Use of an Endothelial Monolayer on a Vascular Graft Prior to Implantation

Temporal Dynamics and Compatibility with the Operating Room

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The temporal sequence of events was examined from initial contact of endothelial cells (ECs) to Dacron® until the establishment of a monolayer. Cultured human adult ECs were radiolabeled, seeded onto Dacron, and adherence was quantified after vigorous washing. Firm adherence of 70% of the seeded ECs was seen by 2 hours to untreated Dacron, by 30 minutes to Dacron pretreated with a combination of interstitial type I/III collagen and an amnion-derived basement membrane (Type IV) collagen surface, and by 10 minutes to plasma-coated Dacron. Parallel samples were examined morphologically by scanning electron microscopy (SEM) to evaluate the adherence of ECs to surfaces. ECs seeded onto plain Dacron exhibited limited adherence, while cells on plasma-treated Dacron exhibited limited cell–cell associations. On basement membrane-treated Dacron, by 30 minutes the ECs exhibited a flat attenuated morphology, completely covering the graft surface. This time-frame is compatible with most vascular procedures, making an immediately endothelialized graft feasible.

THE USE OF prosthetic vascular grafts to bypass arterial occlusive lesions has greatly aided patients with atherosclerosis. The principle limitation of their use has been in the area of small vessel replacement therapy. Experience has shown a poor patency rate when grafts 4 mm and smaller are used, particularly when extensive occlusive disease with outflow obstruction coexists.[1–4] One major contributing factor to the high failure rate has been related to the intrinsic thrombogenicity of currently existing prosthetic materials. A potential solution to complications as a result of thrombogenicity might be the generation of an endothelium on the graft surface. Unfortunately, although this occurs spontaneously at a variable rate in many laboratory animals, it fails to develop in humans.[5–7]

Many investigators have shown that the process of endothelialization can be augmented in animals by autologous endothelial cell (EC) "seeding" of the graft at the time of implantation.[5,8–11] It is not known whether this technique will aid graft endothelialization in humans.[12] However, even if seeding is proven to augment endothelialization in humans, it is not clear that it would improve short-term patency of small caliber grafts. This may be predicted on the basis that 4 to 6 weeks following graft implantation are required for a significant percentage of the graft to be spontaneously endothelialized in most animals. If this time frame is compared with most human lower extremity prosthetic graft clinical series, it is noted that a large percentage of graft failures due to thrombosis occurs within the first month following implantation.[13] Thus, establishment of an intact endothelium on a graft at or near the time of implantation might be necessary before a significant effect on short-term patency could be seen.

For this to be an obtainable goal, one would require a ready source of large quantities of autologous ECs, a prosthetic surface substrate combination receptive to ECs, and knowledge of the attachment requirements and temporal parameters necessary to allow both a functional and shear-resistant monolayer to reliably form. Although all of these factors must be addressed ultimately, in this study we have chosen to determine the temporal sequence of events from ECs seeding on a prosthetic surface to generation of a confluent monolayer. Examining the kinetics of EC attachment and subsequent attainment of confluence is

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useful in that it may reveal whether a vascular graft can be pre-endothelialized at the time of implantation.

Methods

Isolation and Culture of Human Endothelial Cells

Endothelial cells were isolated from vascular tissue procured from brain-dead, heart-beating cadaver renal donors and were cultured according to our previously published procedures.[14,15] In the present study, ECs from adult human iliac veins were used. Briefly, cells were isolated from a fresh iliac vein by treating the luminal surface with collagenase (Worthington Type I; Worthington Diagnostic Systems, Inc., Freehold, NJ) and grown in 25-cm^2 tissue culture flasks precoated with gelatin (1%) in culture medium [medium 199, 20% heat-inactivated fetal calf serum, 90 μg/ml heparin (porcine), and 20 μg/ml endothelial cell growth factor (ECGF)]. Population doublings were calculated during culture by the formula PD = $\log^2$ (number of cells harvested/number of cells seeded × attachment efficiency) and summed to give the cumulative population doublings (CPDs). The EC identity of cultures was established using criteria previously reported,[14] and included positive staining for factor VIII-related antigen, cobblestone morphology, and the expression of EC-specific prostaglandin and angiotensin-convertin enzyme activity.

Preparation of Human Basement Membrane Native Matrix Surfaces

A surface of human basement membrane components was prepared using fresh human placenta.[16,17] All procedures were performed under sterile conditions. The inner amnionic membrane was gently separated manually from chorion, washed three times with sterile distilled H_2O, and incubated with 1 mM n-ethylmaleimide for 1 hour at 4 C. The amnion then was incubated in 4% deoxycholate solution for 2 hours at 37 C. This procedure loosened the epithelial cells from underlying basement membrane and facilitated the denudation of the epithelial cell layer with the addition of gentle agitation using a rubber policeman. The underlying basement membrane was exposed and served as a surface for EC seeding. Prepared amnion was preserved until graft preparation in complete culture media (M 199, 20% heat inactivated FCS, 90 mg/ml L glutamine) supplemented with 1200 μg/ml penicillin/streptomycin and 4 μg/ml Fungizone®. Prior to use, the amnionic membranes were incubated for a minimum of 2 hours in culture media.

