development and/or during pathological processes. Although integrins and growth factor receptors can independently propagate intracellular signals, the synergy of signals provided by the extra cellular matrix (ECM) and growth factors appears to regulate complex processes, including blood vessel development during embryogenesis as well as tumor growth/metastasis and angiogenesis in the adult. Analysis of the crosstalk between the biochemical pathways mediated by integrins and growth factor receptors may ultimately lead to a better understanding of the cell biological processes underlying normal development and the progression of pathological conditions. Several excellent reviews on integrin mediated (Schwartz et al. 1995; Aplin et al. 1998; Giancotti and Ruoslahti, 1999; Hynes, 1999) and growth factor receptor mediated signal transduction (Tallquist et al. 1999; Schlessinger, 2000) have been recently published. These *in vitro* studies help to provide an important insight into the role of integrins in more complex biological questions in vascular biology.

Although integrins are responsible for mediating cell adhesion to the ECM, a role for growth factors in these integrin dependent processes has been emerging gradually. Growth factor–induced cell proliferation, adhesion, and migration in cultured cell models often depend on specific integrins. For example, optimal cell stimulation with epidermal growth factor (EGF), platelet derived

growth factor (PDGF), insulin, or vascular endothelial growth factor (VEGF) (Schneller et al. 1997; Woodard et al. 1998; Soldi et al. 1999; Borges et al. 2000) depends on integrin mediated cell adhesion to the appropriate ECM. Recently, VEGF has been shown to promote the adhesion and migration of cultured endothelial cells via integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ and β_1 (Byzova et al. 2000). Interestingly, basic fibroblast growth factor (bFGF), but neither IGF nor PDGF, enhances endothelial cell adhesion and migration *in vitro*. Integrin $\alpha_v\beta_3$, can also couple with thrombospondin by direct interactions with integrin associated protein to mediate enhanced cell spreading on vitronectin (Lindberg et al. 1996). Thrombospondin and osteopontin can bind IGF-1 binding protein-5 to regulate IGF-1–induced cell growth (Nam et al. 2000). Models of shear stress in endothelial cells indicate that integrins and growth factor receptors can be sensors to transduce mechanical stimuli into chemical signals via intracellular signaling pathways (Takahashi and Berk, 1996; Chen et al. 1999). Taken together, these findings suggest that although a wide variety of cell types may depend on integrin growth factor receptor crosstalk for integrin-mediated cell adhesion and migration, discrete growth factor receptors may be required to provide cell type specific biological responses.

I.2.7. Endothelial Cells in Cardiovascular Tissue Engineering

At Present, the major limitation in the use of biomaterials in blood contacting applications is the occurrence of material induced thrombosis. This complication is particularly acute in small diameter synthetic vascular grafts where occlusive thrombosis occurs rapidly (Burkel, 1988; Conte, 1998). The initial phase of material induced thrombosis is the rapid adsorption of plasma proteins to the artificial surface (Brash et al. 1988). It is generally believed that the nature of the adsorbed protein layer then directs the subsequent cellular response, principally platelet adhesion and activation (Packham et al. 1969; Elam and Nygren, 1992). It is widely recognized that the key to the solution for this problem lies in the surface of the lumen. Most likely, a confluent monolayer of endothelial cells as is found in natural blood vessels is mandatory (Becker et al. 2000).

Endothelial monolayer lining the normal blood vessel serves as a bio-regulator of cardiovascular physiology. The endothelium provides structural

integrity to the blood vessel by forming a continuous, selectively permeable, thromboresistant barrier between circulating blood and the arterial wall (Rubanyi 1993). It also controls blood flow and vessel tone (Furchgott and Zawadzki 1980), platelet activation, adhesion and aggregation (Autio et al 1989), leukocyte adhesion (Cybulski and Gimbrone, 1991), and smooth muscle cell (SMC) migration and proliferation (Casscells, 1992). This is the key rationale behind utilizing autologous ECs to make hemocompatible artificial polymeric surface that will perform the major functions of an intact healthy endothelium that would normally be found in the blood vessel. This will then provide an anticoagulant and antithrombogenic surface to the circulating blood constituents.

Based on clinical experience that autologous tissue, both artery and vein (Szilagyi et al 1957; Wylie, 1965), is the best arterial substitute and that an intact endothelium is a most important element in such substitutes, seeding of endothelial cells upon the fabric grafts was a reasonable next step. The process of lining the ECs to the lumen of the graft is known as seeding (Herring et al 1978; Zilla et al 1987). Seeding of prosthetic implants with host's own endothelial cells contributes to endothelialization and thus reduces thrombus formation and so improves the long-term patency rates in small-diameter grafts (Deutschet al. 1999). To be successful, seeding of grafts so far has required that EC should be cultured for weeks. There have been numerous attempts at creating tissue-engineered vessels composed of prosthetic or biological materials together with autologous cells, which can be readily available on the shelf of any operating theater. The development of such hybrid grafts so far has involved a combination of autologous tissue with scaffolds made of ePTFE, Dacron or polyurethane in order to develop as closely as possible a natural artery wall possessing all the functional and biologic qualities inherent in the patients native vessels.

I.2.8. History of Endothelial Cell seeding

Malcolm Herring is recognized as the "father" of endothelial cell seeding as a result of his 1978 publication with Gardner and Glover (Herring et al. 1978). This early report described the derivation of canine venous endothelial cells from external jugular vein by mechanical disruption. It was rapidly followed by two publications involving the same group that documented the existence of Weibel-Palade bodies, the ultra structural hallmark of endothelium on seeded graft

linings (Herring et al 1979a) and confirmed positive identification of endothelium lining prosthetic grafts by immunofluorescent staining for factor VIII antigen (Dilley et al. 1979). They also studied the proliferation of seeded endothelial cells on 14 different graft materials, including Dacron, Teflon, Orlon and polyurethane-backed graft (Herring et al. 1979b) and concluded that weft-knit Dacron grafts were the most suitable for EC adhesion and it was difficult for cellular elements to adhere on Teflon.

Utilizing enzymatic techniques for EC harvesting from canine external jugular veins, Graham et al. (1980) contributed significantly to the development of EC seeding of vascular grafts. In these studies, the endothelial coverage of luminal surfaces of the implanted grafts exceeded 80% at 4 weeks postoperatively. Graham et al. (1982) also reported the successful seeding of PTFE prostheses in the canine model. These early studies by Herring and Graham guided subsequent efforts of researchers in the field of endothelial cell seeding.

The next phase of the research effort involved evaluating the efficacy of seeding in performances of small caliber grafts (4mm ID). In 1982, Belden and his group reported early data evaluating 4 mm double velour Dacron (Microvel) grafts in the canine artery model (Belden et al. 1982). In addition, in this year, Stanley et al. described an experiment in which 10 cm lengths of EC seeded and non-seeded Dacron grafts evaluated as bilateral iliofemoral bypasses (Stanley et al. 1982). In both of these studies, the thrombus free surface area on seeded grafts was far less compared to non-seeded ones. Further data confirmed theoretical advantages of endothelial cell seeding in maintaining small-caliber vascular graft patency during conditions of acute reduction in blood flow through the grafts (Hunter et al. 1983; Schmidt et al. 1984).

Many investigators conducted experiments to identify the biologic and physiologic mechanisms by which endothelial cell seeding might improve vascular graft performance. Sharefkin et al. performed platelet survival studies in dogs with seeded and non-seeded grafts and concluded that the platelet interaction with Dacron prostheses was reduced and platelet survival was restored when endothelial cell seeding was successful (Sharefkin et al. 1982). Other group studied the prostaglandin biochemistry of seeded and non-seeded

grafts and reported that seeded ECs synthesize PGI_2, but at levels significantly less than the native artery (Sharp et al 1986).

The Tufts University group was the first to publish a laboratory study on non canine graft endothelialization (Callow et al. 1984; Shephard et al. 1984). Platelet deposition on carotid artery grafts placed in baboons was assessed, and it was found that seeded grafts exhibited a significant decrease in platelet adhesion compared to non-seeded prostheses.

Sterpetti and colleagues investigated the effect of endothelial cell seeding on platelet deposition on endarterectomized arteries (Sterpetti et al. 1992). They concluded that endothelial cell seeding is feasible and reduces platelet adhesion.

One of the difficulties in seeding is the relatively low cell density initially applied to the graft. The endothelial cell density on a normal vein is approximately 10^3 ECs/mm^2 (Sipehia et al. 1996). At least 5 x 10^3 ECs/mm^2 has to be seeded initially to get immediate confluent human EC coverage of a small diameter vascular graft, because some of the cells will be washed off after exposure to the flow. To maximize immediate inoculation density, a two stage seeding procedure is developed in which endothelial cells are harvested, cultured *in vitro* to get sufficient cell number, and then seeded and grown to confluence on the vascular graft prior to implantation (Sentissi et al. 1986; Shindo et al. 1987; Prendiville et al. 1991). Disadvantages of this technique are the potential for infection, the alterations of EC phenotype and the requirement of a waiting period of 3-4 weeks.

I.2.9. *In vitro* Culture of Adult Endothelial Cells

To allow routine clinical application of the endothelial cell seeding technique, various limitations need to be resolved. The integrity of endothelial cell (EC) function is compromised with advancing age and directly contributes to age-dependent impairment of angiogenesis and development of vascular diseases (Pili et al 1994; Rivard et al 1999). *In vitro* culture of adult ECs is a difficult task and the efficiency of cell harvesting and *in vitro* culturing depends on factors such as the age and health status of the patient (Sharefkin et al 1986; Watkins et al 1984).Besides the limited number of potentially available cells in autologous

veins such as saphenous vein, both the efficiency of harvest and the degree of adherence were low, whereas during restoration of the blood flow a significant number of seeded cells were lost (Rosenman et al 1985). Aging of the vasculature is also associated with a number of phenotypic and hemodynamic changes commonly observed in cardiovascular disease states (Cooper et al 1994; Kannel, and Gordon, 1980). To increase the number of available ECs, other isolation techniques and other tissues from which ECs can be isolated have been explored.

As alternative for ECs, mesothelial cells (MCs) have been studied for vascular graft seeding. In contrast to ECs, a large number of MCs can readily be made available at the time of surgery. It was shown that Dacron grafts seeded with MCs at the time of graft implantation in dogs released more prostacyclin than unseeded grafts (Bull et al 1988), but thereafter tissue factor expression increased during culture of MCs *in vitro* (Pronk etal 1992). During perfusion with whole blood, fibrin fibers were generated in close contact with seeded cells, and fibrinopeptide-A generation in the perfusate was observed. It was concluded recently, that seeding with MCs does not improve the patency of vascular grafts (Hedeman et al 1998).

Another source that may provide sufficient cells to cover the graft surface are microvessels present in human fat tissue. Over the years several sources of fat tissue have been used to obtain ECs, with omental fat being the most studied tissue. Controversy started when Visser *et al.* (1991) reported that cells derived from omental tissue were predominantly of mesothelial instead of endothelial origin. In later studies, it was clarified that the terms omentum and omental fat have been used interchangably. Cells derived from omentum are mainly MCs, whereas cells isolated from adipose tissue associated with the omentum (omental fat) contain mostly adipocytes and microvascular ECs (Potzsch et al 1990; Williams et 1994).

A major problem in the isolation of human microvascular endothelium is the presence and rapid overgrowth of contaminating cells, such as fibroblasts (Van Hinsbergh et al 1990; Jackson et al 1990; Koyama et al 1996). Although various techniques have been developed to eliminate such contaminating cells, most of these methods are time consuming and/or do not give a reproducible

yield of early passage endothelial cells (Jackson et al 1990). The most promising technique to remove contaminating cells appears to be the repetitive positive selection of ECs with Ulex Europaeus Agglutinin I-coated paramagnetic beads (Jackson et al 1990).

I.2.10. Improving Cell Adhesion to Graft Lumen

Another technical issue related to EC seeding is cell adhesion and retention. If one considers the similarities between the clinical seeding of vascular prostheses with autologous ECs and the conditions necessary to grow EC in culture, the rationale for protein treatment of vascular prostheses becomes obvious. Majority of work on promoting endothelial adherence and growth to synthetic polymers has involved the modification of the surface with coatings of endothelial cell specific adhesion proteins. Many adhesive proteins, such as albumin (Bos et al. 1999a), albumin-heparin conjugates (Bos et al 1998; Bos et al 1999b), collagen (Stansby et al. 1994; Huang et al. 1998; Itoh et al. 2001), collagen-elastin matrices (Goissis et al. 2000), fibronectin (Hess et al. 1992; Honduvilla et al. 1995; Bhat et al. 1998)), gelatin (Hasson et al. 1986; Budd et al. 1989), laminin (Thomson et al. 1991; Schneider et al. 1992), ECM (Ye et al. 2000), RGD-containing peptides (McMillan et al. 2001) and pre clotting with blood, plasma or fibrin glue(Bach et al. 2001), have been applied to the graft surface to improve the seeding efficiency.

Despite the fact that these substances clearly increase the efficiency of endothelial cell seeding, some are non-specific and thus also increase platelet adhesion to the prostheses (Kesler et al. 1986b). This adverse effect can only be reversed by pre treating with anti platelet drugs(Schmidt et al. 1985; Sharp et al. 1986) Endothelial cell loss to shear forces was another significant obstacle. The studies on the kinetics of EC seeding showed that at least 20% of cells were lost during the first hour and even 60% within the first 24 hour (Rosenman et al 1985; Schneider et al 1988; Vohra et al 1991).

I.2.11. EC Retention after Flow

The endothelial cell seeding of prosthetic grafts has improved the blood flow and patency in experimental small artery grafts. However, the technique is plagued at several points by inefficiency. One of the major inefficiencies is the tremendous loss of endothelium from the surface after the

blood flow is re-established *in vivo* (Rosenman et al 1985). An alteration of the graft substrate with protein coatings slows down the rate of loss of cells, but does not prevent the majority of cells from desquamating (Ramalanjaona et al. 1986). The cell losses during *in vivo* perfusion are far greater than the losses that occur *in vitro* with an acellular perfusate (Campbell et al. 1984; Kesler et al. 1986b).

Prolongation of incubation time before exposure to flow has been suggested to allow maturation of the cytoskeleton so as to improve the cell retention on the graft surface (Miyata et al. 1991; Prendiville et al. 1991; Sugawara et al. 1997). Preconditioning the seeded EC monolayer with graded shear stress promotes reorganization of EC cytoskeleton and production of extracellular matrix, which in turn enhances the EC retention after exposure to flow (Ott and Ballermann, 1995). The properties of prostheses also affect EC retention. Dacron expressed higher and easier EC attachment compared to ePTFE when both surfaces were coated with same matrix (Vohra et al. 1990; Sugawara et al. 1997).

Negative charges obstruct the negatively charged ECs from accessing the graft surface because of natural repulsive forces. A technique called electrostatic transplantation has been developed to induce a temporary positive, or less negative, graft surface charge, entailing EC attraction to the graft surface and accelerating their morphologic maturation. Using this technique, Bowlin et al. (1998) achieved no cell loss after a 30 minute exposure to 15 dynes/cm^2 shear stress *in vitro* compared to 30% loss of control grafts.

I.2.12. Clinical Studies

With all the knowledge gained from *in vitro* and animal studies, the true test would be if the endothelial-seeded grafts functioned similarly in humans. Herring and the Indiana group published the first clinical data on the effect of seeding grafts, using mechanically derived human endothelium (Herring et al. 1984). In this study, seeded prostheses were all 6 or 8 mm Dacron velour conduits placed in the axillofemoral, femorofemoral or femoropopliteal positions. In comparison with randomly placed unseeded grafts, overall patency of grafts seeded with mechanically derived ECs were not different among femorofemoral or femoropopliteal bypasses. They updated the data from this study in a 1987 report (Herring et al. 1987). Seeded Dacron graft patency was superior to non-

seeded patency between the first and second postoperative years, but not statistically significant. Zilla et al. (1987) also reported results from a series of 18 patients undergoing femoropopliteal bypass, and on the basis of follow-ups concluded that the degree of endothelialization is not satisfactory.

Ortenwall et al. (1990) seeded one limb of Dacron aortic bifurcation grafts with saphenous vein endothelial cells. Platelets from each patient were labeled with ^{111}indium-oxine and platelet accumulations on each limb of the grafts were measured at 1 and 4 months postoperatively. The report concluded that there was a significant reduction of platelet accumulation on the limbs seeded with endothelial cells.

Herring et al. (1989) reported an increased 1 year patency rate for endothelial cell seeded ePTFE femoral-popliteal grafts as compared to unseeded grafts in the nonsmoking population. But some of the later studies using the same single-stage endothelial seeding technique failed to show a long term advantage of the seeded grafts (Jensen et al. 1994). Magometschnigg et al. (1992) showed a twofold increase in early patency using cultured ECs seeded onto fibrin glue coated ePTFE grafts placed as infrapopliteal bypasses. Utilizing a similar two-stage EC seeding technique, Zilla et al. (1994) demonstrated increased patency and decreased platelet deposition in clinically implanted EC seeded ePTFE femoropopliteal bypass grafts over 3 years as compared to unseeded grafts. This group reported (Meinhart et al. 1997) very encouraging long-term result in a clinical follow-up study involving 109 *in vitro* endothelialized ePTFE grafts. In their initial randomized study, the primary patency rates were 84.7% for endothelialized grafts and 55.4% for control ePTFE grafts after 3 years, 73.8% versus 31.8% after 5 years and 73.8% versus 0% after 7 years. Combining these data with their more recent additional patients, who received EC seeded grafts, the overall 7 year patency was 66% for 107 of endothelialized femoropopliteal ePTFE grafts.

A major concern with EC seeded graft in humans is the potential for intimal thickening. In one case with bilateral above knee grafts seeded with cephalic vein ECs, one of the grafts developed stenosis and had to be replaced 41 months after implantation (Deutsch et al. 1997). The graft had a confluent endothelial lining on a collagen IV positive basement membrane with subintimal

tissue of 1.21±0.19 mm thickness. The unusually thick subendothelial layer has also been found in another case in which a microvascular EC-seeded Dacron graft was placed as a mesoatrial bypass and had to be resected because of external mechanical structure 9 months after implantation (Park et al. 1990).

Thus it can be concluded that, although a myriad of encouraging results have been achieved experimentally, the clinical data of EC seeded grafts have been limited and have shown mixed results. Besides technical problems, concern exists as to the ultimate function of those endothelial cells on the graft surface, the cells having been injured by the process of manipulation and /or chronic exposure to an un-physiologic environment.

I.2.13. Functional Status of Seeded EC

The technology of EC seeding has evolved along with major advances in the understanding of EC physiology. The endothelial monolayer is a dynamic tissue with a repertoire of functions. Specific anticoagulant mechanisms exhibited by ECs include the production of heparin like glycosaminoglycans, prostacyclin production, nitric oxide synthesis, plasminogen activator production, and expression of thrombomodulin. These anticoagulant properties of the vascular endothelium argue in favor of seeding or promoting the growth of an endothelial lining on the lumen of the prostheses. In addition to these anticoagulant properties, however, it is now also appreciated that ECs produce pro-coagulant molecules such as tissue factor, von Willebrand factor, plasminogen activator inhibitor, thrombospondin and collagens. These pro-coagulant functions of endothelial cells are useful physiologically, but may be undesirable in terms of vascular graft performance (Libby, 1987). It is probable that the techniques for the derivation of ECs for vascular seeding, the process of seeding the graft and the evolution of the seeded graft *in vivo* may induce responses in ECs that are different from normal unperturbed cells. It can be theorized that the balance between anti- and pro-coagulant functions of ECs ultimately dictates the thrombogenic potential of the graft. Thus, it is clear that the focus in the evaluation of vascular graft performance must shift to include critical evaluation of the functional status of the endothelial cells at the blood- material interface.

Advances in molecular biology have provided the possibility of applying genetically modified ECs with desirable functions to synthetic grafts. The

first application of this technology to vascular grafting was reported by Wilson et al. (1989). In this initial study, Dacron grafts seeded with retrovirally transduced ECs were containing a lacZ marker gene was implanted into canine carotid artery. Gene expression from modified ECs on the graft surface was identified for a period of 5 weeks. Tissue plasminogen activator (tPA) has been successfully transfected into ECs (Dichek et al. 1991). These modified cells effectively express this fibrinolytic agent after being seeded onto synthetic graft surfaces (Shayani et al. 1994).

Gene transduction is however a developing technology. The effects of gene transduction and of the vector employed on EC biological behavior and on the host response introduce new variables to EC seeding, which remains to be elucidated. Controversial results have been reported related to the adhesion, proliferation, and retention of genetically modified ECs on the surface of the grafts. Messina et al. (1992) observed a lower proliferation rate of lacZ and Tn5 gene transduced canine ECs versus wild type. In contrast, Jaklitsch et al. (1993) and Huber et al. (1995) separately reported that there were no changes in the proliferation rate of t-PA retrovirally transduced cells, but the migration of cells was decreased. A significantly lower percentage of surface endothelialization was detected at 6 weeks on canine thoraco-abdominal aortic ePTFE grafts seeded with lacZ transduced ECs compared with non-transduced control ECs (Baer et al. 1996; Messina et al. 1992). Dunn et al. (1996) reported only 6% retention of ECs that had been t-PA transduced on Dacron grafts after 2 hour exposure to the flow *in vivo*. The similar poor retention of t-PA transduced ECs on ePTFE grafts have been described by other groups (Falk et al. 1998; Huber et al. 1995). For this reason, little has been documented so far concerning the long-term benefits of genetically modified EC-seeded grafts *in vivo*.

I.3. HYPOTHESIS

The cell matrix interactions play an important role in the maintenance of the functional status of EC by modulating the protein expression patterns, in addition to regulating the proliferation and survival. Therefore, biomimetic and biospecific molecules are coated on materials to regulate function of cells on the tissue engineered product. These bioactive molecules can be attached in a

defined spectrum, concentration and spatial distribution in order to control adhesion, growth, viability, differentiation, and function of the cells.

It is the continuous cross-talk between cells and the surrounding matrix environment that leads to the formation of patterns, the development of form (morphogenesis), and the acquisition and maintenance of differentiated phenotype. Therefore, not only to seed EC on biomaterials but also to expand the cell number in tissue culture, it is important to use suitable matrix coated dishes so that they remain viable and functional.

I.3.1. Fibrin as a natural scaffold

Upon tissue damage or blood vessel injury, a blood clot is formed that consists of mainly fibrin, but also thrombin and transglutaminase factor XIIIa. Besides its pro-angiogenic activity (Coughlin, 2000; O'Brien et al. 2001), the fibrin clot also serves as natural provisional matrix for new cell ingrowth and will be replaced by viable tissue during wound regeneration. These characteristics that have been optimized by evolution over a long period of time make fibrin the ideal biomaterial for wound regeneration and angiogenesis (Carmeliet, 2000). This is further established by the wide use of commercial fibrin precursor preparations, termed fibrin glue, (a mixture of concentrated fibrinogen and thrombin, usually derived by cryoprecipitation of human plasma) as tissue sealants in surgery and other applications (Shireman and Greisler, 1998; Dickneite et al 2002).

The fibrin clot then presents a dense network barrier that hinders or inhibits blood leakage, but also supports every stage of blood vessel formation, starting with migration of endothelial cells, primitive endothelial tube formation, to vessel maturation to form a perfused blood vessel network. Finally, fibrin is degraded by cellularly produced fibrinolytic enzymes such as MMPs or plasmin (Sierra, 1993; Mosesson et al 2001). Since fibrin lyses slowly, it can serve as provisional reservoir to deliver proteins (Fasol et al 1994), cells (Bach et al 2001), or DNA of genes (Andree et al 2001).

The formation of an occlusive fibrin clot involves not only assembly of fibrin fibers from soluble fibrinogen precursors; it also involves anchorage of the fibers to the apical endothelial surface (Cheresh, 1987). Endothelial cells adhere, spread and proliferate on both fibrinogen and fibrin *in vitro* through binding to

integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (Cheresh et al. 1989; Suehiro et al. 1997). Like other integrin mediated events, the binding of fibrin to the apical surface can have profound influence on cell function. It can reorganize cyoskeletal elements leading to clot retraction similar to that seen when platelets bind fibrin (Chen et al. 1995). In addition, studies of cultured endothelial cells show that prolonged exposure to polymerized fibrin causes the cells to retract its surface extensions and assume a distinctly different shape (Kadish et al. 1979; Rowland et al. 1984). Dvorak (1987) demonstrated that fibrin gel themselves induce angiogenic response in the absence of tumor cells or platelets and this process was enhanced when chemoattractants or mitogens were included in the gel.

The signal appears to be specific to fibrin as distinct from fibrinogen, its plasma precursor, because endothelial cell binding of soluble fibrinogen does not produce these effects (Weimar and Delvos, 1986). Thus the interest has focused on unique epitopes formed during the assembly and subsequent lysis of fibrin (Rowland et al. 1984; Chang et al. 1995). Spreading of endothelial cells on immobilized fibrin requires cleavage of Bβ chain of fibrinogen, such as would occur during thrombin generated fibrin formation (Bunce et al. 1992).

Although these experiments suggest that integrin occupancy, specifically $\alpha_v\beta_3$, by fibrin is the stimulus for the endothelial cells to fibrin, it is not the only integrin involved. The decrease in intracellular contacts and loosening of the adherence to the matrix, both of which can utilize the β_1 integrins (Dejana et al. 1987; Lampugnani et al. 1991), suggests that occupancy of the β_3 integrins generate intercellular signals that produce an alteration in the binding properties of other integrins. A similar disorganization of the endothelial monolayer can occur when the apical surface is exposed to collagen or fibronectin (Kadish et al. 1979; Weimar and Delvos, 1986). This implies that occupancy of other integrins on the luminal aspect of the cell can produce a change in the integrin composition and binding at the basal surface as well as alteration in cell function. When overlaid with a fibrin clot for prolonged periods, endothelial cells can lose their polar organization, deatch, and migrate into the fibrin clot (Kadish et al. 1979) simulating angiogenesis (Folkman and Shing, 1992a; Brooks et al. 1994b). In addition, cultured endothelial cells, when surrounded by a fibrin gel, will form a tube like structure reminiscent of an angiogenic process (Fournier and Doillon, 1992). Fibrin can also effect phenotypic changes other than migration and

proliferation. Fibrin stimulates synthesis and secretion of tissue plasminogen activator and prostacyclin (Kaplan et al. 1989; Ramsby and Kreutzer, 1993), induces interleukin-8 expression (Qi and Kreutzer, 1995) and suppresses plasminogen inhibitor-1 release (Fukao et al. 1990; Fukao et al. 1995).

I.3.2. Fibrin Growth Factor Interactions

The angiogenic peptide vascular endothelial growth factor (VEGF), also known as vascular permeability factor, stimulates both increased microvascular permeability, which leads to local fibrin formation, and also endothelial cell proliferation needed for angiogenesis (Dvorak et al. 1995). Recent *in vitro* and *in vivo* studies have revealed that the transformation of endothelial cells to an angiogenic phenotype is induced by several factors, including VEGF, fibroblast growth factors (FGFs), hepatocyte growth factor, interleukin-8, and others, (Folkman and Shing, 1992b) but the interaction between VEGF and its receptors is the most important angiogenic event in the physiological and pathological angiogenesis.

The need for fibrin to support endothelial cell spreading, migration, and angiogenesis and the potent stimulation of similar angiogenic responses by VEGF suggest that these processes may be inter related. This concept is supported by evidence that fibrin clots provide a good matrix to support VEGF-stimulated angiogenesis *in vitro* (Brown et al. 1996) and that angiogenic peptide FGF-2 binds specifically to fibrinogen and fibrin (Sahni et al. 1998). Two distinct binding sites for VEGF were identified with both Sepharose-immobilized fibrinogen and fibrin monomer with similar *K*ds, and the maximum molar-binding ratios were 3.8 and 3.6, respectively (Sahni and Francis, 2000). Binding of VEGF to polymerized fibrin differed from fibrinogen or fibrin monomer, as only a single binding site was identified, and the maximum molar-binding ratio of VEGF:fibrin was 0.1:1, indicating that VEGF bound to only 1 in 10 monomeric units (Sahni and Francis, 2000). This is most likely explained by structural changes in fibrin after polymerization, which results in formation of highly organized fibers composed of multiple protofibril units aggregated laterally (Weisel, 1986).

FGF-2, another angiogenic peptide, also binds with high affinity to fibrin and fibrinogen (Sahni et al. 1998), and these binding increases the capacity of FGF-2 to stimulate endothelial cell proliferation (Sahni et al. 1999). Cells cultured

in medium containing 1.5nM FGF-2 and 30nM fibrinogen supported proliferation and did not undergo apoptosis, which is known to occur under conditions of growth factor deprivation (Araki et al. 1990). The binding of VEGF to fibrinogen is not inhibited by FGF-2, thereby indicating that the binding sites on fibrinogen for these 2 angiogenic peptides are independent and separate. The binding affinity for both FGF-2 and VEGF increased after conversion of Sepharose-immobilized fibrinogen to fibrin monomer, suggesting that thrombin-mediated conformational changes affect the binding sites and result in increased affinity for both angiogenic peptides. Insulin-like growth factor–1, another peptide important in wound healing, also binds to fibrinogen and fibrin *K*d values of 0.67 and 0.7 nmol/L, respectively. These high-affinity interactions support a role for fibrin as a reservoir of growth factors at the site of injury.

Integration of cell responses to VEGF and fibrinogen may occur at the receptor and the signal transduction level. Binding of endothelial cells to matrix glycoproteins, including fibrinogen and fibrin, occurs through integrin receptors, and this alters their sensitivity to growth factor–induced signaling (Senger et al. 1996). In turn, VEGF stimulates endothelial cell surface integrin expression (Enaida et al. 1998; Suzuma et al. 1998), thereby regulating the cell response to fibrinogen and fibrin. Also, binding of a complex of fibrinogen and FGF-2 could result in the co-localization of integrin and FGF-2 receptors at the focal adhesion complex, contributing to signal integration (Plopper et al. 1995). Both fibrinogen and fibrin can support endothelial cell attachment through occupancy of $\alpha_v\beta_3$, which results in the formation of a focal adhesion complex that co localizes integrin and VEGF receptors (Takahashi et al. 1999) and fosters signal integration (Hanks and Polte, 1997).

Fibrin glue and heparinized collagen have been used as carrier and local delivery matrix for biological agents such as VEGF, fibroblast growth factor 1 and heparin (Fasol et al. 1994; Kang et al. 1995; Weatherford et al. 1996; Shireman et al. 1999). This will provide retention of VEGF within the fibrin matrix until the matrix is degraded during cell infiltration, releasing the growth factor to become bioavailable (Schense and Hubbell, 1999; Zisch et al. 2001). In this scheme, the release of the incorporated VEGF from the fibrin carrier is dependent upon the rate of degradation of fibrin by cells invading the matrix. Plasmin-mediated proteolysis of fibrin is regulated through cell-derived plasminogen activator (Zisch

et al. 2001). Cell surface receptors for plasminogen activator allow highly localized plasminogen activation, resulting in fibrinolysis directly at the cell surface, or very near to the cell surface, without bulk matrix degradation (Sakiyama et al. 1999). In support of this idea, the mitogenic response of HUVECs evoked by fibrin bound VEGF was unexpectedly stronger (139% over control) compared to the response evoked by soluble VEGF (47% over control) (Zisch et al. 2001).

The growth promoting ability of VEGF modified fibrin surfaces for endothelial cells could be exploited for improving synthetic vascular graft production. *In vitro* lining with cultured autologous endothelial cells, usually of venous origin, provides synthetic arterial grafts with an intact confluent endothelium at the time of implantation (Zilla et al. 1994). The persistence of this confluent endothelial cell layer and its 'fitness' to withstand blood flow without cellular detachment from the synthetic graft material are critical for graft patency and incomplete endothelialization may lead to graft failure. Clinically, fibrin glue has been successfully used for precoating of PTFE grafts prior to endothelialization *in vitro* (Zilla et al. 1989; Deutsch et al. 1997). Several growth factors, such as fibroblast growth factor and VEGF, mixed into fibrin glue have been explored for graft endothelialization (Weatherford et al. 1996; Shireman et al. 1999). For the growth factor to exert its function, it is important that it is not immediately released in the medium during seeding, or removed by blood flow. Immobilization of VEGF into fibrin glue may provide a unique mode for persistent containment that can be expected to withstand flow situations.

I.3.3. Fibrin Interaction with ECM Proteins

Shortly after the tissue damage and during the early stages of wound healing, there is a release of blood contents and tissue factors into the area of the wound, leading to the formation of a vascular plug containing mostly platelets, plasma fibronectin and fibrin. The formation of a fibrin-fibronectin meshwork provides a provisional matrix that serves as a substrate for cell adhesion and migration (Rosales and Juliano, 1995; Wei et al 1997). During the closure of wounds, cells migrate over the provisional matrix primarily composed of fibrin, fibronectin, vitronectin, tenascin and collagen and the cells interact with these ECM molecules through integrins (Juhsz et al., 1993; Gailit et al., 1994). The

sequence of matrix deposition is tightly linked to adhesion and migration of cells to close the wound (Kamei et al 1998).

During the wound closure, fibrin, fibronectin and other ECM molecules such as, hyaluronic acid and collagen in conjunction with growth factors released by the platelets and secreted by cells present in the granulation tissue, provides signals to the cells, leading to their proliferation (Tuan et al 1996; Bissel 1998). The ECM molecules, e.g., fibronectin, specific fragments of laminin and collagen VI, have been shown to stimulate fibroblast and EC proliferation (Bitterman et al 1983; Panayotou et al 1989; Kapila et al 1998). The cooperation between fibrin, ECM molecules and growth factors is well illustrated in the development of new blood vessels of the granulation tissue. Growth factors such as VEGF and FGF associate with fibrin and other ECM molecules and stimulate proliferation of ECs which then migrate to form new micro vessels (Norrby, 1997).

Therefore, it is likely that if biomimetic and biospecific molecules can be coated on materials using fibrin as scaffold, the components in the coated matrix should regulate function of cells on the tissue engineered product. These bioactive molecules can be attached in a defined spectrum, concentration and spatial distribution in order to control adhesion, growth, viability, differentiation, and function of the cells. Therefore, not only to seed EC on biomaterials but also to expand the cell number in tissue culture, it is important to use suitable matrix coated dishes so that they remain viable and functional, without dedifferentiation through several passages required to expand cell numbers.

This study was initiated with a goal to standardize an ECM composition that can be easily coated on tissue culture polystyrene and several other materials that are used for cardiovascular tissue engineering. Such an ECM should support attachment and spreading of endothelial cells from all sources and give signals for cell proliferation and survival and prevent cell apoptosis and dedifferentiation.

I.4. OBJECTIVES

Researchers are still in search of an ideal matrix to support the growth of EC, without any adverse effects on the phenotype. In this scenario, objective of this study is to identify an optimal matrix for culturing EC *in vitro*, and also to act

as a matrix coating on the vascular biomaterials to support EC growth. The effect of cell-matrix interaction on the behavior of ECs were evaluated in terms of adhesion, proliferation, apoptosis and normal expression of prothrombotic and anti-thrombotic proteins, so that an ideal matrix can be selected for cell expansion and use in tissue engineering. The cells thus grown on a composite matrix were further seeded on different cardio vascular biomaterials, to demonstrate whether matrix dependent cell thrombogenicity has reflected on platelet adhesion also.

1. To standardize a matrix composition and the coating technique onto cell culture plates to attain optimum cell adhesion, spreading and proliferation.

2. To compare the composite matrix with conventional gelatin coating by evaluating the effect of passage progression on the cell behavior in terms of adhesion, proliferation, and apoptosis using HUVECs.

3. To evaluate the function of EC monolayer, by analysis of the mRNA expression pattern of pro-thrombotic and anti-thrombotic proteins, using reverse transcriptase-polymerase chain reaction (RT-PCR) and ELISA.

4. To standardize the isolation and culture of HSVECs from cardiovascular patients, and evaluate the behavior of these dysfunctional cells when grown on the composite matrix in terms of cell viability and protein expression pattern.

5. To standardize the composite matrix coating onto different biomaterials used as cardiovascular implants.

6. To compare the composite matrix coated devices with bare devices, in terms of EC adhesion, spreading, proliferation, and survival.

7. To compare the thrombogenicity of bare devices against EC grown conduits by platelet adhesion and coagulation studies.

Chapter II

MATERIALS AND METHODS

II.1. ISOLATION, CULTURE, AND CHARACTERIZATION OF ENDOTHELIAL CELLS

II.1.1. Preparation of crude Endothelial Growth Factor (ECGF) from Bovine Hypothalamus

Hypothalamus from young calf (~1 year old) was used for isolation of growth factors according to the method described by Maciang *et al.* (1979). Bovine calf brain was obtained (aseptically) from a local slaughterhouse in ice cold normal saline. The hypothalamus was removed carefully, without any blood-containing regions, and was cut into pieces of about 1 cm^2. Tissue was then homogenized for 3 min in 0.1 M NaCl (~500 ml), at 2-8°C. The homogenate was stirred for 2h at 4^0C, maintaining the pH at 7 and was subsequently centrifuged for 40 min at 13,800g at 4^0C in a refrigerated centrifuge (Hitachi, SCR20BA). The supernatant was recovered and mixed with streptomycin sulfate to get 0.5% w/v final concentration, and incubated overnight at 4^0C (pH 6.8 to 7) to extract the lipid content. The mixture was centrifuged at 4^0C for 40 min at 13,800g, and the supernatant was dialyzed against 0.1 M NaCl overnight at 4^0C. The dialyzed solution was again centrifuged for 40 min at 13,800g at 4^0C, and the supernatant obtained was dispensed into small aliquots containing 500 micrograms of Lowry`s protein, lyophilized in a freeze dryer (Modulyo 4K Freeze Dryer, Edwards, UK) and were stored at -20^0C for further use.

II.1.2. Quality check and analysis of Protein content in Crude ECGF

The quality of the extracted growth factor was verified by Poly Acryl amide Gel Electrophoresis (PAGE). 20 µg of the sample after digestion in sample buffer

containing dithiothreonate (DTT) and sodium docecyl sulphate (SDS) was subjected to PAGE in 12% gel as per the method of Laemmli (Laemmli U.K, 1970). Commercially available endothelial growth supplement (Product No. E-2759, Sigma Chemical Co., St. Louis, MO, USA) and high molecular weight markers (Sigma Chemicals, USA) were run in parallel lanes for comparison.

For estimation of protein content, the lyophilized growth factor was reconstituted in 1ml of PBS. The total protein content of the sample was estimated according to Lowry's method (Lowry, 1951).

II.1.3. Preparation of Sera

The blood collected from new born calf is allowed to clot for 1h at room temperature (RT) and kept at 4^0C overnight for clot retraction. The released sera were centrifuged, dialyzed against Hank's Balanced Salt Solution (HBSS), heat inactivated by incubating at 56^0C for 30 min, and frozen until use.

Human serum is prepared from freshly collected blood obtained from healthy donors. The sera are dialyzed against HBSS, heat inactivated and frozen until use.

Fetal Bovine Serum (FBS) is purchased from Gibco-BRL, USA. It is stored at -70^0C until use.

Before use the sera are sterile filtered through a 0.22 µm membrane filter (Millex GP, Millipore) at room temperature.

II.1.4. Isolation of Human Umbilical Vein Endothelial Cell (HUVEC)

Human umbilical cord was collected in ice cold Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) containing antibiotics (1000 U/ml of Benzyl penicillin and 1000 µg/ml of Streptomycin). All the veins for the study were collected from the Obstetrics Department of Medical College, Trivandrum, after informed consent. Both distal and proximal ends were tied with sterile silk just before removing the cord from the placenta to ensure sterility within the lumen.

Endothelial Cells are isolated from the umbilical vein using the methods of Jaffe *et al.* (1973) and Gimbrone *et al.* (1974) with slight modifications. The umbilical cord collected was brought to the tissue culture

laboratory within 4h of child birth and all the subsequent procedures were performed under laminar flow hood. The cord was washed with fresh ice cold HBSS and damaged area were discarded. A small piece was cut off at one end of the cord so that a fresh non retracted vein end was obtained, and the vein was cannulated with a custom made stainless steel cannula and secured with a 3-way stopcock. The lumen of the vein was washed thoroughly with ample quantity of ice cold HBSS using a syringe via the 3-way stopcock, to remove the blood. A small piece was cut off from the other end and a stainless steel cannula was inserted and secured with a 3-way stopcock. Subsequently the vein was filled with 0.2% type I collagenase (Sigma Chemicals, USA, and Product No. C-6885) in Medium 199 and the 3-way was closed. After 15 min incubation at 37^0C in a Petri plate containing HBSS, the surface of the cord was gently massaged to dislodge the digested cells. The content of the lumen were collected into a 50 ml sterile centrifuge tube (NUNC, Roskilde, Denmark), using a syringe through the 3-way. The lumen of the vein was flushed with additional 20 ml of M 199 to collect loosely attached cells and is pooled with the initial suspension. Cell suspension was centrifuged at 200g for 5 min at 4^0C in a tabletop refrigerated centrifuge (Biofuge Stratos, Haeraeus), and the cell pellet was washed twice with M 199 by repeat centrifugation. The final cell pellet was re-suspended in complete MCDB 131 medium (Gibco-BRL, USA) containing 20% FBS (Gibco-BRL, USA), 200 µg/ml growth factor mixture, Streptomycin sulfate (100 µg/ml) and Benzyl Penicillin (100 U/ml). The cell suspension was then seeded on 25 cm^2 culture flask (NUNC), pre-coated either with gelatin, or with fibrin composite containing fibrin, gelatin and growth factors. The culture flask was incubated under 5% CO_2 at 37^0C in an incubator (Thermo Forma 3110, USA). The culture was fed with fresh medium every alternate day until confluence.

