Endothelial Linings in Prosthetic Vascular Grafts

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Of all the animals so far studied, man is the only one known to be incapable of healing prosthetic vascular grafts with its own natural endothelium. Consequently there has been considerable effort in the 30 or so years since the first prosthetic grafts became available either to find truly nonthrombogenic artificial materials or to enhance the body's own natural healing processes in order to provide blood-compatible devices. It has been known from the days of the earliest prosthetic grafts that the fibrin-platelet coagulum that typically lines them is an adequate surface only for grafts of 8 mm i.d. or greater. Unfortunately, the need for smaller prostheses is equal to or greater than that for the large. In the early days, emphasis in graft development was focused on different materials, such as nylon, Dacron, Teflon, tanned bovine carotid arteries, or umbilical veins as conduits.

Paralleling the work with prosthetic vessels was the development of the artificial heart and the desire to provide blood-compatible linings for these devices. The idea of cell or tissue fragment seeding of prostheses in order to provide pseudointimal linings originated because of its applicability to artificial hearts (see ref. 2 for a review of this early work). Our own work in this area began with artificial hearts as well, under the auspices of the National Heart, Lung, and Blood Institute and the Artificial Heart Program. At that time the emphasis was on developing microfiber substrates upon which to grow pseudointimal linings and on finding a cell or tissue type that would provide a blood-compatible surface. In order to develop these materials and methods the prosthetic devices were fabricated into nonporous urethane tubes lined with a microfiber scaffold and of a size that could be tested by interposition in the abdominal aorta of a dog (FIG. 1). The cells were introduced into the prostheses and cultivated *in vitro* until a pseudointima developed.[3] The progress was slow; materials were not very cell compatible, and the cells that were easily grown *in vitro* were thrombogenic. However, in the late 1970s several developments occurred that made cell-lined vascular grafts a practical reality. First, we and other groups of investigators finally learned how to isolate and grow adult endothelial cells. Second, we switched from the troublesome microfiber grafts to commercially available vascular prostheses (FIG. 2). Third, a new group of investigators[4] discovered that scrapings from the intima of cutaneous veins, seeded into grafts in clotted blood and then immediately implanted into dogs, would produce endothelial linings in these vessels. With these developments research on endothelial cell seeding greatly increased. In the last few years endothelial cell seeding techniques have developed rapidly to the point where clinical reports of success with the method are beginning to appear[5] (for a complete review, see refs. 6 and 7). In the following pages we will briefly describe the method and the current status of research in this area. This presentation is not meant to be exhaustive, but to highlight some of the more important observations as well as questions still to be answered.

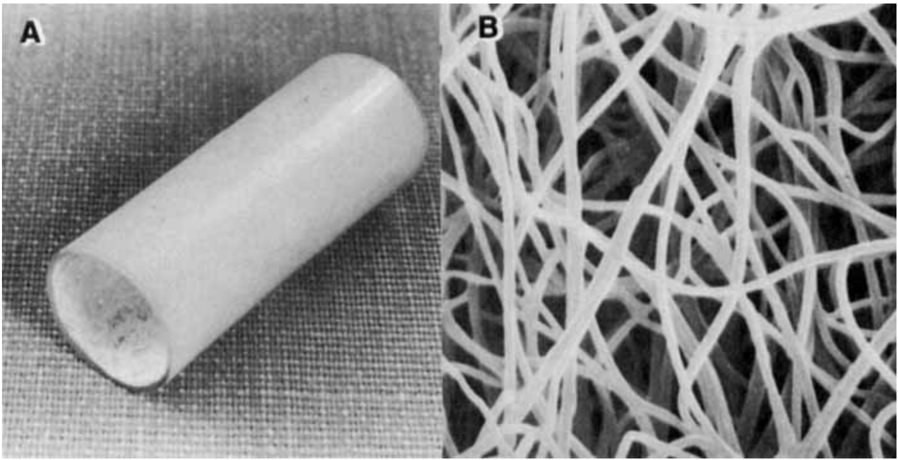

FIGURE 1. (**A**) Urethane tube lined with nonwoven polypropylene microfiber like that used in early seeding experiments (shown actual size). (**B**) SEM micrograph of microfiber lining; magnification: 1000×.