Collagen Preparation

Heterologous interstitial collagen types I and III were prepared from human placenta following the procedures of Madri.[18] Briefly, minced and freeze-dried placenta was pepsin-digested, and a collagen fraction was solubilized with 0.5 M acetic acid. Types I/III collagen were precipitated with 1.4 M NaCl, and the precipitate was collected and dialyzed extensively against buffer. The white flocculent collagen was used either immediately or freeze dried and stored at −20 C until use.

Graft Surface Preparation

The surface of woven Dacron graft was coated with collagen following the solubilization of collagen in 0.0174 M acetic acid and dilution to 0.32% collagen with ice-cold medium 199 and $NaHCO_3$. Deposition was promoted by allowing grafts to sit overnight at 20 C. The surface then was covered with 0.2% glutaraldehyde for 1 minute and rinsed with plain medium. Prepared amnion then was overlayed onto the graft surface, with the basement membrane surface oriented away from the Dacron® surface. This was incubated at 37 C for 2 hours to promote graft-to-amnion adhesion. The amnion-coated graft was then immobilized within a plastic ring (Beem capsule, Polysciences, Fort Washington, PA), providing a 0.5-cm^2 surface area.[19]

Dacron Treatment with Platelet-Rich Plasma

Platelet-rich plasma (PRP) was prepared from anticoagulation (acid-citrate dextrose) whole blood from normal human donors. Just prior to graft treatment, PRP was mixed with 50 mM $CaCl_2$. Grafts were treated with PRP, and a fibrin clot was permitted to form at 37 C. The clot was washed with culture media prior to EC seeding.

Scanning Electron Microscopy of Endothelial Cell Seeded Grafts

ECs derived from iliac veins were grown to confluence in 25-cm^2 flasks and used for cell seeding after two cell passages at a 1:4 split ratio. ECs were treated briefly (1.5 minutes) with trypsin solution (0.25% trypsin with 0.09% EDTA in normal saline), washed once with culture media, and resuspended in complete culture media prior to graft surface seeding. ECs were seeded at a cell concentration sufficient to provide a 100% confluent monolayer of cells on gelatin-coated plastic surfaces. This density is equal to 1×10^5 cells/cm^2.

At appropriate times, seeded graft surfaces were washed free of non- or loosely adherent cells by forcing complete culture media through a Pasteur pipette directed over the seeded graft surface. Graft surfaces were immediately fixed in 1% glutaraldehyde and prepared for SEM.

Scanning Electron Microscopy

Seeded graft surfaces were fixed with 1% glutaraldehyde for 1 hour, 2% glutaraldehyde for 2 hours, washed three

times (20-minute period) with Tyrodes cacodylate buffer (pH 7.4), and dehydrated in a graded series of acetone. The grafts, still immobilized within plastic rings, were critical point dried and coated with gold palladium. Mounted samples were examined in a Phillips scanning electron microscope.

Radiolabelling of EC

ECs were radiolabeled using two separate procedures.

Thymidine labeling. Confluent ECs in a T-25 flask were treated with trypsin solution, washed with culture medium, counted, and replated onto a T-75 flask at 10^4 ECs/cm^2. After 24 hours of growth, 0.5 μCi of triatrated thymidine (Amersham, Arlington Heights, IL) was added to the flask and incubated for 24 hours. The radioactive supernatant was removed and incubated for 24 hours. The ECs were treated with trypsin solution and counted on a scintillation counter.

Indium labeling. ECs were grown to confluence in 25 cm^2 flasks and briefly trypsinized. Released cells were pelleted by centrifugation (100 × g; 3 min) and washed once with phosphate buffered saline (pH 7.4). The cells were resuspended just prior to labeling in 0.5 ml of phosphate buffered saline. The cell concentration was adjusted to 2.5×10^5 cells/ml. Twenty microcuries of indium^{111} (Medi Physics, Emeryville, CA) were added to the cell suspension, and cells were permitted to label for 30 minutes with gentle agitation. Just prior to washing, a 5 μl sample was removed to permit final analysis of labeling efficiency. Labeled cells were washed three times by centrifugation using complete tissue culture medium. The final pellet was resuspended in complete tissue culture medium to a final concentration of 2.5×10^5 cells/ml.

