TISSUE ENGINEERED BIOMATERIAL SURFACES USING ENDOTHELIAL CELLS AND BIO ADHESIVES TO REDUCE THROMBOGENICITY

A Thesis Presented

by

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SREE CHITRA TIRUNAL INSTITUTE
FOR MEDICAL SCIENCES & TECHNOLOGY
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DECLARATION

I, T.R.Santhosh Kumar, hereby declare that I had personally carried out the work depicted in the thesis entitled "TISSUE ENGINEERED BIOMATERIAL SURFACE TO REDUCE THROMBOGENICITY USING ENDOTHELIAL CELLS AND BIOADHESIVES" except where external help sought are acknowledged.

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Submitted

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To my mother..........

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SYNOPSIS

SYNOPSIS

Arterial occlusive diseases caused either by aneurysms or atherosclerosis can be successfully bypassed by autogenous vein grafts, if suitable vein is available. But in most cases suitable autogenous vein may not be available due to various reasons. In such occasion, the only option is to implant a synthetic vascular graft. Large diameter vascular grafts made of polyethylene terephthalate (Dacron) or poly tetra fluoro ethylene(Teflon) have been clinically successful and have long term patency, mainly owing to the high flow rate. However, in the case of small diameter vascular grafts where lower flow rate and higher surface area to blood ratio prevail, even a small thrombus will lead to early graft failure. Therefore, the patency rate with currently available small diameter vascular grafts remains low. The main reason attributed to this is, lack of post implant endothelialization of synthetic vascular graft in humans. Thus, the middle portion of the vascular graft remains as a thrombogenic surface leading to the chances of platelet dependant vascular occlusion.

In this background, knowing the ability of endothelial cell (EC) to act as a thromboresistant surface, Malcolm Herring introduced the idea of EC transplantation on the surface of the biomaterial as a technique to reduce the thrombogenic potential of vascular biomaterials. It has been demonstrated that the successful complete coverage of EC is possible, but this technique has not witnessed the transition from experimental to clinical arena, mainly because of the cumbersome multistage approach involving variable level of inefficiencies during each of the following stages.
1. Harvesting of EC from the donor; 2. Attaching EC to the vascular surface; 3. Replication of the EC to confluency; 4. Efficiency to resist the shear stress once attached to the surface; 5. Maintenance of the antithrombotic function of the transplanted cell.

Each stage in the procedure is critical and refinement of each step will help to solve the complexities associated with this novel idea which had elicited a great deal of enthusiasm from vascular biologists. Fibrin matrix formed during tissue injury as a hemostatic barrier, adheres to surrounding tissue and cells and act as a scaffold for migration of cells during tissue regeneration. Therefore, in this study it is attempted to improve attachment and proliferation of EC to fibrin matrix and to evaluate the nonthrombogenic aspects of the monolayer using human umbilical vein endothelial cells (HUVEC).

Objectives of the study are: 1. Standardization of the composition and coating technique of fibrin matrix on to surfaces to attain optimum HUVEC attachment, spreading and proliferation. 2. Modification of the composition of the matrix using other proteins and growth factors to enhance proliferation; 3. Evaluation of the function of endothelial cell monolayer with respect to its ability to resist platelet adhesion and thrombus formation; 4. Evaluation of the efficiency of seeded cells to resist forces of shear stress; 5. Comparison of cell attachment, proliferation, thrombogenic nature and resistance to shear stress of HUVEC grown on different matrices.

Chapter I, introduces background of the problem and briefly reviews topics such as properties of implant biomaterials, problems with its blood compatibility, methods used for surface modification to reduce thrombogenicity, and in particular, the tissue engineering technique involving endothelial cell seeding to make blood compatible surfaces. The problems faced for successful seeding and proliferation of endothelial cells on polymeric surfaces, the adhesive proteins used to enhance cell adhesion to surfaces, problems with each of the matrix proteins used and the reported advantages of fibrin as matrix protein etc. are also highlighted. Finally, the reason for chosing fibrin as the matrix protein is elaborated based on the existing literature.

Chapter II consists of all the materials and methods used during the study. The methods described are for:

i. Coating of Polystyrene (PS) with gelatin, ii. Preparations of Fibrin coated culture plates, iii. Composite coating of surface, iv. Isolation of ECGF and incorporation of GF with fibrin, v. Retention of proteins/growth factors in fibrin matrix, **vi**. Degradation of fibrin matrix, vii. Cell isolation, characterization, & seeding, viii. Assay of EC spreading: ix. EC Proliferation assay by count and ^{3}H-Thymidine uptake: x. Assay of shear stress effect on the cell monolayer: xi. Thrombogenicity of surfaces by platelet adhesion, aggregation and Nitric Oxide synthesis. xii. Comparisons between bare Dacron, Teflon, and coated surface for cell adhesion, proliferation, thrombogenicity and resistance to shear stress.

Chapter III, consists of the results and discussion which is divided into 10 major sections. In section III.1. the results from umbilical vein and saphenous vein endothelial cell culture and their characterization are presented. By the morphological analysis of the monolayer grown from cell isolates as well as with their cell specific antibody and ligand binding studies, it is demonstrated that both cell types yielded uniform culture, which expressed FVIII in the Weibel palade bodies and LDL receptors on the membrane surface.

The § III.2. Compares the results from the cell adhesion, and spreading on different substrates. HUVEC adhesion was significantly high on fibrin-coated dishes than on bare polystyrene (PS) or gelatin-coated dishes. After 4 h of seeding 91 % of the initially seeded cells were attached and well spread on the fibrin coated surface while only 60- 65 % of cells were found attached on the gelatin or uncoated surface. The percentage adhesion was similar on fibrin matrix with or without growth factor. The matrix markedly influenced the spreading efficiency. After 20 min, the cells on the fibrin coated surface started to spread and by 30 minutes most of the cells on this matrix become flattened with cytoplasmic extensions and on the fibrin matrix with GF also the cell spreading rate was high and the cytoplasmic extensions were widespread and thus occupied more area. Though attached to the surface, the cells in the gelatin remained spread in a rounded morphology for which it took 45 to 70 min.

The section III.3 explains the features of the EC adhesion and spreading on to different vascular materials, coated with adhesive matrices. The major finding was that the EC attachments to gelatin coated vascular materials were poor. The gelatin coating onto biomaterials has failed to reproduce the results obtained from that on the tissue culture polystyrene. Other wise, fibrin and composite coating gave reproducible, uniform cell attachment and spreading on Dacron as well as PTFE. Other materials such as UHWPE, Ti, DLC-coated Ti, were found to support attachment and growth on bare surfaces, while the attachment was stronger when matrices were precoated on to the surfaces of these materials that are candidates for implant devices. Section III.4 to 8 Explains Cell proliferation on different matrices and the effect of the composition of the matrix on doubling time, when grown on coated tissue culture polystyrene and biomaterials. Eventhough, the number of cells attached to gelatin was only 66%, and on fibrin 92%, of seeded cell, after 48 h, the cell number on both the substrates were similar. With a seeding density of 2x 10^4 /cm^2, the cells on the fibrin- and gelatin- coated surfaces showed a cell density of 4.4x10^4 /cm^2 and 3.8x10^4/cm^2, respectively, after 72h. While on GF immobilized fibrin, the cells have grown to confluence after 48h and by 72 h the cell has reached to overconfluent stage with an average density of 7.4x10^4/cm^2. Eventhough the initial attachment was low, the proliferation of attached cells on gelatinized substrate was higher than that on fibrin. However, after ECGF incorporation into fibrin matrix when seeded with half the density of cells, the cells multiplied and reached to near confluent stage on 3rd day. The average doubling time calculated based on cell density over the study period was 31 h for gelatin, 42 h for FG, 24 h for (FG +GF). The proliferation rate monitored with tritiated thymidine showed that there was 23% more proliferation on gelatin-coated surface, and 16% more on fibrin coated surface, as compared to uncoated polystyrene. On the other hand, on ECGF incorporated fibrin, proliferation rate was higher by 150%.

Section III.9, reports results on the coating technique, matrix stability, thrombogenicity and retention of growth factor within the matrix. Thrombogenicity of the fibrin was found minimal compared to the bare PTFE or Dacron, and gelatin coated surfaces. The retention of growth factors within the fibrin matrix after the initial five minutes was about 85-90%. After the next 24 h, around 30 - 40% of the total incorporated protein was still left in the matrix. Then the release has declined which resulted in the retention of about 18 to 28% after 48h. The cumulative release of chromoproteins for a period of 96 h was around 80 to 95%, depending on the initial protein concentration that was incorporated. For cell culture, we incorporated 300 μg of endothelial cell growth factor, and from the release study it is estimated that at 48 h 20% of the incorporated proteins and peptides were retained in the matrix. The fibrin layer was uniform and was highly stable when incubated with either 100% serum, or 20 % serum in culture medium. The adhesion of the fibrin to the surface was obtained uniformly and did not detach during the procedures of cell seeding or trypsinization.

In section III.10. the properties of the monolayer in terms of the maintenance of the normal physiology is discussed. When monolayer grown on gelatin was exposed to flow, 40% of EC grown on gelatin was lost from the surface. The shear stress calculated from the volumetric flow and viscosity of the fluid was between 15-30 dynes/cm^2. Whereas the EC grown on fibrin matrix and composite were oriented in the direction of flow, the loss of cells from the surface was negligible.

In Chapter IV, all the results are further summarized and conclusions are made. In conclusion, the techniques for coating fibrin on the surfaces were standardardized, to a get a uniform layer enmeshed with endothelial cell growth factors. It was found a superior matrix, compared to gelatin, to support adhesion and spreading of endothelial cells (EC), isolated from the human umbilical vein. The ECGF incorporated with this adhesive matrix enhanced proliferation of cells significantly, attaining confluent monolayer in 48 to 72h. The matrix did not degrade during cell seeding or proliferation. The monolayer had typical cobblestone morphology, and ability of the EC grown on fibrin to resist the forces of shear stress was high, compared to the gelatin-coated coverslips. The EC grown on fibrin and composite were also found nonthrombogenic. Using HSVEC, the number of cells and the time required to form a tissue engineered, nonthrombogenic confluent monolayer of autologous surface was determined.

Finally, future research and application prospects are also projected. Since fibrin incorporated with growth factors form a very good substrate for adhesion and proliferation of HUVEC, EC from patients` autologous vein can be isolated and grown on small diameter vascular graft for implantation. The major advantage of monolayer grown on such surfaces will be its ability to resist shear stress. A method for preservation of all properties of the ideal matrix using lyophilisation has been achieved. Thus, a commercial viability of the technique can also be explored in future.

CHAPTER I

INTRODUCTION

INTRODUCTION

I.1. Summary

The worldwide death rate from cardiovascular disease is estimated to be high, especially among people in the age group 36 to 74. Many deaths from cardiovascular diseases are attributed to atherosclerosis, a pathological process affecting the large and medium sized muscular arteries such as aorta, coronary and cerebral arteries. The current modalities of treatment of diseases leading to narrowed blood vessels include atherectemy, angioplasty or bypass of the affected vessel with autogenous or artificial vessel. Autogenous veins have been recognized as the preferred conduit in medium and small diameter vessel reconstruction, for coronary artery and femoropopliteal bypass and patch angioplasty. However, multiple previous operations, inadequate length of the vein, phlebitis or varicosities of the desired vein etc. leave the patients with shortage of suitable veins when there is recurrent occlusion. In such conditions, the only option is to bypass the occluded vessel with biologic or artificial prostheses. Current indications for inserting vascular prostheses are advanced atherosclerosis, aneurysms, arterio-venous dialysis grafts, or traumatic injury.

Many artificial materials have been developed aiming at successful substitution of blood vessel in the human body, but only a few have shown satisfactory results *in vivo*. Thrombus formation at the blood- biomaterial interface is almost inevitable in long-term implants. It is generally accepted that the thrombotic events are initiated by the polymer surface by protein adsorption. These artificial materials promote adhesion and growth of platelet enriched thrombus, which can lead to occlusion of the vascular graft or to embolic complications. Therefore, various efforts have been made to develop new thromboresistant polymeric materials or to modify the blood contacting surface, of the existing, well characterized materials, by physicochemical or biologic methods.

The major efforts made to improve the blood compatibility of currently available vascular grafts and associated problems are reviewed here. The luminal surface of the blood vessel, which is the most suitable blood container, holds the endothelial cell monolayer. These cells play a major regulatory role in maintenance of vessel patency by a balanced action of anticoagulant and procoagulant molecules synthesized and expressed on them. The tissue engineering principles by which endothelial cells can be transplanted on to the vascular biomaterial is considered to be a novel approach for solving problems with thrombogenicity of artificial materials. Inorder to address the challenges involved in the development of tissue engineered, endothelialized artificial conduits, a hypothesis is generated. The importance of bioadhesives like fibrin sealant as a natural scaffold, and role of growth factors to promote proliferation of cells in culture has been exploited in the methods to solve the hypothesis. Finally, the objectives of this study, focuses on the successful seeding of endothelial cells on the biomaterials, to form confluent monolayer in the culture that resist shear stress and thrombosis while maintaining normal physiology.

I.2. Autogenous Vein Graft – The Golden Choice

Currently there is a general agreement that autogenous vein is the preferred conduit in medium and small vessel reconstruction, including coronary artery and femoropopliteal bypasses and patch angioplasties. Five-year patency rates of 80% for aortocoronary and 30-75% for femoropopliteal bypasses exist when autogenous saphenous vein is utilized. Following implantation of the vein in the arterial system, 60-70% of the venous endothelial cells disappear within 48 h (Sottiurai and Batson 1982). Denuded areas are covered by thrombus, and endothelial cell proliferation is seen originating from the remaining cells. By 4 weeks, 80-90 % of the vein graft is reendothelialized. By 12 weeks the venous and arterial endothelium are confluent. During the next 3-6 months fibroblasts progressively infiltrate the vein media and adventitia. The venous internal elastic lamina disappears between 6-9 months following implantation. By one year autogenous vein grafts used as artery grafts lose most of their elasticity. The biochemical mechanism governing this process remain unknown and myointimal fibrosis remain a common defect in the healing of vein grafts. Several investigators suggest that the sites of myointimal hyperplasia will later predispose to development of atherosclerosis (Szilagyi *et al.* 1973). Distal anastomotic stenosis, intimal thickening and atherosclerosis in the vein, fibrotic valves or stenoses in reversed veins are some common failure modalities of saphenous vein grafting. The autogenous larger diameter saphenous vein is unavailable or unsuitable as a graft in 20-30 % patients requiring a lower extremity bypass. Because more bypasses are now being anastomosed distally for limb salvage, only autogenous grafts are considered effective at such locations. Cephalic veins can be used as arterial grafts when no saphenous vein is available although arm veins may have a lower patency rate and higher incidence of dilatation and atherosclerosis compared to saphenous veins (Schulman and Bradley 1982).

Because of its excellent long-term patency, the internal mammary artery is currently considered the best choice for younger patients requiring an aortocoronary bypass. But when this artery is not available or not indicated, the use of right gastric artery or an intercostal artery is preferred.

I.3. Alternate Biologic Approach

In the absence of utilizable autogenous veins for small vessel reconstruction, numerous biologic grafts have been developed with variable success. In 1980, Carrel first reported the use of frozen and formalin preserved arterial xenografts as arterial substitute. The utility of this graft was limited by the supply and complications of aneurysm formation, rupture and thrombosis. Cryopreserved venous allografts have been performed well in animal studies with immunosuppressive therapy while in humans it has met with limited success (Selke *et al.* 1989). Dardik and Dardik (1976), introduced glutaraldehyde treated human umbilical veins for use as arterial substitutes with external Dacron reinforcement. Even though the performance of these graft in femoropopliteal bypasses were comparable to saphenous vein, they tend to form aneurysms beyond 3 year of implantation. Sawyer *et al.* (1986) have developed a negatively charged glutaraldehyde tanned graft that may have improved tensile strength compared to the previous biologic and umbilical vein grafts with equivalent patency rate to saphenous vein for over 7 yr in below knee bypasses in preliminary clinical evaluation.

I.4. Vascular Biomaterial

The motivation for synthetic vascular graft development probably arose in a frustration of attempts to treat expanding abdominal aneurysms in 1940s and 1950s. Subsequently, the development of materials to replace or bypass diseased arterial segments has facilitated substantial advances in vascular surgery over the last 40 years. Voorhees *et al.* first introduced porous cloth tubes as an arterial substitute in 1952. Five years later Edwards and Tapp (1957) fabricated bifurcated aortic grafts of braided nylon that were crimped to prevent kinking. Newer synthetic prostheses have enabled successful reconstructions in large diameter, high flow vessels such as the aorta and its primary intrathoracic and abdominal branches. In medium sized (< 6mm diameter) and small diameter arteries (<4 mm) bypass or replacement with long segments of textile grafts has resulted in short-term patency rates. Small vessel reconstructions presently rely on the use of autogenous veins for coronary artery and below knee femoropopliteal lesions and for micro-vascular repair of digits and cerebral vessels.

The desirable features of a vascular graft are numerous and include handling performance, durability and cost consideration. The prosthetic must be flexible yet resist excessive dilatation and simulate the visco-elastic properties of the vessel to which it is anastomosed. It must be sterilizable and resistant to infection. But the most important requirement is the flow surface should be maximally thrombo-resistant and allow generation of a thin pseudo-intima or complete spontaneous re endothelialization

As a result of the pioneering work of Voorhees and co workers (1952), which demonstrated the efficacy of using a synthetic textile tube as an arterial substitute, considerable research was expended during the 1960s in the quest to find the most suitable synthetic material. It soon became evident that the biostability of nylon, Vinyon N, Orlon, and Ivalon was insufficient for long term vascular grafting. Only polytetrafluoroethylene (PTFE, Teflon) and polyethyleneterephthalate (Polyester, Dacron) have been shown resistant to biodegradation *in vivo.*

The expanded form of PTFE is widely used as an arterial substitute on the femoropopliteal and axillofemoral positions. These grafts have good handling characteristics, are relatively kink resistant and need no pre-clotting before implantation. They are available in diameters ranging from 4 to 30 mm. When implanted above the knee, the results are encouraging and comparable to those obtained with the saphenous vein, whereas below the knee, the implantation of the e-PTFE is less successful. In spite of the positive clinical results, lack of compliance leading to anastomotic intimal hyperplasia, absence of luminal healing and thrombogenic nature under low flow are the disadvantages. Attempts to overcome these limitations such as modification of the internal surface to increase hydrophilicity, treatment with pyrolitic carbon and the addition of an external rigid spiral support etc. have led to inconclusive results.

Vascular prostheses, fabricated as polyester textile tubes are the most frequently used device in peripheral vascular surgery for the replacement of large and medium sized vessels. Long term results representing a follow up period of over 15-20 years have shown satisfactory results when Dacron grafts are implanted in the aortic and iliac sites. Technical developments to improve the device over the years led to the introduction of different designs like, light weight design, velor, woven or knitted and externally supported grafts. Woven Dacron has lower porosity than knitted grafts, does not bleed excessively at implantation and therefore does not require preclotting. Dacron grafts are usually crimped for flexibility and to prevent kinking across angled anatomy. Newer knitted Dacron grafts have filamentous velour construction on their outer and inner surfaces that are thought to facilitate tissue incorporation.

Additional materials have also been evaluated for fabrication of vascular graft especially to address poor patency rate of small diameter reconstructive surgery. Because of the improved thromboresistant property showed by polyurethanes used in intraaortic balloons and artificial heart, this material has got some attention. They also showed resistance to flex fatigue and are thromboresistant when used in acute experimental models (Leake *et al.* 1989). *In vivo* evaluation of polyurethane in vascular substitution resulted in inconsistent success. Polyurethane is susceptible to hydrolysis and oxidation of ester linkages *in vivo* as well as enzymatic degradation by inflammatory cellular response.

I.5. Bioresorbable Grafts.

The underlying principle of the bioresorbable type of graft is that after implantation the synthetic prostheses gets gradually resorbed and simultaneously replaced by the host's native tissues. Polyurethane, polyglycolic and polylactic acids and polydioxanone prostheses are being investigated for this application. Resorption of these biodegradable grafts are characterized by macrophage infiltration and phagocytosis of prosthetic material at a rate parallel to new tissue ingrowth.

Bioresorbable arterial interposition grafts of polyglactin and polydioxanone implanted in rabbits and canines elicit thicker and more cellular, myofibroblast laden inner capsule than do Dacron or PTFE grafts (Greisler et al. 1993). In small diameter arterial positions these composite grafts showed no aneurismal changes and had significantly better patency for up to 1 yr, compared to Dacron or PTFE grafts. The clinical efficacy of these grafts depends on a balance between rapid graft resorption and slower degradation rates required for structural stability. Requirement for extensive and fast tissue ingrowth before significant graft material is resorbed is a prerequisite to minimize aneurismal dilation. The most widely used technique is to compound the bioresorbable material with a nonresorbable material or to combine one or more bioresorbable materials so that the more rapidly resorbed material evokes a rapid tissue ingrowth while the second material provides temporary structural integrity to the graft. Totally resorbable composite graft woven from yarns of 75% PG910 and 26% PDS demonstrated a 100 % one year patency with no aneurysm in the rabbit aorta model (Greisler *et al.* 1988). The regenerated arteries withstood 800 mm Hg of pulsatile systolic pressure *ex vivo* without bursting and holding active non-thrombogenic endothelial cell layer. Van der Lei *et al.* (1987) evaluated grafts prepared from a mixture of 95% polyurethane and 5% polylactide and hypothesized that modification of the graft preparation including smooth muscle cell seeding may help to enhance optimal orientation of smooth muscle cell and prevent aneurysm formation. Though proved effective in animal models where tissue ingrowth and regeneration capacity is high, its clinical applicability in human beings still remains to be evaluated.

I.6. Modes of Graft Failure

The most common failure modes of synthetic vascular graft is thrombosis and pseudointimal hyperplasia. Less common failure modes include infection, generation of pseudoaneurysm and dilatation. The contributing factors of thrombus formation is complex and primarily mediated by biomechanical and bioelectrical potential of materials at blood - material interface, low flow conditions and limited spontaneous endothelialization in humans.

I.7. Blood Compatibility of Biomaterials

The consequences of the interaction of artificial surfaces with the blood may range from gross thrombosis and embolization to subtle effects such as accelerated consumption of haemostatic elements. Thus, thrombosis, thromboembolism, bleeding and infection are the major deterrents to the use and further development of advanced cardiovascular prostheses. In some cases the material's properties may not be the major determinants of the blood-material interactions. Flow parameters, compliance, porosity etc. may also be as important as the blood compatibility of the material itself.

Despite the multitude of attempts over the past several years to correlate material characteristics with relative degree of thromboresistance, the principles defining the properties that endow a material with the ability to resist thrombus formation are not well understood. The role of surface topography and porosity in thromboresistance also has not been well defined. However, there is compelling evidence that the surface physicochemical properties of the materials and devices influence early events such as protein adsorption and platelet adhesion. When placed in contact with blood, most of the artificial surfaces immediately acquire a layer of adsorbed proteins whose composition and mass may vary with time in a complex manner, depending on substrate surface type. Protein-surface reactions involve complex dynamic processes of competitive adsorption-desorption, denaturation and activation. A number of plasma proteins may be important for mediating cell attachment and cell-surface interactions. Fibrinogen and von Willebrand factor has been considered to be of particular importance in the cell adhesion process (Bailly *et al.*, 1996)

In many cases, material properties are constrained by the intended device applications. Thus fabric or porous materials are used to permit construction of anastamoses and tissue anchoring when vascular grafts are developed. The characteristics at the luminal surface of the vascular prostheses are often considered to be of primary importance in modulating the ingrowth and function of cells at the blood material interface. Because adsorbed proteins have different affinities for the graft surface, a low affinity interaction may result in desorption, allowing secondary adsorption of another protein of higher affinity. Circulating blood elements may then interact not only with the bare biomaterial surface but also with the adsorbed proteins. Surface characteristics of grafts, including chemical and physical properties, also contribute to protein adsorption. The initial relative concentration of proteins on the graft surface may have far reaching consequences because of a cascade effect of subsequent interactions. For example, the relative concentration of adsorbed fibrinogen is important, because its RGD region avidly binds the GPIIb-IIIa receptor complex on platelet membranes. Adherent platelets then are capable of releasing potent thrombogenic, chemoattractant, and mitogenic factors, which results in a subsequent cascade of events that may affect the eventual success or failure of the device

Platelet adhesion to the graft surface occurs rapidly and the rate of adhesion is proportional to the concentration of adsorbed fibrinogen (Roohk *et al.* 1976). Platelets also adhere to surfaces devoid of fibrinogen. Platelet adhesion results either from receptor mediated recognition of a binding domain on an adsorbed protein or from conformational changes or partial denaturation of a platelet membrane glycoprotein in response to graft surface. Graft surface texture and hemodynamic characteristics, including flow rate and shear stress, also modulate platelet deposition. Platelet aggregation in the presence of RGD containing proteins, such as fibrinogen proceeds rapidly by interaction with the platelet membrane glycoprotein receptor complex. Following adhesion, cytoskeletal reorganization and pseudopod formation occur. Subsequently, platelets degranulate with the release of bioactive substance from dense bodies and alpha granules. Degranulation results in increase in the local concentration of platelet agonists including seratonin, epinephrine and ADP as well as adhesive protein like fibrinogen, β-thromboglobulin, thrombospondin, von Willebrand factor and fibronectin. During this process, receptors for activated clotting factors are exposed which increases thrombin generation. Other products released include thromboxane A_2, which leads to vasoconstriction, intensive platelet aggregation and neutrophil adhesion. The platelet factor 4 released further increases platelet aggregation and inhibits circulating proteases. Platelet derived growth factor is strongly chemotactic and mitogenic *in vitro* for fibroblast and smooth muscle cells and has been implicated in the development of neointimal hyperplasia.

Platelet deposition and the previously described cascade of events appear to persist long after prostheses are implanted. One year following implantation of Dacron grafts, thromboxane B_2 release remained elevated and systemic platelets counts depressed in a canine model. (Ito *et al.*1990). Indium -111 labeled platelet-imaging studies confirms ongoing platelet deposition in humans as well. Obviously, this persistent activity may lead to graft failure.

Biomaterials may activate both classical and alternative complement pathways resulting in generation of C5a. (Chenoweth 1987). Dacron activates complement to a greater degree than e-PTFE (Sheppard *et al.*

1984). The generated C5a is strongly chemotactic for monocyte. Monocytes likely play a dominant role in both early and late tissue responses to implanted prostheses.

I.8. Cellular Events

Circulating polymorphonuclear leukocytes (PMNL) are attracted early after blood contact to the synthetic graft. C5a and Leukotriene B4 are among the potent chemoattractants, responsible for PMNL recruitment. PMNL products, including products of oxygen metabolism may retard endothelialization of blood contacting surface. Monocytes are attracted to the vascular graft soon after implantation. Macrophages are derived primarily from circulating monocytes that attract, adhere and progressively differentiate in to inflammatory monocytes, and finally activated macrophages. A few of the cells are also derived from resident macrophages present in the tissue surrounding the graft. The activated macrophages attempt to degrade and digest the foreign body. Many plasma derived monocyte recruitment factors are present as a result of the activation of complement and coagulation pathways and platelet activation in response to graft implantation.

I.9. Endothelialization of Grafts

Unlike in other species tested, the inability of human beings to spontaneously endothelialize the blood-contacting surface of currently available prosthetic grafts has been identified as a unique occurrence.

Transanastomotic pannus ingrowth results in endothelium residing on the surfaces within 1 cm of anastomoses to native vessels. Pannus ingrowth is a result of migration and proliferation of the endothelial cells of the adjacent artery. The two other potential sources of endothelium are transinterstitial ingrowth across the prosthetic wall or fallout of circulating endothelial cells or their multipotential primitive mesenchymal cell precursors (a purely speculative source) which have not been reproducibly demonstrated in humans.

I.10. Methods to Reduce Thrombogenicity

Despite the improvement in the graft design and development, no artificial surface currently available is free from the problems of thrombotic interactions between surface and blood and no artificial surface is truly nonthrombogenic. It is generally accepted that the thrombotic events are stimulated by and occur directly on the polymer surface through the initiation of the intrinsic coagulation cascade and the adhesion and aggregation of activated platelets. Subsequently, various efforts have been made to modify the surface of the biomaterial to improve their resistance to thrombosis and embolization, based on the knowledge from the cellular events of blood material interaction that mediate graft occlusion. These techniques may be separated into two major categories: 1. Physicochemical 2. Biological.

I.10.1 Physicochemical Modification

For the modification of polymer surfaces by chemical procedures, there are two basic approaches based on creation of a new surface that has altered blood protein adsorption characteristics or on creation of an activated surface for the subsequent or simultaneous immobilization of biomolecules.

The most common chemical surface modification technique has been radiation grafting to copolymerize selected monomer compositions. This may be followed by bonding of antithrombotic or fibrinolytic agents on to the reactive groups introduced in the graft copolymer (Hoffman 1981). Plasma gas discharge or radio frequency glow discharge RFGD is another important way to modify biomaterial surface (Yasuda & Gazicki 1982). Hydrophobic coatings composed of silicon and fluorine containing polymers as well as polyurethane has got some importance because of their good clinical performance in cardiovascular applications. Polymeric fluorocarbon coatings deposited from a tetrafluoroethylene gas discharge have been found to improve thromboresistance of small diameter Dacron grafts (Garfinkle *et al.* 1984). Polyurethanes have also been synthetically modified under different conditions in attempts to optimize the hard and soft segment ratio and composition at the polymer surface (Lelah and Cooper 1986). Hydrophilic coatings also have been popular because of their low interfacial tension in biologic environments. Polyethylene oxide coated surfaces have been found to resist protein adsorption and cell adhesion and therefore been proposed as potential blood compatible coatings (Mori *et al.* 1982).

I.10.2. Modification with Biological Molecules

Many different groups have studied immobilization of heparin, heparin analogues or heparin prostaglandin or heparin fibrinolytic enzyme conjugates (Kim & Feijen.1985). Heparin has been the first biologically active molecule immobilized on synthetic materials. Heparin has been electrostatically bonded through its anionic groups to the detergent benzalkonium chloride, which had been previously adsorbed on a graphite impregnated base (Gott *et al.* 1963). Novel polyaminoether urethane ureas containing tertiary amino groups in the side or main chains were synthesized, quaternised with different alkyl halides and finally heparinised (Yoshihiro *et al.* 1986). Since ionically bound heparin also desorbs easily from the surface when exposed to plasma, numerous investigators covalently immobilized heparin on to biomedical polymers by using different chemical procedures. Coating of two polyether polyurethane (Pellethane & Biomer) and polyethylene with a heparin–PVA hydrogel has been studied by exploiting the biological activity of heparin along with good hemocompatibility of hydrogels (Ramon and Sefton 1986). But in most methods the immobilized heparin showed a reduced biological activity compared to a solution of heparin as measured by a number of clotting tests and platelet adhesion studies indicating that the procedures significantly reduces the function of biological moiety.

Albumin coated surface have been studied because surfaces that resisted platelet adhesion *in vitro* were noted to adsorb albumin preferentially

(Kim *et al.* 1974). Munro *et al.* (1981) coupled alkyl chains of 16 and 18 carbon residues to polymers to enhance albumin binding to the surface and thus to improve blood compatibility. They demonstrated that albumin binding was substantially enhanced with C16 and C18 alkylation while fibrinogen adsorption was inhibited by C18 alkylation. Dacron vascular prostheses coated with albumin, followed by cross linking either with glutaraldehyde or carbodiimide to increase stability has been found to increase short term blood compatibility by inhibition of platelet adhesion and release (Kottk *et al.* 1989). Albumin has also been utilized with heparin by preadsorption of both molecules or by their covalent coupling which results in heparin albumin conjugate. Investigators have found that heparin albumin conjugate physically adsorbed onto different polymeric surfaces reduced both fibrin formation and platelet aggregation.

Prostacyclin is a potent endogenous antiplatelet agent. A method for preparing immobilized prostacyclin from immobilized prostaglandin F2 α was developed by use of a diaminoalkane spacer arm interposed between the polymer surface and the immobilized prostaglandin (Ebert 1982). This material has been found to inhibit both platelet aggregation and platelet adhesion. Later prostaglandin E1 was also utilized, covalently bound to heparin, to provide the dual pharmacological role of decreasing the extent of platelet aggregation and fibrin formation. But these polymers can be used for only short-term blood compatibility applications.

Fibrinolytic enzymes and various prostaglandins have also been immobilized by various techniques to enhance fibrin dissolution and resist platelet aggregation at the surface (Kim & Feijen 1985). The mimicry of biologically inert surfaces (Hayward and Chapman 1984) is another strategy for the development of blood contacting materials that utilizes the introduction of chemical groups. Since phosphorylcholine –containing phospholipids represent approximately 90% of the total lipids present on the surface of the plasmamembrane of blood cells, a surface layer of phosphorylcholine was chemically linked to the material surface exhibiting characteristics of a biomembrane. Incorporation of microspheres containing NO releasing compounds within the PTFE grafts (Sharon et *al.* 1998) has also been investigated to reduce platelet adhesion as well as activation. Methods to improve the spontaneous endothelialization of the graft after implantation are mainly concentrated around immobilization of growth factors like ECGF and b-Fibroblast Growth Factor along with the graft (H.P.Greisler *et al.* 1993)

Various techniques such as alkylation, plasma discharge and application of thin polymer films have been utilized for impregnation of albumin on the vascular grafts. Knitted Dacron prostheses coated with albumin, gelatin and collagen are currently available for clinical use. The graft protein coating minimizes porosity thereby eliminating the need for preclotting. Unlike albumin coating the efficiency of other protein coating in reducing platelet adhesion and subsequent thrombogenicity still remains inconclusive.

