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ENDOTHELIAL CELL SEEDING OF POLYMERIC VASCULAR GRAFTS

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1. ABSTRACT

Pioneers in the field of small diameter graft development sought to promote graft endothelialization and, thereby, increase patency by transplanting a varying degree of autologous endothelial cells onto vascular grafts prior to implantation. This process has become known as **endothelial cell seeding**. The underlying hypothesis is quite simple; that is, by promoting the establishment of the patient's own endothelial cells on the blood contacting surface of a vascular prosthesis, a "normal" endothelial cell lining and associated basement membrane, together known as the neointima, will form on the graft and counteract the rheologic, physiologic, and biomaterial forces working synergistically to promote graft failure. After 30 years of research in this area, this simple hypothesis has proven to be deceptively naive. The purpose of this review is to summarize the historic context and current base of knowledge regarding many of the technical issues relevant to the endothelial cell seeding process. Special attention is given to electrostatic endothelial cell seeding, the latest research methodology designed specifically to accelerate endothelial cell adhesion and morphological maturation onto expanded poly(tetrafluoroethylene) (e-PTFE) small diameter vascular grafts.

2. INTRODUCTION

As early as the late 1970s, endothelial cell seeding was utilized to help improve the patency of small diameter, polymeric vascular grafts (1). Since that time, many advances have been made toward this goal, with the majority of focus on the tissue engineering of a "living vascular graft." Though the large effort in this relatively new field has not yet come to fruition, tissue engineering research offers the promise of further elucidation of the interactions between endothelial cells and smooth muscle cells. This information is critical for development of a successful polymeric or tissue engineered small diameter vascular graft - a goal often referred to as the "Holy Grail" (2). Until this "ideal" vascular bypass graft is available, however, endothelial cell seeding of traditional polymeric vascular prosthetics can provide a marked improvement in patency rates of graft implants.

A small-diameter vascular graft is defined as being less than 6 mm in internal diameter (I.D.). In clinical usage, these small diameter grafts have low patency rates in humans due to thrombosis and intimal hyperplasia. Both of these processes, at least in part, likely originate due to the lack of development of an endothelial layer on the luminal surface of the graft (3,4) and/or abnormal endothelial and smooth muscle cell direct and indirect communication. Results showing lack of endothelial cell lining development in humans are in sharp contrast to results of the research with other animal species. These data show that neointimal formation on polymeric vascular graft luminal surfaces occurs by endothelial cell proliferation from perianastomotic artery, the microvessels of graft interstices, or circulating progenitor endothelial cells (5).

Endothelial cells are more complex than was originally believed. They do not merely create a single cell lining on the luminal surface of blood vessels. The endothelial cells also release molecules that modulate coagulation, platelet aggregation, leukocyte adhesion, and vascular tone. Upon the absence of these cells, i.e., in the case of the lumen of an implanted synthetic polymeric vascular graft, the host reaction progresses to eventual failure. Loss of patency within the first thirty days post-implantation is due to acute thrombosis. This early stage failure is a consequence of the inherent hostility of the biomaterial's blood-contacting surface, which is non-endothelialized. To date, the only known completely non-

thrombogenic material is an endothelium; any other material that comes into contact with the bloodstream is predisposed to platelet deposition and subsequent thrombosis. The long-term failure mode of small diameter polymeric vascular grafts is anastomotic hyperplasia. The precise mechanisms behind initiation of anastomotic hyperplasia are still being defined; however, endothelial cell and smooth muscle cell dysfunctions and improper communications are likely involved.

Pioneers in the field of small diameter graft development sought to promote graft endothelialization and, thereby, increase patency by transplanting a varying degree of autologous endothelial cells onto vascular grafts prior to implantation. This process has become known as **endothelial cell seeding**. The underlying hypothesis is quite simple; that is, by promoting the establishment of the patient's own endothelial cells on the blood contacting surface of a vascular prosthesis, a "normal" endothelial cell lining and associated basement membrane, together known as the neointima, will form on the graft and counteract the rheologic, physiologic, and biomaterial forces working synergistically to promote graft failure. After 30 years of research in this area, this simple hypothesis has proven to be deceptively naive.