II.1.5. Human Saphenous Vein Endothelial cell (HSVEC) isolation

The residual pieces of saphenous vein obtained from patients undergoing coronary artery bypass grafting (CABG) surgery from the department of cardiothoracic surgery was collected in ice cold HBSS and stored in refrigerator until use. The vein was brought to the tissue culture laboratory and all the subsequent procedures were performed under the laminar flow hood. The lumen of the 2-5 cm long vein was flushed with ~10 ml of serum free M199 medium. The lumen was filled with 0.2% collagenase in serum-free M199 and was incubated

for 15-20 min at 37^{0}C. After gentle massaging, the dislodged cells were collected into a 10 ml sterile centrifuge tube (NUNC), using a syringe through the 3-way. The lumen of the vein was flushed with additional 5 ml of M 199 to collect loosely attached cells and was pooled with the initial suspension. Cell suspension was centrifuged at 200g for 5 min at 4^{0}C in a tabletop refrigerated centrifuge (Biofuge Stratos, Haeraeus), and the cell pellet was washed twice with complete MCDB 131 medium by repeat centrifugation. The final cell pellet was re-suspended in complete MCDB 131 medium, and was seeded into one of the wells of a 4 well tissue culture plate (NUNC), pre-coated with either gelatin, fibrin composite containing fibrin, gelatin and growth factors. A mild wash was given after 6h of seeding to remove loosely attached cells and debris. Fresh medium was added every alternate day until confluence. The culture conditions are same as described above for HUVEC.

II.1.6. Serial propagation of HUVEC and HSVEC

When the cells attain confluence, the cells were sub cultured. For harvesting, cells were washed with fresh warm (37^{0}C) serum free M199 two times and the monolayer was incubated with 0.25% trypsin-EDTA solution (Gibco-BRL, USA) until the cells start to detach (usually 3-5 min), followed by immediate dilution in ice cold complete medium to inhibit further trypsin activity. Slowly mixed the cell suspension using a sterile pipette and transferred into a 10 ml centrifuge tube. The cell suspension was immediately centrifuged at 200g for 5 min at 4^{0}C, re-suspended in warm complete medium and seeded in fresh pre-coated culture dishes at split ratio 1:3.

II.1.7. Characterization of Endothelial Cells

Typical endothelial nature of the cells was assessed by morphological analysis and by using specific ligands/antibodies conjugated with fluorescent probes.

II.1.7.1. Morphology Analysis

Routine evaluation of the quality and growth pattern of the cultured cells was done using an inverted phase contrast microscope (Leica DM IRB, Geramany) at 10x, 20x and 40x magnifications. The cells were characterized by the typical cobblestone morphology on attaining confluence.

II.1.7.2. Uptake of Fluorescent labeled Acetylated LDL

Acetylated LDL is taken up by macrophage and endothelial cells that possess scavenger receptors specific for the modified LDL. Endothelial cells in primary cell culture can be identified by their ability to take up fluorescent AcLDL.

Cells grown on pre-coated cover slip bottom tissue culture plates up to near confluent stage was used for DiI AcLDL uptake studies as per the method described by Voyta *et al* (1984) with some modifications. In the near confluent stage, the medium was replaced with fresh MCDB 131 containing 10% FBS and 10 µg/ml of 1,1-dioctadecyl-3,3,3,3-tetramethyl Indocarbocyanine perchlorate (DiI) labeled Acetylated Low Density Lipoprotein (DiI AcLDL) (Molecular Probes, USA Cat No. L3484) and replaced in the CO_2 incubator. After 4h the culture plate was removed and washed three times with warm serum free M199 and viewed under inverted fluorescent microscope (Leica DM IRB, Geramany), using Rhodamine Filter.

II.1.7.3. Conjugation of Anti Mouse IgG raised in Rabbit with FITC

The polyclonal anti-mouse IgG (Sigma Chemicals, USA) was concentrated to get 2mg/ml of protein. The Fluorescene Isothiocyanate (FITC) (Sigma Chemicals, USA) was dissolved in Dimethyl Sulphoxide (DMSO) at a concentration of 1mg/ml. For each 1ml of protein solution 50 µl of the dye solution was added slowly with gentle stirring. The reaction was allowed to proceed for 8h at 4^0C with continuous stirring. Subsequently, NH_4Cl was added to get 50 mM final concentration and incubated for 2h. The unbound dye was separated from the conjugate by passing through a column of Sephadex gel matrix with an exclusion limit of 20,000 -50,000 (Sephadex G50).

II.1.7.4. Immunofluorescence analysis for vWF

The monolayer of endothelial cells grown on pre-coated 4-well cover slip bottom culture plates (NUNC) was fixed with 3.7% Para formaldehyde in PBS for 30 min. The fixed monolayer was washed with PBS three times and, quenched for 20 min with 0.27% NH_4Cl/0.38% glycine in PBS. Washed again with PBS and permeabilized with 0.5% Triton X-100 (Sigma Chemicals, USA) in PBS. After washing thoroughly with PBS, incubated with monoclonal anti-human von Willebrand Factor (vWF) antibodies (primary antibody) raised in mouse (Dako,

Denmark; 1:200 dilution) in blocking buffer for 30 min. Following this, wells were washed with PBS three times, and then incubated with 50 µl of FITC conjugated polyclonal anti-mouse IgG antibodies raised in rabbit (secondary antibody) in blocking buffer at a dilution of 1:100 for 20 min. Finally the monolayer was washed thoroughly with PBS, and the cells were viewed using a fluorescence microscope (Leica, DM IRB) using FITC filter.

II.2. EFFECT OF MATRIX COMPOSITION ON EC SPREADING, PROLIFERATION, SURVIVAL AND PHENOTYPE

II.2.1. Preparation of matrix coated Tissue Culture Polystyrene (TCPS)

II.2.1.1. Gelatin coated Polystyrene (GCPS)

2% gelatin (Sigma Chemicals, USA) was prepared in PBS and autoclaved for 15 min (121^0C, 15 Psi) prior to use. Can be used immediately or stored in refrigerator for future use up to one month. To prepare gelatin coated polystyrene (GCPS), culture plates (NUNC) of appropriate size were added with 2% gelatin, and incubated for minimum 2h at 37^0C. The excess solution was aspirated out just before cell seeding.

II.2.1.2. Fibrin coated Polystyrene (FCPS)

Bovine thrombine (Merck) was reconstituted in 0.035 M CaCl2 at a concentration of 5 IU/ml. The second component human fibrinogen was prepared from screened pooled human plasma by cryoprecipitation method and stored in the lyophilized form. Just before use, this was reconstituted in distilled water to get 2 mg/ml final concentration (Total protein ~2.7 mg/ml). To make fibrin coated polystyrene (FCPS), culture plates were incubated with bovine thrombin (~ 2.5 IU cm^{-2}) for 30-60 min at RT. After removing the excess thrombin solution, the surface was added with 10 µl of cryoprecipitate for each cm^2 area with 20 µg of added ECGF. Fibrin was allowed to polymerize at 37^oC for 30min and were then frozen at -70^oC for at least 2h and lyophilized. The freeze dried dishes were either used immediately for cell seeding or stored in the refrigerator for later use.

II.2.1.3. Composite coated Polystyrene (CCPS)

For composite coated polystyrene (CCPS), culture plates were incubated with bovine thrombin (~ 2.5 IU cm^{-2}) for 30-60 min at RT. The remaining process

is similar to that for FCPS but cryoprecipitate-ECGF mixture was premixed with 0.02% w/v of gelatin.

II.2.2. EC Adhesion and Spreading

Fluorescent phallotoxins stain F-actin at nano molar concentrations and are extremely water soluble, thus providing convenient probes for labeling, identifying, and quantitating F-actin in cell cultures. Unlike antibodies, the binding affinity does not change appreciably with actin from different species or sources. Non specific staining is negligible, and the contrast between stained and unstained areas is extremely large.

EC was stained for F-Actin with Texas Red Phalloidin (Molecular Probes, USA; cat No. T7471) and microscopically analyzed the actin filament assembly. Third passage cells were used for the study. The cell count was noted using a Haemocytometer and 4 x 10^4 cells were seeded onto GCPS, FCPS and CCPS each in 4-well cover slip bottom tissue culture plates and supplemented with 0.5ml complete medium (each well 2 cm^2). For analysis of cell adhesion behavior, the medium was removed 2h and 24h after seeding, using separate wells for each period, washed with phosphate buffered saline (PBS) and were fixed using 3.7% Para formaldehyde. The fixed cells were permeabilized using 0.1% Triton-X100 (Sigma Chemicals, USA) in PBS. After washing three times with PBS, cells were stained for actin with Texas red Phalloidin for 20 min in the dark at 37^0C as described by Lewis and Bridgman (1992). Finally, washed thoroughly with PBS, and the cells were viewed using a fluorescence microscope (Leica, DM IRB) using Texas red filter. For long-term storage, the cells were air dried and then mounted in a permanent mountant ProLong ® Gold, and stored in the dark at (2–6) °C.

The fluorescence intensity at specific area was estimated by viewing the stained cells under a confocal microscope (TCS SP2 AOBS Laser Scanning Spectral Confocal Microscope, Leica Microsystems, Germany). A series of 'Z' sections of a selected area of each sample was observed and the data was computed to find out the fluorescence intensity at each sections using Leica Confocal Software (LCS Ver. 2.5).

II.2.3. EC Proliferation Assay

II.2.3.1. By Time taken to form a confluent monolayer

Proliferation of endothelial cells was evaluated till 15th passage on both GCPS and CCPS by microscopic analysis for monolayer formation. Time was given for each passage to grow into a confluent monolayer and once the monolayer formation is confirmed by microscopic examination, cells were harvested using trypsin, counted and reseeded in a 1:2 split ratio. Cells were counted using a Haemocytometer to ensure that, same number of cells was seeded into the new flask in each case. The proliferation rates on each of the matrix studied were tabulated and a graph was plotted.

To determine the population doubling time on different substrates, confluent cells at the end of 3, 6, 9, 12 and 15 passages were trypsinized and 2 x 10^4 cells in 0.5 ml each were seeded onto coated 4 well culture plates. The cells were harvested after a specified time period (72 h till passage 6 and 120h for passages 9, 12 and 15). The population doubling time was calculated using the equation as below:

Population Doubling Time (T_D) = 0.693/k

Where, k = $[2.3 \log N_2/N_1] / \Delta t$

N1 = Number of cells at onset (Time T_0)

N2 = Number of cells after Time T_1

Δt = $T_1 - T_0$

II.2.3.2. PCNA Immuno Staining

In addition to microscopic analysis, confluent cells at the end of 3, 6, 9, 12 and 15 passages were trypsinized and 2 x 10^4 cells in 0.5 ml each were seeded onto coated 4 well cover slip bottom culture plates. The cells were fixed after 24h using 4% Para formaldehyde and permeabilized with 0.2% TritonX 100. After labeling with FITC conjugated mouse anti human antibody against PCNA (Serotec, UK), cells were viewed under fluorescent microscope (Leica DM IRB). About 20 cells each from 5 different fields were counted and percentage of PCNA positive cells was estimated.

II.2.3.3. By ^{3}H-thymidine Uptake

To quantify initial attachment and proliferation of EC on different matrices, ECs in log phase were labeled with tritiated thymidine (^{3}H-thymidine) (American Radio Chemicals, USA) by placing 20 µCi ^{3}H-thymidine /25cm^2 flask for 72h at 37°C [Badylak *et al.* 1999]. Cells were then harvested and 4 well flat bottom culture plates coated with different matrices were seeded with 4 x 10^4 cells. After 2h of seeding, the unattached cells were removed, and DNA extracted for ^{3}H counting as per the method standardized by Sivkumar et al. [1992]. Briefly, the cells were solubilized with 0.5 ml of 0.1 M NaOH containing 0.1% SDS and precipitated with 0.5 ml of 10% Trichloroacetic acid (TCA) (Sigma) for 1h at 4°C. Then it was centrifuged at 10,000g for 15 min at 4°C in Biofuge Stratos (Haeraeus, Germany). To the pellet, 1ml absolute alcohol was added and kept in ice bath for 30 min. Centrifuged again at 10,000g for 15 min at 4°C. The resulting pellet was air dried and dissolved in 0.5 ml of 0.1 N NaOH. An aliquot containing 5 x 10^4 cells was also processed in the same way for ^{3}H counting and the count per minute (CPM) was detected using a liquid scintillation counter (Wallac 1409, LKB, Sweden). The number of cells attached was calculated from the counts of unattached cells and the count of known number of ^{3}H-thymidine-loaded cells.

To quantify cell proliferation, after removing unattached cells at 2h of seeding, the attached cells were fed with complete MCDB 131 medium containing 2µCi ml^{-1} ^{3}H thymidine. After 48h, medium was changed with fresh medium containing ^{3}H thymidine and at 72h; DNA was extracted as described above for ^{3}H counting.

II.2.4. EC apoptosis assay

II.2.4.1. Apoptosis Assay by Fluorescence Microscope

Cell viability, on different matrix coated surfaces were analyzed using Vybrant Apoptosis Assay Kit #3 (Molecular probes Inc OR. Cat No. V13242). The kit provides a rapid and convenient assay for apoptosis. The kit contains recombinant Annexin V conjugated to fluorescein Iso Thio Cyanate (FITC annexin V), as well as a ready-to-use solution of the red-fluorescent Propidium Iodide (PI) nucleic acid binding dye. The human anticoagulant, Annexin V, is a 35–36 kD Ca 2+ -dependent phospholipid binding protein that has a high affinity for Phosphatidylserine (PS). In normal live cells, PS is located on the cytoplasmic

surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. Annexin V labeled with a fluorophore can identify apoptotic cells by binding to PS exposed on the outer leaflet. PI is impermeant to live cells and apoptotic cells, but stains dead cells with red fluorescence, binding tightly to the nucleic acids in the cell. After staining a cell population with FITC Annexin V and PI in the provided binding buffer, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence.

To assess the effect of matrix coating on cell viability, confluent cells grown on different matrix coated culture plates at 3, 6, 9, 12 and 15 passages were trypsinized, and a suspension with $2x10^4$ cells in 0.5 ml complete MCDB 131 medium was seeded onto coated cover slip bottom 4 well culture plates (NUNC). 72h after seeding, cells were analyzed for apoptosis using Vybrant Apoptosis Assay Kit according to the manufacturer's instructions. Briefly, the cells after the incubation period were washed in cold PBS, and incubated for 15 min with 50 µl of the annexin V conjugate in annexin- binding buffer supplied with the kit added with 0.5 µl of the 100 µg/ml PI working solution. After staining, the cells were washed thoroughly with PBS and were viewed using an inverted fluorescence microscope (Leica, DM IRB). Percentage of apoptotic cells were calculated from total cells in 10 to 12 fields counted under phase contrast microscopy and the number of fluorescent cells in the respective fields viewed under fluorescence filter.

II.2.4.2. Apoptosis Assay by Flow Cytometry

For Flow cytometric analysis of apoptosis, confluent cells grown on different matrix coated culture plates at 3, 6, 9, 12 and 15 passages were trypsinized, and washed with ice cold PBS. The resulting cell pellet was re-suspended in 100 µl of annexin- binding buffer (cell density ~$1x10^6$cells/ml) and added 5 µl annexin V conjugate and 1 µl of the 100 µg/mL PI working solution. Incubated for 15 min at RT and after the staining, the cells were diluted with FACS sheath fluid and analyzed using a flow cytometer (FACSAria, BD Biosciences) to estimate the percentage of live/apoptotic/dead cells.

II.2.5. EC Phenotype

The phenotypic alterations of ECs as an effect of serial passaging on different matrices were compared by mRNA expression assay for two thrombotic proteins and two anti-thrombotic proteins synthesized by EC, and the release of each of these molecules into the culture media was estimated.

II.2.5.1. mRNA expression study by Reverse transcriptase polymerase chain reaction (RT-PCR)

ECs were grown on 25cm^2 GCPS and CCPS culture flasks and cells were harvested using 0.25% Trypsin-EDTA (Gibco BRL, USA) when 3, 6, 9, 12 and 15 passages attained confluence as described earlier. Isolation of RNA was done using a commercial kit (Stratagene, USA), according to the manufacturer's instructions. After purification, 20 ng of RNA quantified by spectro photometric measurement (Hewlett Packard Diode array 8453, USA) was utilized for RT-PCR using Brilliant RT-PCR kit (Stratagene, USA). After the reverse transcription reaction (at 45^{0}C for 1h, 90^{0}C for 5min), PCR was performed in the same tube (at 94°C for 30 sec, at 60°C for 45 sec, and at 72°C for 1 min), for 30 cycles on a mini PCR system (Master cycler-Personal, Eppendorf, Germany). Primer sequences were determined using Primer3 software. Accession numbers of genes of interests, nucleotide sequences of primers and expected size of amplicons are provided in the Table II.1. PCR products were then fractionated on a 1% agarose gel stained with ethidium bromide. Molecular weight marker (100 bp DNA ladder; Stratagene) were also loaded in adjacent lanes. The ethidium bromide stained DNA in the gel were photographed using a DC 120 Kodak digital camera (Eastman Kodak, Rochester, NY) for the verification of size and analysis of band intensity using Multi Analyst Software (Biorad, USA).

II.2.5.2. mRNA expression study by Real Time PCR

ECs were grown on 25cm^2 GCPS and CCPS culture flasks and cells were harvested using 0.25% Trypsin-EDTA (Gibco BRL, USA) when 3, 6, 9, 12 and 15 passages attained confluence as described earlier. Isolation of RNA was done using a commercial kit (Stratagene, USA), according to the manufacturer's instructions. After purification, 20 ng of RNA quantified by spectrophotometric measurement (Hewlett Packard Diode array 8453, USA) was utilized for reverse transcription (RT) using StrataScript Reverse Transcriptase (Stratagene, USA).

After the RT reaction, real time PCR was performed using DyNamo SYBR Green qPCR kit (FINNZYMES, USA), using 8 µl of the cDNA, 10 µl PCR mastermix and 1 µl each of forward and reverse primers, for 30 cycles (at 94°C for 30 sec, at 60°C for 45 sec, and at 72°C for 1 min), on a real time PCR machine (Chromo 4, MJ Research, USA). Sequences of primers were determined using Primer3 software. Accession numbers of genes of interests, nucleotide sequences of primers and expected size of amplicons are provided in the Table II.1.

Table II-1. Sequences of primers used in RT-PCR

Transcripts	Accession Number	Primer Sequence (5' to 3')	Amplicon size (Base pairs)
vWF	NM_000552	F: caccattcagctaagaggagg R: gccctggcagtagtggata	298
tPA	NM_000930	F: atgggaagacatgaatgcac R: gaaaggggaaggagacttga	319
PAI-1	NM_000602	F: ttggtgaagggtctgctgtg R: ggctcctttcccaagcaagt	485
eNOS	NM_000603	F: agctgtgctggcatacagga R: atggtaacatcgccgcagac	486
β Actin	NM_001101	F: ctatccaggctgtgctatcc R: atgccagggtacatggtg	523

Sequences, amplicon size and accession number of primers used in RT-PCR. Primer sequences were determined using Primer3 software from the Internet http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.

II.2.5.3. Detection of proteins released into the media by ELISA

For ELISA, ECs were grown on coated 25 cm^2 culture flasks and when 2, 5, 8, 11 and 14 passages attained confluence, medium was changed completely with fresh medium and cells were maintained for 24h. 1 ml media was taken for vWF, t-PA and PAI-1 assay. The withdrawn medium was centrifuged at 4^0C to remove particles/debris and stored frozen until the assay was performed.

The Assay was done using ELISA kit (Helena Biosciences, UK) according to the manufacturer's instructions. Briefly, 100 µl of the test sample and serial dilutions of the particular standard provided with the kit were introduced to the microwells coated with monoclonal antibody against the protein of interest. Incubated for 1h at room temperature (RT) and washed 5 times with wash solution provided with the kit using an ELISA plate washer. Then 100 µl of the monoclonal antibody coupled to horse radish peroxidase (HRP) was added and incubated for another 1h at RT. Washed again with the wash solution and immediately after washing, added 200 µl of the HRP substrate, 3,3',5,5' – Tetramethylbenzidine (TMB)/H_2O_2 for color development and incubated exactly for 5 min, and stopped the reaction by adding 50 µl of 0.45 M Sulfuric acid supplied with the kit. Waited for 10 min to allow the color to stabilize and the optical density was measured at 450nm using an ELISA reader (DIGISCAN, Asys Hitech, Austria). The standard curve was generated using DigiWin 3 software and the level of the protein was quantified.

II.2.5.4. Assessment of NO synthesis

Nitrite, which is the stable metabolite of nitric oxide, was estimated by modified Griess reagent (SIGMA, USA). For this assay, ECs were grown on matrix coated 4 well culture plates and when 2, 5, 8, 11 and 14 passages attained confluence, the NO release was analyzed as nitrite content in the medium. To eliminate the protein interference, complete medium from the culture wells were removed 24h before estimation and fresh serum-free, phenol red-free M199 medium (SIGMA, USA) was added. After 24h incubation, medium was collected, centrifuged to remove particulates if any, and added an equal volume of Griess reagent. Incubated for 15 min at room temperature and color developed was read at 540 nm using a diode array spectrophotometer (Hewlett Packard 8053, USA). The amount of nitrite formed was calculated from a calibration curve obtained using serially diluted sodium nitrite as standard.

II.3. EC SEEDING ON BIOMATERIALS: TISSUE ENGINEERING OF CARDIOVASCULAR DEVICES

II.3.1. Study Materials

Woven Dacron Patches and 6mm diameter pieces of Dacron vascular graft (Single velour polyester fabric, Meadox Medicals Inc.), Stainless steel (SS)

of 316L grade (1cm^2 flat pieces), polished (0.1μ Ra) titanium (Ti) discs of 15 mm diameter, ultrahigh molecular weight polyethylene (UHMWPE) of Himount 1900 grade of 15 mm diameter, and Ti discs coated with diamond like carbon (DLC) by plasma enhanced vapor deposition method. The materials were sterilized by autoclaving and washed thoroughly with serum free medium, before cell seeding/matrix coating.

II.3.2. Coating of the materials with gelatin

The graft pieces and other metallic and polymer materials after autoclaving were coated with gelatin by immersing in 2% gelatin in PBS and incubating at 37^{0}C as per ξII.2.1.1 (as done for TCPS coating). The excess solution was aspirated out just before cell seeding.

II.3.3. Coating of Materials with the Composite matrix

The procedure used is more or less same as that used for TCPS coating ξ2.2.1.3. The materials were kept immersed in the thrombin solution for 30-60 min. After removing the excess thrombin solution, the cryoprecipitate-ECGF mixture premixed with 0.02% w/v of gelatin was layered onto the surface and allowed to polymerize for 30 min at 37^{0}C. The coated and freeze-dried materials are validated for immediate cell seeding or can be stored at 4°C for future use.

II.3.4. Endothelial cell culture on bare and Coated Materials

Before seeding endothelial cells, all the materials were washed with serum free medium, ECs harvested from 25cm^2 culture flasks were counted using a hemocytomter and ~ 4 x 10^4 cells/cm^2 were seeded onto each material. After 6h, the unattached cells were removed and fresh complete medium was added. Culture conditions were similar as described earlier. Medium was changed every alternative day, and allowed to grow for 5 days before performing the studies described below. At this seeding density, cells from passage 3-5, grown on gelatin coated culture flasks usually attain confluence by 3-4days.

II.3.5. EC spreading on Materials – Fluorescent staining for actin

HUVEC was stained for F-Actin with Texas Red Phalloidin (Molecular Probes) and microscopically analyzed the actin filament assembly. Third passage cells (2x 10^4 cells cm^{-2} area), were used for seeding each material. For analysis

of cell adhesion behavior, 24 h after seeding, each of the materials was washed with phosphate buffered saline (PBS), the cells were stained for actin as described earlier in ξ2.2.2.

The fluorescence intensity at specific area was estimated by viewing the stained cells under a confocal microscope (TCS SP2 AOBS Laser Scanning Spectral Confocal Microscope, Leica Microsystems, Germany). A series of 'Z' sections of a selected area of each sample was observed and the data was computed to find out the fluorescence intensity at each sections using Leica Confocal Software (LCS Ver. 2.5).

II.3.6. Identification of Focal Adhesion by Vinculin Staining

For immunostaining, third passage HUVECs (2x 10^4 cells cm^{-2} area), were seeded onto bare and coated bio materials, and after 24h in culture, cells were fixed with 3.7% Para formaldehyde in PBS, washed with PBS, and permeabilized with Triton X-100 (0.5%) in PBS. Vinculin was stained by incubation with mouse anti-human vinculin antibody (SIGMA) for 30 min followed by incubation with HRP labeled secondary antibody (SIGMA) for 30min and incubation with HRP substrate DAB (SIGMA). The cells were counter stained using Harry's haematoxylin and were viewed using a Light Microscope (Lieca MPS30, Switzerland).

II.3.7. Cell Attachment and Proliferation on Materials

To quantify initial cell attachment, HUVEC in log phase were labeled with tritiated thymidine (^{3}H-thymidine) (American Radio Chemicals, USA) by placing 20 µCi ^{3}H-thymidine /25cm^2 flask for 72h at 37°C. These cells were harvested and 4 x 10^4 cells cm^{-2} were used for seeding each device/material. After 2h of seeding, the unattached cells were removed, and DNA extracted for ^{3}H counting as per the method standardized by Sivkumar et al. [1992]. An aliquot containing 5 x 10^4 cells was also processed for ^{3}H counting and was detected using a liquid scintillation counter (TRIATHLER Multi-label tester, HIDEX, Finland). The number of cells attached was calculated from the counts of unattached cells and the count of known number of ^{3}H-thymidine-loaded cells.

To quantify cell proliferation, the unattached cells were removed after 2h of seeding, and attached cells were fed with complete MCDB 131 medium

containing 2μCi ml^{-1} ^3H thymidine. After 48h and 96h, medium was changed with fresh medium containing ^3H thymidine and at 120h cells were harvested from separate devices and assayed for ^3H, as described above.

II.3.8. EC Apoptosis on Materials

Cell viability, on bare and composite coated surfaces were analyzed using Vybrant Apoptosis Assay Kit (Molecular probes Inc OR, USA.). Briefly, EC suspension with 2 x 10^4 cells cm^{-2} in complete MCDB 131 medium was seeded on bare and coated surfaces and culture was maintained for 72 h, after which the staining was done. Percentage of apoptotic cells were calculated from total cells in 10 fields counted under phase contrast microscopy at 10x magnification, and the number of fluorescent cells in the respective fields viewed using a fluorescence filter (Leica, DM IRB).

II.3.9. Quantification of mRNA expression by HUVECs grown on study materials by Real Time PCR

ECs were harvested using 0.25% Trypsin-EDTA (Gibco BRL, USA) after 5 days in culture. Isolation of RNA was done using a commercial kit (Stratagene, USA), according to the manufacturer's instructions. 20 nanogram of isolated RNA quantified by spectrophotometric measurement (Hewlett Packard Diode array 8453, USA) was utilized for reverse transcription (RT) using StrataScript Reverse Transcriptase (Stratagene, USA). The rest of the protocol is similar as discussed earlier in section II.2.5.2.

II.3.10. Assessment of NO Release

Nitrite, which is the stable metabolite of NO, was estimated using Griess reagent (Sigma). For this assay, bare (B_Ti, B_DLC-Ti and B_UHMWPE) and composite coated materials (CC_Ti, CC_DLC-Ti and CC_UHMWPE), with HUVEC grown on them for a period of 120h, were used. To eliminate the protein interference, complete medium was replaced with fresh serum-free, phenol red-free M199 medium (Sigma). The rest of the protocol is similar as discussed earlier in section II.2.5.4.

To detect NO release under shear stress, the devices with a monolayer of HUVEC grown on them were placed in a parallel plate flow chamber and exposed to the flow. Phenol red-free M199 medium was perfused at a flow rate of

50 mL/min using a peristaltic pump for 1 h at room temperature (Shear stress ~35 dynes cm^{-2}). The medium was collected after 1h for NO assay.

II.3.11. Effect of shear stress on EC monolayer

Bare and coated Dacron graft pieces of 5cm length and 6mm diameter were seeded with HUCEV at a cell density of 10^5 cells cm^{-2} area and cultured in complete MCDB 131 medium for 72h. Then the monolayer was washed with fresh warm complete medium and exposed to complete medium under dynamic conditions (shear stress 20 dynes cm^{-2}) for 1h, using a peristaltic pump (MIDI-Digital, Cole Parmer, USA). After exposure, the cells were washed with PBS three times, fixed with 0.5% gluteraldehyde (Sigma) for 1h and stained using Maygrunwald stain (Sigma). The graft pieces were cut open and fixed on a slide and viewed under light microscope (Lieca MPS30, Switzerland) to assess the cell detachment on exposure.

II.3.12. Thrombogenecity assessment of the surfaces

Thrombogenic nature of the bare and coated material surfaces were assessed by exposing to platelet rich plasma and determining the platelet consumption, platelet adhesion, and variations in coagulation parameters such as Partial Thromboplastin Time (PTT) and Fibrinogen.

II.3.12.1. Preparation of Platelet Rich Plasma (PRP)

Whole blood obtained from healthy human volunteers was collected in citrate phosphate dextrose (CPD). This was centrifuged at 200g for 10 min (Labofuge 300, Heraeus, Germany) to obtain platelet rich plasma (PRP).

II.3.12.2. Platelet Consumption Study

The materials were fixed in 4 well culture plates, and the surface was added with 1 ml PRP with a count of (2 – 2.5) x 10^8/ml and agitated for 1h in an environ shaker (Labline Instruments Inc, IL, USA) at 37^0C and at a speed of 70 ± 5 RPM. After 1 min and 1h of exposure, PRP samples were taken for analysis using a clinical Hematology Analyzer (Cobas Minos Vet, Roche Diagnostics, France) to determine the platelet counts. The percentage of platelets adhered was calculated from the count reduction at the end of 1h of exposure.

II.3.12.3. Platelet adhesion study

II.3.12.3.1. Qualitative By Scanning Electron Microscope (SEM)

Bare and modified materials such as Dacron graft, Ti, DLC coated Ti, UHMWPE were seeded with endothelial cells (10^5 cells cm^{-2} area) and cultured for 120h to form a monolayer. The materials with a monolayer of EC grown on the surface were exposed to PRP with a count of (2 – 2.5) x 10^8/ml for 1h under agitation in an environ shaker at a speed of 70 ± 5 rpm at 37 ± 2°C. After the exposure period the grafts were washed with PBS, and fixed with 2% gluteraldehyde overnight at 4°C. Then, dehydrated with graded concentrations of alcohol (30%, 50%, 70%, 90%and finally 100%), and then with amyl alcohol, and critical point dried, gold sputter coated and viewed under a scanning electron microscope (Hitachi S2400, Japan).

II.3.12.3.2. Quantification of Platelet Adhesion by Radioscintigraphy

II.3.12.3.2.a. Labeling of Platelets with ^{125}I

The labeling is done according to the method of Resmi *et al.* (2004). Briefly, the PRP was centrifuged at 1000g for 15 min and the platelet pellet was re-suspended and centrifuged (three repetitions) with a modified Tyrode's Buffer (Annexure - I) containing 10% Acid citrate dextrose (ACD). The final pellet was re-suspended in Tyrode's buffer without ACD. Labeling of platelets with ^{125}I (American Radio Chemicals, USA) was done in a ventilated fume hood using lactoperoxidase (Sigma, Cat no. 29H3778) in presence of H_2O_2 (Sigma) ^{125}I and the enzyme was added to the platelet suspension to the required concentrations (0.2 mCi ^{125}I / 10^9 platelets; 5 nM final concentration lactoperoxidase). Iodination reaction was done by addition of 5µl aliquots of 1mM H_2O_2 at 1 min intervals for 5 min. After the reaction, free ^{125}I was removed from labeled platelets using a column of sepharose 2B (Sigma) according to the method of Tangen et al (1971). Platelets are eluted in the void volume. After labeling platelet count was taken using Hematology Analyzer (Cobas Minos Vet, Roche Diagnostics, France).

After each labeling experiment, aliquots of known platelet number were spot dried on a stainless steel template and exposed to phosphor screen. The radioactivity on labeled platelets and in isotope solution used for labeling was

detected using Molecular Phosphor Imaging System (GS505, Biorad, USA). Labeling efficiency (LE %) of each experiment was calculated.

II.3.12.3.2.b. Exposure of materials to labeled PRP

The radio labeled platelets were diluted using autologous plasma to get a platelet count of (2 - 2.5) x 10^8 /ml. Exposure of bare materials and those seeded with EC to radio labeled PRP were carried out in the same way as explained for SEM. After exposure, the materials were washed thoroughly with PBS to remove nonspecifically bound platelets. The adhered platelets were fixed with 2% gluteraldehyde, overnight at 4^0C. After fixation, materials were rinsed with PBS, dried and scanned using Molecular Phosphor Imaging System (GS505, Biorad, USA).

II.3.12.3.2.c. Quantification of Adhered Platelets

Aliquots of labeled platelets with known counts, and the isotope solution used for labeling were spot dried on a stainless steel template. The materials that are fixed and dried after exposed to labeled PRP were fixed on stainless steel template, and were exposed to the phosphor screen together with the dried platelet aliquots and imaged using the Phosphor Imaging System. The mean count per min was obtained and the calibration curve of radioactivity against platelet number was generated using the software Multi Analyst (Biorad, USA) from the radio-image intensities of serially diluted known number of platelet aliquots. The same software detected the number of platelets adhered on the materials, using the calibration curve, based on the image intensity.

II.3.12.4. Coagulation Analyses

The materials are exposed to fresh anticoagulated whole blood as explained above and plasma was separated from blood before and after exposure. The Partial Thromboplastin Time (PTT) and fibrinogen concentration were detected using a coagulation analyzer (STart 4, Diagnostica Stago, France), by strictly adhering to the ISO 10993 Guidelines. These tests are standardized and validated in our laboratory and is accredited against quality system ISO/IEC 17025:2005(E) by COFRAC (France).

II.4. STATISTICAL ANALYSIS

Quantitative data was initially analysed to confirm if the distribution was normal or skewed. Data normally distributed (i.e. with no significant difference between the mean and the median) were analysed by unpaired student's t-test or Anova test. Data whose distribution was skewed (i.e. there was a significant difference between the mean and the median) were analysed by Mann-Whitney U test and the Kruskal-Wallis test. Data were correlated using the MS Excel software.

Chapter III

STANDARDIZATION OF SUITABLE MATRIX FOR HUVEC CULTURE

III.1. ABSTRACT

Importance of endothelium in maintaining a thrombus-free luminal surface is substantial; accordingly *in vitro* seeding of EC to improve blood compatibility of cardiovascular devices is under evaluation in various laboratories. It is understood that many functions of human vascular EC are dynamic and that in long term cultures endothelial cells lose the potential to regenerate with the progress in passage number; however, the related phenotypic alteration is not so well studied. Endothelial cell proliferation response to growth factors is dependent on extracellular matrix and its absence render cells apoptotic. Signaling for growth, survival and differentiation is induced by specific matrix components that are different for each cell type. Therefore, it is important to use suitable composition of matrix coating to expand the cell number in tissue culture and to seed EC on biomaterial surface, so that cells remain viable and functional. A combination of matrix components which supports EC growth and survival in the normal physiological conditions may be the best choice of matrix composition for *in vitro* cell culture.

In this study it is attempted to formulate a composite matrix, containing fibrin, fibronectin, and gelatin with growth factors enmeshed in the matrix. The effect of cell-matrix interaction on the behavior of HUVEC were evaluated in terms of adhesion, proliferation, apoptosis, m-RNA expression and synthesis of prothrombotic and anti-thrombotic factors to assure that cells grown on the

selected matrix maintain a normal phenotype. Once proven as an ideal matrix the composition can be used for cell expansion and use in modification of surfaces by tissue engineering.

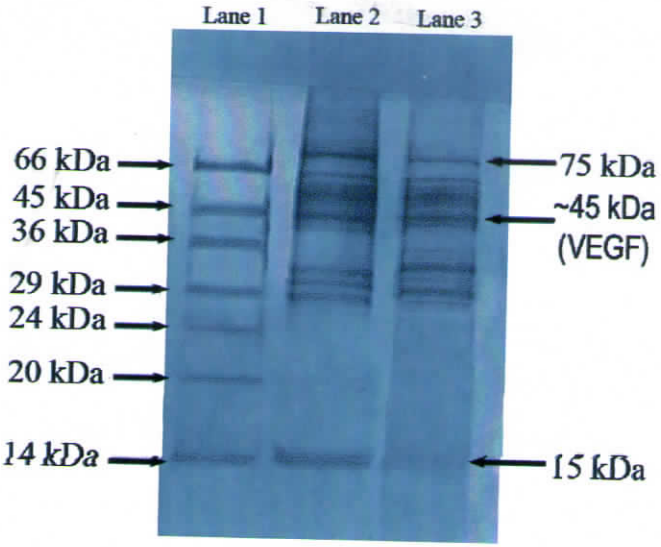
The results of the experiments that are presented in this chapter were carried out with the objectives; 1, compare the influence of composite matrix with conventional gelatin coated surface by evaluating the effect of passage progression on the cell behavior in terms of adhesion, proliferation, and apoptosis using HUVEC, 2, evaluate the function of EC monolayer by analysis of the mRNA expression pattern of pro-thrombotic and anti-thrombotic proteins, using reverse transcriptase-polymerase chain reaction (RT-PCR) and quantification of the synthesized products.

III.2. CULTURE AND CHARACTERIZATION OF HUVECS

III.2.1. Selection of Growth Factor, Serum and Medium

Previous investigations have shown that vascular endothelial cells can be maintained in culture only using a medium supplemented with optimal concentrations of growth factors from bovine hypothalamus or pituitary glands. During this study, isolation of growth factors from bovine source was done in house as per the protocol II.1.1.

Analysis of the isolated growth factor by SDS-PAGE showed that, protein bands were comparable with that in commercial sample (Fig.III.1). Maciang *et al* (1979) reported that, in the crude extract of bovine hypothalamus, human endothelial growth promoting activity is associated with a polypeptide weighing ~75kDa, and fibroblast mitogenic activity with a low molecular weight fraction of ~15kDa. When the quantities of total protein loaded were same, the relative concentrations of both of the fractions were found to be greater in the in-house preparation compared to commercially available growth supplement. In addition, the 15 kDa band seems to be diffused suggesting presence of denatured protein which is likely to cause functional inefficiency due to conformational change of the fibroblast growth factor (FGF) in the commercial sample.



ıre III.1. SDS-Page Pattern of growth factor purified from bovine othalamus. Lane1, Molecular weight marker (Sigma Chemicals, USA); Lane 2, in-house ed Growth Factor; Lane3, Commercially available Growth Factor from Sigma Chemicals,

ne search for potential regulators of angiogenesis has yielded numerous idates: acidic fibroblast growth factor (aFGF), basic fibroblast growth factor F), transforming growth factor-α (TGF-α), TGF-β, hepatocyte growth factor, r necrosis factor- α (TNF-α), etc. (Folkman and Shing, 1992; Klagsbrun and nore, 1991). Although these molecules are able to promote angiogenesis, it been found that the pivotal role is played by a protein called vascular thelial growth factor (VEGF) (Ferrara, 1993). In particular, the recent finding he loss of even a single VEGF allele results in embryonic lethality points to nique role played by this factor in the development and differentiation of the ılar system (Carmeliet et al. 1996; Ferrara, 1996).

EGF is a potent mitogen for micro and macrovascular endothelial cells ed from arteries, veins, and lymphatics, but it is devoid of consistent and ciable mitogenic activity for other cell types (Plou" et al. 1989; Pepper et al. . The denomination of VEGF was proposed to emphasize such narrow cell specificity (Ferrara and Henzel, 1989; Leung et al. 1989). VEGF otes angiogenesis in tridimensional *in vitro* models, inducing confluent vascular endothelial cells to invade collagen gels and form capillary-like ures (Pepper et al. 1992). These studies provided evidence for a potent

synergism between VEGF and bFGF in the induction of this effect (Pepper et al. 1992).

Native VEGF is a basic, heparin-binding, homodimeric glycoprotein of 45kDa (61). These properties correspond to those of VEGF165, the major isoform. From the Fig.III.1, it is clear that the 45kDa VEGF band is more intense in the in-house preparation compared to commercially available growth supplement. These results give a clear indication why the in-house preparation was found to be more potent than the commercially available growth supplement.

Both commercially available Fetal Bovine Serum (FBS) (Gibco BRL, USA) and serum obtained from new born calf, prepared by a method developed in-house were used in the study. Serum from both sources gave reproducible and excellent results in terms of maintenance of cell morphology, cell attachment and proliferation.