Providing biomaterial surface with a biologically active compound eventhough offers numerous possibilities of altering blood -biomaterial interface, the bioactivity of the molecule after processing, and questions concerning long term efficacy of the molecule to resist thrombus formation, still remains as a unexplored aspect. So most of the techniques are considered to be having only short-term application just like heparin bonded material.

I.11. Tissue Engineering using Endothelial Cells

I.11.1. Endothelial Cell: The Natural Blood Container

Endothelial cells are actively antithrombotic and provide best local environment for preventing thrombosis. The endothelium is a confluent monolayer of thin, flattened rhomboid shaped cells lining the intimal surface of all blood vessels and, thus is situated at the vital interface between the circulating blood and the host's body tissue. Endothelial cells can sense changes in the mechanical, chemical and humoral environments, process these signals and respond by the synthesis and release of a myriad of factors (Ryan *et al.* 1992). It is instructive to regard endothelium as a dynamic balance, in which opposing functions are strictly controlled under physiological conditions, but which may be altered to such an extent that a pathological event may result. There are at least four important physiological functions attributed to the endothelium, which involve haemostatic control, vascular tone modulation, growth regulating signals and a central role in regulation of inflammatory response.

The endothelium is nature's most efficient antithrombogenic surface, the maintenance of which depends on the production of numerous factors, acting either as anticoagulant or as promoters of fibrinolysis. These include the synthesis of potent antiplatelet factors, such as nitric oxide (NO) (Radomski *et al.* 1987), prostacyclin, PGI2 (Czervionke et *al.*, 1979) thrombomodulin, heparan sulphate proteoglycan (Keller *et al.* 1987) as well as plasminogen activator of tissue and urokinase type (t-PA and u-PA). Although under physiologic conditions the antithrombogenic activity of EC predominates, procoagulant activity can rapidly be induced by proinflammatory cytokines or bacterial toxins (Nawroth *et al* 1986). Tissue factor is one of the most important endothelial procoagulant factors (Ruf *et al.* 1994). In addition, through the production of plasminogen activator inhibitor–1 (PAI-1), the endothelium can prevent the initiation of the fibrinolytic cascade. Further prothrombogenic products are the coagulation factors V and VIII as well as the receptors for the factor IX and X. The endothelium acts as an important regulator of vascular tone by the production of both vasodilating and vasoconstricting agents. NO, the most potent vasodilator ever known and the PGI_2 are the key players in the maintenance of vasodilator activity, which is of vital importance especially in the arterial tree. Endothelin –1 is the most important vasoconstricting factor synthesized by the EC. (Yanagisawa et *al.* 1988)

I.11.2. Endothelial Cell Seeding: A Historical Perspective

Tissue engineering is a multidisciplinary science that utilizes basic principles from engineering and life sciences to construct tissues from their cellular components (Vacanti& Vacanti,1994). Endothelial cell seeding was the first attempt to implement principles of tissue engineering for the creation of EC coverage on the otherwise nonhealing prosthetic vascular grafts. Although techniques to isolate EC have been available for some time, the concept of EC transplantation on blood contacting surface to reduce surface thrombogenicity was first of all put forward by Malcolm Herring (1978). In the landmark report, they isolated canine venous EC from external jugular veins by mechanical disruption of cells using a wool pledget. These cells were subsequently mixed with whole blood used to preclot porous, 6 mm internal diameter, Dacron prostheses and the grafts were implanted in the infrarenal aorta. The grafts were evaluated at 4 weeks postoperatively. The mean thrombus free surface area of seeded graft was 76% compared to 22% for nonseeded grafts and the glistening luminal surface of seeded grafts histologically resembled endothelium.

Later many laboratories have made intensive efforts to optimize the methods employed in harvesting, growing and seeding of EC and enhancing the attachment and proliferation of seeded cells on the graft surface. Two procedures have been developed to create endothelialized surface. The first approach involves isolation of EC and incorporation within a clot on the surface or direct seeding onto the surface. The density of cell is generally low, necessitating the proliferation of endothelium to create a confluent cell monolayer after implantation. This is unlikely to happen in the highly undefined matrix without enough maturation of the cytoskeletal elements of the few cells attached in the flow surface.

An alternate procedure is the establishment of an endothelial cell monolayer on a vascular graft prior to implantation. This procedure is known as sodding or *in vitro* endothelialization, which differs from seeding technologies in several important respects. The seeding procedure utilizes minimal cell numbers usually isolated by mechanical scraping within the operating theatre, which are placed within plasma or blood and preclotted on the graft. The resulting luminal surface exist as a highly thrombogenic fibrin meshwork.

But the sodding technique involves isolation and culture of cells within the tissue culture laboratory to attain enough cells to completely cover the vascular prostheses followed by seeding the cells after proper precoating to enhance cell attachment and growth. So at the time of implantation the luminal surface presents actively non-thrombogenic cell monolayer with enough cytoskeletal maturation to resist blood flow.

I.12. Review of Literature

I.12.1. Endothelialized Grafts

Malcolm Herring (1979) studied the proliferation of seeded EC on 14 different designs of prosthetic grafts including Dacron, Teflon, Orlon and polyurethane backed grafts and concluded that weft-knit Dacron grafts were most suitable for EC adhesion than Teflon. Later Herring and Graham (1980) enzymatically isolated EC from canine venous segments by sequential incubation with trypsin and collagenase and seeded the cells on 6 mm double Velour Dacron graft either immediately after isolation or 14 days after *in vitro* cultivation and used as thoracoabdominal bypass grafts.

A variety of techniques have since been employed to hasten the development of confluence of seeded EC. Capillary endothelial cells are available in large number and have been utilized for transplantation on to graft surfaces by Jarrel and colleagues (1986). They isolated EC from adipose tissue by collagenase treatment followed by purification in percoll gradient centrifugation and cells were seeded on the Dacron grafts. 50-100% of the seeded cells were capable of resisting shear stress ranging from 0-80 dynes /cm^2. However, of concern was the potential fibroblast contamination resulting in fibroblast overgrowth mediated myointimal hyperplasia. Sterpetti *et al.* (1988) compared the efficacy of endothelial cell seeding with jugular vein endothelial cell versus omental microvascular endothelial cells on to e-PTFE grafts and implanted in to 25 canine aortas. Five weeks after implantation, thrombosis free surface area in both the groups were significantly greater than that found in unseeded e-PTFE controls. But they noted significant thicker subendothelial layer in the microvascular group. These investigators concluded that microvessel endothelial cells could be successfully seeded on the vascular grafts but that optimization of the endothelial cell procurement method is critical.

I.12.2. Adhesive Proteins in EC Attachment

Williams *et al.* (1985) reported experiments evaluating the compatibility of adult human endothelial cells derived from iliac veins with both Dacron and PTFE *in vitro*. They concluded that the adhesion on uncoated grafts were very poor while the adherence increased dramatically on grafts treated with extracellular matrix, plasma or fibronectin. Thus the effect of fibronectin on endothelial cell attachment to prosthetic vascular grafts has received considerable attention.

The kinetics of endothelial cell adhesion to biomaterial surfaces and retention by the surface in flow condition was studied by Rosenman and colleagues (1985). An average of 19.8% of the isolated cells were adherent immediately following seeding. Thirty minutes after restoration of circulation 70.2% of the adherent cells desquamated from the surface. Further cell loss occurred through 24 h, at a rate of 3.7% / h with no significant additional loss. Thus only 4.4 % of initially harvested cells remained on the surface after 24 h, in circulation. Following this disturbing report a large number of investigation was carried out to optimize conditions of cell adhesion, cell retention and cell proliferation following seeding. Foxall *et al.* (1986) seeded adult human saphenous vein endothelial cells on to e-PTFE and Dacron grafts with and without collagen and fibronectin treatment.

Cells adhered more readily on to protein treated surface than on untreated Dacron and PTFE, and proliferated well. When the two biomaterials were compared, a confluent monolayer was achieved within 9 days on e-PTFE, but significantly less cells grew on Dacron surface. In an effort to increase the initial adherence and retention of seeded endothelial cells, Ramalanjaona *et al.* (1986) pretreated e-PTFE surfaces with fibronectin and resulted in increase in adherent endothelial cells with low mean cell loss following restoration of blood flow.

Kesler *et al.* (1986) pretreated both e-PTFE and polyester elastomer surfaces with fibronectin prior to seeding with Indium Oxine labeled human umbilical vein endothelial cells and found that fibronectin led to an increased cell retention when exposed to 15 dynes /cm^2 shear stress but not the initial cell adhesion on either surfaces. Patterson *et al.* (1989) compared the seeding efficiency onto e-PTFE grafts coated with either fibronectin or Matrigel, a commercially available basement membrane which contains laminin, Type IV collagen, heparan sulfate, proteoglycan and entactin. Results demonstrated no difference in seeding efficiency due to these two materials.

Kaehler *et al.* (1989) later compared human endothelial cell attachment to e-PTFE surfaces pretreated with fibronectin, laminin, Type I/III collagen and fibrin glue. Cell adherence and cell spreading were both superior on surfaces pretreated with either the combination of fibronectin and Type I/III collagen or with fibrin glue. However, concern regarding fibrinolysis led these authors for recommendation of fibronectin plus type I/III collagen pretreatment.

Basis of employing the above plasma protein to enhance endothelial cell attachment is the presence of a particular aminoacid sequence Arg-Gly-Asp that serves as the primary binding site for the transmembrane integrin receptors of the EC. *In vitro* studies have shown that EC adhesion can also be improved by directly grafting these peptide sequences on the graft surface. Recently, Bhat *et al.* (1998) introduced a heterogeneous ligand system, which utilizes two adhesion mechanisms addressing both firm initial attachment and rapid cell spreading. The system consist of a extrinsic high affinity receptor –ligand system involving avidin –biotin for specific rapid and firm initial attachment of cell membrane to the graft surface and an intrinsic lower affinity integrin dependant receptor ligand system necessary for cell spreading and actin filament assembly.

A heterogeneous ligand system on glass surface consisting of both avidin-biotin and fibronectin integrin attachment mechanism significantly increased the strength of initial attachment of bovine aortic endothelial cells compared to homogeneous ligand treatment with either avidin–biotin or FN alone. This group has found that HUVEC retention on the heterogeneous ligand treated graft surfaces is significantly higher compared to homogenous ligand treated surfaces for shear stress in the range of 10-30 dynes /cm^2. By this method, the initial EC retention in the small vascular grafts could be increased to greater than 80% compared to 40 % retention rates with FN treatment alone.

I.12.3.Shear Stress Resistance and Thromboresistance of Transplanted EC

The next stage of the development of tissue engineering technology, for vascular conduit has been the evaluation of efficacy of the transplanted cells to resist shear forces of blood flow and thrombosis. Many investigators have conducted experiments to identify the biologic and physiologic mechanisms by which seeding might improve vascular graft performance. Clagett *et al.* (1982) reported platelet serotinin level and normalization of platelet survival times in dogs with endothelial cell seeded grafts, as well as significant differences in luminal surface production of 6 keto PGF 1 α between seeded and nonseeded grafts. But Sicard (1984) did not find a difference in PGI 2 levels between seeded and non seeded vascular grafts but did report that seeding lessened thromboxane A_2 production by the walls of seeded grafts compared to non seeded controls. The suggestion was that an alteration in the ratio between PGI_2 and thromboxane might be responsible for the reduction in platelet deposition onto seeded grafts. But the effect of prostaglandin biosynthesis and its role in reduced thrombogenicity of seeded vascular graft still remains controversial and the subject of ongoing research.

Once adherent to a surface, seeded endothelial cells must resist desquamation in shear stress conditions. Sentissi *et al* (1986) evaluated endothelial cell retention to collagen and fibronectin pretreated e PTFE grafts using bovine aortic endothelial cells labeled with indium Oxine. Seeded grafts were exposed to either 25 ml/min or 200 ml /min flow rates for 60 min *in vitro* and luminal surfaces were evaluated histologically and the retained radioactivity quantitated. Results demonstrated a relatively insignificant loss of labeled endothelial cells during flow and this loss did not significantly differ between high flow and low flow. Rupnick *et al.* (1989) subjected woven Dacron grafts seeded at high density with microvascular endothelial cells to shear stress ranging from 0-20 dyne /cm^2 and found a direct relationship between shear stress and cell desquamation.

I.12. 4. Clinical Studies

Compared to *in vitro* and experimental animal studies, only a few clinical trials have been undertaken. The first clinical trial of ECs of vascular prostheses was reported by Herring *et al.* (1984). In their study mechanically harvested EC-seeded grafts were evaluated in 161 patients and noted improved patency rates but the results were discouraging if the patient happens to be a smoker. Herring *et al* (1987) later reported 2 years patency of seeded PTFE grafts 73.9% of which was not significantly different from patency of vein grafts in a concurrent patient population. Zilla *et al.* (1989) also reported results from a series of 18 patients undergoing seeded PTFE femoropopliteal bypasses. An average of 3.1×10^3 cells /cm^2 were seeded and patients were monitored by a series of platelet functional studies and concluded that endothelialization in the seeded graft was only minor. Fasol *et al.* (1989) evaluated the platelet activation with seeded and non seeded e PTFE graft and found significantly higher platelet activation indices in patients with either seeded or non seeded grafts compared to saphenous vein graft.

Ortenwall *et al.* (1989) used preclotted seeded femoropopliteal PTFE grafts either for the proximal or distal half of the vessel. In 23 patients, the mean thrombogenicity index was significantly reduced in seeded graft segments at 1 and 6 months after implantation while 5 grafts failed to show a response to seeding. Thus the future of endothelial cell seeding on to currently available vascular grafts clinically remains an open question.

Eventhough, 25 years of extensive research in the field has culminated in several clinical trials, the disappointing outcome from some of the initial clinical studies led to the early retreat from the refined idea which has got significant enthusiasm from the clinicians initially. This forced most surgeons to avoid procedural complexities associated with the technique to opt the single stage graft implantation. Some clinical studies did not show improved results with seeded grafts while some reported partially favorable results. It seems obvious from these experiments that the endothelial cell seeding efficiency has to be improved further in all the stages including initial isolation, adhesion of EC on graft surface, their proliferation in *in vitro* environment and finally their efficiency to resist shear stress as well as thrombus formation in contact with blood.

If the mere presence of a confluent endothelial cells on the surface of the blood-contacting surface were enough to address the thromboresistance, the problem would already have been resolved. It is clear that the endothelial cells are not inert and are capable of producing a variety of prothrombotic as well as antithrombotic factors depending on the nature of the surface on which it is growing, and the conditions it is exposed to. The regulation of endothelial cell physiology is a complex interplay of cell-cell, cell-matrix, cell-humoral factors, and cell- hemodynamic interactions. Moreover, the technique of successful transplantation of endothelial cell involves multistage processes starting from isolation and each stage involves a lot of variability and complexity. So optimization of cell harvest, cell growth, cell attachment and proliferation on the material surface without appreciable change in cell physiology and ability to resist blood flow are the important prerequisites to the success of this technique.

I.12.5. Current Strategy and its Drawbacks.

Inorder to optimize the cell attachment on the material surface the requirement of an adhesive protein is imperative. The adhesive proteins so far tried out are fibronectin collagen, vitronectin, whole extracellular matrix, and eventhough these proved to be effective in initial cell adhesion, the role of these adhesives to platelet adhesion and activation turns out to be a demerit. This is because in case of inadequate endothelialization, the substrate-coated surface gets exposed to flowing blood, and the platelet dependant vascular occlusion is likely to take place. Even if at the time of implant, a monolayer is established, one cannot rule out the possibility of derangement of endothelial layer once exposed to flow leading to exposure of the adhesive covered area to blood. Lower proliferation of cell on some adhesive like FN is another adverse point. Even though, precoating of PTFE with high concentration of (5 mg /ml) FN improved the adhesion of HSVEC, cell spreading remained incomplete and with time all the initially adhered cells detached. This may be due to insufficient adsorption of FN. The low affinity of FN for Dacron or Teflon has been reported (Van Wachem *et al.* 1987). Kesler *et al.* (1986) found that coating of polyester elastomers with the adhesive protein, fibronectin of and PTFE did not increase initial attachment of EC but did increase significantly, the percentage of inoculums retained after perfusion in *in vitro*. Subsequently, only few researchers have tried to rectify the complexities involved with the smooth transfer of this technique to practical reality.

I.13. Hypothesis

To make the concept of endothelial cell seeding a practically viable technique, it requires further improvements in all stages of the approach starting from the cell isolation to implantation of endothelialized graft. The actual number of cells available for seeding is low and to make a confluent monolayer on the surface, maximum cells needs to be attached. This also necessitates the *in vitro* culture with high level of multiplication potential to avoid unnecessary waiting time, for the patient.

First of all, a method has to be standardized to get enough cells from the small sized vessel of the patient, and get it multiplied for complete coverage of the vascular graft, in limited time. Next, a reliable and reproducible technique of coating of the graft with adhesive protein matrix to improve cell retention and growth on the biomaterial has to be developed. Such substrate should support adequate cell attachment and proliferation, should be non-thrombogenic by itself and the cells seeded should have enough potential to resist the forces of shear stress. Only if the composition of the substrate coating is suitable for growth of physiologically normal cells, the grown monolyer can be blood compatible and the patency of the conduit can be maintained.

Therefore, standardization of such a matrix composition, and uniform and stable coating of the matrix, and demonstration of the non-thrombogenicity of the coated surface, adequate cell attachment and proliferation on the matrix, demonstration of the non thrombogenic, shear resistant nature of the cells form the major aims of this thesis. Inorder to achieve these objectives, fibrin mixed with gelatin is chosen as the adhesive matrix, while coating the surface with the matrix proteins, immobilization of endothelial cell growth factors is also planned. The retention of GF along with a matrix may have better potential because when the peptides are bound to a substrate, its degradation by proteases might be reduced, and local concentration of the GF available for cells to consume can be increased.

I.14. Fibrin Tissue Adhesive-Prospective Matrix

I.14.1. Composition of Fibrin Glue

Fibrin glue is the concentrated version of fibrin that is formed as a result of physiological blood coagulation that arrest bleeding. This soft tissue adhesive is prepared in a two component form as fibrinogen (I) and thrombin (II). The transformation of fibrinogen into fibrin gel involves enzymatic cleavage and polymerization reactions. These enzymatic reactions are thrombin dependent, which cleaves fibrinopeptides A and B from fibrinogen to form fibrin monomers. Thrombin also activates factor XIII, which is present in the fibrinogen concentrate, into a transaminase (F-XIII a), which in turn catalyzes polymerization of fibrin monomers to form protofibrils. Lateral association of these protofibrils results in branching and formation of three dimensional net works. The latter structure when formed in high proportion, exhibits tissue compatibility, optimal haemostatic and healing properties, elasticity and relatively high adhesive strength. The plasma components associated with fibrinogen comprise fibronectin and plasminogen, which are essential for tissue attachment and biodegradation and wound healing.

Fibrinogen and associated proteins can be isolated from human plasma using cryoprecipitation without any chemical intervention, by critically controlled thermal schedules and centrifugation. Techniques are also well developed that permits preparation of fibrinogen concentrates from autologous plasma, the use of which will prevent viral disease transmissions. The concentrated fibrinogen can be mixed with commercially available bovine thrombin, in 25 mM $CaCl_2$ solution, to form a stable adhesive gel.

I.14. 2. Properties of Fibrin Glue

I.14.2.1. Physical

Fibrin is distinguished from fibrinogen, chiefly by its insolubility under physiological conditions. Electron micrographs show an end-to-end and side-to-side alignment of fibrin units in a clot. The corresponding bands have a periodicity of about 230A°. Due to cross-linking, insoluble fibrin formed in the presence of factor XIIIa cannot be dissolved in urea and other similar solvents. Fibrin in the dry form is stable below 170°C, and requires no special conditions of storage.

I.14. 2.2. Biological.

With respect to toxicity, fibrin is a safe material. Fibrin breakdown products are well established as being toxicologically innocuous. The biodegradation of fibrin products has also been well studied, and the elimination of fibrin from the organism is partly an enzymatic(fibrinolysis), and partly a cellular process (phagocytosis). Various resorption rates have been found for different fibrin products ranging from a few days to several months. A bactericidal effect of fibrin also has been reported.

I.14. 3. Fibrin Glue- A Natural Scaffold for Tissue Regeneration

Apart from a haemostatic function, fibrin is believed to promote wound healing. Accelerated healing was reported after applying fibrin powder on wounds, and fibrin has been thought to have nutritive value in the healing process. The coagulation pathway generates numerous vasoactive mediators and chemotactic factors, which together recruit inflammatory leukocytes to the wounded site. The role of deposited fibrin in the formation of normal granulation tissue is well described. Fibrinopeptides formed during fibrinogen to fibrin conversion, acts as a chemotactic agent for PMNs. Fibrin degradation products formed by proteolytic digestion of fibrin net work, stimulate the migration of monocytes, which convert to macrophages. Stimulated fibroblasts migrate through the fibrin gel structure and deposit collagen. By about a week after injury and clot formation, the wound clot would have been fully invaded and replaced by activated fibroblasts that are stimulated by growth factors to synthesize and remodel a new collagen rich matrix. In other words, fibrin acts as a scaffold promoting the ingrowth of cells.

Fibrin deposition is a consistent early event in solid tumors and healing wounds and precedes new blood vessel ingrowth in both. Harold F. Dvorak

(1989) *et al.* 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 fibrin gel deposited in tumors and wounds serves as a provisional matrix that over a period of time becomes organized in to immature, highly vascular connective tissue or granulation tissue. Generation of granulation tissue requires the coordinated activation and migration of vascular endothelial cells, macrophages and fibroblasts into the fibrin gel with the resulting new blood vessel formation and deposition of interstitial collagen. The factors regulating fibrin deposition in tumors and wounds are now fairly well understood (Dvorak , 1985).

Fibrin based matrices are involved in peripheral nerve repair, and can be introduced into the nerve gap to enhance regeneration efficacy as it can be measured by the number of axons and rate of axon growth. Matrix properties such as degradability, porosity and stiffness are related to fibrin micromorphology and in principle could affect tissue regeneration.

I.14.4. Advantages of Fibrin Sealant in Endothelial Cell Seeding

For tissue engineering applications this wound healing matrix provide a three dimensional scaffold which can serve as a substrates for cell adhesion and migration. Schrenk et al. (1987) reported that the use of fibrin glue is superior to the use of blood preclotting and that an increased number of cells adhered to the graft surface in the early period of the seeding. Mazzucotelli et al.(1991) found that coating of PTFE or Dacron vascular graft with biological sealant transglutaine which contains other adhesive protein in addition to fibrinogen and v WF significantly improved endothelialization *in vitro*. But Zilla *et al.* (1987) noted apparent cell loss from fibrin glue coated endothelialized vascular graft 2 days after exposure to flow due to fibrinolysis. Some other workers have noted poor proliferation of EC on two commercially available fibrin Tissucol and Beriplast . The variability in results with different fibrin may be due to different composition or different method of formation of the gel because it is known that the integrity of clot formed is dependant on various factors like concentration of the components fibrinogen and thrombin, presence of calcium and other factors. The cell proliferation may also be affected by the migratory capacity of the cell after initial adhesion. In a clinical trial, fibrin coated PTFE grafts were lined with autologous EC and implanted at confluency in the femoropopliteal position. While no cells attached to the graft in 27 % of the attempts, a three year followup revealed a significantly improved patency of these grafts as compared to control non lined grafts (Zilla *et al.* 1991).

Rubens *et al.* (1992) demonstrated that platelet accumulation on thermally denatured fibrin surface was significantly decreased as compared to native fibrinogen The thrombogenicity of fibrin still remains as a matter of controversy and it is well known that the polymerizing fibrin is highly reactive to platelet compared to completely polymerized fibrin. Although the involvement of GPIIb/IIIa is always recognized, there is some disagreement in the literature regarding the state of platelets that is required for their adhesion to fibrin. Hantgan *et al.* (1985) stated that platelet activation is essential for these cells to bind to polymerized fibrin fibers, while Jens *et al.* (1991) have suggested that fibrin interacts not only with activated platelets but also with resting platelets. If unreacted thrombin is entrapped within the fibrin it is likely to enhance platelet accumulation by the activation of circulating platelets. This may be the reason behind variable results published so far. Hence, the method of preparation of fibrin and its coating technique remains critical in determining the thrombogenicity.

I.15. Endothelial Cell Proliferation and Growth Factors

Endothelial cells are intimately involved in a vide range of biological and pathological processes. Generation of a vascularised network requires angiogenesis, which is controlled by autocrine and paracrine signals in a natural setting. In the absence of a stimulus the endothelial cell layer exists in a quiescent state with a turnover time exceeding thousands of days (Folkman, 1996). Currently, the growth factors like vascular endothelial cell growth factor, fibroblast growth factors, platelet derived growth factors, transforming growth factor- β and osteonectin are found to have high level stimulatory effect on endothelial cells. The growth factors are extracellular proteins present in minute concentrations *in vivo* that activate target cells through receptor binding. Normally, angiogenic growth factors possess the ability to induce neovascularisation *in vivo* and can typically be classified in to four families viz. epidermal growth factors, fibroblast growth factors, platelet derived growth factors and transforming growth factors. From endothelial cells two VEGF receptors have been isolated and characterized; Flt-1 and Flk-1. Both the receptors are transmembrane tyrosine kinases. EC ingrowth, whether trans-interstitial for graft implantation or from the adjacent uninjured artery is regulated by complex interactions with growth factors and cytokines released by the cells and extracellular matrix within the local microenvironment. Among these the potent EC mitogens of the FGF family, FGF–1 and FGF–2 are the best characterized. At least 4 high affinity and one low affinity FGF receptors have been so far identified. The mitogenic activity of FGF-1 for EC's is potentiated by heparin, which additionally protects it from proteolytic degradation by circulating proteases.

The impregnation of vascular grafts with therapeutic agents contained within controlled delivery vehicle has got some attention to improve spontaneous endothelialization. Greisler *et al.* (1993) used a double sandwich technique, first affixing human plasma fibronectin to both Dacron and PDS graft surfaces at concentration of 10 μg/ cm^2 followed by heparin 20μg/ cm^2 and FGF –1. The release kinetics shows that the Dacron retained 43 % and 25% of its applied GF at 7 and 14 days respectively. But the study has not evaluated the functional state of the GF retained for enhancing the proliferation of cells.

I.15.1. Human Umbilical Vein EC as the Model.

There have been numerous efforts to isolate and culture endothelial cells from both animal and human tissues in order to investigate functional properties and variations of these cells in biology and pathology. Mostly human umbilical vein endothelial cell has been used and it has two major advantages: 1. The vein is easily available, 2. The growth rate is usually high. Once a homogenous cell isolate is obtained by enzymatic digestion, the major challenge is to get a good proliferation rate. The medium is often supplemented with whole human serum, which results in optimal growth at a serum concentration as high as 50%. However, a good growth rate in EC culture is attained by exogenous mitogens isolated from hypothalamus or pituitary glands of other animals, or tumour conditioned medium and show maximal growth when heparin is also added (Folkman et al 1979). When the culture medium is supplemented with growth factors isolated from bovine hypothalamus and pituitary glands, a good growth curve was observed by many workers.

Now, knowing the importance of the adhesive matrix that should be immobilized on vascular biomaterials for getting tissue engineered, blood compatible conduits immobilization of growth factors along with the fibrin adhesive is planned. The ability of the matrix to retain the growth factors for upto 72h should generate a local microenvironment with adequate concentration of mitogenic factors required for cell migration, and proliferation on the matrix. Since the fibrin has a porous microfilament nature, retention and controlled release of growth factors by simple diffusion become practical.

I.16. Objectives

In the above elaborated background, the main objectives of this study are

1. Standardization of a matrix protein composition to improve endothelial cell adhesion and proliferation.
2. Development of a coating technique to make ready to use blood compatible materials.
3. Establish stability of the matrix formulated and coated on the surface in the presence or absence of cell.
4. Evaluation of the thrombogenicity of the matrix coated surface by platelet adhesion and activation studies.
5. Standardization of cell isolation from human umbilical cord vein, and culture conditions.
6. Characterization of the monolayers using specific marker molecules.
7. Comparative evaluation of cell adhesion on matrix coated as well as uncoated vascular biomaterials.
8. Improve proliferation of cells by growth factor immobilization along with the matrix, and establish the effective retention of GF in the matrix for the required multiplication period.
9. Establish normal physiological status of the monolayer, by NO assay and the ability of monolayer on the matrix to repair mechanical damage.

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8. Improve proliferation of cells by growth factor immobilization along with the matrix, and establish the effective retention of GF in the matrix for the required multiplication period.
9. Establish normal physiological status of the monolayer, by NO assay and the ability of monolayer on the matrix to repair mechanical damage.

10. Evaluation of EC monolayer grown on different substrates to resist the forces of shear stress *in vitro*.
11. Study of thromboresistance of the monolayer by platelet adhesion and activation.
12. Study of cell attachment and growth on different vascular biomaterials, and the shear stress and thromboresistance.
13. Standardization of cell number to surface area ratio, w.r.t. time taken for monolayer formation using adult human saphenous vein EC.

CHAPTER II

MATERIALS AND METHODS

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II.1. Endothelial Cell Isolation, Culture and Characterization

II.1.1. Purification of Endothelial Cell Growth Factor (ECGF)

Crude endothelial cell growth factor was prepared from bovine hypothalamus according to the method of Maciang *et al.* (1979). Bovine brain was removed aseptically and the hypothalamus was cut into small pieces of 1 cm^2. The pieces of brain was homogenized for 3 min in 0.1 M NaCl in an ice cold homogenizer with ~500 ml of NaCl. The homogenate was stirred for 2h at 4°C, maintaining pH at 7. The homogenate was centrifuged subsequently for 40 min at 13800g and 4°C in a refrigerated centrifuge (Hitachi, SCR20BA). The supernatant was mixed with streptomycin sulfate to get 0.5% (w/v) final concentration and incubated at 4°C for 4 h to extract lipids. After repeat centrifugation at 13800 g for 40 min, the supernatant was dialyzed against 0.1 M NaCl for 16h at 4°C. The dialyzed solution was centrifuged, freeze dried in a lyophilizer (Modulyo 4K Freeze Dryer, Edwards, UK) and stored at –20°C for further use.

II.1.2. Analysis ECGF

Total protein content of the sample was estimated according to Lowry's method (Lowry 1951). 20 μg of sample after digestion in sample buffer containing DTT and SDS, was subjected to Polyacrylamide Gel Electrophoresis in 12% gel as per the method of Laemmli UK (Laemmli UK, 1970). Commercially available endothelial cell growth supplement (product No.E-2759, Sigma Chemicals, USA) and high molecular weight markers (Sigma Chemicals, USA) were run in parallel lanes for comparison.

II.1.3. Human Umbilical Cord Collection

Human umbilical cord was collected in ice cold Ca^{2+} and Mg^{2+} free Hank's Balanced Salt Solution (HBSS) containing antibiotics (1000U/ml of streptomycin and 1000μg/ml of benzyl penicillin) (Annexure 1). Both distal and proximal ends were tied with sterile silk just before removing the cord from the placenta to ensure sterility within the lumen. The vein was brought to the laboratory within 2h of collection. All the veins for this study were collected from the Obstretics Department of Medical College, Trivandrum.

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

Human Umbilical Cord Vein Endothelial Cell was isolated as per the method of Jaffe *et al.* (1973) with slight modification. The umbilical cord vein collected immediately after child birth as in §II.1.3 was brought to tissue culture laboratory and all the subsequent procedures were done under laminar flow. The cord was washed with fresh HBSS and then the umbilical vein was cannulated with a custom made stainless steel cannula or 16 G i.v cannula and secured with a 3-way stopcock. The lumen of the vein was washed thoroughly, with ample quantity of sterile HBSS with a syringe via the 3-way stopcock, to make free of blood. Subsequently, the vein was filled with 0.2% type-I collagenase (Sigma Chemicals, USA, Product No.C6885) in Medium 199 (Annexure-1) by closing the 3-way at both ends. After 15 minutes incubation at 37°C the surface of the cord was gently massaged to dislodge the digested cells. The dislodged cells with the enzyme solution were collected in a sterile 50 ml centrifuge tube (NUNC) using a syringe through the 3-way. The lumen of the vessel was flushed with additional 20 ml of M 199 to collect loosely attached cells and added to the initial cell suspension. The cells were washed twice by centrifugation at 200 *g* for 5 min in a tabletop refrigerated centrifuge (Haeraeus, Biofuge Stratos). The final cell pellet was resuspended in complete medium RPMI 1640 or MCDB 131(Sigma chemicals ,USA) containing 20% New Born Calf Serum (NBCS, prepared in the laboratory as per Annexure 1), 150 µg/ml ECGF, heparin sulfate (100U/ml), streptomycin sulfate (100µg/ml), strepto-penicillin (100U/ml) and amphotericin (2.5µg/ml). The cell suspension was then seeded on gelatin coated 25cm^2 culture flask (NUNC) at a density of 2x10^4/cm^2 and incubated under 5% CO_2 at 37°C in an incubator (NARCO 5100, USA). The culture was fed with fresh medium every second 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 surgery was collected in HBSS and used for EC isolation. The luminal side of 2-4cm long vein was flushed with 2-5 ml of serum free RPMI medium. Then the lumen was filled with 1ml of 0.2% collagenase in serum-free medium for 20min at 37°C. After gentle massaging, the dislodged cells were flushed with additional 5ml of medium and washed two times in complete medium by centrifugation as in §II.1.4. The final cell pellet was resuspended in complete medium containing 20% human serum (Annexure 1), 200μg/ml of ECGF from hypothalamus and seeded into two of 24 wells of the culture plate (NUNC), precoated with gelatin. A mild wash was given after six hours of seeding, to remove loosely attached cells and debris. Fresh medium was added every 2 days until confluence.