The results of early animal studies were quite promising. Historical perspectives of endothelial cell seeding research including early animal and clinical studies were published (6,7). More recently, the early optimism was tempered by clinical results that were often disappointing, and it was clear that modifications to traditional methodologies to promote neointimal development via endothelial cell seeding were needed if this approach was to be technically successful and reach its goal of significantly improving patency rates of polymeric vascular grafts. The previous twenty-five years of research in endothelial cell seeding has shown researchers the complexity of the biology of the cells forming the vascular wall and proven that technically successful seeding of endothelial cells does not necessarily result in a positive outcome clinically.

The purpose of this review is to summarize the historic context and current base of knowledge regarding many of the technical issues relevant to the endothelial cell seeding process. Special attention is given to electrostatic endothelial cell seeding, the latest research methodology designed specifically to accelerate endothelial cell adhesion and morphological maturation onto expanded poly(tetrafluoroethylene) (e-PTFE) small diameter vascular grafts. The wealth of animal studies and clinical trials is reviewed elsewhere (6,7) and will not be detailed in this review.

3. VASCULAR GRAFTS AND ENDOTHELIAL CELL SEEDING HISTORY

The motivation behind the development of polymeric vascular grafts was based on the rationale that there is a need to replace large diameter arteries such as the aorta, which have been compromised due to disease or trauma. Shortfalls of other replacement solutions, such as the lack of a sufficient autologous source and the limited success of homo- and xeno-grafts, led researchers to explore more practical options (8). The use of a polymeric (Vinyon "N" cloth) vascular prosthetic was first published in 1952 by Voorhees, Jaretski, and Blakemore, who presented the concept of a synthetic conduit as a replacement for deficient natural blood vessels (9). In 1954, the first published clinical results of this research demonstrated that the polymeric conduits could be used as an arterial replacement/substitute (10). This advance obviously sparked the race to develop the optimum polymeric vascular graft. Quickly, two polymeric compositions for large (>10 mm) and medium (6-10 mm) inner diameter (I.D.) vascular grafts were developed and continue to be used clinically today with great success. The first, and most satisfactory in terms of mechanical strength and thromboresistance, of these two compositions is expanded poly(tetrafluoroethylene) (e-PTFE). The second is poly(ethylene terephthalate) (PET), better known as Dacron®. As noted, these materials function with success at diameters above 6 mm I.D. However, the patency rates for small diameter (<6 mm I.D.) vascular prostheses configured from these same materials are unacceptable when utilized clinically due to acute thrombus formation (11,12) and chronic anastomotic hyperplasia (13-15).

Dr. Malcolm Herring pioneered the technology of endothelial cell seeding in the late seventies (1) by proposing that, if small diameter vascular grafts could be made more biocompatible, they could be of enormous clinical importance in peripheral and coronary bypass grafting procedures (1). This lofty goal has yet to be realized; today, polymeric small diameter vascular grafts are used only in last resort cases. It is estimated that there are over 1.4 million surgical procedures performed each year in the United States that require the use of an arterial prosthesis, the majority of which require the application of a small diameter (<6 mm I.D.) prosthetic. Of those small diameter prosthetics required, most are used for coronary artery bypass procedures, but many are used for reconstruction of various body parts in conjunction with autologous tissue transplants.

Due to limited success in clinical trials, endothelial cell seeding has been largely experimental. Significant technical success has been demonstrated in preclinical trials with animal models; however, this success has not been transferred thus far to human vascular surgery. Surgeons have long expressed concerns about the many technical issues that must be overcome in order to successfully seed endothelial cells in an acceptable time frame for human surgery. These concerns will be addressed throughout this review with particular attention to how electrostatic endothelial cell seeding overcomes many of these issues. More importantly, though it is so desperately needed clinically, the long-term benefits of an endothelial cell seeded polymeric vascular graft in clinical applications have not yet been demonstrated.

Over the past twenty-five years, the majority of the research efforts focused upon process issues in an effort to

maximize cell harvesting and seeding efficiencies in a clinically relevant time frame. This review briefly overviews the history of these studies and then describes the novel technology of electrostatic cell seeding, which offers solutions to many of the deficiencies of other approaches for seeding endothelial cells onto polymeric vascular grafts.

