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Endothelial cell seeding

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Endothelial cell seeding is a technique that has developed over the past 15 years in response to the need for a high performance synthetic vascular graft. This review details our present knowledge of seeding and examines the various problems that have hampered its introduction into clinical practice.

Small vessel occlusive arterial disease is bypassed most successfully by autogenous vein grafts[1,2]. However, when vein is not available surgeons are obliged to implant synthetic grafts. Factors that are relatively unimportant in large vessel replacement, where synthetic grafts are satisfactory, are highly significant in small vessel bypass. In the latter blood flow is reduced and there is a rapid adsorption and accumulation of blood proteins on to the graft surface[3], with the formation of a thrombogenic pseudointima of compacted fibrin[4]. Because an endothelial cell lining never develops on artificial surfaces in humans[4,5], the pseudointima is retained throughout the life of the prosthesis and may be important in subsequent graft occlusion.

Endothelial cells are actively antithrombotic[6] and provide the best possible local environment for preventing thrombosis. The endothelial lining occupies a pivotal position in the homoeostasis of the vascular tree[7] synthesizing prostacyclin, thromboxane A_2, endothelin and other chemicals that maintain a delicate balance between factors promoting and opposing thrombosis. In addition to haemostasis, endothelial cell physiology contributes to early atherosclerotic change, haematogenous tumour metastasis, transplant rejection and white blood cell emigration to sites of inflammation. Further elucidation of endothelial cell biology may eventually bring therapeutic benefits to patients with widely differing diseases.

The development of an artificial graft lined by active, vascular endothelium (the living prosthesis) would combine the virtues of endothelium with the availability of synthetic materials and could result in a conduit with a performance much nearer to that of autogenous vein. However, seeded artificial grafts may never be as good as autogenous vein, because other important factors such as the chemical nature of prostheses and compliance influence their fate. Despite these limitations, coronary artery bypass grafting, synthetic heart valves, artificial organs and peripheral vascular surgery are all areas that could exploit improvements.

Endothelial cell seeding has developed in response to the need for an improved prosthesis and consists of various techniques to promote the growth of vascular endothelial cells on the luminal surface of artificial grafts. Over a period of 15 years extensive research has culminated in several clinical trials. In this review we discuss the development of endothelial seeding, the problems associated with its implementation, and its possible role in surgical practice.

Isolation and harvesting of endothelial cells

Although techniques to isolate endothelial cells have been available for some time[8], the development of identification procedures and of long-term, *in vitro* culture were important advances[9–11]. Subsequently, endothelial cells were isolated from many different sources[12] but umbilical cord was the first and, because of its availability and convenience, remains the most commonly used source for laboratory work.

There are two principal methods of harvesting large vessel endothelium[12,13], mechanical and enzymatic. Cells can be mechanically harvested by scraping them from the intima, but this may lead to cell damage and contamination with smooth muscle cells. Mechanical harvesting is probably more unreliable and certainly less popular than the enzymatic techniques that have superseded it. These latter techniques bathe the endothelium in collagenase, allowing separation of the cells from the extracellular matrix and basement membrane. In the eversion method a vessel is turned inside out and bathed in enzyme[14], whereas in the infusion technique the enzyme is flushed into the vessel. Both are widely used.

Although endothelial cell harvesting is now reported routinely, the success and yield vary with the source. Gimbrone *et al.*[15] found that at 12 h after seeding on to tissue culture plastic only 20–50 per cent of harvested cells had attached, and an adequate growth of cells may be possible in only 33–81 per cent of specimens[16–18]. Human long saphenous vein produces a lower yield of viable cells with a longer doubling time compared with cells from animal large vessel endothelium[19]. In addition, non-smokers provide significantly better cell harvests[20].

When large amounts of tissue are available, large numbers of cells can usually be obtained[21]. These cells will grow and migrate most efficiently on tissue culture plastic coated with fibronectin or gelatin[22] and require a culture medium containing glutamine, serum, endothelial cell growth factor and heparin[23–25]. Using these supplements cells may be cultured for long periods.

There have been few attempts to standardize the harvesting of endothelial cells. In one study[26] the average harvesting efficiency was only 12–15 per cent, possibly because even fresh veins have an incomplete endothelial lining. Although cell isolation with the infusion technique may be more efficient than with the eversion method[27], in general present harvesting methods are inefficient and yield insufficient cells for confluent coverage of a graft surface[28–30]. Twenty-four hours after seeding a graft, as little as 0·27 per cent of the surface may be covered by cells[30]. If this problem is to be overcome an abundant source of endothelial cells is required. Although the long saphenous vein is a good source, if sufficient cells are available to line a prosthesis the vein itself is likely to be adequate for arterial bypass. At present no sufficiently abundant source of endothelial cells exists in humans to provide reliable confluent coverage of a prosthetic graft. Current methods of harvesting are relatively inconsistent and empirical and so clinical studies have taken place against a background of poorly defined experimental techniques.