Radiolabeled EC Seeding and Adherence Quantitation

The ECs were seeded onto matrix-coated Dacron immobilized in Beem capsules and incubated for specified time intervals (t = 1, 5, 10, 20, 30, 60, 120 minutes). At each time interval, the following samples were obtained. The first sample, designated the "supernatant," was obtained by pipetting off the supernatant from the Beem capsule. The second sample, designated "loosely adhered," was obtained by vigorously washing the graft surface by forcefully pipetting culture medium three times onto the surface. The medium used to perform these washings was pooled, and the entire specimen formed the second sample. The third sample, designated "adhered," was obtained by removing all adherent ECs from the graft surface. This was performed by solubilizing EC samples in triplicate in 0.2 ml of 0.3% sodium dodecyl sulfate and transferring the resulting solution to filter paper. Each filter paper was transferred to 10% ice cold TCA, and precipitated material was counted. The individual sample counts were normalized to percentages of the total number of counts in all three samples and plotted as % EC in each fraction *versus* time in minutes. ECs labeled with tritiated thymidine were counted in a scintillation counter, and ECs labeled with indium were counted in a gamma counter. Each data point represents the mean of two separate samples. Each adhesion curve was replicated in at least three separate experiments.

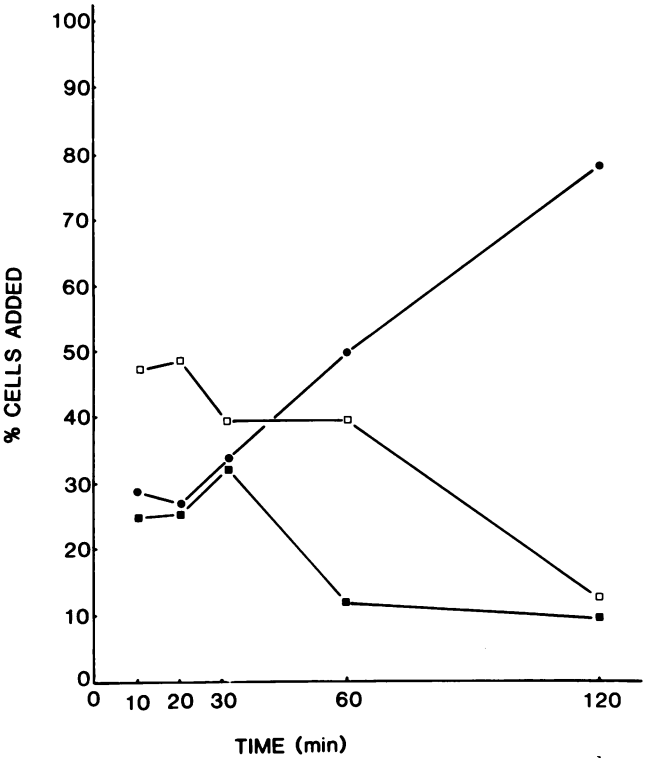


FIG. 1. Adherence of human adult endothelial cells to untreated Dacron grafts. Endothelial cells were radiolabeled and cell association quantified as described in the methods. At designated times, ECs that remained free in the supernatant (■–■) were quantified. The graft surface then was washed by expelling media from a Pasteur pipette over the surface and cells loosely adhered (□–□) and firmly adhered (●–●) were quantified. Briefly cultured and minimally trypsinized endothelial cells exhibit a time-dependent adherence to untreated Dacron.

Results

Quantitative Evaluation of EC Adherence

The adherence of ECs to graft surfaces was evaluated using both thymidine-labeled and indium-labeled cells. Both of these procedures provided similar results, indicating that the method of cell radiolabeling does not affect the kinetics of cell adhesion. Human endothelial cells exhibited a time-dependent adherence to untreated woven Dacron graft surface (Fig. 1). By 10 minutes, approximately 30% of the added cells were observed to be firmly adhered to the Dacron surface. The number of firmly ad-