4. AUTOLOGOUS ENDOTHELIAL CELL HARVESTING

Before endothelial cell seeding can occur, harvesting of endothelial cells from an appropriate autologous source is necessary to prevent rejection of the implanted foreign tissue. Sources of autologous endothelial cells most often used experimentally include: 1) non-essential vessels and 2) omental or subcutaneous adipose tissue. Endothelial cells from non-essential vessels such as the saphenous vein have been harvested by two basic techniques: 1) mechanical scraping (16) and 2) enzymatic digestion (17,18). Mechanical scraping uses mechanical disruption to dislodge the endothelial cells from the luminal surface of the vessels. This technique, however, tends to effect endothelial cell damage and also usually leads to scraping of a significant number of smooth muscle cells located below the thin endothelial layer, causing contamination of the desired endothelial cell aliquot (1,16). Enzymatic harvesting of cells from the vascular lumen requires the action of proteases such as collagenase and/or trypsin to digest the basement membrane proteins and cause release of the endothelial cells (19-21). This process, if done incorrectly, can damage cellular proteins, which, in turn, affects endothelial cell viability and subsequent ability to adhere to a polymeric vascular prosthetic surface (22,23). Mechanical scraping results in a greater than 75% efficiency for endothelial cell harvesting (17), and enzymatic digestion results in an 80-100% overall efficiency (17). It should be noted, however, that the total number of endothelial cells harvested by these methods is limited by the total length and surface area of source non-essential vessel available.

Another concern with the above techniques is that, due to prior usage and existing disease conditions, availability of non-essential autologous vessels is extremely limited or even nonexistent in many patients, thus limiting the number of autologous endothelial cells that can be utilized in surgery. This concern prompted investigators to seek alternative sources that could adequately supply autologous endothelial cells. With only a few possible choices available, the alternative source that has been most intensively studied is the microvasculature, which can potentially provide a more than adequate number of endothelial cells for most clinical applications. Microvascular endothelial cells can be harvested from the micro-vessels (arterioles, capillaries, and venules) found in omental adipose tissue (19,24,25). Briefly, the technique begins by mincing the adipose tissue and placing it in an enzymatic solution for a designated period of time. The final suspension of enzymatic solution, tissue, and endothelial cells is then 1) centrifuged using a Percoll density gradient (19), 2) filtered (26), or 3) purified with Dynabeads® (Dynal; Oslo, Norway) coated with lectin Ulex europaeus I (UEA I) (27) to separate the components and isolate the microvascular endothelial cells. This method results in approximately an 84% overall harvesting efficiency, which translates to approximately $2.5x10^6$ endothelial cells per gram of adipose tissue (28,29).

Another approach to deriving large numbers of autologous endothelial cells from patients with limited sources is tissue culture expansion. The tissue culture approach for cell population expansion for vascular graft seeding was first reported by Graham et al (30). In 1973, Jaffee et al (20) reported that the doubling time for human endothelial cells in the first *in vitro* cultures was 92 hours, which means a prolonged culture period to achieve a substantial quantity for clinical use. Since this time, there has been much improvement in cell culturing of endothelial cells, in particular the supplements added to the culture media. These advancements have led to a reduced doubling time of approximately 24 hours. Thus, tissue culture can result in a relatively large number of endothelial cells from limited autologous inocula in a time span of a few days to weeks.

However, tissue culture expansion of endothelial cells for clinical application has raised some issues. First, the still lengthy culture period makes the process impractical for emergency bypass procedures. Second, the exposure of endothelial cells to tissue culturing media containing an undefined serum, e.g., fetal bovine serum, presents the potential for both genotypic and phenotypic modulation during an *in vitro* culture period (31). In addition, the human manipulation of these tissue culture systems for periodic media refreshing and frequent cell passaging introduces the potential for contamination. If undetected in culture, contamination could lead to vascular graft infection, subsequent failure, and possibly eventual amputation or death.

5. ENDOTHELIAL CELL SEEDING TECHNIQUES

Numerous methodologies for endothelial cell seeding onto prosthetic graft surfaces have been reported in the literature. These techniques can be differentiated and categorized based upon the unique physical force utilized in each seeding process: (1) gravitational (1, 32-35), (2) hydrostatic (36,37), and (3) electrostatic (38-43). The hypothesis, just as originated by Dr. Herring, for each of these approaches is that the seeding of endothelial cells on a graft luminal surface will promote, with time after implantation or culture period *in vitro*, the development of a mature, physiologically appropriate confluent graft luminal endothelial cell lining or pseudo-intima.