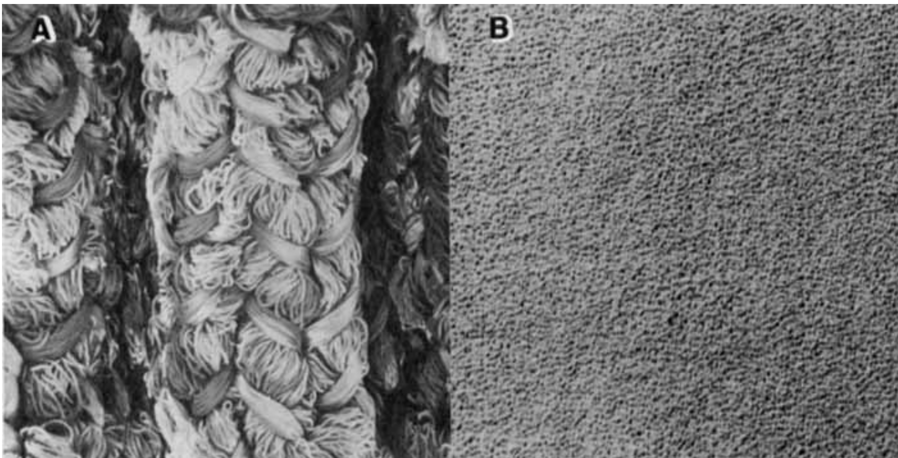

FIGURE 2. (**A**) Surface of a clinically used knitted Dacron, velour, crimped graft; magnification: 25×. (**B**) Surface of expanded Teflon (ePTFE) graft at the same magnification as **A**; note the relative smoothness.

THE METHOD

Endothelial Cell Isolation

Most commonly, endothelial cells (EC) are isolated from subcutaneous veins using proteolytic enzymes. In dogs external jugular veins are isolated, removed, and everted over stainless steel rods[8] in order to prevent exposure of nonendothelial cells to the

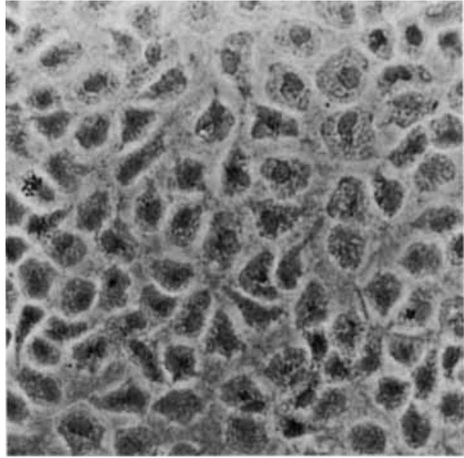

FIGURE 3. Phase contrast micrograph of typical canine venous endothelial cells in tissue culture; magnification: 300×.

enzymes. After washing in calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS), the veins are incubated successively in 0.1% trypsin-0.125% EDTA, pH 8, and 0.5% collagenase (CLS type II), pH 8, each for 10 minutes at 37°C. The rods are then spun in Medium·199 (M199) to dislodge the EC and the exfoliated cells washed in M199 in preparation for graft seeding or tissue culture (FIG. 3).

A good cell harvest will yield 1.0–1.5 × 10^6 cells from a 10 × 0.5 cm segment of vein.

