JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 13 (2002) 751–755

Fibrin-mediated endothelial cell adhesion to vascular biomaterials resists shear stress due to flow

T. R. S. KUMAR
Thrombosis Research Unit, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Poojapura, Thiruvananthapuram – 695012, India

L. K. KRISHNAN*
BMT Wing, SCTIMST, Trivandrum – 695011, India
E-mail: lissykk@sctimst.ker.nic.in

In vitro endothelial cell (EC) seeding onto biomaterials for blood-contacting applications can improve the blood compatibility of materials. Adhesive proteins adsorbed from serum that is supplemented with the culture medium intercede the initial cell adhesion and subsequent spreading on material surface during culture. Nevertheless, physical and chemical properties of vascular biomaterial surface fluctuate widely between materials resulting in dissimilarity in protein adsorption characteristics. Thus, a variation is expected in cell adhesion, growth and the ability of cell to resist shear stress when tissue engineering on to vascular biomaterials is attempted. This study was carried out with an objective to determine the significance of a matrix coating on cell adhesion and shear stress resistance when cells are cultured on materials such as polytetrafluoroethylene (PTFE, Teflon) and polyethyleneterephthalate (Dacron), ultra high molecular weight polyethylene (UHMWPE) and titanium (Ti), that are used for prosthetic devices. The study illustrates the distinction of EC attachment and proliferation between uncoated and matrix-coated surfaces. The cell attachment and proliferation on uncoated UHMWPE and titanium surfaces were not significantly different from matrix-coated surfaces. However, shear stress resistance of the cells grown on composite coated surfaces appeared superior compared to the cells grown on uncoated surface. On uncoated vascular graft materials, the cell adhesion was not supported by serum alone and proliferation was scanty as compared to matrix-coated surface. Therefore, coating of implant devices with a composite of adhesive proteins and growth factors can improve EC attachment and resistance of the cells to the forces of flow.

1. Introduction

Cell attachment, spreading and cytoskeletal organization on the implant surface is thought to play a decisive role on *in vivo* performance of prosthetic devices. In order to achieve blood compatibility the material surface should be inert towards the circulating cells like platelets while allowing adhesion and growth of endothelial cells. Generally, cell adhesion to biomaterial surface is mediated through the interaction of transmembrane integrins and plasma derived adhesive proteins adsorbed on to the surface [1]. However, reports show that EC do not adhere to currently available vascular graft materials like Dacron and PTFE [2, 3]. Thus poor patency of small diameter vascular graft in humans is a major failure modality. The concept of designing a material surface with improved EC adhesion and reduced platelet adhesion is still a challenge for material scientists.

EC transplantation or seeding on the surface of vascular biomaterial after precoating with an adhesive matrix has been a promising approach to reduce the thrombotic potential of material surface and has been widely investigated for the small diameter vascular grafts [4–6]. Although there has been considerable progress with respect to improving EC attachment and growth on vascular graft materials, little is known about the requirement of a matrix protein coating on other implant materials, such as mechanical heart valve and intravascular stents. *In vitro* endothelialization of heart valve prostheses have been reported to reduce thrombosis and tissue hyperplasia by the reestablishment of the natural

*Author to whom all correspondence should be addressed. Currently at Division of Cancer Biology, Rajiv Gandhi Centre for Biotechnology, Jagathy, Thycaud P.O., Thiruvananthapuram – 695014, India

barrier between prosthetic material and blood components [7, 8]. It has been shown that human endothelial cell can be transplanted on the surface of mechanical heart valve [9]. However, the durability of the monolayer in the highly complex hydrodynamic environment is a major concern. An approach to seed bio-prosthetic heart valve by tissue engineering is initiated by some investigators [10, 11] and the technology is likely to get widespread acceptance in the near future especially in the area of ventricular assist devices.

Cell adhesion to an artificial material is mediated only through an initially adsorbed layer of proteins. Nature of proteins that are adsorbed to material surface when it is exposed to blood, plasma or serum will determine the extent of cell adhesion, spreading and proliferation [12]. In addition, if the physical and chemical properties of the surface impede adsorption of proteins that are required for cell adhesion, such surface is not likely to get endothelialized soon after implantation. Therefore, precoating of cardiovascular devices of varying physical, chemical and topographical properties should mediate uniform cell adhesion and growth of EC for creation of tissue engineered devices or to augment endothelialization after implantation.