As has been highlighted, much of the historic work on endothelial cell seeding has been driven by the perceived need to increase the total number of endothelial cells that adhere to the grafts as well as the morphologic development stage of the adhesion in order to reduce loss upon implantation. A normal endothelium in the lumen of a blood vessel is a simple squamous epithelium consisting of a single layer of flat cells. Upon harvesting of the endothelial

cells and resuspension in seeding media, the endothelial cells assume a spheroid morphology. During the seeding procedure, the endothelial cells initially adhere as a spheroid with a point adhesion to the graft substrate. After a lag period that depends upon cell-substrate interactions, the endothelial cells mature and flatten to a discoid shape and then continue maturation into a completely flattened phase with a central hill (region of the cell nucleus) on the adhesion substrate. Again, the phase maturation is important for increasing the streamlining as well as the amount of contact between the endothelial cells and the graft material, thus reducing the number of endothelial cells lost upon implantation and subsequent exposure to fluid shear stress. The increased spreading (morphological maturation) during endothelial cell seeding is an important step for the success of these methods. A study by Pratt et al (44) has shown that endothelial cell adherence to a vascular graft exposed to flow conditions is dependent upon the endothelial cell-surface interaction and the time allowed for adhesion and morphological maturation, or spreading, prior to the initial exposure to fluid shear stress.

5.1. Gravitational Endothelial Cell Seeding

The most basic and extensively studied of the three seeding techniques utilizes gravitational forces to deliver endothelial cells to a vascular graft luminal surface. The generic concept involves filling the graft with harvested endothelial cells resuspended in seeding medium, which can be various forms of tissue culture media, whole blood, or blood plasma. The filled graft is maintained horizontally and rotated periodically or continuously over a prolonged seeding time. Coating the graft surface prior to seeding with biological glues such as fibronectin is commonly used with this technique to promote endothelial cell attachment and morphological maturation on the synthetic graft material. However, there is a major disadvantage to use of biological glue in this technique: any region that is not endothelialized during seeding becomes even more thrombogenic upon implantation than the native graft material (12). Biological glues are discussed further below.

A significant disadvantage of early attempts at gravitational endothelial cell seeding was that, if the seeding time was short (<2 hours), the adhered endothelial cells were in a spheroid morphology upon completion of the seeding procedure, leading to a significant loss of endothelial cells when blood flow was restored through the graft. The cells had minimal time to mature and flatten and did not have enough contact with the graft to withstand the shear forces induced by blood flow. Endothelial cell losses of up to 95% in the first 24 hours postimplantation were observed (45). To overcome this limitation, attempts were made to endothelialize the graft luminal surface via the traditional method but follow with subsequent *in vitro* tissue culture of 7-14 days to allow morphological maturation prior to implantation (33,34,46-49). This modification did improve results and minimize loss of endothelial cells upon exposure to blood flow. However, as previously mentioned, the practicality of prolonged *in vitro* culture of endothelialized grafts remains a concern of surgeons and researchers. As discussed, this is not an acceptable option in emergency situations, and the potential for genotypic and/or phenotypic changes and introduction of infection (cell culture contamination) are of major concern to both proponents and adversaries of clinical use of endothelial cell seeding.

5.2. Hydrostatic Endothelial Cell Seeding

Hydrostatic seeding techniques use a pressure differential, either internal pressure (32,36) or external vacuum (37), across the microporous graft wall to force harvested endothelial cells suspended in the chosen medium onto the luminal surface of the vascular graft material. Because whole blood is typically used as the base medium, heparin is usually added to the medium to mitigate clotting and subsequent blocking of pores during seeding. Once seeding is completed, however, preclotting to close off the pores is necessary prior to implantation. To date, experience has shown that there are at least three major limitations of hydrostatic seeding techniques. The first limitation is that any immobilized heparin from the seeding medium remaining on the graft surface can hamper preclotting. Furthermore, in patients that are receiving systemic heparin therapy, this technique is not adequate; the problems with preclotting are compounded in these cases. Heparin interacts with the Antithrombin III molecule, which inhibits the action of thrombin to form clots within the graft pores, and hemostasis is difficult to maintain (50). Second, the surface may be inherently rough and thrombogenic immediately following the preclotting procedure, leading to activation of platelets and possible thrombus formation (32). Finally, immediately following seeding, adhered endothelial cells on the graft surface are in a spheroid morphology (100% spheroid shaped); the cells are merely trapped at the luminal surface of the graft straight from the seeding suspension. Prior to implantation, an *in vitro* culture period of greater than two hours is required for optimal endothelial cell adhesion and morphological maturation and to minimize cellular loss upon exposure to vascular physiological shear forces (37).