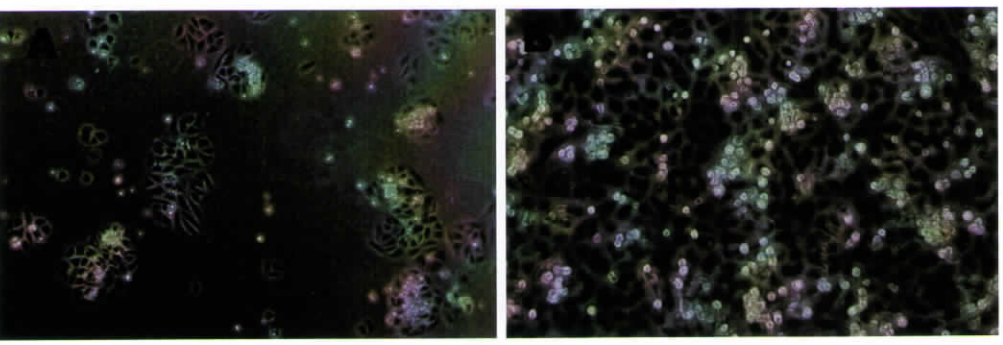
The commercially available media used in this study were MCDB 131 and M199 (Gibco BRL, USA). During the initial 2-3 passages, the morphology and proliferation rate were not satisfactory when M199 was used; whereas with MCDB 131, the growth pattern was consistently good, even if 10% serum is used till 5-6 passages. Therefore, MCDB 131 with 20% serum, 200µg/ml of growth factor and antibiotic-antimycotic solution (Gibco BRL, USA) were used as the standard culture medium throughout the study, unless specified other ways.

III.2.2. Isolation and Subculture of HUVECs

Collagenase digestion of about 15-20cm long cord vein yields ~5-8 x 10^5 cells. Among the isolated cells about 60-70% was in the form of aggregates of 20-100 cells. About 50-60% of the initially seeded cells attach in 2h of incubation of cell suspension in complete medium. Occasionally, a few individual elongated, spindle shaped cells were observed in some cell isolates. But during the initial washing of the culture plate after 2h with complete medium, most of the contaminating cells were eliminated. The attached cells started to migrate and proliferate within 24h of seeding (Fig.III.2A). When an average seeding density of ~20000 cells/cm^2 is maintained, the cells attained confluence within 4-5 days on both GCPS and CCPS. Initial cell adhesion was comparable on all the matrices

tudied. But the proliferation rate was found to be lower on FCPS and took ~7-9 lays to attain monolayer. The cell density was ~60-70x10^3cells/cm^2 at onfluence. Fig III.2B shows a confluent monolayer which is ready to be split for ubculture.

When the cells attain confluence subculture was done as mentioned in II.1.6. he split ratio was 1:2 and cells were transferred to a fresh coated flask of the ame type (from GCPS to GCPS; FCPS to FCPS; and CCPS to CCPS). Cells arvested between 2nd and 6th passages usually take 3-4 days to reach onfluence at this split ratio on both GCPS and CCPS. On FCPS, the proliferation ate was slower and minimum 6-8 days is required for monolayer formation. Cells ere maintained up to 15 passages for the study and the morphology was omparable on GCPS and CCPS until 6th passage. Subsequently, proliferation ate was found to be slightly deteriorated on GCPS with the appearance of peri uclear vacuoles. Comparison of morphology and proliferation rate is discussed n detail in following sections, of this chapter.



igure III.2. Phase contrast microscopic image of a representative field from HUVEC culture. A, cells showing migration and spreading, 24h after seeding; B, HUVEC Monolayer formed in the primary culture, ready to be sub cultured.

II.2.3. Characterization of Endothelial Cells

Primary culture of HUVEC by enzymatic isolation from cord vein is likely to be ontaminated by other cell types such as smooth muscle cells and fibroblasts. In rder to confirm the identity of EC, the typical morphology was studied under hase contrast microscope and the presence of specific molecules such as cavenger receptor for acetylated low density lipoprotein (AcLDL) and von Willebrand factor (vWF) were detected in cultured cells.

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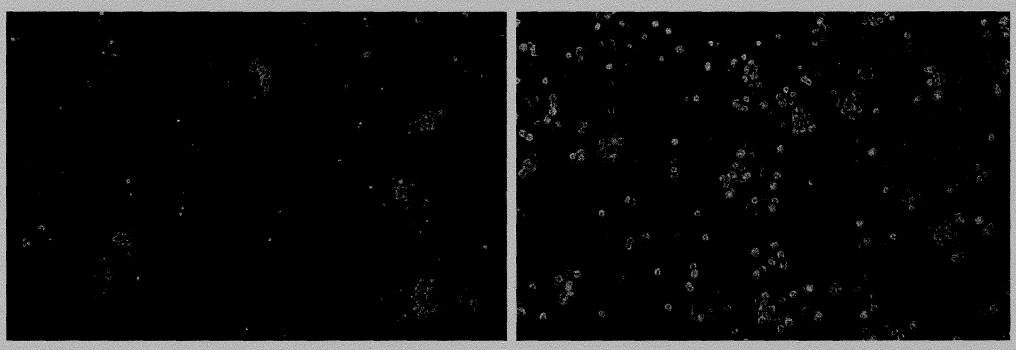

Figure III.2. Phase contrast microscopic image of a representative field from HUVEC culture. A, cells showing migration and spreading, 24h after seeding; B, HUVEC Monolayer formed in the primary culture, ready to be sub cultured.

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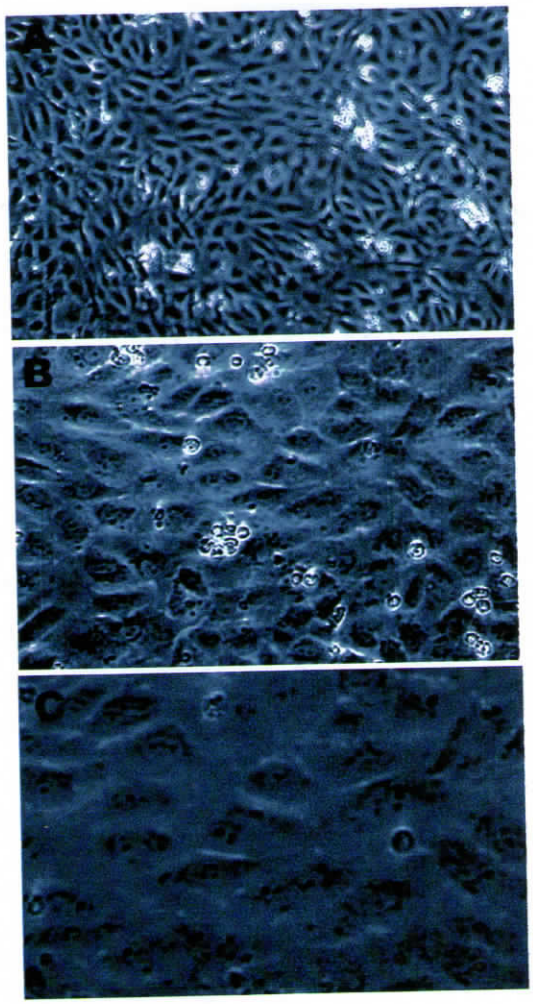
III.2.3.1. Morphology

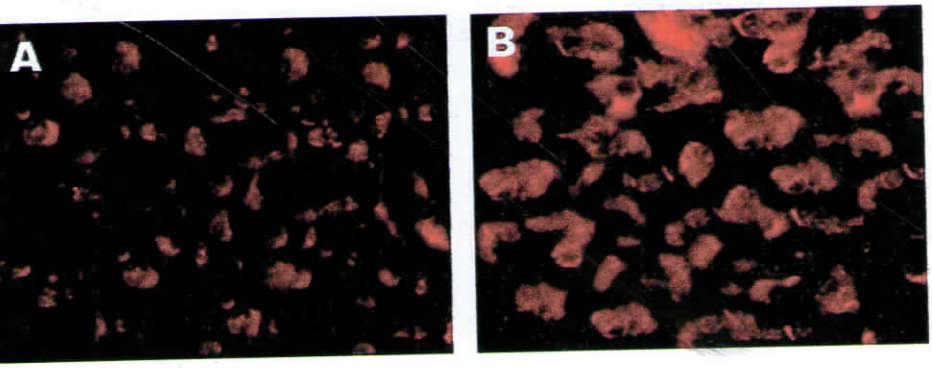
On reaching confluence, EC should show typical cobble stone morphology with closely packed monolayer without any visible intercellular space under phase contrast microscope (Jaffe *et al* 1973). The typical cobble stone morphology of HUVEC observed under phase contrast microscope at different magnifications is shown in Fig.III.3. The likely contaminant fibroblast cells should be usually stellate shaped without noticeable contact inhibition, upon confluence. The smooth muscle cells appear as elongated spindle shaped with evident cytoplasmic muscle striations and exhibit a hill and valley pattern on confluence. Both these population were not seen in the culture when viewed under phase contrast microscope. When the HUVEC were maintained in the complete medium for prolonged period after confluence, the cells start to infiltrate above monolayer. This kind of sprouting behavior is considered to be one of the unique features of ECs (Jaffe *et al* 1973).

III.2.3.2. AcLDL Uptake

HUVEC grown on both matrices showed uniform distribution of fluorescence in the cytoplasm when incubated with DiI labeled AcLDL. The AcLDL that is bound to the scavenger receptors on the cell membrane is internalized and appear as granular structure throughout the cytoplasm, surrounding the nucleus. The fluorescent probe DiI remains in the cytoplasm even after the LDL is metabolized. The scavenger receptor for AcLDL is not expressed in smooth muscle cells and fibroblasts, even though they have receptors for LDL. Hence this technique confirms the homogenous nature of HUVECs that were propagated. During the initial passages, there was no difference between cells grown on GCPS and CCPS. In the later passages, the internalization process deteriorated with less level of fluorescence in cells grown on GCPS. HUVECs from passage 12 grown on GCPS and CCPS are shown in the Fg.III.4.



ure III.3. Phase contrast microscopic images of representative fields n HUVEC culture at different magnifications. The typical cobble stone phology of HUVEC is evident. A, 10x; B, 20x; C, 40x.



gure III.4. Fluorescent Micrographs of HUVEC showing DilAcLDL uptake. GCPS 12P; B, CCPS 12P. Original magnification 40X.

III.2.3.3. vWF Identification

The vWF is a high molecular weight protein present in endothelial cells and platelets, but not seen in any other cell types. So the detection of this protein using immunofluorescence is one of the widely employed specific tests to characterize EC in culture. vWF is stored in intracellular granules of EC, named Weibel Palade bodies, located around the nucleus.

The perinuclear distribution of rod shaped Weibel Palade bodies is clearly evident in cells grown on both the matrices stained with antibodies conjugated with FITC. During the initial passages, the expression of vWF was comparable in cells grown on GCPS and CCPS. In the later passages, the cells grown on GCPS over expressed vWF, and the Weibel Palade bodies were seen diffuse throughout the cytoplasm (Fig.III.5). It has been reported that in culture after 5th passage the vWF expression is diffuse in the EC monolayer (Prudence and Bicknell, 1993), which again seems to be dependant on the culture conditions used.

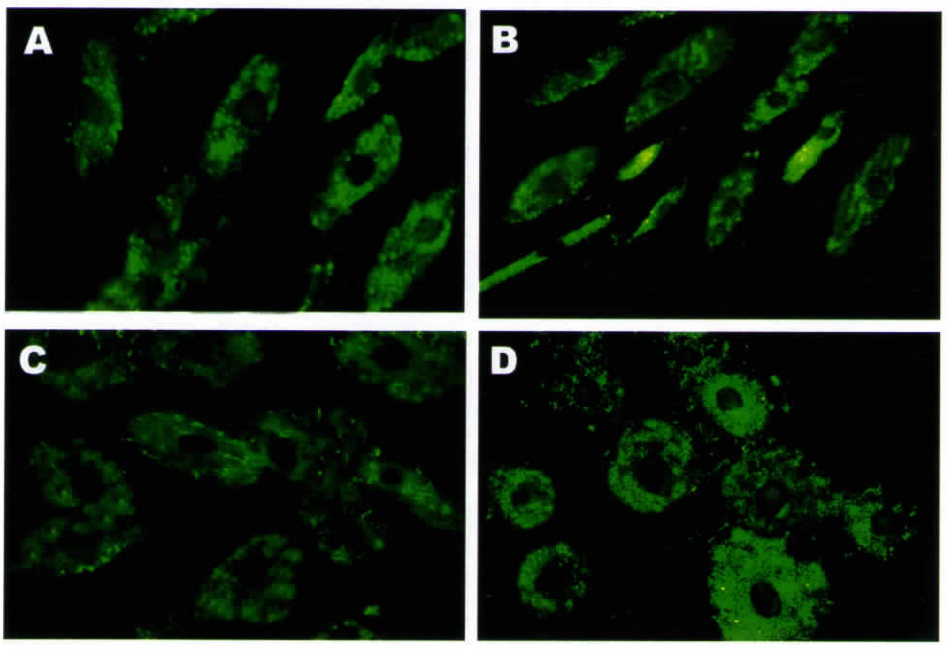


Figure III.5. Fluorescent Micrographs of HUVEC stained for vWF using FITC conjugated antibodies. A, CCPS 3P; B, GCPS 3P; C, CCPS 12P; GCPS 12P. Original Magnification 40X.

The results indicate that after repeated passages, the physiology of cells grown on GCPS in culture must have changed. On CCPS such problems were not seen till 12th passage (Fig.III.5), which indicates that the matrix composition used is ideal to maintain the normal physiology of the cells.

III.3. EFFECT OF MATRIX COMPOSITION ON HUVEC ADHESION, PROLIFERATION AND SURVIVAL

III.3.1. HUVEC Adhesion and Spreading

Comparison of HUVEC adhesion and spreading by F-actin staining of cells seeded on three types of matrices illustrate that on GCPS, EC spread well in 2h, subsequently by 24h they multiply, each cell occupying relatively large surface area as seen in fig.III.6.A&B. On FCPS, EC adhere with good actin staining, whereas poor spreading and proliferation with low surface area under each cell is evident (Fig.III.6.C&D). Attempt to get better actin organization and proliferation by combining gelatin with fibrin, give promising results with good cell adhesion, spreading and proliferation on CCPS (Fig.III.6.E&F). It is evident from fig III.6 A&B that cell adhesion on gelatin does not bring about sufficient actin organization especially along the periphery of cells compared to the cells on CCPS after 24h (Fig.III.6.F).

Since actin organization is subject to cell adhesion, the intensity of actin stain is compared on all three matrix coated surfaces using confocal microscopy. Figure III.7 A-C show representative cross sections (z) of selected area from cell cultures on each matrix. The micrographs clearly illustrates that intensity of fluorescence is highest on CCPS > on FCPS> on GCPS. The fluorescence intensity at an area that contains 6 cells on each matrix is presented in fig.III.7.D The intensity at each of the 10 z-sections made is shown and is similar to what is seen in the micrograph, the fluorescence is highest on CCPS >FCPS>GCPS.

Even though, gelatin is a good substrate for cell growth, its uniform distribution and retention on surfaces is uncertain (Balcells and Edelman, 2002). This can be the reason why the EC microfilament assembly was poor on GCPS, giving a round morphology to the cells. However, the gelatin binding sites on fibronectin should enhance gelatin retention and its uniform distribution in the composite matrix. Since fibrinogen and fibronectin are the natural constituents of

the wound bed at the sites of vascular injury and new vessel formation (Dejana et al, 1990), the interaction with these proteins when coated on material surfaces could enhance EC seeding.

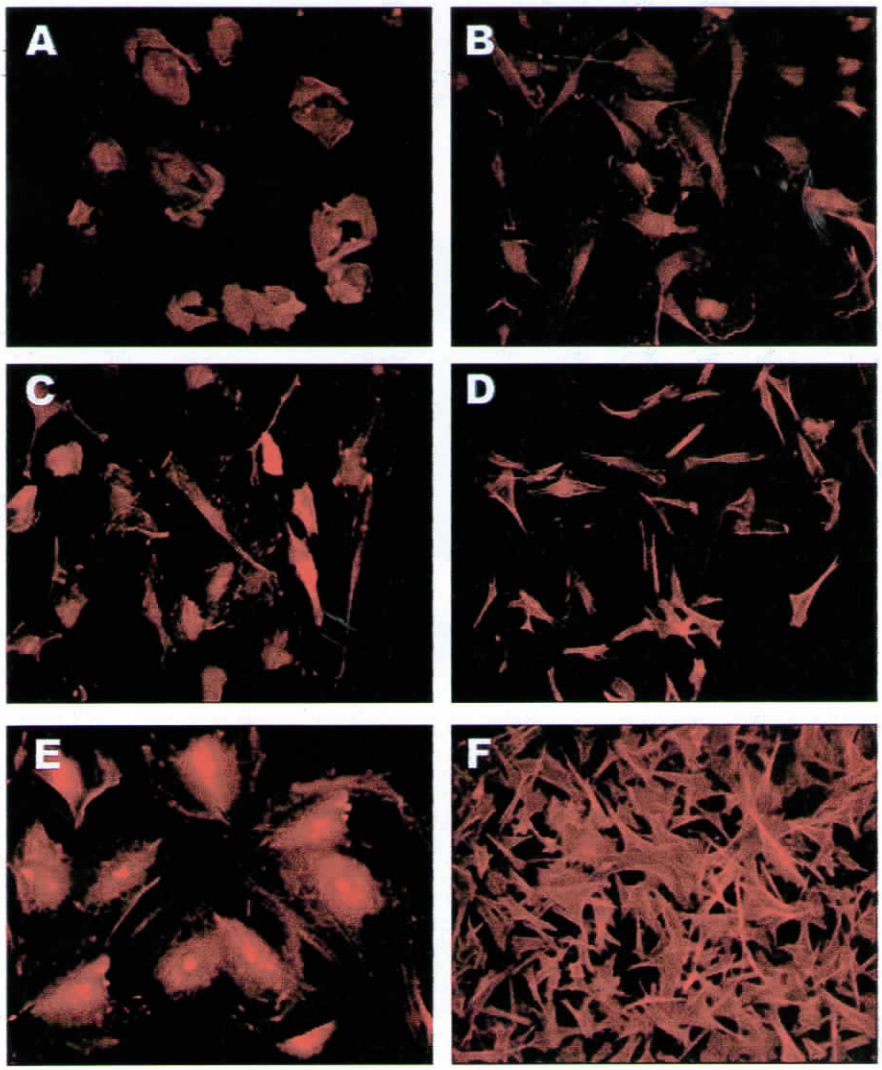


Figure III.6. Actin Stained HUVEC on Study Matrices. A& B are representative fields of HUVEC on GCPS 2h and 24h after seeding, respectively. HUVEC on FCPS and CCPS are in C-D and E-F, respectively at 2h and 24h after seeding. All cells used for seeding was from the 3rd passage. Original Magnification 40x.

The microfilament assembly was found to be satisfactory on FCPS with intense actin staining, however, sufficient spreading and growth was not found. It has been reported that endothelial cells cultured as monolayers on collagen gels or Matrigel align along the axis of matrix tension, but not in fibrin gels (Korff and

Augustin, 1999). The microfilament assembly was found to be satisfactory on FCPS with intense actin staining, however, sufficient spreading and growth was not found. It has been reported that endothelial cells cultured as monolayers on collagen gels or Matrigel align along the axis of matrix tension, but not in fibrin gels (Korff and Augustin, 1999).

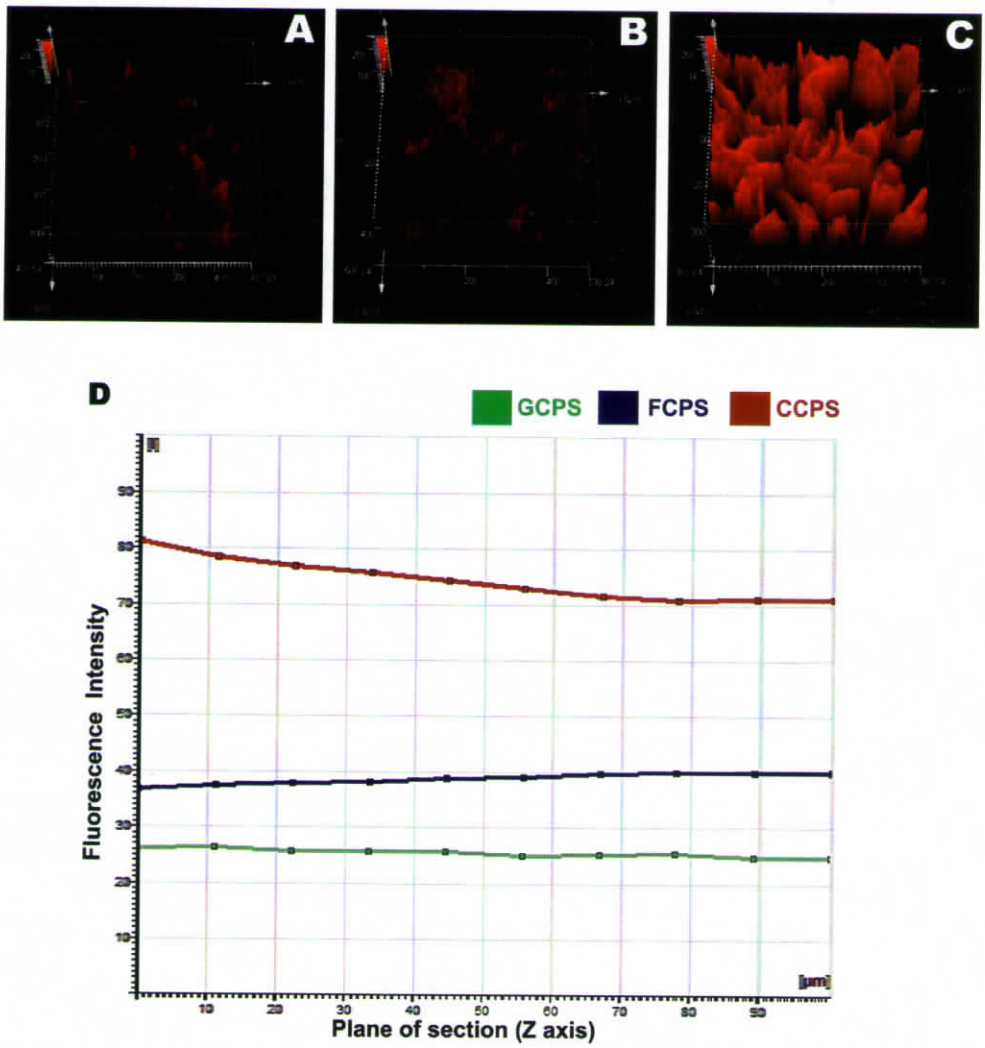


Figure III.7. Quantitative Confocal Microscopy: 3D reconstruction of confocal microscopic images of HUVECs stained for F-actin with Texas Red Phalloidin. 10 z sections of 10 µm thickness were taken and a 3D image was constructed using confocal software. A, GCPS; B, FCPS; C, CCPS. D. Graphical representation of fluorescence intensity of a selected field containing 6 cells on each matrix, graphs plotted for cells on GCPS, FCPS and CCPS are super imposed. Cumulative fluorescence intensity shown on y-axis is against cross section distance (total of 10 z-sections of 10 µm thickness) as shown in x-axis.

Therefore, gelatin was added with FCPS and this technique seems to have modulated the tensional forces resulting in good cell spreading and microfilament assembly. Appropriate orientation of the signaling machinery of the cell is dependent on the structure of the focal adhesion complex, which provides an intricate inter relationship between the ECM, the cytoskeleton and signaling cascades (Craig and Johnson, 1996). Actin cytoskeleton is a dynamic structure that rapidly changes shape and organization in response to stimuli and cell cycle progression. Therefore, any disruption of its normal regulation may lead to cell transformations. Here by mixing gelatin with fibrin, optimization of cell spreading and microfilament assembly was attained. No specific markers for focal adhesion were used in this study; however, the actin microfilament reorganization on CCPS is suggestive of good focal adhesion points.

III.3.2. HUVEC Proliferation

III.3.2.1. Effect of matrix on HUVEC during serial passaging

As the cell adhesion and actin organization may influence cell proliferation, the effect of matrix composition on HUVEC in serial passages was compared. During the initial 3-4 passages, proliferation rate was similar on GCPS and CCPS. Cells harvested between 2nd and 4th passage usually takes 3days to reach confluence on both GCPS and CCPS. But, on FCPS, the proliferation rate was slower and it takes 6-8 days to reach confluence. This may be due to a stronger adhesion, which prevents migration and spreading. Considering this and the above data on cell spreading, FCPS was omitted from further studies, and comparison was drawn between GCPS and CCPS only.

It is found that the time taken to form a monolayer on GCPS increases from 4th passage number, but on CCPS doubling time is gradually affected only by 10th passage. When the proliferation rate was evaluated till 15th passage on GCPS and CCPS, it was found that, the time taken to form 15 passages of confluent monolayer on GCPS is ~ 85 days whereas on CCPS in ~ 50 days 15 passages of monolayer are formed (Fig.III.8A).The ability of EC to regenerate is found to be deteriorating passage by passage when cells are grown on GCPS. From Fig.III.8B, it is clear that by 12th passage, the time taken for cells on GCPS to

form monolayer is almost the double when compared to similar passages on CCPS.

The data from population doubling time analysis correlates well with the above finding. From Fig III.8C, it is clear that till 3rd passage the doubling time was similar on GCPS and CCPS. But after that the doubling time was found to be increasing passage after passage when grown on GCPS. By 6th passage itself there is considerable difference in the proliferation rate and by 12th passage, the doubling time was almost the double when grown on GCPS compared to cells on CCPS.

The results were found to be statistically significant in all cases ($p < 0.05$) when analyzed by unpaired student's *t* test.

III.3.2.2. PCNA Immunostaining

HUVECs grown on GCPS and CCPS were immunoassayed for PCNA to quantify the number of proliferating cells. The table III.1 shows a comparison of PCNA +ve cells from passages 3-15 on GCPS vs. CCPS.

During the initial 6 passages, the number of proliferating cells is comparable on both the matrices, but by 9th passage, there was a considerable difference (p, 0.001) in the rate of proliferation. In the case of CCPS, cells started to show senescence by 12th passage only. By 15th passage, the number of PCNA +ve cells was 20% on GCPS while on CCPS, still 65% of cells showed signs of proliferation. Fluorescent microscopic images of PCNA stained cells grown on GCPS and CCPS from passages 3, 9 and 15 are given in Fig.III.9.A-F. This data correlates well with the above experiment wherein a gradual decrease in the proliferation rate was noted on GCPS from 6th passage onwards.

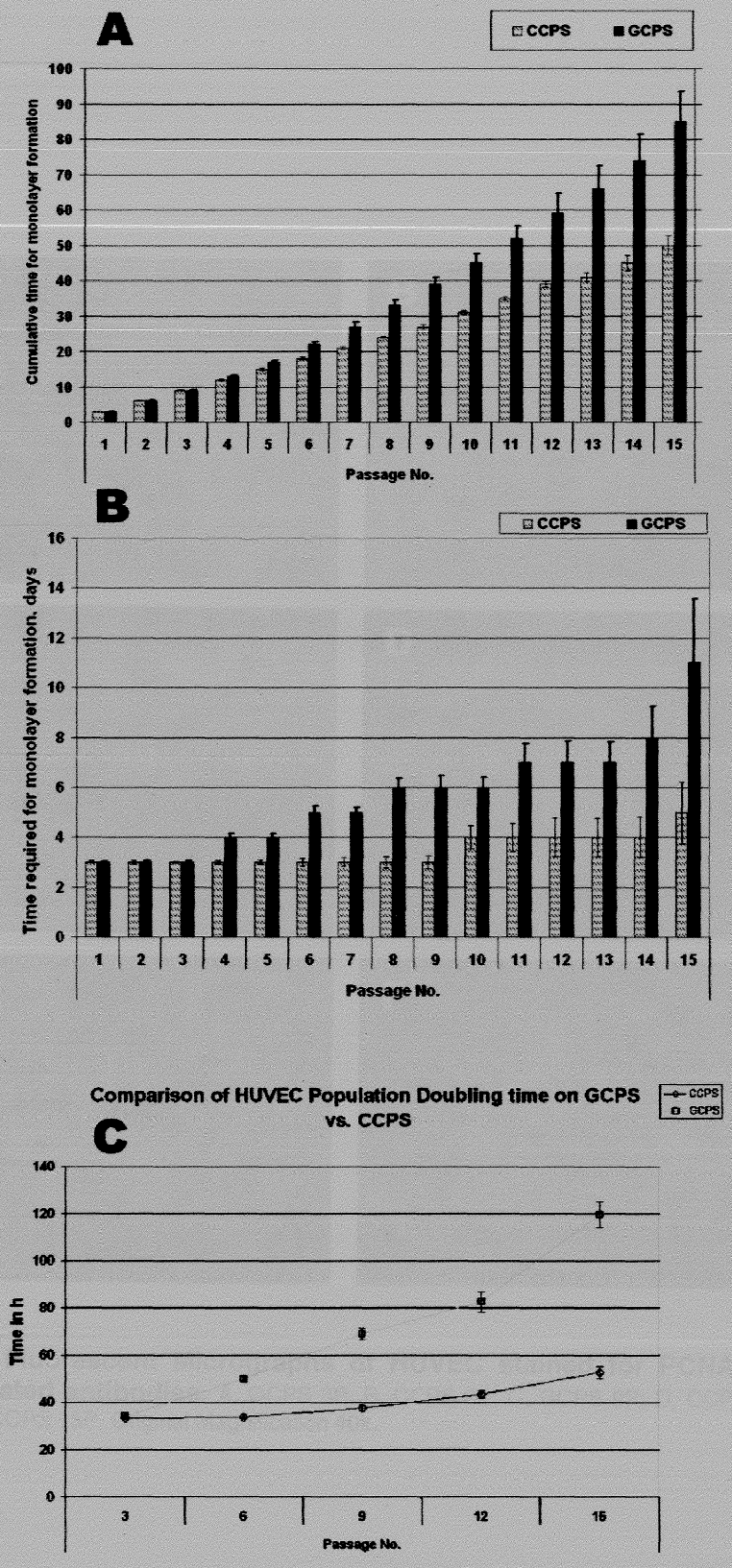


Figure III.8. Graphical representation of proliferation rates of HUVEC on GCPS and CCPS. A: Cumulative time for monolayer formation till 15 Passages, B: Time required for each passage to reach confluence C: Comparison of Population doubling time. (n=6).

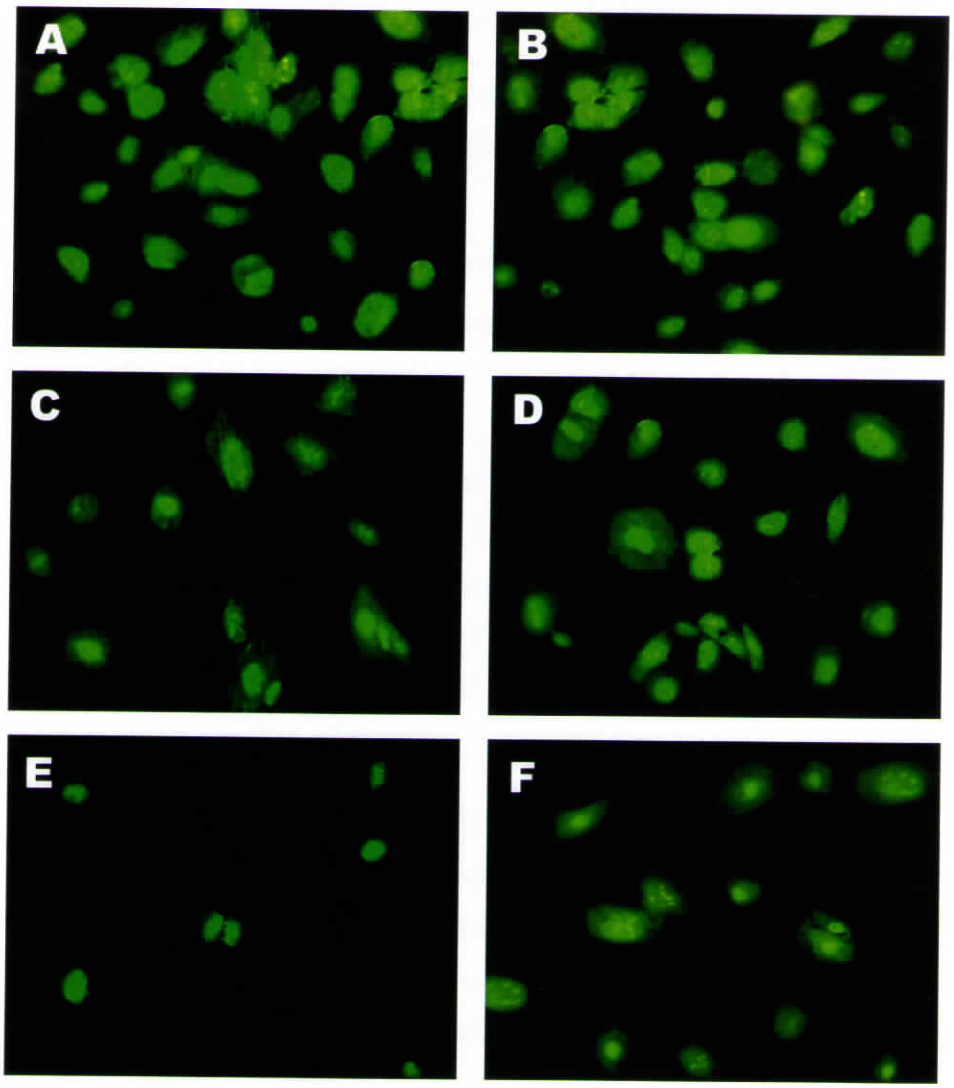


Figure III.9. Fluorescent Micrographs of HUVEC stained for PCNA using FITC conjugated antibodies. A, GCPS 3P; B, CCPS 3P; C, GCPS 9P; D, CCPS 9P; E, GCPS 15P; F, CCPS 15P. Original Magnification 40x.

Table III-1. Comparison PCNA +ve cells in HUVEC cultures grown on GCPS and CCPS. The semi quantitative method of PCNA staining of EC grown on GCPS and CCPS was used as an indicator of proliferation in different passages. Average and standard deviation were obtained by repeating the experiment with HUVEC isolated from three separate umbilical cord veins in coated 4 well plates.

Passage No.	Percentage of PCNA +ve cells grown on:	
	GCPS (n= 3)	CCPS (n=3)
3	89±4	88±5
6	83±6	87±3
9	65±5	85±4
12	47±3	78±3
15	20±2	65±4

III.3.2.3. Proliferation Assay by ^{3}H-thymidine Uptake

This study was done to substantiate the data observed with the PCNA immuno staining. Only the proliferating cells would take up thymidine for the DNA synthesis during the cell cycle. Radio labeled thymidine was provided in the medium and after 72h DNA was extracted from the cells and counted using scintillation counter. Only the 6th and 12th passage cells on GCPS and CCPS were compared. It was found that, there was no considerable difference in the proliferation rate during passage 6, and on both matrices, almost two population doubling were noted within 72h. But by 12th passage, the proliferation rate on GCPS was only about 60% of that seen on CCPS (Fig.III.10).

The observed poor proliferation rate on GCPS is possibly due to either the lack of well formed focal assembly or because of poor adsorption of gelatin to the material surface (Balcells and Edelman, 2002) and absence of immobilized growth factor. However, on FCPS, even in the presence of growth factors, adequate cell spreading and proliferation was absent at early passage itself which may be due to the tensional forces generated when HUVEC bind to fibrin. By mixing gelatin with fibrin, optimization of cell spreading and microfilament assembly was attained and the incorporation of functional growth factors and regulators with ECM and their controlled release might have improved cell behavior.

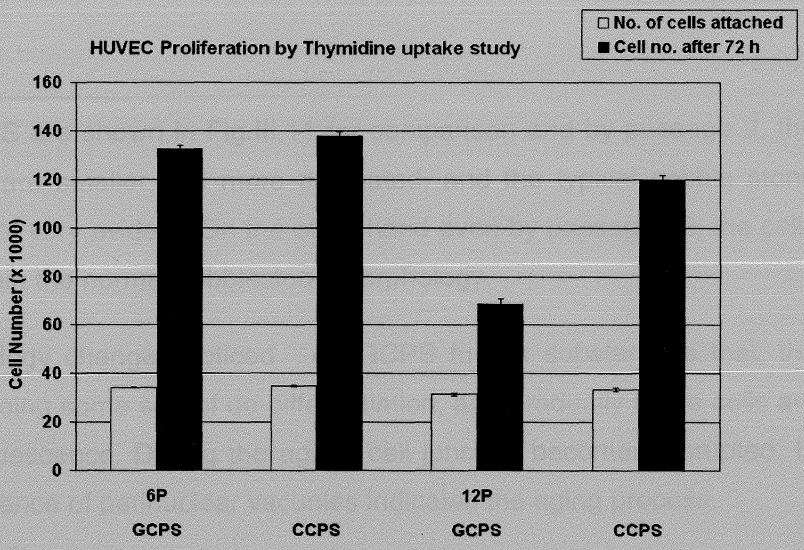


Figure III.10. HUVEC Proliferation rate on GCPS and CCPS analyzed by thymidine uptake study. Y-axis shows the cell number and X-axis, the Passage number and type of matrix on which the cells are grown. (n=3).

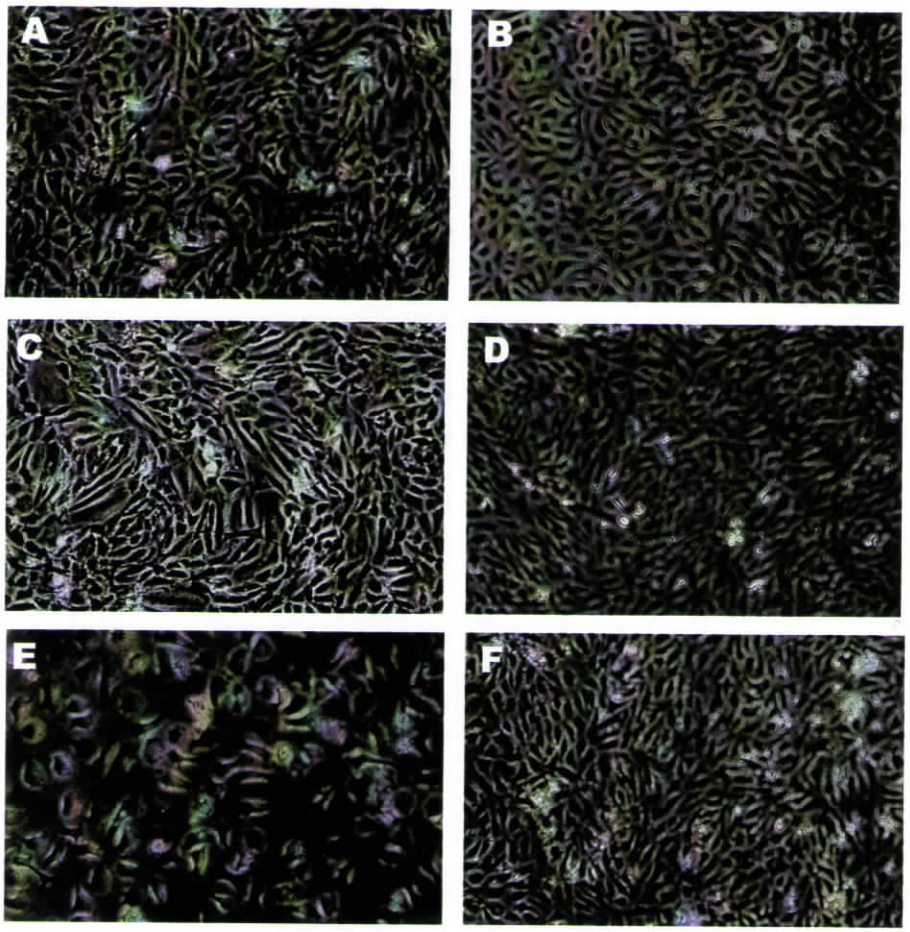
Fibrin as a natural scaffold has sites to bind and retain growth factors that are released based on the cell-demand (Ehrbar et al 2004). The cryoprecipitate which was used for making the composite contains fibronectin and transglutaminase (FXIII), hence cross linking of fibrin to fibronectin should take place. The role of fibronectin in cell adhesion, migration, and signaling for proliferation and survival is well demonstrated. Therefore, the effect on endothelial cell growth that is seen on composite coated surface is probably contributed by fibrin and VEGF bound to it, fibronectin cross linked to fibrin, gelatin covalently attached to fibronectin, and in addition the mitogenic fibrino peptides released on fibrinogen to fibrin conversion.

III.3.3. Comparison of HUVEC Morphology

Before trypsinization of HUVEC for subculture, morphology of the monolayer was photographed using a digital camera to detect morphological distinctions between the cells grown on specific matrices. In the initial 6 passages, the morphology was comparable on GCPS and CCPS. In later passages on GCPS a slow transformation in the morphology was noticed. The cells appeared smaller with the presence of perinuclear vacuoles. The changes in the morphology noticed are well in agreement with the decline in the proliferation rate as described above. Photomicrographs of HUVECs from passages 6, 9 and 12 on

GCPS and CCPS are shown in Fig.III.11 for comparison and by passage 9, the cells on GCPS are smaller and more elongated, and the typical cobble stone morphology is not very evident. On the other hand even by passage 12, the cells on CCPS maintain the normal cobblestone morphology.