Endothelial Cell Seeding

The EC, either immediately after isolation or after *in vitro* cultivation, are seeded into vascular grafts which have been precoated with autologous blood or some other adhesive, such as fibronectin.[9–11] With Dacron grafts, which are usually porous, the cells are suspended in 0.5 ml of M199 and mixed with 10 to 15 ml of whole blood, the

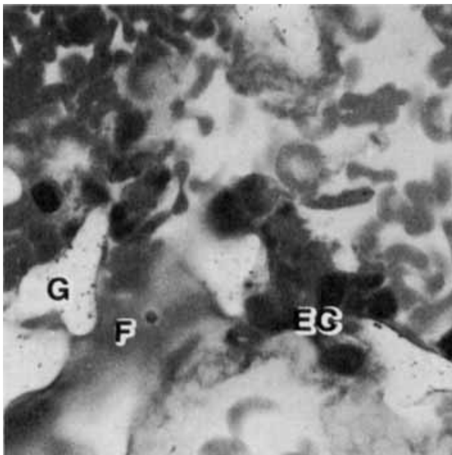

FIGURE 4. Photomicrograph of a section of a Dacron graft seeded with endothelial cells; *EC*, endothelial cells; *G*, graft fiber; *F*, fibrin. Magnification: 600×.

distal end of the graft clamped, and the cell-blood mixture injected into the graft, as for preclotting. The blood is forced through the graft wall filtering out the endothelial cells, which are thus seeded into the interstices (FIG. 4).[9,12] With grafts that are nonporous to blood, such as expanded Teflon (ePTFE), the cells are mixed with a small amount of tissue culture medium, injected into the graft, which has been pretreated with blood or some other adhesive, the ends clamped, and the graft left to sit horizontally.[13] Periodic rotation of the graft during the seeding process allows for even distribution of cells. If blood has been used in the seeding process, the grafts are flushed with a small amount of heparinized blood.[19] The grafts are implanted in the experimental animal using standard surgical procedures, with care taken to prevent dessication. In experiments using EC that have been cultivated prior to seeding,[9] the procedures are the same following trypsinization of the culture flasks to obtain a cell suspension. As an alternative, grafts may be cultivated *in vitro* after seeding in order to obtain a confluent endothelial lining prior to implantation. The methods used are similar to those described in ref. 2. This procedure may be more useful for grafts placed in small vessels or in the venous circulation,[14] which with low flow are more susceptible to thrombosis.

A variety of graft types have been used for EC seeding studies, such as knitted Dacron, with or without velour surface, crimped or noncrimped, externally supported or not. Expanded Teflon grafts are seeded equally well, whether of standard or greater than standard porosity. Sites for implantation and testing of the seeded grafts have included: thoracoabdominal bypass grafts, infrarenal, aortoiliac, iliac, iliofemoral, femoral, and carotid arteries as well as inferior vena cava. Small diameter grafts (<6 mm i.d.), whether seeded or not, have usually required pharmacological intervention to maintain early patency.[14]

RESULTS

Endothelialization (healing) of the graft surface proceeds rapidly after graft implantation, the rate depending upon the number of cells that are seeded and how tightly adherent they are to the graft surface. Small clusters of EC, appearing much like colonies of cells in a tissue culture flask, are visible on the graft surface between 1 and 4 days (FIGS. 5 and 6).[12] These cells grow rapidly and by 14 days as much as 75% of the graft surface is covered with endothelium (FIG. 7). Within the next month the remainder of the surface becomes covered with EC (FIG. 8). Initially this neointima is a monolayer of cells (FIG. 9), but with time subendothelial smooth muscle cells appear to form a layer of variable thickness (FIG. 10) depending upon the graft type and length of implantation.[15] The lining cells display all of the characteristics of endothelium, including cobblestone morphology and tight intercellular junctions,[15] Weibel-Palade bodies,[16] the presence of factor-VIII-related antigen[17] (FIG. 11), nonthrombogenicity,[18] and lack of platelet adhesion and production of prostacyclin (PGI_2).[19] In addition, platelet serotonin levels[19] and platelet survival return to normal significantly faster in seeded grafts.[20,21]

Nonseeded grafts, in contrast, become lined with a fibrin-platelet coagulum shortly after implantation that is frequently several hundred micrometers in thickness and which may persist indefinitely (FIG. 12). Eventually most nonseeded porous grafts in experimental animals heal from cells growing into the graft as pannus from the natural vessel, from tissue surrounding the graft, or perhaps from circulating EC spontaneously seeded from the bloodstream.

