A CELLULAR LINING FOR ePTFE VASCULAR GRAFTS

Studies using adult human endothelial and mesothelial cells

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SUMMARY

The main aims of the work reported in this thesis were to assess, using laboratory techniques, the clinical feasibility of the currently popular concept of immediate cell seeding of prosthetic vascular grafts, and to compare the cell/prosthesis interaction of two cell types which have been recommended for this purpose.

The thesis describes a series of laboratory studies investigating several aspects of immediate seeding of cultured adult human endothelial and mesothelial cells onto ePTFE vascular graft material.

A comparison was made beween mechanical and enzymatic harvesting of adult human endothelial cells from segments of long saphenous vein. The enzymatic method, using collagenase, virtually always succeeded in releasing large numbers of cells, which subsequently grew to confluence and could be subcultured thereafter through several passages. The mechanical technique consistently failed to provide sufficient viable cells to grow in culture. A technique for harvesting microvascular endothelial cells from subcutaneous and omental fat was assessed, but success using this method was limited.

Endothelial cells, derived enzymatically from long saphenous vein, and mesothelial cells, derived enzymatically from omentum removed at laparotomy, were grown in culture. Comparison was made of the growth characteristics of these two cell types on the substrates fibronectin, type 4 collagen, laminin (all basement membrane extracellular matrix proteins), basement membrane gel, and uncoated culture flasks. Basement membrane gel proved unsatisfactory for the growth to confluence of both cell types. Both endothelial and mesothelial cells grew equally well on all the other substrate proteins, although the former required special growth supplements in the medium, whereas the latter did not. Endothelial cells failed to thrive on uncoated culture flasks, whereas mesothelial cells grew well on this surface. Both cell types had similar life spans in culture.

A series of experiments was performed in order to assess the time course of attachment of the two cell types to standard tissue culture polystyrene. Fibronectin coating of the culture surface gave the most rapid attachment of both cell types, with laminin giving the poorest adhesion rates. Irrespective of substrate coating used, the majority of attached cells had become adherent within one hour of plating. Mesothelium attached more slowly to all substrates than endothelium.

The ability of cultured endothelial and mesothelial cells to attach to uncoated, protein coated, and "pre-clotted" ePTFE after 1 hour's incubation was assessed using two different techniques. Adhesion to this surface was slower than to tissue culture polystyrene, irrespective of the pre-treatment used, but, again, fibronectin coating gave the highest average attachment efficiency. The most consistent attachment rates, however, were obtained using pre-clot as the substrate for seeding, although average attachment was marginally lower.

Scanning electron microscopy of all the endothelial seeded protein coated specimens demonstrated large numbers of attached and flattened cells, but with large areas of exposed ePTFE, irrespective of seeding density. Pre-clotted specimens demonstrated virtual complete coverage after 1 hour's incubation, if a high seeding density of cells was used. Attached cells were at various stages of spreading. Although Scanning electron microscopy of mesothelial cell seeded specimens showed many attached cells, these were mostly rounded up, only a small number being fully spread.

The conclusion from these findings is that pre-clotted blood is the best substrate with which to line ePTFE prior to seeding with endothelial cells. If seeding cell density is sufficiently high, this treatment enables the surface to form a confluent monolayer of cells within a period of one hour. Pre-coating with extracellular matrix proteins is patchy, and results in incomplete cell coverage.

The microvascular harvesting technique requires further development as a means of greatly improving cell yields.

Mesothelial cells attach to ePTFE more slowly than adult endothelial cells, whether the surface is pre-coated with protein or pre-clotted with whole blood.

An artificial flow circuit was then designed, in order to subject seeded cells to a shear stress similar to that which would be experienced *in vivo.* ePTFE graft segments were pre-clotted with whole blood and seeded with endothelial cells as in the earlier experiments, the graft segments then being inserted into the flow circuit and exposed to flow for 60 minutes. Cell retention during the period of flow was assessed quantitatively by means of indium-111-oxine labelling of the cells, and the post-flow appearance of the endothelial monolayer was examined by scanning electron microscopy.