This morphology changes noticed on GCPS further substantiate that, the cells are undergoing some sort of de-differentiation, and gradually some cells are approaching senescence. During the aging, cell junction becomes disrupted. In addition, appearance of perinuclear vacuoles indicates the aging process.



igure III.11. Photomicrographs of HUVEC showing the morphology hanges during the serial passaging. A, GCPS 6P; B, CCPS 6P; C, GCPS 9P; D, CPS 9P; E, GCPS 12P; F, CCPS 12P. Original Magnification 10x.

GCPS and CCPS are shown in Fig.III.11 for comparison and by passage 9, the cells on GCPS are smaller and more elongated, and the typical cobble stone morphology is not very evident. On the other hand even by passage 12, the cells on CCPS maintain the normal cobblestone morphology.

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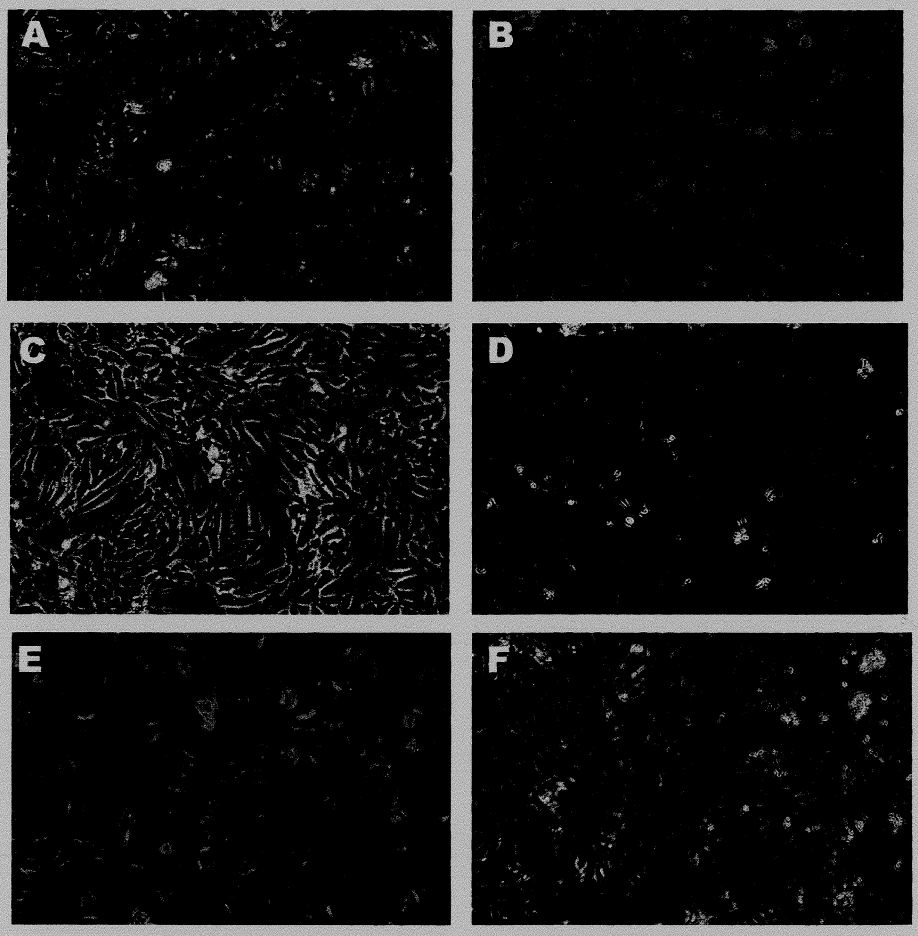
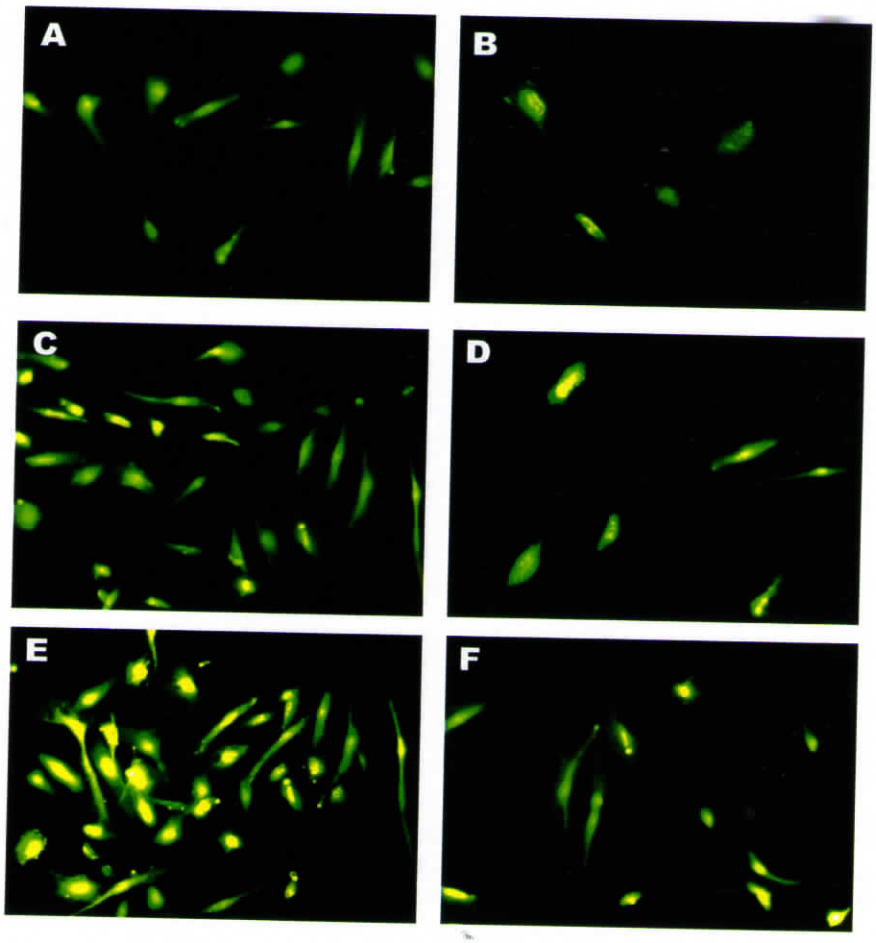


Figure III.11. Photomicrographs of HUVEC showing the morphology changes during the serial passaging. A, GCPS 6P; B, CCPS 6P; C, GCPS 9P; D, CCPS 9P; E, GCPS 12P; F, CCPS 12P. Original Magnification 10x.

III.3.4. HUVEC Apoptosis on GCPS vs. CCPS

It is observed that the cells are more apoptotic on GCPS compared to that on CCPS. During the initial 6 passages, the apoptosis was negligible on both of the matrices. Fig.III.12 shows Annexin V stained cells from passage, 9, 12 and 15 grown on GCPS and CCPS. When cells from similar passage numbers are compared, more cells show apoptosis on GCPS than that on CCPS. Percentage of apoptotic cells in GCPS is about 30 % in 9th passage and by 15th passage it increases to ~80 % , however, in CCPS it seems to be ~ 15 % and ~30 % in 9th and 15th passages, respectively.



igure III.12. Representative Fields of Annexin V stained cells. A, GCPS 9P; B, CPS 9P; C, GCPS 12P; D, CCPS 12P; E, GCPS 15P; F, CCPS 15P. Original Magnification 0x.Staining was done 72 h after seeding.

When the apoptotic and dead cells were distinguished and quantified using flow cytometric assay the data correlates well with the above experiment. But here since the assay is more sensitive, a real distinction could be made between the apoptotic/dead cells. It was found that, on GCPS, by passage 9 ~25% cells are apoptotic and ~10% are dead. On CCPS, only ~5% were apoptotic and 8% were dead. By passage 15, almost 60% of the cells turned apoptotic and 20% were dead on GCPS; whereas on CCPS the figure was only 15% and 12% respectively (Fig III.13 and Table.III.2).

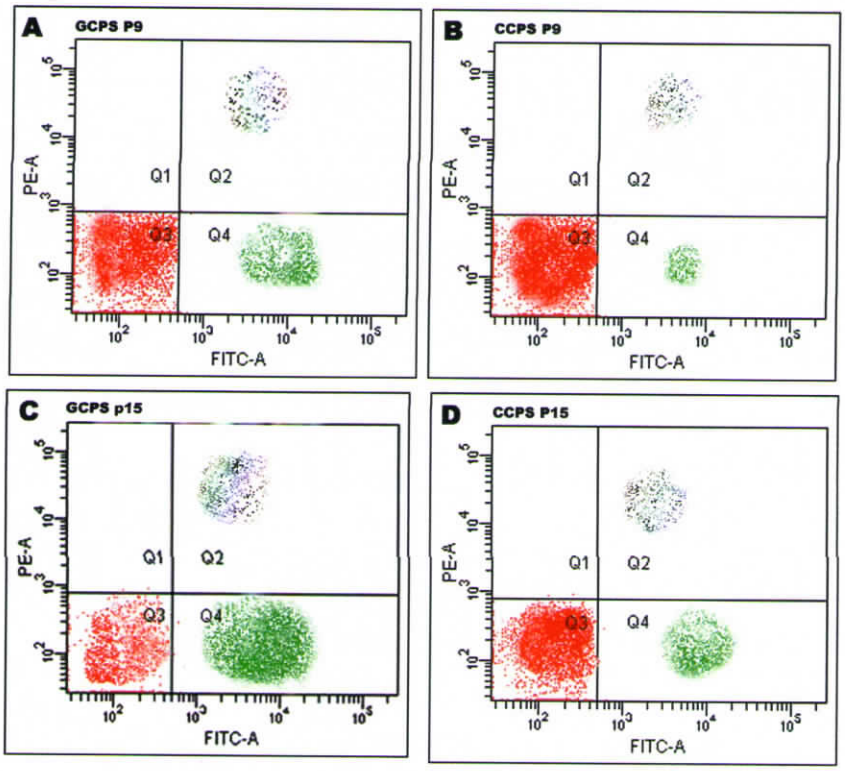


Figure III.13. Quantification of live/apoptotic/dead cells when grown on GCPS and CCPS. *Representative flow cytometry analysis data of HUVECs from one cord* ein is shown. The dot plot with quadrant gate is saved as jpg file. Q3 shows live cells, Q4 poptotic cells and Q2 dead cells. A: GCPS Passage 9; B: CCPS Passage 9; C: GCPS Passage 5; D: CCPS Passage 15.

The data on apoptosis correlates well with the above discussed results on ell proliferation. Interactions of matrix molecules with cells modulate the cellular esponse to growth factors and are critical for cell cycle progression. Adhesion to ne extracellular matrix activates intracellular signal transduction pathways

important in the regulation of cell growth (Howe et al., 1998), death (Frisch et al., 1997) and differentiation (Roskelley et al., 1995).

Table III-2. Comparison of HUVEC Apoptosis on GCPS vs. CCPS. Quantification of Live/ Apoptotic/ Dead cells by Flowcutometric analysis. Assay was performed with HUVEC isolated from two different umbilical veins.

Passage No:	Percentage of Live/Apoptotic/Dead Cells when grown on					
	GCPS (n=2)			CCPS (n=2)		
	Live	Apoptotic	Dead	Live	Apoptotic	Dead
3	95.2±1.3	0.9±0.1	3.8±0.5	96.5±2.2	0.8±0.2	2.6±0.7
6	94.6±3.2	1.5±0.6	4.8±1.5	94.8±3.5	1.9±0.3	3.3±1.2
9	64.6±2.3	25.6±1.8	9.2±1.9	86.5±2.9	5.1±1.3	7.9±0.9
12	48.5±5.3	37.2±3.7	16.7±3.2	79.5±3.2	11.6±2.4	8.2±3.2
15	21.3±6.5	58.7±4.5	20.1±0.9	73.4±5.3	15.3±2.3	11.9±1.3

Cells which are anchorage-dependent for proliferation require both adhesion to the extra cellular matrix and growth factor stimulation to progress through the cell cycle (Howe et al., 1998; Assoian and Zhu, 1997). So the poor spreading and insufficient actin organization observed on GCPS might be responsible for the apoptotic tendency. Also the growth factor immobilization and release in the case of the CCPS, along with good spreading and cytoskeletal organization is thought to play a critical role in providing good growth signals. These findings indicate that spreading and perhaps integrin-mediated cytoskeletal organization, are also intimately linked to viability.

III.4. EFFECT OF MATRIX COMPOSITION ON HUVEC PHENOTYPE

The transcription level of prothrombotic vWF and PAI-1; antithrombotic t-PA and NO synthesizing enzyme eNOS in HUVECs isolated from 6 different umbilical veins at different levels of serial passaging on GCPS and CCPS were compared. Down/up regulation of mRNA was assessed by the increase in band intensity in samples obtained from different cultures that were grown on different matrices through serial passages. The release of these molecules by HUVECs was also quantified. Release of t-PA, vWF and PAI-1 into the culture media was analyzed by ELISA, and the release of NO was quantified by nitrite estimation using modified Greiss reagent.

III.4.1. Expression and release of vWF

It was found that the m-RNA expression for the pro-thrombotic molecule, vWF increases in cells grown on GCPS by passage 6 and as the passage number progressed to 15, the level got amplified further (Fig.III.14). However, only at a later passage (12p) in EC grown on CCPS detectable change of vWF expression was seen. Also, the increase in the expression with the progression of passage was found to be marginal in the case of cells grown on CCPS ($p < 0.01$ when compared to GCPS).

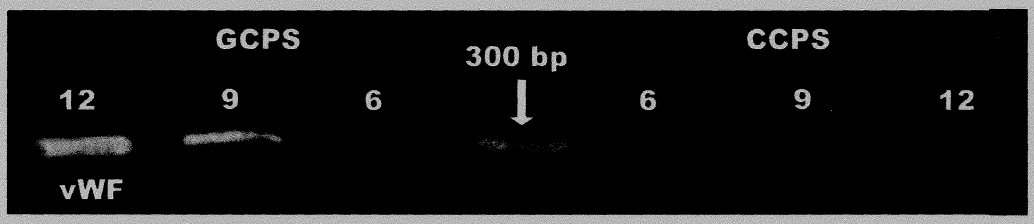


Figure III.14. Relative abundance of vWF mRNA on serial passaging of HUVEC: Representative picture of gels showing amplicons from HUVEC grown on GCPS and CCPS. Passage numbers after which RNA was isolated from HUVEC are as numbered in each figure. The 300 bp band of the ladder is shown by arrow head.

Release of vWF into the culture media was analyzed by ELISA and it was found to be high when the HUVEC was grown on GCPS as compared to that was grown on CCPS (Table.III.3). There is no detectable level of vWF release into the medium from HUVEC grown on CCPS in both 2nd and 5th passage. As the passage progressed, the release of vWF from HUVEC grown on GCPS went onto further higher levels. But on CCPS the release was much lower and steady till the passage 14 (Table.III.3).

During the characterization experiment (described in section III.2.3.3), it was found that in the initial passages, the expression of vWF was comparable in cells grown on GCPS and CCPS. In the later passages, the cells grown on GCPS over expressed vWF, and the Weibel Palade bodies were seen diffuse throughout the cytoplasm (Fig.III.5). The mRNA expression data and release of vWF into the medium also correlates well with this finding.

von Willebrand factor (vWf), is a large adhesive glycoprotein circulating in plasma as a series of heterogenous multimers, of molecular weight 0.4-20 x 10^6, which is synthesized exclusively in ECs and megakaryocytes and stored in Weibel-Palade bodies in ECs and in platelet α granules (Mannucci, 1995). As carrier for coagulation factor VIII in the plasma, vWf coordinates the formation of fibrin and platelet-rich thrombi. Under physiological conditions, vWf is released from ECs into the plasma and to the abluminal cell surface, where it binds to the subendothelium. Here it mediates initial platelet adherence to the subendothelium by linking to specific platelet membrane receptors (glycoprotein Ib-IX complex) and to constituents of subendothelial connective tissue.

Table III-3. ELISA data for vWF: effect of matrix during passaging. ELISA data for vWF released into culture medium from HUVECs grown on GCPS and CCPS. Average and standard deviation are calculated from six experiments using HUVEC isolated from six different veins.

Passage No:	vWF ng/ ml (n=6)	
	GCPS	CCPS
2	2.76±0.56	N.D.
5	5.46±0.69	N.D.
8	7.84±0.73	4.30±0.85
11	11.42±0.74	4.96±0.40
14	14.84±0.59	6.18±0.53

vWF is secreted by more than one pathway (Miller et al., 1998). In cultured ECs, the majority of newly synthesized vWF is secreted constitutively (Loesberg et al., 1983) and additional release can be provoked by various agents such as thrombin, fibrin, histamine, epinephrine, vasopressin, endotoxin, cytokines, components of the complement systems, and shear stress (Lopes and Maeda, 1998). Also there are reports suggesting that, vWF is heterogeneously distributed throughout the vasculature, transcriptional control in response to the tissue microenvironment being responsible for local variations in endothelial cell levels of vWF (Zanetta et al., 2000).

Chronically raised levels of vWF occur in patients with diseases involving vascular pathology (Savage et al., 1991; Heywood et al., 1996) and in these cases the constitutive synthesis and secretion of vWF may be adaptively raised

in response to some components of the disease process. Studies indicate that the constitutive phenotype of ECs is unstable and their behavior can change rapidly once explanted. Commonly used culture conditions may activate or otherwise alter the endothelial phenotype (Grant et al., 1989).

In this study, it is found that the expression level and secretion of vWF is accelerated upon serial passaging on GCPS. This may be because of the changes in the microenvironment and the altered signaling between the ECM and cells. This gives a clear indication that after repeated passages, the normal anti-thrombotic phenotype of cells grown on GCPS must have changed. On CCPS such problems were not seen, and altogether the data indicates that the matrix composition used is ideal to maintain the normal physiology of the cells.

III.4.2. Expression and release of PAI-1

The mRNA expression of PAI-1, which also is a pro-thrombotic molecule, was found to be up-regulated in cells grown on GCPS by passage 6. The level went further higher as the passage number progressed. (Fig.III.15). However, in EC grown on CCPS, the increase in the expression with the progression of passage was found to be negligible when compared to cells grown on GCPS ($p < 0.01$).

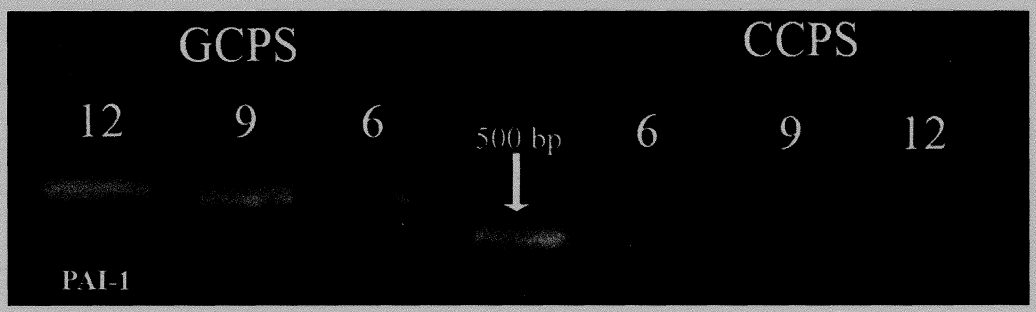


Figure III.15. Relative abundance of PAI-1 mRNA on serial passaging of HUVEC: Representative picture of gels showing amplicons from HUVEC grown on GCPS and CCPS. Passage numbers after which RNA was isolated from HUVEC are as numbered in each figure. The 500 bp band of the ladder is shown by arrow head.

The release of PAI-1 into the medium was comparable till the 3rd passage, but then the protein released into the medium by the EC grown on GCPS showed a steady increase as passage number progressed. On the other hand, the PAI-1 release by EC grown on CCPS remains almost unchanged until the 14th passage (Table.III.4).

Table III-4. ELISA data for PAI-1: effect of matrix during passaging. ELISA data for PAI-1 released into culture medium from HUVECs grown on GCPS and CCPS. Average and standard deviation are calculated from six experiments using HUVEC isolated from six different veins.

Passage No:	PAI-1 ng/ ml (n=6)	
	GCPS	CCPS
2	268±12	257±8
5	290±16	189±13
8	350±26	195±19
11	595±14	181±17
14	746±26	210±14

Plasminogen activator inhibitor type 1 (PAI-1), the primary regulator of plasminogen activation *in vivo*, appears to have major clinical relevance (Loskutoff, 1991). PAI-1 is the major inhibitor of plasminogen activation by tissue-type (t-PA) and urokinase-type (u-PA) plasminogen activator. PAI-1 belongs to the family of serine protease inhibitors (SERPINs).

Under physiological conditions, PAI-1 is released into the circulation and the extra cellular space, which results in plasma levels of only 5 to 20 ng/ml of active PAI-1, sufficient to control fibrinolysis and extracellular proteolysis. Under pathological conditions, however, ECs secrete quite large amounts of PAI-1 (Bernd et al., 2002).

Elevated levels of plasma PAI-1 activity are associated with thrombotic disorders, such as deep venous thrombosis (Juhan-Vague et al., 1987), disseminated intravascular coagulation (Suffredini et al., 1989), coronary artery disease (Huber et al., 1990), and myocardial infarction (Hamsten et al., 1987).

Therefore PAI-1 seems to be a key molecule in thrombotic vascular diseases. In this study it was found that the expression of PAI-1 is significantly up-regulated when ECs were passaged on GCPS. This clearly indicates that during long term culture on GCPS, ECs turn to an activated phenotype with more prothrombotic molecule expression. This is not seen when cells are grown on CCPS.

III.4.3. Expression and release of tPA

During initial 3-6 passages, mRNA expression of tPA, an anti thrombotic molecule, was found to be similar and low in cells grown on both the GCPS and CCPS (Fig.III.16). As the passage number progressed to 12, down regulation of tPA transcription was noted in cells grown on GCPS with barely detectable bands, whereas, the m-RNA expression was unaffected in cells passaged on CCPS.

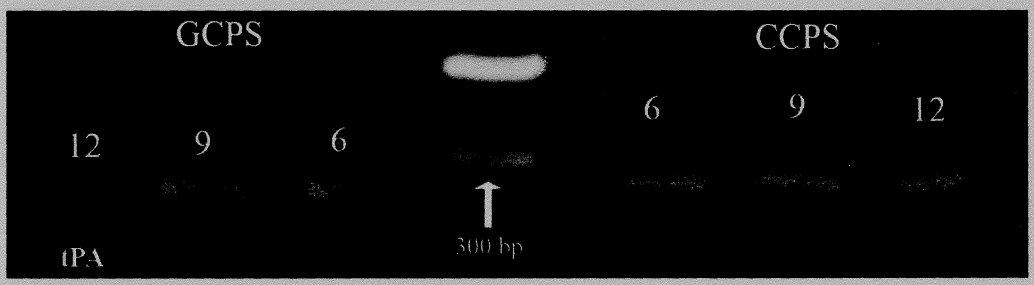


Figure III.16. Relative abundance of tPA mRNA on serial passaging of HUVEC: Representative picture of gels showing amplicons from HUVEC grown on GCPS and CCPS. Passage numbers after which RNA was isolated from HUVEC are as numbered in each figure. The 300 bp band of the ladder is shown by arrow head.

When the release of tPA into the culture media was analyzed by ELISA, and it was found that by 2nd passage itself, there was considerable difference in the release of t-PA by HUVEC grown on GCPS, when compared to cells grown on CCPS ($p < 0.01$). The synthesis and release of the protein by cells on GCPS further diminished and gradually went onto negligible levels by 11th passage, whereas the release by HUVEC grown on CCPS remained steady till 14 passages (Table III.5).

Tissue-type plasminogen activator (tPA) is one of the two physiological activators of the pro-enzyme plasminogen, converting the latter into its active derivative plasmin (Collen and Lijnen, 1991). tPA has been primarily implicated in clot dissolution via its fibrin specificity (Carmeliet et al., 1994).

Table III-5. ELISA data for tPA: effect of matrix during passaging. ELISA data for tPA released into culture medium from HUVECs grown on GCPS and CCPS. Average and standard deviation are calculated from six experiments using HUVEC isolated from six different veins.

Passage No:	tPA ng/ ml (n=6)	
	GCPS	CCPS
2	0.36±0.04	0.48±0.05
5	0.32±0.07	0.49±0.09
8	0.21±0.02	0.48±0.07
11	0.09±0.004	0.47±0.08
14	0.04±0.006	0.46±0.07

tPA production by cultured ECs is regulated by a variety of external stimuli at the level of gene transcription and cellular release (Levin et al., 1989). Measurements of plasma tPA levels suggest such regulated production/secretion occurs *in vivo* as well (Cugno et al., 1989). Intracellular signaling pathways operative in stimulated tPA release have been described *in vitro* (Levin and Santell, 1991). The mechanisms that control EC tPA production *in vivo* are less well understood, but clearly such regulation occurs (McCrae et al., 1994).

It was found that both the transcription of tPA mRNA and the release of the protein were down regulated in ECs serially passaged on GCPS. This further makes the ECs more thrombogenic. But the ECs grown on CCPS maintained a steady level of tPA synthesis and release till 15 passages. This further confirms that the surrounding extracellular matrix plays a key role in regulating the behavior of cells.

III.4.4. Expression of eNOS and release of NO

The mRNA expression data for eNOS, the enzyme that synthesize the vasodilator and anti-thrombotic molecule NO, was found to be similar in cells grown on GCPS and CCPS during initial 3-6 passages. As the passage number progressed to 12, down regulation of the eNOS mRNA was clearly evident in cells grown on GCPS with barely detectable bands (Fig.III.17). On the other hand, the m-RNA expression was found to be unaffected by serial passaging when grown on CCPS.

When the NO release into the medium by ECs grown on GCPS and CCPS through several passages was quantified by nitrite estimation, it was found that the release of NO from cells grown on both matrices was comparable up to the 5th passage. But the EC grown on GCPS slowed down NO synthesis from 8th passage and by 14th passage, the release was found to be almost negligible (Fig.III.18). However, the cells grown on CCPS maintained their ability to synthesize and release NO through all the 14 passages (Fig.III.18).

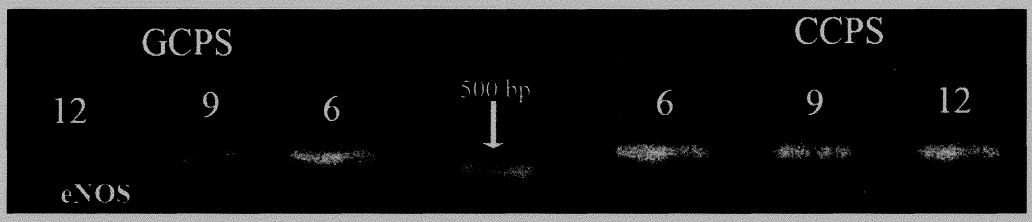


Figure III.17. Relative abundance of eNOS mRNA on serial passaging of HUVEC: Representative picture of gels showing amplicons from HUVEC grown on GCPS and CCPS. Passage numbers after which RNA was isolated from HUVEC are as numbered in each figure. The 500 bp band of the ladder is shown by arrow head.

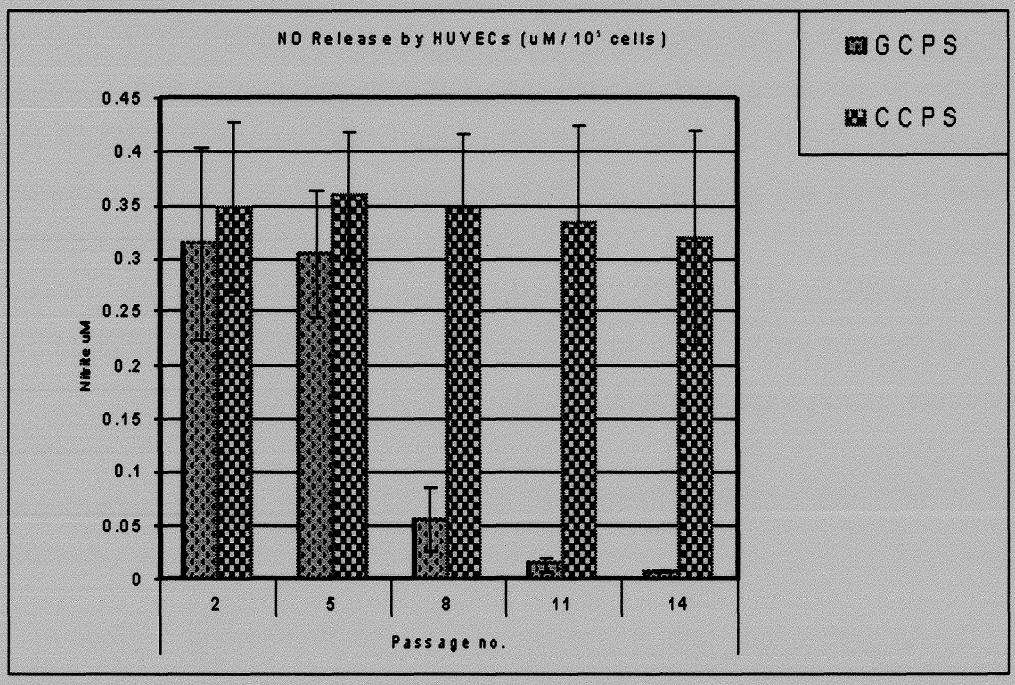


Figure III.18. Graphical representation of NO release by HUVECs grown on GCPS and CCPS; Cells at 2, 5, 8, 11 and 14 passages were incubated for 24h in serum free medium after attaining confluence, and NO released in to the medium was assayed using Griess reagent. The Y-axis is nitrite in μM/10^5 cells. X-axis indicates passage number. (n=6).

Nitric oxide synthase (NOS) is an enzyme responsible for the production of NO, a heterodiatomic free radical product generated through the oxidation of L-arginine to L-citrulline (Stamler at al., 1992). The endothelial isoform, eNOS or the NOS3 gene product, is constitutively active in ECs but is stimulated further by receptor-dependent agonists that increase intracellular calcium and perturb plasma membrane phospholipid asymmetry (Venema et al., 1995). Receptor-dependent agonists that stimulate eNOS include thrombin, adenosine 5'-diphosphate, bradykinin, substance P, and muscarinic agonists, in addition to shear stress (Topper et al., 1996) and cyclic strain (Awolesi et al., 1994). The increase in eNOS activity evoked by shear stress contributes to the phenomenon of flow-mediated vasodilatation, an important autoregulatory mechanism by which blood flow increases in response to exercise (Loscalzo and Vita, 1994). This is in part a result of shear-induced transcriptional activation due to the presence of a shear response consensus sequence, GAGACC, in the promoter of the NOS3 gene (Venema et al., 1994).

EC-derived NO has several important effects on the vasculature. First, NO maintains basal tone by relaxing vascular smooth muscle cells (Loscalzo and Welch, 1995) through the binding of NO to the heme prosthetic group of guanylyl cyclase. Endothelial-derived NO also inhibits platelet adhesion, activation, secretion, and aggregation and promotes platelet disaggregation, in part through a cyclic GMP dependent mechanism (Mendelsohn et al., 1990). NO also inhibits expression of P-selectin on platelets and, by inhibiting the agonist-dependent increase in intra platelet calcium (Mendelsohn et al., 1990), suppresses the calcium sensitive conformational change in the heterodimeric integrin glycoprotein IIb-IIIa required for fibrinogen binding (Michelson et al., 1996). Additionally, NO appears to promote platelet disaggregation indirectly by impairing the activity of phosphoinositide 3-kinase, which normally supports conformational changes in GP IIb-IIIa, rendering its association with fibrinogen effectively irreversible (Pigazzi et al., 1999).

In addition to these effects on the vasculature, endothelial derived NO inhibits leukocyte adhesion to the endothelium (Kubes et al., 1991; De Caterina et al., 1995), and inhibits smooth muscle cell migration (Marks et al., 1995) and

proliferation (Garg and Hassid, 1989). These latter effects serve to limit neointimal proliferation that occurs after vascular injury and, combined with its stimulatory effect on EC migration and proliferation (Fukuo et al., 1995), suggest that NO helps to sustain vascular reparative mechanisms.

Various studies suggests that in humans, NO-mediated endothelial function is deficient in preatherosclerotic states such as hypercholesterolemia, diabetes mellitus, hypertension and smoking (Cooke and Dzau, 1997; Harrison, 1997) and correlates with risk factor profile (Vita et al., 1990). More importantly, prospective studies identify deficient NO-mediated endothelial function as a quantitative, independent predictor of adverse cardiac events (Schachinger et al., 2000; Heitzer etal., 2001). Our studies shows that the eNOS transcription levels and NO release data correlates well with the above discussed release of proteins into the culture media by HUVEC and the dedifferentiation observed when they are serially passaged on GCPS turning them to a pro-thrombogenic state.

Compared to the gelatin coated polystyrene that up regulate thrombogenic molecules and down regulate anti-thrombogenic molecule, the composed bio mimetic matrix was found to maintain normal cell differentiation. Graded functional transformation of cells grown on GCPS was evidenced here with an over expression of the pro-thrombotic and under expression of anti-thrombotic molecules. Beta actin was used as housekeeper gene to ensure that the difference in the expression noticed was not contributed by differences during the isolation procedure or due to some other factors which alter the physiology of the cells altogether.

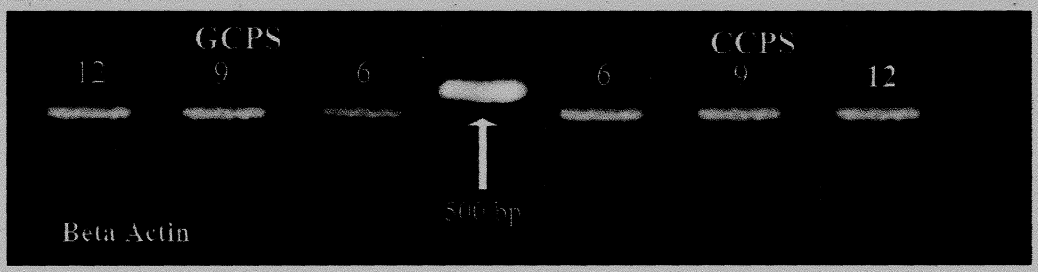


Figure III.19. Relative abundance of the house keeping gene beta actin mRNA levels on serial passaging of HUVEC: Representative picture of gels showing amplicons from HUVEC grown on GCPS and CCPS. Passage numbers after which RNA was isolated from HUVEC are as numbered in each figure. The 500 bp band of the ladder is shown by arrow head.

And it was found that, the expression level of housekeeper gene β-actin was steady and comparable throughout the study (Fig.III.19).

It was very evident from the HUVEC adhesion and spreading study that, cell adhesion on gelatin does not bring about sufficient actin organization. The focal adhesion complex plays a major role in integrating the integrin mediated signaling events from the matrix. Appropriate orientation of the signaling machinery of the cell is dependent on the structure of the focal adhesion complex, which provides an intricate inter relationship between the ECM, the cytoskeleton and signaling cascades (Garcia et al 1999; Aplin, 2003). Integrins communicate with many specific structural and signaling molecules, and these proteins act as linkers between the integrin receptors and the cytoplasmic actin cytoskeleton, which is associated with nuclear membrane, membranes of cellular organelles as well as with various enzymes and thus influences intracellular processes that are important for cell behavior including transport and secretion of various molecules, endocytosis, and decision between cell proliferation and differentiation or apoptosis. So the dedifferentiation of HUVECs and the subsequent phenotypic alterations which make the cells pro-coagulant when grown on GCPS can be attributed to poor spreading, lack of well formed focal assembly and the resulting failure in the appropriate orientation of the signaling machinery. On the other hand, the composite matrix used was found to support good actin organization and transduce signals for proliferation, maintenance of viability and cell function. It has been reported that apoptotic endothelial cells become procoagulant (Bombeli et al 1997). Thus from this study it is evident that, HUVECs grown on GCPS gradually become apoptotic and lose their normal anti-thrombotic potential.

Chapter IV

BEHAVIOR OF DYSFUNCTIONAL HSVEC ON COMPOSITE MATRIX

IV.1. ABSTRACT

A number of inherited primary thrombophilic states were found to be associated with abnormalities of endothelial cell–mediated anticoagulant membrane functions, and some pathological clinical conditions were found to be associated with the conversion to a non-constitutive activated endothelial state with an inflammatory thrombogenic phenotype. Dysfunctional endothelium appears to contribute significantly to systemic vascular disorders such as atherosclerosis, and it has been suggested that the tissue microenvironment surrounding blood vessels controls endothelial cell phenotype. Some of these phenotypic alterations have been found to occur in ECs undergoing senescence *in vitro* and include increased expression of proteolytic inflammatory markers and vasoconstrictors and reduced expression of vasodilators and vaso protective factors.

The vascular ECM not only maintains vascular wall structure, it also regulates key events in vascular physiology. Alterations of endothelial cell (EC) phenotype in cardiovascular diseases (CVD) as an effect of alteration of extra cellular matrix (ECM) composition have not been well understood. For all types of macro and micro vascular ECs, growth and formation of a monolayer is observed when cells are cultured on fibronectin or gelatin substrate in the presence of EC growth factor and heparin, however, they tend to dedifferentiate

in repeated subcultures and change their morphological and biochemical characteristics.

The objective of this study was to use *in vitro* cell culture on a bio mimetic matrix model to characterize phenotype of human saphenous vein endothelial cells (HSVEC) that is harvested from CVD patients. A fibrin composite matrix that has been already demonstrated to maintain the HUVEC phenotype in culture through several passages was used and compared its effect with the effect of conventionally used gelatin. Parameters studied were m-RNA expression of von Willebrand factor (vWF), plasminogen activation inhibitor (PAI), tissue plasminogen activator (t-PA) and nitric oxide synthetase (eNOS) and release of these molecules into the medium. Proliferation and apoptosis of HSVEC cultures obtained from 8 different patients were compared on two matrices until passage 12. Results suggest that on the model matrix HSVEC showed good proliferation rate and survival through several passages. It is demonstrated that matrix composition can influence switching of EC phenotypes into pro/anti thrombotic states. This matrix model may be suitable to study the effect of exogenous factors on EC dysfunction with respect to CVD.

IV.2. SELECTION OF PATIENTS FOR THE STUDY

Vein from randomly selected male patients of age between 40 and 60 undergoing coronary artery bypass grafting (CABG) at the hospital wing of our institute were used for the experiment. The collection of samples was done in concurrence with the requirements of the Institute Ethics Committee, and the baseline characteristics of 8 patients from whom the veins were obtained is given in TableIV.1. The base line characteristics were blinded from the lab till the study was completed.

IV.3. ISOLATION AND CHARACTERIZATION OF HSVEC

After pilot isolation and standardization of technique EC isolation from all 8 saphenous vein subjects was successful. Samples of ~2 cm length was received in the laboratory 8-12 h following vein harvest, subsequent to confirming that it was no longer required for the by pass repair. HSVEC in initial culture took longer period for reaching confluence, and subculture was possible only after 10 to 15 days after initial seeding. The cells obtained from the initial cultures were

split into two and equal parts were seeded on GCPS and CCPS, which was numbered as passage 1. The passage after passage transfer of cells from GCPS was to GCPS and that from CCPS was to CCPS.

Table IV-1. The Baseline Characteristics of Patients selected for the study. Age, sex, Major diagnosis and risk factors of the patients are listed in the table. AOE: Angina on exertion; ASMI: Antero Septal Myocardial Infarction. CAD: Coronary artery disease: COPD: Chronic Obstructive Pulmonary Disease; DM: Diabetes Mellitus; DOE FC II: Dyspnea on exertion Functional Class II; DVD: Double Vessel disease; HTN: Hypertension; IHD: Ischemic Heart Disease; LMCA: Left major coronary artery; MOD: Multi organ disease; PND: Paroxysmal Nocturnal Dyspnea; TIA: Transient ischemic attack; TVD: Triple vessel disease.

Serial No.	Age	Sex	Diagnosis	DM	Angina	IHD	HTN	Dyslipi-daemia	Smoking	Other Findings
1	56	M	CAD, TVD	+	+	+	+	-	+	ASMI, TIA
2	50	M	CAD, TVD	+	+	-	+	+	+	LMCA Disease
3	47	M	CAD, TVD	+	+	-	+	+	+	MOD, DOE FC II
4	58	M	CAD, DVD	+	+	+	+	+	+	PND, Stroke, TIA, COPD
5	52	M	CAD, TVD	+	-	-	-	-	-	AOE Class II
6	57	M	CAD, DVD	+	+	+	+	+	+	PND, AOE Class III
7	49	M	CAD, TVD	+	+	–	+	+	+	Stroke, COPD, ASMI
8	51	M	CAD, DVD	+	+	–	+	+	+	ASMI, LMCA Disease, PND

HSVEC grown on both matrices internalized DiI labeled AcLDL, which appear as granular structure throughout the cytoplasm, surrounding the nucleus. This confirms the homogenous nature of HSVEC isolated. During the initial 3-4 passages there was no difference in the uptake between cells grown on GCPS and CCPS. But after that the uptake of AcLDL found to be deteriorated in cells grown of GCPS. But cells grown on CCPS showed no considerable reduction

even by 12th passage. Figure IV.1 shows comparison of LDL uptake by cells grown on GCPS and CCPS from passages 3 and 9.