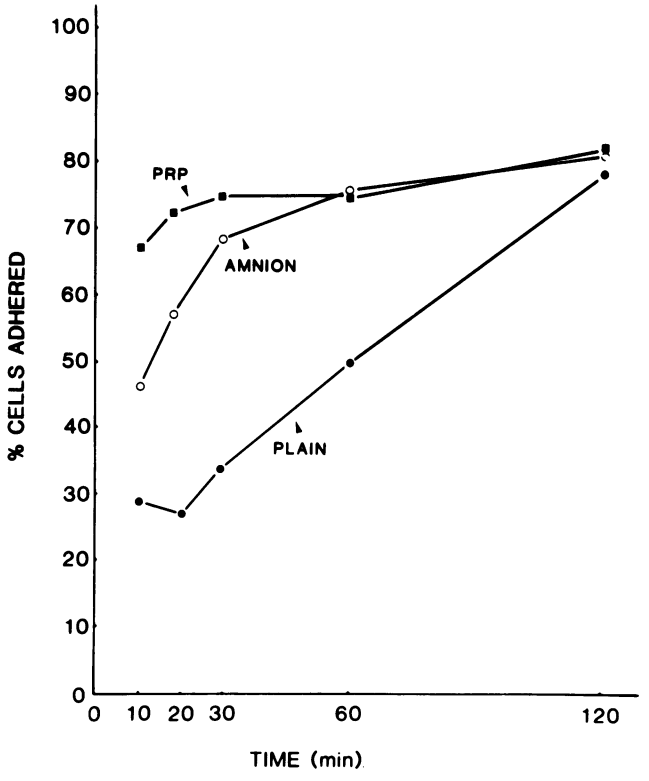


FIG. 2. Comparison of human adult endothelial cell adherence to untreated Dacron and Dacron treated with either platelet-rich plasma or human amnionic membrane. Establishment of a protein surface on Dacron accelerates the adherence of endothelial cells to the surface. PRP = surface resulting from clotting platelet rich plasma on Dacron. AMNION = surface resulting from the bonding of acellular amnionic membrane to the Dacron surface. The basement membrane surface of the amnion was oriented away from the Dacron surface. PLAIN = untreated Dacron weave. Cellular adherence was quantified as described in Methods.

hered cells gradually increased with time, with 50% firmly adhered by 1 hour and 80% of the added cells firmly adhered by 2 hours. Simultaneous quantitation of loosely adhered and nonadhered cells (Fig. 1) revealed that a major proportion of the cells that were not firmly adhered to the graft surface were free in suspension. By 2 hours of association with the graft, surface cells that were either loosely adhered or free in suspension accounted for approximately 20% of the total cells added to the graft.

When the graft surface was pretreated to produce a surface of either a platelet-rich plasma (PRP) clot or a natural basement surface prepared from human amnion, the rate of association of human endothelial cells was dramatically accelerated (Fig. 2). The PRP clot facilitated the rapid association of ECs, with 70% of the added cells firmly adhered following 10 minutes of incubation. The number of cells adhered to PRP increased to a maximum of 80% by 2 hours.

The initial adherence of HAEC to human basement membrane (amnion)-coated Dacron was intermediate between untreated Dacron and PRP-treated Dacron (Fig. 2). The number of cells adhered increased with the time until the number of cells adhered to amnion equalled the number adhered to PRP-treated Dacron, sometime between 30 and 60 minutes. As seen in Figure 2, all the surfaces exhibited approximately the same number of adherent cells after 2 hours of incubation.

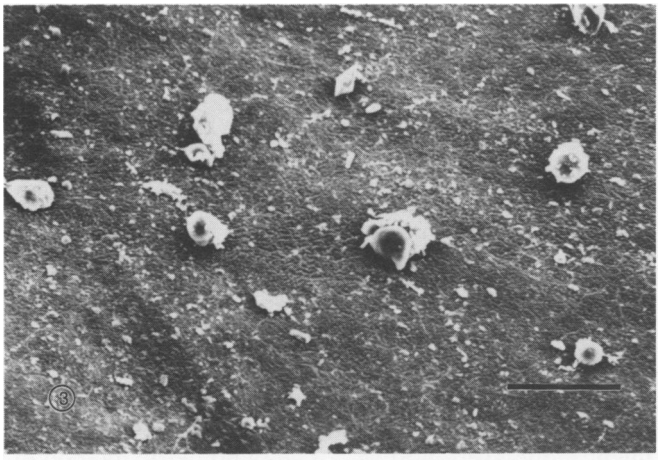
Morphological Evaluation of EC Adherence to Graft Surfaces

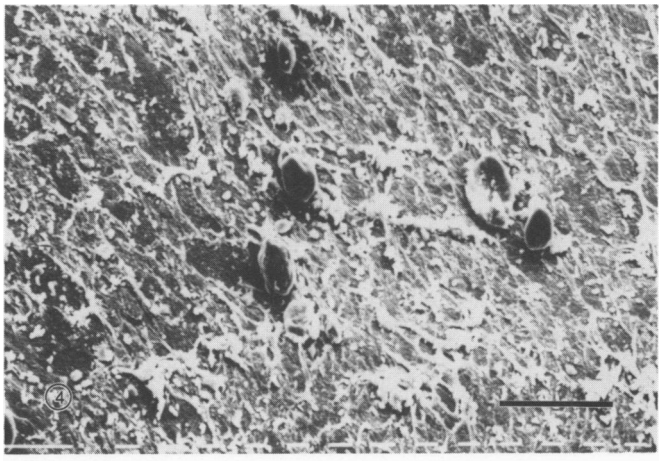
The quantitative analysis of EC-graft interaction provided an analysis of the rate of EC adherence to the surface, but questions remained as to the form of interaction of ECs with graft surfaces. Therefore, we evaluated morphologically the adherence of ECs to basement membrane-treated and PRP-treated graft surfaces, and to untreated Dacron itself. The temporal sequence of HAEC adherence to basement membrane treated grafts are shown in Figures 3 to 7. Following the addition of ECs, graft surfaces were washed at several intervals between 1 and 120 minutes and evaluated by SEM. After just 1 minute, round ECs were observed to be firmly adhered to the comparatively smooth amnion surface. Following 10 minutes of incubation (Fig. 4), ECs still maintained a round appearance, suggesting focal adherence to a limited area of the cellular basal surface.