Microvascular endothelial cells and mesothelium

In an effort to overcome the problems associated with procuring large vessel endothelium, researchers have turned to microvascular endothelium, which can be isolated in large quantities

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from omentum[12] and tolerates prolonged culture[31]. Isolation entails enzymatic digestion of the tissue, followed by either separation on a centrifugation gradient[32] or graded meshes[33]. Although the cell harvests seem to be lower after centrifugation, equivalent results with both methods are obtained after seeding grafts *in vivo*[34].

The only alternative cell source is the lining of the peritoneal cavity. Peritoneal mesothelial cells are actively antithrombogenic and produce prostacyclin[35]. Preliminary work on their role as vascular graft linings has been undertaken.

A problem that must be faced in the isolation of microvascular endothelium from omentum is contamination with mesothelial cells. Most studies confirm the endothelial origin of their cells by staining for factor VIII-related antigen. However, some workers[36,37] doubt the origin of omentally isolated cells and one study was virtually unable to show any difference between pulmonary microvascular endothelial cells and pericardial mesothelial cells[38]. Recently, monoclonal antibodies against cytokeratins 8 and 18 have been recognized as markers for mesothelial cells. Studies using these markers have suggested that omentally derived cells are mesothelial, not endothelial[39].

Seeding studies

In vitro *studies*

Once the isolation and culturing of endothelial cells became routine, attempts to apply cells to vascular prostheses (seeding) rapidly followed[40]. However, the methods used to isolate and culture cells vary and seeding densities in the laboratory are often at levels presently unachievable in humans. Cells are not always subjected to physiological conditions and the resulting published literature is somewhat confusing and contradictory.

Adhesion on to many artificial surfaces is biphasic[41]. Initial adherence is rapid and virtually complete by 15–30 min. There is then a second phase when adhesion still increases, but at a much slower rate. Although the raw surfaces of Dacron® (E.I. du Pont, Wilmington, Delaware, USA) or polytetrafluoroethylene (PTFE) do not encourage endothelial cells to adhere[42], precoating with a substrate, such as fibronectin or gelatin, improves coverage[43–45]. Platelet-rich plasma, amnion-coated Dacron, fibrin glue and combinations of collagens and fibronectin or laminin have also been found to be effective[41,46,47], but with short seeding times seeding efficiencies can be disappointingly low[47]. However, once attached to the graft material, the cells will usually grow successfully[48], especially on PTFE[42].

Once attached to the graft surface the endothelial cells must withstand the shear stresses of circulating blood. Cells are lost rapidly from a seeded graft within the first 30 min, and by 24 h only 4 per cent of the seeded cells may remain[28]. Fibronectin can improve endothelial cell retention in conditions of shear stress[49–51], but this advantage may be offset by its tendency to cause increased platelet deposition on the graft surface[52].

Leucocytes may be partly responsible for endothelial cell detachment. If tissue culture medium is used in an artificial circuit more cells remain attached than when blood is used[43], and endothelial cell retention can be vastly improved in seeded prostheses implanted into leucopenic dogs[53].

Laboratory studies on microvascular endothelial cells are more limited but have shown that initial cell adherence to grafts is rapid and that the cells withstand high shear stresses[54]. Plating microvascular endothelial cells on to a pre-established clot at supraconfluent densities (sodding) has produced confluent graft coverage at 3 h[55].

In vivo *studies*

Animals. Seeding synthetic prostheses for *in vivo* use with endothelial cells was first attempted in 1975[40]. Subsequently, seeded grafts have been evaluated using a number of functional assessments. Initially, platelet deposition appears to be greater on seeded grafts than on controls, but by 2 weeks after implantation seeded grafts show less deposition, and platelet survival soon returns to normal, unlike on control grafts in which it remains shortened[56,57]. The use of antiplatelet agents in the early postoperative period, combined with seeding, seems to be an especially effective combination[56,58,59].

The thrombus-free surface area is increased and the surface thrombogenicity is reduced in seeded Dacron grafts in dogs and the rapid development of an endothelial monolayer by 4 weeks is associated with thin inner capsules[60–62]. Knitted Dacron is better than woven Dacron at supporting endothelial cell growth[63] and preclotted PTFE grafts also develop good endothelial coverage[64].

The healing of seeded grafts has been closely examined in dogs. The University of Michigan group[14,65] showed that initially both seeded grafts and controls are covered with a platelet carpet and scattered white blood cells. By the fourth day the first signs of endothelialization are present on seeded grafts and from 7 days there is enlargement of the endothelial patches. Seeded grafts are grossly clean and are 60 per cent covered with endothelium by 2 weeks; at 1 month a confluent monolayer with vasa vasorum is present. Similar work[66] confirmed the above findings up to 15 days, but failed to show a significant difference between seeded and unseeded Dacron grafts from 15 days onwards.

Improved patency rates have been demonstrated in small-diameter seeded prostheses of Dacron and PTFE, and have been associated with superior platelet survival, clot-free surface area and the development of endothelium[67–71].

Microvascular endothelium from omentum has been used to seed grafts *in vivo*, and produces a rather thick subendothelial layer, although thrombus-free areas are probably equivalent to seeding with large vessel endothelium[72,73]. Seeding with mesothelial cells can also lead to the development of a cellular lining which secretes prostacyclin[74,75].