5.3. Endothelial Cell Seeding – Biological Glue

As mentioned, many investigators have attempted to increase the number of endothelial cells adhered to synthetic grafts, as well as enhance their degree of surface adhesion and morphological maturation, by placing an adhesive protein on the luminal surface prior to seeding to act as a "biological glue" (44,51-59). Significant research efforts have focused on glue formulations including fibronectin, extracellular matrix, collagen, laminin, fibrin, fibroblast matrix, and plasma (33,52,53,54,56-61). The most commonly investigated biological glue is fibronectin. Fibronectin is an adhesive glycoprotein found in the basement membrane to which endothelial cells attach in native blood vessels. This glycoprotein is also required for attachment of endothelial cells to culture flasks *in vitro* and, thus, it was a logical choice for the principal biological glue.

Problems with using fibronectin and other glues arise immediately upon implantation. Any glue surface that has not been endothelialized or has lost its endothelial coverage due to flow-induced shear forces over morphologically immature cells is thrombogenic. Since it

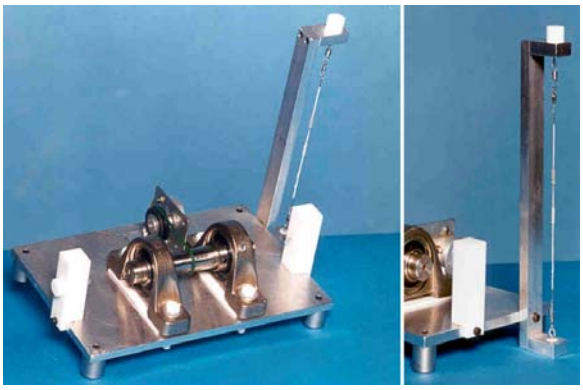

Figure 1. The prototype electrostatic endothelial cell seeding apparatus (43).

is difficult to harvest a sufficient number of cells for seeding relative to total graft surface area and seeding techniques are inefficient, implantation of a completely endothelialized graft is difficult to achieve and control (62). This glue surface is, thus, attractive to platelets and can promote the thrombotic events that lead to acute graft failure (12). Ramalanjaona et al. (52,63) were able to show that, using a fibronectin glue, the number of adhered endothelial cells was increased, these cells displayed enhanced adherence and morphologically maturation, and the loss of cells upon exposure to shear stress was reduced. However, complete endothelialization was not achieved, and any non-endothelialized areas were thrombogenic upon implantation; experimental subjects required anti-coagulant therapy to reduce the complications caused by thrombus formation.

5.4. Electrostatic Endothelial Cell Seeding

One of the more challenging technical issues related to endothelial cell harvesting from autologous sources and subsequent seeding is the time required for proper application of these processes. It has been the collective experience of those in the field that a minimum of 45-60 minutes is required for harvesting and seeding of endothelial cells onto a prosthetic graft for direct implantation into a patient. This time frame, however, does not include that needed for extensive cellular adhesion or morphological maturation of the cells to occur prior to implantation and restoration of blood flow through the graft. Conversely, some investigators have suggested that a time greater than two hours from cell harvest to seeded graft implantation is definitely not acceptable clinically due to the chance of genotypic and/or phenotypic changes. In addition, an increased procedure time translates into a longer time that a patient is under anesthesia. Though the length of time and dosage of anesthesia and its safety are difficult to generalize since multiple drugs, techniques, anesthetists, and patients are all interrelating factors, the longer the exposure, the greater the chance for complications (64,65).

The necessity for an incubation period to allow significant cell adhesion and morphological maturation of seeded endothelial cells is related to the basic nature of the electrostatic interactions between the polymeric graft materials and the cells. The clinically successful vascular prosthetics such as e-PTFE are highly negatively charged. This negative charge repels endothelial cells (66,67) and platelets (68-72), which are also negatively charged. Thus, initial adherence of seeded endothelial cells must overcome the long-range negative-negative charge repulsive forces between the cells and graft material in order for the seeding procedure to result in significant cellular adhesion and morphological maturation and be successful. Experiments using platelets have demonstrated that the cellular adhesion of platelets on a negatively charged substrate is one order of magnitude (ten times) less than expected by gravitational settling alone due to this electrostatic repulsive interaction that can only be overcome via a stochastic process (73-75). Even when cells successfully overcome the initial long-range repulsive forces and attach to the surface, similar short-range repulsive interactions subsequently alter cellular morphologies by preventing or slowing morphological maturation.