Apart from an initial cell loss immediately after commencing flow through the endothelialised graft segments, the cell loss during the 60 minutes flow was low at each of three different flow rates assessed (25, 100 and 200 ml/minute). Scanning electron microscopy demonstrated persistence of a virtually intact monolayer after 60 minutes of flow at these flow rates.

It is concluded that, once endothelial cells are attached to pre-clotted ePTFE grafts, the cell/substrate and substrate/graft bond is sufficiently strong to resist the shear stress likely to be encountered when such a graft is implanted *in vivo.*

CHAPTER 1

SMALL CALIBRE VASCULAR GRAFTS

THE NEED FOR IMPROVEMENT

1.1. Introduction

Peripheral vascular disease secondary to atherosclerosis continues to present a major health problem in the Western world, estimates suggesting the presence of symptomatic disease in as many as 2-3 per cent of males aged 45-60, and 1% of females aged 50-65 in the United Kingdom (Boobis & Bell 1982). Although, as with any pathological condition, the best cure is prevention, it will undoubtedly be many years before the complexities of the aetiology and the factors affecting the natural history of atherosclerosis are resolved, thereby permitting effective preventive measures. Efforts are still therefore required to identify adequate means of treating the already established, and symptomatic disease.

Although many patients with Fontaine stage 2 disease improve significantly with conservative measures, such as stopping smoking, losing weight, taking more exercise and avoiding fat and cholesterol in their diets (Imparato et al 1975), there are those with more severe ischaemia in whom these measures are less effective, and disabling claudication may progress to rest pain and gangrene. As attempts to influence the natural history of peripheral vascular disease by pharmacological means have to date been universally unsuccessful (Boobis & Bell 1982), the only means of relieving symptoms in a large number of these patients and, in many cases avoiding amputation, remains surgical bypass.

Since the turn of the century, when surgeons first became seriously interested in the concept of replacing or bypassing occluded arteries, they have been striving to identify the ideal arterial substitute. Although many biological and synthetic materials have been evaluated for this purpose, many of which perform well when applied to the large diameter intra-abdominal arteries, most have proved unsatisfactory when applied to small diameter, low flow situations such as those pertaining below the inguinal ligament. By far the most satisfactory material in this regard is autogenous vein, which has been found to give

satisfactory limb salvage and long term patency rates when used to bypass femoro-popliteal occlusions (Mehta 1979). Because of this, autogenous vein has tended to be the "gold standard" against which all other graft performances are compared.

In recent years there has been a growth of interest in the concept of promoting endothelialisation within prosthetic graft surfaces, an idea which was initially conceived following the observation that prosthetic grafts in human subjects consistently fail to develop an endothelial lining, other than perhaps a few "islands" throughout the length of the graft (Sauvage et al 1975). This represents a fundamental difference between prosthetic and autogenous venous grafts, and may contribute in a large way to the discrepancy between the results of the different graft types.

The justification for continuing research into developing a better vascular prosthesis may be illustrated by a brief review of the many possible alternatives which have been assessed over the last 40 years or so.

1.2 Autogenous Vein as an Arterial Substitute

The early pioneering work concerning the use of vein to replace diseased or occluded arteries was carried out at the beginning of the present century. Although there were several individual reports of successful grafting in a variety of different arteries (Gluck 1898; Goyanes 1906; Lexer 1907), the first definitive work on the subject was that of Alexis Carrel, who developed basic suturing techniques and demonstrated, by detailed histological follow-up of veins implanted into animal models, the histological changes of "arterialisation" which begin almost immediately following exposure of vein to arterial pressures (Carrel 1906).

Further reports of clinical success followed (Bernheim 1916; Murray 1941), but it was only after Kunlin, in 1949, described the use of reversed

saphenous vein to bypass arterial obstructions, that the use of vein as a replacement artery came into widespread use (Kunlin 1949).