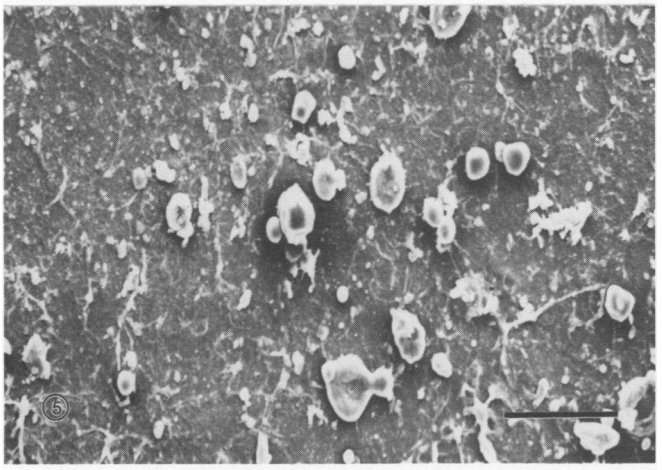
Evaluation of EC adherence to basement membrane after 20 minutes of incubation provides the first evidence of cellular shape change (Fig. 5). While spherical cells persist, cells with a more flattened morphology are observed. The edges of the more compressed cells are still rounded, suggesting limited association of cells at their distal surfaces. An increase in the number of cells adherent after 20 minutes also is evident. Figure 6 illustrates numerous morphologies of ECs adhering to the amnion surface. Most easily identifiable are the persistence of round cells adhered to both partially and fully spread ECs. The most numerous but least identifiable cell morphology is the extensively spread ECs, which provide a near complete cover to the original amnion surface. ECs that have not completely attenuated are also identifiable.

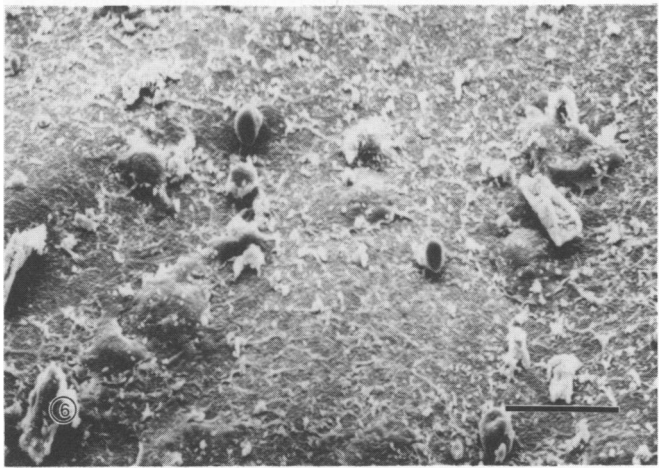
A complete morphological maturation of the EC-seeded amnion surface is observed 1 hour after the onset of cell association (Fig. 7). ECs have covered the amnion surface, and the loss of membrane ruffles (compare the cell surfaces in Figs. 6 and 7) results in a smooth EC monolayer surface. The close association of ECs makes the identification of cellular borders difficult; however, the occasional presence of incompletely attenuated cells (arrow) provides a point of reference for the evaluation of the cellular nature of this monolayer due to the topology of the underlying Dacron fibers (Figs. 7 and 9).

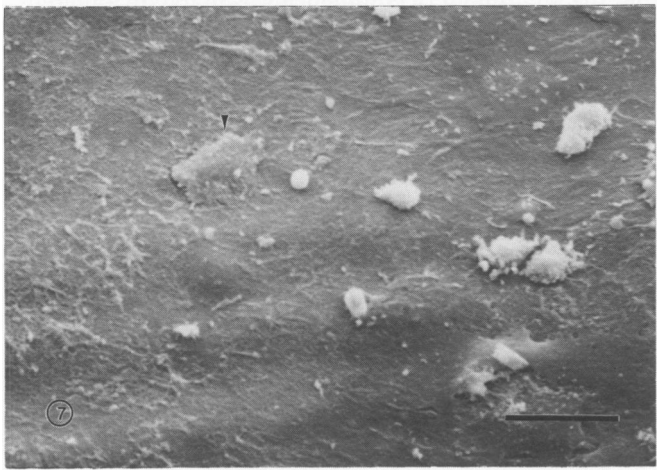
The morphology of cells adhered to PRP (Fig. 8) and untreated Dacron (Fig. 9) was also evaluated after 60