II.1.6. Subculture of Endothelial Cells

The medium was removed at confluence and washed with fresh warm serum-free M199. Then the monolayer was incubated with trypsin-EDTA 0.125-1% solution (fill volume of 3ml for $25cm^2$ culture flask and 0.5ml for each well of 24 well plate) for 5min, followed by immediate dilution in ice-cold complete medium. The cell suspension was immediately centrifuged and resuspended in fresh warm complete medium and seeded on fresh bottles at 1:3 split ratio.

II.1.7. Characterization of Endothelial Cells

Typical endothelial nature of cell was assessed by morphological analysis and using specific ligands and antibodies conjugated with either fluorescent or enzyme markers.

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 (Biostar 1820, American Opticals) at 100x, 200x, and 450x magnifications. The cells were characterized by the typical cobblestone morphology of endothelial cell on confluence.

II.1.7.2. Uptake of Fluorescent Labeled LDL

Cells grown on gelatin coated coverslip up to near confluent stage was used for Dil Ac LDL binding studies as per the procedure of Voyata et al (1984) with some modification. In the near confluent stage the growth medium was replaced with fresh growth medium containing 10% serum and 10µg /ml of Dil (1,1-dioctadecyl-3, 3,3-tetramethylindo-carbocyanine perchlorate) labeled acetylated low density lipoprotein (Molecular Probes L3484,USA) and incubated at 37°C and 3% CO_2 for 4-12h. Then the coverslip was removed and washed three times with warm serum-free M199 and viewed under fluorescent microscope (Nikon, Epi-fluorescent Microscope, E-600) using Rhodamine filter.

II.1.7.3. Immunostaining for Factor VIII Related Antigen

The cells grown on gelatin coated coverslip in the near confluent state were used for this staining. Initially the medium was removed and the monolayer was washed several time with warm serum-free M199. This was followed by fixing the cells in 80% ice-cold acetone for 30min. After washing the fixed cells with cold M199 the monolayer was incubated with 50µl of polyclonal antibody to human von Willebrand Factor (1:200 dilution) for 30min (Sigma Product No. F-3520). Then the antibody was removed and washed three times with M199 followed by incubation of 50µl of secondary antibody tagged with alkaline phosphatase diluted at 1:200 in tris buffered saline for 30min. After washing three times with serum free medium, 5 ml of freshly prepared substrate was added (Bromochloroindolyl Phosphate/ Nitro Blue Tetrazolium, BCIP/NPT) and allowed to develop colour at room temperature with agitation, until the stain is suitably dark. After rinsing with PBS containing 20 mM EDTA, the monolayer was viewed under light microscope and photographed.

II.1.7.4. Immunofluorescence with FITC Conjugated Secondary Antibody

Immunostaining of the EC was also done using rabbit antibodies as primary and anti rabbit goat secondary antibodies. The second antibodies were raised in-house by immunization of goat with purified rabbit antibodies.

II.1.7.4.1. Conjugation of Anti Rabbit IgG Raised in Goat with FITC

The antirabbit IgG raised in goat was purified by passing through Protein A column as the method of Harlow and David (1988). The IgG fraction from the column was concentrated to get 2mg/ml of protein. The Fluorescene Iso Thio Cyanate (FITC) was dissolved in Dimethyl Sulphoxide (DMSO) at a concentration of 1mg/ ml. For each 1ml of protein solution 50μl of the dye solution was added slowly with gentle stirring. The reaction was allowed to proceed for 8h at 4°C with continuous stirring. Subsequently, NH_4Cl was added to get 50mM final concentration and incubated further for 2 hrs. The unbound dye was separated from the conjugate by passing through a column of gel matrix with an exclusion limit of 20,000-50,000 (Sephadex G50).

II.1.7.4.2. Indirect Staining of EC with FITC Conjugated Second Antibodies

The monolayer of endothelial cells grown on gelatin coated microscopic coverslip was fixed in cold acetone for 30min. The fixed monolayer was washed and incubated with 50μl of polyclonal antibody (primary antibody) to human von Willebrand Factor (1:200 dilution) for 30min (Sigma Product No.F-3520). After thorough washing of the primary antibody, the monolayer was incubated with 50μl of FITC conjugated secondary anti-rabbit IgG at a dilution of 1:100 for 20 min. Finally, the monolayer was washed and viewed under fluorescent microscope (Nikon, Epi-fluorescent Microscope, E-600) using green filter.

II.2. Cell Seeding on Different Substrates

II.2.1. Preparation of Substrate Coated Tissue Culture Polystyrene (TCPS)

II.2.1.1 Gelatin coated TCPS

24-well culture plates and 25cm^2 culture bottles were used (NUNC) for the study. 2% gelatin was prepared in PBS and autoclaved for 15min prior to use. All the surfaces were incubated with 2% gelatin (Sigma Chemicals, USA) in phosphate buffered saline (PBS) for at least 1h at 37°C. Excess solution was drained off just before cell inoculation.

II.2.1.2. Fibrin coated culture plates

Bovine thrombin (Merck) was reconstituted in 0.025 M $CaCl_2$ at a concentration of 20U/ml. The second component human fibrinogen was prepared from screened pooled human plasma by cryoprecipitation method, which is in the lyophilized form. Just before use, this was reconstituted in distilled water to get 20mg/ml final concentration. The wells of 24-well culture dishes or 25cm^2 culture flask were initially incubated with a thin layer of 20U/ml of bovine thrombin in 0.025M $CaCl_2$ for 30-60min at room temperature. The remaining thrombin solution was removed and the surface was added with a thin layer of human fibrinogen concentrate reconstituted in serum-free M199 at a concentration of 20mg/ml and allowed to polymerize for 30 min at 37°C.

II.2.1.3. Incorporation of Growth Factor with Fibrin

After immobilization of thrombin on the surface, as described in §II.2.1.2, 20mg/ml fibrinogen containing 2mg/ml of ECGF was added and allowed to polymerise for 1h at 37°C. A 25cm^2 flask consumed around 5mg of fibrinogen and 500μg of ECGF.

II.2.1.4.Composite Coated Surface

The TCPS surface was incubated with 20U/ml of Bovine thrombin in 0.025M $CaCl_2$ for at least 30min. After removing the excess solution, the surface was added with a thin layer of fibrinogen 20mg/ml containing 0.01mg gelatin and allowed to polymerize at 37°C. To make GF-incorporated composite, the second component fibrinogen and gelatin containing 2mg/ml of ECGF, was used.

II.2.1.5. Preparation of Freeze Dried Matrix

The polystyrene culture dish was incubated with thrombin solution as in §II.2.1.2., and excess thrombin was removed. Then the surface was added with thin layer of fibrinogen 20mg/ml containing GF as §II.2.1.3 and §II.2.1.4., with or without 0.01mg of gelatin. The surfaces were allowed to polymerize for 37°C and on the completion of polymerization, the dishes were frozen at –70°C for at least 24h and lyophilized. The freeze-dried dishes can either be used immediately for cell seeding or stored in the refrigerator for later use.

II.2.2. Analysis of the Surface Texture of the Matrices

All the surfaces coated with different substrates before and after freeze-drying were analyzed under phase contrast microscope after soaking in complete medium. For scanning electron microscopy (SEM), the matrix was fixed in PBS containing 2% Glutaraldehyde, followed by dehydration in graded series of alcohol. After critical point drying, the samples were gold coated and viewed under Scanning Electron Microscope (Hitachi, Japan).

II.2.3. Cell Attachment Assay

The EC attachment efficiency was assayed in 24-well culture plates prepared as described in §II.2.1. Before seeding the cells, each well was washed with serum-free medium two times to remove unreacted thrombin if any. The third to fifth passage HUVEC was harvested as described in §II.2.1.6. The cell count was noted using Haemocytometer and resuspended in complete medium to get 10^5 cells/ml. The cell suspension was seeded at a density of $2x10^4$ cells/cm^2 per well. Following seeding, the cells were incubated at 37°C in 5% CO_2 for 2h and subsequently the non adherent cells were removed by washing with serum-free medium. The attached cells were harvested with Trypsin-EDTA solution. The cells were counted to calculate the percentage of cells attached. The average of counts in five wells were taken for each substrate.

II.2.4. EC Spreading Assay

The spreading of cells were assessed in each well by viewing under the phase contrast microscope at 20X and 45X magnification at randomly selected fields after 4h of seeding. One hundred randomly selected cells from each well were analyzed at time periods 20min, 30min, 1h, 2h and 4h. Percentages of cells spreaded on each matrix, with respect to seeded cells, were calculated in each case. The proportion of total number of cells which had attached and those had started to spread (i.e. showing deviation from a round, phase bright morphology with cytoplasmic extensions on to the substratum) was determined by observing under the phase contrast microscope and photographed.

II.2.5. EC Proliferation Assay

II.2.5.1. By Direct Counting

The second passage cells were seeded on 24-well culture dishes coated with different substrates as given in §II.2.1, with a seeding density of $2x10^4/cm^2$. The cells were harvested separately from each well at every 24h till 72h and counted. The proliferation rate and doubling time on each substrate were calculated.

II.2.5.2. By ^{3}H-thymidine Uptake

For ^{3}H-thymidine uptake study, 96-well flat-bottomed culture dishes (NUNC), coated with different substrates were used. After 4h of cell seeding, non-adherent cells were washed with serum-free medium and fresh medium containing tritiated thymidine 0.5 µCi/ml was added. The culture was fed with fresh medium containing tritiated thymidine after 48h. 72h after seeding, the plates were washed and cells were precipitated by the addition of 5% ice cold Trichloroacetic acid (TCA). The resultant precipitate was dissolved in 1N NaOH, neutralization with 1N HCl and washed extensively with water. The count per minute (cpm) of ^3H was measured using a liquid scintillation counter (Wallac-1409, LKB, Sweden). The uptake of thymidine was then calculated.

II.2.6. Effect of Shear Stress on Cell Monolayer

Glass coverslips coated with fibrin, composite and gelatin, prepared as described in §II.2.1, were used for the study. The cells were seeded at a density of $2x10^4/cm^2$ and grown to confluence. The monolayer was washed with serum free medium and the cover slip was fixed onto the parallel plate flow chamber fabricated (Engg.Service of our Institute) with slight modification of the model suggested by Mc Intire *et al.*(1987). The EC monolayer was exposed to the complete medium at the rate of 15ml min^{-1} for 1h at 37°C with the help of a peristaltic pump (Pharmacia LKB Pump P-1, Sweden). For one-dimensional laminar flow, the wall shear stress T_w (dyne /cm^2) is related to the volumetric flow rate Q (cm^3/S) as follows

$$T_w = 6\,\mu Q \;/\; w\,H\,(x)^2$$

Where **μ** is the media viscosity, **w** is the width of the flow channel and **H(x)** is the height of the flow chamber as a function of position along the microscope slide (Bhat and Trusky 1998).

Accordingly, the wall shear stress exerted was calculated from the volumetric flow rate (cm^3 s^{-1}), media viscosity, width of the flow channel and height of the flow chamber. After the exposure, the monolayer was washed with serum-free medium and immediately viewed for morphological alterations and cell loss using phase contrast microscope.

II.2.7. Thrombogenicity of Endothelialized Surface

The cells grown to confluence on different matrices in culture dishes were incubated with platelet rich plasma (PRP) from human blood (count of $2x10^3 ml^{-1}$) for 1h at 37°C. The PRP was collected after the incubation, and aggregatory response of the platelets to the agonist adenosine diphosphate (ADP) was evaluated using 560-Ca Whole Blood Lumi Aggregometer (Chronolog Corp, USA). The monolayer was washed with PBS to remove nonspecifically bound platelets and viewed under phase contrast microscope to assess the platelet adhesion.

II.2.8. Assessment of Nitric Oxide (NO) Synthesis

The stable metabolite of nitric oxide synthesized by the cells, nitrite was estimated using Greiss reagent as per the method of Marletta *et al.* (1988). The formation of nitrite (NO_2) and nitrate (NO_3) was detected in cell culture supernatants. This assay determined the total NO based on the enzymatic conversion of NO_3 to NO_2 by nitrate reductase (Sigma Chemicals), and detection of nitrite as an azo dye product of the Greiss reaction. The results were presented in nanomoles of NO_3^-/NO_2 per 10^6 cells. For this assay, cells grown on $25cm^2$ culture flask on different matrices in the near confluent stage were used. To eliminate the protein interference, 24h before the estimation the complete medium from the bottle was removed and fresh serum and phenol red free RPMI 1640 was added and incubated for 24h. Subsequently the medium was collected and immediately the nitric oxide formed was reduced to nitrite by adding 20U/ml of nitrate reductase at 25ºC for 30min. 200µl of the reduced sample was added with equal volumes of freshly prepared Greiss reagent (1% sulfanilamide/0.1% N- (1-naphthyl) ethylenediamine dihydrochloride in 2% phosphoric acid) and incubated at 37°C for 20 min. The colour developed was estimated at 540 nm in a Diode array spectrophotometer (Hewlett Packard, 8053, USA). The nitrite in the solution was quantified using a standard curve prepared from known concentration of sodium nitrite 0-20nmol/ml. The data was analyzed by the software Chemstation (Hewlett Packard).

II. 3. Growth of Cells on Different Substrates

Woven Dacron Patch material (Single Velour Polyester fabric, Meadox Medicals, Inc) and Small Diameter PTFE (Gore tex) grafts were cut to desired size to fix within the wells of 24 culture dishes and autoclaved before use. The sterilized pieces were fixed in the wells with the help of a custom made acrylic ring and washed thoroughly with serum free culture medium before coating with substrates.

II.3.1. Preparation of graft surfaces

II.3.1.1. Gelatin Coated Graft

Graft pieces either PTFE or Dacron woven patch material fixed on the 24 culture dishes after washing and drying were incubated with 2 % gelatin in PBS as per §II.2.1.1 (as done for TCPS coating). The excess fluid was drained off just before cell seeding

II.3.1.2. Fibrin Coated Graft

The procedure used is more or less same as that used for TCPS coating §II.2.1.2. The fixed graft material as above were incubated with thrombin solution for 30min. followed by fibrinogen containing ECGF layering on the surface.

II.3.1.3. Composite Coated Graft

Thrombin in $CaCl_2$ was immobilized on the graft surface as above. A thin layer of 20mg/ml of fibrinogen containing 1mg of ECGF and 0.01mg of gelatin was added and allowed to polymerize for 30 min at 37°C.

II.3.1.4. Uncoated Graft

The graft pieces fixed in the wells with the custom made acrylic ring were washed dried and used for seeding of cells

II.3.2. Cell Adhesion study

All the surfaces were seeded with HUVEC at a density of $2x10^4$ cells $/cm^2$ and incubated for 37° C for 2 h in the incubator in the complete medium. After 2h, unattached cells were removed and counted in counting chamber to calculate the percentage of cell adhesion on each graft surface. In addition, the attached cells were washed with serum free medium, fixed with 2% Glutaraldehyde and stained with May Grumwald stain (Sigma Chemicals). The entire surface was viewed under light microscope.

II.3.3. Cell Proliferation Study and Kinetics of Monolayer Formation

The graft surfaces coated with the matrix as above were seeded with second to seventh passage HUVEC as well as HSVEC at different densities at 2×10^4, 4×10^4, $6 \times 10^4 / cm^2$ and incubated in complete medium for 4 days, 2 days and 1 day respectively. In order to assess the confluency of the cell, the graft pieces were washed and fixed with 2 % glutaraldehyde in PBS for 4 h. After thorough washing the monolayer was stained with May Grunwald stain and viewed under light microscopy.

II.3.4. Effect of Shear Stress on the Cell Monolayer on Materials

The graft pieces prepared as per the section §II.2.3.1 were seeded with 6 $\times 10^4$ HUVEC /cm^2 and cultured in complete medium for 24h. Then the monolayer was washed with fresh complete medium and fixed in the wells of the parallel plate flow chamber and complete medium was circulated over the monolayer with the help of a peristaltic pump at flow rate of 15ml/min for 1 h. The effluent was collected and counted to assess the cell loss during flow. All the monolayers after exposure to flow were fixed and processed for microscopy.

II.3.5. HUVEC Growth on the Freeze Dried Composite Coated Conduits

Eight mm PTFE and knitted Dacron grafts were autoclaved and filled with 20U/ml of thrombin solution in 25mM $CaCl_2$ after closing both ends with a surgical silk. Then the graft pieces were inserted into a sterile 2ml syringe one end of which was made blunt by cutting. The luminal surface was added with a thin layer of 20mg/ml fibrinogen, containing 0.01mg /ml of gelatin and GF. Excess of protein solution was removed immediately. Both the grafts were incubated at 37°C for 30min for complete polymerization. The grafts were freeze dried and were seeded with a cell density of 6×10^4 cells/cm^2. The syringes were incubated at 37°C after closing both ends with a sterile polyethylene plug. The graft pieces were rotated at 12 rev/h manually for 4h and subsequently the cell suspension within the graft was removed and counted to assess the cell adhesion. Then the graft pieces were kept in horizontal position in the incubator for 24h, filled with complete medium. After 24h, the medium was removed and the grafts were washed with warm PBS and fixed in 2% glutaraldehyde in PBS for 4h. After rinsing the glutaraldehyde, thè grafts were stained with May Grunwald Stain, cut open and fixed on a glass slide and mounted with DPX and viewed under light microscope.

II.3.6. Cell Growth on Other Biomaterials for Vascular Applications

In this study, the Ultra High Molecular Weight Poly Ethylene (UHMWPE), the tilting disc used for Chitra Heart Valve, Titanium (Ti) and Diamond like carbon coated Ti (DLC) indented for new heart valve disc development, were used for cell seeding experiments with or without composite coating. The discs were seeded with 6 x10^4 HUVEC/cm^2 and cultured in the complete medium at 37°C in a humidified incubator. After 24h, the monolayer was washed and fixed with 2% glutaraldehyde. After washing off the glutaraldehyde, the monolayer was stained with May Grunwald Stain and viewed under incident light. (Leica, DMR, Japan)

Representative samples meant for shear stress study were grown to confluence and fixed in the wells of the parallel plate flow chamber and complete medium was circulated at a flow rate of 15ml/min for 60min. The monolayer was then fixed with glutaraldehyde, stained and viewed under incident light.

II.4. Repair of Monolayers of HSVEC

The fifth passage HSVEC cells were grown on composite coated 8.8 cm^2 petri dishes to confluence. A superficial longitudinal scratch over the monolayer was made by using the tip of a vascular forceps. After the removal of all the damaged cells by thorough washing, the damage made was analyzed under phase contrast microscope and photographed. The monolayer was incubated with complete medium up to 3 days. The repair efficacy was noted every 24h.

II.5 PROPERTIES OF MATRIX

II.5.1.Stability

II.5.1.2. In the absence of cells:

The fibrin and composite matrices were prepared as mentioned in §II.2.1.2. in 25cm^2 tissue culture flask. The freeze-dried matrix were added with 2ml of complete medium containing 10% serum and incubated at 37°C. After every 48h, the medium was collected and fresh medium was added. The integrity of the matrix was evaluated under phase contrast microscope everyday for up to 30days. The presence of fibrin degradation product was analyzed in the medium periodically at every 48h by agglutination method using latex particles coated with monoclonal antibody to human FDP. (FDP Plasma Cat No 00540 Diagnostic Stago).

II.5.1.3 In the presence of EC

The freeze-dried matrices were seeded with $2x10^4$ cells /cm^2 (either HUVEC or HSVEC) and allowed to grow for 8 days in the incubator. After every 2days, the medium was collected for assay of FDP and fresh medium was added. After confluence, the cells were further maintained 4days in the overconfluent stage to assess for any further degradation. Then the cells were trypsinized and the stability of the matrix was assessed under phase contrast microscope during trypsinization. The cells were washed and reseeded in the same dish and cells were allowed to grow for further 6 days.

II.5.2. Retention of Proteins in Fibrin Matrix

Two chromoproteins cytochromeC, of molecular weight 12.0 kDa and methemoglobin, of molecular weight 64.0 kDa, obtained form Sigma Chemicals, USA, were used, to demonstrate the retention of peptides in the matrix. A standard curve was prepared using known concentrations of these chromoproteins against absorbance at 405 and 450 nm, for cytochromeC and methemoglobin, respectively. After treating the culture plate (24 well) surface with thrombin as described for fibrin coating, it was incubated with 0.25ml of fibrinogen, containing 600μg of chromoprotein, and allowed to polymerize for 1h at 37°C. After polymerization, each surface was washed with serum free medium and the presence of chromoprotein in the washing was quantitated using the standard curve, by measuring the A_{405} and A_{415}. The quantity of incorporated peptide was calculated to be around 500μg. The wells were incubated with 0.5 ml of medium containing 5% serum and incubated at 37°C, which was then replaced with fresh medium at the intervals of 5 min, 24h, 48h, 72h and 96h. The chromoprotein content in the medium recovered at each time interval was estimated.

II.5.3. Thrombogenicity assessment of surfaces

II.5.3.1. Preparation of graft surface.

Dacron, woven vascular grafts (Bard, USA) and e PTFE (Gore Tex) grafts were cut to desired size to fix within the wells of a 24 culture dishes (NUNC). These pieces were fixed within the wells with the help of a custom made acrylic ring. After fixation the luminal surface was washed with distilled, deionised water three times, dried and used for precoating. The study surfaces such as uncoated polystyrene, Dacron or PTFE, after washing and drying, were coated with desired matrix as described in §II.2.1.to §II.2.2.5. Fibrin glue-coated and composite-coated surfaces were washed with PBS in order to remove excess thrombin if present.

II.5.3.2. Preparation of PRP and Treatment with Surfaces:

Blood was collected in Acid Citrate Dextrose (ACD) solution from healthy human volunteers. By differential centrifugation at 200*g* for 10min, on a high-speed centrifuge (SCR20BA, Hitachi, Japan), platelet rich plasma was collected. Platelet poor plasma (PPP) for use with aggregation measurement was collected by centrifugation at 1000*g*, for 10min.

II.5.3.3. Platelet Consumption Study

All the surfaces were incubated with 1 ml PRP with a count of $2x\ 10^5/\ mm^3$, for 1h at 37°C, with occasional agitation, in a petri dish. Platelet counts were noted periodically, using an automatic hematology analyzer, Cobas Minos Vet (Roche Diagnostics, France). Platelet consumption per mm^2 area was calculated from the count reduction noted at the end of 1h incubation.

II.5.3.4. Aggregation Response of Platelets after Contact with Substrates

At the end of the experiment, the changes caused to the platelet in contact with the substrate was evaluated by assessing the functional status of the platelets in suspension. The normal ability of the platelets to respond to the physiological stimuli, ADP was evaluated as per the method of Cardinal DC and Flower RJ (1980) in an aggregometer (Chronolog 560-Ca Lumi Aggregometer Chronolog Corp., USA). For this 0.45ml of PRP was taken into a siliconised glass cuvettes and base line was set with platelet poor plasma (PPP). Platelets were stimulated by addition of 100μM ADP. The rate and amplitude of aggregation were calculated using the software AGGLINK. The data was compared with the aggregatory response of PRP without any material contact.

II.3.5. Evaluation of Spreading of Platelets on the Substrates by SEM

Nature of attachment and spreading of platelets specifically bound to the substrate was evaluated by SEM. All the surfaces were washed three times with PBS to remove nonspecifically bound platelets. The attached cells were fixed with 2% glutaraldehyde in PBS for 24h at room temperature. The fixed samples were washed three times with PBS and dehydrated with graded concentrations of alcohol, (30%, 50%, 70%, 100%) critical point dried, sputter coated with gold, and viewed under a Hitachi Scanning Electron Microscope (Hitachi, Japan).

CHAPTER III

RESULTS & DISCUSSION

RESULTS AND DISCUSSION

III.1. Cell Culture and Characterization of Human Endothelial Cells

III.1.1. Growth Factor Selection

Previous investigations have shown that human and bovine vascular endothelial cells could be grown and maintained in culture provided the medium is supplemented with optimal concentrations of growth factors from bovine hypothalamus or pituitary glands (Gospodarowiez D *et al.*,1978). During this study when growth factor obtained from commercial source, was used at a concentration of 150 μg/ml in the culture medium, the proliferation rate was not satisfactory. Therefore, isolation of growth factors from bovine source was attempted as per the protocol (II.1.1). From 500 gm of hypothalamus the yield was about 500 mg of Lowry's protein. The fat extraction with streptomycin sulfate was effective and a clear supernatant was obtained after the final centrifugation. About 100 mg was dispensed into freezing vials and lyophilized product was stored at –70°C. Once reconstituted with serum-free medium, it was stored frozen in the solution form. The growth factor activity was found stable for two and half years in the lyophilized form. ?

Analysis of the isolated growth factor by polyacrylamide gel electrophoresis showed protein bands that were comparable (Fig.III.1) to that in commercial sample. Maciang *et al.* (1979) reported that, in the crude extract of bovine hypothalamus human endothelial cell growth promoting activity was associated with a polypeptide having a molecular weight of ~75 kDa, and fibroblast cell mitogenic activity was associated with a low molecular weight fraction of ~15 kDa. When the quantity of total protein loaded on PAGE were same, the relative concentration of ECGF in the isolated extract is found greater in the in-house preparation compared to the commercially available growth supplement, whereas the presence of b-FGF is similar in both samples.

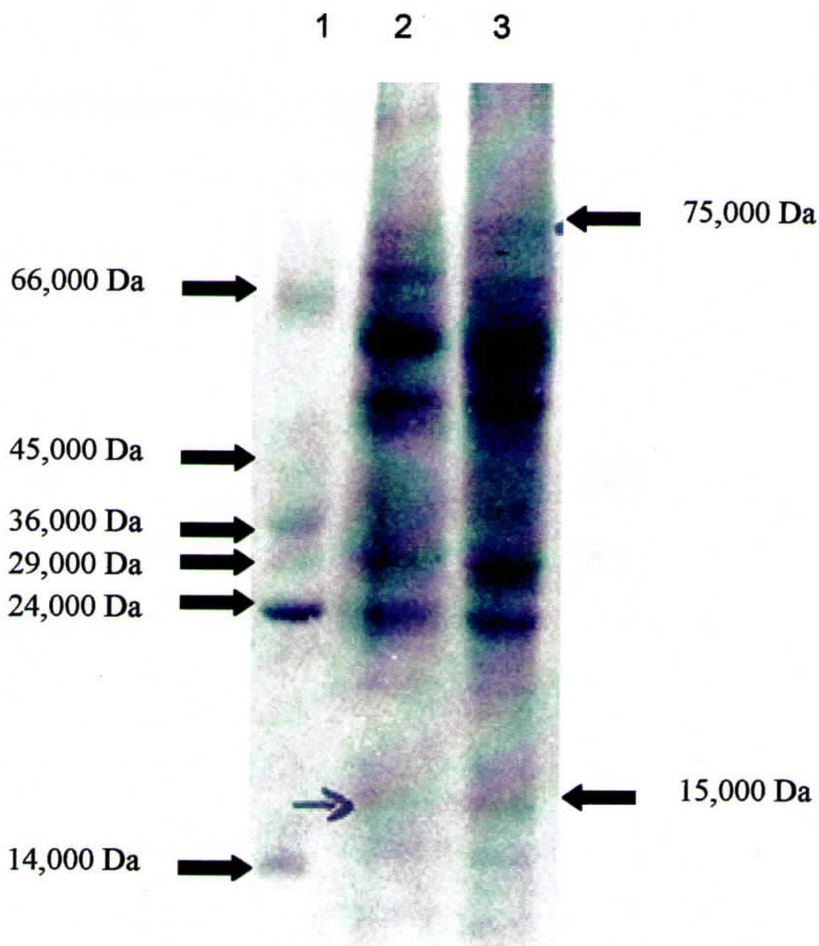

Fig.III.1. SDS-PAGE pattern of growth factor purified from bovine hypothalamus. Lane1, Molecular weight Markers; Lane 2, Commercially available Growth Supplement from Sigma Chemicals, USA ; Lane 3, Purified Growth Factor .

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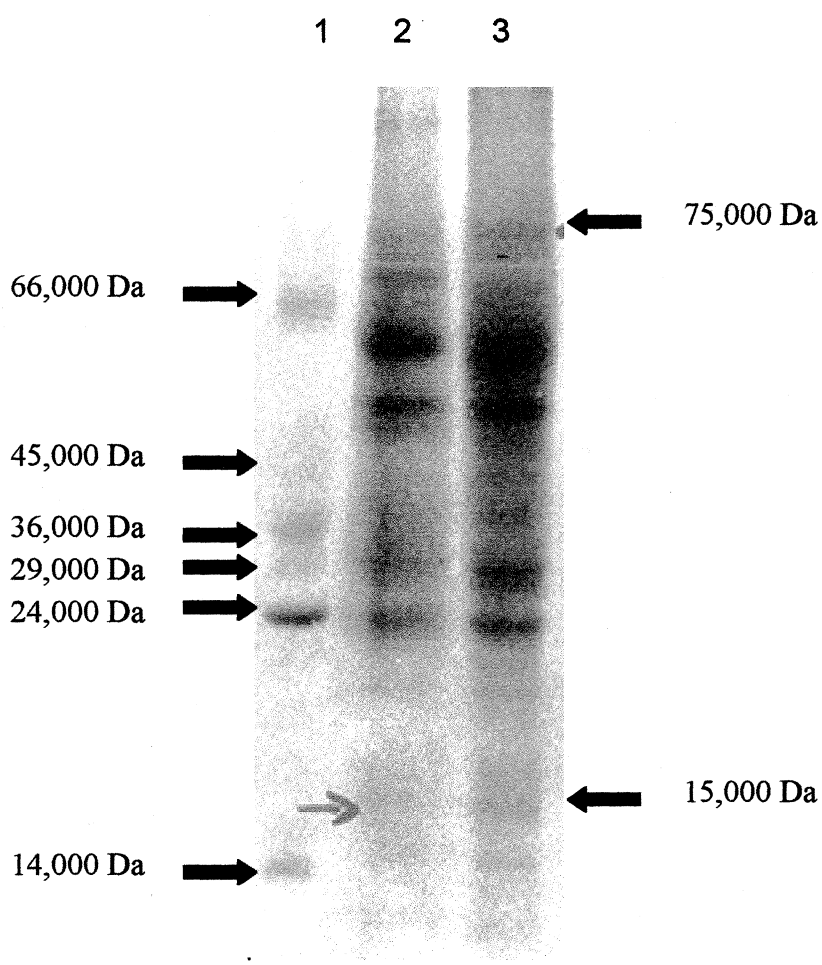

Fig.III.1. SDS-PAGE pattern of growth factor purified from bovine hypothalamus. Lane1, Molecular weight Markers; Lane 2, Commercially available Growth Supplement from Sigma Chemicals, USA ; Lane 3, Purified Growth Factor .

III.1.2. Serum Selection

Commercially available serum was not used for the cell culture during this study. Serum obtained from new born calf, prepared by a method developed in-house, and human serum prepared from voluntary donors, were supplemented with the tissue culture medium of choice. The adhesive proteins are usually present in the serum which is known to contribute to the initial cell attachment to the culture plates. The growth factors released from platelets upon blood clotting is known to support mitogenesis and chemotaxis. Therefore, the choice of serum plays a significant role in getting good cell attachment and proliferation. Majority of the cultures were done using new born calf serum, whereas for most of the studies with HSVEC, human serum was used. Serum from both sources gave reproducible and excellent results in terms of maintenance of cell morphology, cell attachment and proliferation.

III.1.3 Medium Selection

The commercially available media used in this study were M 199, RPMI 1640 and MCDB 131. There was no marked difference between any of these media when supplemented with 20% serum (either derived from new born calf or human) containing 150 μg /ml of ECGF, in terms of cell growth. The morphology and growth pattern was consistently good in MCDB, even with 10 % serum. Therefore, MCDB with 10 or 20% serum, 150 μg/ml of ECGF, 100U/ml of heparin sulphate , 100 U/ml of streptomycin , 100 μg/ml of benzyl penicillin and 2.5 μg /ml of amphotericin has been used as the standardized medium, throughout the study unless specified.

III.1.4. Cell Isolation and Culture

III.1.4.1. Yield of EC from Umbilical Vein

Collagenase digestion of about 15 cm long umbilical cord vein yielded ~ 5-8 x 10^5 cells. Among the isolated cells about 50-60% of the cells were in the form of aggregates of 50- 200 cell mass and the remaining were individual cells. About 50-60 % of the initially yielded cells were attached on the gelatin coated flask, after 4 h of incubation in complete medium. The cells attached on the surface appeared as patches and newly growing cells started migrating from underneath the patches. Occasionally, few individual, elongated, spindle shaped cells were observed in some cell isolates. But during the initial washing of the plate after 4 h, with complete medium, most of these cells were eliminated, yielding a homogenous cell monolayer culture. The attached cells started proliferation and migration after 18 h of seeding. With an average seeding density of around 20,000 cells/cm^2, the cells attained confluency within 6-7 days. The cell density was ~ 60 to 70 x 10^3 cells/cm^2 at confluence.

III.1.4.2. Yield of EC from Saphenous Vein

The average number of cells harvested from 4-5 cm of saphenous vein was 6 to 8x10^4 cells. Unlike that of HUVEC the cells detached from the vein as smaller patches of 10-12 cells. Majority of the cells were singles or doublets. Out of the yielded cells 60 % attached on the gelatin coated TCPS in the presence of serum containing medium making a density of around 2x 10^4 cell /cm^2. At this density it took around 7 days to make a confluent monolayer which come to ~6- to 7 x 10^4 cells/cm^2.