There subsequently followed a number of reports describing satisfactory results using this technique to save lower limbs which were otherwise facing amputation. Dale, for example, reported a limb salvage rate of 65% in 41 patients undergoing femoropopliteal bypass for severe ischaemia, and Ray et al. described a salvage rate of 77.5% in 40 patients with disease of similar severity (Dale 1967; Ray et al 1970). The latter authors' overall long term patency rate, including those procedures which were carried out for claudication only, was 84.6%. Some of the successful grafts occurred despite the presence of very low flow rates and poor radiological run-off, leading to the conclusion that the rate of flow required to maintain patency in an autogenous vein graft is low. Tyson and Reichle in 1972 similarly reported good results, with a limb salvage rate of 51 out of 70 femoro-tibial bypasses for severe ischaemia (Tyson and Reichle 1972).

More recently, long term patencies of up to 72% at 5 years have further demonstrated the suitability of autogenous vein as an arterial substitute, although the success rate does to some extent depend on the severity of the disease, those with rest pain and gangrene tending to do less well than those with intermittent claudication (Mehta 1979; Veith et al 1986).

As the above results have not been bettered by any graft material subsequently assessed, most vascular surgeons would consider autogenous vein as the first choice conduit for femoro-popliteal or more distal bypass.

1.3 Alternatives to Saphenous Vein

The problem presented by the 30% or so of cases in which there is no suitable long saphenous vein has been addressed by many workers throughout the history of vascular replacement surgery, and, to date, no completely satisfactory solution has been found.

Broadly speaking, three approaches have been taken:

a. Utilise some other form of "biological" conduit. Into this category falls arterial and venous homografts, arterial heterografts, and the mandril technique investigated by Sparks and other workers in the early 1970's.

b. Develop a synthetic material which has chemical properties which render it sufficiently thromboresistant to support the low rates of blood flow often encountered within smaller calibre vascular grafts, and which may be responsible for their failure. This approach included a variety of measures to induce an electrical charge across the wall of the graft, and attempts to bond heparin within the graft structure.

c. Develop a synthetic material which, when made into a vascular graft and implanted, would allow endothelium to grow along the graft lumen from the ends, and possibly also through the substance of the graft from ingrowth of connective tissue capillaries, working on the premise that if a naturally lined biological material was not available, then the next best thing is, logically, a conduit which is still lined by the physiologically normal blood-surface interface. This was the basis for the development of the porous arterial graft.

a. Biological Arterial Substitutes

Biological grafts include alternative autogenous veins, arterial and venous homografts, heterografts, autogenous "fibro-collagenous" tubes and glutaraldehyde tanned human umbilical vein.

All of these possible artificial conduits have proved less than ideal, although in virtually all cases, initial results were encouraging.

Arm Veins

Arm veins have not been widely accepted as the solution to the problem, mainly because of concern that these veins are rather friable, thin walled and difficult to handle, their friability rendering them more susceptible to aneurysm formation. Few large series of these grafts exists, however, to confirm or refute these difficulties, although Campbell and colleagues reported good patency rates using arm veins routinely in the absence of suitable saphenous vein (Campbell, D.R. et al 1979).

Arterial and Venous Homografts

In the late 1940's the possibility of using arterial homografts to replace diseased arteries received attention. Gross and colleagues demonstrated that all cellular elements in homografts disappeared following implantation, the homograft thereafter acting purely as a mechanical conduit, rather than a living prosthesis (Gross et al 1949). Although initial results were good, as longer term results became available a high incidence of late failure and aneurysm formation became apparent (Warren 1956; Linton 1958; Meade et al 1966), problems which have been attributed to immunologically induced ischaemia and degeneration of the graft wall. Attempts to minimise this effect were unsuccessful, and these grafts are no longer used.

The possibility of utilising venous homografts for small vessel replacement or bypass has also been explored, but clinical experience is sparce and results appear to be significantly inferior to those for autogenous vein (Oschner et al 1971; Tice & Zerbino 1972).