This situation has been confirmed by endothelial cell and fibroblast studies that prove the dependence of cell adherence on substrate surface charge (76-81). These studies used several substrates with differing surface charges to study cell adhesion, morphological maturation (spreading), and contact regions between the cells and the substrates. The overall results from these studies indicated that an increasingly positively charged surface leads to enhanced adhesion, spreading, and degree of contact regions. The results on increasingly negatively charged substrates indicated the inverse, with inhibited adhesion, reduced spreading, and reduced contact regions.

The only conclusion that can be drawn from evaluation of the first 15 years of research effort related to endothelial cell seeding techniques is that few concrete technical advancements have been made. This situation changed with the introduction of a novel device and methodology that has shown potential value for improvement of the efficiency of endothelial cell attachment and minimization of cellular loss upon implantation (38-43). The method is called electrostatic endothelial cell seeding. This technique has been evaluated *in vitro* and *in vivo* using the prototype apparatus shown in Figure 1 (43). The key to this technique is enhancement of endothelial cell adhesion by inducing a temporary positive surface charge or "temporary glue" on the typically negatively charged e-PTFE graft luminal surface. Following cell transplantation, the e-PTFE graft luminal surface resumes its inherent highly negative charge. Upon restoration of blood flow, any non-endothelialized graft surface or exposed graft surface that lost endothelial cells due to shear forces remains non-thrombogenic, at least to the extent of the material composition.

The electrostatic technique answers the fundamental question underlying traditional endothelial cell seeding techniques - "How can the surface potential of the graft be altered to attract endothelial cells without rendering the surface thrombogenic?" The electrostatic endothelial cell seeding technique takes advantage of the dielectric properties of the graft material. When a dielectric material

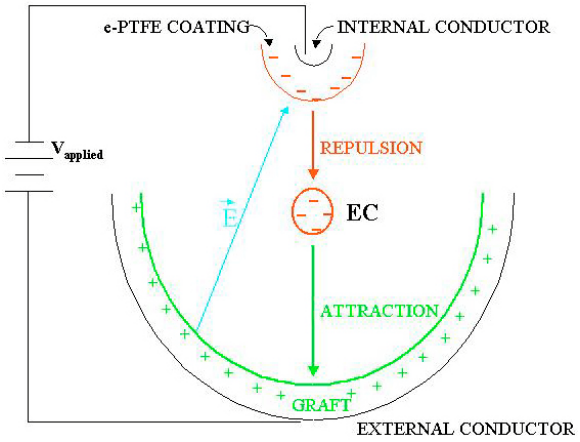

Figure 2. Illustration of the phenomena occurring within the electrostatic endothelial cell seeding apparatus (Figure 1) demonstrating the applied electrical field (E), the induction of a positive, less negative, surface charge on the surface of a polymeric vascular graft, and the attractive/repulsive forces acting on the endothelial cells contained within the seeding suspension.

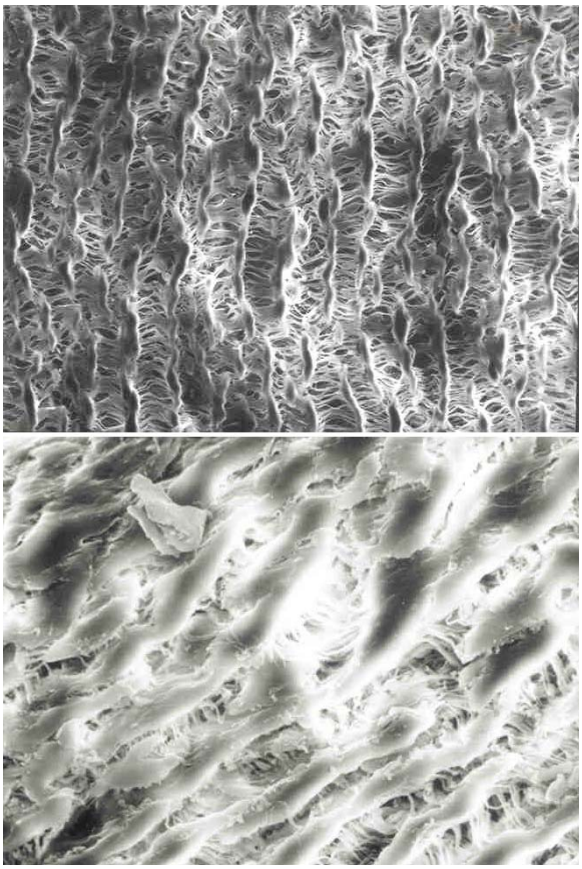

Figure 3. Scanning electron micrographs immediately after the electrostatic seeding of human umbilical vein endothelial cells (+1.0 Volt applied for 16 minutes) on ePTFE (GORE-TEX®; 30 μm internodal distance) illustrating the complete, morphologically mature coverage of the nodal areas (Magnification 260 and 750x, respectively).