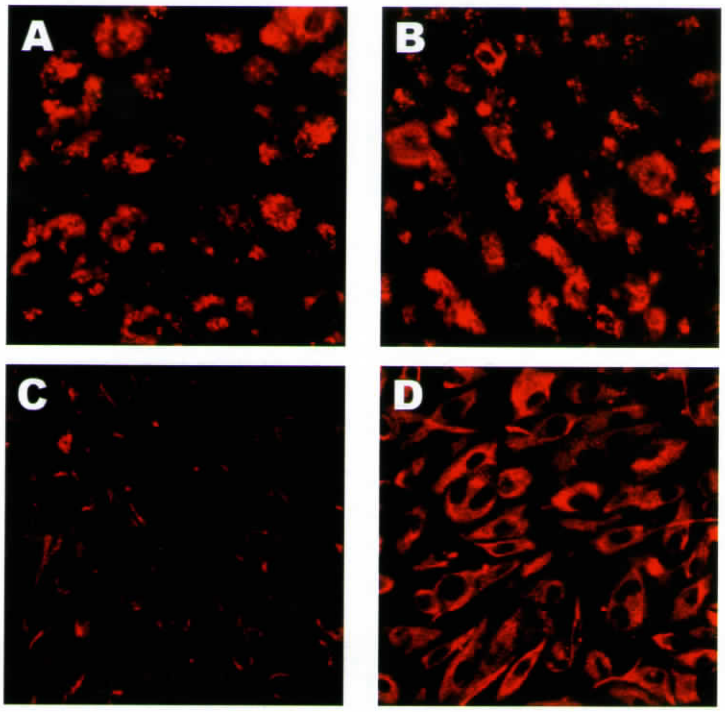


Figure IV.1. Fluorescent Micrographs of HUVEC showing DilAcLDL uptake: Comparison of LDL uptake by cells grown on GCPS and CCPS. A, GCPS 3P; B, CCPS 3P; C, GCPS 9P; D, CCPS 9P. Original magnification 40X.

When the cells were immuno stained for vWF using FITC conjugated antibodies, the perinuclear distribution of vWF in rod shaped Weibel Palade bodies was clearly evident. A representative confocal laser scanning micrograph of cells on GCPS and CCPS is given in Fig.IV.2. These experiments confirm the homogenous nature of isolated cells. The expression of vWF was very high even during the initial passages, compared to HUVECs. During the first 3-6 passages, the expression was comparable on both of the matrices. But in cells grown on GCPS, vWF expression went onto further higher levels with progressive passages; where as in cells on CCPS, the expression was found to be down regulated with passage progression. The data is discussed in detail along with the results of mRNA expression and release of vWF into the medium, later in this chapter.

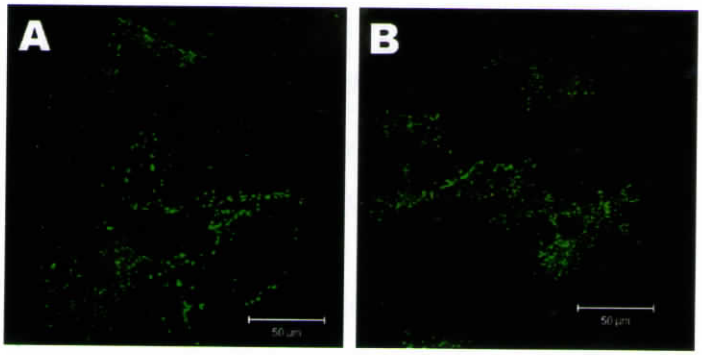


Figure IV.2. Confocal Micrographs of HUVEC stained for vWF using FITC conjugated antibodies. Micrographs show rod shaped Weibel Palade bodies. A, GCPS 2P; B, CCPS 2P.

The typical cobble stone morphology of endothelial cells was evident in the initial passages, on both GCPS and CCPS. A morphology change was seen after passage 6 when cells were grown on GCPS and in the later passages morphology changed drastically, whereas on CCPS typical EC morphology was maintained till passage 12 (Fig.IV.3).

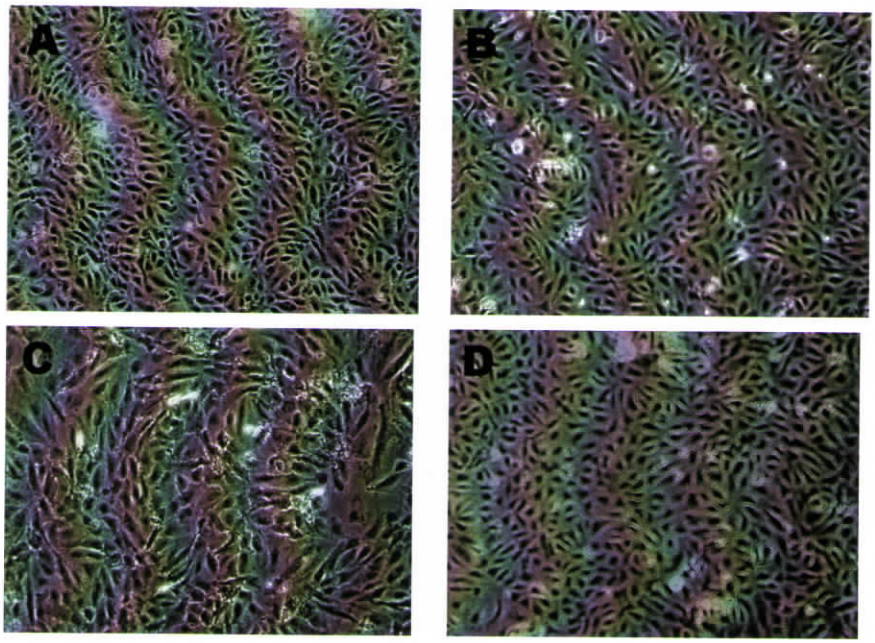


Figure IV.3. Phase contrast micrographs of HSVEC grown on GCPS and CCPS. A: GCPS Passage 3; B: CCPS Passage 3; C: GCPS Passage 12; D: CCPS Passage 12. Original magnification 10x.

IV.4. EFFECT OF MATRIX COMPOSITION ON HSVEC PROLIFEARTION AND SURVIVAL

IV.4.1. HSVEC Proliferation

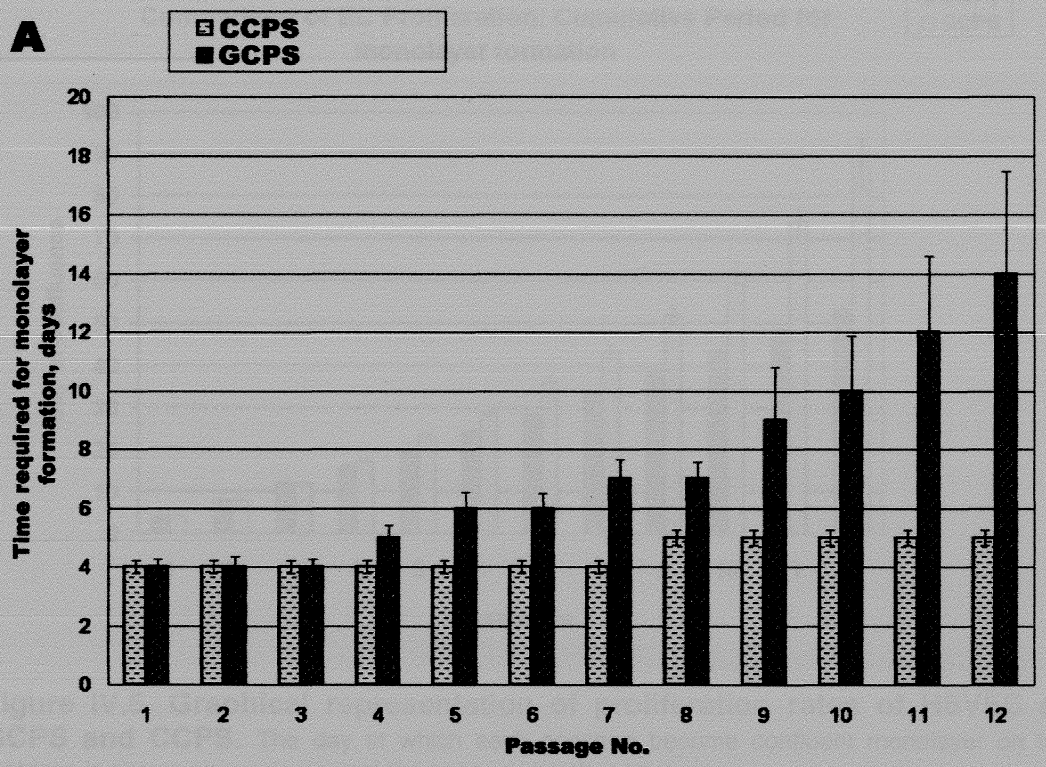
IV.4.1.1. Effect of matrix composition on serial passaging of HSVECs

Proliferation rate of HSVEC obtained from 8 patients were compared till 12th passage on GCPS vs. CCPS. While the proliferation rate was similar on both matrices till 3rd passage, cells grown on GCPS deteriorated from passage 4 onwards and the time taken to attain confluent monolayer was unacceptably high from 9th passage (Fig.IV.4A). The number of days taken for monolayer formation on CCPS was similar till passage 7, and even after that a steady proliferation rate was seen till passage 12. From Fig.IV.4A, it is clear that by 9th passage, the time taken for cells to form monolayer when grown on GCPS is almost the double when compared to similar passages on CCPS and by 12th passage it was 3 fold higher. Results from the doubling time analysis is well in agreement with the above finding (Fig.IV.4B). By 6th passage itself there is considerable difference in the proliferation rate and by 12th passage, the doubling time was found to be three fold higher when grown on GCPS compared to cells on CCPS.

When the cumulative time period for monolayer formation was evaluated till 12th passage on GCPS and CCPS, it was found that, the time taken to form 12 passages of confluent monolayer on GCPS is ~ 85-90 days whereas on CCPS in ~ 50-55 days 12 passages of monolayer were formed (Fig.IV.5).

IV.4.1.2. PCNA Immuno Staining

HSVECs grown on GCPS and CCPS were stained with FITC conjugated anti-PCNA antibodies to quantify the number of proliferating cell. Stained cells from passages 3, 6, 9 and 12 were analyzed using fluorescence microscope. It was found that till passage 3, the number of PCNA +ve cells were comparable on GCPS and CCPS (Fig.IV.6). But by 6th passage, percentage of proliferating cells reduced on GCPS and by 9th passage, only 40% of the cells stained +ve for PCNA. By 12th passage the proliferating cells were only ~ 20% on GCPS. On the other hand, cells on CCPS maintained the proliferating potency till 12th passage. From the Fig.IV.6, it is evident that even by 12th passage, almost 80% of the cells were +ve for PCNA when grown on CCPS.



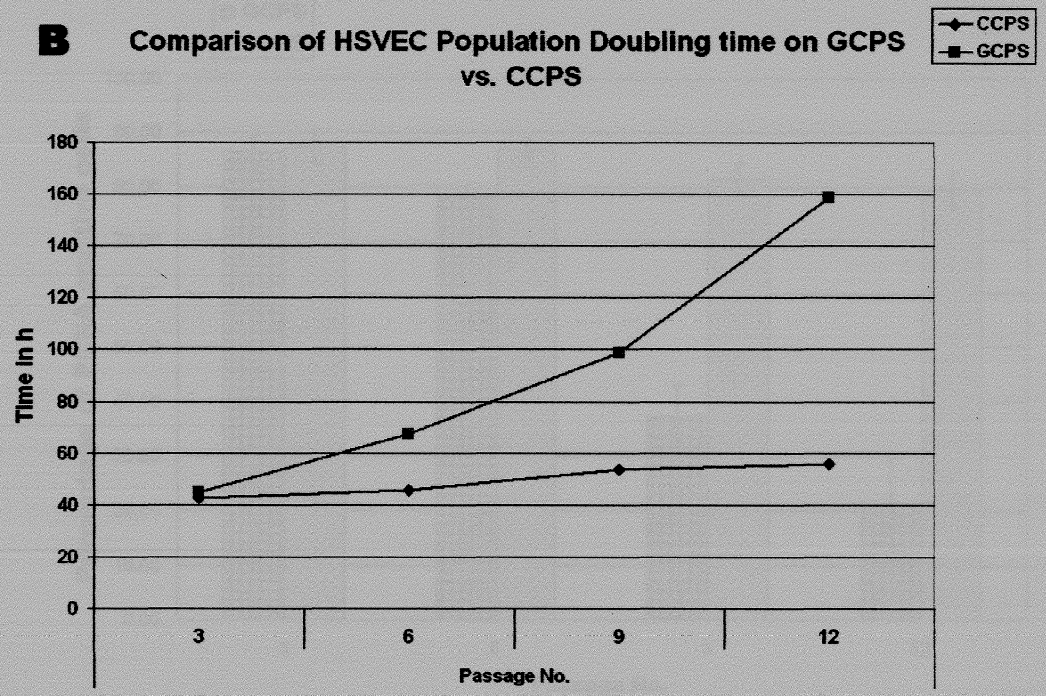


Figure IV.4. Graphical representation of proliferation rates of HSVEC on GCPS and CCPS. A: Time taken for each passage to form confluent monolayer on two matrices is shown on y-axis against the passage number on x-axis. B: Comparison of Population doubling time.

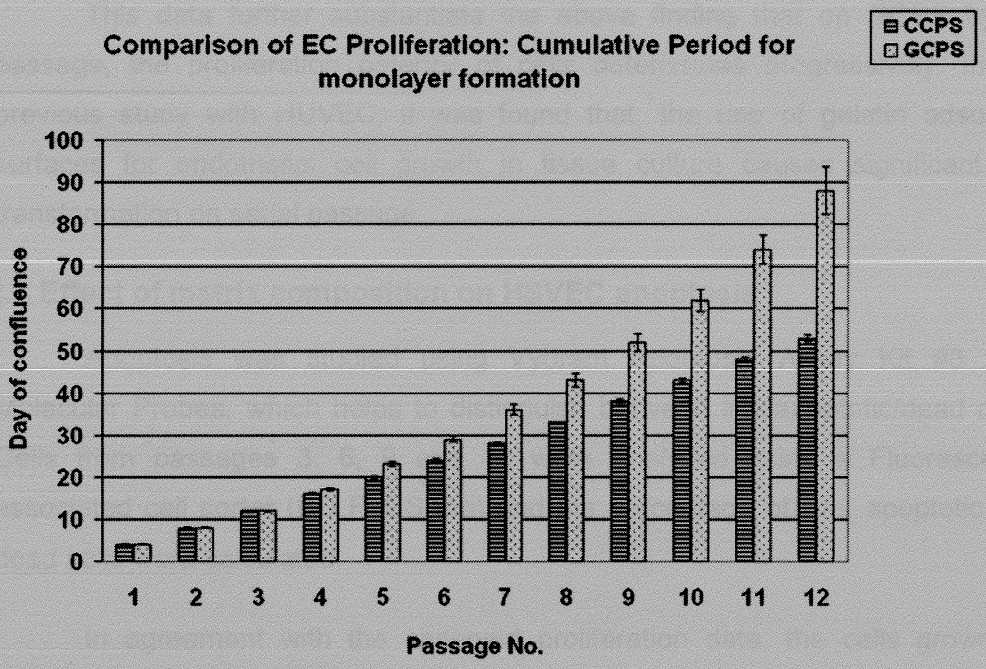


Figure IV.5. Graphical representation of proliferation rates of HSVEC on GCPS and CCPS. The day at which each passage become confluent monolayer on two matrices is shown on y-axis against the passage number on x-axis.

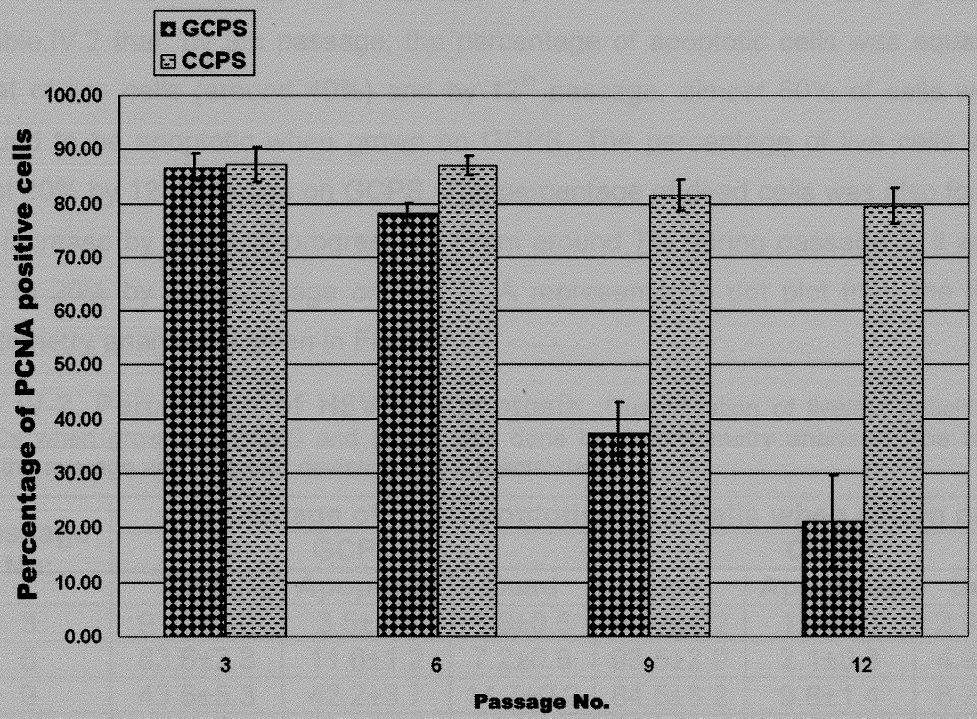


Figure IV.6. Comparison of PCNA +ve cells in HSVEC cultures grown on GCPS and CCPS. The semi quantitative data of PCNA staining of HSVEC grown on GCPS and CCPS was detected in different passages. Average and standard deviation were obtained by repeating the experiment with HSVEC isolated from 8 patients.

This data further substantiate the above finding that on GCPS by 6th passage, the proliferation potency of cells deteriorates progressively. In the previous study with HUVEC, it was found that, the use of gelatin adsorbed surfaces for endothelial cell growth in tissue culture causes significant cell transformation on serial passage.

IV.4.2. Effect of matrix composition on HSVEC apoptosis

Apoptosis was studied using Vybrant Apoptosis Assay Kit #3 from Molecular Probes, which helps to distinguish between live/apoptotic/dead cells. Cells from passages 3, 6, 9 and 12 were analyzed using a Fluorescence associated cell sorter (BD FACSAria) and the percentage of live, apoptotic and dead cells were calculated.

In agreement with the observed proliferation data, the cells grown on GCPS showed an apoptotic tendency by 6th passage. While more than 90% of the cells were live during the 3rd passage on both of the matrices, by 6th passage more than 10% of cells turned apoptotic on GCPS, and the percentage of live cells went down to 83% (Table.IV.2). It is evident from the data given in Table.IV.2 that, by 9th passage, the percentage of apoptotic cells was equal to that of live cells (around 40%) and by 12th passage, almost 60% of cells were found to be apoptotic when grown on GCPS. The percentage of live cells was just 20% by 12th passage on GCPS. The percentage of dead cells was also found to increase by passage progression. From around 7% during passage 3, it went up to 20% by 12th passage on GCPS. A representative dot plot from the flow cytometry analysis is given in Fig.IV.7.

Table IV-2. Percentage of HSVEC Apoptosis . Quantification of live/apoptotic/dead HSVECs when grown on GCPS and CCPS was done by flowcytometry analyses. The table shows the average and standard deviation of 8 subjects' data.

Passage No:	Percentage of Live/Apoptotic/Dead Cells when grown on					
	GCPS			CCPS		
	Live	Apoptotic	Dead	Live	Apoptotic	Dead
3	91.2±3.2	3.5±0.2	6.8±0.5	94.2±4.2	1.9±0.3	3.9±0.7
6	83.6±2.3	11.6±1.2	7.2±0.9	93.5±2.9	2.1±0.3	4.9±0.3
9	43.5±5.3	42.2±3.7	14.7±0.2	84.5±3.2	9.6±1.4	6.2±1.2
12	22.60±6.5	58.7±4.5	19.1±0.5	74.4±5.3	16.6±1.3	10.8±2.3

However, the tendency towards apoptosis was minimal in cells grown on CCPS. Even by 12th passage, the percentage of apoptotic cells were just above

5%. And the increase in the dead cells also was less when grown on CCPS compared to that on GCPS (Table.IV.2).

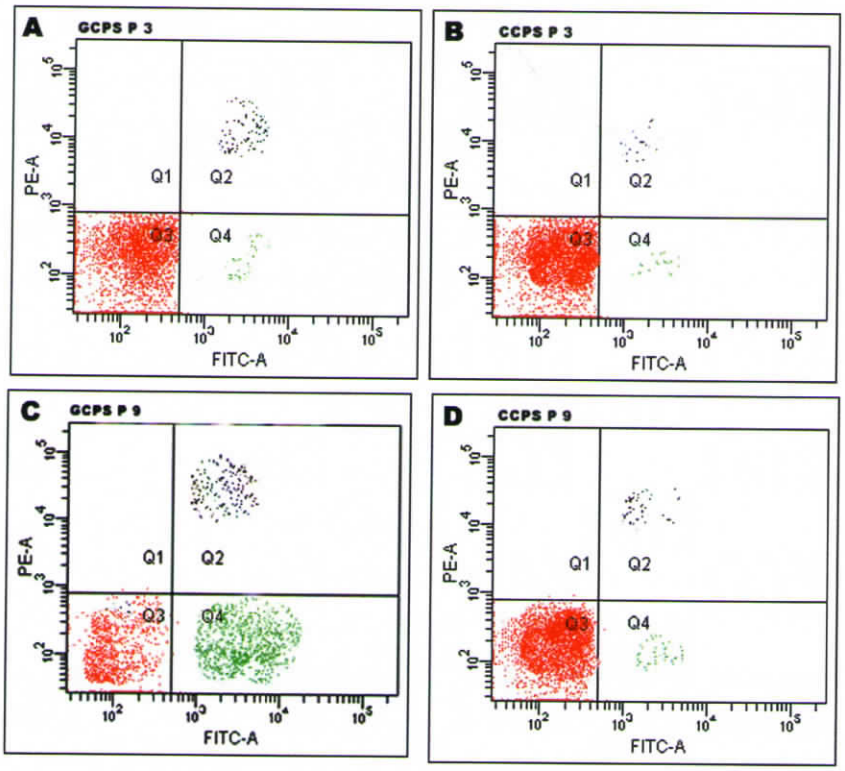


Figure IV.7. Quantification of live/apoptotic/dead cells when grown on GCPS and CCPS. Representative flow cytometry analyses of HSVECs of one patient are shown. The dot plot with quadrant gate is saved as jpg file. Q3 shows live cells, Q4 apoptotic cells and Q2 dead cells. A: GCPS Passage 3; B: CCPS Passage 3; C: GCPS Passage 12; D: CCPS Passage 12.

The apoptotic tendency noticed in the HSVEC culture is in agreement with what we observed with the HUVEC. On GCPS, the lack of well formed focal adhesion complex and failure in the signaling mechanism in providing the required signals for cell proliferation and survival is thought to play a major role in turning the cells apoptotic. It is interesting to note that electron microscopic studies of occluded human saphenous vein grafts showed severely abnormal endothelial cell structure with wide intercellular junctions, which was suggestive of apoptosis (Kockx et al 1994). It has been reported that apoptotic HUVECs, either adherent or in suspension, become pro coagulant by increased expression of PS and the loss of anticoagulant membrane components. In addition an area

of apoptotic endothelial cells *in vivo* may lead to significant exposure of sub endothelial matrix (Bombeli et al 1997).

IV.5. MATRIX COMPOSITION AND THE HSVEC PHENOTYPE

To find out the effect of matrix composition on the expression and synthesis of molecules regulating the anti-thrombotic behavior of endothelial cells, the expression level and release of two thrombotic and two anti-thrombotic molecules were analyzed. The mRNA expression level was studied by conventional RT-PCR and subsequent analysis by agarose gel electrophoresis. Also a semi quantitative difference in the expression levels was studied by real time PCR analysis. Beta actin was used as house keeping gene. The release of these proteins by HSVECs into the culture media was also studied. Release of t-PA, vWF and PAI-1 into the culture media was analyzed by ELISA, and the release of NO was quantified by nitrite estimation using modified Greiss reagent.

IV.5.1. Expression and release of vWF

When the transcription level of vWF mRNA was compared in cells grown on GCPS and CCPS by RT-PCR, it was found that the expression was comparable on both matrices during the initial passages (Fig.IV.8A). While, the proliferation rate, apoptosis and cell death were similar for cells isolated from all 8 subjects, subject # 5, in table IV.1 seems to have a different pattern of m-RNA expression of vWF. The other entire 7 subjects showed similar pattern, where both vWF and PAI were relatively high in cells of passage number 3, grown on both GCPS and CCPS. In cells on GCPS the expression increased as the passage number progressed, whereas in cells on CCPS, expression of vWF and PAI reduced as the passage number progressed. Representative result for vWF & PAI from one subject out of the group of 7 is given in Fig.IV.8A.

In the case of subject # 5, the mRNA expression of vWF was lower compared to all other 7 subjects during the 3rd passage on both GCPS and CCPS. On GCPS, the expression level showed a steady increase with passage progression, whereas on CCPS, the expression remained almost steady throughout the 12 passages (Fig.IV.8B).

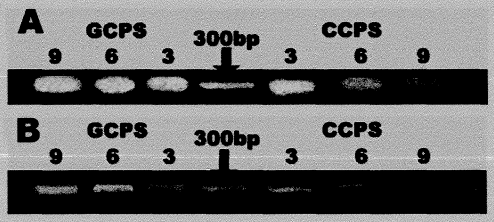


Figure IV.8. Relative abundance of vWF mRNA on serial passaging of HSVEC: Representative picture of gels shows amplicons from HSVEC grown on GCPS and CCPS. Passage numbers after which RNA was isolated are as numbered in each figure. The 300 bp band of the ladder is shown by arrow head. A, Representative picture of one subject out of the group of 7; B, Gel image of subject # 5.

In addition to the qualitative analysis of PCR products using agarose gel electrophoresis, a semi quantitative Real Time PCR measurement was carried out and the data is summarized in TableIV.3. Lower Ct value indicates that the mRNA expression is high. The higher the Ct value, the lower is the mRNA expression.

Table IV-3. Real Time PCR data for vWF: Effect of matrix during passaging. The table shows, quantification of the vWF transcript expression by real-time PCR in HSVECs grown on GCPS and CCPS. Beta Actin is used as the house keeping gene. The cycling time (Ct) values are given as average ± SE from 7 subjects. The Ct value of subject #5 is given in bracket.

Passage No:	vWF Ct Value	
	GCPS	CCPS
3	19.2±0.2 (21.3)	19.1±0.3 (21.4)
6	18.7±0.3 (21.1)	20.3±.0.5 (21.2)
9	18.1±0.2 (20.4)	20.9±0.4 (21.7)
12	17.4±0.5 (19.8)	20.8±0.5 (21.6)

Cycling time (Ct) values were not affected by the matrix and were similar in the passage number 3. But as the passage number increased, Ct value for vWF decreased on GCPS and increased on CCPS. Clearly the vWF mRNA seems to be over expressed as the passage number increased on GCPS, and the expression was found to be reduced on CCPS as passage progresses.

Here also, it was found that data from subject # 5 was different compared to the rest 7 subjects. The expression level was lower when compared to other 7 subjects, indicated by a higher Ct value. Expression was steady and same on CCPS even when passage number increased, but on GCPS they were affected by the increase in passage number. Thus no significant shift was seen in cells from subject #5, if cells were grown on CCPS and the Ct values of cells from 7 other subjects after 9 or 12 passages on CCPS match with that of subject #5 in passage 3.

To substantiate the reverse transcriptase PCR data, synthesis and release of vWF by HSVECs into the culture media was measured. Table.IV.4 gives the data of ELISA for vWF, average ± S.D of seven subjects and the values from cells of subject #5 in bracket.

The release of vWF was similar in all subjects studied during passage number 3 on both CCPS and GCPS. But as the passage number progressed, the release of vWF got amplified further in cultures on GCPS and was reduced in cultures on CCPS, indicating that on the former cells turn more thrombotic. Compared to subject #5, cells from all the 7 subjects showed high vWF even in passage number 3, but as passage number progressed on CCPS, values became similar to that of subject #5 in passage 3. Thus it is seen that cells growing continuously on CCPS turn in to a less thrombogenic phenotype.

Table IV-4. ELISA data for vWF: Effect of matrix during passaging. ELISA data for vWF released into culture medium from HSVECs grown on GCPS and CCPS. Average and standard deviation are calculated from seven experiments using HSVEC isolated from seven different subjects. The data of Subject #5 is given in bracket.

Passage No:	vWF ng/ ml	
	GCPS	CCPS
3	34.2±4.54 (9.8)	29.54±5.67 (9.6)
6	45.57±6.34 (15.8)	14.13±7.2 (7.6)
9	78.48±3.34 (42.5)	8.4±2.31 (6.4)
12	84.68±13.58 (62.7)	8.8±1.56 (6.9)

During the characterization of HUVECs it was noted that in cells immuno stained for vWF, the fluorescence intensity was high and there were more rod shaped Weibel Palade bodies seen in the cytoplasm even during the early passages, on both GCPS and CCPS. But as the passage progressed, the expression got further amplified in cells grown on GCPS, and Weibel Palade bodies were seen diffused throughout the cytoplasm, where as in cells on CCPS, the expression was found to be down regulated with passage progression (Fig.IV.9

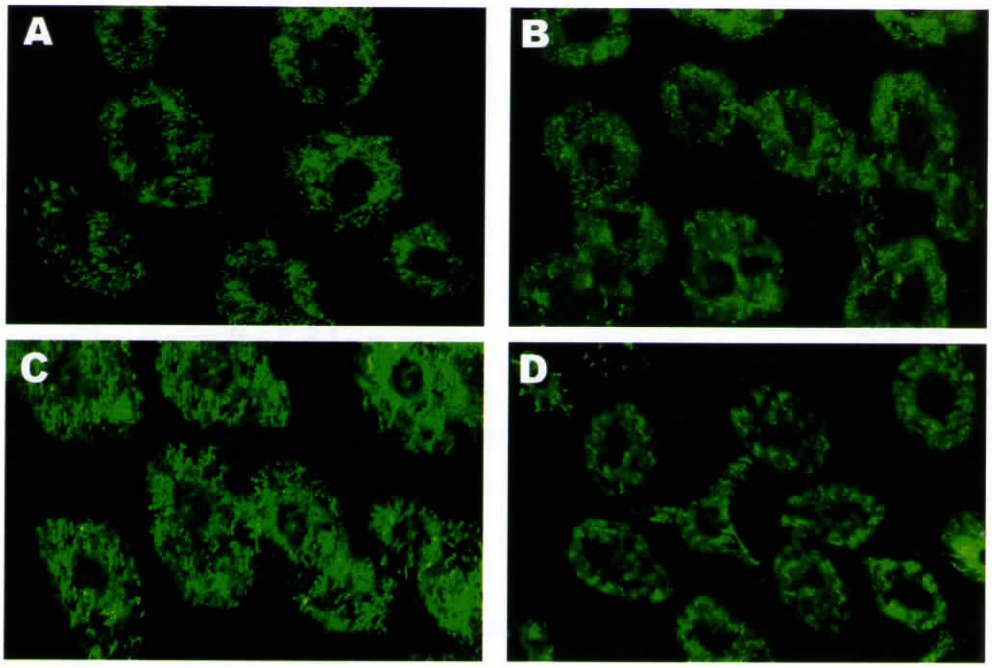


Figure IV.9. Fluorescent Micrographs of HSVEC stained for vWF using FITC conjugated antibodies. A, GCPS 3P; B, CCPS 3P; C, GCPS 9P; CCPS 9P. Original Magnification 40X.

From all these data, it is clear that HSVECs isolated from all the 7 subjects were more of a thrombogenic phenotype compared to HUVEC, during the initial passages itself. vWF is regarded as an acute phase thrombogenic protein which predicts severe coronary events complicating unstable coronary artery disease (Thompson et al., 1995). *In vivo* an up-regulation of vWf expression in acute respiratory failure, acute and chronic renal insufficiency, hypertension, diabetic nephropathy, and vasculitis (Cucuianu *et al.,* 1983; Carvalho *et al.,* 1982; Janson *et al.,* 1980; Pedrinelli *et al.,* 1994; Stehouwer, 1995; Kahaleh, 1981; Marasini,

et al., 1980; Pedrinelli *et al.,* 1994; Stehouwer, 1995; Kahaleh, 1981; Marasini, 1992) has been reported. Recently, an elevated level of von Willebrand factor has been reported to be a risk factor for coronary heart disease with other hemostatic factors (fibrinogen and factor VIII) (Folsom et al., 1997).

High levels of vWF is a (poor) prognostic indicator for myocardial infarction, re-infarction and mortality (Jansson et al., 1991; Bickel et al., 2002). vWF is also a prognostic indicator of other cardiovascular events such as stroke and the requirement for arterial surgery in patients with hypertension, intermittent claudication, angina and ischaemic heart disease (Blann and McCollum, 1999). In addition, high vWF predicts the development of thromboembolic events and poor prognosis in patients with rheumatoid arthritis and systemic sclerosis (Ames et al., 1997; Blann et al., 1997).Endothelial injury in atherosclerosis and inflammatory vascular disease is also accompanied by increased levels of procoagulant vWF (Blann, 1993; Blann and McCollum, 1994).

Thus it appears that in all 7 subjects except subject #5, EC was dysfunctional with critical pro thrombotic nature, and there is a gradual change in the expression and synthesis of vWF depending on the matrix on which cell subculture was done. In all the seven cases expression of the pro-thrombotic molecule vWF, reduced if the cells were grown on CCPS. In the case of subject #5, molecular expressions remained steady on CCPS but dedifferentiation into a more pro thrombotic phenotype was evident on GCPS.

IV.5.2. Expression and release of PAI-1

When the expression of PAI-1 mRNA was studied by RT-PCR, it was found that the pattern was similar to that of vWF expression. All seven subjects except subject # 5 showed a similar pattern and the expression was high on both GCPS and CCPS during initial passages. In cells grown on GCPS, PAI-1 transcription level went onto further higher levels on serial passaging, where as in cells on CCPS, the expression got reduced with passage progression (Fig.IV.10A).

In cells from subject # 5 also the trend was similar to that of vWF. During the initial passages, the expression level of PAI-1 was less compared to other 7 subjects on both the matrices. On GCPS, the expression got amplified with

passage progress; while on CCPS, the transcription level remained almost steady till 12th passage (Fig.IV.10.B).

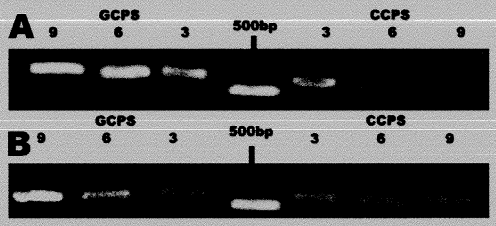


Figure IV.10. Relative abundance of PAI-1 mRNA on serial passaging of HSVEC: Representative picture of gels shows amplicons from HSVEC grown on GCPS and CCPS. Passage numbers after which RNA was isolated are as numbered in each figure. The 500 bp band of the ladder is shown by arrow head. A, Representative picture of one subject out of the group of 7; B, Gel image of subject # 5.

The Real Time PCR data also follows the same trend. The Ct values were similar during the initial passages on both matrices indicating that the expression level of PAI-1 mRNA was similar in cells on GCPS and CCPS during the initial 3 passages. But, in cells grown on GCPS, the expression got amplified with increase in passage number which is clear from the Table.IV.5 showing the Ct values. The Ct values got reduced from 18.7 to 17.2 by 12th passage. On the other hand, on CCPS, the expression level got reduced during the serial passages.

Table IV-5. Real Time PCR data for PAI-1: Effect of matrix during passaging. The table shows, quantification of the PAI-1 transcript expression in HSVECs grown on GCPS and CCPS. Beta Actin is used as the house keeping gene. The cycling time (Ct) values are given as average ± SE from 7 subjects. The Ct value of subject #5 is given in bracket.

Passage No:	PAI-1 Ct Value	
	GCPS	CCPS
3	18.7±0.5 (20.4)	18.5±0.4 (20.2)
6	18.1±0.2 (19.9)	19.2±0.3 (20.4)
9	17.6±0.3 (19.1)	19.8±0.2 (20.3)
12	17.2±0.2 (18.5)	19.7±0.1 (19.9)

In the case of PAI-1 expression also, it was found that data from subject # 5 was lower compared to other 7 subjects, indicated by a higher Ct value.

Expression remained almost the same on CCPS even when passage number increased, but on GCPS the transcription level of PAI-1 mRNA got amplified as passage progressed. It was found that the expression of PAI-1 by cells from 7 other subjects after 9 or 12 passages on CCPS match with that of subject #5 in passage 3.

When the release of PAI-1 by HSVECs into the medium was studied by ELISA, it was found that the protein release by cells from all subjects except subject #5 was similar and higher during initial passages. Upon serial passaging on GCPS, the release got further higher, while when grown on CCPS, the PAI-1 release into the medium was found to be reduced passage after passage ($p < 0.001$). In the case of subject # 5, the PAI-1 in the medium was lower on both the matrices during initial passages compared to the rest 7 subjects. When grown on GCPS, the release was found to be increasing with passage number, while on CCPS, the release remained almost steady.

Table IV-6. ELISA data for PAI-1: Effect of matrix during passaging . ELISA data for PAI-1 released into culture medium from HSVECs grown on GCPS and CCPS. Average and standard deviation are calculated from seven experiments using HSVEC isolated from seven different subjects. The data of Subject #5 is given in bracket.

Passage No:	PAI-1 ng/ ml	
	GCPS	CCPS
3	405±16 (168)	398±8 (157)
6	548±21 (286)	257±16 (149)
9	657±14 (406)	195±24 (143)
12	694±18 (568)	197±32 (147)

In addition to this apparent key role within thrombosis and hemostasis, PAI-1 seems to contribute to the development of atherosclerosis and other vascular diseases. Studies of PAI-1 expression in normal and atherosclerotic vessels have suggested an association between increased PAI-1 expression and the progression of atherosclerotic disease (Raghunath et al., 1995). Schneiderman et al (1992) demonstrated increased levels of PAI-1 mRNA levels

in severely atherosclerotic aortic tissue, compared with normal and mildly affected arteries.

Furthermore, in patients with coronary artery disease as well as early atherosclerosis, high PAI-1 plasma levels have been found (Hamsten et al., 1987; Huber et al., 1990; Salomaa et al., 1995). In fact, an increase in plasma PAI-1 as well as enhanced expression of PAI-1 mRNA has been implicated in the process of restenosis after balloon dilatation (Huber et al., 1992; Sawa et al., 1994). Therefore, it was assumed that high PAI-1 levels would attenuate fibrinolysis and in turn create a prothrombotic state that might result in pathological fibrin deposition, thus promoting accumulation of extracellular matrix and atheroma formation.

From the data on expression level of mRNA and release of the protein into the medium, it was evident that cells from all subjects except subject # 5 were more of a pro-thrombotic phenotype with high PAI-1 expression during the initial passages itself. This further confirms the dysfunctional nature of ECs isolated from those subjects. When grown on GCPS, the cells turned more thrombotic, whereas cells grown on CCPS turned less thrombotic. This further suggests that cell matrix interaction plays a major role in giving proper signaling for the maintenance of normal phenotype. The composite matrix was found to be successful in giving signals for cell proliferation, survival and differentiation.

IV.5.3. Expression and release of tPA

In the case of the anti thrombotic molecule tPA, the effect of matrix was different. In all 7 subjects other than #5, the expression was less in cells of passage 3, irrespective of the matrix. As they were continuously passaged on CCPS, expression got augmented while it lessened in cells on GCPS (Fig.IV.11A). In the case subject #5, expression of tPA was similar on both matrices during passage3, but higher when compared to all 7 subjects in the study group. When grown on CCPS, cells from subject #5 showed a steady expression of tPA m-RNA through 12 passages, whereas on GCPS, the expression level deteriorated (Fig.IV.11B).

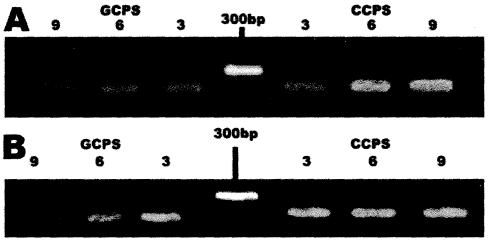


Figure IV.11. Relative abundance of tPA mRNA on serial passaging of HSVEC: Representative picture of gels shows amplicons from HSVEC grown on GCPS and CCPS. Passage numbers after which RNA was isolated are as numbered in each figure. The 300 bp band of the ladder is shown by arrow head. A, Representative picture of one subject out of the group of 7; B, Gel image of subject # 5.

The Real time PCR data was also well in agreement with the above finding. Ct values were similar during the 3rd passage on both the matrices, which indicates that the expression level of mRNA by cells grown on GCPS and CCPS were comparable during the initial passages. But while passage number progressed, the Ct value went up on GCPS; while on CCPS, the Ct values got reduced on serial passaging. This shows that the expression of tPA mRNA got down regulated while passaging on GCPS and on CCPS the transcription level of tPA improved on serial passaging (Table IV.7).