These results have been duplicated in both large (>6 mm i.d.) and small diameter

FIGURE 5. SEM micrograph of a cluster of EC on a Dacron graft shortly after implantation; magnification: 400×.

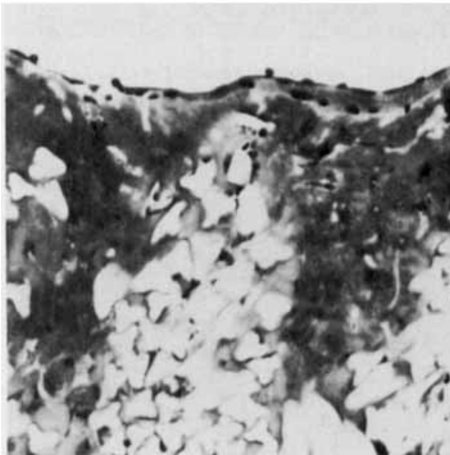

FIGURE 6. Light micrograph of a seeded Dacron graft 4 days after implantation; the cells have begun to form a monolayer on the surface; magnification: 200×.

(<6 mm i.d.) arterial grafts as well as venous grafts. In addition, EC seeding has been shown to be effective in man[5] as well as baboons[22] and rhesus monkey.[23]

DISCUSSION

Cell Sources

As pointed out earlier, a wide variety of tissue sources were initially evaluated[2] in attempts to find a cell type that was readily available, easily isolated, and that possessed the necessary nonthrombogenic characteristics. The obvious choice for lining grafts was the endothelial cell, but routine methods for obtaining large quantities of cells necessary for seeding had not been developed. The publication of a routine method

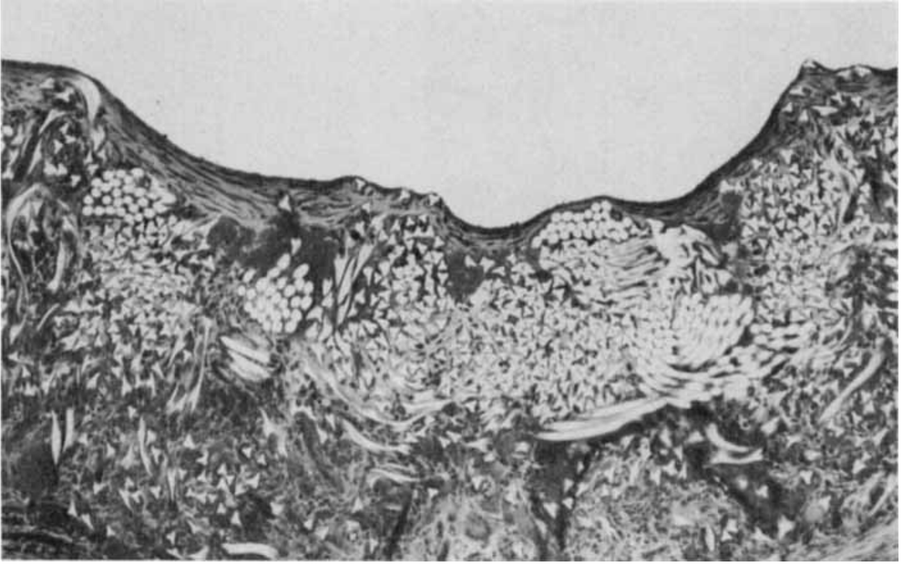

FIGURE 7. Light micrograph of a seeded Dacron graft 2 weeks postimplantation; the EC lining and subjacent smooth muscle are well developed; magnification: 100×.