III.1.4.3. Subculture of the Monolayer

After washing the trypsinized cell suspension, the cells were seeded at 1:3 split ratio. The cells harvested between 2nd and 7th passage, usually takes around 4 days to reach confluence in this split ratio. The cells were maintained up to 12 passages without any significant morphological change. After 13 passages in 60 days of culture the HUVEC proliferation rate slightly deteriorated with appearance of few enlarged cells having highly pronounced cytoplasmic extension and perinuclear vacuoles. The HUVEC could be maintained up to 90 days in culture. But in the case of saphenous vein EC the senescent tendency was noted even from 5th passage cells just after 35 days of culture.

III.1.5. Characterization of Endothelial Cells

Enzymatic isolation of specific cells from tissues is likely to be contaminated with other cell types. In order to confirm the identity of endothelial cells, the morphology, presence of specific molecules, such as LDL receptor and von Willebrand factor were detected in the cultured cells. Immunofluorescent staining against Factor VIII and binding of fluorescent probe Dil tagged low density lipoproteins has confirmed the homogenous nature of the endothelial cells cultured in this study.

III.1.5.1. Morphology

On confluence, the EC showed typical cobblestone 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 different magnification is shown in figures III.1.2 - III.1.4. The likely contaminant fibroblast should be irregular shaped or usually stellate shaped without noticeable contact inhibition, upon confluence. The smooth muscle cell is expected to appear as elongated spindle shaped and evident cytoplasmic muscle striations and hill – valley pattern. Both these populations were not seen in the culture, when viewed under phase contrast microscope. When the HUVEC were maintained in the complete growth medium for prolonged period after confluence, the cells start to infiltrate above the monolayer (Fig. III.1.5.). But upon trypsinization and reseeding the cells regain its normal morphology. This kind of sprouting behavior is considered to be one of the unique features of endothelial cells (Jaffe *et al.* 1973).

No marked difference in morphology was observed in monolayers of HSVEC (Fig.III.1.6 & Fig.III.1.7), compared to the HUVEC. But the relative size of the cell seems to be slightly smaller in the early passages of HSVEC necessitating more number of cells to make a monolayer. After the third passage the cell size becomes comparable to the HUVEC. However, the contact inhibition of monolayer is much pronounced in HSVE. HSVEC can be maintained in the confluent state without much sprouting for 3-4 days, after confluence. In this stage proliferating cells organize themselves as monolayer by close apposition and shortening.

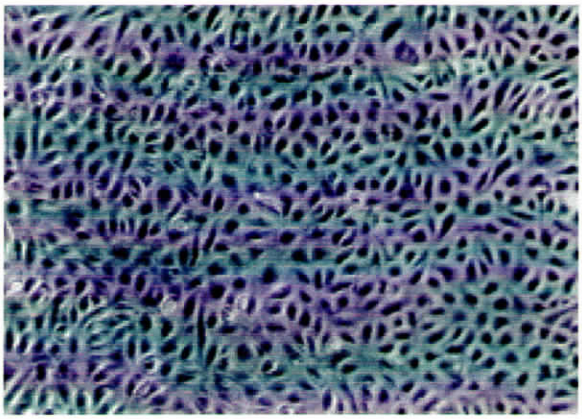

Fig.III.1.2. Photomicrograph of a representative field from HUVEC culture. Typical cobblestone morphology is evident. (Mag.100x)

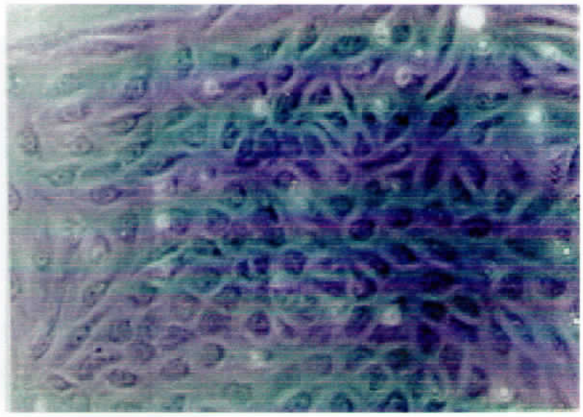

Fig. III.1.3. Photomicrograph of a HUVEC culture, magnified. Polygonal closely packed morphology is seen. (Mag.200x)

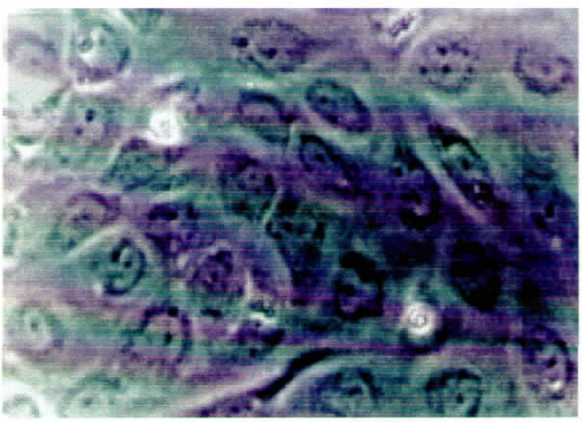

Fig.III.1.4. Photomicrograph of HUVEC, higher magnification. Under the phase contrast microscope tight inter cellular junctions are evident (Mag. 450x).

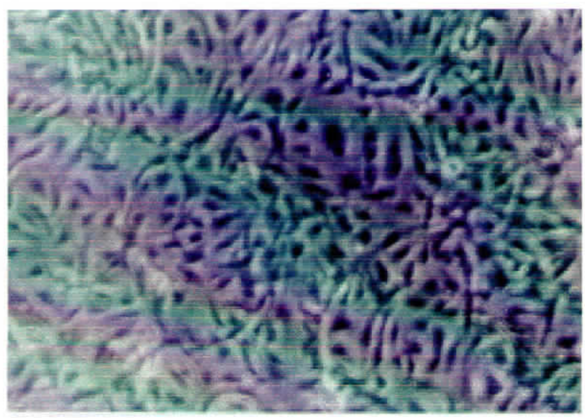

Fig.III.1.5. The photomicrograph of sprouted monolayer Highly packed cells are seen, migrating above the plane of the monolayer (Mag 100X).

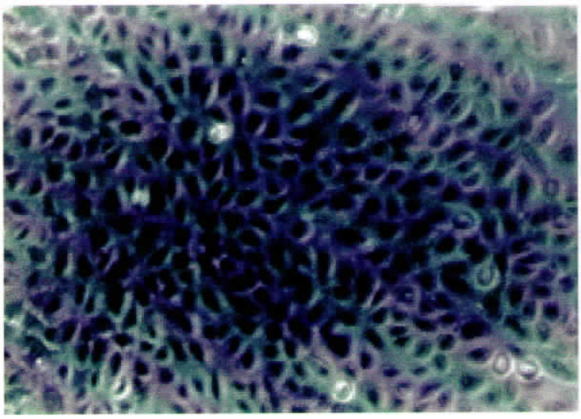

Fig III.1.6. Photomicrograph of HSVEC, under low magnification. Confluent monolayer with cobblestone morphology as seen under PC microscope (Mag.100x)

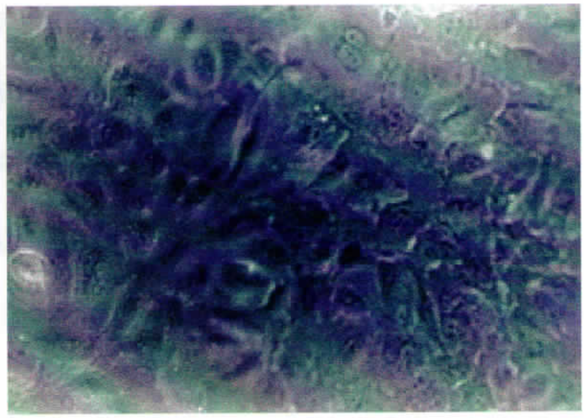

Fig.III.1.7. Confluent Human Saphenous Vein Endothelial cells, magnified. (Mag 450x)

III.1.5.2. Localization of FVIII on the EC

Two methods have been employed to stain the cells with antibodies against Factor VIII. The first one using commercially available secondary antibody conjugated with the enzyme alkaline phosphatase and the other one with FITC conjugated secondary antibody raised in goat. The perinuclear rod shaped Weibel palade bodies is clearly evident in FITC stained samples under high magnification in both cell types (Fig III.1.8-III.1.9). The typical perinuclear distribution of FVIII on HUVEC and HSVEC stained with alkaline phosphatase enzyme conjugated secondary antibody is also seen in the fig III.1.10-III.1.11. Only upto to 7th passage, the presence of FVIII in the cytoplasm in both HUVEC and HSVEC was tested and in all tests done, the presence was evident without any detectable change in the intensity of fluorescence . The intensity of FVIII is relatively poor in senescent cells from saphenous vein after 5th passage compared to those from HUVEC. This is consistently evident in those samples stained with fluorescent labeled antibody and with alkaline phosphatase conjugated antibody.

The factor VIII related antigen – vWF is a high molecular weight protein present in both endothelial cells and platelets but not seen in any other cell types. So the detection of this protein using immunostaining is one of the widely employed specific test to characterize EC in culture.

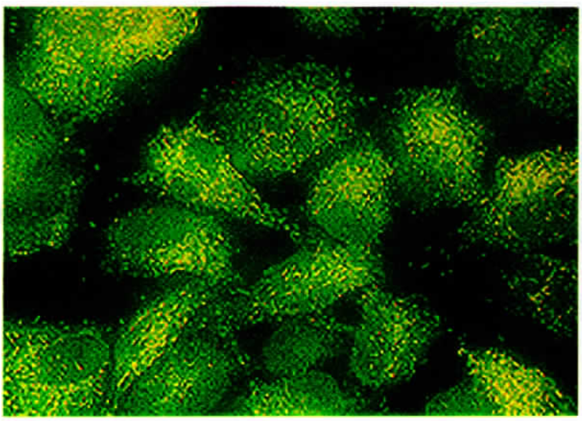

Fig.III.1.8. Fluorescent micrograph of HUVEC stained for Factor VIII. FITC conjugated secondary antibody was used. (Mag 400x.)

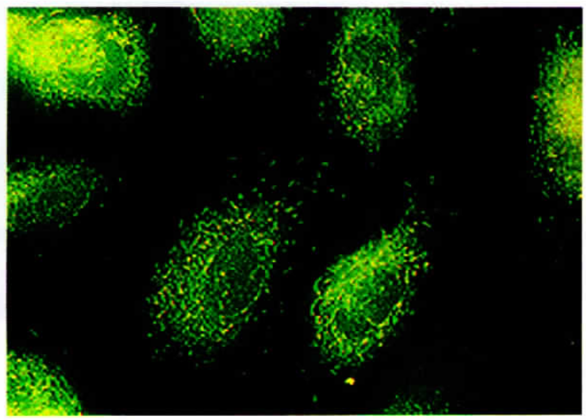

Fig.III.1.9. Fluorescent micrograph of HSVEC stained for Factor VIII. FITC conjugated secondary antibody was used. (Mag 400x.)

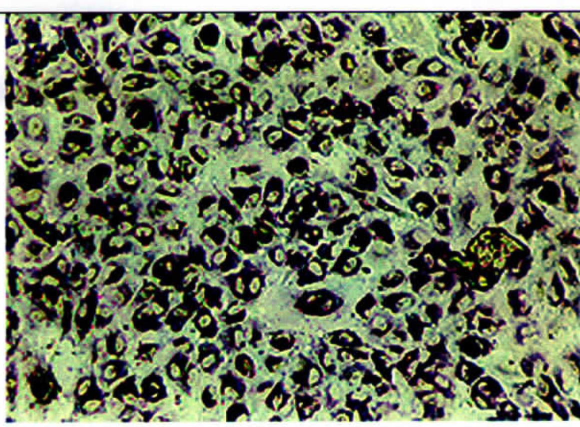

Fig III.1.10. HUVEC culture Immuno stained for Factor VIII with alkaline phosphatase. Indirect staining with enzyme-linked secondary antibody was done. (Mag 100x)

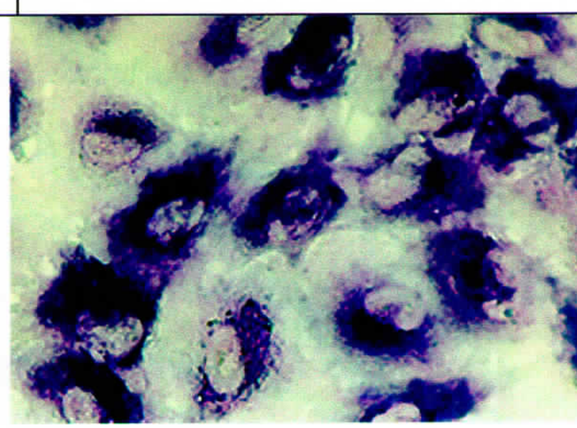

Fig III.1.11. HSVEC Immuno stained for Factor VIII with alkaline phosphatase. Enzyme-linked secondary antibody was used.(Mag 400x.)

Von Willebrand factor is stored in intracellular granules of EC, named Weibel palade bodies, located around the nucleus. Once activated, the FVIII gets expressed on the membrane surface or gets released depending on the extent of stimulation. In the case of platelets, they are stored in $\propto$- granules and gets released into the medium upon activation of cells, and it is an irreversible process. Whereas in the case of EC, the FVIII reappears in Weibel Palade bodies, possibly because either it gets endocytosed or it is re synthesized. It has been reported that in culture after 5^h passage the FVIII is diffuse in the EC monolayer (Prudence and Bicknell, 1993), which again seems to be dependent on the culture conditions used. This indicates that after repeated passages, the physiology of cells grown must have changed. In this study such problems were not seen till 7^{th} passage, which indicates that the matrix on which it is grown and the medium used are ideal to maintain the normal physiology of cells.

III.1.5.3. Uptake of Low-Density Lipoprotein

Both HUVEC and HSVEC showed uniform distribution of the fluorescence in the cytoplasm of samples incubated with Dil labeled LDL (Fig.III.1.12 & III.1.13). In this study the acetylated low density lipoprotein is labeled with a fluorescent probe which may remain in the cytoplasm even after the LDL is metabolized. The photomicrographs shown are cells that internalized LDL during 7^{th} and 10^{th} passages, in case of HSVEC and HUVEC, respectively. In the later passages the internalization process deteriorated with less level of fluorescence in both cell types. The LDL that is bound to the receptors on the cell membrane is internalized and appear as granular structures throughout the cytoplasm, surrounding the nucleus. The receptor for low-density lipoprotein can be seen, other than endothelial cell, in smooth muscle cell membranes also. But here, the homogenous nature of endothelial cells is proven, by morphological and FVIII staining methods. Hence, this technique further confirms the normal physiological status of the cell, whereby LDL receptor is active and the bound ligand is internalized for subsequent metabolism. While FVIII staining is done in fixed cells, LDL uptake is studied in growing culture.

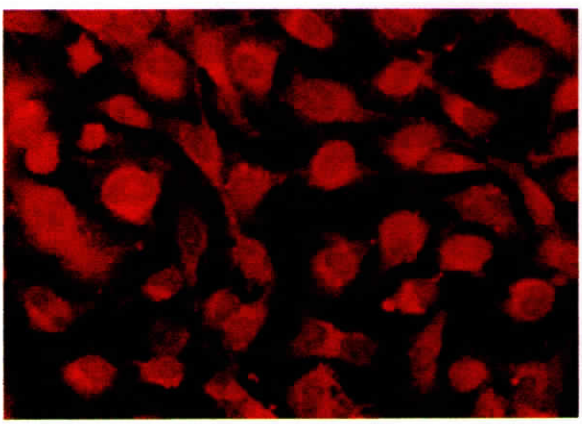

Fig. III.1.12. Fluorescent micrographs of HSVEC, showing Dil. Internalized low density lipoprotein labeled with fluorescent probe Dil shows granular features surrounding the nucleus. (Mag 200x).

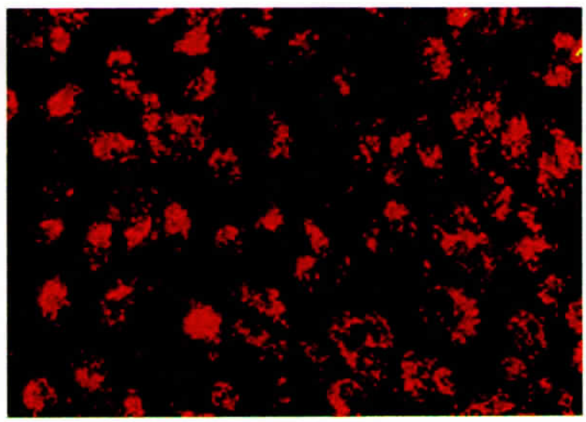

Fig. III.1.13. Fluorescent micrographs of HUVEC showing Dil. The granular distribution of internalized Low Density Lipoprotein labeled with fluorescent probe Dil is seen around the nucleus. (Mag 200x).

From the above reported results form the morphological analysis, and identification of specific antigen such as FVIII, the culture is characterized as 100% pure endothelial cells. The binding studies of the ligand, LDL additionally confirms the active physiology of the grown cells. In the case of HUVEC and HSVEC, the latter passage cultures consisted of senescent cells showing variable morphology with vacuoles. Such cell also showed poor staining efficiency with both FVIII antibodies and Dil labeled LDL. This result enabled the use of the cells from 4^{th} to 7^{th} passage cells for standardization of tissue engineering techniques, for seeding biomaterials .

III.2. Cell Adhesion and Spreading on Different Matrices

III.2.1. Gelatin Coated PS

Of the total number of cells seeded around 66% attached after 2 h of seeding on gelatin coated PS, compared to 62 % on uncoated PS. By this time, most of the attached cells were not fully spread and were still in a spherical morphology. In this matrix the cells nearly took 10-18 h for complete spreading. The representative spreading pattern of the EC on this matrix is shown in the fig.III.2.1. From the picture it is clear that during spreading the contact of the cytoplasmic extension with the matrix is not so intimate such that the periphery of the cytoplasm can be easily demarcated under phase contrast microscope. In the uncoated TCPS also the spreading remains incomplete by this time (Fig III.2.2).

III.2.2. Fibrin Coated PS

The HUVEC adhesion was significantly high on fibrin coated dishes compared to bare TCPS or gelatin coated TCPS. After 2 h of seeding ~92 % of the initially seeded cells were attached. Within this period, around 50% of the cells were completely spread on the surface and by 4 h the remaining attached cells were also uniformly organized with the underlying matrix (Fig .III.2.3). It is clearly seen that cytoplasm occupied much more area on these surface. Because of the high interaction of the cell with the matrix it is very difficult to demarcate the cytoplasmic periphery which got interspersed with the substrate, unlike that is seen in fig.III.2.(1&2). The percentage of adhesion and spreading was similar on fibrin matrix irrespective of the presence of growth factor within the matrix. Similar attachment and spreading pattern was observed even when the matrix was freeze dried and stored before seeding the cells. Because freeze dry technique can be effective in improving the shelf life of the matrix it may have advantages in the application of these surfaces stored precoated for ready use.

III.2.3. Composite coated PS

Almost like fibrin, 96 % of the seeded cells were found attached after 2 h of seeding on this matrix. The attachment of cells on these surface is much faster compared to any other substrate used in this study. The cytoplasmic extension migrated towards the substrate within 20 min after seeding and by 2 h majority of the cells were completely spread. Compared to gelatin coated surface the cells occupied much more area on the matrix by extensive spreading along the matrix. Because of strong adhesive interaction between the cytoplasmic periphery with matrix, the boundary between the matrix and cell is not well pronounced under phase contrast microscope. No significant change in adhesion and spreading was seen here even after freeze drying of the matrix(Fig. III.2.4- & III.2.5).

In summary, out of the 4 surfaces evaluated for cell attachment, a high level of adhesion was seen on fibrin and composite, followed by gelatin and least was on the bare PS.

It has been reported in a number of studies that the attachment of cells to synthetic polymers is determined by the adsorption of proteins followed by cell membrane receptor interaction with the adsorbed protein (Horbett, 1994). This adsorption differs from material to material depending on the physicochemical characteristics and surface texture. The commonly used cell culture substratum, tissue culture polystyrene, has oxygen containing groups incorporated on to the basic polymer, probably by radio frequency plasma modification (Curtis, *et al.* 1983).

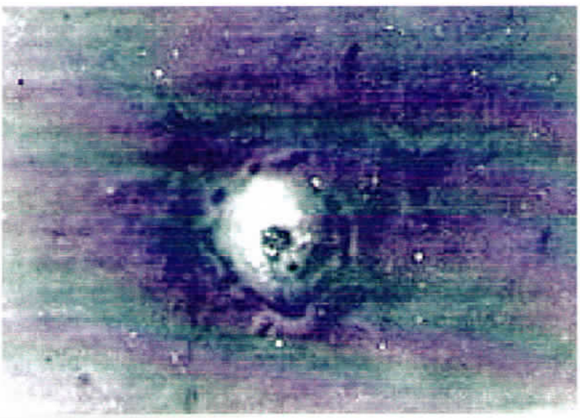

Fig.III.2.1. Photomicrograph of a spread HUVEC on uncoated TCPS. The picture was taken 4h after seeding(Mag 450x)

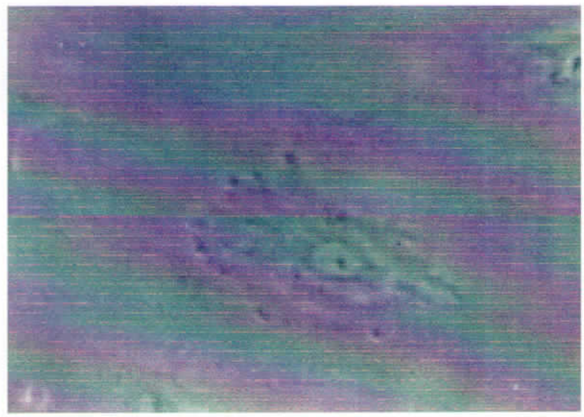

Fig.III.2.2. Photomicrograph of HUVEC on Gelatin coated TCPS. The picture was taken 4h after seeding(Mag 450x)

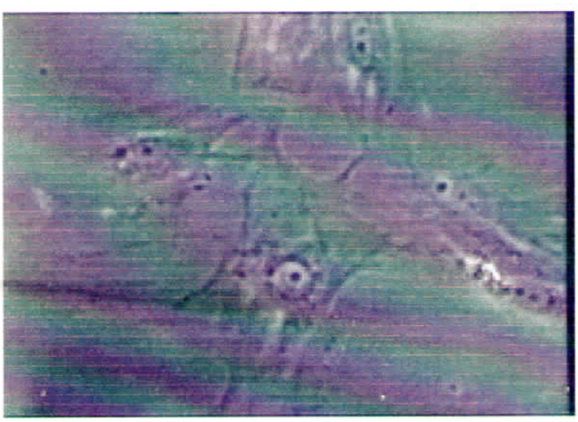

Fig.III.2.3. Photomicrograph of HUVEC on TCPS coated with fresh fibrin. The picture was taken 4h after seeding (Mag 450x)

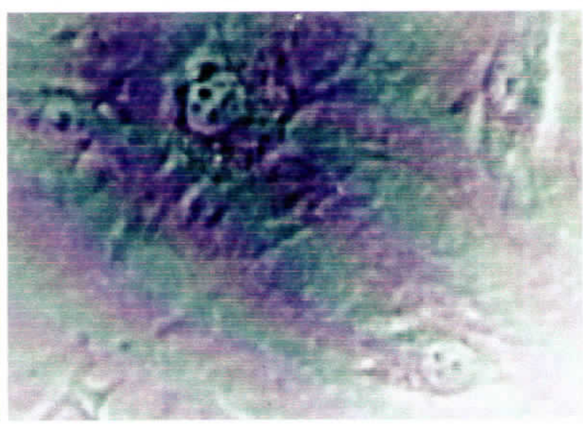

Fig.III.2.4. Photomicrograph of HUVEC on TCPS coated with freeze dried fibrin. This was viewed 4h after seeding (Mag 450x)

The increased oxygen content of tissue culture polystyrene and the resultant hydrophilicity is likely to enhance selective adsorption of adhesive proteins such as characteristics and surface texture. The commonly used cell culture substratum, tissue culture polystyrene, has oxygen containing groups incorporated on to the basic polymer, probably by radio frequency plasma modification (Curtis, *et al.* 1983).

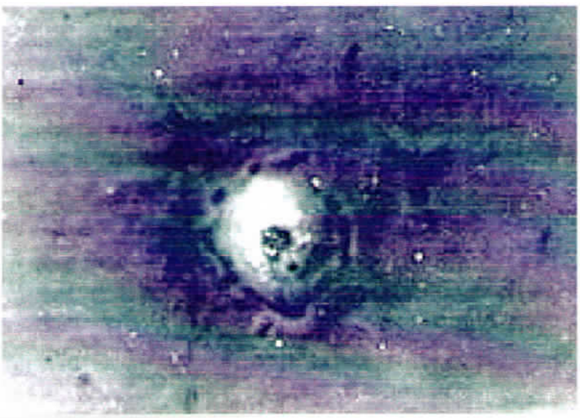

Fig.III.2.1. Photomicrograph of a spread HUVEC on uncoated TCPS. The picture was taken 4h after seeding(Mag 450x)

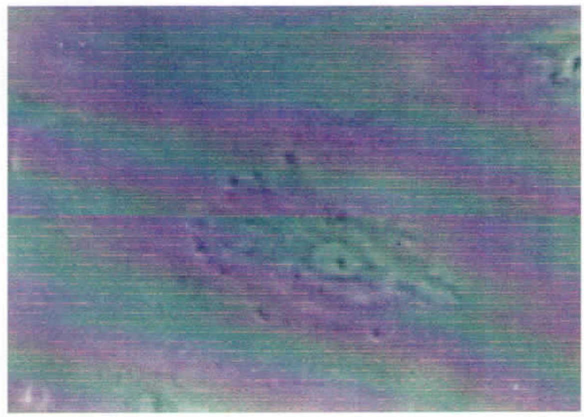

Fig.III.2.2. Photomicrograph of HUVEC on Gelatin coated TCPS. The picture was taken 4h after seeding(Mag 450x)

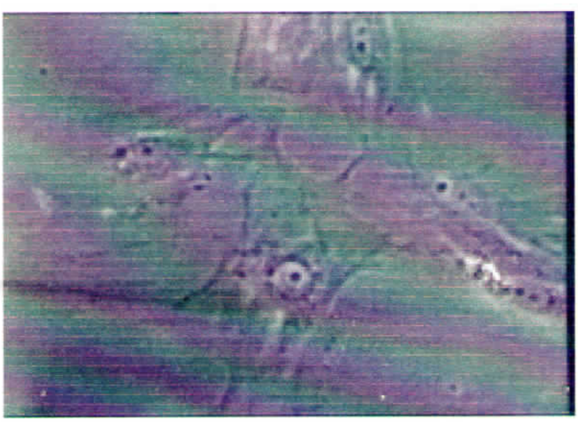

Fig.III.2.3. Photomicrograph of HUVEC on TCPS coated with fresh fibrin. The picture was taken 4h after seeding (Mag 450x)

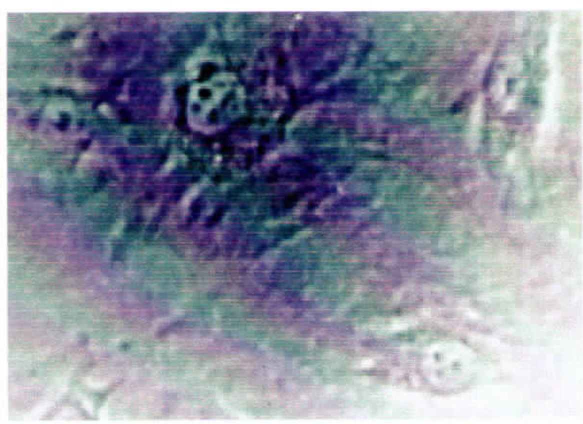

Fig.III.2.4. Photomicrograph of HUVEC on TCPS coated with freeze dried fibrin. This was viewed 4h after seeding (Mag 450x)

The increased oxygen content of tissue culture polystyrene and the resultant hydrophilicity is likely to enhance selective adsorption of adhesive proteins such as vitronectin and fibronectin thereby supporting cell attachment In this study 20% serum is present in the medium and around 62% of cells were attached on to TCPS. However, the spreading was poor compared to fibrin and composite.

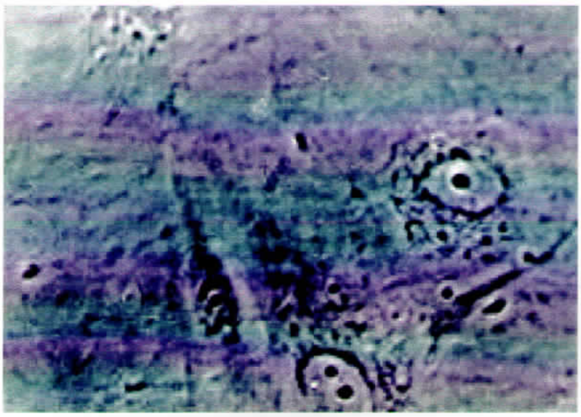

Fig.III.2.5. Photomicrograph of HUVEC spreading on fresh composite 4 h after seeding (Mag 450x)

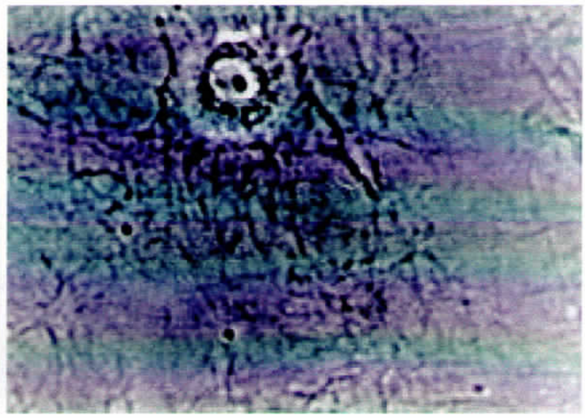

Fig.III.2.6. Photomicrograph of HUVEC spreading on freeze dried composite 4 h after seeding (Mag 450x)

No	Substrates	Percentage of adhesion Mean ± SD
1	Uncoated TCPS	62 ±6
2	Gelatin	66±4
3	Fibrin	92±4
4	FD Fibrin	96±2
5	Composite	94±6
6	FD Composite	96±2

Table.III.1. The percentage adhesion on each substrate is shown in the table. Values are mean ± SD.

Some earlier studies have reported that the adsorption of fibronectin on to tissue culture polystyrene is inhibited by other serum proteins. (Steele , *et al.* 1992).

Recently a chemically modified polystyrene Primaria ^{TM}, which is commercially available has been described as having advantages than TCPS for the culture of endothelial cells, neurons and other fastidious cells. (Klein-Soyer *et al*,1989). But later analysis of mechanism of cell attachment on this surface shows that the amount of fibronectin adsorbed onto primaria is supraoptimal for the attachment of HUVEC and fibroblast. Therefore, the reason behind poor initial cell spreading on uncoated polystyrene may be suboptimal adsorption of adhesive protein from the supplemented serum.

III.3. Cell Adhesion on to Vascular Biomaterials

The cell attachment on different adhesive coated vascular graft material is shown in the fig III.3.1. With the same seeding density of cells, as that was used for attachment assay on matrix coated TCPS, the cell retention on the uncoated PTFE and Dacron were low. On uncoated PTFE about 15% of seeded cells were found attached and on uncoated Dacron around 18% cells were retained 4h after seeding.

But the matrix compositions such as fibrin and composite enhanced cell attachment on both PTFE and Dacron, similar to that of similarly coated TCPS. On the other hand, the gelatin coating has not significantly effected cell adhesion and spreading on to these biomaterials. (Fig .III.3.1. and Fig.III.3.2.)

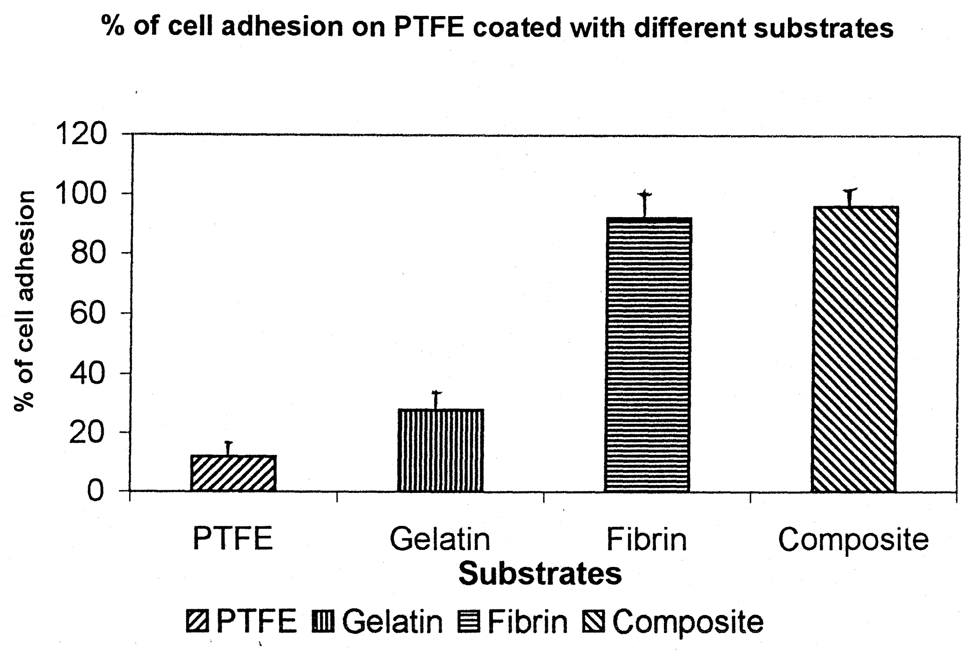

Fig.III.3.1. Graphical representation of percentage of adhesion of HUVEC on PTFE coated with matrix proteins. Values are ±SD (n=5)

This indicates that gelatin may not have got adsorbed onto these highly hydrophobic polymers though the technique employed to coat TCPS and the biomaterials is same. But, relatively good amount of fibrin and composite could be coated on to these surfaces uniformly, irrespective of the material properties of the surfaces. Simple adsorption such as in the case of gelatin is mainly controlled by the physical and chemical nature of the surface. The mechanism of coating of the matrix with fibrin/composite in the currently explained method is not completely dependant on the adsorption characteristics of the material. Here the initially adsorbed or retained thrombin polymerizes the fibrinogen concentrate layered onto it. Retention or entrapment of other adhesive proteins present in the cryoprecipitate obtained from human plasma is also likely to take place.

