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ELECTRON MICROSCOPY OF ENDOTHELIAL CELL - BIOPOLYMER INTERACTION

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Abstract

Vascular endothelial cells form a natural antithrombogenic lining on all blood vessels. Replacement or bypass of small diameter blood vessels with artificial polymeric grafts has not been clinically acceptable due to the thrombogenic nature of polymeric material. One approach to improving the patency of vascular prosthetic devices has been the establishment of endothelial monolayers on the blood flow surface using the technique known as seeding. Scanning electron microscopy has been a major tool in evaluating the interaction of endothelial cells with polymeric surfaces resulting in a basic understanding of forces and structures regulating endothelium-polymer interactions. In-vitro and in-vivo studies have established the feasibility of using endothelial cell seeding technology in human clinical trials. This tutorial describes the development of endothelial cell seeding technology and illustrates how scanning electron microscopic evaluations have furthered our understanding of endothelial cell-polymer interactions.

Key Words: Endothelial Cells, Vascular Grafts, Atherosclerosis, Vascular Surgery, Polymers, Biocompatability, Seeding, Antithrombogenicity

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Introduction

Synthetic vascular grafts gained clinical acceptance in the 1950's as an arterial replacement in reconstructive surgery. Since that time the clinical experience has shown that large diameter grafts used in high flow, low resistance conditions exhibit highly acceptable long term patency rates. As the diameter of the vascular graft used for arterial replacement drops below 6 mm, the failure rates are deemed unacceptable (Veith et al., 1986; Darling and Linton, 1972). While synthetic vascular grafts provide unacceptable patency rates in small diameter positions, autologous natural vessels, specifically saphenous vein, provide more acceptable results (Bergan et al. 1982). The major difference between the patency rates observed for natural saphenous vein replacements as compared to artificial synthetic vessels appears to be the presence of endothelial cells which line the luminal surface of native blood vessels (Gimbrone, 1986; Jaffe, 1985). A major hypothesis of many research laboratories, including ours, has been that re-establishing a natural endothelial cell lining on synthetic vascular graft surfaces will improve both the short term and long term patency rates of these artificial blood vessels.

Dr. Malcolm Herring, working at the University of Indiana, first reported the successful creation of an endothelial cell lining on a vascular graft using a process he termed endothelial cell seeding (Herring et al. 1978). Since that time numerous research groups have evaluated the process of endothelial cell prosthetic graft interaction focusing their attention on two major areas. First, the source and techniques for the isolation of endothelial cells from vascular tissue has received extensive attention (Graham et al. 1980 a,b; Graham et al. 1982; Sharefkin et al. 1987; Sharefkin et al. 1982; Maciag et al. 1981; Thornton et al. 1983). Mechanical and enzymatic procedures have been improved to optimize the quality and quantity of endothelial cells which can be procured from large vessel sources (Jarrell et al. 1984; Sharefkin et al. 1987; Jarrell et al. 1987). The use of autologous fat to procure microvessel endothelial cells has recently shown great promise as an easily obtainable source of autologous endothelial cells (Jarrell et al. 1986; Radomski et al. 1987). With the discovery of substances which stimulate the proliferation of endothelial cells in culture, the use of cultured cells for endothelial cell seeding has also been suggested (Jarrell et al. 1984; Watkins et al. 1984; Franke et al. 1987; Zilla et al. 1987).

A second major area of research interest has been the evaluation of surfaces which exhibit enhanced adhesiveness for endothelial cells (Williams et al. 1985; Schmidt et al. 1987; Seeger and Klingman, 1985; Baker et al. 1985). Our basic understanding of the interaction of endothelial cells with extracellular matrix proteins has led to the development of vascular graft surfaces which mimic the natural surface upon which endothelial cells normally reside (Baker et al. 1985). In order for endothelial cell seeding technology to gain widespread clinical acceptance, the procedures for the isolation and establishment of endothelial cell layers upon vascular grafts must become compatible with the constraints of an operating room. More importantly, endothelial cell seeding techniques must result in the rapid establishment of functional endothelial cell monolayers on the surface of currently available synthetic vascular grafts.