Heterografts

Bovine heterografts, introduced by Rosenburg in the late 1950's, were also associated with difficulties which only became apparent some years after they came into use. The problems with these grafts, elucidated by Dale and Lewis, were a disturbingly high rate of early and late thrombosis, an unacceptibly high infection rate, and an aneurysm rate of 5% (Dale & Lewis 1976). These grafts were also difficult to re-explore on account of their soft and non-resistant wall.

Fibrocollagenous Tubes

Peirce introduced the concept of the fibro-collagenous tube as an arterial conduit in 1953. The channels resulting from the presence of polyethylene tubes in the rectus sheaths of dogs for a period of five weeks were found to give good patency rates when implanted as canine aortic grafts. Histological examination of these grafts 12 months after placement revealed a compact fibrous layer with circular orientation of collagen bundles, with a thin lining of normal endothelium (Peirce 1953). Various modifications to both materials and technique were made (Eiken & Norden 1963; Schilling et al 1964) until Sparks applied the process clinically in the early 1970's. Although Sparks' results were good (Sparks 1973) long term patency rates were poor, and an inordinately high complication rate, especially aneurysm formation, subsequently became apparent, precluding these grafts from further clinical use (Hallin & Sweetman 1976). An additional problem with these grafts was that a period of some weeks was required for maturity to be reached, obviously a disadvantage in cases of critical ischaemia.

Human Umbilical Vein Grafts

In 1976, Herbert Dardik described the use of human umbilical veins, removed from the cord and "tanned" by treatment with glutaraldehyde (Dardik et al 1976). By 1980, Dardik's group had amassed experience of the use of 552 such

grafts implanted in patients. They found 5 year cumulative patency rates of 60% in the femoropopliteal position, the 4 year patencies in the femorotibial and femoroperoneal positions being 38% and 28% respectively (Dardik et al 1982). These grafts were found to be biocompatible, nonantigenic and mechanically equivalent to artery, the most consistent feature of which is a continuous internal elastic membrane, but without an endothelial lining.

The conclusion reached by Dardik and his co-workers was that these grafts gave long term results equivalent to those for autogenous vein. Unfortunately, these results have not been duplicated by other authors, and the human umbilical vein graft has not displaced saphenous vein as the first choice arterial substitute (Bergan et al 1982). With increasing experience of these grafts, concern over the incidence of aneurysm formation has recently arisen (Nevelsteen et al 1987).

Conclusions

Investigation of all the above possible solutions to the problem of developing an ideal biological arterial graft has therefore fallen short of providing the answer. Although, in theory, one might expect the best vascular conduits to be biological in origin, most of these grafts are plagued by poor long term patency rates, or prohibitively high complication rates. One major factor contributing to the consistent superiority of autogenous vein may be the lack of a viable endothelial lining on any of the alternatives.

b. Prosthetic Vascular Grafts

Other workers, realising the potential usefulness of having available "off the shelf" artificial grafts, turned their attention to developing a suitable synthetic graft material. The first attempt to bridge an arterial defect with a prosthetic graft was by Alexis Carrel in 1912, an attempt which failed (Carrel 1912). It was not until some years later that the true potential of prosthetic grafts was realised.

In this field, two approaches have been taken. One was to attempt to develop a material which, although it would never endothelialise, would be intrinsically anti-thrombogenic enough to resist occlusion by thrombosis. The alternative was to try to manufacture grafts in such a way as to encourage ingrowth of the patient's own endothelium, working on the assumption that endothelium is the optimal surface to support blood moving at the slow rates of flow occurring in small calibre graft situations.

Non-thrombogenic Surface

The first of these approaches has to date met with limited success, since all prosthetic surfaces so far evaluated possess an inherent thrombogenicity. Attempts to minimise this by passing an electrical current through them, by carbon coating, and by bonding heparin into their structure, each gave good early experimental results (Sharpe et al 1965; Sharpe et al 1966; Sharpe et al 1968; Scott et al; Hufnagel et al 1967). Unfortunately, each has fundamental problems which have to date precluded its routine use in human subjects.