FIGS. 3–7. Scanning electron micrographs illustrating the association of human adult endothelial cells with amnion-treated Dacron as a function of time. Cells were permitted to associate with the amnion surface for 1 minute (Fig. 3), 10 minutes (Fig. 4), 20 minutes (Fig. 5), 30 minutes (Fig. 6), or 60 minutes (Fig. 7). Nonadherent cells were removed by washing and samples fixed for morphological examination. All micrographs are printed at identical magnifications, and the bar is equal to 30 microns.

minutes of adherence. EC adherence to PRP-treated Dacron (Fig. 8) was observed to involve areas both devoid of cells and areas where cells exhibited EC characteristic cell–cell interaction. Also of interest is the observed apparent deposition of fibrin on the surface of flattened ECs. Since this fibrin layer was formed prior to cell seeding, we suggest that ECs are exhibiting the ability to partially migrate under the fibrin lining prior to their complete adherence and flattening. Most importantly is the common observance of areas that totally lack endothelium and therefore expose the fibrin layer. Finally, Figure 9 represents EC adherence to untreated Dacron surfaces

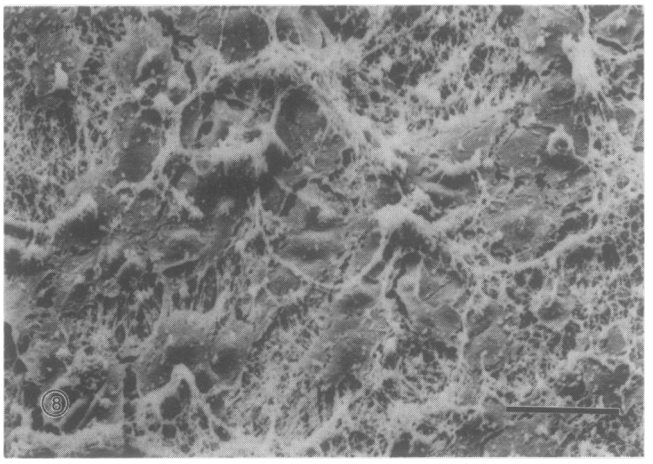


FIG. 8. Scanning electron micrograph of human adult endothelial cell association to PRP-treated Dacron. PRP was forced into the Dacron surface and excess PRP removed. A fibrin clot was permitted to form and washed prior to EC seeding. Bar = 30 microns.

after 60 minutes. To resist the forces generated during washing prior to fixation and during sample preparation for microscopic examination, ECs must wrap partially around and across individual Dacron fibers. Patches of multiple ECs were not observed on untreated Dacron surfaces.

Discussion

Vascular graft endothelialization potentially can be produced by low density EC seeding followed by EC proliferation, high density EC seeding, or spontaneous ingrowth of ECs onto a surface following implantation. High density EC seeding with establishment of a confluent monolayer at the time of implantation offers the best possibility of a nonthrombogenic graft in the first several weeks following surgery when the risk of thrombosis is greatest. The present study therefore was undertaken to examine whether high density EC seeding was capable of producing a morphologically normal appearing endothelial monolayer within time parameters compatible with an operating room vascular procedure. Experimental conditions were chosen based on our previous observations on EC-graft interactions.[16,19] However, unlike our previous studies, early passage ECs with only two prior exposures to trypsin were exclusively used.[20,21]

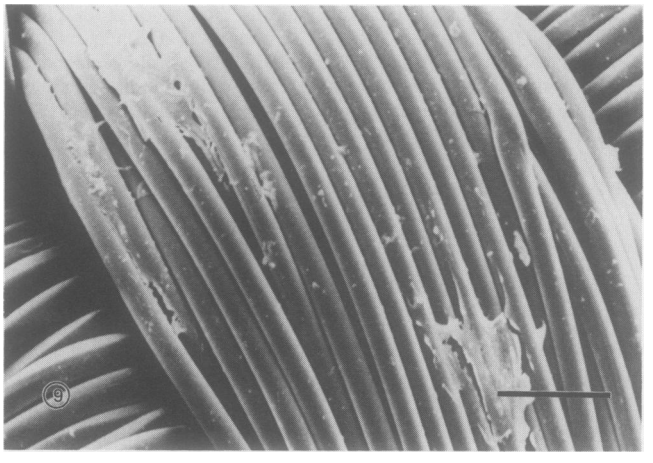


FIG. 9. Scanning electron micrograph of human adult endothelial cell adherence to untreated Dacron graft. Bar = 30 microns.