Materials and Methods

Human Endothelial Cell Isolation and Culture

Techniques for the isolation and culture of human microvessel and large vessel endothelial cells have recently been reviewed (Williams 1987). The complete technique for the isolation and culture of human adult large vessel endothelial cells can be found in the work of Jarrell et al. (1984). The isolation and culture procedures for human adult microvascular endothelial cells can be found in the work of Williams et al. (1987) and Jarrell et al. (1987). Briefly, our techniques use tissue obtained from human donors according to our Institutional Review Board approved protocol. Large blood vessels are transported directly from the operation room to our tissue culture facility. Endothelial cells are removed from the luminal surface using a collagenase enzymatic digestion. Isolated endothelial cells are seeded onto gelatin coated polystyrene tissue culture plates and cultured in the presence of endothelial cell growth supplement and heparin in media 199E containing 15% fetal calf serum. Cultures are split at 1:4 split ratios and used at a cumulative population doubling level of 20 or less. Microvascular endothelial cells are isolated from fat using collagenase digestion followed by centrifugation to remove vascular tissue from buoyant adipocytes. Microvascular endothelial cells are either used directly or cultured using identical conditions described for human large vessel endothelial cells above.

Scanning Electron Microscopy (SEM)

The interaction of endothelial cells with polymeric surfaces in vitro have been performed on graft materials immobilized within a modified embedding capsule (Williams et al. 1985). Materials from in vitro and in vivo studies were processed identically for scanning electron microscopic evaluations. Samples were fixed with 3% glutaraldehyde in 0.05M PIPES (Piperazine-N,N'-bis[2-ethane-sulfonic acid]) buffer (pH 7.4) for two hours. Following fixation the samples were dehydrated in graded steps of acetone and critical point dried in a Polaron critical point dryer with carbon dioxide. The dried samples were mounted on SEM specimen stubs and sputter coated with gold in a polaron sputter coater. The samples were then examined and photographed using the following scanning electron microscopes: JEOL JSM 35C; JEOL 820; ISI ABT55; Amray 1200; Amray 1810.

Transmission Electron Microscopy (TEM)

Samples were fixed in 3% glutaraldehyde buffered with 0.05M PIPES at pH 7.4. The samples were then post-fixed with 1% osmium tetroxide in 0.05M PIPES buffer at pH 7.4. Following dehydration in graded steps of acetone the samples were embedded in Spurrs low-viscosity media. Silver sections were cut with a Diatome diamond knife on a Sorvall MT2-B microtome and picked up on uncoated copper grids. They were post-stained with 1% uranyl acetate and counter stained with Satos lead. The sections were examined and photographed in a Joel 100CXII transmission electron microscope.

Results and Discussion

Electron Microscopy of Native Blood Vessel Endothelium

The blood contacting surface of the vascular system is uniformly lined by a layer of extremely attenuated endothelium. With the exception of highly specialized microvascular beds, this endothelial cell lining can be characterized as a uniform layer of endothelial cells with overlapping tightly associated junctions. This layer completely covers the underlying basement membrane [Figure 1] synthesized by these endothelial cells during development. The luminal plasma membrane of endothelium is antithrombogenic and maintains normal blood homeostasis by inhibiting the adherence of platelets, leukocytes, and other blood borne elements while maintaining a transport function in order to provide nutrients to the underlying cellular layers. SEM [Figure 2] reveals the highly attenuated but complete coverage of endothelium on a native blood vessel surface. In this scanning electron micrograph the vessel has been allowed to undergo spasm resulting in constriction of vascular smooth muscle cells. The endothelial cell layer maintains complete coverage of this lining under these conditions. TEM [Figure 3] reveals typical endothelial cell structures as identified by endothelial cell tight junctional associations as well as numerous micropinocytic vesicles within the cytoplasm. The structure and function of vascular endothelium has progressed from its original description as a thin semi-permeable cellophane lining on blood vessels to our current understanding of the cell type as a metabolically active cell with numerous complex, ultrastructurally defined features which maintain it as a selective permeability barrier. The endothelial cell has now been realized as a critical player in numerous physiologic as well as pathophysiologic conditions.

Endothelial Cell Interaction With Commercially Available Vascular Graft Surfaces

At the present time the two most widely used synthetic vascular graft materials are expanded polytetrafluoroethylene [ePTFE] and poly-ethyleneterephthalate [dacron in both woven and knitted forms]. Scanning electron micrographs of these materials [Figures 4A and 4B] illustrate that these are highly unnatural surfaces for the association of endothelial cells as compared to the normal basement membrane material upon which endothelial cells normally reside [Figure 1]. These polymers were originally chosen for vascular reconstruction due primarily to their relative inertness with respect to interaction with normal body tissue, their lack of aneurysm formation, their relative ease of surgical handling, and the general