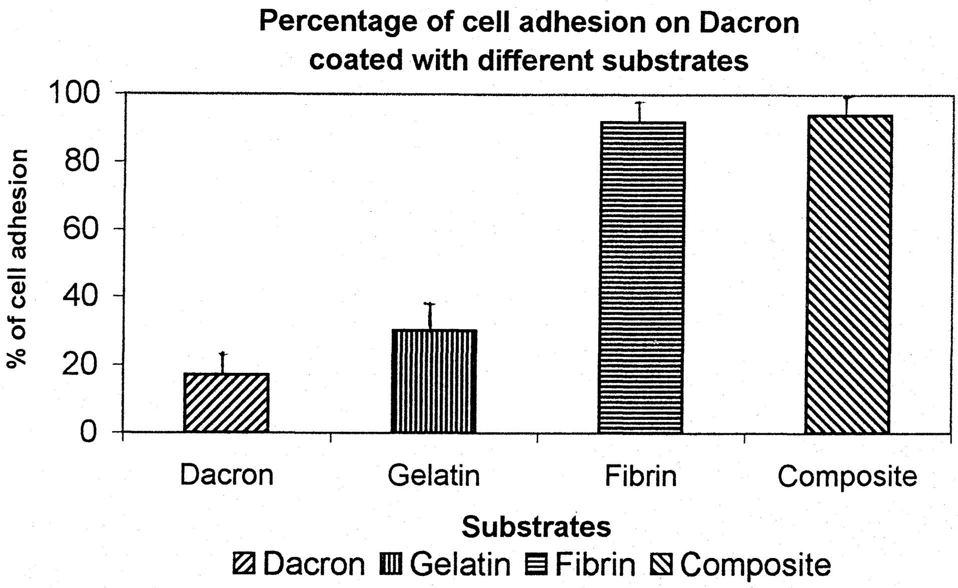

Fig.III.3.2. Graphical representation of percentage of adhesion of HUVEC on bare Dacron and matrix coated Dacron. Fibrin and Composite are Freeze dried after coating . Values are ± SD(n=5)

In most of the reported studies, fibrinogen is allowed to adsorb first on the surface which is followed by thrombin addition. Thus, the protein composition on the surface is dependent on the initially adsorbed fibrinogen, which is likely to have undergone conformational changes, depending on the surface characteristics. This might lead to poorly defined fibrin formation, and may not be able to immobilize other adhesive proteins. In the presently used technique, because adsorbed thrombin is known to retain its enzymatic activity, the results obtained were very promising, as high and consistent cell adhesion could be attained irrespective of the properties of the material surface.

In general, most of the highly anchorage dependent cells adhere to the adhesive protein when immobilized on the surface. This initial adhesion is a prerequisite for any such cell for growth and is primarily mediated by interaction of integrin class of cell adhesion receptors with adhesive proteins. (Ruoslahti *et al.*, 1987). Most of the proteins listed as adhesins have a specific recognition sequence- RGD- and the integrin binding to these sequence provide a signal for cell adhesion, spreading and growth. In the case of fibrinogen which holds two RGD sequence in the domain of the molecule and because thrombin cleavage exposes the sequence, cell adhesion may be mediated by the integrin. Moreover, the fibrinogen concentrate is likely to contain other adhesive proteins like fibronectin and vWF which may be trapped in the polymer network, making multiple receptor binding possible. In case of composite, it also traps the gelatin which serves to provide additional integrity as well as binding strength, as gelatin is derived from an extracellular matrix protein, collagen, which provides adequate number of binding sites. The poor cell adhesion to the gelatin coated surfaces may be due to poor immobilization of gelatin on the surface. Once immobilization is enhanced by the entrapment inside the fibrin gel, gelatin also contributes to cell attachment. This explains the increased attachment and spreading on composite, compared to that on gelatin or fibrin alone.

III.4. Cell Growth on Matrices

Significant difference in the growth of initially attached cells were observed depending on the composition of the matrices. This observation was verified by two methods and reproducible results were seen in both techniques.

III. 4.1. By direct count

The proliferation rate as assessed by counting the number of cells on different matrix, at different time intervals is plotted as growth curve (Fig.III.4.1). Even though the number of cells attached to gelatin was only 66%, after 48 h, the cell number in fibrin and gelatin were more or less similar. With an initial seeding density of 2x 10^4 cells/cm^2, the cells on the fibrin and gelatin coated surfaces showed a cell density of 4.9x10^4 /cm^2 and 4.6x10^4/cm^2, respectively, after 72h. While on growth factor (GF)

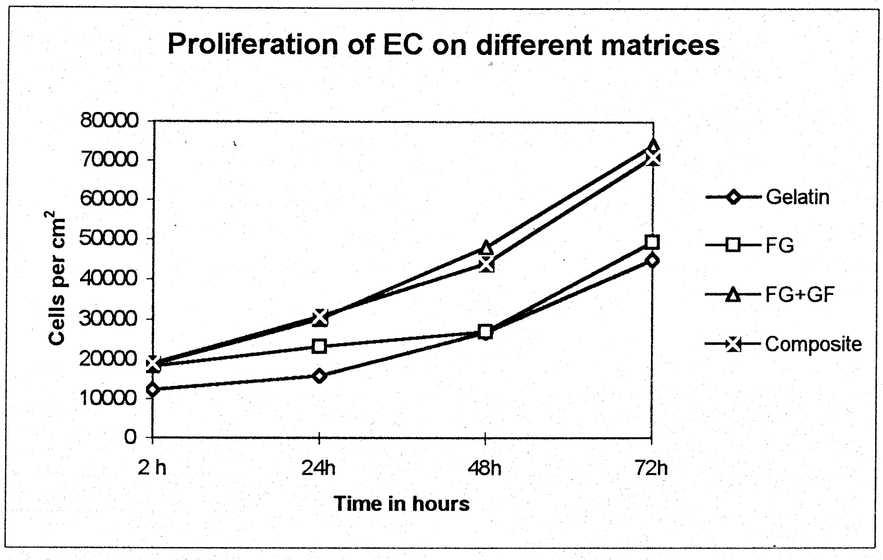

Fig.III.4.1. Graph showing the proliferation of HUVEC on different matrix coated TCPS .

immobilized fibrin the cells have grown to confluence after 48 h and by 72 h the cell has reached to an over confluent stage with an average density of 7.4x10^4/cm^2. Likewise, on the composite coated TCPS the final density after 72 h reached around 7.1x10^4 from a initial density of 1.9x10^4/cm^2.

Representative light microscopic fields of EC layer on gelatin, fibrin, fibrin with GF and composite are shown in fig.III.4. 2 to fig III.4.9. In spite of the low attachment the proliferation of cells on gelatinized surface was satisfactory.

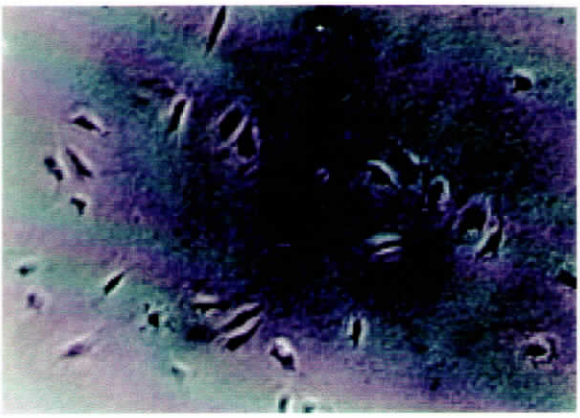

Fig.III.4.2. Photomicrograph of HUVEC, 24 h after seeding on Gelatin. The Gelatin coated TCPS shows low attachment density (Mag 100x).

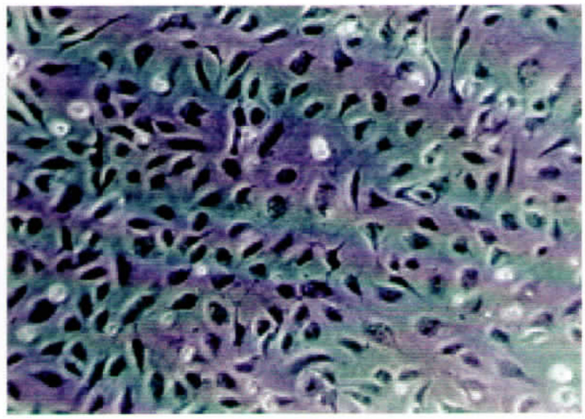

Fig.III.4.3. Photomicrograph of HUVEC, 72 h after seeding on Gelatin. The Gelatin coated TCPS holds a confluent monolayer (Mag 100x).

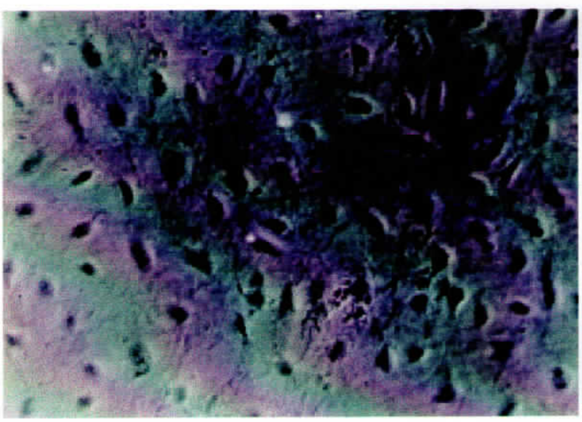

Fig.III.4.4. Photomicrograph of HUVEC 24 h after seeding on Fibrin. Fibrin coated TCPS shows more number of cells compared to Gelatin (Mag 100x).

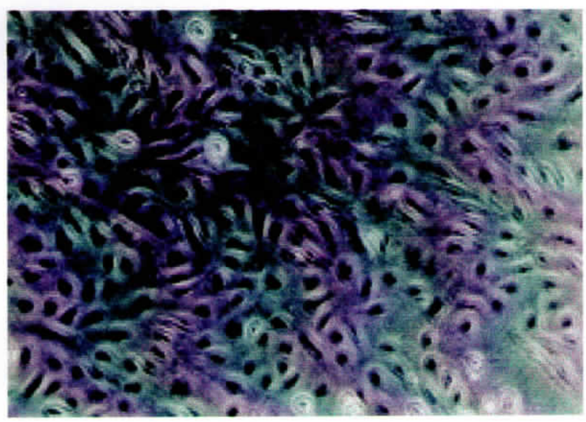

Fig.III.4.5. Photomicrograph of HUVEC 72 h after seeding on fibrin . The fibrin coated TCPS holds a confluent monolayer of EC(Mag 100x).

After seeding, the cells grown on Gelatin reached near confluent state on 3rd day. (Fig III.4.2.& III.4.3).The growth curve shows that with a cell density of $4x10^4$ /cm^2, the cells on (F + GF) reached a near confluent stage by 48 h and the cells showed sprouting morphology due to overgrowth and lack of space at 72 h (Fig III.4.6 & III.4.7).

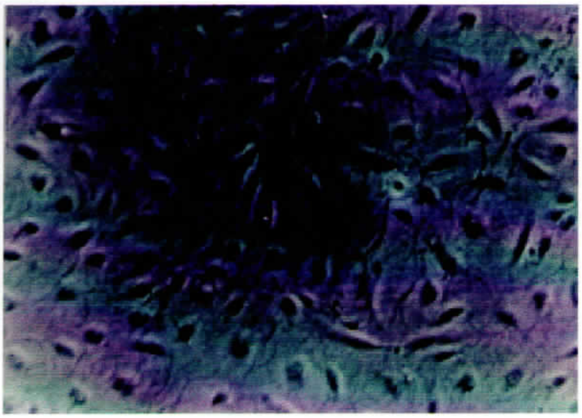

Fig.III.4.6. Photomicrograph showing status of EC seeded on Fibrin+GF. The cell attachment on this matrix immobilized with GF is similar to that on fibrin (Mag 100X).

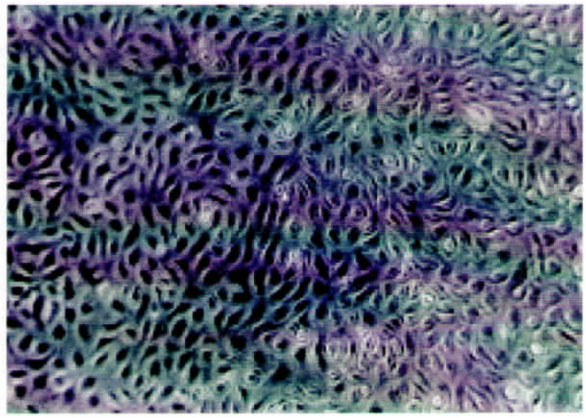

Fig.III.4.7.Photomicrograph of EC monolayer on (Fibrin + GF), 72 h after seeding. The GF immobilized with Fibrin on TCPS resulted in good cell growth (Mag.100X).

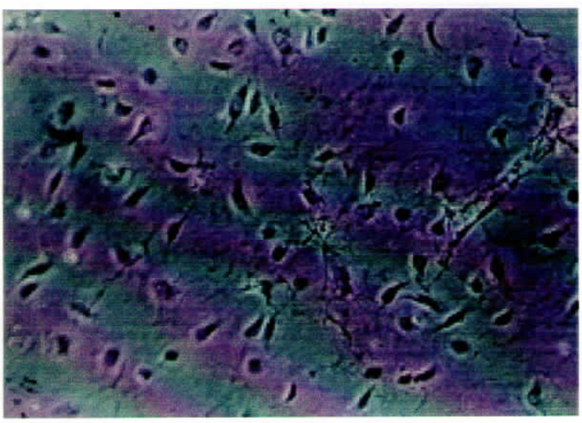

Fig.III.4.8. Photomicrograph of EC 24 h after seeding on composite of Gelatin,Fibrin and GF. The status is comparable to that on fibrin+GF (Mag 100x).

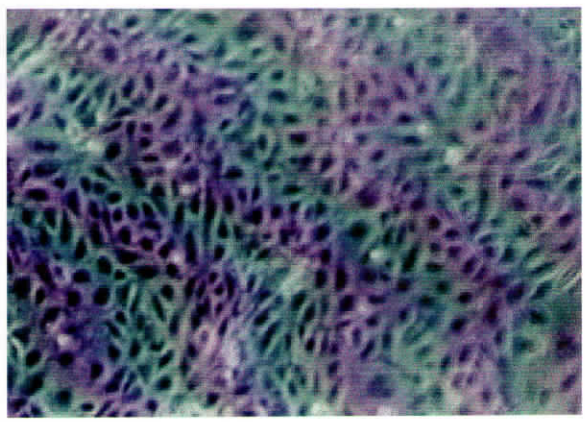

Fig.III.4.9. Photomicrograph of EC, 72 after seeding on the composite. The monolayer show an overconfluent growth on TCPS immobilized with GF, Gelatin and Fibrin. (Mag 100X).

While on fibrin without GF, only 60-70 % of the area was covered with EC by 48h (not shown) by 72 h on both gelatin and fibrin matrices the cells have reached to the confluent stage(Fig.III.4.3 & III.4.5). In the case of composite the cell density has reached to an over confluent stage (Fig.III.4.8 & III.4.9).

The average doubling time calculated for the cells grown on fibrin alone was 42 h and least on fibrin containing GF with 24h (Table No. II.2). For the cells on gelatin and composite, the doubling time was 31h and 26h, respectively, with the seeding density as indicated above . So the doubling time of cells grown on composite and fibrin +GF were similar.

III.4.2. By Uptake of Tritiated Thymidine (^3H).

The proliferation rate monitored with tritiated thymidine showed that there was 23% more proliferation on gelatin coated surface, and 16% more on fibrin coated

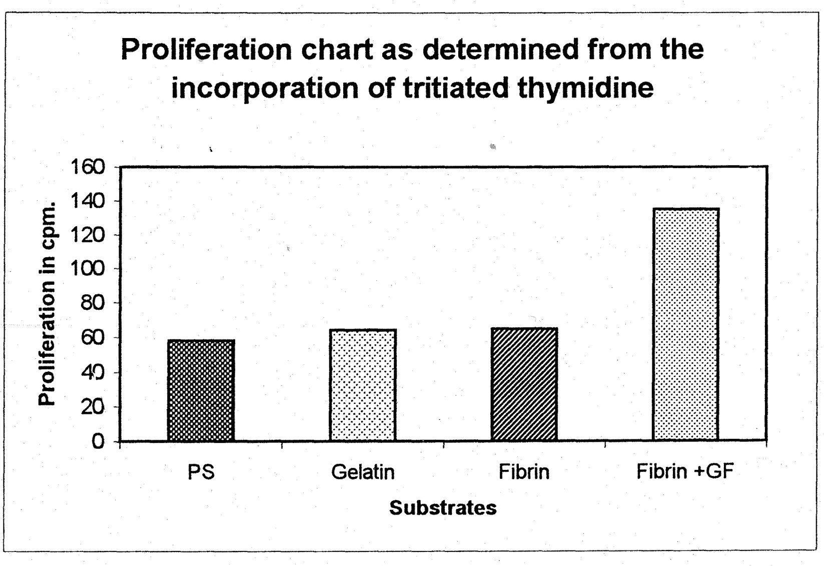

Fig.III.4.10. Bar diagram showing the proliferation of HUVEC on different substrate-coated TCPS as determined from the uptake of tritiated thymidine.

surface, as compared to bare polystyrene. On the other hand, on ECGF incorporated fibrin, proliferation rate was higher by 150% (Fig.III.4.10).

III.5. Cell Growth on Freeze Dried Matrices

A slight improvement in HUVEC attachment and spreading was noted on the freeze dried and stored matrix compared to freshly prepared matrices, irrespective of its composition. The cell attachment ranged from 93-97 % of the seeded cells even after storage of the matrix coated flask at 4° C for 3 months. Occasionally, minute microscopic bare areas were noted on the FD samples. These are created probably by the rupture and displacement of bubbles formed during polymerization which were subsequently broken during freeze-drying process. In these areas, though the cell attachment was poor initially, later on the cells migrated to fill the gap.

The proliferation rate of cells on FD matrix was found better than that of the fresh matrix containing GF. The doubling time calculated for the HUVEC grown on FD fibrin containing GF was 21.4 h which is slightly lower than that on the similarly coated fresh matrix. Such difference in doubling time was noted in FD composite, where the doubling time was improved from 26 h to 19.5h due to freeze drying. Starting from a cell density of 1.8×10^4 /cm^2, the density of the HUVEC reached to 8.2×10^4 /cm^2 after 72 h, showing a clear overconfluent monolayer in the freeze dried fibrin containing GF matrix (not shown). But in the case of freeze dried composite the cell density attained 9.0×10^4 /cm^2 after 72 h of seeding (Fig.III.5.1)

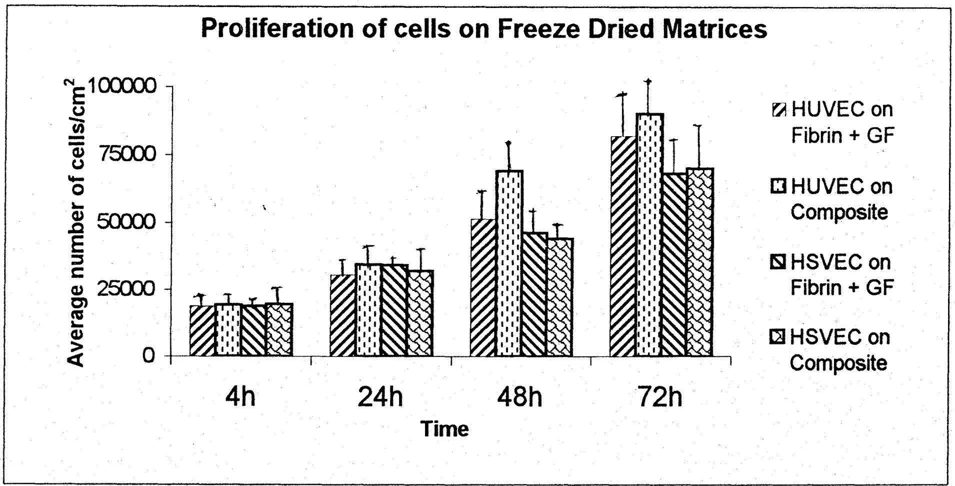

Fig.III.5.1. Bar diagram showing the proliferation of HUVEC and HSVEC on Freeze dried fibrin containing GF and composite coated TCPS. Values are average ± SD (n=5).

In the case of saphenous vein endothelial cells, there was no significant level of difference in the attachment of cells on the different substrate coated TCPS. The proliferation of 2-5 passage cells also yielded more or less comparable cell proliferation on this matrix . The only difference noted was the minimum number of cells required for getting a monolayer was slightly higher (7-8 x10^4 cell/cm^2). The doubling time calculated for the cells grown on the matrices (fibrin + growth factor), and composite were 27.1 and 27.6 respectively. Thus it is established that the growth rate of HSVEC, was slightly slower compared with HUVEC when same matrix and conditions of growth were maintained. The freeze drying technique enables preparation and storage of ready- to-use matrix coated biomaterials for tissue engineering applications.

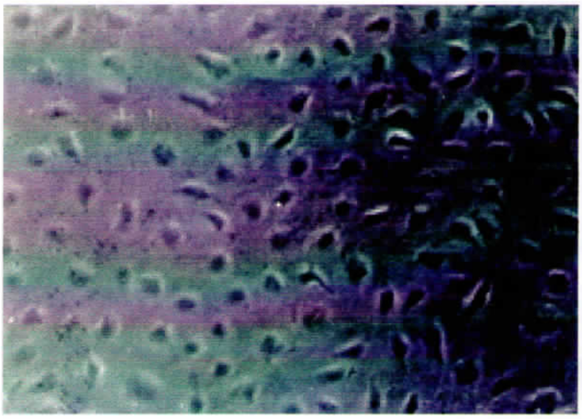

Fig. III.5.2. Photomicrograph of HSVEC, 24 h after seeding on Freeze Dried (F+GF). The cell attachment to this matrix-immobilized TCPS is intact with good spreading.(Mag 100x)

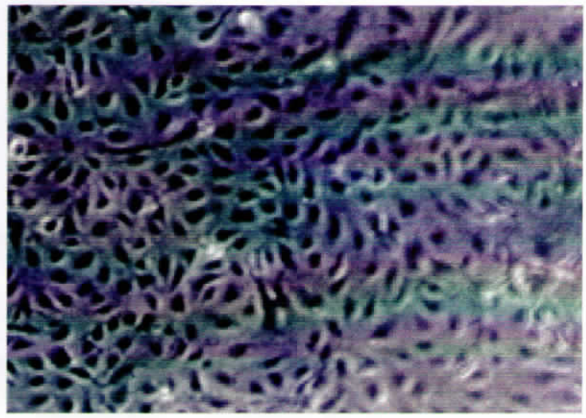

Fig. III.5.3. Photomicrograph of HSVEC, 72 h after seeding on Freeze Dried(Fibrin+ GF). The proliferation on this matrix-immobilized TCPS resulted in confluent monolayer.(Mag 100x).

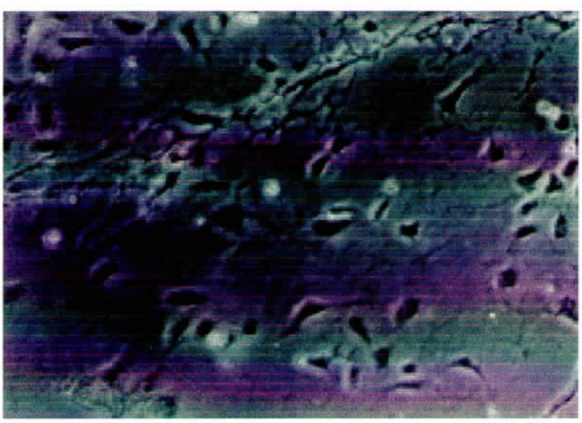

Fig. III.5.4. Photomicrograph of HSVEC, 24 h after seeding on Freeze Dried composite. The matrix supported good cell attachment and growth (Mag 100x).

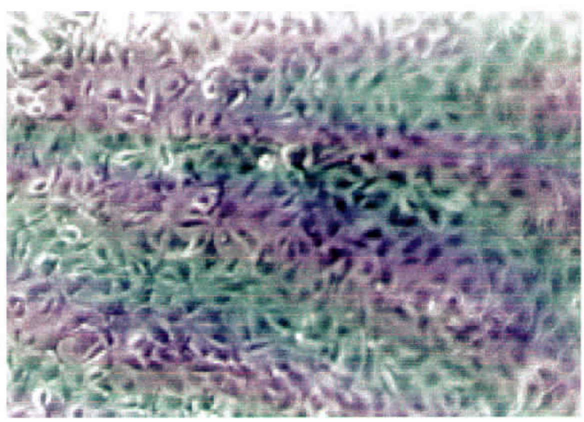

Fig. III.5.5. Photomicrograph of HSVEC, 72 h after seeding on FD Composite. The proliferation on the composite coated TCPS resulted in a tightly packed monolayer (Mag 100X).

Type of cell	Matrix	Average doubling time (hrs)
HUVEC	Gelatin	31.0
	Fibrin	42.0
	(Fibrin+ GF)	24.0
	Composite	26.0
	Freeze dried (F+GF)	21.5
	Freeze dried Composite	19.5
HSVEC	Freeze dried (F+GF)	27.1
	Freeze dried Composite	27.6

Table.III.2. Average doubling time calculated for HUVEC and HSVEC. The cells grown on different matrix coated TCPS for a period 72 h with a initial cell density of $2x10^4$ cells/cm^2, were harvested and counted to calculate the data given.

III.6. Kinetics of Monolayer Formation On FD Matrices

The cell densities used to plate and the time required for formation of a monolayer on fibrin with gelatin and GF (FD) is shown in the table III.3. With an initial cell density of 6.5 x 10^4/cm^2 is plated, HUVEC monolayer can be obtained within 4 h while for HSVEC it requires nearly 7.5 x10^4 cells/cm^2 for getting the monolayer within 4 h. Actually, this is only the time required for sufficient attachment , spreading and maturation of the cytoskeletal elements because this density doesn't necessitate the proliferation to become a monolayer. With a cell density of 2x 10^4 cells/cm^2, it nearly took around 50 h for HUVEC and 72 h for HSVEC for complete endothelialization. ?

Seeding density/cm^2	HUVEC	HSVEC
2 x 10^4	~ 50h	~72h
4 x10^4	~24 h	~48h
6 x 10^4	~4h	~56h
7 x 10^4	~4h	~4h

Table.III.3. Time interval for monolayer formation w.r.t. seeding density for HUVEC and HSVEC. Both the cells were grown on FD composite.

The HUVEC has been used to standardize the effectiveness of the matrix in providing a non thrombogenic, stable scaffold for the cell adhesion and proliferation, where as *in vivo* application one has to use autologous endothelial cells from the available blood vessel. Table.III.3 shows that there is marked difference in proliferation rate of HSVEC on both the standardized matrices compared to that of HUVEC. Starting from a cell number of 4 x10^4 cells, the cell density reached to 24x10^4 after 7 days. Ten more days culture in composite coated TCPS yielded ~ 30x10^5 cells which is found enough to cover nearly 50 cm ^{2} surface area. Thus a 5 cm piece of HSVEC yielded enough cells to seed 20 cm of 4 mm diameter vascular graft within 17 days of *in vitro* culture. As it is always better to give 24-48h for the finally seeded cells on the graft for cytoskeletal maturation, the overall time required to get an endothelialized graft of length 20 cm, (4 mm diameter) is estimated to be 18 to20 days, with the standardized culture condition.

III.7. Monolayer Repair of HSVEC

The scratch injury made on the monolayer (Fig III.7.1) was found repaired by 24h (Fig.III.7.2). The result from this experiments enables to estimate the response to injury of the monolayer transplanted on the graft that might occur during surgical procedure. Wounds that are only a few cells wide, repair themselves through cell spreading. Medium sized wound require both migration and proliferation of the cells to fill in the defect(Reidy *et al.* ,1981). However, as in this study it took only ~ 24 h to fill a 30 cell wide injury on the composite coated surface, where the multiplication time for the cell in the particular monolayer is around 27 h (Table. III.2). Therefore, it is likely that the migration as well as spreading may be the major mechanisms in the healing process. In the initial 3 h after injury the cells located at the leading edge of the wound undergo well characterized architectural and cytoskeletal changes noted during directed cell migration.

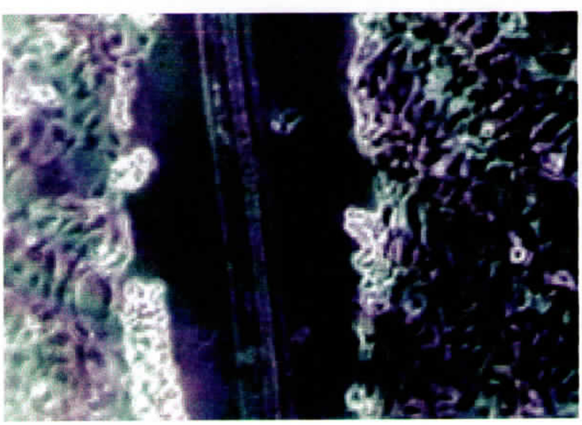

Fig III.7.1. Monolayer of HSVEC grown on TCPS Coated with FD composite after induction of injury (Mag 100x)

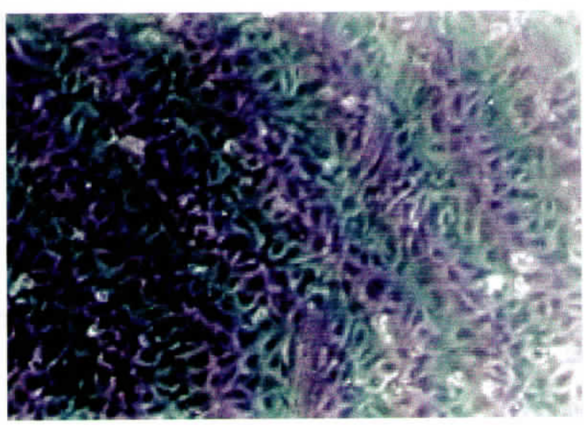

Fig III.7.2. Repaired monolayer of HSVEC grown on TCPS Coated with FD composite 24 h after injury. The arrow indicates the mark formed on the TCPS during induction of scratch injury, under the monolayer (Mag100x)

However, it is not clearly understood whether a damage will lead to enhanced proliferation on the injured site mediated by locally released GF from the cell. This is possible because the area filled by the repaired cell is much more than that can be repaired by mere migration and spreading.

III.8. Cell Growth on Biomaterials

III.8.1. Vascular graft material

The correlation between the confluent cell density and the time to get monolayer on different substrates has been standardized as per § III.5 & § III.6. As the attachment of cells on uncoated vascular biomaterial was poor, this substrate has not been included for further studies. Even though, the attachment and proliferation of HUVEC on gelatin coated graft was poor, this has been evaluated only for a comparative study. Likewise, the proliferation obtained with fibrin without GF was not satisfactory, and this adhesive has also been eliminated from further studies further study. It has also been noted that the attachment and proliferation of cells on both the freeze dried samples are better than that of similar compositions of fresh matrices and hence only the FD matrices is evaluated for cell proliferation studies on biomaterials.

The biomaterials, Dacron and PTFE could be endothelialized successfully in a flat piece or in a tubular form, provided the appropriate matrix proteins are immobilized (Figs. III.8.1 to III.8.12)

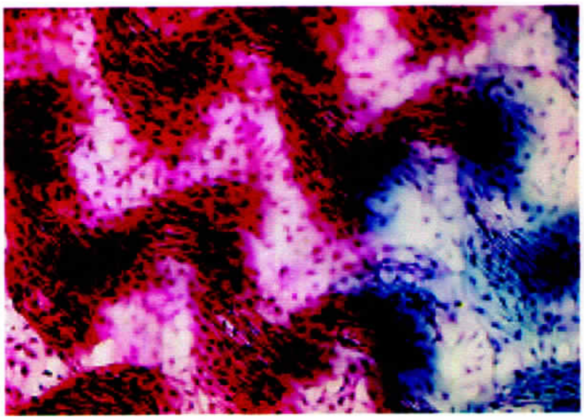

Fig.III.8.1. Photomicrograph of HUVEC grown on (Fibrin + GF)-coated Dacron graft pieces. Seeding was done with an initial density of $3x10^4$ cells/cm^2 and monolayer is viewed after 48 h of plating (Mag 50X)

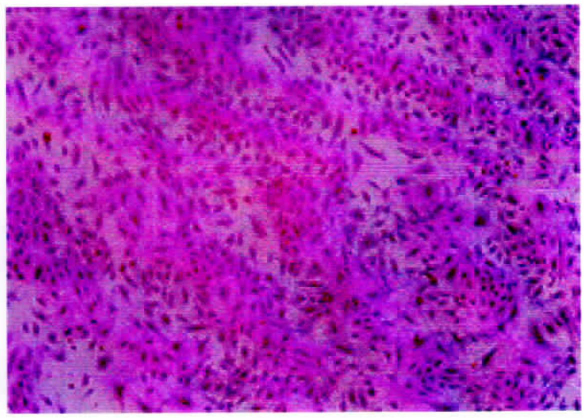

Fig.III.8.2. Photomicrograph of HUVEC grown on (Fibrin + GF) coated PTFE graft pieces. Seeding was done with an initial density of $3x10^4$ cells/cm^2 and monolayer is viewed after 48 h of plating (Mag 50X).