Table IV-7. Real Time PCR data for tPA: Effect of matrix during passaging. The table shows, quantification of the tPA transcript expression in HSVECs grown on GCPS and CCPS. Beta Actin is used as the house keeping gene. The cycling time (Ct) values are given as average ± SE from 7 subjects. The Ct value of subject #5 is given in bracket.

Passage No:	tPA Ct Value	
	GCPS	CCPS
3	23.2±0.04 (21.2)	23.1±0.05 (21.1)
6	23.6±0.06 (22.4)	22.6±0.09 (20.9)
9	23.9±0.1 (23.2)	21.8±21.1 (21)
12	24.3±0.2 (23.9)	21.9±0.1 (21.4)

In the case of subject #5, the tPA expression was almost 4fold higher compared to the rest 7 subjects during passage 3 on both the matrices. On CCPS, the expression remained steady; whereas when grown on GCPS, the

mRNA expression of tPA got worsened and reached similar levels found in other 7 subjects.

When the release of tPA into the medium was studied, in all 7 subjects, tPA level was found to be much less in passage number 3, as compared to subject #5, but as the passage number of these cells increased on CCPS, more tPA was detected in the medium and was almost close to the value in passage number 3 of the subject #5 (Table.IV.8). On the other hand, release of tPA into the medium by cells passaged on GCPS got further reduced with passage progress. Even in the case of subject #5, when grown on GCPS, the release level went down drastically to reach the same level compared to other 7 subjects by 12th passage. On CCPS the release of tPA by cells from subject #5 remained steady throughout the study.

Generally, tPA is considered to be a protective factor for the development of thrombotic disease. A decrease in the fibrinolytic activity is linked to an increase in risk of myocardial infarction (Verheugt et al., 1987; Hamsten et al., 1987). It has been found that tPA antigen is a risk factor for the long term mortality in patients with angina pectoris and coronary artery stenosis (Margaglione et al., 1994). A large scale prospective study has shown the predictive power of tPA antigen on myocardial infarction and stroke (Scarabin et al., 1998).

Table IV-8. ELISA data for tPA: Effect of matrix during passaging. ELISA data for tPA released into culture medium from HSVECs grown on GCPS and CCPS. Average and standard deviation are calculated from seven experiments using HSVEC isolated from seven different subjects. The data of Subject #5 is given in bracket.

Passage No:	tPA ng/ ml	
	GCPS	CCPS
3	0.05±0.004 (0.48)	0.05±0.005 (0.47)
6	0.038±0.007 (0.35)	0.25±0.09 (0.51)
9	0.036±0.002 (0.08)	0.42±0.07 (0.58)
12	0.035±0.004 (0.04)	0.41±0.08 (0.56)

Paradoxically the most convincing association between fibrinolysis and cardiovascular risk was an increased level of the profibrinolytic enzyme itself

(Ridker et al., 1993; Juhan-Vague et al., 1996). Blann et al. (1995) reports an increased tPA antigen activity in atherosclerosis. However, to understand the apparently paradoxical association of ischemic events with high plasma levels of tPA, one should remember that the above normal levels are reported in subjects with high plasma PAI-1 (Padro et al., 1995; Raghunath et al., 1995). An increase in tPA is thought to reflect an inhibitory effect of PAI-1 on tPA activity, so that high levels of either factor reflects reduced fibrinolysis (Margaglione et al., 1994). Alternatively, the increased plasma tPA may be the product of the activity of another cell such as the platelet or the hepatocyte (Blann et al., 1995).

The biological mechanisms underlying the association of elevated tPA/PAI-1 or vWF with recurrent myocardial infarction are not yet clear. But conventional dogma suggests that at least part of the tendency towards coagulation and reduced tendency towards fibrinolysis in atherosclerosis may be related to increased PAI-1 activity and reduced tPA activity and that this could explain the greater risk of thrombosis in these patients. Wiman et al. (2000), provides further confirmation of the association of hemostatic variables with cardiovascular risk. It remains to be established; however, to what extent measurement of these factors may help to identify patients with elevated cardiovascular risk and whether this may have therapeutic consequences.

The finding that the expression of tPA by HSVECs grown on GCPS is negligible, further substantiate that, the cells on GCPS turns more thrombogenic during passaging. However, when grown on CCPS, they not only down regulates the expression of pro-thrombotic molecules such as vWF and PAI-1, but also up regulates the expression of anti-thrombotic tPA, turning them more towards the normal anti-thrombotic behavior. tPA production by cultured ECs is regulated by a variety of external stimuli at the level of gene transcription and cellular release (Levin et al., 1989). So this finding clearly suggests that the matrix on which ECs are cultured has far reaching influence on the cell phenotype.

IV.5.4. Expression of eNOS and release NO

The enzyme eNOS is the endothelial isoform of nitric oxide synthase which is responsible for the production of NO, a potent anti-thrombotic molecule and vasodilator. When the transcription level of eNOS mRNA was assessed by RT-PCR, we found that the expression is very low on both the matrices during

the initial passages, except for the subject #5. On GCPS, the expression level went down further on passaging. But strikingly, the expression of eNOS mRNA was found to be increasing with passage progression when cells were passaged on CCPS (Fig.IV.12). In the case of subject #5, the expression was similar and almost comparable with the expression level of HUVECs during passage 3. But on progressive passages on GCPS, the expression level went down and resembled the expression profile of the rest 7 subjects. In cells passaged on CCPS however, there was a slight improvement rather than reduction in the transcription level.

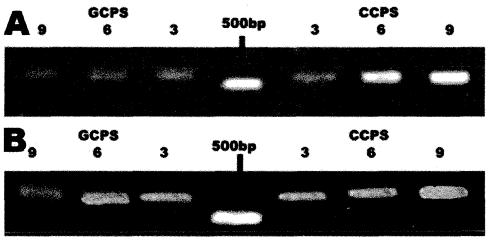


Figure IV.12. Relative abundance of eNOS mRNA on serial passaging of HSVEC: Representative picture of gels shows amplicons from HSVEC grown on GCPS and CCPS. Passage numbers after which RNA was isolated are as numbered in each figure. The 500 bp band of the ladder is shown by arrow head. A, Representative picture of one subject out of the group of 7; B, Gel image of subject # 5.

When the expression level was monitored by Real-Time PCR to confirm the above results, it was found that the Ct values were higher during the initial passages on both matrices, indicating a low level of expression in cells from all 7 subjects except subject #5 (Table.IV.9). The Ct values went further higher when cells were passaged on GCPS, and on contrary when grown on CCPS, the Ct values came down by almost 2 units, which indicates a 4fold increase in mRNA expression by passage 9 which remained study till passage 12 (Table.IV.9).

In the case of subject #5, the Ct values were higher during passage 3 on both GCPS and CCPS, compared to rest 7 subjects, and the expression level was almost double as indicated by the Ct value (Table.IV.9). The expression went down by almost 4 fold, when passaged on GCPS; while the expression level was almost steady in cells grown on CCPS even by 12th passage.

Table IV-9. Real Time PCR data for eNOS: Effect of matrix during passaging.
The table shows, quantification of the eNOS transcript expression in HSVECs grown on GCPS and CCPS. Beta Actin is used as the house keeping gene. The cycling time (Ct) values are given as average ± SE from 7 subjects. The Ct value of subject #5 is given in bracket.

Passage No:	eNOS Ct Value	
	GCPS	CCPS
3	25.4±0.05 (24.3)	25.2±0.06 (24.2)
6	25.8±0.1 (24.9)	24.1±.0.1 (23.8)
9	26.1±0.2 (25.7)	23.4±0.2 (23.6)
12	26.8±0.1 (26.1)	23.5±0.1 (23.9)

To further confirm the mRNA expression data of eNOS, the release of NO, which is the end product and the molecule responsible for the anti-thrombotic effect, was assayed in the culture medium. The finding was well in agreement with the mRNA expression data in that, the NO release was almost negligible during the passage 3 in cells from all 7 subjects except subject #5 (Fig.IV.13). When passaged on GCPS, the release was found to be reduced further by passage 12. On the other hand, in cells passaged on CCPS, the release of NO went up several folds by passage 6, and then again it doubled by passage 9, and remained almost steady till 12th passage.

In the case of subject #5, the release of NO was much higher than that of rest 7 subjects during passage 3 and was comparable on both matrices (Fig.IV.13). But on serial passaging on GCPS, the release went down drastically to reach almost comparable level with that of other 7 subjects by passage 12. On contrary, the release of NO remained steady till passage12 in cells passaged on CCPS.

Nitric oxide (NO) is recognized as an important endothelium-derived vasodilator in several vascular beds (Moncada et al., 1991). Impaired endothelium-dependent coronary and peripheral vasodilation has been reported during heart failure in both humans and animals (Drexler et al., 1992; Katz et al., 1993; Wang et al., 1994).

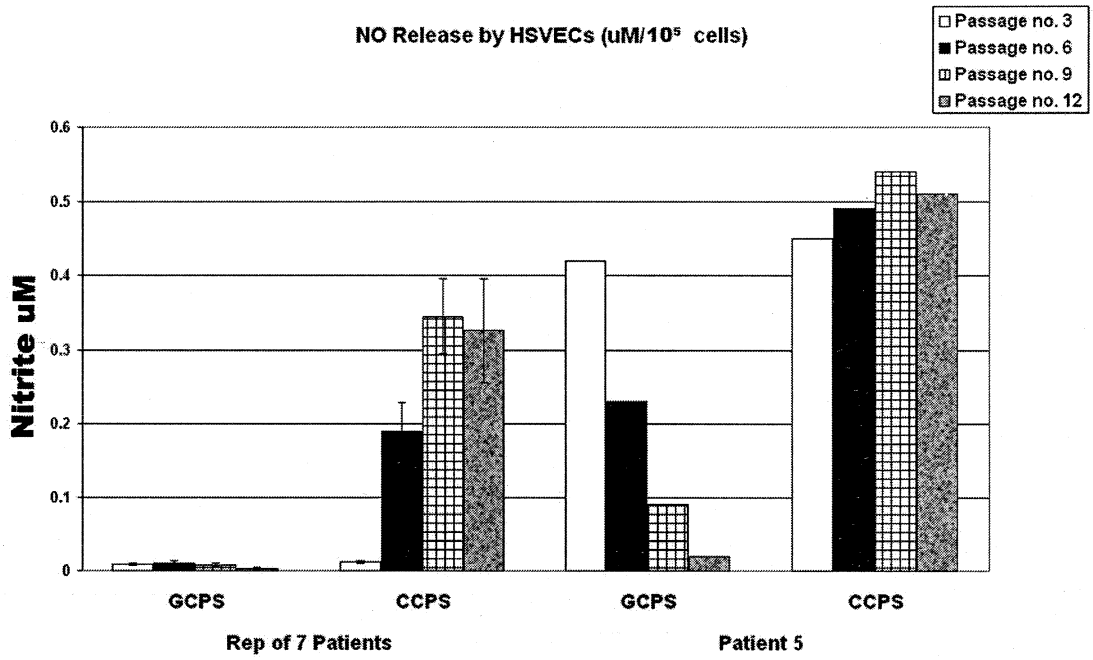


Figure IV.13. Graphical representation of NO release by HSVECs grown on GCPS and CCPS: Cells at 3, 6, 9 and 12 passages were incubated for 24h in serum free phenol red free medium after attaining confluence, and nitrite in the medium was assayed using Griess reagent. The Y-axis is nitrite in $\mu M/10^5$ cells. The Average ± SE of 7 subjects are shown on the left side. Subject #5's data is on right side.

The observation of deficient NO-mediated vasorelaxation in hypercholesterolemia suggests that loss of NO bioactivity is an early feature of atherosclerosis (Shimokawa and Vanhoutte, 1989; Cooke et al., 1992). In animal models, treatment with NOS inhibitors potentiates neointimal proliferation (Naruse et al., 1994; Cayatte et al., 1994), whereas supplemental L-arginine (Cooke et al., 1992; Candipan et al., 1996) or NO adducts (Guo et al., 1994; Marks et al., 1995) improve endothelial function and limit neointimal proliferation. Mice with targeted deletion of the eNOS gene are hypertensive (Huang et al., 1995) and respond to vascular injury with increased intimal hyperplasia and abnormal remodeling (Rudic et al., 1998; Mashimo and Goyal, 1999).

NO also appears to play a significant role in human atherogenesis. Atherosclerosis is associated with reduced endothelial NO production (Oemar et al., 1998), and in patients with coronary artery disease, even angiographically normal coronary segments show paradoxical constriction to acetylcholine (Ludmer et al., 1986). Endothelial function is also deficient in preatherosclerotic conditions such as hypercholesterolemia (Zeiher et al., 1993), diabetes mellitus (Williams et al., 1996), hypertension (Calver et al., 1992), smoking (Zeiher et al.,

1995), and normal aging (Zeiher et al., 1993). Treatment of hypercholesterolemia (Egashira et al., 1994), dietary supplementation with L-arginine (Drexler et al., 1991; Clarkson et al., 1994), or antioxidant therapy (Levine et al., 1996) improves the functional deficit. These studies suggest that loss of normal NO bioavailability precedes progression to more advanced lesions.

The mechanisms that result in loss of NO bioactivity in vascular disease have remained less clear. Data from experimental models suggest that increased production of reactive oxygen species such as superoxide anion (OO-) rapidly react with NO and contribute to the NO deficit in vascular diseases (White et al., 1994; Harrison and Ohara, 1995). Further complexity in the relationships between NO and superoxide in the vascular wall is suggested by the observation that eNOS itself can generate superoxide rather than NO under conditions of substrate or cofactor deficiency (Xia et al., 1996; Cosentino et al., 1998) or in response to atherogenic stimuli such as hyperglycemia (Cosentino et al., 1997) or LDL particles (Pritchard et al., 1995). These findings have led to the concept of NOS uncoupling, where the activity of the enzyme for NO production is decreased, in association with an increase in NOS-dependent superoxide production.

Several studies suggest a role for Tetrahydrobiopterin (BH4) in regulating vascular NO bioavailability. Endothelial dysfunction is improved by addition of BH4 to vessel rings from animals with atherosclerosis, diabetes or hypertension (Cosentino et al., 1998). Vascular superoxide production appears to be at least partially mediated by BH4 dependent eNOS uncoupling in several vascular disease animal models, including atherosIcerosis, (Laursen et al., 2001) diabetes (Meininger et al., 2000; Hink et al., 2001) and hyperhomocysteinemia (Lang et al., 2000). There are reports suggesting that, eNOS dysfunction is in part regulated by protein kinase C (PKC) signalling, since PKC inhibitors abrogate the increase in superoxide production in vessels (Hink et al., 2001; Guzik et al., 2002) and improves NO-mediated endothelial function (Beckman et al., 2002).

From this it is clear that the HSVECs isolated from the subjects except subject #5, are dysfunctional in terms of NO synthesis. From Table.IV.1, it is clear that all of them have multiple risk factors, which may be associated with decrese in the eNOS activity. The results from our study clearly substantiate that the cells

which are already dysfunctional in terms of eNOS expression and NO release, when grown on GCPS turns more thrombogenic on serial passaging. And the most striking observation was that the dysfunctional HSVECs from 7 subjects, showed signs of improvement in terms of synthesis of anti-thrombotic molecules, when grown on CCPS.

To eliminate any discrepancy in the isolation procedure which may affect the overall mRNA content, we have used Beta Actin as house keeping gene. It was found that expression level of beta actin was similar in cells from all 8 subjects. There was no difference in trend noticed in the case of subject #5 in the expression of Beta Actin. Also the transcription level of beta actin was steady throughout 12 passages (Fig.IV.14), on both GCPS and CCPS, when RT-PCR was started with same quantity of RNA (20ng). The total RNA isolated was less during the later passages on both matrices. In the case of cells on GCPS, the quantity of RNA isolated was much less during passages 9 and 12 which may be due to the apoptotic tendency exhibited by cells, which is discussed in detail earlier in this chapter.

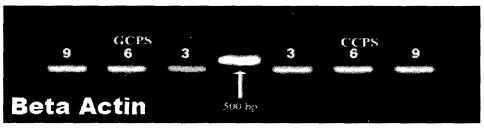


Figure IV.14. Relative abundance of the house keeping gene Beta Actin mRNA on serial passaging of HSVEC: Representative picture of gel shows amplicons from HSVEC grown on GCPS and CCPS. Passage numbers after which RNA was isolated are as numbered in each figure. The 500 bp band of the ladder is shown by arrow head.

The Real Time PCR data again confirms this observation in that, the Ct value remained steady throughout the study till the passage 12 on both GCPS and CCPS (Table.IV.10). The Ct values for Beta Actin was much lower compared to the other molecules studied. This is because of a high level of constitutive expression of Beta Actin by the cells, which is a well recognized fact.

Table IV-10. Real Time PCR data for Beta Actin: Effect of matrix during passaging. The table shows, quantification of the Beta Actin transcript expression in HSVECs grown on GCPS and CCPS. Beta Actin is used as the house keeping gene. The cycling time (Ct) values are given as average ± SE from 8 subjects.

Passage No:	Beta actin Ct Value	
	GCPS	CCPS
3	16.4±0.2	16.5±0.1
6	16.5±0.08	16.6±0.2
9	16.4±0.1	16.4±0.3
12	16.8±0.3	16.5±0.2

Expression of pro thrombotic and anti thrombotic molecules under different conditions of the *in vitro* culture of HSVEC passaged on GCPS and CCPS is summarized in Table IV.11. It appears that in all 7 subjects EC was dysfunctional with critical pro thrombotic nature, and there is a gradual change in the expression and synthesis of vWF, PAI, t-PA and NO depending on the matrix on which cell subculture was done. Dysfunctional endothelium appears to contribute significantly to systemic vascular disorders such as atherosclerosis (Schnitzer, 1998), and some pathological clinical conditions were found to be associated with the conversion to a non-constitutive activated endothelial state with an inflammatory thrombogenic phenotype (Stern et al 1988; Stern et al 1991). It has been suggested that the tissue microenvironment surrounding blood vessels controls endothelial cell phenotype (Nachman and Jaffe, 2004).

In all the seven cases HSVEC changed in to a better phenotype if the cells were grown on CCPS. In the case of subject #5, molecular expressions remained steady on CCPS but dedifferentiation into a more pro thrombotic phenotype was evident on GCPS. In summary, out of 8 subjects, HSVEC from one subject was not pro thrombotic but all the other seven were pro thrombotic. It is evident that cells dedifferentiate in cultures grown continuously on gelatin but on the model matrix cells gradually shifted into a phenotype with normal cellular function in comparison with HUVEC or cells from subject #5.

Table IV-11. Gradation of pro-/anti- thrombotic molecule expression Relative mRNA expression and release kinetics of Pro- and anti-thrombotic molecules by HSVECs when grown on GCPS and CCPS. The grades are given arbitrarily to indicate the raise/drop in the expression. The mRNA expression data is based on Real Time PCR experiment. The release data is based on ELISA for vWF, PAI &t-PA and nitrite assay for NO.

Passage number & Matrix	mRNA vWF/PAI		Release of vWF/PAI		m-RNA t-PA/eNOS		Release of t-PA/eNOS	
	Group of seven subjects	Subj-ect#5	Group of seven subjects	Subj-ect #5	Group of seven subjects	Sub-ject #5	Group of seven subjects	Subj-ect#5
P 3 CCPS	+++	+	+++	+	+	+++	-	+++ +
P6 CCPS	++	+	++	+	++	+++	++	+++ +
P9 CCPS	+	+	+	+	+++	+++	+++	+++ +
P12 CCPS	+	+	+	+	+++	+++	+++	+++ +
P 3 GCPS	+++	+	+++	+	+	+++	-	+++ +
P6 GCPS	++++	+	++++	++	+	++	- -	++
P9 GCPS	+++++	++	+++++	+++ +	-	+	- -	+
P12 GCPS	++++++	+++	+++++	+++ ++	- -	-	- -	-

Chapter V

EC SEEDING ON BIOMATERIALS: TISSUE ENGINEERING OF CARDIOVASCULAR DEVICES

V.1. ABSTRACT

Incidence of thrombogenesis and occlusion of cardiovascular implants is likely to be reduced by endothelial cell (EC) growth promotion on the biomaterials used for device fabrication. However, proper signaling between the matrix proteins deposited on the device surface and the cells grown on it is a prime requirement for growth and functionality. It was demonstrated earlier that a composition of matrix proteins that include fibrin, fibronectin, gelatin and growth factors maintain a steady proliferation potential and prolong the survival of endothelial cells in *in vitro* culture. In this study the assessment of the same matrix to promote endothelialization of biomaterials used for fabrication of cardiovascular implants were carried out.

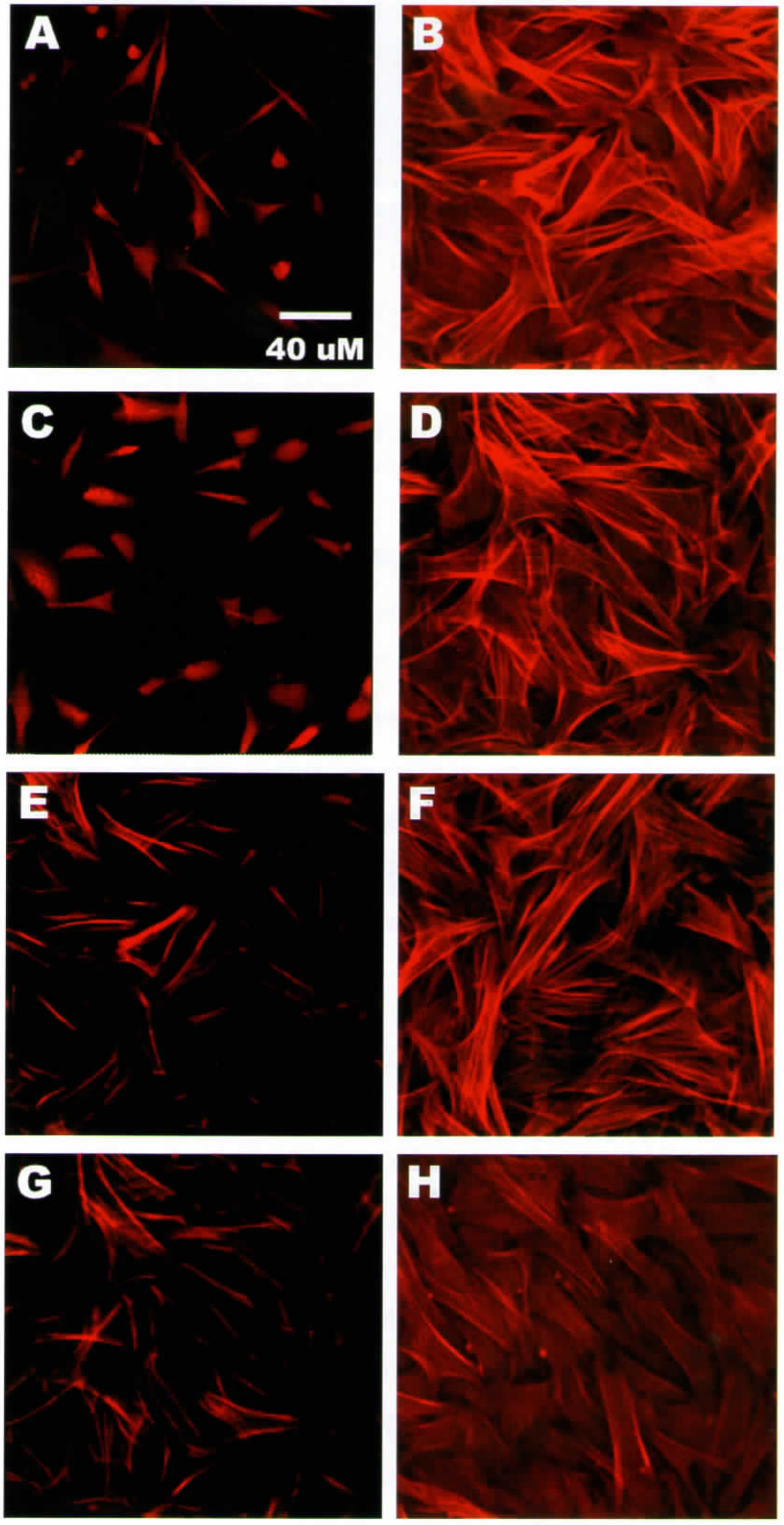
The specific objectives of this study were: 1) To standardize the composite matrix coating onto different biomaterials used as cardiovascular implants; 2) To compare the composite matrix coated devices with bare devices, in terms of EC adhesion, spreading, proliferation, and survival; and 3) To compare thrombogenecity of ECs grown on bare materials with the ECs on a biomimetic composite matrix by mRNA expression analysis, platelet adhesion and coagulation studies. The results suggests that the designed composition of bio mimetic adhesive proteins and growth factors is suitable for EC growth, survival and functional integrity, thus making it suitable for cardiovascular tissue engineering that requires *in vitro* EC culture.

V.2. EC SPREADING AND FOCAL ADHESION FORMATION ON MATERIALS

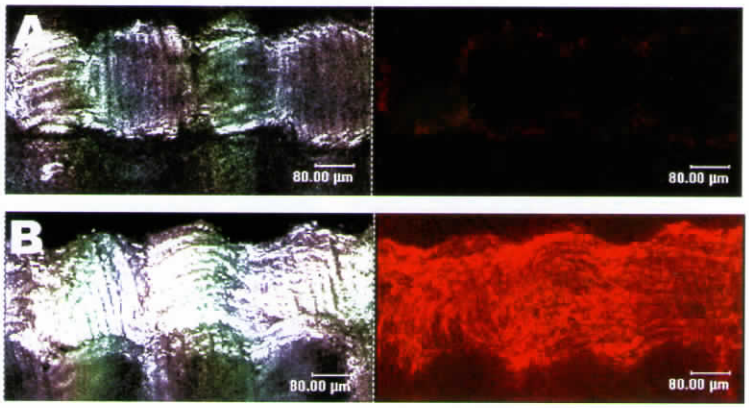
V.2.1. Cell spreading on materials by F-Actin staining

Actin organization is an important event in cell adhesion, spreading and proliferation. Therefore, a comparative analysis was done among five different materials that have intended applications in cardiovascular device fabrication. From the Fig.V.1, it is evident that, on all of the bare materials studied, cell spreading is poor as seen from the actin organization. Cells were not spread and remained elongated. After the composite coating on all base materials actin organization was found to be much improved (Fig.V.1 B, D, F, and H). It was seen that compared to bare Ti, DLC-coated Ti is better for cell spreading as indicated by the actin organization (Fig.V.1A, C). Since Dacron is porous and that the material surface is irregular, cells could not be viewed under ordinary fluorescent microscope. It was viewed under confocal microscope and a series of z sections were taken. The sections were then superimposed. From the Fig.V.2, showing the confocal microscopic image of actin stained cells on bare and composite coated Dacron, it is evident that on bare material, the spreading is poor. On coating with the composite matrix improvement in spreading was seen (Fig.V.2B).

Since actin organization is dependent on cell adhesion and focal adhesion organization, the intensity of actin stain was quantified on composite coated material against the bare using scanning confocal microscopy. Confocal micrographs of EC on bare and coated materials are shown in Fig.V.3. After taking a series of 18 sections of 5μM thickness, the average of all images was constructed using software. From this figure, it is evident that spreading is very poor and the fluorescence intensity per each cell was also very less which indicates poor organization of the actin cytoskeleton.



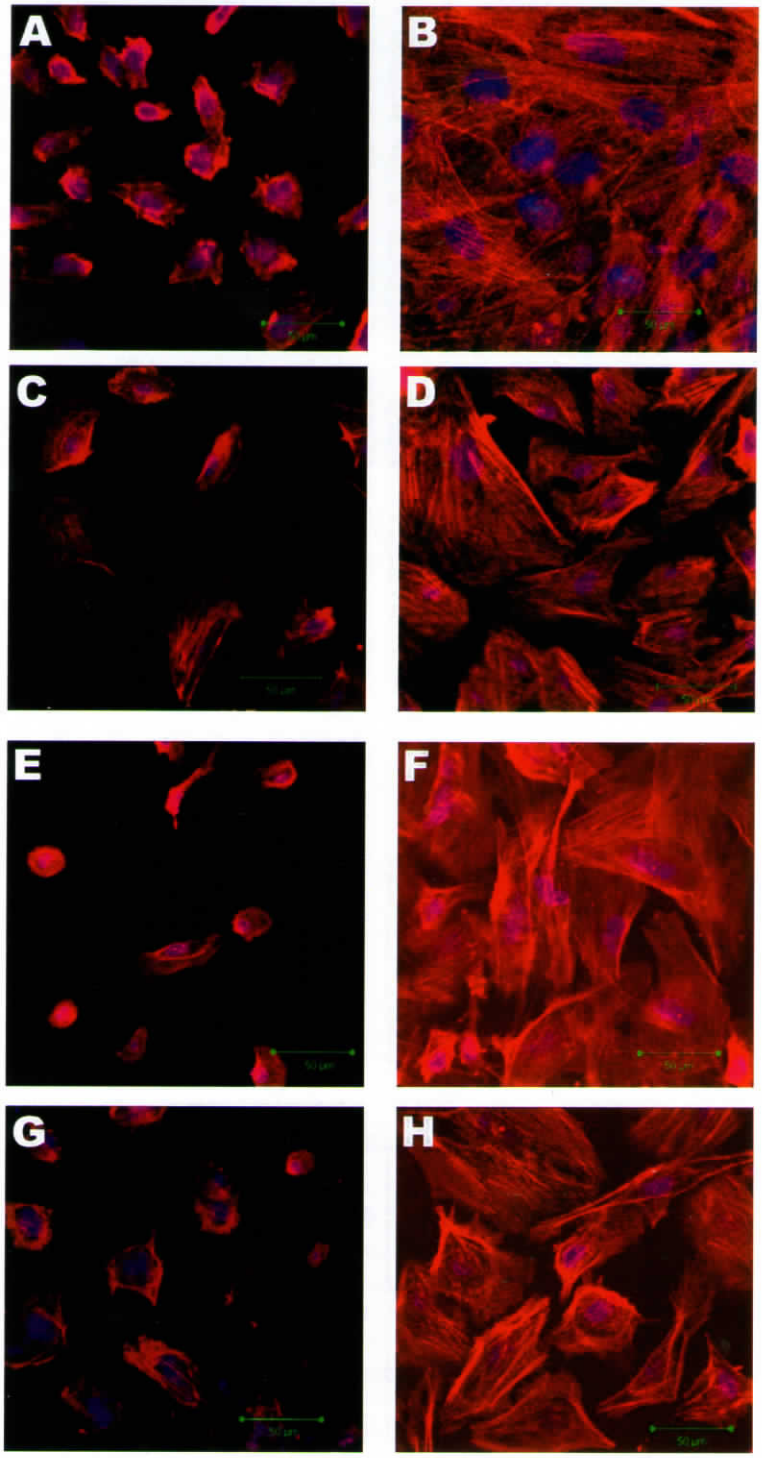
ıre V-1. F-Actin stained HUVEC on study materials: Staining was done after of cell seeding. A, C, E, G; Bare materials (A: Ti, C: DLC_Ti, E: SS G: UHMWPE) and B, D, Composite coated (B: Ti, D: DLC-Ti, F: SS, H: UHMWPE). All cells used for seeding was the 3rd passage. Original magnification 40x.



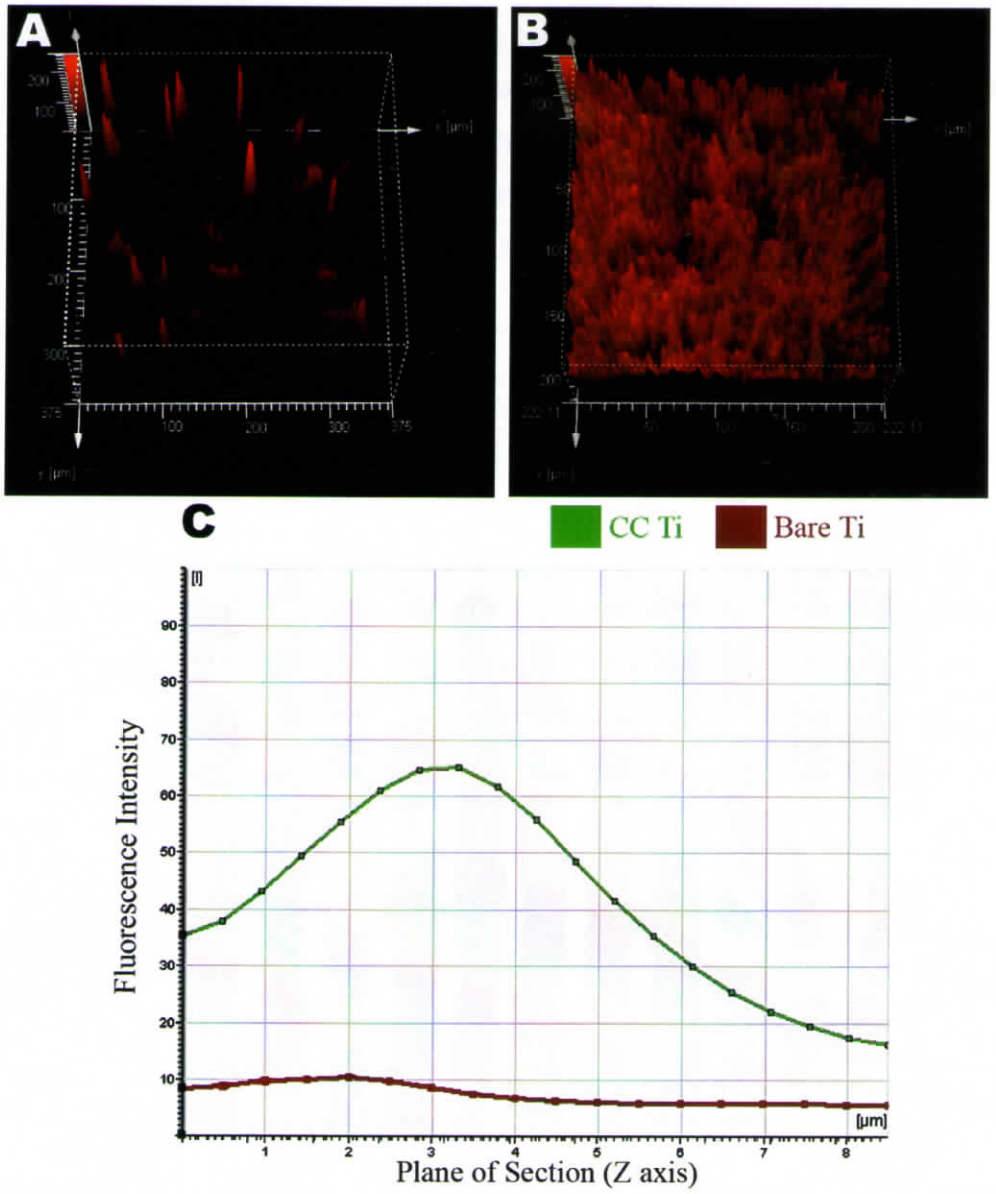
ure V-2. Confocal Microscope images of F-Actin stained HUVECs. A, Bare ron; B, CC_Dacron. After taking a series of 20 sections, average of all the sections was ed with the help of software.

The 3D view of the materials with cells stained for actin clearly showed that the fluorescence intensity is significantly different on bare and composite materials in each case. Thus, for detection of the quantitative difference in fluorescence intensity, a field containing ten cells each on bare and composite materials was selected and a graph is plotted using the software. The Fig.V.4 shows a representative 3D image of cells on bare vs, coated Ti and the fluorescence intensity graph for the same. Though only Ti data is shown here, in the case of all other materials, similar results were obtained.

A major design criterion for cardiovascular implant materials is appropriate biological interaction with the host tissue. Endothelial cell adhesion remains an elusive goal in the engineering of all synthetic vascular implants for improving the tissue integration of device materials. Seeding of blood contacting implants with endothelial cells should result in the development of a confluent cell monolayer to mimic the function of the normal vascular endothelium. The benefit to this approach has not yet realized as most of the materials used for cardiovascular prosthesis are not designed to promote cellular interaction. Appropriate orientation of the signaling machinery of the cell is dependent on the structure of the focal adhesion complex, which provides an intricate inter relationship between the ECM, the cytoskeleton and signaling cascades (Garcia et al 1999; Aplin, 2003).



ıre V-3. Representative data from Confocal Microscopic analysis of VEC on materials: After taking a series of 18 sections, average of all the sections was ed with the help of software. A, C, E, G; Bare materials (A: Ti, C: DLC_Ti, E: SS G: WPE) and B, D, F, H; Composite coated (B: Ti, D: DLC-Ti, F: SS, H: UHMWPE). All cells for seeding was from the 3rd passage.



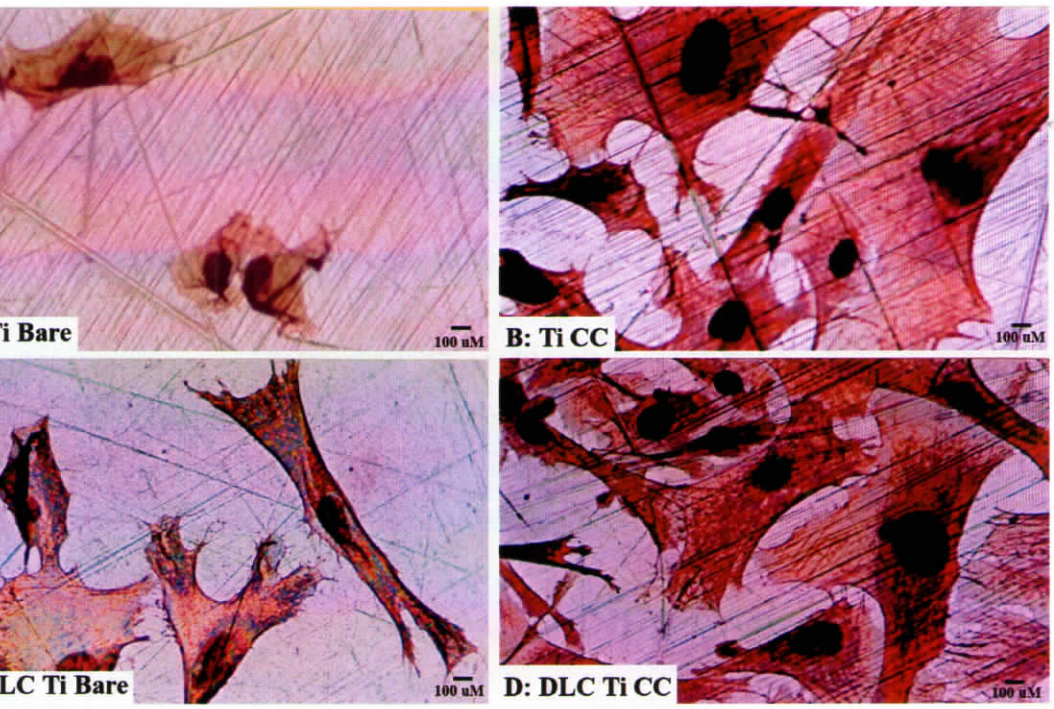
ure V-4. Representative data from Confocal Microscopic analysis of VEC on Titanium: After taking a series of 18 sections, a 3D image was constructed using software. A, Bare_Ti; B, CC_Ti. C, Graphical representation of fluorescence intensity of a cted field containing ten cells on each matrix, graphs plotted for cells on Bare Ti, Bare DLC-Ti composite coated Ti are super imposed. Cumulative fluorescence intensity shown on y-axis ainst Z cross-section (total of 18 z-sections of 5 µm thickness) as shown in x-axis.

To improve biocompatibility of medical implants, the most advanced strategy aims at the creation of materials that promote attachment, migration, proliferation, differentiation, long-term viability and cell functioning. Functional receptor mediated signal transduction and cell adhesion on a conventional biomaterial is mediated by the extracellular matrix (ECM) molecules such as fibronectin, vitronectin, collagen or laminin that are adsorbed to the material

rom the culture medium. So the poor adhesion and actin organization bserved on bare materials may be because of the lack of proper ECM omponents in mediating cell adhesion and spreading. It is demonstrated that ne use of a bio mimetic matrix that contain adhesion molecules and growth actors support focal adhesions and associated actin assembly.

2. Identification of Focal Adhesion by Vinculin Staining

Apart from staining for F-actin, the localization of vinculin at the focal ontacts was also examined to get a clear idea regarding the focal adhesion omplex formation. One metallic material (Ti) and a modified metal (DLC_Ti) were chosen for this study. EC seeded on the composite coated materials xpressed improved levels of vinculin, and most of it was found to be ocalized sharply at the focal adhesion points through out the well spread cells Fig.V.5 B, D). In contrast, vinculin localization was poor on uncoated naterials and was seen concentrated almost exclusively in the perinuclear egion (Fig.V.5 A, C).



re V-5. Light Micrographs of HUVEC Focal Adhesions on Materials. narker used is vinculin and the HRP conjugated secondary antibody was visualized using All cells used for seeding was from the 3rd passage. A: Bare Ti; B: Composite coated Ti; C: DLC-Ti; D: Composite coated DLC-Ti. Original magnification 50x.

When the two bare surfaces were compared for cell adhesion and spreading, DLC-coated titanium was found to support more focal adhesions, compared to bare Ti.