In this background, this study was conducted in order to explore the possibility precoating the currently used cardiovascular materials to make a difference in EC adhesion, proliferation and subsequently to measure the ability of cells to resist the forces of shear stress compared to the EC that is grown on bare materials.

2. Materials and methods

2.1. Endothelial cell isolation and culture

The cells used were derived from human umbilical vein (HUV) as essentially described by Jaffe *et al*. [13] with some modification. The umbilical vein was cannulated and thoroughly washed with sterile Hanks balanced salt solution (HBSS) followed by incubation in 0.2% collagenase in serum-free Medium 199 for 20 min at 37 °C. The dissociated cells were washed in RPMI 1640 medium containing 10% newborn calf serum (NBCS) by centrifugation for 7 min at 100 *g*. The final pellet was resuspended in complete RPMI medium (GIBCO BRL, USA), containing 20% NBCS, 100 µg/mL of streptomycin sulfate, 100 U/mL of benzyl penicillin, 100 U/mL of heparin, 150 µg/mL endothelial cell growth factor (ECGF) and seeded on gelatin coated tissue culture polystyrene flasks. The cells were maintained in a humidified incubator at 37 °C in 5% CO_2. The cells were sub-cultured in the near confluent stage at 1 : 3 split ratio, and was characterized by staining for vWF.

2.2. Preparation of vascular materials for cell seeding

PTFE (Gore Tex) and woven Dacron vascular graft (Bard) were cut open longitudinally and 1 cm^2 square pieces were prepared. The graft pieces were fixed in the 24 well plates with the help of a custom-made acrylic ring. The surface was washed thoroughly with de-ionized water before pre-coating or cell seeding. Titanium (Ti) and UHMWPE discs of 15 mm diameter size were washed extensively with de-ionized water, dried and ETO-sterilized before use. They were placed within the wells of a 24 well culture plate and coated with substrate as described in Section 2.3.

2.3. Matrix coating

The material surfaces prepared as in Section 2.2 were washed with serum-free medium 199 before substrate coating or cell seeding. Initially the surface was incubated with a thin layer of thrombin solution 20 U/mL in 0.025 M $CaCl_2$ for 1 h. After aspiration of the excess thrombin solution, thin layer of a solution of fibrinogen, gelatin and ECGF was added on to the surfaces and were allowed to polymerize for at least 1 h at 37 °C. The coated material surfaces were freeze-dried using a lyophilizer (Edward Modulyo, UK).

2.4. Cell seeding

HUVEC obtained between third to fifth passages by trypsinization was used for cell seeding and growth assay. The seeding density was maintained at 4×10^4 cells per cm^2 and incubated at 37 °C in the CO_2 incubator. After 2 h, the medium from each well was collected to remove the unattached cells and was washed with fresh medium. The unattached cells were counted to calculate the percentage of cells attached on each surface. The materials with attached cells were incubated with fresh complete medium in the incubator. After 48 h, the cells on the material surface were fixed with 2% glutaraldehyde in phosphate buffered saline (PBS) for 4 h followed by washing and staining with May-Grunwald Stain. The graft pieces and UHMWPE were viewed under a light microscope in transmittance mode while the Ti samples were viewed under reflected light mode (Lieca epifluorescent Microscope).

2.5. Effect of shear stress

The efficiency of the cell monolayer that were grown on matrix coated and bare surface to resist the shear stress of flow was evaluated in a custom made parallel plate flow chamber which is a modified version as described by McIntyre [14]. The monolayer grown on material surface for 48 h was washed with fresh medium and was immediately placed on the basal plate of the flow chamber. Complete medium was circulated over the monolayer using a peristaltic pump for 1 h at a shear stress of 2.5 dynes/cm^2. The circulated medium was collected, the cells were concentrated by centrifugation and count was noted using a Neubauer Counting chamber. The monolayer of cells grown on material surfaces was immediately washed in PBS and was fixed with 2% glutaraldehyde. After staining with May-Grunwald stain viewed as described in Section 2.4.