Two separate methods to study adherence were used because we have previously observed that a cell number compatible with a contact-inhibited confluent monolayer (*i.e.*, 10^5 ECs/cm^2) does not always correlate with a confluent monolayer on SEM.[19] The radiometric method of quantitating cell adhesion, using both tritiated thymidine and indium^{111}, was used as an accurate method to measure the number of ECs that are either not adherent, firmly adherent, or in the process of attaching to the surface. Using these tools, we have observed that firm adherence takes place within minutes to plasma-coated Dacron. In spite of this rapid adherence property, progression to a morphologically normal-appearing monolayer is delayed. The ECs at 60 minutes demonstrate few cell-to-cell interactions and have a "stellate" morphology rather than a "cobblestone" morphology. Although the nonthrombogenic characteristics of this surface have not been examined, the abnormal morphology suggests that these ECs are not experiencing ideal conditions and may not tolerate the effects of flow. EC adherence to the amnion collagen-coated Dacron graft was slower than for the plasma coated, but the attainment of confluence was markedly different. Although the adherence was focal at the early time points, by 20 minutes the ECs were forming many attachment points to the surface and were in the process of flattening and spreading. The process of flattening was maximal by 30 minutes, and many cell-to-cell interactions were present. This resulted in a confluent monolayer on SEM that appeared morphologically similar to native vessel endothelium. This exciting observation suggests that briefly cultured ECs have the capability of becoming a monolayer within a time-frame compatible with the surgical dissection time prior to inserting a vascular graft. Thus, one could seed a graft at the beginning of the procedure and have a confluent monolayer by the time blood flow was restored.

The adherence study on amnion/collagen-coated Dacron reveals that 77% of the seeded ECs adhered to the surface by 30 minutes. This indicates that the majority of briefly cultured ECs possess the ability to adhere to the surface and that, most likely, no subgroup of ECs with special attachment properties is present or necessary. Since the ECs were seeded at a density equivalent to that of a confluent contact-inhibited monolayer (10^5 ECs/cm^2), it

is also notable that subconfluent attachment (*i.e.*, 77%) still allowed the attainment of a morphologically confluent layer. Thus, the minimum number of seeded ECs necessary to produce complete coverage of a graft without growth may be less than 7.7×10^4 ECs/cm^2.

Confluence in this study is defined as complete EC coverage of the prosthetic surface as seen on SEM. Cell-to-cell associations appear normal, but further studies with transmission electron microscopy are ongoing to determine the type of junctions present, as well as the type of association between the ECs and graft substrate combination. Substrate is known to have an effect on cell morphology and function and will have to be examined in this experimental setting before concrete conclusions can be drawn.[22-25] In addition, the use of amnion as a graft substrate requires further investigation. It is a biologically derived material not readily applicable to clinical use. In addition to Type IV collagen, it also contains other cellular attachment factors such as fibronectin and laminin. The effect of these variables in this system is not known, but they could play a key role in the establishment of a monolayer.

Although the EC monolayer appears normal morphologically, it remains to be shown if it possesses other functional characteristics of normal endothelium, particularly with reference to nonthrombogenicity. It must also be shown that the monolayer is able to withstand physiological arterial shear stresses and maintain contained adherence.[26,27] This must be demonstrated not only for the EC-basement membrane adhesion but also the basement membrane-vascular graft interaction as well as EC-to-EC attachments.

We conclude from this study that the majority of briefly cultured human adult large vessel ECs possess the ability to rapidly adhere within 10 minutes to plasma-coated Dacron and within 30 minutes to amnion/collagen-coated Dacron. Adherence to plain Dacron requires longer periods of time before significant adherence has taken place. Although adherence to amnion/collagen-coated Dacron is slower, the net result after 30 minutes is a monolayer of ECs that completely covers the substrate and that appears similar to normal vessel endothelium on SEM. Complete graft coverage does not occur on the plasma coated Dacron or plain Dacron within the 2-hour time frame. The data from the amnion/collagen-coated graft indicates that generation of an EC monolayer while in the operating room is feasible if a receptive graft-substrate combination is used.

Summary

Briefly cultured human adult arterial and venous ECs were seeded at high density onto Dacron vascular graft. The graft was either untreated, pretreated with platelet rich plasma, or coated with a combination of interstitial (Type I/III) and amnion-derived basement membrane (Type IV) human collagen. Adherence studies using radiolabeled ECs demonstrated a high percentage of firm adherence to plasma-coated Dacron by 10 minutes, to amnion/collagen coated Dacron by 30 minutes, and to plain Dacron by 2 hours. Morphological studies using SEM demonstrated complete graft coverage with many cell-to-cell interactions by 30 minutes in the amnion/collagen-coated graft. This did not occur on untreated or plasma-coated Dacron.

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Discussion

DR. ROBERT ZEPPA (Miami, Florida): I would like to ask one question of Dr. Jarrell who is going to close.

Have they tried the experiment with plasma-rich material onto the collagen substrate, which would be yet another intermediate, and I wonder what the timing would be on that?