The poor localization of vinculin in the focal adhesion regions in the case of bare materials is well in agreement with what was observed earlier with actin staining. During the signaling event, integrin receptors are recruited into distinct dot-like or streak-like nano- or micro- domains on cell membrane, called focal adhesion sites or focal adhesion plaques. In these regions the integrins communicate with many specific structural and signaling molecules. The focal adhesions are represented by proteins of membrane associated cytoskeleton, such as talin, α-actinin, filamin, paxillin and vinculin. These proteins act as linkers between the integrin receptors and the cytoplasmic actin cytoskeleton which is associated with nuclear membrane, membranes of cellular organelles as well as with various enzymes and thus influences intracellular processes that are important for cell behavior including transport and secretion of various molecules, endocytosis, and decision between cell proliferation and differentiation or apoptosis is determined.

So the poor vinculin distribution found in the case of bare materials further confirms that the focal adhesion assembly is very poor when cells are grown on bare materials which make a decisive role on further behavior of cells after contacting a biomaterial i.e. switching between cell proliferation and differentiation, survival or apoptosis and other functions. The adsorption of cell adhesion-mediating ECM molecules in appropriate amount, spectrum, spatial conformation, flexibility and their accessibility by the integrin receptors is markedly influenced by the physicochemical properties of the material (Scorcik et al., 2002). Since fibronectin, vitronectin and fibrinogen were found to be the main soluble matrix proteins in blood and thus in serum containing medium that the cells can recognize, one possible explanation for different biological reactivity on different materials might be distinct adsorption and conformation of these proteins. Due to such variability, one can not rely on the natural protein adsorption to support cell adhesion, proliferation and survival on various vascular implant materials. The key finding in this study is that the

deposition of ECM molecules through a fibrin scaffold provides a uniform distribution of adhesion molecules and growth factors on the material surface and thus promotes cell adhesion, spreading, and focal adhesion formation.

V.3. EC PROLIFERATION AND SURVIVAL ON MATERIALS

V.3.1. Quantification of cell attachment and proliferation by ^{3}H thymidine uptake

The number of cells attached per centimeter square area on composite coated materials was compared against the respective bare materials by seeding endothelial cells on them which were already labeled with thymidine (Section.II.3.7). 40 x 10^3 cells were seeded per each cm^2 area of the material. It was found that the initial cell attachment cm^{-2} area was found to be similar on all materials, both on bare and composite coated (Fig.V.6). On all materials more than 80 % of the cells were found to be attached by 2h of seeding.

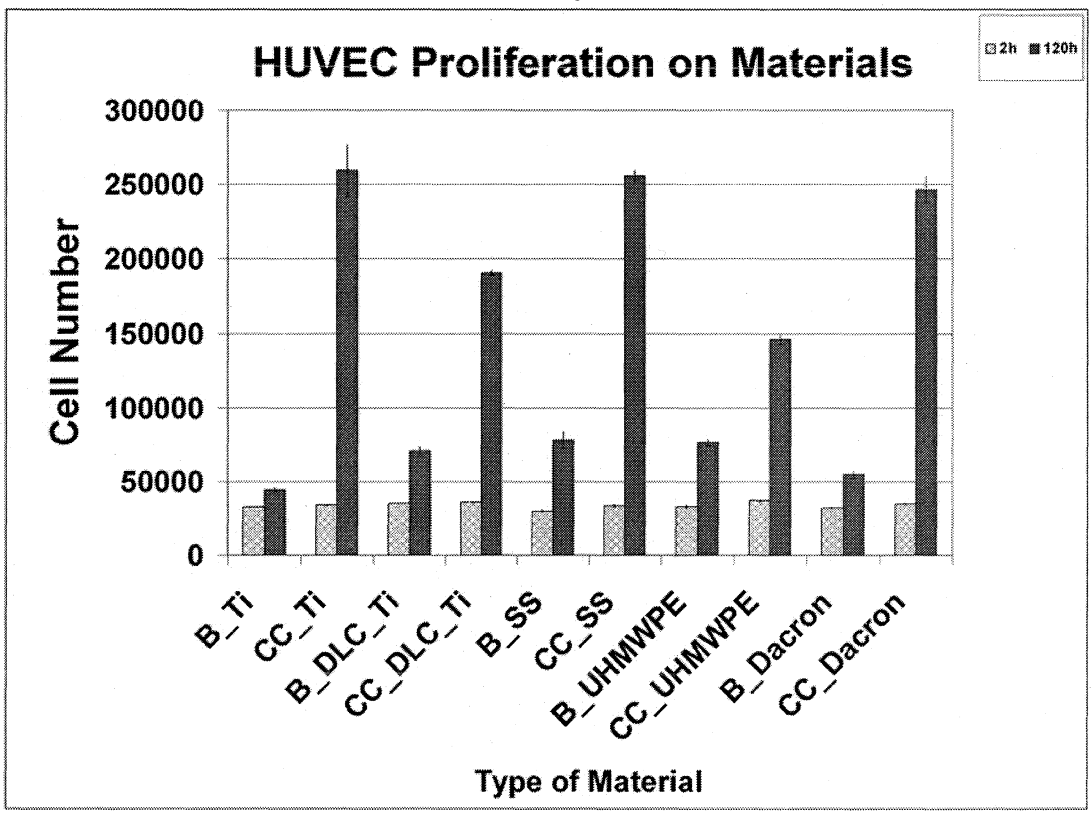


Figure V-6. Quantification of attachment and proliferation of EC using ^{3}H-thymidine uptake. The initial attachment of HUVECs detected at 2h after seeding, and the proliferated cells after 120 h on each of the bare and coated materials are given side by side. The count of cells is adjusted for 1 cm^2 area for each material. The assay was done in 4 replicates experiments and standard deviation (n =4) is given as error bar.

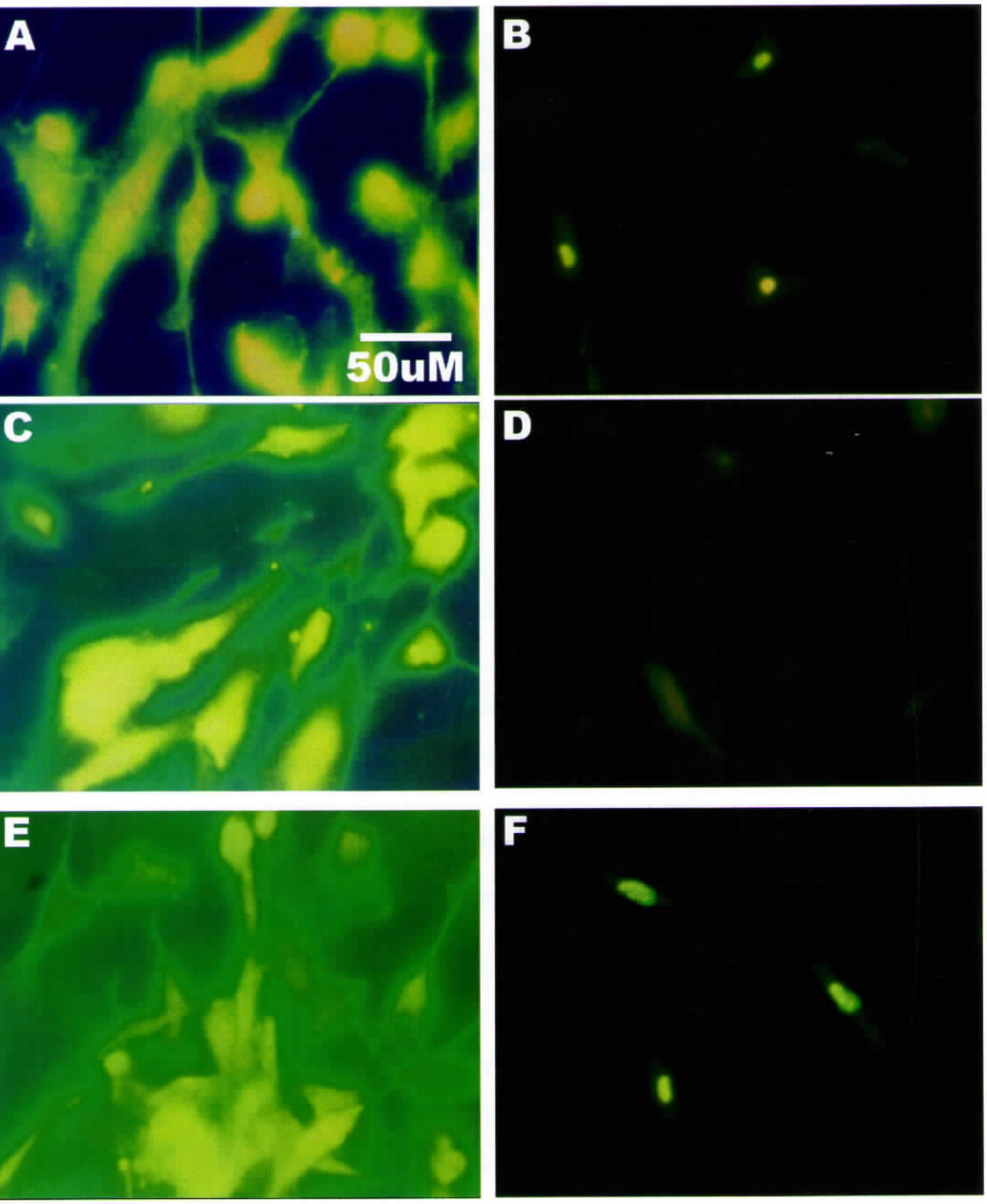
However, cell proliferation after 120h post seeding was significantly enhanced upon coating on all the materials tested (Fig.V.6). Of the bare materials, the proliferation was negligible on bare Ti and bare Dacron whereas on all the other bare materials one population doubling has occurred by 120h. The coating was found to be most effective on Ti, Dacron and SS, compared to DLC coated Ti and UHMWPE. While there were 3 populations doubling by 120h on CC_Ti, CC_Dacron and CC_SS, on Ti-DLC_CC and UHMWPE_CC it were only 2 populations doubling (Fig.V.6).

V.3.2. HUVEC Apoptosis on study materials

As the focal adhesion and actin organization have a direct influence on cell viability, cells grown on uncoated and coated materials after 72h of seeding were stained for Annexin V to detect apoptotic tendency. This study was conducted on one metal (Ti); one modified metal (DLC_Ti) and one polymer (UHMWPE). It is observed that almost 75% of the cells on uncoated materials turned apoptotic by 72h of seeding (Fig.V.7 A, C & E). On the other hand, apoptosis was hardly seen on composite coated materials (Fig.V.7 B, D & F). Apoptotic cells were counted in 10 different fields (10x) and the percentage of apoptotic cells detected on each material is summarized and shown in Table V.1. Of the bare surfaces, apoptosis was found most significant on Ti which correlates well with the proliferation data where it is found that the proliferation rate was also much lower on B_Ti compared to B_DLC_Ti and B_UHMWPE.

Table V-1. Percentage of EC Apoptosis on Study materials. Comparison of Percentage Apoptosis in HUVEC cultures on Bare and composite coated materials is shown. The semi quantitative difference in Apoptosis of HUVEC was detected by counting 10 different fields at 10x magnification. Average and standard deviation were obtained by repeating the experiment from five separate seeding on each type of materials (n=5).

Material name	Bare	Composite coated
Titanium	86±6	15±2
DLC coated Ti	65±9	16±4
UHMWPE	76±7	13±5



ure V-7. Fluorescent micrographs of FITC-Annexin V stained cells: A, C, E are bare materials (A, B_Ti; C, B_DLCTi; E, B_UHMWPE). B, D, and F are composite ted materials (B, CC_Ti; D, CC_DLC_Ti; F, CC_UHMWPE). The cells are of passage number each case and staining was done 72 h after seeding. Original magnification is 50x. Apoptotic s were counted in 10 different fields at 10x magnification and the percentage of apoptotic cells ected in each of the culture surface is summarized in Table V.1.

The observed poor proliferation rate and apoptotic tendency of cells grown on bare materials can be justified because, earlier it was found during the spreading and focal adhesion study that the bare materials does not support EC spreading and focal adhesion assembly. Unlike integrin-mediated cell adhesion, non-receptor mediated cell adhesion on artificial surfaces

cannot ensure the transmission of adequate signals from extra cellular environment into cells and survival of anchorage dependent cells.

The interaction between the integrin receptors and the cytoplasmic actin cytoskeleton, as well as with various enzymes influences intracellular processes that are important for cell behavior and the decision between cell proliferation and differentiation or apoptosis. Cells which are anchorage-dependent for proliferation require both adhesion to the extracellular matrix and growth factor stimulation to progress through the cell cycle (Howe et al., 1998; Assoian and Zhu, 1997). It has been reported that, when endothelial cells are completely denied from adhesion to the substrate they lose viability within 10 hours (Re et al. 1994). Even though the data shows that initial attachment is similar on both bare and coated materials after 2h of seeding the cell spreading and migration were very poor and focal adhesion assembly was lacking. This could be the reason for the observed poor proliferation rate. The deposition of composite matrix provides a uniform distribution of adhesion molecules and growth factors on the material surface and there by promotes cell adhesion, proliferation, and survival.

V.4. EFFECT OF COMPOSITE MATRIX ON HUVEC PHENOTYPE

V.4.1. mRNA Expression Study by *Real Time* PCR

The mRNA expression profiling of two pro-thrombotic molecules (vWF and PAI-1) and two anti-thrombotic molecules (tPA and eNOS) were compared in HUVECs grown on composite coated materials against those on bare materials. For this study one metal (Ti); one modified metal (DLC_Ti) and one polymer (UHMWPE) were selected. The mRNA isolated from HUVECs grown on 6 replicates of each of the materials was used to demonstrate repeatability. Normalization was done using Beta Actin as house keeping gene.

The mean cyclining time (Ct) values from the real time PCR carried out are given in the Table V.2, which show that on all three bare materials vWF and PAI are expressed at early cycling times such as 16 for vWF and 17/18 for PAI. The anti thrombotic molecules tPA and eNOS were expressed later

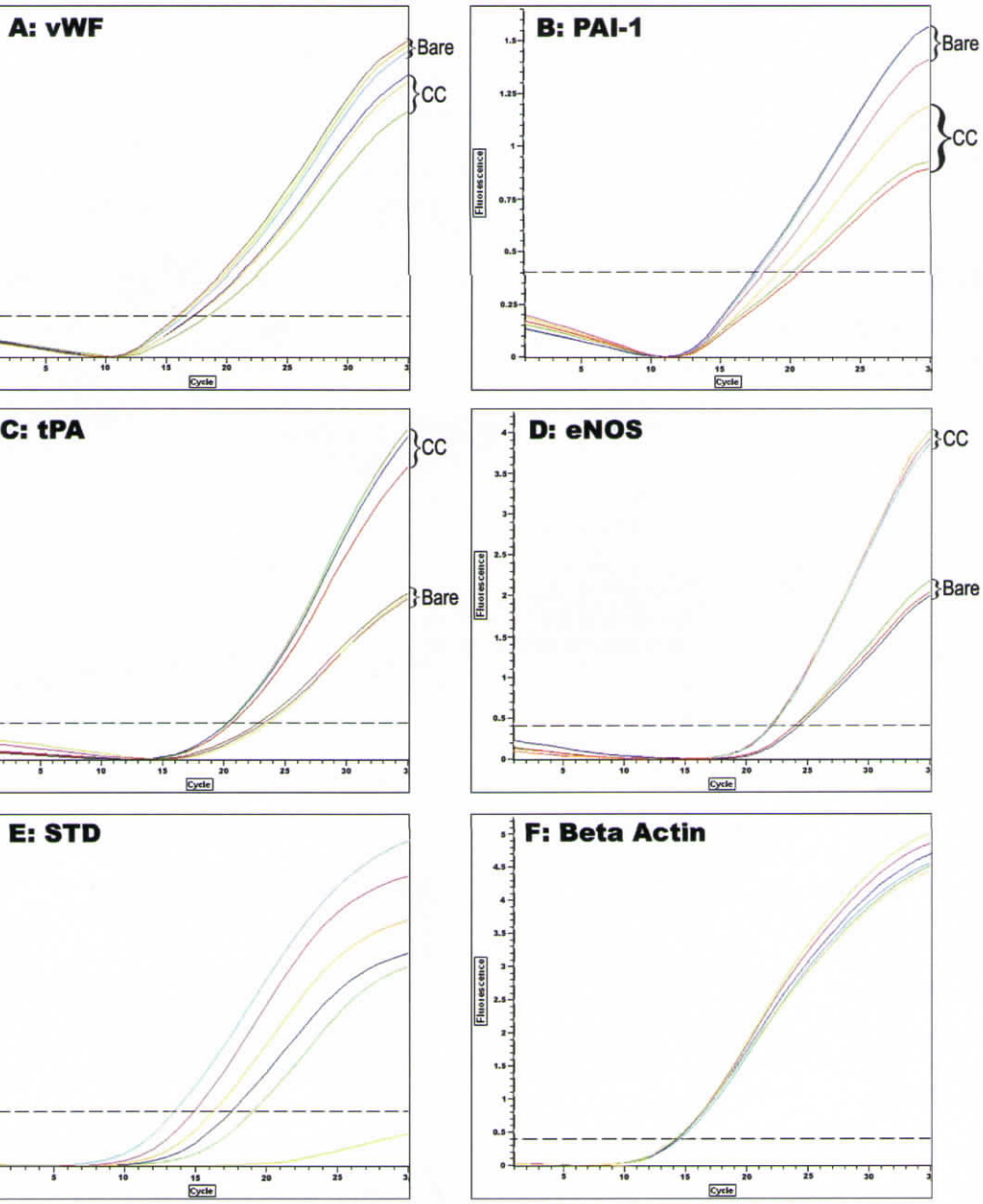
on bare materials such as Ct of 23 for the former and 24 for the latter. It is also seen clearly in the quantitative data presented in Table V.3 that for vWF value is as high as 7.0 pg, and for PAI it is 3.0/2.0 pg for the cells on given materials. On the other hand tPA is as low as 0.1 pg and e-NOS is as low as 0.06pg and value is almost similar on all bare materials.

Table V-2. Comparison of Real-Time PCR Ct Values: Bare Materials Vs. Composite coated Materials The table shows, the Ct values which represent mRNA expression levels in HUVECs grown on Bare and Composite Coated materials. Using specific RT-PCR primers, the expression level of vWF, PAI-1, tPA and eNOS were analyzed. Beta Actin is used as the house keeping gene. The cycling time (Ct) values are given as average ± SE from 6 Replicates. * $p < 0.01$.

Test Material	vWF	PAI-1	tPA	eNOS	Beta ACtin
B_Ti	16.27±0.04	17.44±0.06	22.97±0.23	24.05±0.19	14.26±0.03
B_DLCTi	16.16±0.03	17.62±0.04	23.24±0.17	24.08±0.26	14.25±0.07
B_UHMWPE	16.06±0.08	18.12±0.16	23.39±0.26	24.26±0.21	14.22±0.06
CC_Ti	18.24±0.24 *	20.21±0.08 *	20.23±0.11 *	22.04±0.04 *	14.24±0.04
CC_DLCTi	17.13±0.15 *	19.15±0.19 *	20.30±0.09 *	21.97±0.12 *	14.19±0.06
CC_UHMWPE	17.16±0.09 *	20.85±0.23 *	20.65±0.13 *	22.02±0.07 *	14.23±0.03

However, when EC was grown on composite coated materials, a reversal effect is seen and expression of both prothrombotic molecules was reduced with a higher Ct value of 17/18 for vWF and 19/20 for PAI (Table V.2) and was quantitatively lower such as 2/3 for vWF and 0.5/1.0 for PAI (TableV.3). Similarly expression of antithrombotic molecules t-PA and e NOS increased with lower CT values such as 20.0 for t-PA and 22 for eNOS (Table V.2) and higher quantities such as 0.45 pg for t-PA and 0.25 pg for e-NOS for the given material area (Table V.3). One representative RT-PCR data graph of each of the 4 target genes along with the housekeeping gene, beta actin and standard cDNA is shown in Fig.V.8. Thus from the Fig V.8 and Tables V.2 and V.3 it is obvious that the expression of the two pro-thrombotic molecules - vWF and PAI-1 were high and the expression of both of the anti-thrombotic molecules – tPA and eNOS were low in ECs grown on bare materials. There was a striking effect when ECs were grown on the composite matrix coated materials. The expression of pro-thrombotic molecules went down to significantly lower levels ($p< 0.01$), and the mRNA synthesis of anti-thrombotic

olecules went up considerably (p<0.01). From the multiple bars diagram ig. V.9) showing the fold change in mRNA expression as normalized to Beta ctin it is evident that there was a 2-4 fold decrease in the expression of vWF on matrix coating.



re V-8. A representative data graph showing the amplification of scripts. Quantification of the mRNA expression by real-time PCR in HUVECs grown on and Composite Coated materials were done. 6 Replicate experiments were done and ge Ct values are calculated. **A**, vWF; **B**, PAI-1; **C**, tPA; **D**, eNOS; **E**, STD; and **F**, B actin.

molecules went up considerably (p<0.01). From the multiple bars diagram (Fig. V.9) showing the fold change in mRNA expression as normalized to Beta Actin it is evident that there was a 2-4 fold decrease in the expression of vWF upon matrix coating.

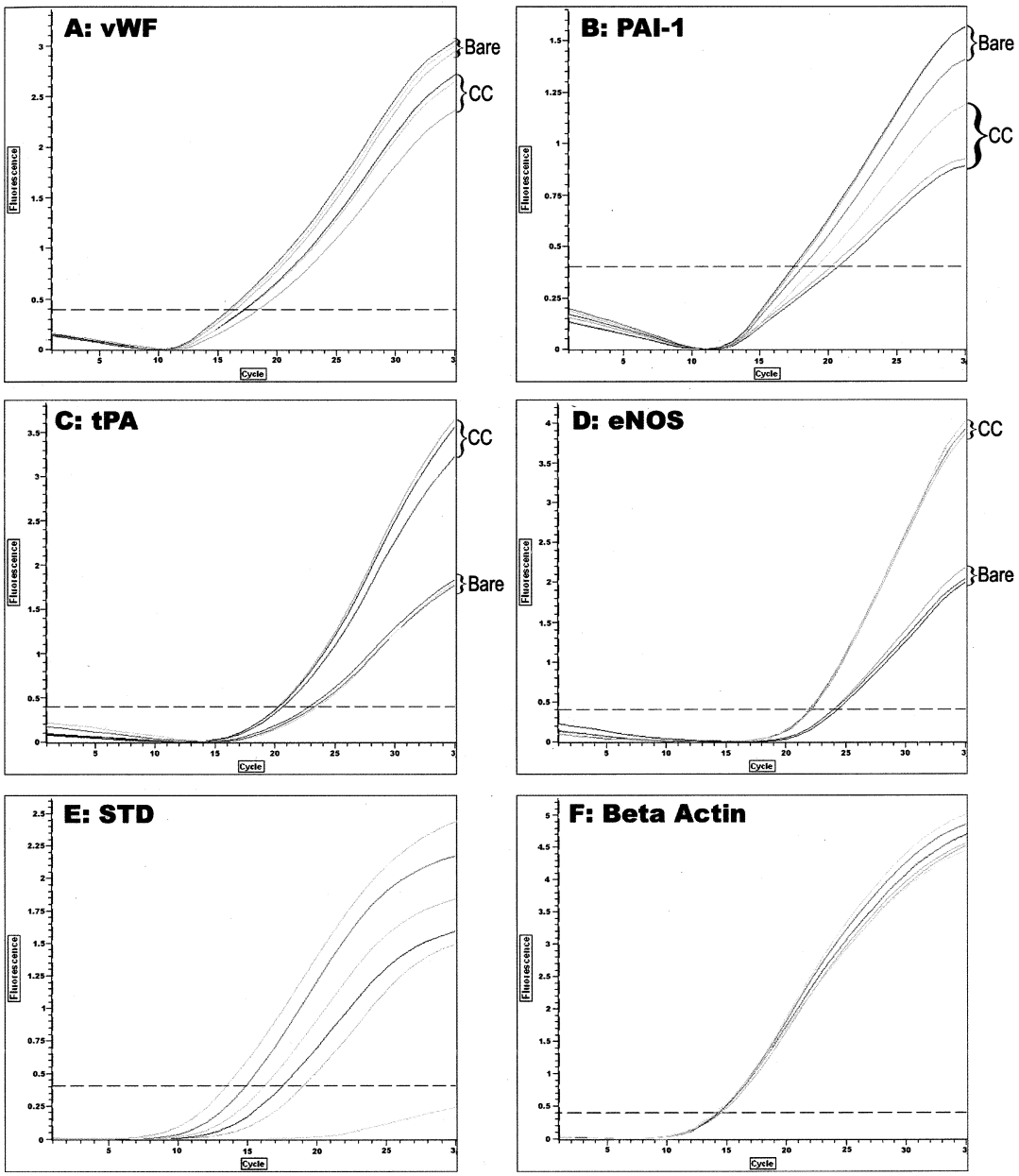


Figure V-8. A representative data graph showing the amplification of transcripts. Quantification of the mRNA expression by real-time PCR in HUVECs grown on Bare and Composite Coated materials were done. 6 Replicate experiments were done and average Ct values are calculated. **A**, vWF; **B**, PAI-1; **C**, tPA; **D**, eNOS; **E**, STD; and **F**, B actin.

The decline in expression was more striking in the case of PAI-1, which were 4-7 folds as compared to bare. It is remarkable to note that the down regulation was most significant on CC_Ti, on which the vWF was down by 4 fold and PAI-1 by 7 fold. The expression of both of the anti-thrombotic molecules – tPA and eNOS went up by 4-5 folds when ECs were grown on composite coated materials.

Table V-3. Quantification of mRNA Expression by Real-Time PCR. The table shows, quantification of the transcript expression by real-time PCR in HUVECs grown on Bare and Composite Coated materials. Using specific RT-PCR primers, the expression level of vWF, PAI-1, tPA and eNOS were analyzed. Beta Actin is used as the house keeping gene. Values are given as average ± SE from 6 Replicates. * $p < 0.01$.

Test Material	vWF (pg)	PAI-1 (pg)	tPA (pg)	eNOS (pg)	Beta Actin (pg)
B_Ti	6.764±0.14	3.738±0.22	0.105±0.02	0.069±0.01	31.354±0.96
B_DLCTi	7.326±0.11	3.639±0.14	0.084±0.02	0.063±0.01	31.428±2.24
B_UHMWPE	7.826±0.29	2.196±0.29	0.075±0.03	0.057±0.01	31.496±1.92
CC_Ti	1.843±0.22 *	0.516±0.07 *	0.474±0.06 *	0.243±0.01 *	31.452±1.28
CC_DLCTi	3.38±0.54 *	1.035±0.10 *	0.459±0.05 *	0.258±0.02 *	31.516±1.92
CC_UHMWPE	3.612±0.32 *	0.468±0.12 *	0.429±0.07 *	0.252±0.01 *	31.479±0.96

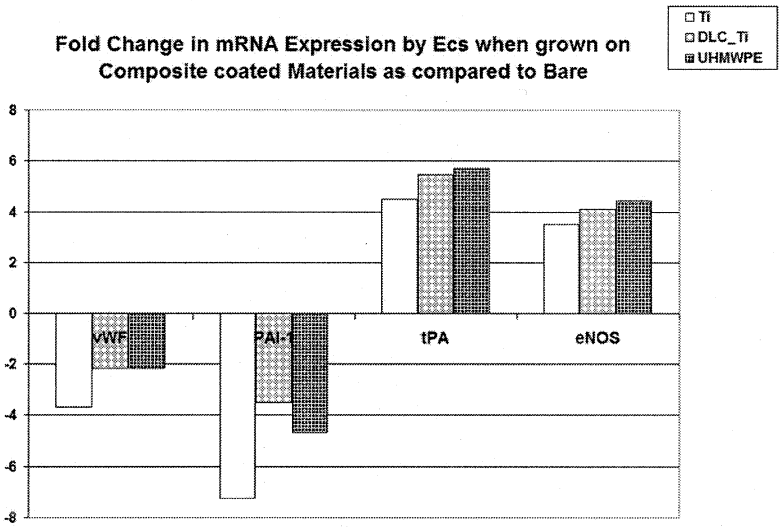


Figure V-9.A graphical representation of fold increase/decrease in mRNA expression. Amplification of mRNA from HUVECs grown on composite coated materials with respect to the HUVEC grown on respective bare materials is shown. Beta actin is used as house keeping gene for normalization of expression.

During the standardization of matrix using HUVECs (discussed in chapter III), it was demonstrated that endothelial cells that are grown on

gelatin coated polystyrene dishes for long term gets dedifferentiated with increase in passage number. The data suggested that, it is important for EC to get signals from the extracellular matrix for cell proliferation, survival and to maintain normal anti thrombotic phenotype. In this study, it is shown that attempts to seed endothelial cells on materials used for fabrication of cardiovascular diseases may fail the purpose because the EC grown on such materials get dedifferentiated and might turn pro thombotic by up regulation of m-RNA expression of prothrombotic molecules such as vWF and PAI, and down regulation of anti thrombotic molecules such as t-PA and nitric oxide synthetase. Real Time PCR data gives a clear indication of phenotypic alteration of EC grown on bare materials into more pro thrombotic form. It has been reported that apoptotic endothelial cells turn procoagulant and that, even floating apoptotic HUVEC increase thrombin formation in plasma (Bombeli et al. 1997). In this study it is found that most of the cells turned apoptotic when they were grown on bare materials (Section V.3.2).

V.4.2. Comparison of NO release by HUVECs grown on Materials

The release of NO by the ECs correlates well with mRNA expression study. From the graphical representation (Fig.V.10), it is evident that the release of NO is almost undetectable when ECs were grown on any of the 3 bare materials studied (< 0.01 $\mu M/cm^2$ area for all materials), and the shear induced NO release was also negligible. Remarkably, the matrix coating enhances the release of NO by the cells grown on them to considerably high levels (Fig.V.10) (p< 0.01 in all the cases). The release of NO under static condition ranged from 0.3 to 0.4 $\mu M/cm^2$ area for the study materials and upon exposure to a shear stress of ~35 $dynes/cm^2$, there was a 5 fold increase in the release of NO. Among the different materials used for the study, the composite coating was found to be most effective on Ti.

From the mRNA expression study, it is evident that eNOS transcription level is very low in cells on bare materials. The results of NO release further confirm that observation. It was found that, cells even lose their ability to respond to shear stress when grown on bare materials by up regulating NO

synthesis. Thus it is clearly evident that the cells do dedifferentiate and lose the normal protective behavior when they are grown on bare materials.

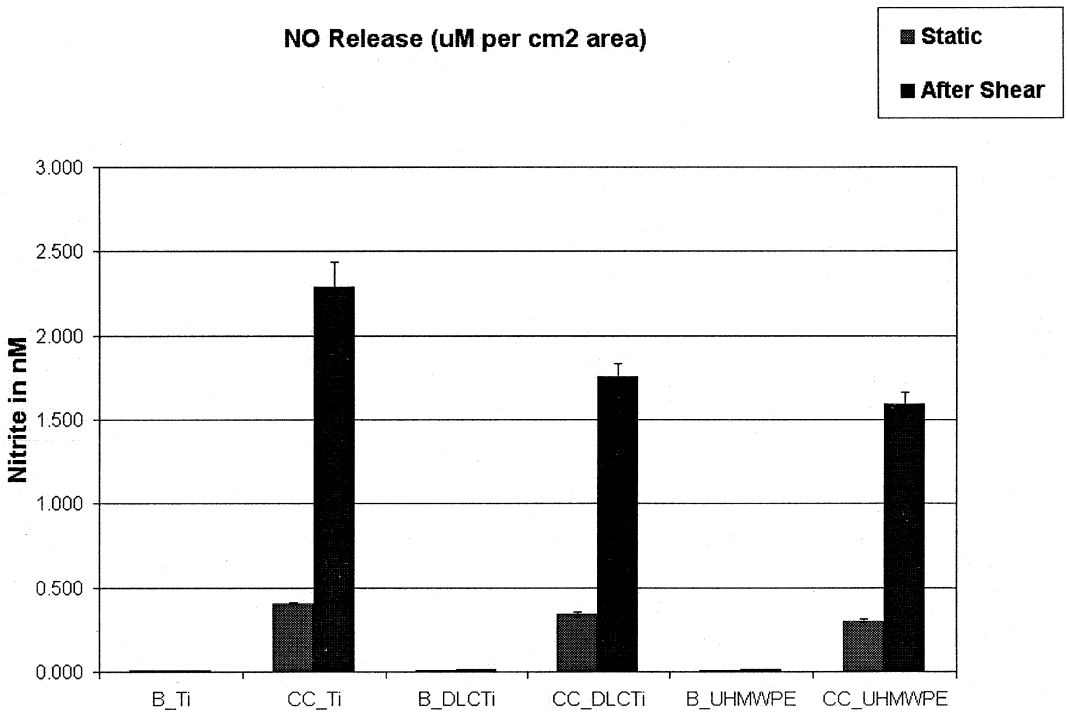
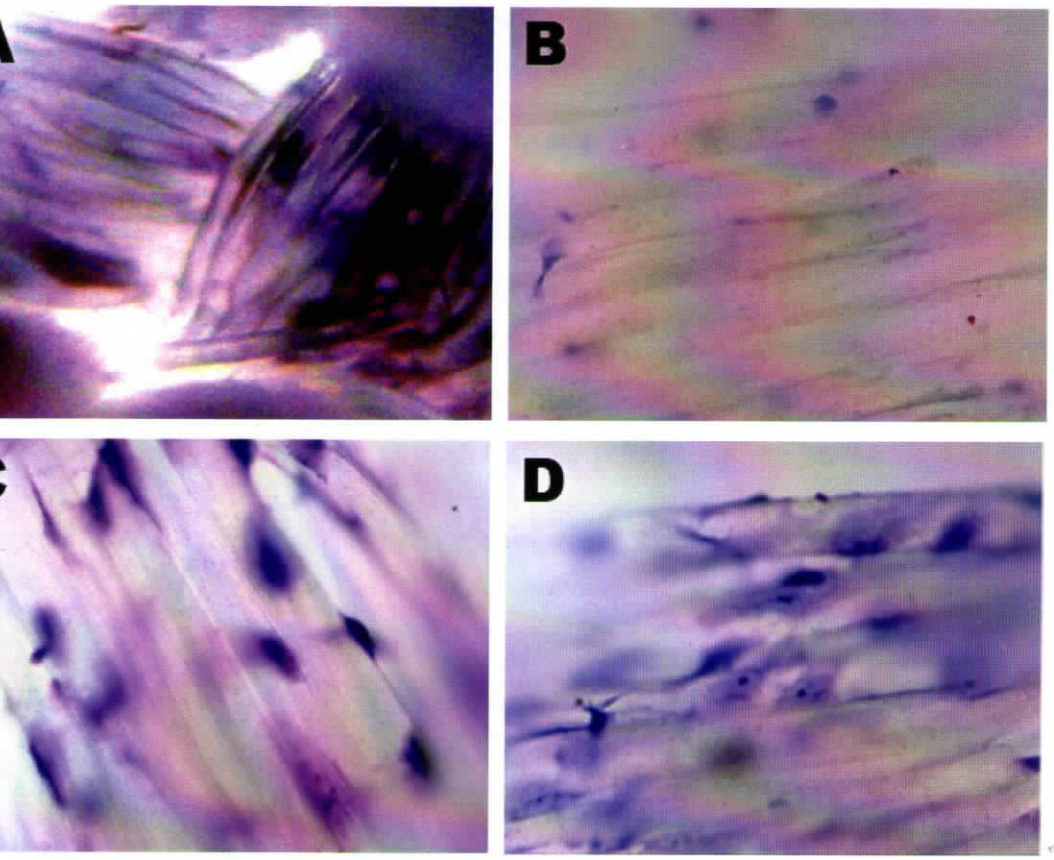


Figure V-10. Graphical representation of NO release by HUVECs grown on bare and composite coated materials. Cells after 120h in culture were incubated for 1h in serum-free, phenol red-free M199 medium, and nitrite in the medium was assayed using Griess reagent. For shear induced release devices with cells was perfused at a flow rate of 50 mL/min using a peristaltic pump for 1 h at room temperature with phenol red-free M199 medium (Shear stress ~35 dynes cm^{-2}), and nitrite in the medium collected after 1h was assayed. The Y-axis is nitrite in $\mu M/cm^2$ area. The Average ± SE of 6 replicates are shown.

V.5. EFFECT OF SHEAR STRESS ON HUVEC MONOLAYER

There are many reports that endothelial cells grown on vascular prostheses get detached when they are exposed to dynamic flow conditions. Even under the physiologic flow rate the cells may get detached if the adhesion and spreading is poor. So to find out whether the matrix coating helps the cells to withstand the shear stress, experiment was carried out with commercially available Dacron vascular graft prostheses (section II.3.1). HUVECs seeded on bare and composite coated 5cm pieces of 6mm diameter Dacron vascular graft were exposed to a shear stress of ~20dynes/cm^2 (Section II.3.11). It was found that by 1h most of the cells on bare Dacron were detached. On the contrary, cells that were grown on composite coated

Dacron were able to sustain the shear stress and the cell detachment was negligible (Fig.V.11).



gure V-11. Photomicrographs of HUVEC stained with May-Grunwald's n. A: EC on Bare Dacron before perfusion; B: EC on Bare Dacron after perfusion; C: EC on posite coated Dacron before perfusion; D: EC on Composite coated Dacron after perfusion.

. THROMBOGENICITY ASSESSMENT OF SURFACES

To examine how this pro-thrombotic phenotype of ECs grown on the bare materials reflects on the *in vitro* blood compatibility of the devices, tests were selected from the list recommended in ISO10993 part4. These tests are standardized and validated in our laboratory and is accredited against quality system ISO/IEC 17025 by COFRAC (France). 1cm^2 patches Dacron vascular graft material, Ti, DLC_Ti and UHMWPE were used for the study (Section II.3.1). HUVECs were grown on bare and composite coated materials as described in section II.3.4.

V.6.1. Platelet Adhesion Studies

V.6.1.1. By Platelet count analysis using Hematolgy Analyzer

When the HUVEC grown on bare and composite coated materials were exposed to PRP and the count reduction in PRP after 1h of exposure were analyzed using a hematology analyzer, significant reduction in platelet count was observed. Table V.4 gives the platelet count in PRP before and after exposure and the count reduction after 1h as % reduction in platelet count. On all the EC grown bare materials, the count reduction after 1h was significantly different compared to composite coated materials with EC monolayer ($p < 0.01$).

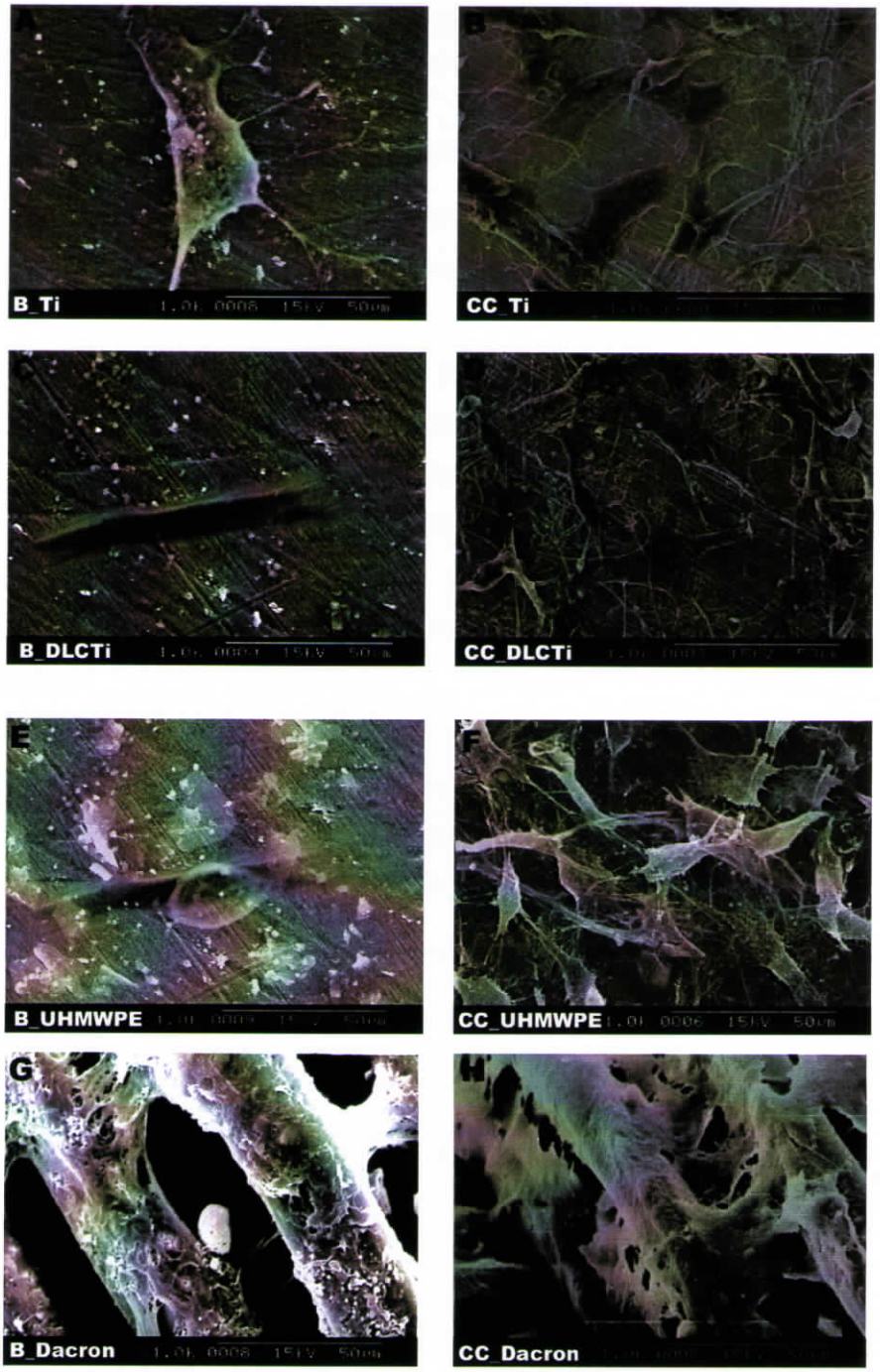
Table V-4. Percentage of platelet count reduction on Bare Vs. Composite coated materials with EC monolayer. One milliliter PRP was exposed to each device in separate wells of polystyrene cell culture plates and the platelet count in the sample at 1h was subtracted from the values obtained in the initial sample. The initial and final values are given in the table along with the reduction. The Average ± SE of 4 replicates are shown. * $p < 0.01$.