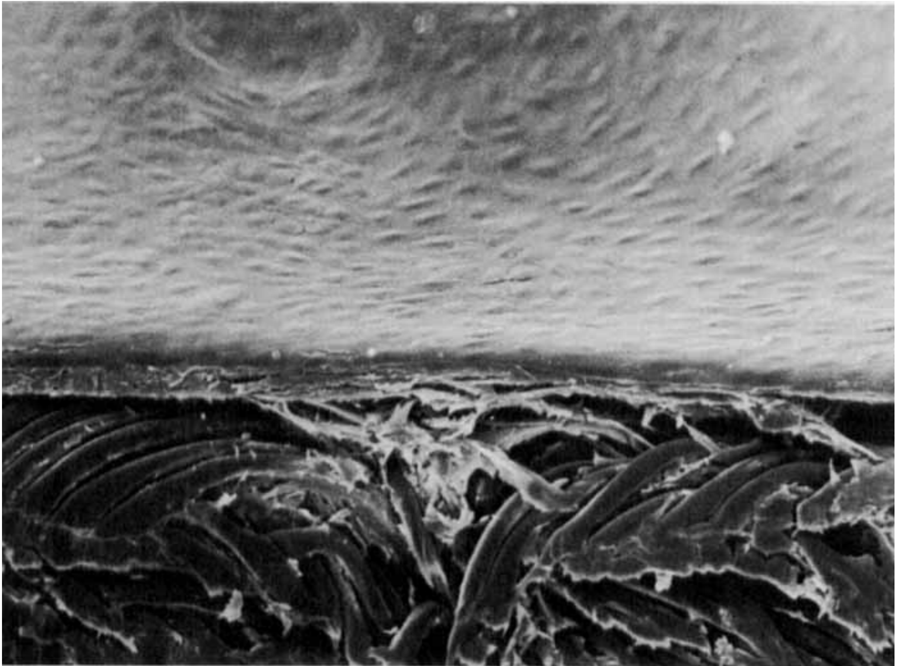

FIGURE 8. SEM micrograph of the cut edge of a seeded Dacron graft 8 weeks after implantation; magnification: 300×.

FIGURE 9. Light micrograph of a seeded a PTFE graft 1 month after implantation; the EC are in a monolayer; magnification: 300×.

for obtaining EC from human umbilical veins by Jaffe *et al.*[24] spurred interest in this cell type. With the demonstration that the method could be modified to harvest bovine aortic EC,[25] two models were available and *in vitro* research flourished. While adequate to harvest embryonic and bovine EC, these methods worked only poorly on adult vessels and the adult EC derived were difficult to grow in culture.

In addition, to prevent rejection, autologous EC were needed for seeding the grafts. Over the next few years the above methods were successfully modified to isolate adult EC from several different types of animals and vessels, including: human iliac arteries; human and bovine pulmonary arteries and veins; porcine aortas, inferior vena cavas, and pulmonary veins; rabbit aortas and marginal ear vessels; guinea pig aortas and

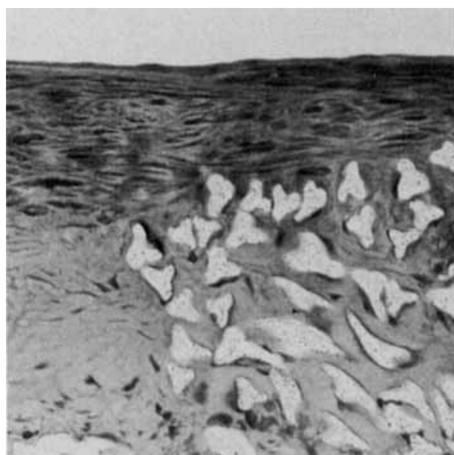

FIGURE 10. Light micrograph of a seeded Dacron graft at 1 year. The original monolayer lining has thickened considerably due to growth of the underlying smooth muscle cells; magnification: 150×.

FIGURE 11. Fluorescence micrograph of a seeded Dacron graft stained for factor-VIII-related antigen and photographed by epi-illumination. The white dots are clusters of stained factor VIII in individual cells; magnification: 100×.