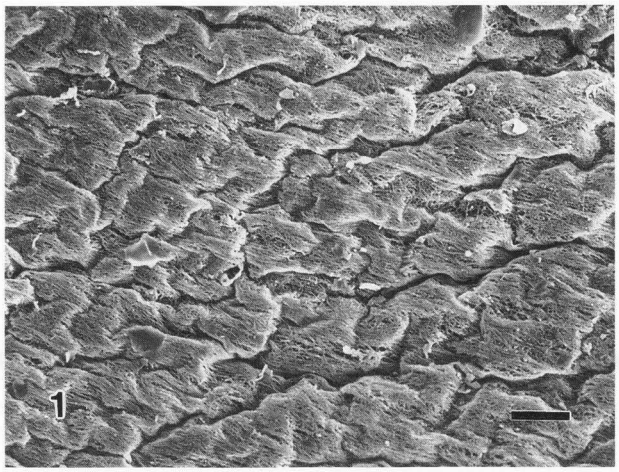
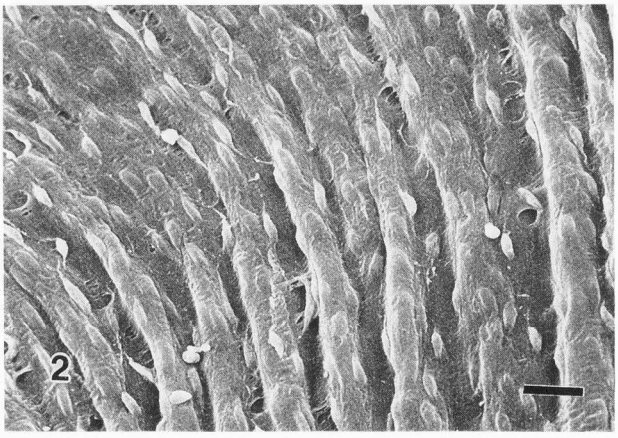


Figure 1 (top left). Scanning electron micrograph of native extracellular matrix underlying large vessel endothelial cells. Bar = 33 micrometers.

Figure 2 (middle left). The natural lining of all blood vessels exhibits a layer of highly spread endothelium. This scanning electron micrograph illustrates a canine iliac artery fixed with glutaraldehyde after the vessel exhibited vasospasm. The undulations represent the effect of smooth muscle cell constriction. Bar = 11 micrometers.



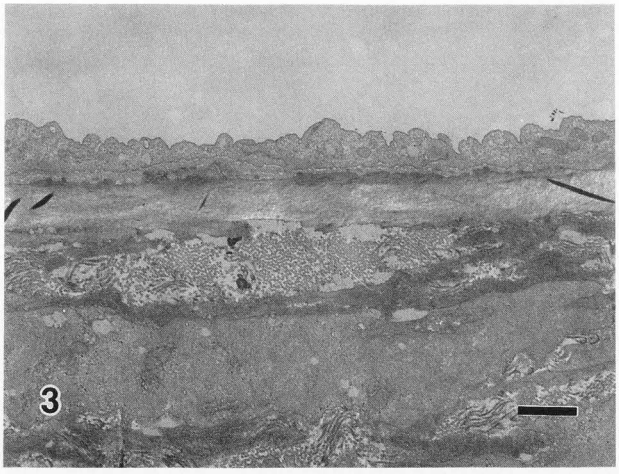
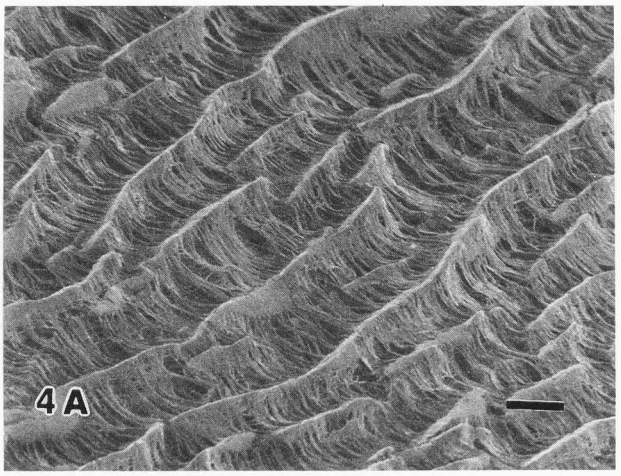


Figure 3. This transmission electron micrograph illustrates the ultrastructure of the endothelium which lines a rat carotid artery. The endothelial cells exhibit characteristic close association of junctional processes and numerous micropinocytic vesicles. Bar = 0.6 micrometers.