3. Results

3.1. Cell adhesion on different surfaces

The percentage of initial cell adhesion on materials was found in the order Ti > UHMWPE > PTFE > Dacron. Cell adhesion was $< 35\%$ on Dacron and $\sim 40\%$ on

PTFE, more than 75% on UHMWPE and more than 85% on titanium. Irrespective of the material characteristics, coating with composite resulted in 90–95% cell adhesion within 2 h of seeding with EC.

3.2. Proliferation and stability of EC

Cells retained the typical cobblestone morphology on Ti and in 48 h of seeding, surfaces were nearly confluent (Fig. 1(a)). From the Fig. 1(b) it is clear that the cells on the composite coated Ti surface are well-attached and organized so that it could maintain the integrity with uniformly organized cytoskeletal elements even after fixation and staining. Whereas, the cells that were grown on uncoated materials showed a tendency to contract during the fixation process. When the cells that were grown on bare Ti and composite coated Ti were exposed to the flow, there was about 30% loss of cells from uncoated Ti (Fig. 1(c)) whereas the monolayer remained intact on composite coated surface (Fig. 1(d)).

On UHMWPE, cells grew with elongated spindle-shaped morphology and after 48 h the surface was almost 90% confluent (Fig. 2(a)), whereas when the discs were coated with composite the cells maintained normal morphology (Fig. 2(b)). Shrinking of cells were observed on fixation of monolayer that was grown on bare UHMWPE, and under flow more than 40% of the cells were lost (Fig. 2(c)) as compared to the retention of almost all cells of the monolayer that was grown on composite-coated material (Fig. 2(d)).

In the case of PTFE, eventhough $\sim$ 40% cell adhesion was initially observed; in 48 h not even 20% of the area was confluent with the EC layer (Fig. 3(a)). However, on composite coated PTFE confluent monolayer was obtained within 48 h (Fig. 3(b)). After exposure to flow, hardly any cell with normal morphology was retained on the bare PTFE (Fig. 3(c)), whereas the monolayer that

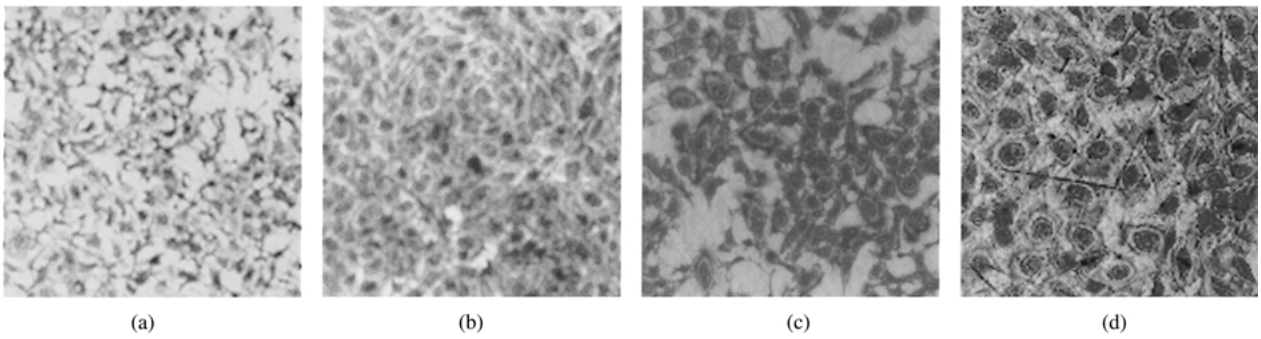

Figure 1 Photomicrographs of endothelial cells grown on titanium. (a) Cells grown on bare Ti for 48 h; (b) Cells grown on composite coated Ti for 48 h; (c) Cells grown on bare Ti for 48 h and exposed to flow; (d) Cells grown on composite coated Ti for 48 h and exposed to flow.