The Ideal Neointima

In 1951 Voorhees made the incidental observation that a piece of silk suspended in the heart of a dog developed an endothelial-like lining. This stimulated investigation into a number of materials constructed in a porous fashion, to establish whether their porous nature would allow eventual endothelialisation of the inner surface. Voorhees carried out a series of canine aortic replacements using "Vinyon N" cloth, and reported promising results with regard to patency rates and behaviour of the graft wall as judged by histological examination after removal at varying time intervals. Voorhees found that the grafts developed a smooth transparent lining composed of multiple layers of flattened cells and collagen fibres. In addition, there appeared to be no foreign

body reaction, and fibroblasts were observed growing in through the interstices of the material (Voorhees et al 1952).

These results led to the investigation of alternative substances, the major ones being Dacron, Orlon, Ivalon sponge, and Teflon. In 1958 Harrison reported the results of a series of aortic, femoral, and carotid replacements using these materials, and concluded that the only satisfactory results were obtained when Teflon was used for large vessel replacement, none of these substances performing well in the case of arteries less than 5 mm in diameter (Harrison 1958).

As a result of his investigations, which involved histological examination of the grafts at varying intervals up to 6 months post implantation, Harrison proposed the following events during the process of "healing" of prosthetic graft materials:

1. A fibrous tissue capsule develops outside the graft, gradually becoming adherent.

2. A layer of fibrin is deposited on the inner surface. This is initially loosely attached, and gradually increases in thickness, to 1-3 mm at three weeks, depending on the graft material used.

3. The fibrin lining is gradually replaced by fibrous tissue growing in through the interstices of the graft.

4. Healing occurs when the graft is lined by a smooth glistening surface, grossly similar to the host intima, but composed of a flattened layer of fibrous tissue.

Although the above sequence of events seems to occur in all species studied, Sauvage and colleagues have demonstrated an enormous variation in the speed and completeness of "healing", being very rapid in the pig, calf and baboon, and extremely slow in man (Sauvage et al 1974). If complete "healing" is defined as including endothelialisation of the luminal surface as suggested by Sauvage, then complete healing has never been observed in man, although in many animal species endothelialisation does eventually occur along the length of the graft. In humans, endothelial coverage generally extends only for a maximum of 6-10 mm "pannus ingrowth" from either anastomosis. More extensive coverage than this is rare and patchy (Sauvage et al 1975). "Healing" in the context of human prosthetic vascular grafts therefore refers simply to the organisation of the rapidly deposited fibrinous layer, with its eventual replacement with fibrous tissue, this then being referred to as the "neo-intima".

Despite modifications to Dacron and Teflon designed to encourage endothelial ingrowth and maximise attachment of the "neo-intima", no endothelialisation resulted, although knitted grafts did show improved connective tissue ingrowth with stronger anchoring of the neo-intima than was the case with their woven counterparts. The addition of an internal velour surface helped this attachment even further, but failed to promote endothelialisation.

The importance of porosity was emphasised by Wesolowski, who in 1961, on the basis of the results of 350 graft implantations into animals, concluded that porosity is the one most important factor determining the outcome of a graft procedure, the more porous the graft, the higher the chance of developing a viable neo-intima (Wesolowski 1961).

1.4. Prosthetic Grafts Applied to Small Diameter Vessels

Although all the above graft materials perform well when applied to large diameter, high flow situations such as aorto-iliac replacement (Papadopoulos

1976), their performance when used for small vessel bypass is very much inferior to that for autogenous vein, the average cumulative patency for Dacron used in the femoro-popliteal situation, for instance, being in the region of 42% at 5 years, in comparison to a rate of approximately 65% at 5 years when saphenous vein is used (Mehta 1979).