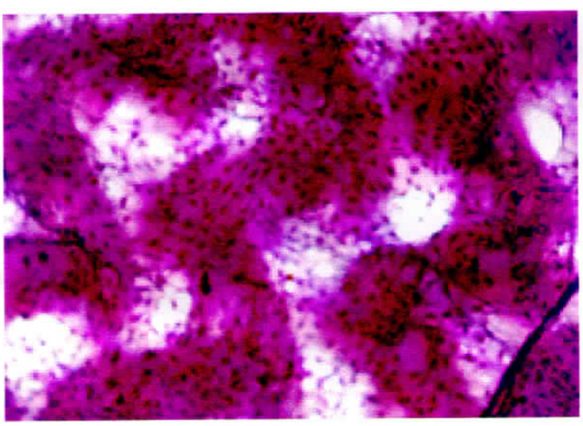

Fig.III.8.3. Photomicrograph of HUVEC grown on FD Composite coated Dacron graft pieces. .After seeding with an initial density of $3x10^4$ cells/cm^2 , the cells were grown for 48 h (Mag 50X).

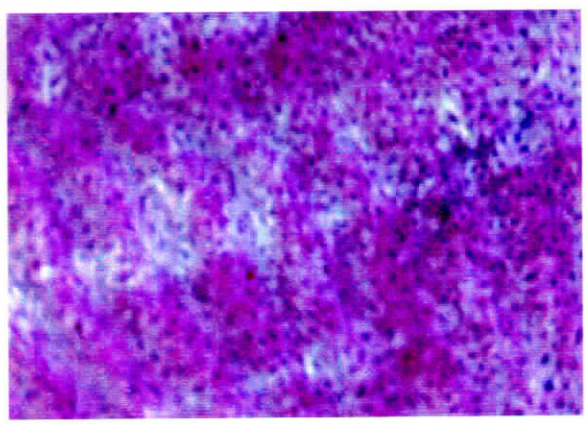

Fig.III.8.4. Photomicrograph of HUVEC grown on FD Composite coated PTFE graft pieces. After seeding with an initial density of $3x10^4$ cells/cm^2, the cells were grown for 48 h (Mag 50X).

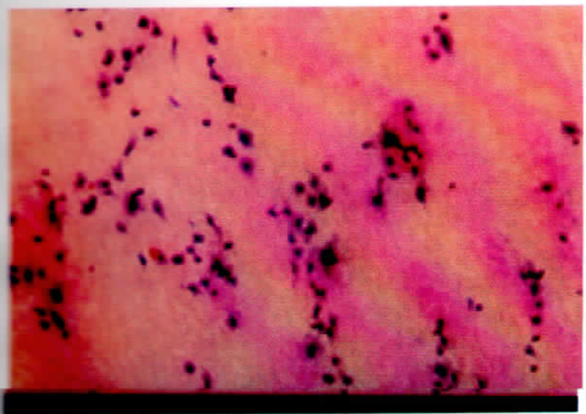

Fig.III.8.5. Photomicrograph of HUVEC grown on Gelatin coated PTFE graft pieces. Cells were viewed 48 h after seeding done with an initial density of 30×10^3 cells/cm^2. (Mag 50X).

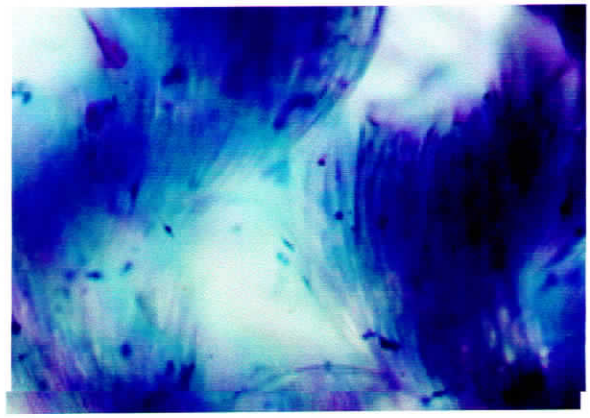

Fig.III.8.6. Photomicrograph of HUVEC grown on Gelatin coated Dacron graft pieces. Cells were viewed 48 h after seeding with an initial density of 30×10^3 cells/cm^2 (Mag 50X).

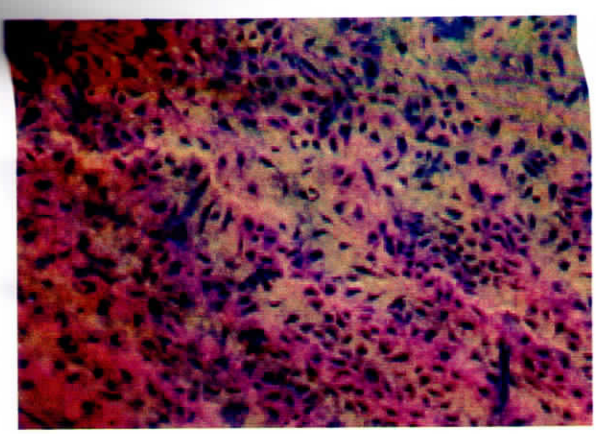

Fig.III.8.7. Photomicrograph of HUVEC grown on Composite(FD) coated PTFE graft in 6mm vascular conduit. The seeding was done with an initial density of 7×10^4 cells/cm^2 and grown for 48 h. The crack in the composite may be caused during fixation of the sample on to the slide. (Mag 50X)

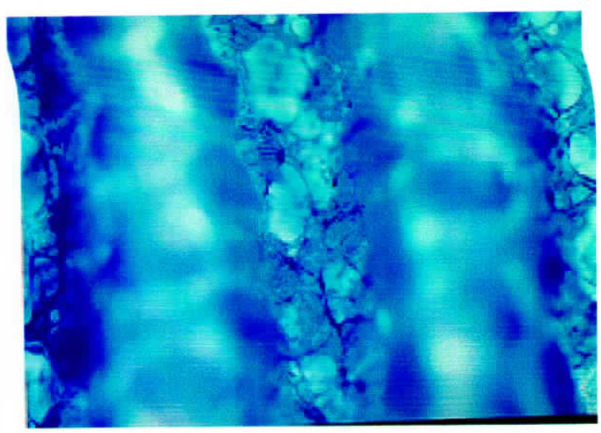

Fig.III.8.8. Photomicrograph of HUVEC grown on Composite (FD) coated knitted Dacron graft conduit. Seeding density of 7×10^4 cells/cm^2 was grown for 48 h. As the graft is knitted the cells occupy on different plane making it difficult to be focused in the same field. (Mag 50X)

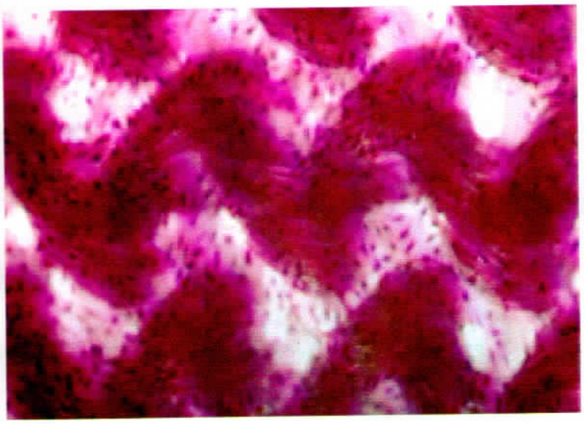

Fig.III.8.9. Photomicrograph of HSVEC grown on Fibrin + GF (FD) coated Dacron graft pieces. Seeding was done with an initial density of $3x10^4$ cells/cm^2 for 48 h .

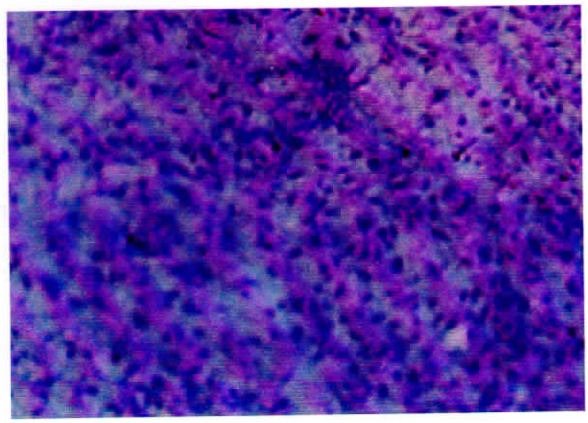

Fig.III.8.10. Photomicrograph of HSVEC grown on Fibrin + GF (FD) coated PTFE graft pieces. Seeding was done with an initial density of $30x10^3$ cells/cm^2 for 48 h .

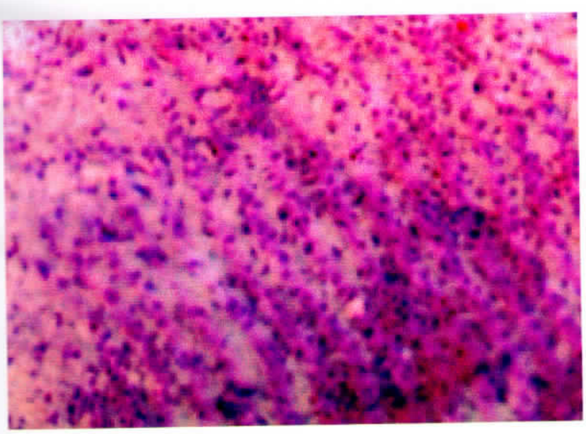

Fig.III.8.11. Photomicrograph of HSVEC grown on Composite (FD) coated PTFE graft pieces. An initial density of $3x10^4$ cells/cm^2 was seeded and viewed after 48 h (Mag 50X)

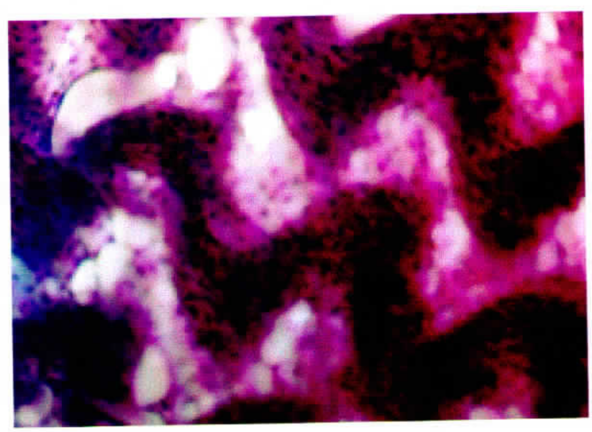

Fig.III.8.12. Photomicrograph of HSVEC grown on Composite (FD) coated Dacron graft pieces. Plating was done with an initial density of $3x10^4$ cells/cm^2 and grown for 48h (Mag 50X).

There was no noticeable difference in cell growth on FD (Fibrin + GF) or composite coated graft compared to the growth on the similarly coated TCPS, irrespective of the cell type. This indicates that when the standardized conditions were used, cell growth was dependent on the biomaterial used, but the matrix compositions influenced the growth. Mainly, it is the composition and the coating technique and stabilization of the matrix that matters for successfully attaining the monolayer. The major difference noted was the poor adhesion as well as proliferation on gelatin coated vascular grafts (Fig .III.8.5.& III.8.6), compared to that of gelatin coated TCPS (Fig.III.4.2 & 4.3). Even though gelatin has not got much attention as a coating substrate for endothelialization of biomaterials, the insoluble form of collagen is reported to have good cellular adhesion and spreading (Bordenave *et al.*, 1999). The use of gelatin for coating of TCPS has been a general practice for culture of EC, for other studies. The reason for the difference of adhesion on graft and TCPS coated with gelatin may be because of the differential adsorption of gelatin to both the surfaces.

The advantage of the composite is that irrespective of the physico chemical properties of the material, it could be immobilized. The difference in time and density noted in getting a monolayer between HSVEC and HUVEC, may be explained as the variations in the physiological status of both the cells prior to isolation.

The cells grown on knitted Dacron and PTFE in tubular form also showed complete endothelialization under light microscope. But because the cells on knitted Dacron occupies different plane after fixation on microscopic slide it was not possible to photograph even under small magnification (Fig.III.8.8). The PTFE graft was mostly covered with the monolayer, the occasional cracks noted on the surface, is probably caused during stretching of the graft, when prepared for microscopy (Fig.III.8.8.).

III.8.2. Other Vascular Material

The cell growth on composite coated and bare UHMWPE heart valve disc, Titanium and Diamond like carbon coated Titanium heart valve discs are compared . The representative photomicrograph of different material with out any coating, after 48 h of seeding with a density of 3-4 x 10^4 cells/cm^2 are shown in the figs.III.8.13 to III.8.18. There was no significant change in attachment and proliferation of cells noted between coated and non coated materials in the presence of serum containing medium. The only difference noted is in the nature of monolayer organization. From the picture it seems that the cells on the composite coated surface are well attached and organized so that it could maintain the integrity with uniformly organized cytoskeletal elements even after fixation. While the cells grown on uncoated materials have tendency to contract during the fixation process probably because of lack of enough adhesiveness with the surface. But compared to vascular graft polymeric materials the cell attachment and growth are more or less same as that of composite coated surface. This may be because of the difference in protein adsorption from the serum supplemented with the culture medium as well as the surface topography. The surface of the vascular graft materials have been developed to reduce the adhesive protein binding in order to reduce the platelet activation and adhesion. While the protein adsorption status of the metals especially Ti and DLC-coated Ti is not well understood. It seems that these surfaces may retain optimal quantity of proteins for the subsequent cell adhesion and proliferation. Unlike the vascular graft, the surface of these discs are highly polished and being metals, are likely to adsorb adhesive proteins like fibronectin from the serum.

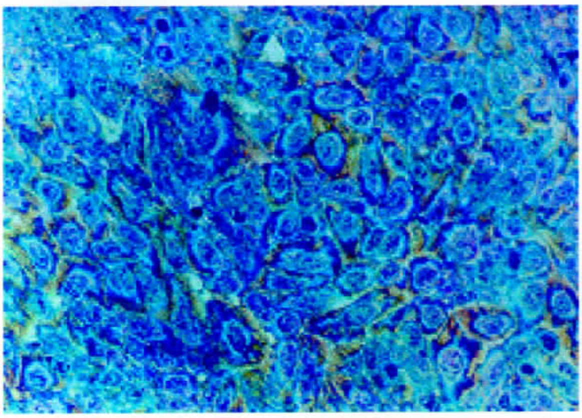

Fig.III.8.13. HUVEC grown on bare DLC-Ti. An initial cell density of 4×10^4 /cm^2 was seeded and 48h later fixed, stained and looked under incident light. (Mag 100X)

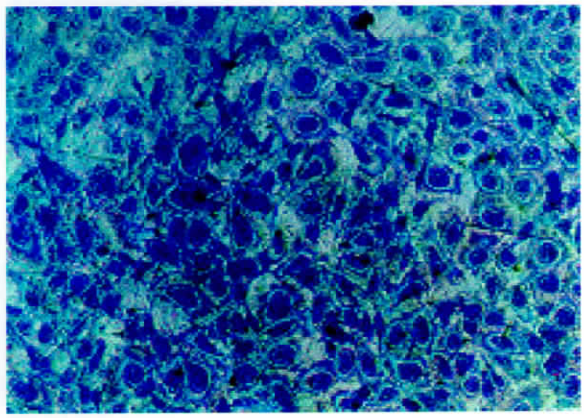

Fig.III.8.14. HUVEC grown on composite coated DLC-Ti. An initial cell density of 4×10^4 /cm^2 was seeded and 48h later fixed, stained and looked under incident light. (Mag 100X)

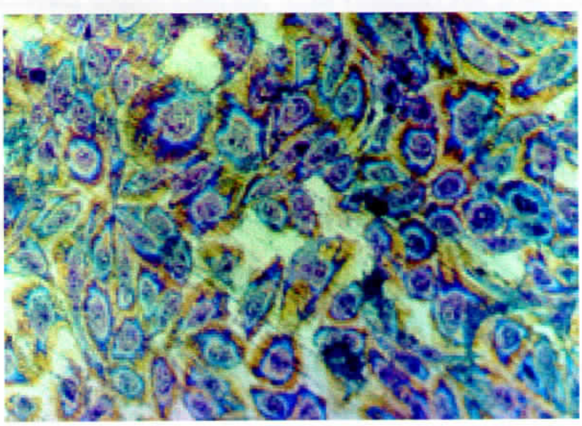

Fig.III.8.15. HUVEC grown on bare Ti with a initial cell density of 4×10^4/cm^2 for 48 h seen under incident light.
(Mag 100x)

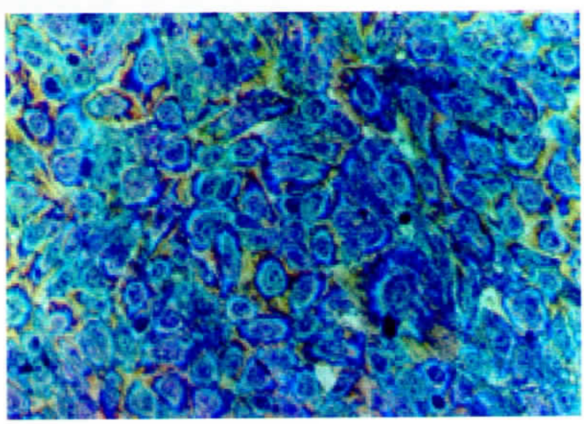

Fig.III.8.16. HUVEC grown on composite-coated Ti with an initial density of 4×10^4 cell/cm^2 for 48 h seen under incident light.
(Mag 100x)

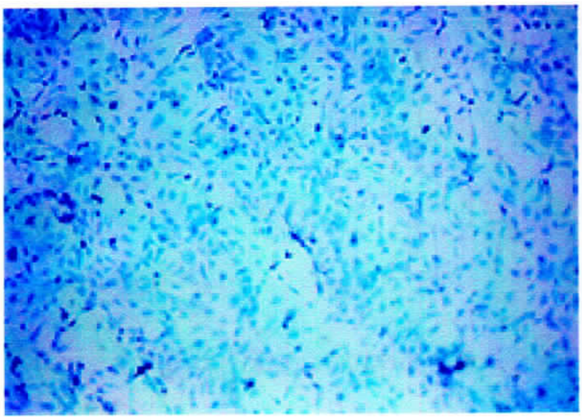

Fig.III.8.17. HUVEC grown on bare UHMWPE disc. An initial cell density of $4x10^4$ cell/cm^2 were seeded and 48h later cell were stained and viewed under incident light. (Mag 50X)

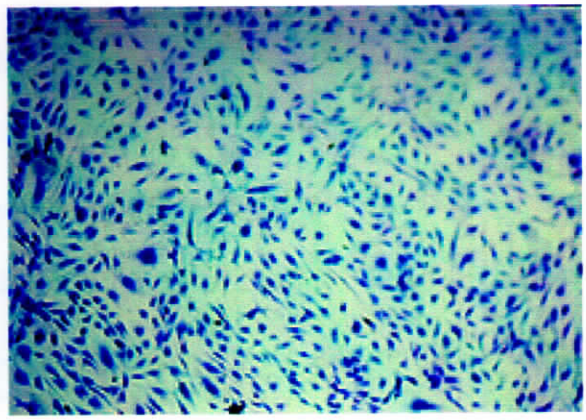

Fig.III.8.18. HUVEC grown on composite coated UHMWPE disc. An initial cell density of $4x10^4$ cell/cm^2 were seeded and 48h later cell were stained and viewed under incident light (Mag 50X).

While the morphology of EC grown on the metals maintained cobblestone morphology independent of the presence of precoated adhesive matrix, the cells on UHMWPE, appeared with varied elongated morphology, on both bare and matrix coated experiments. The reason for this behavior is currently not understood.

Some of the earlier experimental studies of EC seeding has been done with high seeding densities (Zilla *et al.* 1987). In such a situation because of the near confluent density, the cells may be able to synthesize its own ECM and enough growth factors thereby modulating its attachment, spreading and proliferation irrespective of the protein coating. But for real clinical *in vivo* situation the number of cells actually available is very few and so optimum attachment and proliferative conditions should be provided exogenously while low seeding densities such as less than 2 $x10^4$/cm^2 only is possible. In such a situation in addition to the optimum cell adhesion , the matrix should promote the proliferation of the cell optimally to reduce the un necessary waiting time to get the monolayer. Kaehler *et al.* (1989) compared human endothelial cell attachment to PTFE surfaces coated with either fibronectin, laminin, type 1/111 collagen or fibrin glue and found that cell adhesion and spreading was superior on surfaces coated with a combination of fibronectin and collagen and fibrin glue. However, they noted considerable fibrinolysis and supplemented ε-aminocaproic acid to inhibit fibrin degradation products (FDP). In our experience no detectable level of FDP was present in the culture medium up to, 12 days after the coating in the presence or absence of cells when HUVEC was used. Microscopically also intact fibrin network was found after seeding and growing EC. Mazzucotelli *et al.* (1991) mechanically harvested Human Saphenous Vein Endothelial cells and seeded on PTFE and Dacron coated with trans glutamine which contains fibrinogen, von Willebrand Factor and other proteins and reported the advantage of this matrix over that of fibronectin. Henrich *et al.* (1995) compared two commercially available fibrin sealant for HUVEC adhesion and proliferation. They have reported that Factor XIII from Beriplast did not affect adhesion but reduced the proliferation rate.

Here also it is noted a reduction in growth during the initial 48 h for cells seeded on fibrin compared to that on gelatin. Currently, it is not clear why EC adhered to fibrin proliferates slowly. On the fibrin with out GF, the proliferation of HUVEC is not found satisfactory even in the presence of 20% serum especially in the initial stage of seeding with lower density. The proliferation rate on gelatin coated surface was slightly better than that of fibrin in the early stages. The reason for slow proliferation of cells, even though adhesion was high, cannot be fully explained on the basis of high adhesive strength of the matrix. In the same matrix, when incorporated with GF the proliferation rate could be significantly increased. Therefore, strong anchorage dependent inhibition of proliferation may not be the likely mechanism.

It is known that fibrin gels are involved in wound healing and angiogenesis by accelerating the proliferation and migration of cells which are actively involved in wound repair process like endothelial cells. But during the *in vivo* wound healing process, the wounded area is highly complex with localized presence of various chemotactic and mitogenic factors released probably by the cellular components. On the other hand, the fibrin matrix acts as a scaffold to entrap the released components thereby localizing the factors needed for cell growth in addition to acting as a cell support on which the cells can migrate. These are some of the important facts which has not properly taken into account by earlier reported studies. More importantly, the preparation of fibrin for clinical application involves two components and one of the components the thrombin is a known protease, having wide range of activation potential for various cells. Earlier, other workers have evaluated fibrin as a layering medium for *in vitro* and *in vivo* endothelialization prepared by standard method. *In* one of the studies Heinrich *et al.* (1992) used fibrin glue as layering medium for *in vitro* endothelialization. The technique used was forcing the fibrinogen solution through the luminal side followed by immersing in thrombin. This procedure is followed by one more dipping in thrombin solution. But this technique is likely to trap excessive thrombin by the initially formed fibrin which may adversely affect the cell and the surface thrombogenicity . So the preparation of fibrin for coating purposes should be done carefully. Based on the pilot studies studies it was decided to first saturate the surface with thrombin (Santhosh & Krishnan, 1998) and subsequently the adsorbed thrombin was used to polymerize the fibrinogen layered over it. This technique reproducibly yielded thin and uniform coating of fibrin or composite irrespective of the material surface. In this method the cells are unlikely to be exposed to thrombin as most of the adsorbed thrombin may have been consumed for fibrin generation and polymerization.

Earlier, Zilla *et al.* (1987) has successfully seeded EC on vascular grafts that had been pretreated with another fibrin sealant, but they used higher seeding density, and noted considerable cell loss due to apparent fibrinolysis of the fibrin glue under shear stress. Here no fibrin degradation was detected even 7 to10 days of growth in the matrix in static conditions with HUVEC. But in the case of HSVEC there was slight quantity of FDP detected after three days of culture of cells in the near confluent stage. But the degradation was less than 0.1% and the matrix retained its intact morphology even during trypsinization. The discrepancy may be because of the difference in the method of preparation of fibrin and variability of composition. But by this time, ECM may have been laid on the surface that might enhance adhesion.

When the matrix was in freeze- dried condition the proliferation rate was slightly enhanced than that of the freshly prepared matrix. This may be because in the dried condition, it may take up more GF from the medium which is unlikely to happen in the case of wet freshly formed fibrin or composite. The local high concentration of GF available within the matrix has considerably improved the proliferation and on all the graft pieces the same level of cell proliferation is attained. This demonstrate that, for such situations, fibrin glue with ECGF immobilized in the fibrin net work or composite can be an excellent atmosphere for cell attachment and growth on any vascular biomaterial.

Cell adhesion, spreading and proliferation are determined by multiple factors. For e.g. low proliferation of EC on fibronectin even with good cellular adhesion has been noted by various workers. Precoating of PTFE with FN improved the adhesion of HUVEC, but the cell spreading remained incomplete and with time all the initially adhered cells detached. This may be because of insufficient FN adsorption on biomaterial. The low affinity of FN for Dacron or Teflon has been reported(Van Wacem *et al* . 1987). If the adhesion is too high the cells cannot retreat the pseudopodia to enter into mitotic stage. This definitely affects the migration of cells and subsequent proliferation. In this study if the cells were grown on gelatin alone, the proliferation was slightly better than that on fibrin alone even though the adhesion was not sufficient on gelatin- coated TCPS and worst on Gelatin coated Dacron or PTFE.

Promoting

Whole extracellular matrix has gained much attention because of its high attachment as well as proliferative capacity. The presence of fibronectin, collagen, some glycosaminoglycans and GF are documented within the ECM (Hugo, 1987). The highly thrombogenic nature of some of the components of the ECM such as collagen, and difficulty in obtaining the matrix are two important drawbacks. But still this emphasizes the importance of the presence of multiple factors for cell adhesion as well as growth. In the composite that is developed in the present study, that contain in addition to GF, gelatin also, yielded much better results in terms of cell proliferation irrespective of the material surface used. In this matrix also the proliferation rate was better on FD matrix compared to the fresh matrix. One of the remarkable difference noted with the use of matrix is that the cells seeded on composite organized its cytoskeletal elements in very limited time period. The cell spreading and organization on the FD composite coated graft was complete by 8 h when a near confluent density was provided (Fig. III.8.19).

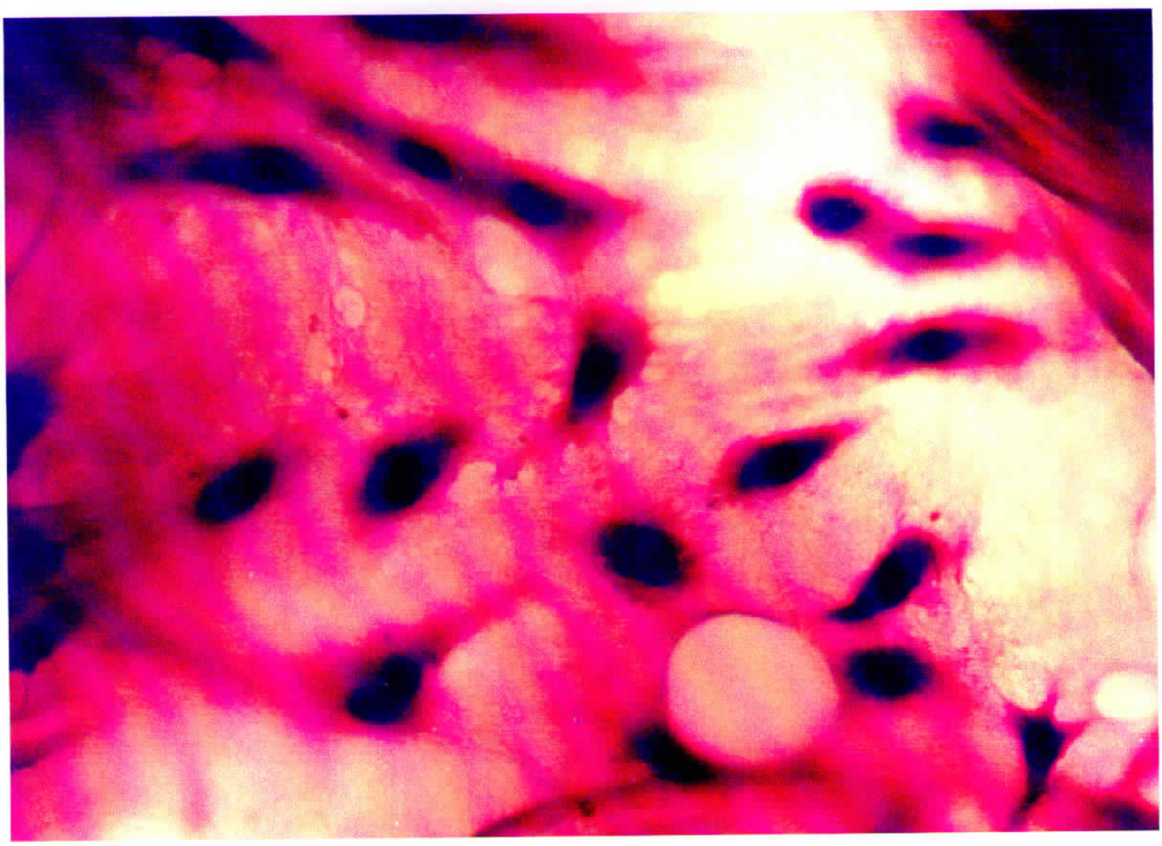

Fig.III.8.19. Nature of spreading of HUVEC on composite coated Dacron 8 h after seeding in near confluent state. (Mag 200X)

Endothelial cells express multiple integrins including $\alpha_v\beta_3$ which mediates adhesion to a number of glycoproteins including vitronectin , thrombospondin, vWF, fibrin, collagen etc. (Leslie *et al.* 1992). As the composite matrix contains most of these proteins including gelatin, the strength of attachment and spreading will be possibly enhanced than that of the fibrin or gelatin alone.

Eventhough, superficially some gaps formed during freeze drying has made discontinuity of the plane, this area may have underlying matrix which cannot be focused in the field. So with time, cells migrate and completely cover these gaps as observed in other photographs (III. 8.3).

III.9. Characteristics of Matrices

III.9.1. Structural analysis of the matrix on TCPS

Qualitative analysis of freshly formed fibrin and composite under phase contrast microscope showed tightly interconnected fibers throughout the surface as shown in figs. III.9.1 & III.9.2. Dense, cross-linked, fibrin threads are tightly woven through out the surface making inter fiber distance minimal. In some areas, the bundles appear denser and such conformational change may be dependendent on the fibrin structure. The polymerization time ranged from 30 second to 2 minutes with the concentration of fibrinogen and thrombin indicated in the methods. Compared to the fibrin matrix, the fiber bundles formed on the composite are slightly denser and the number of bundles/mm^2 is less making inter bundle space slightly wider. This indicates that the gelatin incorporated with the fibrinogen is not only uniformly distributed with the matrix during polymerization, each fiber bundles are also uniformly impregnated with the gelatin thereby increasing the thickness of the bundles.

The scanning electron microscopic picture of the fibrin and composite showed the surface texture of the fibers more clearly (Fig.III.9.5 & III.9.6). Compared to fibrin, the fiber bundle thickness is more on composite while the density of network is higher on fibrin matrix. Around 60-70% of the fiber bundle thickness in fibrin distributed in the rage of 100-200 nm while in composite most of the fibers were distributed around 150-300 nm bundle thickness. During polymerization, the enzyme thrombin cleaves fibrinopeptides from fibrinogen creating fibrin monomers that assembles in a half–staggered manner to form protofibrils with two monomer thickness. The protofibrils then aggregate laterally to form fiber bundles that branch to make a network or gel. The gel density is directly proportional to the concentration of fibrinogen (Morrison *et al.*, 1947). However, the distribution of fiber bundle diameters depends on the kinetics of the self-assembly process which is affected by the concentration of fibrinogen, thrombin, other proteins that may be present and ions especially Ca^{++} (Weisel and Nagaswamy, 1992). The actual significance of the bundle thickness and density of fibers on endothelial cell attachment and proliferation has not been worked out. However Herbert *et al.* (1998) has studied the micro morphology of fibrin gels on neurite growth and arrived at the conclusion that increase in fibrinogen concentration caused a decrease in the average fiber bundle thickness and increase in the number of fiber bundles and a marked decrease in neurite length. This indicates the importance of micro morphology of the matrix in the adhesion and growth of other anchorage dependant cells also. Moreover, there is evidence that with decrease in fiber bundle diameter, fibrinolysis is retarded compared to thicker bundles (Gabriel *et al.* 1992). The fibrinolysis also is likely to influence cell migration and proliferation.