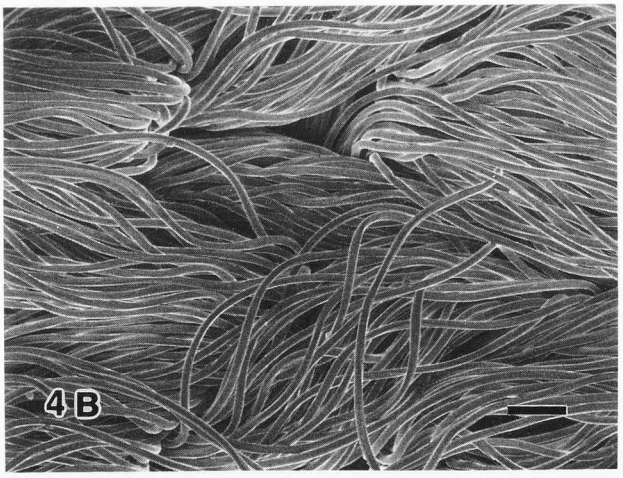
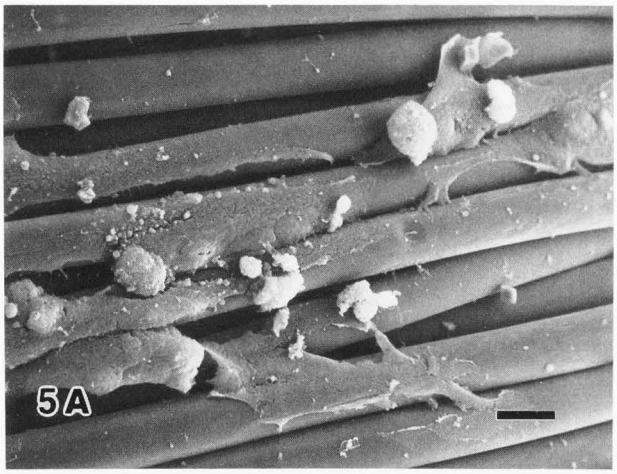


Figure 4 (top). Polymers used for artificial vascular prostheses exhibit a surface topology quite different from the native basement membrane of blood vessels (cf Figures 4A, 4B and Figure 1).

4 A. Scanning electron micrograph of uncoated ePTFE at an accelerating voltage of 1.0 kV. Bar = 11 micrometers.

4 B. Scanning electron micrograph of uncoated Dacron graft material at an accelerating voltage of 0.8 kV. Bar = 56 micrometers.



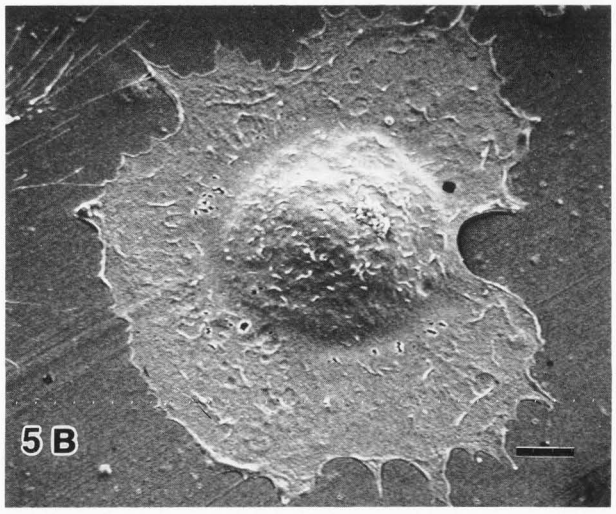


Figure 5. Human endothelial cells exhibit the ability to adhere and spread on polymeric surfaces treated with extracellular matrix proteins. **A.** Human endothelial cells adherent to dacron graft surface. Bar = 11 micrometers. **B.** A single human endothelium exhibits the ability to spread on Poly (ethyleneterephthalate) film. Bar = 4.0 micrometers.

belief that these polymeric surfaces exhibited reduced thrombogenicity as compared with other experimental polymers. The use of these materials for endothelial cell seeding techniques, therefore, has two inherent difficulties, namely the relative structural differences between these polymers and the native basement membrane surface, and secondly the realization that these surfaces were developed to exhibit reduced cell adherence characteristics.