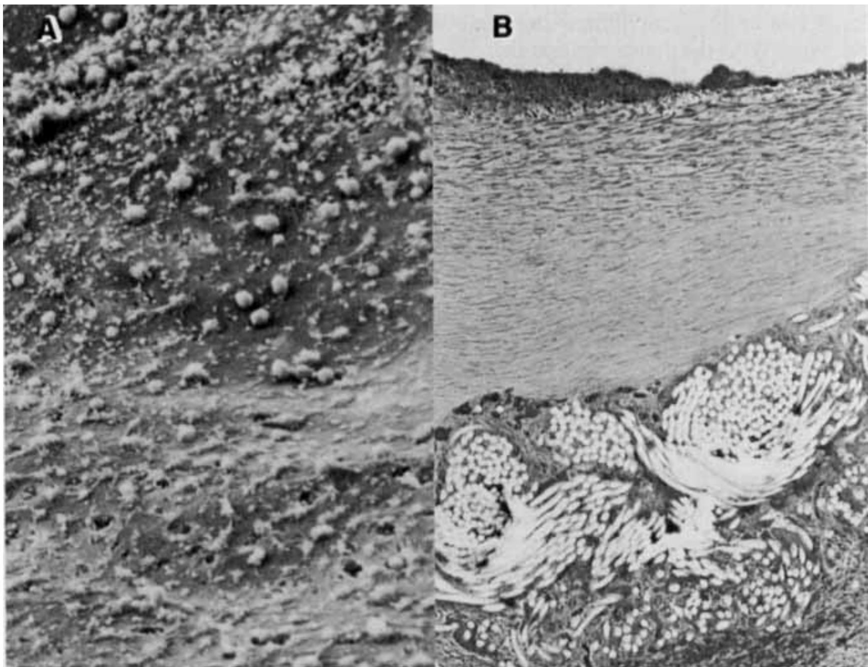

FIGURE 12. (**A**) SEM micrograph of a nonseeded Dacron graft 1 month after implantation, and (**B**) light micrograph of a similar graft 6 months after implantation. The surface of both is composed of a fibrin platelet coagulum. Magnifications: (**A**) 800×; (**B**) 150×.

portal veins; and canine jugular veins and aortas (see ref. 26 for further discussion). Of most importance for graft seeding purposes were methods that could be applied to adult subcutaneous veins that are expendable and potentially useful for clinical application of the seeding technique. Most experiments involving graft seeding have been done with canine jugular veins, a model for human saphenous vein, which is the current choice for clinical application.

While subcutaneous veins such as saphenous can provide large numbers of cells (potentially 1×10^5 cells/cm^2 of vein surface), microvascular EC from adipose tissue is even more abundant (potentially 1×10^6 cells per gram of fat) as well as expendable. With this quantity of cells it may be possible to seed enough cells at the time of implantation to line the graft completely.[27,28] While abundant cells are available from microvasculature, there is a possibility of considerable contamination of the isolates with perivascular or smooth muscle cells. The effect of these cells on the ultimate fate of a seeded graft is unknown.

Recently, mesothelial cells derived from omentum have been used for graft seeding and have been found to produce an intimal lining similar to endothelium.[29] These cells produce large amounts of PGI_2, possess fibrinolytic activity, and do not adhere platelets. Like microvasculature from fat, mesothelial cells are abundant and easily isolated, thus sparing subcutaneous veins for possible coronary bypass if necessary. No long-term studies of grafts seeded with these cells have been done, however.

It has been reported that grafts seeded with nonautologous EC give rise to endothelial linings.[30–32] Considering the known antigenic nature of EC, it seems unlikely that the cells lining these grafts could be from the donor. Grafts were examined only at 2 months postimplantation, more than long enough for spontaneous healing to occur.

Endothelium has been observed in the middle of nonseeded grafts used as controls as early as 2 weeks.[12] Until it has been proved that the cells lining these grafts were actually from the donor, these reports must be viewed with some skepticism. It seems possible, however, that something in the inoculum, such as growth factors from the donor cells (before their loss), could have hastened healing.