The addition of gelatin and growth factors along with the fibrinogen to prepare composite slightly prolonged the polymerization time compared to fibrin alone, not exceeding 3 min. This means that incorporation of gelatin along with fibrin is not affecting the kinetics of polymerization significantly. In this study the concentration of thrombin and fibrinogen was standardized from the initial pilot studies aiming at a uniform layer of matrix. When the concentration of thrombin or fibrinogen was increased, the polymerization time was very less leading to thick fibrin bundles at some areas leaving the other areas with loosely formed gel. With the currently used thrombin concentration it is not clearly known how much activity is bound on the surface. As always a thin layer of thrombin may be present on the surface after aspirating the excess thrombin, and polymerization rate was controlled, there was adequate time for uniform distribution of the thin layer of fibrinogen added. This helped in attaining a uniformly distributed matrix throughout the surface.

After freeze drying the matrix looked uneven under phase contrast microscope but when rehydrated with medium the gel imbibed with the fluid and regained its initial texture (Fig.III.9.3 & Fig.III.9.4). There was no change in bundle thickness or distribution noted after freeze drying process, while the surface appears slightly foggy probably because of infiltration of the viscous medium through out the matrix. The structure of freeze-dried composite as seen from the phase contrast microscope is more or less same compared to non FD composite.

The SEM pictures show the uniformity as well as thin nature of the matrix on the graft (Fig III.9.5 & III.9.6). In both composite as well as fibrin coated graft the underlying Dacron mesh is also visible underneath the matrix because of the thin nature of the coating. The thin layer should enhance cell adhesion and growth and proliferating cells synthesize its own ECM, in parallel to the degradation of the matrix thereby modulating its physiology. However, in the case of PTFE because of highly hydrophobic nature of the surface the time of initial incubation with the thrombin has increased to 1 h for attaining optimum adsorption. In the pilot studies, with 30 min of thrombin incubation incomplete coverage with the matrix was attained.

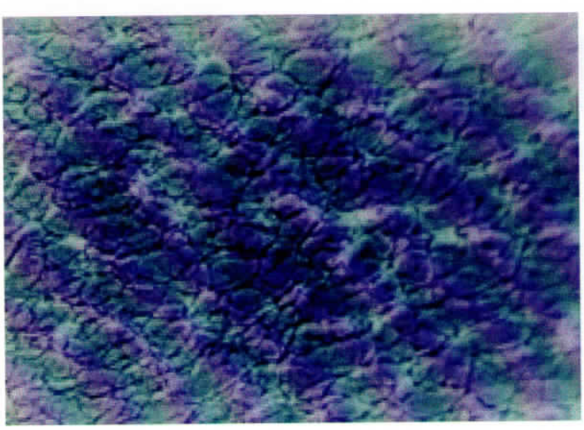

Fig.II.9.1.Phae contrast micrograph of fresh Fibrin formed on TCPS. The network looks uniform (Mag 200x).

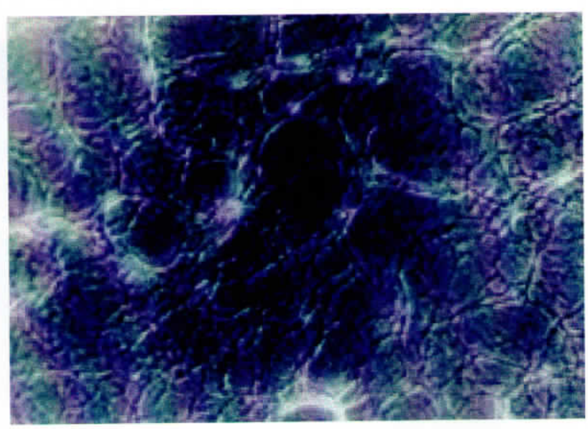

Fig.II.9.2. Phase contrast micrograph of fresh Composite on TCPS. The network looks intact with thicker bundles (Mag 200x).

The SEM pictures show the uniformity as well as thin nature of the matrix on the graft (Fig III.9.5 & III.9.6). In both composite as well as fibrin coated graft the underlying Dacron mesh is also visible underneath the matrix because of the thin nature of the coating. The thin layer should enhance cell adhesion and growth and proliferating cells synthesize its own ECM, in parallel to the degradation of the matrix thereby modulating its physiology. However, in the case of PTFE because of highly hydrophobic nature of the surface the time of initial incubation with the thrombin has increased to 1 h for attaining optimum adsorption. In the pilot studies, with 30 min of thrombin incubation incomplete coverage with the matrix was attained.

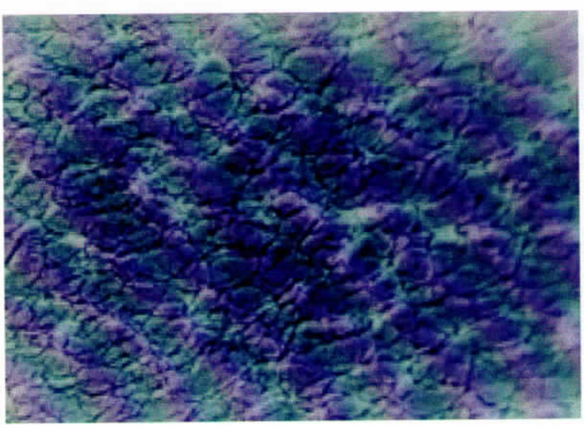

Fig.II.9.1.Phae contrast micrograph of fresh Fibrin formed on TCPS. The network looks uniform (Mag 200x).

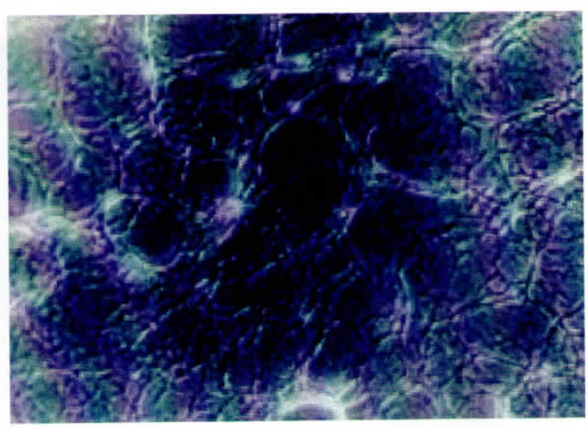

Fig.II.9.2. Phase contrast micrograph of fresh Composite on TCPS. The network looks intact with thicker bundles (Mag 200x).

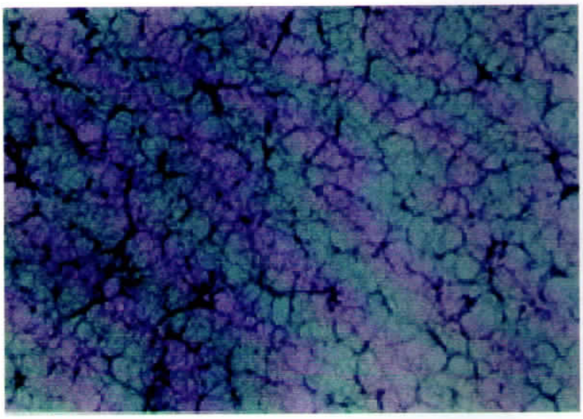

Fig.II.9.3.Phase contrast micrograph of freeze Dried Fibrin on TCPS. After re-hydrating with complete medium, the fibrils looked intact without any cracks (Mag 200x).

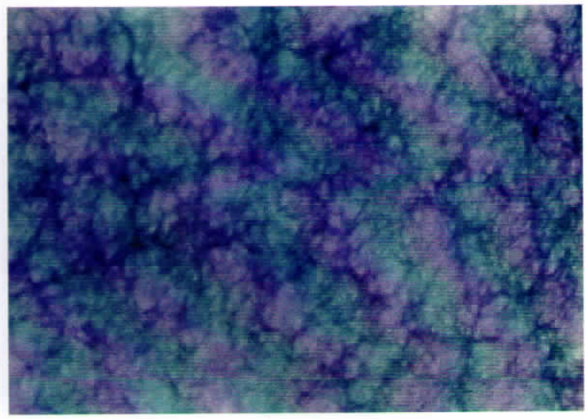

Fig.II.9.4. Phase contrast micrograph of freeze dried composite on TCPS. After rehydrating in complete medium, the network is stable and uniform (Mag 200x)

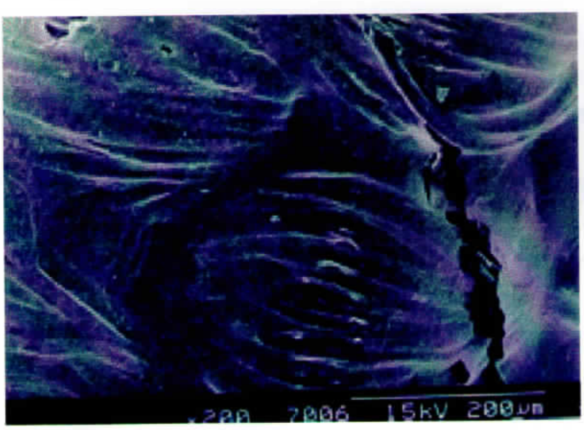

Fig.III.9.5. SEM picture showing surface texture of Dacron coated with Composite. The crack seen is probably caused during fixation of the sample on the stub for SEM (Mag 0.2 K)

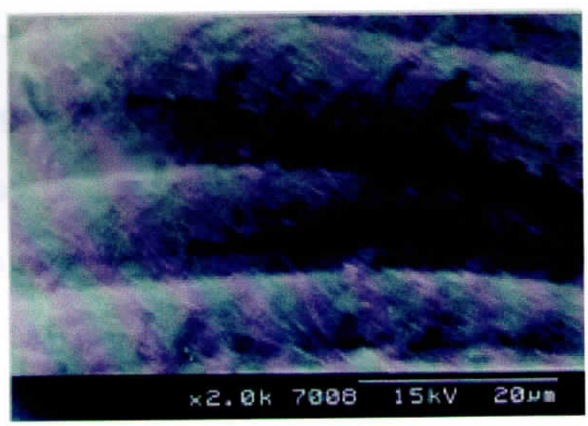

Fig.III.9.6. SEM picture showing surface texture of Dacron, coated with Fibrin. A thin, uniform coverage of the material surface is attained.

Duration	Fibrin (% of release)	Composite (% of release)
1Week	<2.5μg/day	<2.5μg/day
2 Week	<2.5μg/day	<2.5μg/day
3 Week	5-10μg/day (0.7-1.4)	10-20μg/day 1.4-2.8
4 Week	20-25μg/day (2.8-3.5)	25-30μg/day (3.5-4.2)
Cumulative for final14 days	175- 245 μg/day (3.5-4.9)	245-350/day (4.9-7)

Table.III.9.1. The FDP released from FD composite and Fibrin matrices to the complete medium in the absence of cell. The percentage of released FDP w.r.t. initial immobilized quantity, in a 7 day period is shown within the bracket.

In the presence of HUVEC also there was no detectable level of FDP noted up to 6 days of cell growth on both matrices. Later, inorder to assess the role of cells in fibrinolysis confluent monolayer was maintained for 3days on both matrices and the level of FDP was again undetectable. This indicates that HUVEC grown on fibrin and composite are not influencing the fibrinolysis.

However, both matrices when seeded with HSVEC yielded detectable level of FDP, which was directly proportional to the density of the cell on the matrix. The FDP released from fibrin matrix by the confluent monolayer over a period of 2 days was ~ 6 μg, which is less than 0.1% of the immobilized matrix. Compared to HUVEC, it seems that HSVEC is causing slight degradation of the matrix depending on the density of the cell. Eventhough, with high cell density around 2% of matrix has been degraded in 20 days, the matrix microscopically looked intact in the presence of cells even after repeated trypsinization and passaging. This may be because of the ECM that is laid on the matrix during cell growth. In both cell type the matrix with stood action of the enzyme trypsin. The fig III.9.9. & fig III.9.10 shows the matrix after 2 consecutive trypsinization and harvesting of HSVEC grown on composite. The cells are detached from the matrix and have become rounded and still the integrity of the matrix is evident underneath.

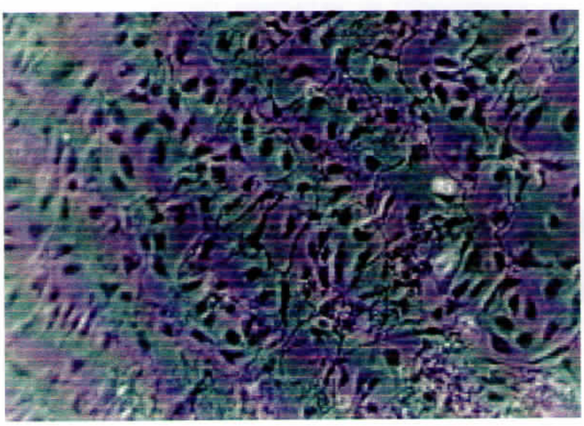

Fig.III.9.9. Phase contrast micrograph of HSVEC on composite coated TCPS just before trypsinization (Mag 100x)

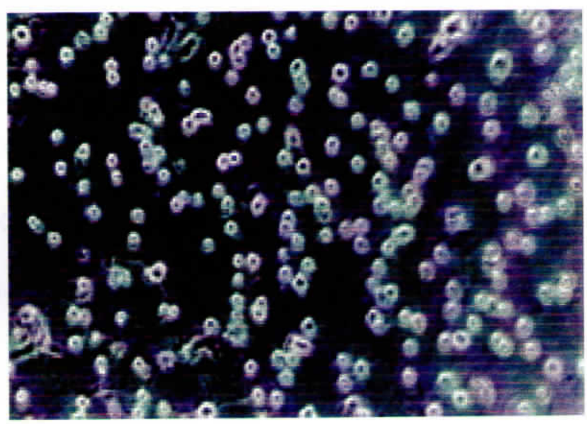

Fig.III.9.10. Phase contrast micrograph of HSVEC on composite coated TCPS, 5 min after trypsinization. All the cells become rounded. Still the intact matrix is seen (Mag 100x)

III.9.3. Release Kinetics of the Matrix

The results throughout the study consistently showed a significant improvement of cell proliferation, when ECGF was incorporated either with fibrin alone or with (Fibrin +Gelatin). The ECGF added with medium did not show such a marked effectiveness on cell multiplication and migration. This clearly indicates the local high concentration of ECGF is the determining factor. Inorder to prove that, the ECGF immobilized with matrix was sustained for enough period in adequate concentrations for the cell to consume peptide immobilization and its release kinetics was studied.

Endothelial cell ingrowth whether transinterstitial following graft implantation or after *in vitro* cell seeding is regulated by complex interactions among growth factors and cytokines released by the cells and the extracellular matrix within the local microenvironment. Among these are the potent EC mitogen of the fibroblast growth factor family which include seven related polypeptides of which acidic FGF-1 (a FGF) and basic FGF-2 (b FGF) are the best characterized.

The impregnation of vascular biomaterial with therapeutic agents contained within a controlled delivery vehicle have clinical significance for various reasons. Growth factors play an important role in regeneration of lost tissue both naturally and therapeutically. It is known that most of the growth factor will retain its optimum activity when it is bound to some matrix protein rather than in soluble form and it is less amenable to proteolytic degradation. In the GF incorporated matrix, cell growth was significantly enhanced leading to the attainment of monolayer within limited period. The GF in the microenvironment is readily accessible for cell surface receptors to bind and induce stimulus response.

Inorder to study the release kinetics from the matrix, two representative chromoproteins of comparable molecular weight, to that of known growth factor has been selected. The one of the GF which is known to have growth promoting activity is b-FGF of molecular weight 15,000 Dalton. To represent this factor cytochrome C of molecular weight 12 kDa was opted. The other component in the crude GF having cell mitogenic potential is ECGF of molecular weight 75,000 Dalton. In order to represent that component the chromoprotein Methemoglobin of molecular weight 69.000 Dalton was used.

The pattern of release of the respective chromoproteins from the matrix in different time interval is given in the fig III.9.11. After removing the chromoprotein that are not enmeshed, the incorporated chromoprotein was estimated to be 500μg in each well. During the initial 5 min there was around 10 % of the incorporated protein released and in the next 24 h, 40-50 % of the incorporated protein got released. There is no significant difference in release pattern between the two chromoproteins. Even after 96 h, the matrix retained 20-25% of the incorporated chromoprotein along with the matrix for the consumption of cells. It indicates that about 100 μg of chromoprotein retained by the matrix should be an adequate concentration in the microenvironment.

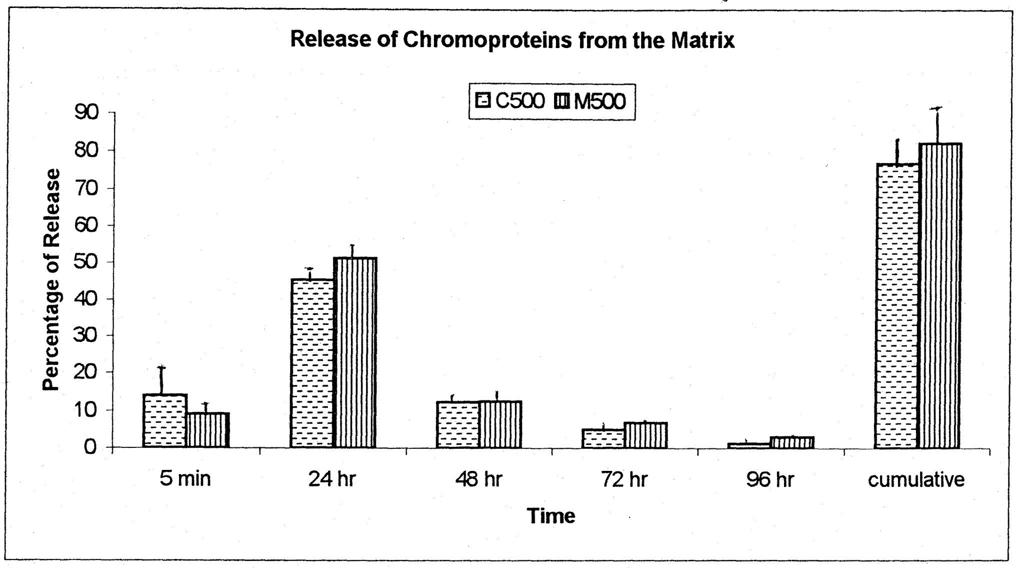

Fig.III.9.11. Graphical representation of release kinetics of chromoproteins from the fibrin matrix in to the medium. C 500 and M 500 are cytochromeC and methemoglobin respectively. Values are mean±S.D.(n=5)

The mechanism of release from such a matrix can occur through two different ways, the dissociation of peptide from the matrix and subsequent diffusion in the medium or by the proteolytic degradation of the matrix. In the present study, it seems that the release of GF from the matrix is predominantly controlled by diffusion because the degradation of the matrix in the initial 15 days was found to be significantly low as noted from the FDP assay. (III.9.2).

III.9.4. Thrombogenicity of Matrix

Success of endothelialization can be judged only if the matrix and the monolayer grown on it show nonthrombogenic nature at the time of implantation. Because, there is no reliable technique to demonstrate that the graft surface to be implanted is completely covered with EC, the nonthrombogenicity of the matrix is significant.

Various parameters like platelet retention by the substrate coated on different materials after *in vitro* platelet rich plasma contact, is an important determinant of the thrombogenicity. The functional damage undergone by the platelet in contact can also predict the platelet activation induced by the matrix. The number and nature of irreversibly attached platelets on the surface were analyzed by scanning electron microscopy after removing the non-specifically bound platelets.

III.9.4.1. Platelet Consumption

The average number of platelets consumed by the different substrates after 1h of incubation is shown in the graph (Fig.III.9.12.). At the end of 1h incubation untreated bare Dacron surface consumed 446 platelets per mm^2 compared to gelatin 166 /mm^2, fibrin glue 125/mm^2 and the composite 234/mm^2.

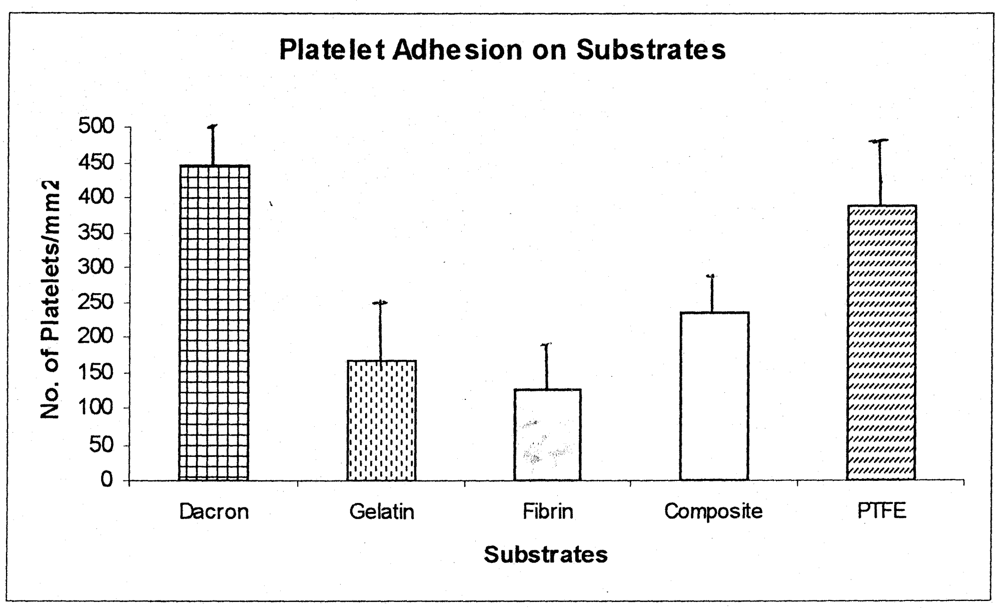

Fig.III.9.12. Graphical representation of platelets consumed by different substrates in static condition. Values are Mean ± SD (n=5)

PTFE also consumed much more platelets than any of the protein matrix used. With all the surfaces, there was a marked reduction in count in first 10 min then remained steady after 20 min up to 1h except in bare Dacron and uncoated PTFE that was continuously consuming platelets.

Platelet retention plays a significant role in thrombosis of, medium and small diameter, vascular grafts. The initial step in the retention of platelets on the surface of these grafts is platelet adhesion, which may be followed by platelet aggregation and fibrin deposition due to activation of coagulation. However, the initially bound platelets on the surface may get detached from the surface in flow situation depending on the kinetics of interaction between platelet and substrates. This initial adhesion appears to be high on uncoated Dacron and Uncoated PTFE. The fibrin coated surface consumed less number of platelets during the study period.

III. 9.4.2. Aggregatory response of platelets

The functional status of the platelets remaining in the suspension was evaluated by inducing the platelets with its physiological agonist ADP in aggregometer. The platelets without contact to any substrates served as control. The aggregatory response of platelets after contact as measured from increase in light transmission against time is shown in the graph. (Fig.III.9.13.).

The control platelets aggregated with an amplitude of 78 % and slope of 77. After exposure to bare Dacron and gelatin, the extent of aggregation was 44 % and rate was 44 while with fibrin coated and the composite coated surfaces the aggregatory response was slightly better with an extent of 49% and rate 48%. whereas due to uncoated PTFE contact the PRP response was reduced to 40% aggregation. This shows that the platelets are undergoing activation and is refractory to the addition of agonist. The activated platelets tend to adhere to the initially adhered layer on the surface and this explains the increase in consumption of platelets observed in §III.9.4.1.

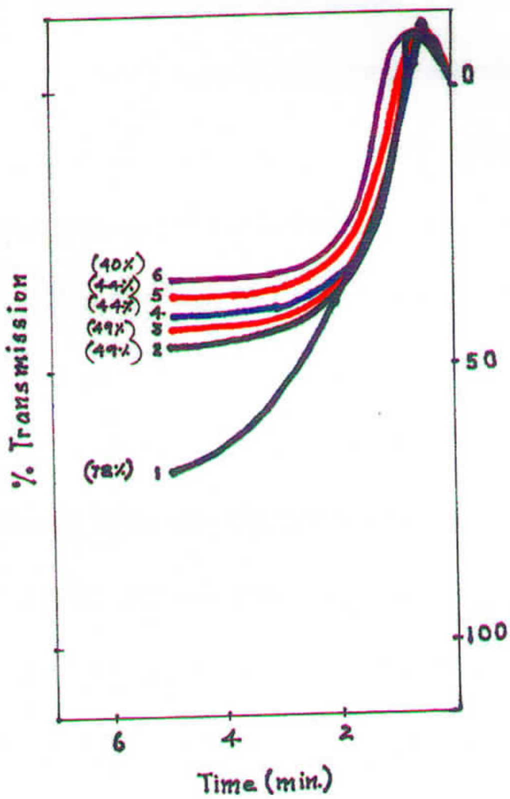

Fig.III.9.13. Tracing of the aggregatory response of platelets after contact of PRP with materials and matrices. The tracings were analyzed by the soft ware AGGLINK .1.Control 2.Fibrin coated Dacron 3. Composite coated Dacron 4. Gelatin coated Dacron 5.Uncoated Dacron 6. Uncoated PTFE.

III.9.4.3. Platelet Spreading

Dense cross-linked network of fibrin was evident on fibrin coated surface making a uniform layer all over the surface under SEM (Fig III.9.18). While on composite coated graft the meshwork looks much stronger with gelatin reinforced fibrin meshwork (Fig.III.9.17). The number of platelets adhered was in the order uncoated Dacron > Uncoated PTFE > gelatin > composite > FG. On the uncoated Dacron, mass of aggregated platelets along with polymerized fibrin is seen (Fig.III.9.15). The highly developed pseudopod of the platelets along the periphery of the thrombus indicates that most of the platelets on the surface spread well which can attract more platelets and enhance thrombus growth. Such events ultimately lead to the failure of the device especially in small diameter low flow areas. Likewise, on the uncoated PTFE also massive thrombus with fibrin formation is seen eventhough it is not spread over the surface (Fig.III.9.14), probably because of highly hydrophobic nature of the surface. These figures show that both uncoated Dacron and PTFE are highly thrombogenic in nature. The TCPS, Dacron or PTFE coated with gelatin also induced high level of platelet spreading as seen in the SEM. The nature of spreading of platelets on Gelatin coated TCPS is represented in the fig.III.9.16. However, in all the material surface coated with fibrin, only few platelets were found attached and most of the attached platelets were intact without much pseudopod formation(Fig.III.8.19). Even the platelets enmeshed within the matrix retained its discoid shape indicating nonthrombogenic nature (Fig.III.9.17). All the surfaces coated with composite also showed less number of platelets. Though gelatin is present in the composite, the non-thrombogenicity is maintained (Fig.III.9.18). So, the thrombogenicity expressed by the gelatin coated surfaces may not be by the gelatin molecule itself. Since, the coating may be nonuniform the bare material surface exposed to PRP may induce platelet adhesion and activation.

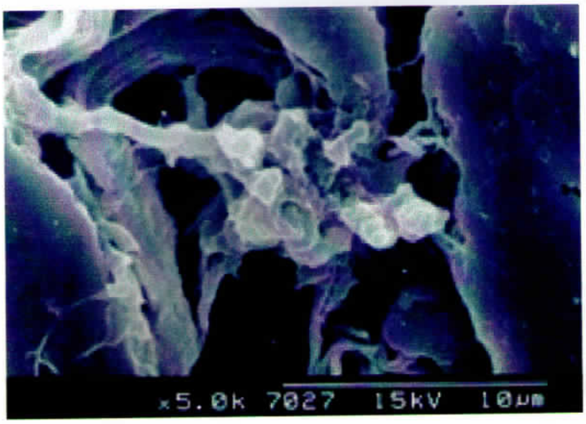

Fig.III.9.14. SEM picture of platelet adhesion on uncoated PTFE. Massive thrombus interconnected with fibrils is seen.

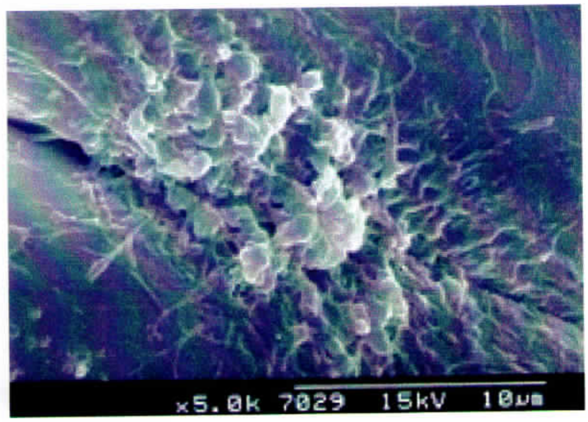

Fig.III.9.15. SEM picture of platelet adhesion on uncoated Dacron. Highly spread thrombus with platelet pseudopods is seen.

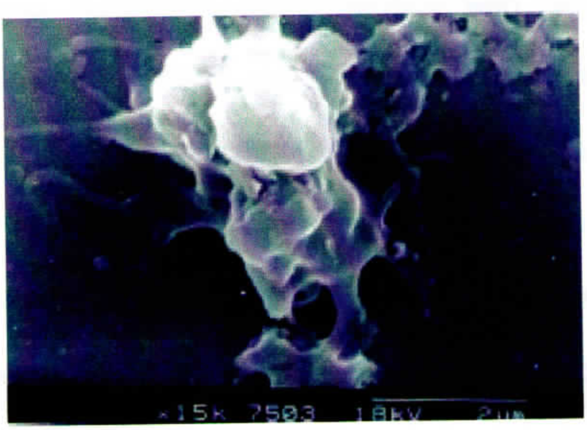

Fig.III.9.16. SEM picture showing the nature of spreading of platelets on gelatin coated TCPS

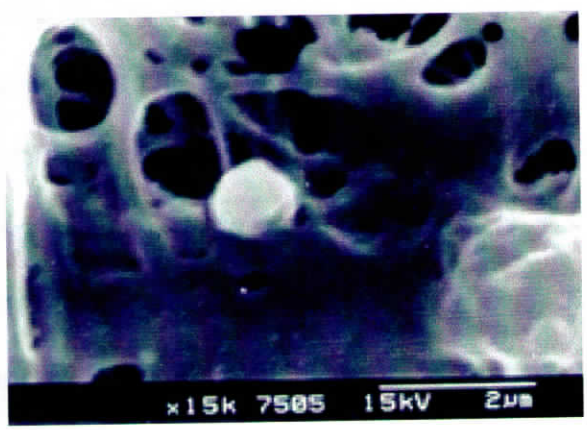

Fig.III.9.17. SEM picture of platelets showing the nature of spreading on Fibrin coated TCPS

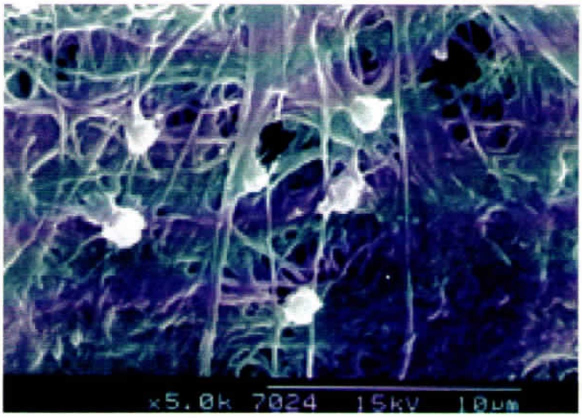

Fig.III.9.18. SEM picture of platelet adhesion on composite coated TCPS.

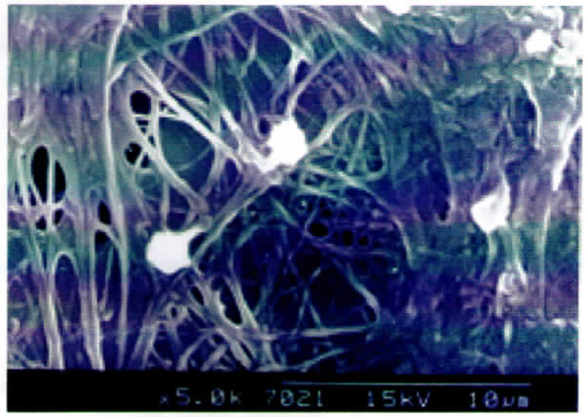

Fig.III.9.19. SEM picture of platelet adhesion on fibrin coated TCPS

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. The various adhesive proteins so far used for enhancing EC attachment such as collagen, gelatin, laminin, fibronectin, whole extracellular matrix proteins etc have been found to be highly thrombogenic. In the present study, fibrin is evidenced as the most nonthrombogenic surface with respect to the parameters studied such as platelet retention, platelet activation and spreading. Despite extensive investigation, the thrombogenicity of fibrin remains as a controversial issue. It has been observed that the pseudointima formed on human vascular graft as part of normal healing response is characterized by the presence of compact cross-linked fibrin. Some evidence suggests that Factor XIII mediated fibrin network formation results in reduced platelet adhesion due to alteration of the primary binding sites in the D domain of polymerized fibrin (Mc.Manama *et al.* 1986). But Jen and Lin (1991) observed that platelets show high affinity towards partially polymerized fibrin than towards fully polymerized fibrin. They observed greater levels of platelet adhesion on fibrin-coated surfaces compared to fibrinogen. One of the reasons for this discrepancy may be the method of preparation of fibrin. Usually the fibrin is formed by mixing fibrinogen concentrate and thrombin in the presence of $CaCl_2$. The thrombin is a potent platelet activator and if some unreacted active thrombin is exposed, it will lead to activation of platelets. Once all the thrombin is consumed for polymerization the mature fibrin acts as a surface with minimal reaction to the hemostatic elements. The polymerizing fibrin is thrombogenic because it is in the stage of conversion of fibrinogen to fibrin in the presence of thrombin while after complete polymerization it will become nonthrombogenic provided no active thrombin is enmeshed within the network. On uncoated bare grafts, platelet adhesion and activation were high and activation of blood coagulation has resulted in the formation of fibrin. The technique standardized in this study, where thrombin is immobilized first on the surface followed by layering excess fibrinogen has the advantage that no unreacted thrombin will be left on the surface. Moreover, it has been reported that the fibrinogen adsorption is dependant on the physical properties of the material used and in most of the cases, the adsorbed protein will undergo denaturation leading to change of its activity. This may be likely to lead to the formation of poorly defined fibrin meshwork on the material surface. While it has been reported, that thrombin adsorbed on different surfaces retained its enzymatic activity despite differences in physical characteristics (Williams and Bagnell.1981)

Rubens *et al.* (1995) also reported previously that compared to fibrinogen-coated surfaces, platelet adhesion to the fibrin-coated surfaces is lower. This may be because, the RGD sequence on the fibrinogen molecule readily recognizes its receptors on the platelet membrane once it is adsorbed on a surface. However, once fibrin network is formed, the RGD sequence may not be accessible for platelets and hence adhesion is lower. Although the involvement of GPIIb-IIIa is recognized in the binding of platelets to fibrinogen, the number of binding sites on activated platelets increases considerably. Hantgan *et al.* (1985) have reported platelet activation is essential for the binding to fibrin while Jen *et al.* (1991) suggested that fibrin interacts both with activated and non-activated platelets. Inorder to avoid the possibility of thrombin induced platelet activation, the coated surfaces were washed extensively. This may be the reason for the reduced platelet adhesion on fibrin-coated surfaces observed in this study.