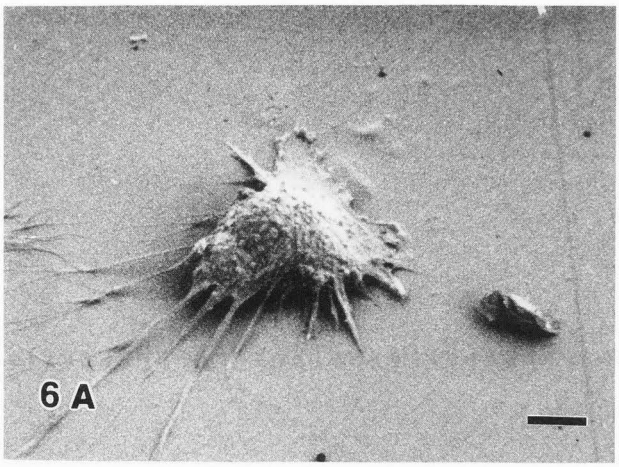
Endothelial Cell-Polymer Interactions

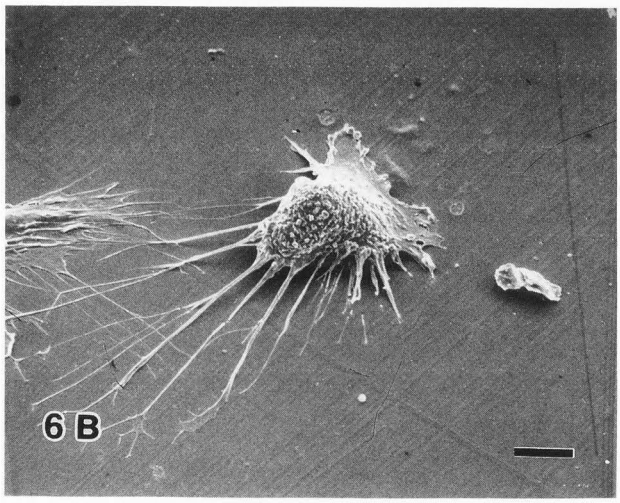
The first studies of endothelial cell-polymer interaction actually can be traced to the early development of endothelial cell culturing techniques. The need to coat polymeric surfaces with extracellular matrix proteins is now a common laboratory procedure used to enhance the adherence and multiplication of anchorage dependent cells (Gospodarowitz et al. 1980; Kleinman et al. 1981; McAuslan et al. 1982). Endothelial cells, especially those derived from human adult large vessel and microvessel sources, exhibit a matrix dependent specificity for their subsequent adherence and proliferation (Madri and Williams, 1983; Kleinman et al. 1981). With the availability of techniques for the isolation and purification of individual extracellular matrix proteins the ability to treat synthetic vascular graft surfaces with these proteins became a reality (Williams et al. 1985; Schnittler et al. 1987; Jarrell et al. 1984; Pratt et al. 1988). While human endothelial cells exhibited only minimal affinity for synthetic vascular graft surfaces, pre-coating of these vascular grafts with extracellular matrix proteins dramatically enhanced both, quantitatively and qualitatively, the adherence and spreading characteristics of endothelium (Pratt et al. 1988; Rupnick et al. 1989). Scanning electron micrographs [Figures 5A and 5B] illustrate the interaction of human adult endothelial cells with woven Dacron, and polyethylenetetrapthylate film. While these electron micrographs indicate that a thin film of extracellular matrix protein will stimulate the adherence and spreading of endothelial cells onto their surfaces, the extent of this endothelial cell interaction must be considered suboptimal.

The ability to utilize lower accelerating voltages has numerous advantages for the visualization of endothelial-polymer interaction. Figures 6A-C illustrates the interaction of a single human adult endothelial cell with a polymeric surface at three different accelerating voltages. These three micrographs represent a series of accelerating voltages which were evaluated with respect to the resulting ultrastructural features. We observed optimal visualization of delicate membrane structures at an accelerating voltage of 7.5 kV [Figure 6B]. At higher accelerating voltages [Figure 6C] over penetration of electrons resulted in decreased definition of plasma membrane structure. At extremely low accelerating voltages [Figure 6A] almost total loss of surface definition was observed.

These results illustrate that for biological materials the principle that the highest resolution is obtained with the highest accelerating voltages is not always true. Higher accelerating voltage results in deeper penetration of the beam which results in secondary electrons being produced deeper within the sample. This results in reduced resolution of surface structure. Two additional effects of higher accelerating voltage are edge effects and charging. Charging is an especially difficult problem with the study of endothelial cell-polymer interactions where cellular material must be observed on polymers which are inherently nonconductive. This often requires heavier deposition of conductive coatings resulting again in reduced resolution of surface structures.

A major area of continued research has been the development of techniques to improve endothelial cell-polymer interaction. By optimizing the surface structure of polymeric materials used for endothelial cell seeding techniques it is hoped that rapid deposition of endothelial cells can be achieved. One such surface we have utilized is a thick basement membrane surface adhered to a synthetic vascular graft (Baker et al. 1985). This surface supports the production of an endothelial cell monolayer following seeding techniques [Figure 7]. The deposition of a thick (Baker et al. 1985) or thin (Williams et al.