Jacobson, in 1963, suggested, on the basis of a series of canine grafts, ranging from 1-4 mm in diameter, and 3-4 cm in length, that prosthetic grafts should not be used for vessels less than 4 mm in diameter (Jacobson et al 1963). He based this statement on the finding of 100% patency when arterial homografts, arterial autografts, or venous autografts were used, as opposed to a patency rate of 0% in the case of prosthetic grafts. This figure was somewhat improved if the diameter of the prosthesis was made 2-3 times the diameter of the vessel it replaced, but even then, patency rates were poor, at 20% and 62% respectively.

Several clinical studies have confirmed the disappointing performance of Dacron grafts in the small vessel situation, with 5 year patency rates varying from 10% to 50% (Reinhold et al 1979; Szilagyi et al 1965). The poor results reported by most authors applied irrespective of the type of Dacron used (knitted, woven, velour).

1.5. Prosthetic Graft Occlusion

In order to improve prosthetic graft performance, it is clearly relevant to consider possible reasons for prosthetic graft failure. Esquivel and Blaisdel, in a review of the reasons for prosthetic graft failure, stated that "the characteristics of the graft lining are probably the most critical factors in the outcome of a vascular prosthesis", the other main reasons for failure being blood alterations and decreased flow through the graft (Esquivel & Blaisdel 1986). Apart from the

inherent thrombogenicity of all prosthetic materials, the phenomenon of neo-intimal hyperplasia contributes to a large extent to the failure of the grafts. Although the exact cause of this condition is as yet unknown, the most popular theory is that the process is due to a combination of factors, including compliance mismatch, anastomotic turbulence, and release of platelet derived growth factors which stimulate proliferation of smooth muscle cells.

As the first hours and days after implantation are important in determining the subsequent performance of an arterial graft, the events occurring when the surface comes into contact with flowing blood are of crucial significance. Irrespective of the actual graft material used, the reactions occurring between a newly implanted synthetic vascular graft and the circulating blood are relatively consistent, and commence almost immediately the graft material is exposed to the circulation. The relative importance of each of the events and the ultimate fate of the graft depend to a large extent on the physico-chemical properties of the material, the presence or absence of anticoagulant and the velocity of the blood flow through it. This latter factor depends on the length and diameter of the conduit and also the quality of the distal "run off" vessels.

1.6. Blood-Surface Interactions

The initial event, occurring within the first few seconds of exposure of blood to a synthetic surface, is the absorption of blood proteins, notably fibrinogen, into the substance of the material. This stimulates the attachment of platelets, which in turn stimulates platelet release reactions, setting in motion the enzyme cascade leading to fibrin formation. The presence of fibrin leads to the adherence of more platelets, which undergo release reactions, and thus a "viscious cycle" is set up, with subsequent growth of the platelet-fibrin aggregate (Bartlett & Anderson 1982).

In the absence of any anticoagulant activity the above reaction proceeds to the development of a macroscopic clot. In a prosthesis of large diameter, with arterial blood flowing rapidly through it, the surface clot is constantly "washed clean", and patency maintained until the graft "heals" by the ingrowth of fibrous tissue and capillaries. If, on the other hand, the diameter of the graft is small, and the rate of flow through it slow, then the resulting clot may become large enough to result in graft occlusion.

These events are modified by the presence of anticoagulant to the extent that the platelet aggregates are not "glued together" by the formation of fibrin, and are much less adherent to the graft surface. Small micro-emboli are therefore more likely, leading to a reduction in platelet survival time, but without clinical relevance.

Other factors which then come into play in determining the long term fate of the graft are the thickness of the neo-intima and the development of pseudo-intimal hyperplasia at the anastomoses. Neo-intimal thickness depends directly on the porosity of the graft material (Wesolowski et al 1961), while neo-intimal hyperplasia, although as yet poorly understood, is believed to result from smooth muscle cell stimulation by platelet release factors produced by platelets aggregated at the anastomoses.