The reduction in thrombogenicity of vascular graft due to surface modification is also evidenced from the aggregatory response of platelets after surface contact. The aggregatory response of platelet was better after contact with fibrin glue coated surface and composite coated surface compared to the response of platelets after contact with Dacron and gelatin. Overall, the response was reduced after contact with all surfaces compared to that of the control. However, aggregation results showed that the platelets underwent minimal damage on contact with fibrin and the composite, compared to uncoated and gelatin alone.

The study concludes that the thrombogenicity of fibrin glue coated surface is lower than that of the gelatin coated surface. The addition of gelatin with fibrin glue, has sustained the nonthrombogenic property of the fibrin glue.

III.10. Physiological Characterization of the Monolayer

III.10.1. Effect of shear stress

The representative photograph of the EC monolayer grown on different substrates followed by 1 h exposure to flow is shown in the fig.III.10.1. to III.10.8. From the gelatin coated coverslip after the flow about 30-40% of the cells were lost as noted from the cell count in the effluent. While there was no significant cell loss on either fibrin or composite coated surface. This indicates that the ability of the cell to resist the forces of shear stress is markedly influenced by the substrate on which the cells are growing. On all the samples the cells have aligned towards the flow indicating the ability of the cells to respond to flow as in real *in vivo* environment. The shear stress achieved with the flow chamber is around 15 dynes/cm^2 which is comparable to the shear forces prevailing in small diameter arteries.

In fibrin and composite coated surface, EC showed more intensive cellular spreading with increased distribution of cytoplasmic extensions along the substrate. Such cells are likely to have a higher number of adhesive bonds to increase its attachment strength. Whereas in gelatin coated surface, cells occupied less area compared to fibrin or composite. Such poor adherence during the initial stages of seeding has reflected on the resistance to the shear stress of the confluent monolayer also.

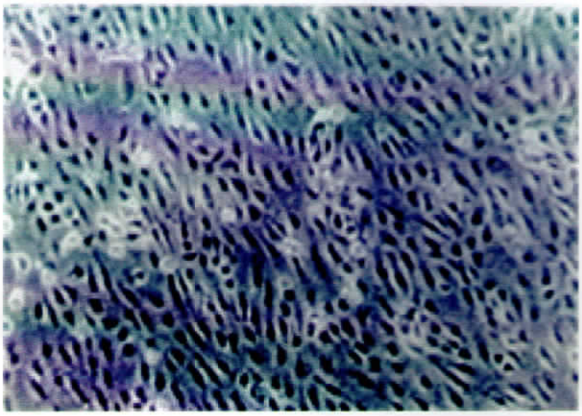

Fig.III.10.1. Monolayer of HUVEC on fibrin coated coverslip after exposure to flow. The cells have aligned to the direction of flow, and no cell loss is apparent. (Mag 100X)

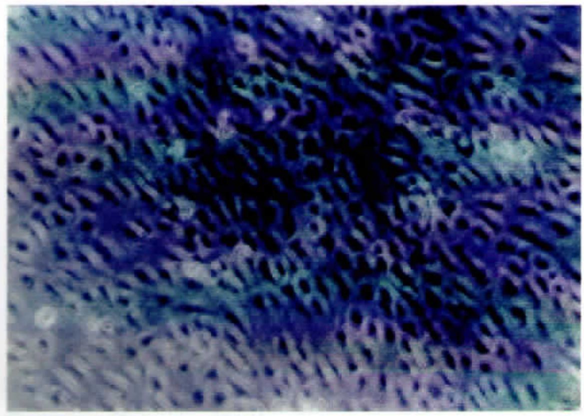

Fig.III.10.2. Monolayer of HUVEC on composite coated coverslip after exposure to flow. The cells are intact on the surface while aligned to the direction of flow. (Mag 100X)

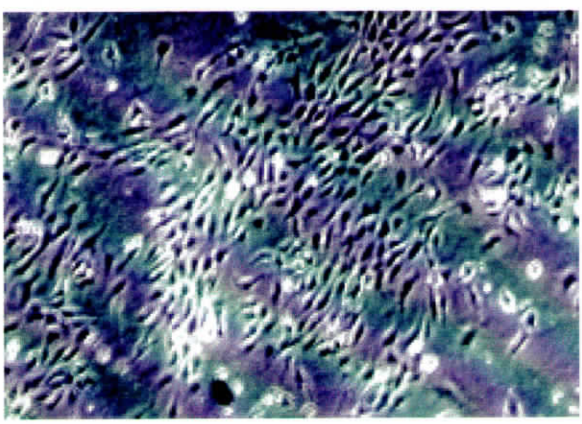

Fig.III.10.3. Monolayer of HUVEC on Gelatin coated coverslip after exposure to flow. Areas from where the cells have detached is clearly seen, while the cells remaining on the surface has aligned to the direction of flow. (Mag 100X)

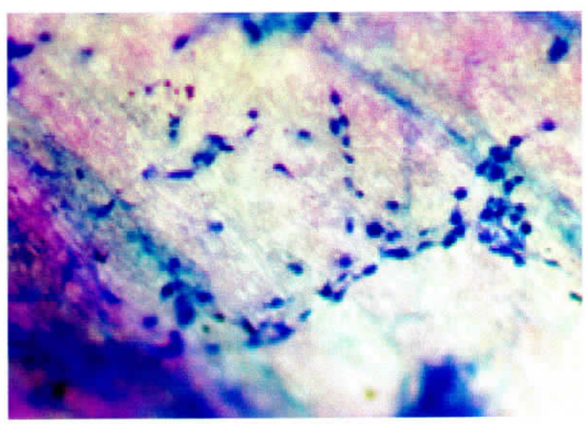

Fig.III.10.4. HUVEC on Gelatin coated PTFE after exposure to flow. Most of the area is bare with out any cells. (Mag 100X)

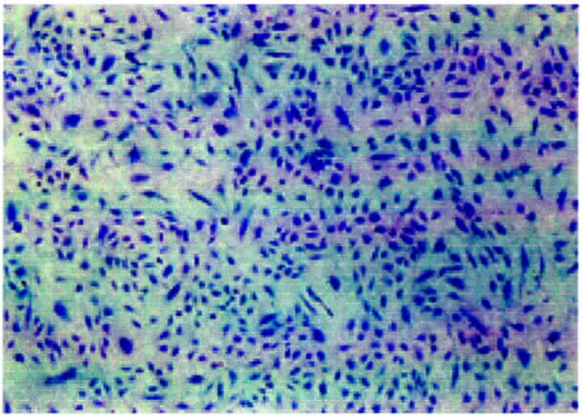

Fig.III.10.5. Monolayer of HUVEC on Composite coated PTFE. Compared to the gelatin coated PTFE (10.4) the composite coated PTFE retained most of the cells after exposure to flow. (Mag 50X)

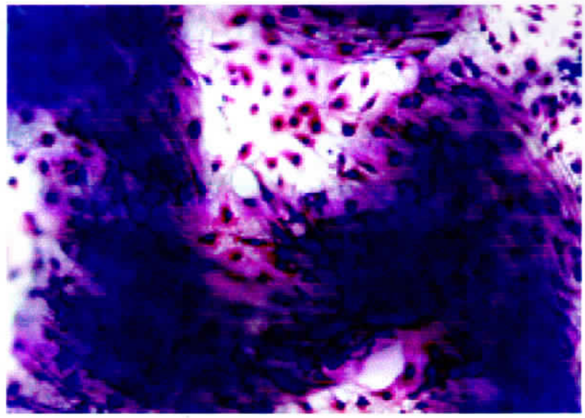

Fig.III.10.6. Monolayer of HUVEC on Composite coated Dacron graft after exposure to flow. Mostly, cell are intact, but because of differences in the plane of the field, all the cells cannot be focused simultaneously. (Mag 100X)

The fig.III.10.5 and fig.III.10.6 represent the monolayer of cells on composite coated graft pieces after exposure to shear forces for 1 h. There was negligible or no cell loss when coated with composite on both the grafts. While on gelatin coated graft only few cells remained after exposure to flow (fig.III.10.4). Unlike the coverslip, the cell alignment was not much evident probably because during fixation with glutaraldehyde the cells slightly contract leading to slight change in morphology. While for phase contrast micrographs cells are viewed live just after exposure to flow.

In the case of Titanium and DLC eventhough, the cell attachment and growth was good on bare surface, there was marked difference when both the samples were exposed to flow. After exposure to flow around 20-30 % of the cells were lost from the uncoated surface compared to composite coated surface. This is evident from the stained samples viewed under incident light. A representative photograph of monolayer of cells on bare and composite coated Titanium discs, after exposure to flow are shown in the figures (Fig.III.10.7. aand III.10.8). Apart from the cell loss from the bare disc, even the attached cells are not well organized and show tendency to detach from the surface.

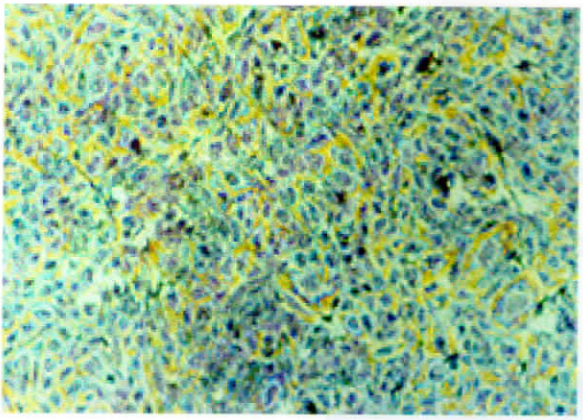

Fig.III.10.7. Monolayer of HUVEC on Composite coated Ti disc after exposure to flow. (Mag 50 X)

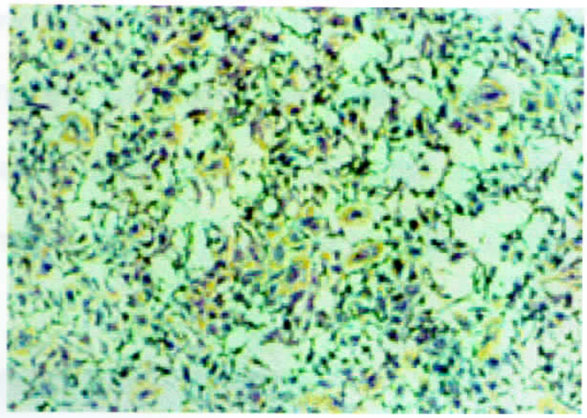

Fig.III.10.8. Monolayer of HUVEC on bare Ti disc after exposure to flow. under incident light. (Mag 50X)

The cells also slightly contracted during fixation probably because of lack of enough adhesive strength with substratum. While on composite coated disc even after flow, the cells still maintain its organized morphology without any cell loss.

III.10.2.Characterestics of the Monolayer

III.10.2.1. Platelet adhesion and activation

To evaluate blood compatibility, the monolayer of EC was exposed to the confluent monolayer with PRP at a count of 2.0 x 10^5 for 1 h at 37°C in static conditions. The platelet adhesion on the monolayer was minimal when grown on all the matrices and there was no visible platelet clumps. A representative photomicrograph of monolayer of cells on composite matrix after exposure to PRP

From the platelet function studies, it seems that the monolayer is capable of acting as a thromboresistant surface, and there is no consumption of hemostatic elements.

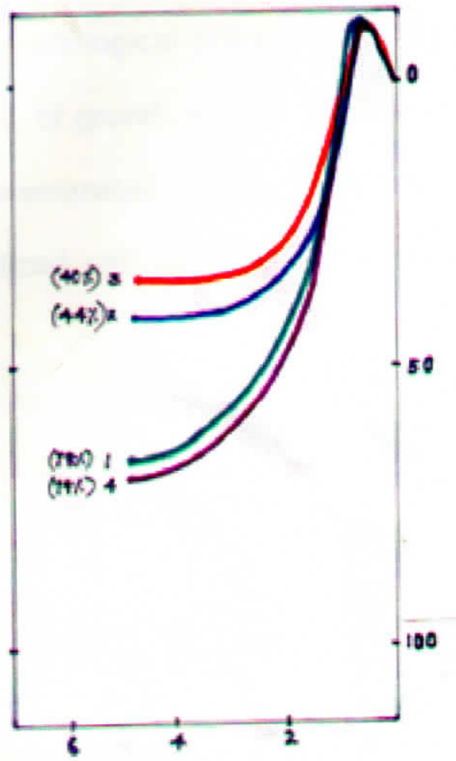

Fig.III.10.10. Pattern of Aggregatory response comparison of PRP after contact with Dacron or PTFE and EC monolayer on composite.
1. Control 2. PTFE 3. Dacron. 4. EC monolayer on composite

III.10.2.2. Nitric Oxide production by the monolayer

The nitric oxide synthesized by the monolayer of cells grown on different substrates over a period of 24 h is shown in the fig.III.10.11. The nitrite concentration determined using Greiss reagent shows significant difference on different materials . The Nitrite level is significantly high on Fibrin+ GF incorporated matrix compared to gelatin or composite coated surface.

viewed under higher magnification is shown in the fig.III.10.9.The monolayer of cell could be passaged after contact with the platelets with out any change in normal morphology. As the technique involves the transplantation of cells on a new environment and the cells may be capable of synthesizing both prothrombotic and antithrombotic factors, it is essential to know the thrombogenicity of cell layer grown on the matrix. It has been earlier reported that the substrates modulate the physiology of the cell in different ways and it is not clearly understood what is the acceptable level of various prothrombotic and antithrombotic factors produced by cells to resist the thrombus formation.

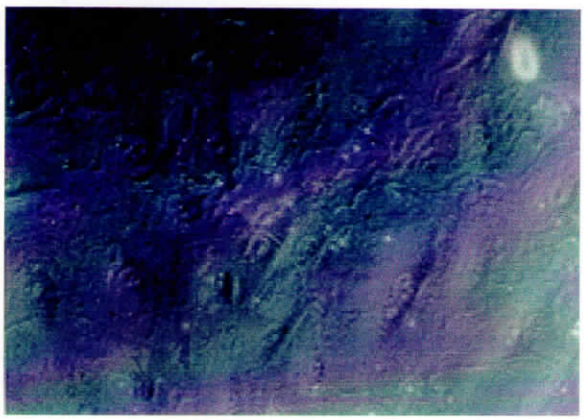

Fig.III.10.9.Phase contrast micrograph of confluent monolayer of HUVEC grown on composite coated TCPS after 1 h exposure to PRP. No visible adhered platelets are seen.(Mag 450x)

The aggregation curve of platelets after contact with the monolayer is compared with that of uncoated surface (Fig. III.10.10). After 1 h of incubation with the monolayer, the platelets aggregated similar to that of control with a light transmission of 72%. While the platelets after contact with bare Dacron or PTFE aggregated with a light transmission of 40% and 44% respectively as seen in fig III.10.10 . This indicates that the monolayer of cells grown on matrix is maintaining its normal physiology.

Inorder to avoid serum mediated interferences in the measurements, serum-and, phenol red- free RPMI was added to the confluent monolayer and maintained in this medium for 24 h. In this condition the monolayer can be maintained for 48 h without detectable level of morphological change. Later on, the cells tend to detach from the surface due to lack of growth promoting medium. So the nitrite released during a period of 24 h only is estimated. From the results it is evident that, when cells are grown on fibrin immobilized with GF, the normal physiology was adequately maintained.

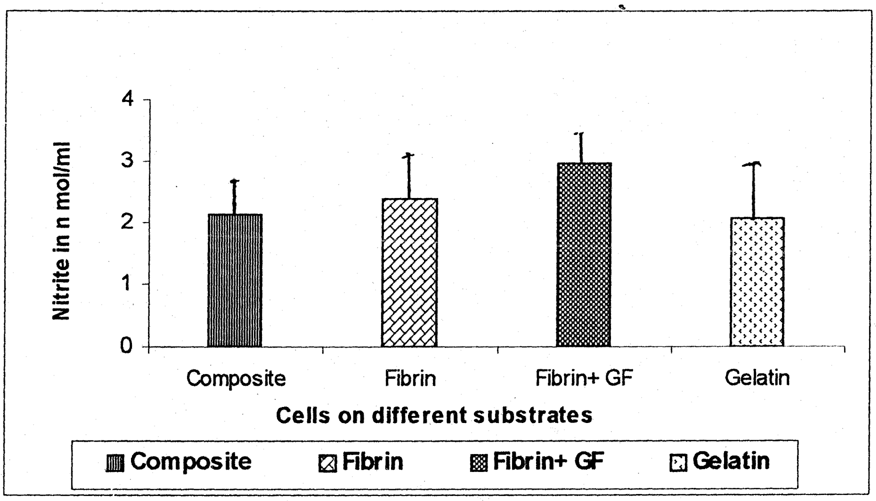

Fig.III.10.11. Bar diagram showing average Nitrite level of tissue culture supernatant from the monolayer of HUVEC on different matrices. Values are mean ± S.D.(n=3)

Thus the cells grown the standardized matrix produced NO better than that were grown on gelatin or bare surfaces. Platelet adhesion and activation were also found minimal when PRP was exposed to the monolayer grown on this matrix. Moreover, the attachment strength of the monolayer to the matrix was adequate to resist forces of flow.

CHAPTER IV

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

IV.1. Restatement of Problem

Endothelial cell is the most blood compatible natural surface known till date, and tissue engineering of biomaterials with EC to reduce thrombogenic complications of implants has been suggested. Efforts are being made by several groups to make it a clinical success. While the vascular materials are best suited in terms of other physico-chemical characteristics, they are highly hydrophobic and EC attachment to these surfaces is a major challenge. Various adhesive proteins have been tried as candidates for enhancing EC attachment to the materials, but drawbacks exist with each of these proteins. Therefore, this study was undertaken to standardize a matrix to implement tissue-engineering principle into practice.

IV.2. Approach to the Problem

The major objective of the study was to immobilize an adhesive matrix to biomaterials such that endothelial cell adhesion and monolayer formation onto the material can be achieved. The matrix protein should compose of an optimal concentration of proteins to form a stable and uniform coating, which is biocompatible and biodegradable, and metabolic products should be nontoxic. The coating technique should be easy to perform, and the protein should be non-thrombogenic and should resist stress at least till the cell monolayer formed is stabilized on their own extracellular matrix proteins.

With this approach, the first attempt was to standardize a matrix composition, and second, to develop a coating technique. Fibrin was the major adhesive protein of choice, which is known to have all the properties as a natural scaffold in the biological system. Since gelatin is also known as the suitable matrix for tissue culture of endothelial cells, which is a denatured, non-antigenic, and biodegradable form of collagen, the possibility of a combination of fibrin and gelatin was thought. As fibrin gels are capable of holding other small molecules, possibility of retaining ECGF in the microenvironment was also addressed. The retention rate of the growth factor was worked out, and in order to stabilize the coated matrix on the surface a freeze-dry cycle was conducted.

Extensive studies were carried out to assess advantages of the coated matrix as a non-thombogenic surface in comparison with the currently used vascular biomaterials. Since the major problem of blood material interaction is associated with platelet adhesion and thrombus formation, PRP was prepared and treated with surfaces. The retention of platelets by bare and modified surfaces after contact with PRP, and the spreading pattern were assessed. The ability of platelets to respond to agonists was also evaluated, as the surface induced activation of these cells can accelerate the adhesion to the already deposited platelet layer on the surface. The aggregatory response of platelets to ADP, after contact with the substrate was used to evaluate its thrombogenicity. The stability of the surface in the tissue culture medium containing serum was tested, to find out whether excessive fibrinolysis occur, that might affect the integrity of the cell monolayer.

The cells were isolated from human umbilical vein and seeded on all the matrices under evaluation. The parameters assessed were, the cell adhesion within 4h of seeding, the cell spreading, and the proliferation. The seeding densities were correlated to the growth rate. Differences in cell spreading capabilities on different matrices were compared to the respective bare materials such as tissue culture dishes and biomaterials (including polymers and metals). The difference in attachment, spreading and proliferation, between HUVEC and human saphenous vein EC were also studied. The number of cells required for making a seeded conduit was calculated from the growth rate observed on the standardized substrate.

The physiological status of the monolayer grown on the standardized matrix was studied. The ability of the monolayer to resist shear stress when exposed to flow was assessed by analyzing the cell detachment into the medium. Comparison of the shear stress resistance when the cells were grown on bare materials and on matrix coated surfaces were conducted. The matrix standardized during this study was established ideal for tissue engineering of EC by assessing the effectiveness of the matrix to maintain several passages of the cell for a prolonged period. Finally, assessment was also made to see if the monolayer influenced the matrix stability.

IV.3. Summary of Results

The results of the study are mainly divided into four parts: 1. The culture and characterization of endothelial cells from umbilical vein and saphenous veins; 2. Standardization of a composition and coating technique of the proteins that can enhance cell adhesion and growth; 3. Evaluation of the matrix stability and non-thrombogenic behavior; 4. Evaluation of the monolayer for normal physiology, non-thrombogenicity, and shear stress resistance.

The isolated cells were found to maintain cobblestone morphology, irrespective of the substrate on which they were grown. The FVIII related antigen was localized on the cell by using either enzyme-linked/FITC conjugated antibody. The Weibel palade bodies in which the FVIII is located is found under fluorescence microscope as well-defined rod shaped granules. All the granules were found within the cytoplasm, which is the expected location in the normal EC. The LDL uptake was also evident when growing cells were treated with Dil labeled LDL. The LDL may get metabolized after it is internalized through the receptor channel, but the fluorescent probe Dil remained throughout the cytoplasm.

Initially TCPS was used for standardization of the composition and coating technique of matrix proteins that support good cell adhesion, spreading and proliferation. The matrices evaluated were gelatin, fibrin, fibrin+ECGF, fibrin+gelatin+ECGF and the best results were obtained with

F+GF and composite (F+GF+gelatin). The growth rate monitored was maximum with both these matrices and hence, for evaluation of cell seeding on to biomaterials, only these two were used.

The coating technique yielded uniform adhesive matrix, with fine bundles of fibrin network. The fibrin formed was stable and not detectable levels were degraded to generate fibrin degradation products. Even microscopically, it was seen to maintain the surface morphology of the coated matrix intact for several days after soaking in the complete medium. There was no shrinking or cracks seen at any stage of evaluation. The freeze drying turned it more storable, as it could be shelved for several months without any damage or degradation of matrix properties, and it could be re-hydrated to get a uniform net work when needed for cell seeding. The growth factors were retained in the matrix, even after freeze drying, which got released into the medium in 96h of soaking with serum containing medium.

The surfaces were studied for its thrombogenic potential and it was found that bare Dacron and PTFE are highly thrombogenic, as more platelets are retained, spread and grew into a thrombus on them. The platelets in contact with these surfaces also lost their ability to respond to agonists, considerably. The fibrin and fibrin+gelatin coated surfaces exhibited a low platelet retention, spreading and activation of the platelets in suspension. However, the surface coated with gelatin was observed to consume more platelets than the fibrin matrix that contained gelatin. The activation of platelets was also reduced even if gelatin is present in the matrix with fibrin. Even though, few platelets are found adsorbed on the matrices, fibrin and composite, they were not spread, and no pseudopodia were generated. This shows that not much thrombus formation is likely.

The matrix was stable even when HUVEC monolayers were grown on fibrin-gelatin composite for several days. In contrast, HSVEC mediated slight fibrinolysis, which was directly proportional to the number of cells present. The degradation, however, has not adversely affected the integrity of the matrix up to 30 days and the cells could be maintained up to 3 passages by repeated trypsinization.

The monolayers grown on the standardized matrix was also found non-adherent to platelets. This establishes that the EC grown maintains the cell in its normal physiological status, where non-thrombogenic molecules are expressed on the cell membrane. This result was substantiated by the amount of nitric oxide that was produced by the monolayer grown on composite.

While the attachment of the cell monolayer on to the composite matrix was optimal for enhancing the cell migration and proliferation, it was also adequate also to resist the forces of shear stress induced by flow. The cells grown on fibrin+GF resisted the shear stress, whereas those were grown on gelatin and on bare surfaces were lost into the medium after exposure to the flow. Irrespective of the nature of matrix, all the cells got aligned to the direction of flow after the monolayers were exposed.

Other candidate biomaterials for vascular applications tested for EC adhesion and proliferation were Titanium, DLC-coated titanium, and ultrahigh molecular weight polyethelene. EC attached on all the three materials, without any matrix coating, while the cells grown after matrix coating on these materials showed better resistance to shear stress.

The fibrin matrix with GF-incorporated is found ideal for cell culture because up to 15 passages of HUVEC could be maintained on this matrix, without any senescence of cells. The later passages grown on gelatinized surfaces tend to senesce with enlarged morphology having large vacuoles, but when these cells are harvested and seeded onto the composite, the cells revived with normal morphology. The ability of the monolayer of HSVEC on the composite matrix to repair the damage to the monolayer is also demonstrated.

IV. 4. Conclusions

The composition of an adhesive matrix is standardized, which consisted of fibrin and gelatin that can be coated to the biomaterials, to support cell adhesion, spreading and migration. The incorporation of growth factor with the above matrix proteins, enhanced cell proliferation significantly. A coating method is standardized to get a uniform network and the growth factor retention within the matrix for an optimum period is achieved. The immobilized protein matrix is found stable without any excessive degradation for a period of 30 days, when incubated with tissue culture medium supplemented with serum. The matrices were non-thrombogenic compared to the bare biomaterials.

The endothelial cells isolated from human umbilical vein and saphenous vein were found homogenous, with typical EC morphology. The rod shaped Weibel Palade bodies could be visualized in both cell types when stained with enzyme/FITC conjugated antibodies. The LDL receptors on the growing cells recognized the Ac.LDL that was labeled with DiI, and was internalized. This LDL uptake demonstrate the normal physiological status of the cells when they are maintained in serum-free medium also.

The cell adhesion and spreading was superior on all fibrin containing matrices. The presence of GF or gelatin has not affected cell attachment, on the other hand they improved cell proliferation. The gelatin coating may leave uncoated areas in the TCPS, and biomaterials whereby platelet adhesion is higher and EC adhesion is poorer. The composite prepared from GF, gelatin and fibrinogen was found the best matrix in terms of fibril morphology, cell adhesion, spreading and proliferation.

The monolayer formed on this ideal matrix was non thrombogenic. The NO production was adequate when the cells were grown on composite or F+GF. This indicates normal physiology of the cells grown on fibrin containing matrix. The cells grown on each of these two matrices showed resistance to shear stress whereas the cells grown on gelatin coated or bare surfaces such as UHMWPE, Ti and DLC-coated Ti, were detached at varying degrees, on exposure to flow. The cell loss from gelatinized glass coverslip, Dacron and PTFE were significantly high. The monolayer exposed to the flow got aligned to the direction of flow.

The advantage of the use of fibrin as the matrix protein is that it is effective in immobilizing GF to retain a high local concentration for the cells, and it could incorporate gelatin within the matrix, uniformly. Both fibrin and gelatin are natural biodegradable scaffold proteins, that can support cell adhesion and migration.

To sum up, a standardized matrix is formulated during this study that served as an excellent tissue engineering matrix for modifying the thrombogenic biomaterial surfaces for blood compatibility. The behavior of the cells are generally dependent on the matrix on which they grow and the study establish that the tissue engineered EC monolayer on thrombogenic material surfaces such as Dacron and PTFE, maintains normal physiology.

IV.5. Future Directions

Using saphenous vein EC, the number of cells and time interval required for making a tissue engineered vascular conduit is estimated. HSVEC from more patients should be harvested and grown to standardize the technique. This is important because depending on the underlying cause of the vascular disease in each patient, the yield of cell harvest, adhesion and proliferation may vary. A method has to be standardized for making tissue engineered vascular grafts consistently.

Once the technique is standardized, animal experimentation has to be conducted. Though the effect of shear stress was studied by *in vitro* techniques, the data cannot be translated directly into clinical conditions. The *in vivo* studies in animal models will give a better understanding on the effects of shear stress, and the influence of other physiological mechanisms to maintain the patency of the implanted conduit.

The tissue engineered vascular grafts should be brought to clinical practice at the earliest in order to cope up with the advancing technology worldwide.

The major limitation in the application of the technology is the requirement of a tissue culture facility in all the centers were vascular surgery is routinely done. Therefore efforts may be done to cryopreserve the patient`s vessel, which can be sent to the tissue culture facility where experts can modify the grafts using the autologous EC and send back to the surgeon for implantation.

The cell were observed to adhere and grow well on other biomaterials such as UHMWPE, Ti and DLC coated Ti, that are candidates of other blood contacting devices. Therefore, the technology can be used for other implants also such as stent, heart valve sewing ring and cages, that are difficult to get endothelialized.

Since the standardized matrix composition behaves like a natural scaffold by maintaining the normal physiology of cells, this matrix can be used to culture EC for understanding mechanisms and modulations of the cell functions in physiology and pathology. This matrix can also be used for evaluation of other cell types such as epithelial cells and neurons.

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ANNEXURE 1.

List of Media and Buffers.

1. Water for Tissue Culture

   Tap water distilled three times, deionised by passing through Millipore filters, autoclaved and used immediately.

2. Hank's Balanced Salt Solution (HBSS)(Ca^{2+} and Mg^{2+} free, 1X)

   5.4 mM KCl, 4.4 mM KH_2PO_4, 136.9 mM and 2.7 mM Na_2HPO_4. The prepared solution is autoclaved for 30 min at 120°C and used.

3. Collagenase Solution for Subculturing

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

4. O.25 % Trypsin EDTA Solution. (100 ml)

Trypsin	0.05 gm
NaCl2	0.8006 gm
Na HCO3	0.03612 GM
KCl	0.04025 gm
D Glucose	0.09 gm
EDTA	0.0024.

   After dissolving all the components sterile filtered , aliquoted and frozen below –20 ° C

5. New Born Calf Serum

   The blood collected from New Born Calf is allowed to clot for 1 h at 37° C and the released serum was collected centrifuged, dialyzed against HBSS, heat inactivated and frozen until use.

6. Human Whole Blood Derived Serum

   Human Serum is prepared from freshly collected blood obtained from healthy donors. Before use, the sera are dialyzed against HBSS, heat inactivated, filtered, and frozen at –70°C in 50 ml tubes.

7 M.199

The powdered medium is dissolved one litre of sterile Distilled water and after adding 0.35 gm of Na HCO_3 the p H is adjusted to 7.3.

8. RPMI 1640

The powdered medium dissolved in 1 litre of tissue culture grade water and by adding 2 gm of Na HCO_3

9. MCDB131 with L- Glutamine

The powdered media is dissolved in one litre of sterile water (I) . After adding 1.18 gm of $NaHCO_3$, the pH is adjusted to 7.3 stored at 4° C in dark and used within 4 weeks

10. PBS

150 mM NaCl, 10 mM Na2HPO4 and 1.5 mM KH_2PO_4 (Ph.7.4)

Abbreviations

Short form	Expansion
Ac. LDL	Acetylated Low density Lipoprotein
ACD	Acid Citrate Dextrose
ADP	Adenosine Di Phosphate
C5a	Complement factor 5a
Dacron	Polyethylene Terephthalate
DLC	Diamond Like Carbon
EC	Endothelial Cell
ECGF	Endothelial Cell Growth Factor
EDTA	Ethylene Diamine Tetra Acetate
e-PTFE	Expanded PTFE
FD	Freeze Dried
FDP	Fibrin Degradation Products
FGF	Fibroblast Growth Factor
FITC	Fluorescein Iso Thio Cyanate
FN	Fibronectin
GF	Growth Factor
HUVEC	Human Umbilical Vein Endothelial Cell
LDL	Low Density Lipoprotein
NBCS	New Born Calf Serum
NO	Nitric Oxide
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffered Saline
PC	Phase Contrast
PDGF	Platelet Derived Growth Factor
PDS	Poly Dimethyl Siloxane
PG	Poly Glycine
PGF-1α	ProstaglandinF-1α
PGI2	Prostaglandin I2

PL	Poly Lactic acid
PMNL	Poly Morpho Nuclear Leukocytes
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PS	Poly Styrene
PTFE	Poly Tetra Fluoro Ethylene
RGD	Arginine-Glycine-Aspargine
SEM	Scanning Electron Microscope
TCA	Tri Chloro Acetic acid
TCPS	Tissue Culture Poly Styrene
Ti	Titanium
TX	Thromboxane
UHMWPE	Ultra High Molecular Weight Poly ethylene
VEGF	Vascular Endothelial Growth Factor