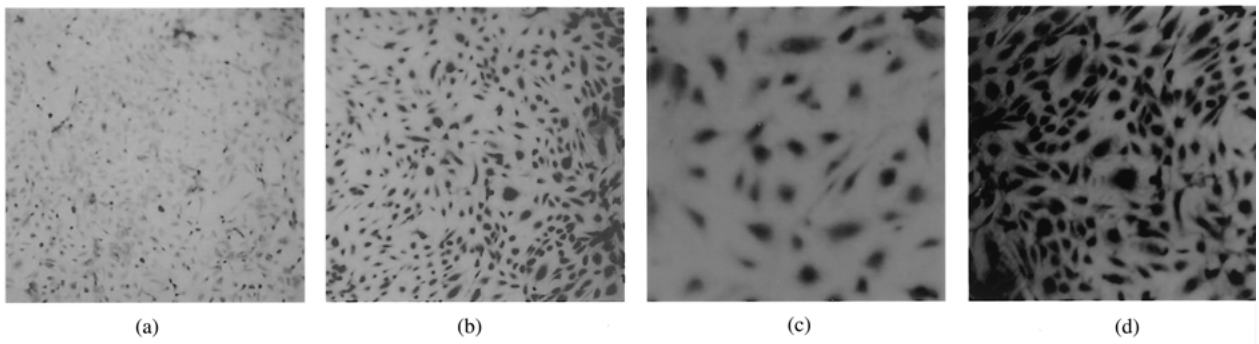

Figure 2 Photomicrographs of endothelial cells grown on UHMWPE. (a) Cells grown on bare UHMWPE for 48 h; (b) Cells grown on composite coated UHMWPE for 48 h; (c) Cells grown on bare UHMWPE for 48 h and exposed to flow; (d) Cells grown on composite coated UHMWPE for 48 h and exposed to flow.

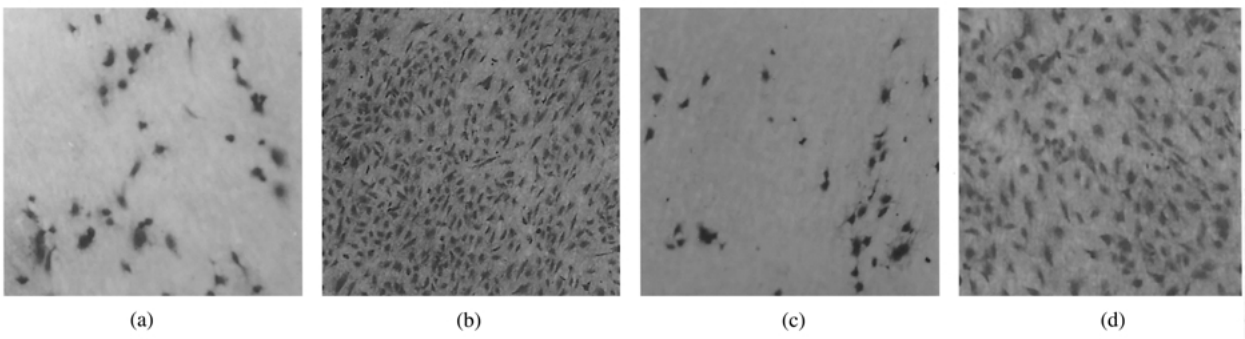

Figure 3 Photomicrographs of endothelial cells grown on PTFE. (a) Cells grown on bare PTFE for 48 h; (b) Cells grown on composite coated PTFE for 48 h; (c) Cells grown on bare PTFE for 48 h and exposed to flow; (d) Cells grown on composite coated PTFE for 48 h and exposed to flow.

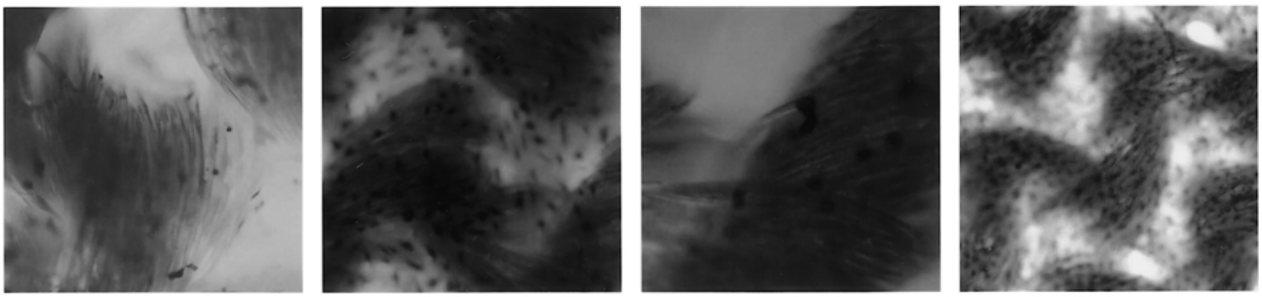

Figure 4 Photomicrographs of endothelial cells grown on Dacron. (a) Cells grown on bare Dacron for 48 h; (b) Cells grown on composite coated Dacron for 48 h; (c) Cells grown on bare Dacron for 48 h and exposed to flow; (d) Cells grown on composite coated Dacron for 48 h and exposed to flow.