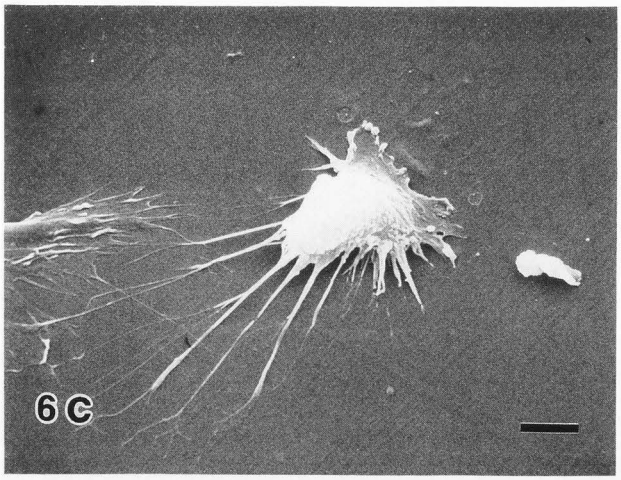


Figure 6. Scanning electron microscopy of endothelial cell to polymer interactions is facilitated by the use of specific accelerating voltages. **A.** 1 kV. **B.** 7.5 kV. **C.** 30 kV. Bar = 3.0 micrometers.

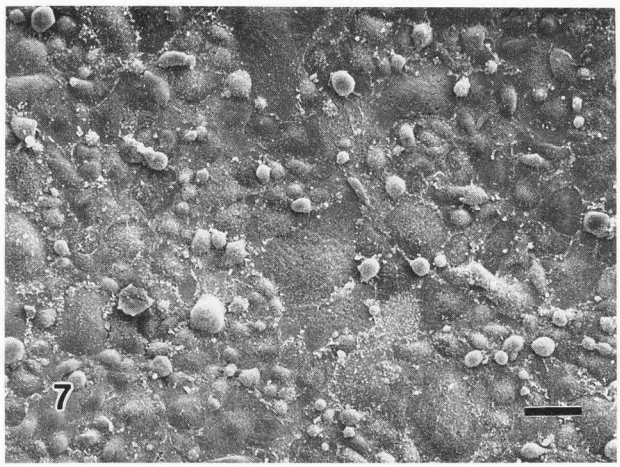


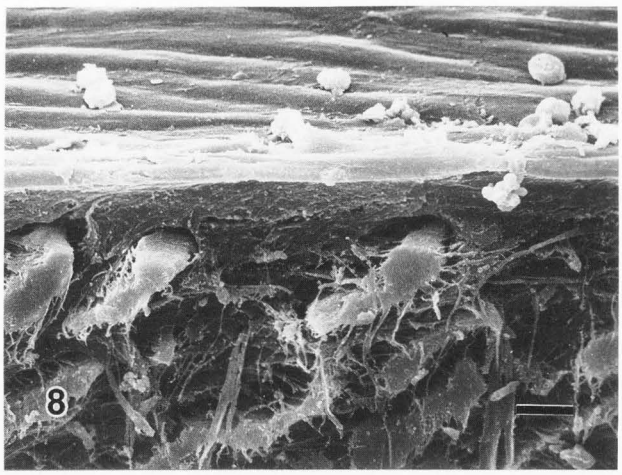
Figure 7. The natural basement membrane found on the epithelial surface of human amniotic membrane exhibits enhanced adherence capacity for human endothelium. Bar = 11 micrometers.

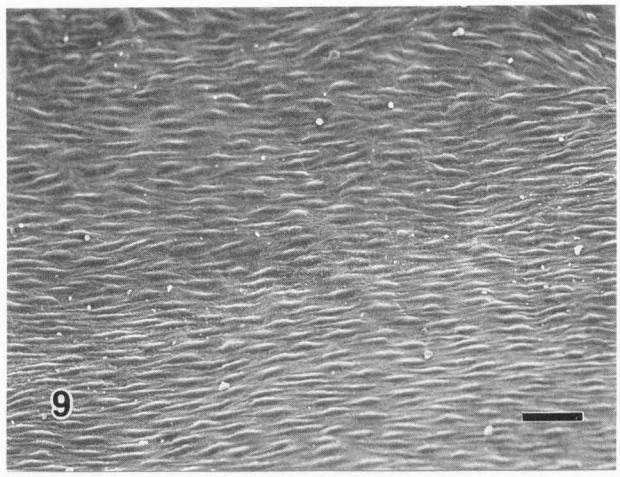
1985) extracellular matrix on vascular grafts improves endothelial adherence and spreading by masking the inherent "anti"-cellular characteristics of bare polymer. Future studies will undoubtedly focus on direct modification of the surface chemistry of available polymers (Pratt et al. 1988) or development of new polymers with enhanced affinity for endothelial cell adherence and spreading.