(graft material) is placed within a capacitor (electrostatic seeding apparatus), the electrons of the atoms and ions that make up the dielectric material near its surface are attracted to the capacitor surface, which has accumulated a positive charge (Figure 2). The nuclei of the dielectric material are attracted to the negatively charged capacitor surface. These small displacements, or polarizations, are what induces the surface charge or "temporary glue" on the graft luminal surface. It should be noted that the electrons in a dielectric material are not free and have no current carrying capacity since the material is actually an insulating material; the displacements of the electrons are very slight. Also, the interior volume of the dielectric graft material remains unchanged, thus leaving a net charge of zero over the total dielectric material (82,83).

Several *in vitro* studies have been performed utilizing the electrostatic seeding technique. When human umbilical vein endothelial cells were seeded onto 4 mm I.D. e-PTFE grafts using the electrostatic cell seeding technique, complete nodal area coverage of morphologically mature (completely flattened) endothelial cells (73,540 cells/cm^2) was obtained in 16 minutes (+1.0 Volt applied to apparatus) (40). Minimal cellular membrane damage or other effects on endothelial cell viability were apparent. A section of optimum electrostatically seeded e-PTFE is illustrated in the scanning electron micrographs in Figure 3. The *in vitro* evaluations also revealed no significant losses of endothelial cells upon exposure of the graft to a wall shear stress of 15 dynes/cm^2 for up to 120 minutes immediately following seeding (41). Using a traditional gravitational seeding technique, the majority of endothelial cell loss (up to 30%) occurred within the first 30 minutes of implantation (45,52). To date, no *in vitro* or *in vivo* evaluations of the hydrostatic endothelial cell seeding technique have been performed. It is speculated, based on the fact that the majority of the endothelial cells are in the spheroid state within this 16 minute seeding period, that the retention of endothelial cells would be minimal upon shear stress exposure. Thus, the electrostatic seeding procedure is superior to the gravitational and hydrostatic seeding procedures in terms of the seeding time required, magnitude of endothelial cell adhesion (attachment), and cellular retention.

It is speculated that the electrostatic endothelial cell seeding technique can also rearrange the actin microfilaments, which make up the endothelial cell cytoskeleton and which possess their own electrostatic potential. Activation of this mechanism can assist in the maintenance of endothelial cell adhesion by acting as an anchoring system; this has not yet been demonstrated in our experiments (84,85). It is also hoped that the enhanced cell attachment resulting from electrostatic endothelial cell seeding will enable the seeded endothelial cells to synthesize the necessary fibronectin (<3 days) and basement membrane collagen (<1 week) needed to maintain adhesion on the graft for the long term (86-88).

Preliminary *in vivo* preclinical studies using a canine femoral artery implantation model were performed

to further support the promising *in vitro* results and demonstrate the efficacy of the technique (38,39). More specifically, the purpose of the first of these *in vivo* studies was to evaluate the persistence of electrostatically seeded endothelial cells lining an e-PTFE graft after one week exposure to *in vivo* circulation in a canine femoral artery bypass model (37). Furthermore, this study was performed to confirm that the source of the endothelial cells lining the graft were from the initial inoculum. These objectives were accomplished by visualizing a PKH26 red fluorescent label placed in the endothelial cell membranes prior to the electrostatic seeding procedure. Briefly, this procedure consisted of harvesting autologous canine jugular vein endothelial cells, PKH26-labeling the cells, electrostatically seeding the e-PTFE grafts (4 mm GORE-TEX®, Length = 6 cm), implanting the grafts for one week, and explanting the grafts for light, fluorescent, and scanning electron microscopy evaluations of the luminal surface. The results of this study showed that unseeded control grafts had a mean surface coverage of 6.82 $\pm$ 7.19% given by fluorescence, while the endothelial cell seeded grafts had a mean of 90.3 $\pm$ 14.3%. These results are statistically significantly ($p<0.001$) different. Overall, the seeding time including endothelial cell harvesting and PKH26 labeling was approximately 75 minutes. In summary, the electrostatically seeded endothelial cells persisted after implantation of the graft as demonstrated by the PKH26 labeling data. The data also demonstrated that after one week implantation the pseudo-intima formed by endothelial cell luminal surface coverage was in fact derived from the cells initially seeded, as determined by the abundance of the labeled cells.