was grown on composite-coated PTFE was undisturbed after exposure to flow (Fig. 3(d)).

On bare Dacron with $\sim 35\%$ initially adhered cells, the migration and multiplication was found very low (Fig. 4(a)). On coating the material with composite, the cell spreading and proliferation was as good as all the other surfaces coated with composite. In Fig. 4(b), it is seen that the EC distribution is uniform, though due to the woven texture of the material with uneven planes, not all the cells are focused simultaneously on viewing under light microscope. Even the few cells that were present after 48 h on bare Dacron have got flushed into the medium on exposure to the flow (Fig. 4(c)). On the other hand, the cells that were grown on composite coated surface remained intact after exposure to flow (Fig. 4(d)). On comparison of all bare surfaces, spreading and growth of EC with normal cobblestone morphology was seen only in the case of titanium. However, the cells that were grown on titanium also was shrunk on fixation and $\sim 30\%$ cell was lost on exposure to flow suggesting inadequate adhesive strength. Though the attachment and proliferation rate of EC on UHMWPE was comparable to that of Ti, the morphology of EC on bare UHMWPE was unusual.

The growth rate and resistance of the cells to shear stress were unacceptably poor on both PTFE and Dacron. Even with varied behavior of cells on each of the bare surface under study, coating with composite has resulted in uniform proliferation and resistance to shear stress of monolayers that were grown on all four substrates.

4. Discussion

Irrespective of the chemical and physical nature of the biomaterials that were studied here, uniform coating with a composite of fibrin, gelatin and endothelial cell growth factor was achieved on all substrates. The coated proteins had good adherence to all surfaces and the matrix layer supported good cell adhesion, proliferation and stability of the monolayer that was grown on them.

The success of cardiovascular device is judged by its non-thrombogenicity after it is implanted. Once the blood-contacting surface is endothelialized *in vivo*, it is unlikely to induce thrombus formation. Nevertheless, to get the surfaces endothelialized is the major challenge for all cardiovascular implant devices. Transplantation of cultured autologous endothelial cells on to the blood-contacting surface has tremendous application in the vascular implant biology [4–6]. The currently used vascular biomaterials in various devices include polymer to metals with differing physical properties. We have evaluated the effect of adhesive protein coating on a metal and polymers.

The comparative evaluation of the cell proliferation and morphology on materials without any coating showed higher proliferation rate on Ti compared to uncoated UHMWPE. The morphology as seen under the incident light also favored Ti over bare UHMWPE with typical closely apposed cobblestone morphology. The proliferation rate of EC on both the bare vascular graft material was unacceptable. The morphology of the EC on the entire composite coated surface was similar with well-spread polygonal morphology.

Significance of a matrix coating for tissue engineering application was evident from the proliferation analysis and flow experiments where the presence of the matrix influenced cell growth and retention on all surfaces. Cell adhesiveness is related to the log of the affinity constant of the receptor ligand system [15]. The increased strength of cell attachment on the composite coated surface may be due to the multiple receptor binding possibilities provided by the adhesive that include integrin- and nonintegrin-mediated cell interaction. Such multiple binding to the substrate is likely to improve the cell spreading thereby contributing higher initial strength of attachment and resistance to forces of shear stress. However, in the case of uncoated surface where the cell adhesion is predominantly mediated by adsorbed proteins from serum that vary from material to material depending on the physical property, mostly this is mediated by adsorbed fibronectin where multiple binding is not possible. Even though binding of a cell surface receptor to protein ligand provides cell attachment, it is not sufficient to produce cellular spreading. Previous experiments by other researchers using a combination of integrin-mediated serum protein treatment have improved cell adhesion compared to individual protein treatments [16, 17]. We have demonstrated that a composite of fibrin, gelatin, and endothelial cell growth factor (ECGF) can form an excellent matrix coating on tissue culture polystyrene (TCPS) to enhance cell adhesion and proliferation [18].