Some studies[76,77] have been less favourable, especially the endothelial cell seeding of interposition grafts in veins. Two studies have failed to show improved patency using PTFE grafts in dogs[78,79], although in one[79] there was less luminal clot, decreased platelet accumulation, more endothelium and a thinner inner capsule on seeded grafts. The origin of the neointima that forms on the seeded grafts is also the subject of controversy as the process of seeding may create a milieu suitable for host endothelial cell growth[80]. As one would expect, autologous seeding seems to be most effective, supporting seeded cells as the origin of the neointima, but xenograft seeding may also be successful[81]. Placing seeded and unseeded grafts in the same animal, combined with variations in the use of antiplatelet medication, may be possible reasons for these results.

Clinical trials. Besides the drawbacks of investigating endothelial cells *in vitro*, many of which also apply *in vivo*, animal studies are compounded by one other crucial problem. Although there are marked differences between species[5,82], all experimental animals develop an endothelial cell lining on a prosthetic graft whether or not it is seeded. In contrast, in humans a neointima is probably never formed[4], and there are fundamental differences in the way that animals and humans heal artificial grafts. Despite this, encouraging results from animal work have provoked studies in humans.

Any comparison between seeded and unseeded synthetic femorodistal grafts, in patients with inadequate autogenous vein, presents formidable problems. The small numbers of patients appropriate for this type of surgery, and the large numbers required to construct a trial of sufficient power to establish a difference, would require a multicentre study with facilities for cell harvesting and seeding in each centre. Finally, the time required to confirm the long-term advantages of seeding, if graft occlusion was used as the endpoint, probably precludes such a trial being mounted.

Four centres, at Indianapolis, Dundee, Vienna and Göteborg, have individually undertaken limited trials, using

large vessel endothelium for seeding. Because of the many confounding variables and the small numbers of participants in these trials, the overall results are inconclusive. While some have demonstrated that endothelium can develop on seeded grafts in humans[83,84] this is not a consistent finding[85]. Recently endothelium has been positively identified 9 months after implantation of a Dacron mesoatrial graft, seeded with endothelial cells from subcutaneous fat[86]. Clinical trials, though, are hampered by the inability to observe the development of endothelium directly. Instead, platelet deposition and the thrombogenicity index have been used as the primary measurable features.

The first clinical study[87] examined 18 endothelial cell-seeded femoropoliteal knitted Dacron grafts. There was no overall difference in patency between seeded and unseeded grafts, although autogenous vein grafts fared better than prosthetic grafts. However, the patency rates of seeded grafts were significantly better in non-smokers. A subsequent report[88] demonstrated an early trend towards improved patency and a reduced uptake of platelets in seeded femoropopliteal PTFE grafts. Unfortunately, these early findings never achieved statistical significance.

The earliest studies used mechanical methods of harvesting cells, and a later trial using enzymatic harvesting of cells and antiplatelet therapy[89], demonstrated cumulative patency rates at 3 months of 93 per cent for seeded and 84 per cent for unseeded femoropopliteal grafts. At 1 year the patency rates were significantly different, with 81 per cent for seeded and 31 per cent for unseeded grafts, and all but one of the seeded graft occlusions occurred in patients with a history of smoking or high carboxyhaemoglobin blood levels. Unfortunately, there were only 17 seeded and 14 unseeded grafts in this trial and similar success has not been repeated.

Zilla *et al.*[90] implanted nine seeded and nine unseeded femoropopliteal and femorocrural 6 mm PTFE grafts coated with fibrin glue. They were unable to find differences in platelet accumulation or platelet survival and relied on platelet factor IV, β-thromboglobulin and shape-changed platelets to indicate advantage in the seeded group[29].

Ortenwall *et al.*[30] used Dacron aortic bifurcation grafts in which one limb was seeded and the other acted as control. They demonstrated an early decrease in platelet accumulation on the seeded side and in follow-up studies[91] have shown that platelet deposition on the seeded graft limb has remained reduced, over a period of 12 months. Other studies[92] used preclotted femoropopliteal PTFE grafts, seeding either the proximal or distal half of the grafts with endothelial cells. In 23 patients the mean thrombogenicity index was significantly reduced in seeded graft segments at 1 and 6 months after implantation, indicating a reduction in platelet deposition. However, five grafts failed to show a response to seeding.

Conclusions

Technical innovations have not produced a prosthetic surface to rival vein and there has been no real progress since the introduction of PTFE around 1976. There is now sufficient expertise with endothelial cells to suggest that further developments in materials might best be directed towards developing flow surfaces conducive to the seeding and growth of endothelial cells.

Given the right surface the best outcome would follow if seeding were to be performed using a large number of cells. Cell isolation and culture techniques need to be improved to facilitate high density seeding. Genetic manipulation of endothelial cells[93] and cryopreservation of cells[94] may be important in the future.

The development of an off-the-shelf graft, prelined with cells, would combine an active antithrombogenic lining at the time of implantation with a possible reduction in infection[95,96]. This would eliminate the time required for vein harvesting in peripheral vascular surgery, and the availability of such a graft would encourage distal bypass. However, until techniques improve, the adoption of seeding into routine surgical practice cannot be supported outside clinical trials.

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