Test Material	Initial Count (x 10^6)	Final Count (x 10^6)	% Reduction
B_Ti	2.16±0.032	1.61±0.049	25.2±3.3
B_DLCTi	2.18±0.024	1.92±0.042	12.1±1.6
B_UHMWPE	2.17±0.015	1.71±0.051	20.9±2.5
B_Dacron	2.16±0.022	1.52±0.056	29.8±1.8
CC_Ti	2.15±0.021	1.98±0.365	8.1±1.1*
CC_DLCTi	2.14±0.028	1.97±0.171	7.7±0.9*
CC_UHMWPE	2.19±0.023	2.04±0.285	7.1±0.4*
CC_Dacron	2.18±0.025	2.05±0.264	5.9±0.6*

Though this study gives only a qualitative idea about the platelet adhesion due to possible non-specific adhesion to the test material and containers, the results clearly indicated that the bare materials with EC monolayer consume more platelets. To study the nature of platelet adhesion in detail SEM analysis was carried out and to quantify the actual number of platelet adhered per unit surface area Radioscintigraphic analysis was done using radiolabeled platelets.

V.6.1.2. Platelet adhesion study by SEM

Platelet adhesion studies by SEM indicate that on all of the EC grown bare materials there was considerable adhesion of platelets.



igure V-12. Electron micrograph of Bare and composite coated naterials exposed to platelet rich plasma. A, C, E, and G are bare material (Ti, LC_Ti, UHMWPE and Dacron respectively); and B, D, F and H are composite coated aterials in the same order. Magnification is 1K. Scale bar is shown in each figure.

It is evident from the Fig.V.12, that platelets adhered throughout the aterial and it is interesting to note that many platelets are adhered to the

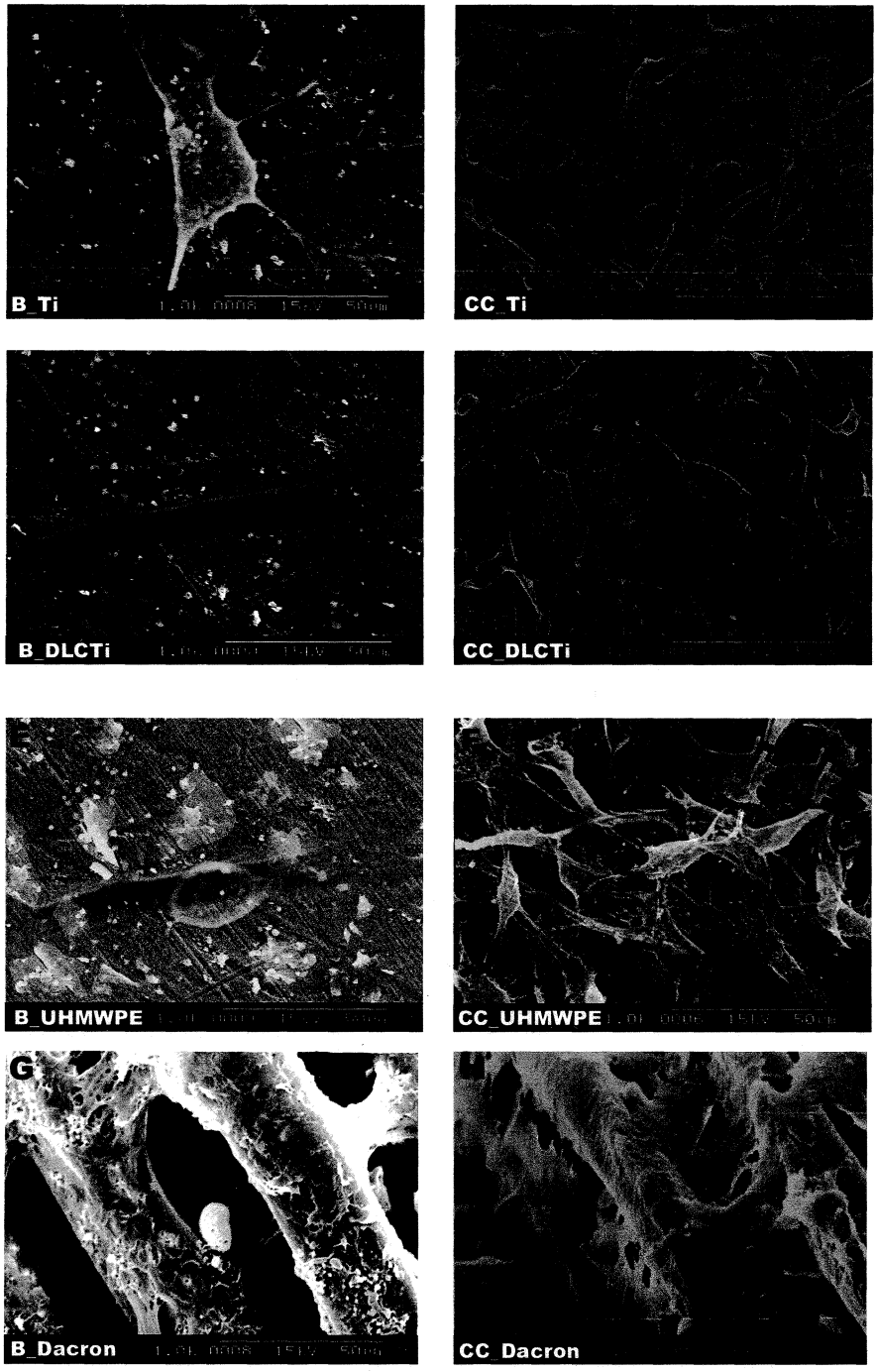


Figure V-12. Electron micrograph of Bare and composite coated materials exposed to platelet rich plasma. A, C, E, and G are bare material (Ti, DLC_Ti, UHMWPE and Dacron respectively); and B, D, F and H are composite coated materials in the same order. Magnification is 1K. Scale bar is shown in each figure.

It is evident from the Fig.V.12, that platelets adhered throughout the material and it is interesting to note that many platelets are adhered to the

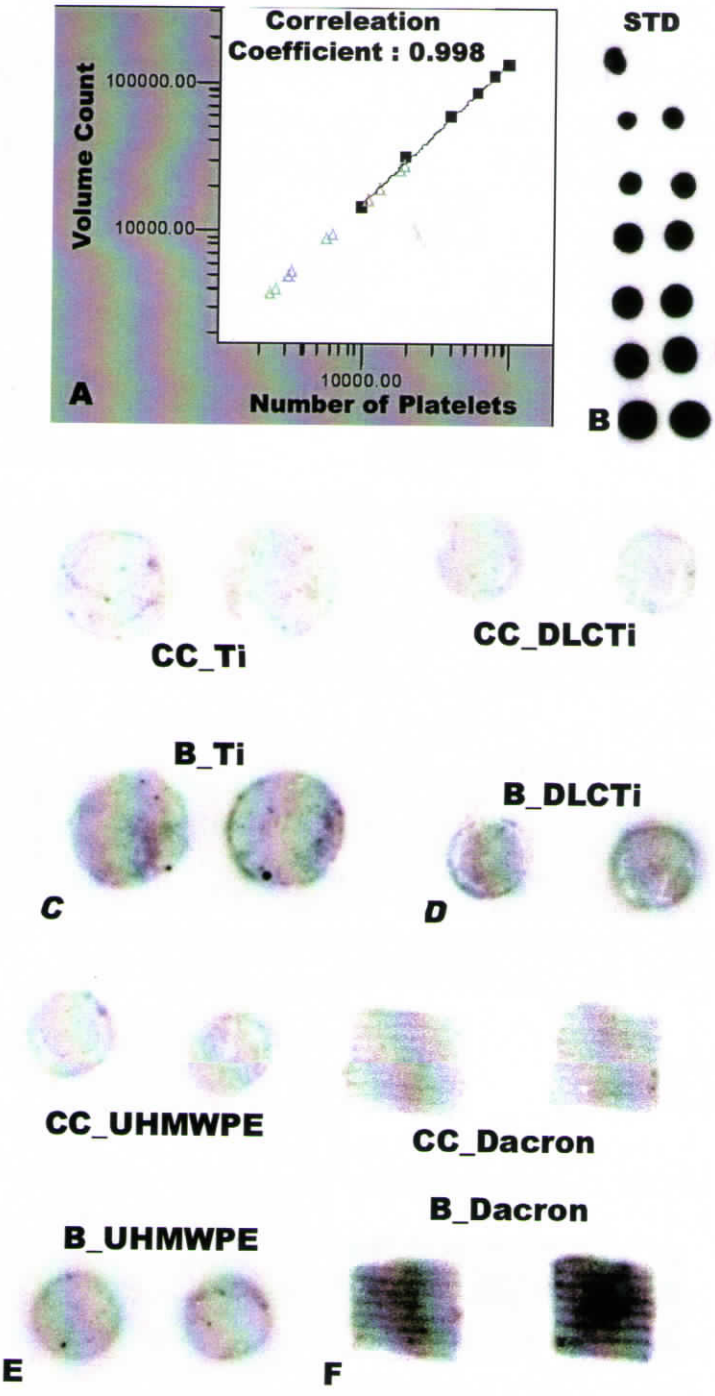
endothelial cell on these bare materials, other than that are adhered directly to the material surface. Whereas, on the EC grown on composite coated materials, platelets are seen very rarely, both on cells and exposed material surface that is not covered with EC monolayer. The most striking effect was the difference between EC on bare Ti and coated Ti.

V.6.1.3. Quantification of Platelet adhesion by Radioscintigraphy

To substantiate the SEM data on platelet adhesion, quantification of platelet adhesion was done by exposing the devices with radio labeled platelets. The radio images of materials shown in Fig.V.13 along with the calibration graph clearly indicate that there is a significant difference in platelet adhesion between the EC seeded bare and coated surfaces. Quantitative data of platelet adhesion on the bare and composite coated EC grown surfaces are summarized in Table V.5. Platelet number adhered was found be reduced to 20% or 25 % on the EC grown composite coated devices, compared to the respective bare material with EC monolayer ($p< 0.001$). The platelet adhesion was very high in the case of EC grown bare Dacron compared to other bare surfaces. However, on coated Dacron with EC monolayer, the adhesion was very minimal and comparable to other coated surfaces. This clearly confirms that composite coating has a beneficial effect on the EC in terms of thromboresistance.

Table V-5. Quantification of Platelet Adhesion by Radioscintigraphy. Number of platelets adhered on each surface is given as cells attached / cm^2 surface area. Intensity of radio images of bare and composite coated materials, based on which platelets deposited was calculated are shown in Fig.5. (n=2). * $p < 0.01$.

Test Material	Number of Platelets Adhered (x 10^3 / cm^2 Area)
B_Ti	11239±1120
B_DLCTi	12998±954
B_UHMWPE	18907±1434
B_Dacron	39,546±2546
CC_Ti	3223±118 *
CC_DLCTi	2476±153 *
CC_UHMWPE	4545±131 *
CC_Dacron	3563±236 *



gure V-13. Calibration graph and radio images based on which mber of platelets deposited was calculated. A, Calibration graph plotted using erial dilution of known number of platelets; B, The images of serially diluted platelets in licates used as STD for plotting calibration graph. The spot at the top corner is that of ted isotope from the count of which, the efficiency of labeling was calculated; C, 2 icate each of coated and bare Ti; D, 2 replicates each of coated and bare DLC_Ti; E, 2 icate each of coated and bare UHMWPE; and F, 2 replicate each of coated and bare cron.

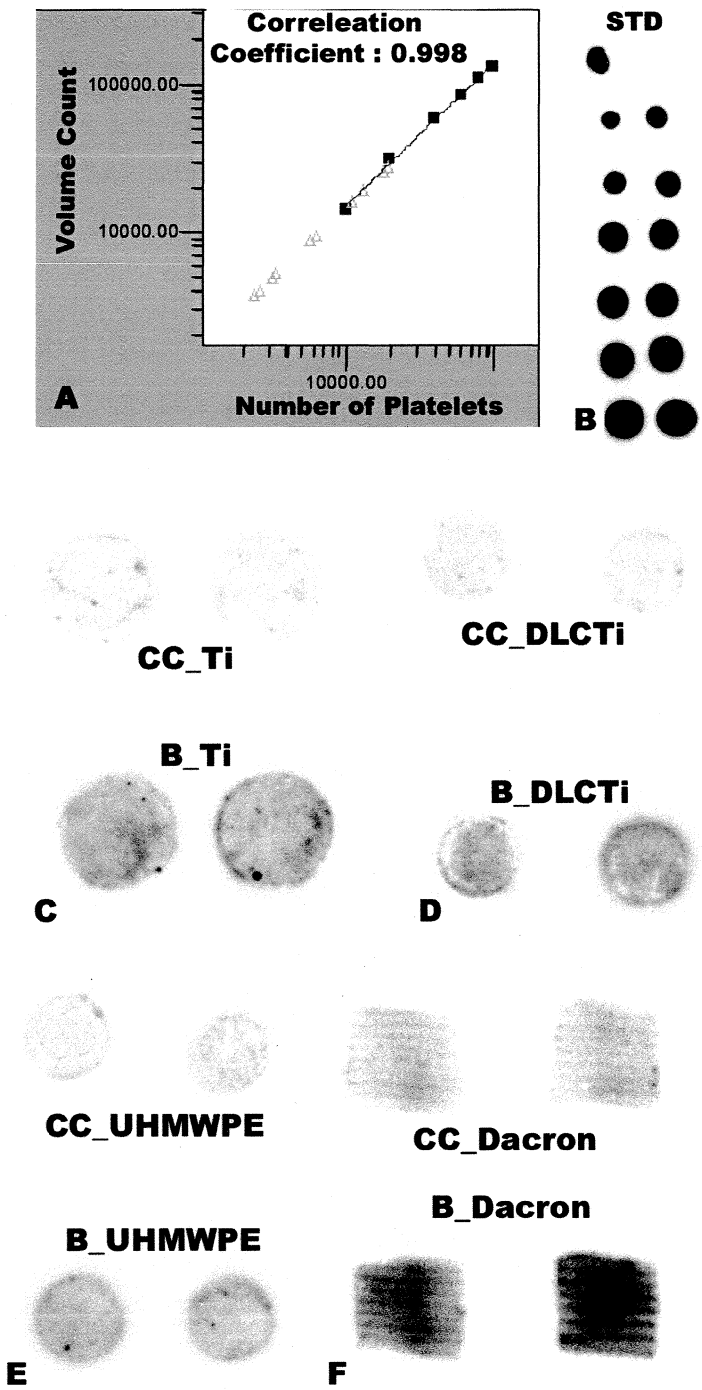


Figure V-13. Calibration graph and radio images based on which number of platelets deposited was calculated. A, Calibration graph plotted using a serial dilution of known number of platelets; B, The images of serially diluted platelets in duplicates used as STD for plotting calibration graph. The spot at the top corner is that of diluted isotope from the count of which, the efficiency of labeling was calculated; C, 2 replicate each of coated and bare Ti; D, 2 replicates each of coated and bare DLC_Ti; E, 2 replicate each of coated and bare UHMWPE; and F, 2 replicate each of coated and bare Dacron.

The results from the platelet adhesion studies suggest that, growing of endothelial cells on bare materials does not improve the thrombogenicity of the materials. The mRNA expression studies indicates that ECs grown on bare material surface get dedifferentiated and turn pro thombotic by up regulation of m-RNA expression for prothrombotic molecules such as vWF and PAI, and down regulation of anti thrombotic molecules such as t-PA and nitric oxide synthetase. Therefore, it is likely that the increased expression of vWF may be contributing to the pro thrombotic tendency of EC causing more platelet adhesion.

Also, it has been reported that endothelial cells undergoing apoptosis become proadhesive even for non activated platelets (Bombeli et al. 1999). Though the mechanism of adhesion of platelets was not understood in their study, data suggested that collagen, fibronectin and RGD-dependent ligands were not involved. It was found in the study that cells grown on uncoated and coated materials after 72h of seeding were stained for positive for Annexin V indicating apoptotic tendency and it was estimated that almost 75% of the cells on uncoated materials turned apoptotic during the study period. On the other hand, apoptosis was hardly seen on composite coated materials. In this study it was observed that platelets bind not only to the exposed material surface but also to endothelial cells grown on bare materials. So it is likely that the up regulation of vWF and its increased synthesis together with apoptosis would result in exposure of vWF mediating platelet adhesion to EC.

V.6.2. Coagulation Studies

Partial thromboplastin time (PTT) which is standardized to pick up the effect of material surface on whole blood to activate extrinsic coagulation pathway was distinctive between the bare-EC and CC-EC surfaces. Coagulation time, PTT was found to be shortened considerably after 1h exposure to all EC grown bare materials that were studied (Table V.6). On the other hand, the shortening of PTT was minimal in the cases of EC grown composite coated materials ($p< 0.01$). Clearly EC grown bare surfaces activate coagulation pathway and thus reflects on PTT. The fibrinogen adsorption was found to be negligible on all the devices and there was no

significant difference between the bare and composite coated EC grown materials (Table V.7).

Table V-6. Partial Thromboplastine Time in Seconds. One milliliter anti-coagulated blood was exposed to each device in separate wells of polystyrene cell culture plates and the clotting time in the sample at 1h was subtracted from the values obtained in the initial sample. The initial and final values are given in the table along with the % shortening. The Average ± SE of 4 replicates are shown. * $p < 0.01$.

Test Material	Initial	Final	% Shortening
B_Ti	130.50±2.12	100.50±1.43	22.97±1.51
B_DLCTi	133.50±3.41	96.50±1.52	27.71±0.89
B_UHMWPE	133.50±3.54	91.50±0.82	31.44±0.45
B_Dacron	130.00±1.67	79.50±3.54	38.87±2.31
CC_Ti	133.00±1.96	125.00±1.34 *	6.02±0.92 *
CC_DLCTi	135.00±1.41	123.50±1.97 *	8.89±0.67 *
CC_UHMWPE	134.50±2.83	120.00±1.34 *	9.77±0.54 *
CC_Dacron	133.00±1.41	117.00±2.83*	12.01±1.23*

Table V-7. Fibrinogen Concentration in g/L. One milliliter anti-coagulated blood was exposed to each device in separate wells of polystyrene cell culture plates and the fibrinogen concentration in the sample at 1h was subtracted from the values obtained in the initial sample. The initial and final values are given in the table along with the % reduction. The Average ± SE of 4 replicates are shown.

Test Material	Initial	Final	% Reduction
B_Ti	1.97±0.04	1.87±0.22	4.84±1.10
B_DLCTi	1.96±0.06	1.83±0.15	6.63±1.05
B_UHMWPE	1.96±0.03	1.90±0.16	2.81±1.07
B_Dacron	1.97±0.02	1.87±0.14	5.33±0.32
CC_Ti	2.05±0.01	1.97±0.08	3.90±0.43
CC_DLCTi	2.03±0.02	1.94±0.06	4.68±0.38
CC_UHMWPE	2.02±0.01	1.96±0.09	2.97±0.26
CC_Dacron	2.05±0.03	1.96±0.07	4.39±0.46

The data from the coagulation studies further confirms that ECs grown on bare material surface turns prothrombotic. The mRNA expression results give a clear indication of phenotypic alteration of EC grown on bare materials into more pro thrombotic form. There are a number of studies showing that endothelial cells can rapidly shift the hemostatic balance from an

antithrombotic to prothrombotic state. This may be caused by increased binding and activity of coagulation factors, loss of anticoagulant surface components, or more actively, by expression and/or release of prothrombotic substances, such as tissue factor (TF), factor V, platelet activating factor (PAF), vWF and PAI (Rodgers, 1988; Nachman, 1992; Martin et al. 1995).

There are reports that apoptotic cells become more procoagulant (Bombeli et al. 1997). Even without cell detachment, stimulation of apoptosis has been found to increase collagen exposure from 5% to 30% within 6 h of cell death. It has been investigated by them to find out if the ECM exposure is the cause of procoagulant effect seen with apoptosis, but it is found that even floating apoptotic HUVEC increase thrombin formation in plasma (Bombeli et al. 1997).

Results of this study indicate that more than covering the cardiovascular device surface with endothelial cells, it is important to make sure that seeded cells should prevent platelet adhesion and activation of blood coagulation. It is demonstrated here that though bare materials give wrong signal to the EC seeded on the surface and turn them prothrombotic, if these surfaces are pre-coated with bio mimetic molecules they help to maintain normal EC phenotype and reduce the risk of platelet adhesion and activation of blood coagulation.

Chapter VI

SUMMARY AND CONCLUSIONS

VI.1. RESTATEMENT OF THE PROBLEM

Recent insights into the pathogenesis of vascular disease have opened up a new research frontier and vasculature has been redefined as a vital organ that can regulate its own tone and structure via numerous cellular mechanisms. A "blood compatible" material would perfectly substitute for the endothelium so that soluble and cellular blood components would remain entirely naïve of its foreign presence. Although biomaterial science has advanced sufficiently in the past 50 years, true biocompatibility of artificial surfaces remains a relatively distant and elusive goal. Understanding the importance of endothelium in maintaining a thrombus free luminal surface, these cells are widely used in the tissue engineering of vascular grafts and other blood contacting devices.

The importance of a functionally intact endothelium is firmly established. Less well characterized, however, is the role of the endothelium in extracellular matrix metabolism, despite the observation that the extracellular matrix comprises the bulk of the media in health and the bulk of the abnormal neointima during disease. In early 1980s, growing endothelial cells in *in vitro* culture was thought to be the most difficult among all cell cultures. Later gelatin coated polystyrene plates were found to support the EC growth in culture, however, it has been well known that in long term cultures and repeated splitting to obtain large number of cells, endothelial cells lose the potential to regenerate as the passage number progresses; however, related phenotypic alteration is not so well studied. Therefore in this study, efforts were made to understand the phenotypic changes that take place when EC is grown continuously through several passages on

gelatin. Further the question was asked how we maintain cells for long term in culture without altering the cell behavior.

Extracellular matrix is essential for endothelial cell survival and proliferation response to growth factors, rendering cells apoptotic in the absence of matrix. Also, it has now become equally clear that the surrounding matrix actively participates in modulating endothelial function and those EC products, in turn, can regulate matrix content and structure. It is the continuous cross-talk between cells and the surrounding matrix environment that lead to the formation of patterns, the development of form (morphogenesis), and the acquisition and maintenance of differentiated phenotype. Therefore, to expand the cell number in tissue culture, it is important to use suitable matrix coated dishes so that they remain viable and functional. As much is it important to have a matrix with optimum composition to get EC attachment, spreading, proliferation and differentiation on biomaterial surface so that the modification is effective to prevent device thrombosis.

Therefore, this study was undertaken to standardize a suitable matrix to normalize differentiation of HUVEC in culture so that the most suitable matrix composition may be used for vascular tissue engineering technique that involve endothelial cell culture. The criteria for suitable matrix were defined as the composition on which cells do not dedifferentiate even if they are cultured for long term in continuous passages. Molecular level monitoring of dedifferentiation was targeted by selecting pro and anti thrombotic markers. The suitability of the standardized matrix for growing adult EC was also focused, so that autologous cells can be generated for tissue engineering applications. Further, use of the matrix to seed endothelial cells on biomaterials and significance of the matrix for maintenance of normal EC phenotype was also taken up.

VI.2. DESCRIPTION OF PROCEDURES

The Major objective of this study was to identify an optimal matrix for culturing EC *in vitro*, and also to act as a matrix coating on the vascular biomaterials to support EC growth. Fibrin, an extracellular matrix component, is occupied in hemostasis, wound healing, and cancer. Fibrin is formed at the sites

of tissue injury and provides a temporary matrix to support initial response of ECs needed for tissue repair. Other than acting as a scaffold for cell attachment, it holds other extra cellular components that are major integrin receptors such as fibronectin, thrombospondin that anchor the cells to the injured tissue along with bound growth factors to generate signals for optimal cell proliferation, survival and expression of appropriate functional proteins. In the light of this information this study was carried out to evaluate the effectiveness of a composite of fibrin, fibronectin, gelatin and vascular endothelial growth factor (VEGF)

The effect of cell-matrix interaction on the behavior of ECs were evaluated in terms of adhesion, proliferation, apoptosis and normal expression of prothrombotic and anti-thrombotic proteins, so that an ideal matrix can be selected for cell expansion and use in tissue engineering. After the isolation, and characterization of Human Umbilical Vein Endothelial Cell (HUVEC) and Human Saphenous Vein Endothelial Cell (HSVEC), the cell adhesion, spreading, focal adhesion organization, proliferation rate and apoptosis on the designed matrix were compared against conventional gelatin coating.

The physiological status of the monolayer grown on the standardized matrix was studied. For phenotypic expression molecules analyzed were; vWF – a prothrombotic molecule, nitric oxide (NO)- an antithrombotic and vasodilator molecule, tissue plasminogen activator (t-PA) – a fibrinolytic factor, and plasminogen activator inhibitor (PAI)- an antifibrinolytic factor. Analysis was done by reverse transcriptase polymerase chain reaction (RT-PCR) for detection of m-RNA expression. The mRNA expression by HSVECs and EC grown on biomaterials were quantified by Real Time PCR. ELISA was performed to detect quantitative variation in release of the molecules into the culture medium. NO release by ECs was detected using a commercial kit. Shear stress resistance of the EC monolayer was studied by perfusion of the seeded biomaterials.

Extensive studies were carried out to assess the advantages of growing EC on the coated matrix to provide a non-thrombogenic surface in comparison with the EC grown on bare vascular device materials. To examine how the phenotype of ECs grown on the biomaterials reflects on the *in vitro* blood compatibility of the devices, tests were selected from the list recommended in ISO10993 part4. These tests are standardized and validated in our laboratory

and is accredited against quality system ISO/IEC 17025 by COFRAC (France). The thrombogenicity of EC grown materials was assessed by coagulation studies, qualitative platelet adhesion using scanning electron microscopy and quantitative assay using radiolabeled platelets (radioscintigraphy).

VI.3. MAJOR FINDINGS

The study was carried out in three parts: 1. Standardization of suitable matrix using HUVEC. 2. Standardization of HSVEC isolation and study the growth and behavior of HSVECs on the Composite Matrix Vs. conventional gelatin coating. 3. Evaluation of the effectiveness of the standardized matrix for EC seeding on biomaterials.

The objective of the first phase of this study was to compare how HUVEC gets transformed when they are serially passaged on long-established gelatin matrix or on a composite of fibrin, fibronectin and growth factors. The key finding was that the cells deteriorate passage by passage on GCPS whereas on CCPS that consists of ECM proteins, gelatin and growth factors, cell phenotype is maintained. Functional receptor-mediated and signal transmitting cell adhesion on biomaterials is mediated by extracellular matrix (ECM) molecules and the incorporation of functional growth factors and regulators with the ECM, and their controlled release might improve cell behavior.

The composite that is used should impart properties of various molecules and their synergistic effect might be responsible for cell adhesion, proliferation, survival and cell differentiation. Fibrin as a natural scaffold material has sites to bind and retain growth factors that are released based on the cell-demand (Ehrbar et al 2004). Focal adhesions are specialized adhesive complexes containing structural and signaling molecules that regulate cell migration, survival, cell cycle progression and differentiation (Sastry and Burridge, 2000). The focal adhesion complex is the multifunctional structure required for cell to integrate integrin mediated signaling events from the matrix with the dynamic cytoskeletal meshwork that mechanically couples the cell with extracellular matrix. Appropriate orientation of the signaling machinery of the cell is dependent on the structure of the focal adhesion complex, which provides an intricate inter relationship between the ECM, the cytoskeleton and signaling cascades (Garcia et al., 1999; Aplin, 2003). Thus the observed poor proliferation

rate and enhanced apoptosis on GCPS is possibly due to either lack of well formed focal assembly or because of poor adsorption of gelatin to the material surface.

In this study it is demonstrated that the use of a bio mimetic matrix that contain adhesion molecules and growth factors support focal adhesions and associated actin assembly, cell proliferation, survival and differentiation. Compared to the gelatin coated polystyrene that up regulate thrombogenic molecules and down regulate anti-thrombogenic molecule, the composed bio mimetic matrix is found to maintain normal cell differentiation. This matrix being an ideal one to maintain normal cell function during *in vitro* cell culture, if these can be deposited on various materials EC should grow on them to form cell monolayer with normal function as well. Matrix selection is important not only for coating the biomaterial/scaffold but also for the tissue culture plates that are used for cell multiplication to get functionally normal cells for tissue engineering.

The results presented clearly suggest that, the use of gelatin adsorbed surfaces for endothelial cell growth in tissue culture is found to cause significant cell transformation on serial passage; and a matrix composed of gelatin, fibrin, fibronectin and the growth factors support good actin organization and transduce signals for proliferation, maintenance of viability and cell function.

The second phase was initiated with an objective to isolate and grow endothelial cells from saphenous vein to understand the cell phenotype in CVD patients. A model matrix which was proven for its support for HUVEC adhesion, spreading, proliferation and survival was used, with an objective to understand how the matrix would influence cell differentiation in continuous culture. Vein samples were collected randomly with out knowing diagnosis or radiological/laboratory findings, from patients undergoing bypass surgery.

The results from this study show that when HSVEC is isolated from CVD patients, in most of the cases pro thrombotic molecules are over expressed. When the results were analyzed, it was found that HSVEC from one patient behaved differently, in terms of molecular expression of pro thrombotic and anti thrombotic molecules in one of the initial passages itself. When the data is correlated with the background information of the patients the major differences seen were that this subject had only one risk factor i.e. Diabetes Mellitis, and that

he suffered from angina on exertion (AOE) only and there was no previous history of MI.

It is interesting to note that the EC from subject #5 behaved more or less similar to that of HUVEC, when it was sub cultured through several passages on CCPS and GCPS. The pro thrombotic factor vWF remained stable on CCPS whereas on GCPS it was over expressed as the passage number progressed. Measurement of PAI, t-PA and eNOS in HUVEC through several passages was similar to that of HSVEC in subject #5. It indicates that HSVEC of subject #5 was not dysfunctional in terms thrombogenicity. Whereas the HSVEC from all other subjects were more pro thrombotic signified by amplified vWF & PAI m-RNA expression and synthesis, and less anti thombotic pointed out by reduced t-PA and eNOS m-RNA expression and synthesis by cells in early culture passage.

The effect of matrix on the proliferation and apoptosis of HSVECs were found to be similar to that on HUVECs. The proliferation rate deteriorated passage by passage on GCPS and apoptotic tendency was found increasing. This is thought to be because of lack of well formed focal adhesion complex and the poor signaling which is already discussed earlier in *Chapter III* in detail. The most significant finding from this study was the reversal of dysfunctional EC from almost all subjects into a phenotype with normal expression of pro thrombotic and anti thrombotic properties comparable to HUVEC as discussed in *Chapter III*.

In summary, it is likely that the change in ECM composition due to generation of phenotypic SMC after vascular injury might lead to divergence that turn EC into more apoptotic, pro thrombotic and less anti thrombotic phenotype. The *in vitro* model developed in this study sustains by providing the optimum matrix and growth factors such as CCPS, dysfunctional EC can change into normal EC. But if EC is allowed to grow continuously on matrix such as GCPS, they become more apoptotic and pro thrombotic. Thus switching of EC phenotype is likely to be dependent on the ECM composition. Therefore this may be a good model to understand the underlying mechanism of EC dysfunction in pathology. Further it can be useful to understand the effect of risk factors and drugs on modulation of EC function.

The aim of the third phase was to demonstrate that if endothelial cells are grown on bare material, they lose their normal phenotype and become more

prothrombotic than it should be to prevent thrombotic tendency. Various researchers attempt endothelialization of biomaterials with an intention to reduce the risk of thrombotic complications. But, once the materials influence the cell phenotype and turn them to be pro thrombotic, endothelialization does not serve the purpose. Therefore more than covering the cardiovascular device surface with endothelial cells, it is important to make sure that such cells grown should prevent platelet adhesion and activation of blood coagulation.

Studies with HUVECs suggested that it is important for EC to get signals from the extracellular matrix for cell proliferation, survival and to maintain normal anti thrombotic phenotype. This study shows that HUVEC grown on bare materials used in cardiovascular device fabrication over express m-RNA for vWF and PAI and down regulate t-PA and eNOS. In addition, increased adhesion of platelets to EC grown on bare materials and acceleration of plasma clotting are also detected. Earlier reports suggest that endothelial cells undergoing apoptosis become more pro-coagulant and proadhesive even for non activated platelets. It was observed that, the cells grown on uncoated materials after 72h of seeding were stained positive for Annexin V indicating apoptotic tendency and it was estimated that almost 75% of the cells on uncoated materials turned apoptotic during the study period. On the other hand, apoptosis was hardly seen on composite coated materials. Thus the results of this study show that seeding of endothelial cells to all surfaces may not turn out to be successful to reduce thrombogenicity of materials because cells undergo phenotypic alterations and turn pro adhesive for platelets and activate coagulation pathway. However, irrespective of the nature of the material, bio mimetic fibrin composite can be coated on surfaces and HUVEC grown on such matrix is able to maintain normal phenotype with minimal platelet adhesion and without activation of coagulation.

VI.4. CONCLUSIONS

- ✓ **Use of gelatin adsorbed surfaces for endothelial cell growth in tissue culture is found to cause significant cell transformation on serial passaging.**

- ✓ **Matrix characteristics have influenced the expression of functional proteins considerably, which may be due to good/poor signaling through focal adhesion points.**

- ✓ **Matrix composed of fibrin, fibronectin, gelatin and the growth factors support good actin organization and transduce signals for proliferation, maintenance of viability and cell function.**

- ✓ **It is not an adverse effect of gelatin that causes dedifferentiation of EC in culture, but gelatin alone is not enough to hold growth factors and elicit signals required for EC to spread, proliferate, survive and function.**

- ✓ **The dysfunctional EC isolated from cardiovascular patients were regenerated to express a normal anti-thrombotic phenotype when grown on the composite, which may due to the right signals received from the matrix.**

- ✓ **The matrix composition proves to be a good model to grow endothelial cells in culture and to study the mechanisms and processes of endothelial dysfunction and to understand the effects of various drugs.**

- ✓ **This study suggests that adherence of the endothelium to the matrix is a dynamic interaction allowing endothelium to change and adapt to various physiological and pathological states.**

- ✓ **Since the matrix favors the maintenance of normal phenotypic expression, the composite matrix can be used for the tissue engineering of cardiovascular conduits, to promote rapid endothelialization.**

- ✓ **Dysfunctional EC isolated from patients autologous vein can be grown on small diameter vascular graft for coronary artery bypass grafting**

- ✓ **The major advantage of monolayer grown on the composite coated device will be normal anti-thrombotic phenotype, resistance to shear stress, and minimal platelet adhesion**

VI.5. RECOMMENDATION FOR FURTHER INVESTIGATION

Over the last two decades we have been observing growing interest in the actual role of haemostatic system disturbances in the development of such conditions as atherosclerosis, deep vein thrombosis, coronary artery disease and so on. The complexity of coronary artery disease genetics arises from the diversity of clinical phenotypes and from the many biological pathways that

contribute to atherosclerotic plaque biology, inflammation, endothelial function, oxidative stress and thrombosis. Cells may express different constellations of genes and therefore vary phenotypically, depending on their environment.

Indeed, it is a widely held view that impaired endothelial function is also the initial step in atherogenesis, which is largely responsible for ischaemic heart disease and thrombotic strokes decades later. Impaired endothelial function is also associated with hypertension, diabetes mellitus and heart failure (regardless of etiology), although whether as a cause or a consequence is undetermined. Hence, understanding endothelial function is likely to be a key to modifying risk factors of cardiovascular disorders and their sequelae. Nevertheless, an ideal system to assess endothelial physiology (and, therefore, pathology) remains uncertain. Though detailed study of endothelial function first became feasible with the development in the 1970s of techniques to culture ECs *in vitro*, the limitations of this approach have become apparent with the realization that conventional methods of cell culture perturbs ECs from their quiescent *in vivo* state to an activated phenotype with loss of specialized functions associated with diverse vessels and organ systems.

In this context, the findings of this study acquire great significance. The matrix composition standardized in this study proves to be a good model to grow endothelial cells in culture and to study the various mechanisms and processes involved in endothelial pathophysiology.

Another important field of application is Tissue engineering. The emerging field of tissue engineering is focusing on the in-vitro generation of functional organ replacements using living endothelial cells and other vascular cells for which non degradable or biodegradable scaffold base materials are used. One of the major hurdles faced by tissue engineers in the field of vascular biology is the maintenance of the functional status of seeded EC. Since the composite matrix favors the maintenance of normal phenotypic expression, the matrix composition can be used for the tissue engineering of cardiovascular conduits, to promote rapid endothelialization.

Some of the important cues for future investigations coming out from this study are highlighted below:

- ✓ The standardized matrix can be used for *in vitro* studies to understand the etiology of endothelial cell dysfunction and related pathological conditions.

- ✓ Involvement of the ECM in the EC dysfunction and the associated pathologies needs to be further evaluated by histopathological analysis of vessels from cardiovascular patients.

- ✓ In this study only the pro- and anti- thrombotic molecules were evaluated. ECs play a major role in mediating the inflammatory responses in disease progression. So how the ECM influences the synthesis of various inflammatory markers by EC and the subsequent interaction between EC and leukocytes needs to be further investigated.

- ✓ Internal mammory artery (IMA) is one of the frequently used conduits for CABG. Since in this study only the saphenous vein EC was examined, endothelial cells isolation from IMA and phenotyping needs to be addressed in future.

- ✓ EC isolated from patients autologous vein or arteries can be grown on small diameter artificial vascular graft after coating with the composite matrix. The feasibility of this type of artificial blood vessel substitutes needs to be evaluated by animal experiments.

- ✓ Tissue engineering: A matrix on which EC can be grown without affecting physiological properties will provide thrombo-resistant artificial cardiovascular devices.

- ✓ Since the data from this study suggest that the approach of coating the designed composition of bio mimetic adhesive proteins and growth factors is suitable for cardiovascular tissue engineering using any material of choice as base material or scaffold, future work should focus on the coating of biodegradable polymeric scaffolds to achieve completely biological cardiovascular conduits.

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Annexure –I

List of Media and Buffers

1. Water for tissue culture

Tap water distilled three times, deionised by passing through Millipore water purifying system, autoclaved and used fresh.

2. Collagenase enzyme solution for EC isolation

Type II collagenase is dissolved in serum free M199 medium to get 0.2% solution (w/v). Sterile filtered through 0.22µ filter, aliquoted and stored frozen.

3. M199 medium

The powdered medium is dissolved in one liter of sterile water and after adding 2.2g $NaHCO_3$ the pH is adjusted to ~7.2. Sterile filtered through 0.22µ filter, and stored at (2-8)^{0}C.

4. MCDB131 Medium

The liquid media is supplied with 10mM final concentration of L-Glutamine. Sterile filtered through 0.22µ filter, and stored at (2-8)^{0}C.

5. HBSS (Ca^{2+} and Mg^{2+} free)

KCL	5.4 mM
KH_2PO_4	4.4 mM
NaCl	136.9mM
Na_2HPO_4	2.7 mM
Distilled water	upto 1000 ml
pH	7.2

The prepared solution is autoclaved for 30 min at 121^{0}C. Glucose solution autoclaved separately was added to the buffer (1% final concentration) and stored at (2-8)^{0}C. Used within one week.

6. PBS

NaCl	150 mM
KH_2PO_4	1.5 mM
Na_2HPO_4	10 mM
Distilled water	upto 1000 ml
pH	7.4

7. Acid Citrate Dextrose (ACD)

Citric acid, monohydrate	4.2 mM
Trisodium Citrate, dihydrate	7.5 mM
Dextrose	13.9mM
Distilled water	upto 100 ml
pH	7.4

8. Tyrodes Buffer

Glucose	5.5 mM
MgCl2	2.09 mM
KCl	5.39 mM
NaCl	140 mM
Tris	14.49 mM
Distilled water	upto 1000 ml
pH	7.4

9. Digestion buffer for PAGE

SDS	0.1%
Tris-HCl	0.05 M pH 7.9
EDTA	0.1 M
DTT	5 mM
NaCl	0.15 M