This study also provides additional information regarding the requirement of a multiple adhesive coating

for improved cell adhesion, proliferation and cell retention to resist forces of shear stress on all vascular material surfaces.

Acknowledgments

We are thankful to our Director, Prof. K. Mohandas and the Head, BMT Wing, Dr G. S. Bhuvaneshwar for the encouragement we have received during the study. We would also like to thank Mr C. V. Muraleedharan, Scientist, Division of Artificial Internal Organs, for providing the titanium and UHMWPE disks for the study. PTFE grafts were a kind gift from Dr A. P. Chauker, cardiovascular Surgeon, Mumbai. The study was partly supported by a senior research fellowship from CSIR and a grant from DST, Government of India.

References

1. R. SINGHVI, A. KUMAR, G. P. LOPEZ, G. N. STEPHANOPOULOS, D. I. C. WANG, G. M. WHITESIDES and D. E. INGBER, *Science* **264** (1994) 696.
2. K. J. PRATT, B. E. JARREL, S. K. WILLIAMS, R. A. CARABASI, M. A. RUPNINICK and F. A. HUBBARD, *J. Vasc. Surg.* **7** (1988) 591.
3. S. WILLIAMS, B. JARREL and L. FRIEND, *J. Surg. Res.* **38** (1985) 618.
4. J. MEINHART, M. DEUTSCH and P. ZILLA, *ASAIO J.* **43** (1997) M515.
5. H. R. LAUBE, J. DUWE, W. RUTSCH and W. KONERTZ, *J. Thorac Cardiovasc. Surg.* **120** (2000) 134.
6. P. ZILLA, R. FASOL, P. PREISS, M. KADLETZ, M. DEUTSCH, H. SCHIMA, S. TSANGARIS and P. GROSCURTH, *Surgery* **105** (1989) 515.
7. M. GRABENWOGER, M. GRIMM and E. EYBEL, *J. Card. Surg.* **7** (1992) 79.
8. L. BENGTSSON, K. RADEGRAN and A. HAEGERSTRAND, *Eur. J. Vasc. Surg.* **7** (1994) 393.
9. L. BENGTSSON and A. HAEGERSTRAND, *J. Heart Valve Dis.* **2** (1993) 352.
10. G. ZUND, C. K. BREUER, T. SHINOKA, P. X. MA, R. LANGER, J. E. MAYER and J. P. VACANTI, *Eur. J. Cardio-Thoracic Surg.* **11** (1997) 493.
11. T. SHINOKA, C. K. BREUER, R. E. TANEL, G. ZUND, T. MIURA, P. X. MA, R. LANGER R, J. P. VACANTI and J. E. MAYER, *Ann. Thorac. Surg.* **60** (1995) 513.
12. J. D. ANDRADE and V. HLADY, *Adv. Polym. Sci.* **79** (1986) 1.
13. E. A. JAFFE, R. L. NACHMAN, C. G. BECKER and C. R. MINICK, *J. Clin. Invest.* **52** (1973) 2745.
14. L. V. MC INTIRE, J. A. FRANGOS, B. G. RHEE, S. G. ESKIN and E. R. HALL, in ''Blood in Contact with Natural and Artificial Surfaces'', edited by Edward F. Leonard, Vincent T. Tritto and Leo Vroman (Annals of New York Academy of Sciences 516 1992) p. 513.
15. V. D. BHAT, G. A. TRUSKY, W. M. REICHERT, *J. Biomed. Mater. Res.* **40** (1998) 57.
16. J. MAZZUCOTELLI, C. KLEIN-SOYER, A. BERETZ, C. BRISSON, G. ARCHIPOFF and J. CAZENAVE, *Biomaterials* **14** (1991) 482.
17. J. KAEHLER, P. ZILLA, R. FASOL, M. DEUTSCH and M. KADLETZ, *J. Vasc. Surg.* **9** (1989) 535.
18. T. R. SANTHOSHKUMAR and L. K. KRISHNAN, *Biomaterials* (in press).

Received 12 September
and accepted 22 October 2001