DR. ROBERT W. BARNES (Little Rock, Arkansas): I appreciated this presentation and the invitation to review the excellent manuscript by Dr. Jarrell and his colleagues. They have made substantive contributions to the feasibility of endothelial cell coating of vascular prostheses, which may become a practical clinical reality in the near future. Although one of my former mentors expressed ambivalance about the clinical utility of endothelial cell coating, stating that "grafts don't fail in the middle, they fail at the ends," I believe that the explosive advances in this field not only may make a small vascular prosthesis available soon, but also will advance our understanding of endothelial cell function in our native circulation.

Dr. Jarrell and his colleagues have appropriately initiated their exciting study in an *in vitro* model system. Although he alludes in his manuscript to many further logical steps at expanding this research, I hope that Dr. Jarrell might comment on some of the obvious questions raised by their paper: (1) What practical source of autologous endothelial cells do you envision for future clinical application? (2) Do you feel that freshly harvested cells can be used for graft coating without antecedent culture? (3) Does amnion have any antigenic or other potentially adverse properties for human use? (4) Do you think your pipette washing model adequately reflects the shear stresses expected in pulsatile blood flow circumstances? (5) Do you have evidence of normal function of the endothelial cell monolayer?

Again, I laud the authors for their valuable contribution, and I look forward to further creative developments from their laboratory.

DR. B. E. JARRELL (Closing discussion): We and others have spent considerable time trying to examine whether establishing an endothelial monolayer on a prosthetic surface in humans was possible and whether it would ever be useful. Most investigators have taken the approach that one can collect a few endothelial cells from a donor vessel and then seed them on a graft at low density and wait. What you are waiting for is the endothelial cells to grow out and ultimately cover the surface.

Although there may be advantages to this approach, we have had concerns about this as a practical methodology in humans. Among these concerns, certainly there is the well established fact that even in properly designed animal trials, 6 to 8 weeks are required from the time of seeding to the time that a stable endothelial monolayer has been generated.

There are some problems with that. If you compare that time period to most clinical series, you will note that the majority of graft failures in small vessel prostheses occur within the first several weeks. Thus, obviously with seeding experiments, the monolayer is not present at the critical, most thrombogenic period of the graft.

We wanted to determine in this study, and as a major thrust in our lab, whether an alternative approach was possible. You have seen the beginning part of that approach today.

Basically, could we set up a confluent monolayer within an hour or two that was compatible with most operating room scenarios? For that to be possible, we had to look at three different areas.

The requirements to do this would be, first, to have a large number of endothelial cells available at the time of implantation; two, to have a graft surface that was receptive to these cells; and three, knowledge of the temporal factors and other chemical parameters that allow this monolayer to form and completely cover the surface.

We will address Dr. Barnes' question of the source of endothelial cells in a later paper. However, I would add that we feel that it is possible without culture and without using a large vessel as a source of endothelial cells to get enough cells to put on a graft. The way that we are accomplishing this is to isolate capillary endothelial cells from fat. We can actually do that process on a routine basis. We do it several times a week in the laboratory from several grams of fat; thus, we feel that we have the source of cells. We will tell you more about this technique in the future.

We feel now that this study demonstrates what the temporal dynamics of monolayer formation are. I would point out that the work presented today represents these specific surfaces. But there are many other surfaces that are available that have not been studied. We have begun to look at some already, and we do feel that these temporal dynamics can be reproduced.

I would point out to you that there is a major thread in this study different from the seeding experiments. We feel adherence is the critical variable to look at, not growth. We spent many months looking at growth but feel that this new approach has more merit.

This is a beginning study. It demonstrates that the cells have a remarkable capacity to sit down on a surface and to connect with one another and totally cover that surface and that this can potentially be done in the operating room at the time of implantation.

We have not completely examined the functional characteristics demonstrated by these cells when placed graft material. We are looking at that currently, but I would hasten to add that when you compare the platelet rich plasma cells with the amnion covered cells, I would certainly be willing to guess that the cells on amnion are going to function better than the cells on platelet-rich plasma. We think that morphology is a very important first step in predicting what the functional characteristics of these cells will be.

In answer to Dr. Barnes' specific questions, the amnion that we use is human amnion. It does not appear to be allergenic, although this would be examined if it were going to be a potential graft application.

We are in the process of looking at flow experiments with this surface. Using freshly isolated cells, it appears that they have a remarkable capacity to remain adherent in fairly high physiological flow conditions. We have not looked at pulsatility yet.

Lastly, to Dr. Zeppa's question, we have not done a time sequence of plasma on collagen. We do routinely use platelet-rich plasma, but my suspicions are that it will not be as good as amnion. In our extensive growth studies with plasma it works, but it is not nearly as good as amnion.

I thank you very much for the privilege of discussing this paper.