Cell Isolation

The earliest and most common methods of isolating EC have been through the use of proteolytic enzymes that attack either the cell junctions or substrate to which the cells are attached. Jaffe *et al.*[24] used collagenase injected into cannulated umbilical veins. While this works well for easily detached EC, it works less well for adult vessels, particularly canine. Rapid perfusion of fluids through the vessel helps in removing cells, but for dog vessels we prefer a method that offers even more agitation or shearing forces to dislodge cells.[8] This is accomplished by everting the vein over a stainless steel rod, suturing it in place, and then after incubation in the enzyme solutions, spinning it with a stirring motor while submerged in M199. Although collagenase has been widely used experimentally, there has been some concern raised about its clinical use since it may contain undesirable DNases.

Other enzymes, such as trypsin without EDTA, chymotrypsin, pronase, and elastase have been used with less success. The neutral protease Dispase (grades I or II) has been suggested by the manufacturer to be good for cell isolation. Thilo *et al.*[33] used it for umbilical vein cells with good results.

Mechanical removal of EC from vessels with steel wool pledgets was used by Herring *et al.*[4] in their early seeding studies. Most likely smooth muscle cells from the

tunica media of the veins were removed as well, however, and this method would appear to give mixtures of cell types that may be undesirable. Ryan[34] has long advocated the use of mechanical removal of EC either through gentle scraping of the vessels or by using polystyrene beads.

While these mechanical methods preserve cell surface coatings and receptors on EC, their use does not seem practical for EC seeding.

The isolation of microvascular endothelium from fat requires collagenase digestion of minced tissue and a series of filtering and sedimentation steps to get capillary fragments.[27] While the method produces large quantities of cells, the procedure is somewhat more lengthy than that used for large vessel endothelium; mixtures of EC, pericytes, and smooth muscle are likely and this may therefore be less desirable. Mesothelium from omentum, in contrast, was removed by collagenase digestion and can be done quite rapidly, but may have the same contamination problems.

Seeding of Grafts

Application of EC to the grafts has been done in a variety of ways. In their first report of successful endothelial cell seeding Herring *et al.*[4] mixed the cells with whole blood that was used to preclot the grafts.

Subsequently several other investigators have used this method on both large-and small-diameter Dacron and ePTFE grafts.

When using this method with Dacron grafts, the cells are trapped in the interstices of the graft wall and 50–75% of the cells remain in the graft when blood flow is restored.[35,36] Grafts of ePTFE are not usually preclotted, since they are relatively impermeable to blood, but introducing cells into the grafts using blood as an initial adhesive produces a healing in the grafts at a rate comparable to that of Dacron even though only 3–4% of the initial inoculum remains after blood flow is restored.[11] Since the rate of healing depends upon the number of cells in the graft per unit area that contributes to the endothelial lining and large numbers of cells may not be available for clinical seeding, it is important that all of the cells introduced into the graft remain.

To accomplish this, two things are necessary: (1) to get the cells to the graft surface, and (2) to provide a suitable substrate to which the cells can tenaciously attach. Simple settling of cells on the surface by gravity is the simplest method, but is slow and even distribution is difficult to obtain. Introducing cells in blood and trapping them in a clot also works, but many cells are lost when blood flow is restored since the cells are not really attached to the graft itself. Forcing the cells against the graft surface by centrifugal force quickly provides almost 100% cell attachment,[3] but is somewhat impractical for long small-diameter or bifurcated grafts. Although much work has been done on cell adhesives for grafts, little work has been done on getting the cells directly on the surface and evenly distributed.

Providing the best surface for cell attachment has been a goal since the earliest days of seeding.[2] Clearly the reason that recent EC seeding on standard Dacron[4,9] and ePTFE[15] is successful is that the cells do not come directly in contact with the graft, but with blood proteins coating the graft. This has been shown in studies on EC adhesion with Dacron[27] and ePTFE,[37] where bare grafts gave poor adhesion while coatings of collagens, or plasma,[27] or fibronectin[37] greatly increased cell retention. In addition, the DNA content of EC attached to bare ePTFE decreased with time, indicating a lack of cell proliferation.[38] On ePTFE grafts coated with glucosaminoglycan-keratin cell adhesion was promoted, but like the naked graft cells showed minimal proliferation.[39]

Sentissi[38] reported that EC seeded on ePTFE coated with fibronectin and type I

collagen gave good growth and were highly resistant to shear in an *in vitro* shunt. In *in vivo* studies, Koeveker *et al.*[41] found that fibrin glue, used to make grafts impermeable, provided a poorer surface for EC healing than did whole blood coatings.