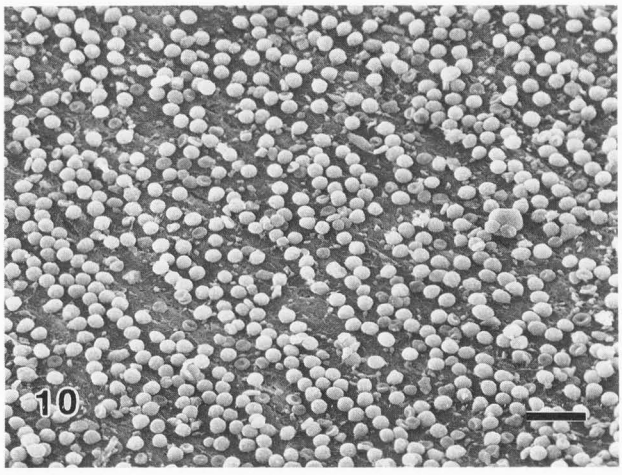
In-vivo endothelial cell monolayer production

Important to the discussion of in-vivo production of endothelial cell monolayers on vascular grafts is the realization that, to date, the optimal animal model for the study of vascular graft performance has not been developed. A major example of this difference between animals and the human species is the fact that while vascular grafts of high porosity implanted into animals exhibit the spontaneous production of an endothelial cell lining (Graham et al. 1982; Herring et al. 1984; Clowes et al. 1986), spontaneous endothelialization of a vascular graft implanted into a human has never been unequivocally reported. Nevertheless, both the canine and the baboon model have provided extremely important insight into the process and effectiveness of endothelial cell monolayer formation following seeding techniques (Graham et al. 1979; Callow et al. 1984; Schmidt et at. 1984; Allen et al. 1984; Stanley et al. 1982; Graham et al. 1980 a,b; Herring et al. 1979). Vascular grafts of 5 mm or less internal diameter seeded with autologous endothelial cells and implanted into the arterial position of dogs exhibit extensive monolayer formation in as little as two weeks. In contrast, control grafts placed in the contralateral arterial position do not exhibit spontaneous endothelialization until at least five weeks of implantation. These animal models have established that endothelial cell seeding dramatically accelerates the formation of endothelial cell monolayers on the luminal surface of vascular grafts.

The new endothelial cell lining of these vascular grafts exhibits many of the characteristics of the normal intimal lining of a natural blood vessel. As shown in Figure 8, the lining of antithrombogenic cells on the synthetic surface can be relatively thin







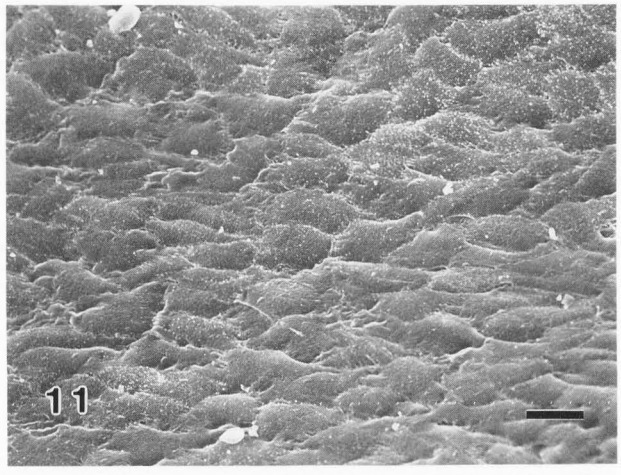


Figure 8. Vascular grafts seeded with autologous canine endothelial cells exhibit a confluent layer of endothelial cells after 5 weeks of implantation. This scanning electron micrograph represents a cross section through an explanted graft showing the antithrombogenic endothelial cell lining resting on the polymeric vascular graft. Bar = 6.0 micrometers.

Figure 9. Scanning electron micrograph of a seeded vascular graft following 5 weeks of implantation in canine model. Bar = 11 micrometers.

Figure 10. A vascular graft implanted in canine artery illustrates the normal disposition of platelets and white cells on this thrombogenic surface. This non-endothelialized surface is in stark contrast to an antithrombogenic endothelial cell lining. (cf Figures 10 and 9). Bar = 11 micrometers.

Figure 11. The antithrombogenic endothelial cell lining of seeded graft represents one of the first successes of "tissue engineering" where cells can be transplanted to a new site in the body to overcome a pathologic condition. Bar = 10 micrometers.