The purpose of the second *in vivo* study of electrostatic endothelial cell seeding was to evaluate the extent of luminal coverage of endothelial cells composing the pseudo-intimal lining and the thromboresistance of electrostatically seeded small diameter e-PTFE vascular grafts (39). During this study, the acute healing of the vascular graft in terms of smooth muscle cell migration through the graft wall was examined in response to endothelial cell seeding. More specifically, this evaluation consisted of harvesting autologous canine jugular vein endothelial cells, electrostatically seeding the e-PTFE grafts (4 mm GORE-TEX®, Length = 6 cm), implanting the grafts in a canine femoral artery model for six weeks, and excising the graft for histological and scanning electron microscopy evaluations of the mid-graft sections. For the acute healing examination, photographic slides were made of each mid-graft section at a magnification (~150X) which rendered the graft wall cross-section as filling the slide image field. The images were then projected and the graft wall cross-section divided into two equal halves (Adventitial and Luminal halves) with each half encompassing a 0.007 mm^2 sampling area. The number of stained smooth muscle cells within each section was then totaled. The results of the histological evaluation at the mid-graft region indicated that electrostatic endothelial cell seeding significantly affected pseudo-intimal development ($p<0.01$), as well as the degree of thrombus formation ($p<0.001$), within the endothelial cell seeded grafts versus the untreated control grafts. The scanning electron microscopy examination demonstrated a mature, confluent endothelium with a cobblestone appearance on the seeded graft luminal surface. The control grafts demonstrated an equal distribution of smooth muscle cells through the thickness of the graft wall while the electrostatically seeded grafts exhibited an uneven smooth muscle cell distribution that was skewed toward the graft luminal surface. The overall conclusion from this *in vivo* study was that the utilization of electrostatic endothelial cell seeding significantly ($p<0.01$) enhanced the development of a pseudo-intima and reduced the incidence of thrombosis in e-PTFE grafts implanted in a canine femoral artery model. Results of the mid-graft smooth muscle cell migration measurements indicate that electrostatic endothelial cell seeding had a significant ($p<0.001$) impact on the acute healing of the standard wall e-PTFE vascular graft specimens.

6. CONCLUSIONS AND RETROSPECTIVE

A wealth of information has been obtained from research protocols investigating the potential of endothelial cell seeding for improvement of small diameter vascular graft function. Realization of the hypothesized potential of this technique for patients has remained elusive to date. This may be due in large measure to the complex biology of the vascular wall, the disruption created by the graft implantation process, and subsequent interactions of the graft with the physiological system. The question is raised, however, of whether or not that potential has yet been fully tested, as various traditional methodologies and techniques for endothelial cell seeding have all been, to one extent or another, inadequate when applied clinically. Given the historic context of endothelial cell seeding, electrostatic endothelial cell seeding offers the potential to circumvent or at least alleviate many of the technical issues which have prevented clinical application of vascular graft seeding. It is with renewed enthusiasm then that we are revisiting the technology of endothelial cell seeding of vascular grafts, armed with new technical approaches and insights into the biology of vascular cells.

For researchers in the field of vascular tissue engineering, (which encompasses endothelial cell seeding as one of its simplest therapies), one question remains with today's attempts at developing a complete or partial vascular construct - "Where does endothelial cell seeding fit in regards to clinical necessity?" Endothelial cell seeding has the potential to improve the patency of small diameter polymeric vascular grafts composed of the same materials that are currently available and which are used extensively in large and medium diameter vascular surgeries. While the procedure does not currently render a long-term patency rate of 100%, it has been shown to significantly improve the long-term patency rates of small diameter synthetic vascular grafts even at low seeding efficiencies. This technique could serve as a critical bridge between what is clinically manageable today for small diameter vascular surgery and the time when the ultimate solution of a tested and approved tissue engineered living vascular graft is developed. Such a graft is particularly important for coronary bypass procedures but is a minimum of 10 to 15 or more years from becoming a clinical reality. Thus, endothelial cell seeding technology would have an

important role in improving the quality of life for patients in need of small diameter vascular grafts in the meantime.

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