Extracellular matrices similar to those underlying natural endothelium would seem intuitively to be the best graft coatings.[27] Dacron grafts coated with a combination of interstitial collagens (types I/III) and amnion-derived basement membrane (collagen type IV) gave firm adherence to 70% of seeded cells within 30 min. Interestingly, grafts treated with platelet-rich plasma alone gave similar attachment in only 10 min.

In *in vivo* studies with ePTFE grafts, Ramalanjoana *et al.*[11] found that 47% of EC attached to grafts coated with fibronectin in comparison to 19.8% on grafts in which cells were seeded in blood. When implanted in the carotid artery, fibronectin did not prevent cell loss within the first 30 min, but at 24 hours six times as many cells were present in the coated compared to the noncoated grafts (21.3 vs. 3.4%). Despite the increase in cell adhesion by coatings with extracellular matrix proteins, the loss of 80% of the seeded cells leaves much room for improvement, and much work still needs to be done on both coatings and seeding methods.

If all of the seeded cells remained attached to the grafts they would heal in a matter of days instead of weeks as is now the case.

There is no agreement on the best physical design of a graft for EC seeding, with some[6] advocating less porous and others[42] more porous grafts. There does seem to be general agreement that a relatively smooth surface is better.[43] The graft material seems to be less important than the surface coating. With current knowledge of extracellular matrix proteins and growth factors, it should be possible in the near future to design a graft ideally suited to endothelial cell seeding.

The addition of EC growth factors to graft surfaces to enhance healing may also be desirable. Greisler *et al.*[44] have recently reported attempts to bond endothelial cell growth factor (ECGF) to biomaterials. Heparin-bonded biomaterials have been available for several years. It is of interest that fibronectin binds heparin as well. Heparin has been shown to be synergistic with ECGF in promoting EC growth,[45] and in addition inhibits smooth muscle cell proliferation.[46] Smooth muscle growth in the body of grafts may be of little consequence, but it has been implicated in the development of anastomotic intimal hyperplasia (IH), said to be the cause of late failure in small diameter grafts. Why IH occurs is not known, but it has been suggested that platelet adhesion with subsequent release of platelet-derived growth factor (PDGF) stimulates the smooth muscle to proliferate. Antiplatelet drugs have been reported to decrease IH in experimental animals, but their efficacy in man remains to be proved.[47] It is clear that antiplatelet drugs are necessary to maintain patency in small diameter grafts in the short term.[48] Aspirin and dipyridamole have been the most commonly used, but other cyclooxygenase and thromboxane synthetase inhibitors have been used as well. The most effective appears to be aspirin alone, although the reasons are unclear.[49,50] Whether the rapid endothelialization of grafts by seeding will reduce IH has not been proved. It has been shown that EC continue to turn over and smooth muscle cells continue to proliferate at anastomoses even after grafts are healed.[51] It has been suggested that this is due to chronic EC injury. It has been shown that smooth muscle cells secrete growth factors acting on neighboring smooth muscle cells and that normal EC produce a heparin-like inhibitor of smooth muscle proliferation.[52] It has been suggested that injured EC may be incapable of secreting this inhibitor, and the result is IH.[50]

It is clear that EC seeding has dramatically improved small diameter graft patency in both animals[48] and man[5] in the short term. Whether it will improve the long-term failure rate is unknown at this point. In the long run the solution to small vessel grafting

will most likely be a combination of better grafts, enhancement of healing by seeding or other means, as well as pharmacological intervention.

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