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and comparable in thickness to the normal intimal lining. The healing process results in an incorporation of the graft both on the abluminal surface as well as the direct interaction of cellular and matrix components with the luminal polymeric surface of the graft. For this reason, the newly established endothelial cell monolayer can resist the forces of shear while exhibiting normal endothelial cell phenotype. Normal phenotype is characterized by the lack of platelet and leukocyte deposition as well as a lack of visualization of fibrin deposition. The establishment of an antithrombogenic endothelial cell monolayer on the surface of a polymeric synthetic vascular graft [Figure 9] is in stark contrast to the highly thrombogenic surface which results when a control graft is placed in the contralateral side. As shown in Figure 10, non-seeded synthetic vascular grafts in the arterial position exhibit the deposition of cellular and proteinaceous materials composed of platelets, white cells, and fibrin polymers. As discussed previously, in high flow rate, large diameter grafts, this deposition of material in non-endothelialized grafts does

not significantly reduce the patency rates of these implants. However, in small diameter applications, with reduced flow rates, the deposition of platelets and fibrin is presumably the major cause of the loss of graft patency during the early stages of implantation (Mansfield et al. 1975; Dryjski et al. 1983; Darling and Linton 1972). Figure 11 illustrates another electron microscopic view of an endothelial cell monolayer established on the surface of a synthetic vascular graft. The development of this new flow surface represents a dramatically accelerated developmental process whereby endothelial cells seeded onto this flow surface at the time of implantation are able to perform numerous cellular functions. These functions include the migration of cells into areas where cell seeding densities may be lower, proliferation of cells to increase the density to be equal with that observed in normal blood vessels, and the establishment of interendothelial junctional associations common to the native endothelial monolayer. What is remarkable is this complete developmental process takes place in a period of two weeks or less in the presence of arterial flow. Presumably the methodology for the establishment of endothelial cell monolayers in the clinical setting will be established long before the mechanisms involved in endothelial cell adherence, migration, proliferation, junction formation, and metabolic activities related to antithrombogenicity are elucidated.

Summary

The study of endothelial cell seeding of vascular graft surfaces has grown at nearly the same explosive rate as our general understanding of endothelial cell structure and function. From the pioneering work of Herring and co-workers, major methodological breakthroughs have taken place which have improved our understanding of endothelial cell-polymer interaction. The ability to isolate and establish long term cultures of both non-human as well as human adult endothelial cells has provided great insight into the structure and function of endothelial cells. Our understanding of the importance of extracellular matrix proteins for the adherence and establishment of endothelial cell monolayers has provided great insight into improving the methodologies for establishing endothelial cell monolayers on vascular grafts.

Future Directions

While the efficacy of endothelial cell seeding principles for the establishment of endothelial cell monolayers on vascular grafts has been established, the surfaces currently available for the adherence of endothelial cells must be considered suboptimal. This suboptimal endothelial adherence is not surprising in light of the fact that these polymers were originally chosen for their relative non-thrombogenic nature. The use of different extracellular matrix proteins to coat the surface of these vascular grafts is actually a simplistic attempt to increase the adherence characteristics of these polymers for endothelial cells. A great deal of future research will undoubtedly focus on mechanisms for enhancing the adherence characteristics of base polymers utilized for endothelial cell seeding techniques. With the availability of new technologies to enhance the endothelial cell interaction with synthetic polymer surfaces, and the availability of autologous endothelial cells, the expanded use of endothelial cell technology in clinical practice will undoubtedly occur. The clinical efficacy of endothelial cell seeding procedures will most assuredly be established over the next few years as results of multiple seeding trials, already begun worldwide, are published (Herring et al. 1987; Risberg et al. 1987; Fasol et al. 1987). Scanning electron microscopy will assuredly remain the major morphologic tool to evaluate endothelial cell-polymer interactions.

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Discussion with Reviewers

S.P. Schmidt: What is the appearance of the endothelial lining, both in vitro and in vivo, when microvascular endothelial cells are seeded onto biopolymers?

Authors: The appearance of the endothelial lining which forms on the vascular grafts seeded with either microvascular or macrovascular endothelium is remarkably similar. The ability of microvascular endothelial cells, which exist as tubes 4 to 20 micrometers in internal diameter, to form a flat monolayer on a 4 mm internal diameter graft is not surprising. During development all large blood vessels are derived from microvascular sprouts.

G. Pasquinelli: Aren't the Weibel-Palade bodies the only typical structures of endothelial cells?

Authors: We do not agree. While venous endothelium contain an abundant amount of these structures, microvascular endothelium contain very few.