The presence of endothelium on the luminal surface of prosthetic grafts is yet another factor which favourably affects their performance in animal models, although this has yet to be achieved in man (Herring et al 1978; Graham et al 1980). Theoretically, an endothelialised surface should be able to withstand much reduced velocities of blood flow better than a prosthetic material or the usual "neo-intima". For the reasons described above, an endothelialised surface which develops within hours of graft implantation is desirable. Even better would be a prosthesis endothelialised at the time of implantation.

1.7. Expanded PTFE as a Vascular Prosthesis

1976 saw the introduction to clinical use of expanded polytetrafluoroethylene (ePTFE), a modification of Teflon with the rather unique "nodes and fibrils" structure, and with the favourable property of being hydrophobic (and therefore less thrombogenic), as well as microporous. Many hopes were raised by Matsumoto's report of 100% patency rates at 4-11 months using this material in canine femoral artery replacements, as opposed to a 0% patency rate for standard Teflon (Matsumoto et al 1973). Although initial clinical results suggested patency rates almost equivalent to those for autogenous vein (Campbell, C.D., et al 1979; Veith et al 1978; Burnham et al 1978), more recent reports, with longer length of follow-up, have revealed disappointing long term patencies when used in the femoropopliteal position, especially in the presence of poor distal "run-off" (Klimach et al 1984; Haimov et al 1979; Echave et al 1979). As with all grafts, long term results deteriorate, the further distal is the distal anastomosis. Mehta, reporting on his statistical analysis of the published literature up until 1979, described overall cumulative patencies for ePTFE of 65% at 3 years in the femoro-popliteal position, and 40% at 2 years in the femoro-tibial or femoro-peroneal position. The corresponding figures for saphenous vein are 68% and 55% respectively. More recently, Klimach and colleagues found 1 year patency rates of 66% for claudicants having ePTFE grafts in the femoro-popliteal situation, and only 29% when carried out for pregangrene (Klimach et al 1984). A cumulative patency rate of 58% at 2 years was achieved in the presence of three vessel "run-off" as opposed to 33% if only one or two distal vessels were patent. The 3 year cumulative patency for the entire group was only 36%.

Veith and co-workers conducted a randomised trial comparing autogenous vein with ePTFE for infra-inguinal arterial grafts. Although patency rates at 2 years for femoro-popliteal grafts were equivalent, beyond this stage the

performance of the vein grafts was significantly better, with 4 year cumulative patencies of 68% for vein, and 47% for ePTFE (Veith et al 1986).

Although comparison between series is difficult owing to wide differences in surgical selection criteria, the general experience of most authors suggests a significantly higher rate of late failure of PTFE grafts compared to autogenous vein, especially in the presence of risk factors such as poor run off, below-knee distal anastomosis, or pre-gangrene as the indication for surgery. Despite this, the favourable handling properties of ePTFE, together with its reasonable performance in comparison with other graft materials, have made it, for many surgeons, the first choice synthetic graft material for use when suitable autogenous vein is unavailable (Bergan et al 1982).

1.8. Endothelialisation Within Prosthetic Grafts

None of the above synthetic graft materials, irrespective of porosity or structure, has resulted in an endothelialised luminal surface, even after many years of implantation. The flow surface of human grafts therefore persists as a fibrinous lining. Although the extended duration of patency achieved in some cases attests to the fact that fibrin is a reasonably non-thrombogenic surface, the persistence of abnormal platelet kinetics in patients with prosthetic vascular grafts confirms the continuing thrombogenicity of the prosthetic neo-intimal surface (McCollum et al 1981), a factor which may be important in predisposing to failure of the graft. The relevance of graft thrombogenicity is confirmed by the demonstration of an association between enhanced platelet adhesion and a higher failure rate of Dacron femoro-popliteal grafts (Evans & Irvine 1966).

Since the most thromboresistant surface known is endothelium, it seems a logical approach to try to achieve a graft lined with this surface. Endothelium owes its thromboresistance to the production of a number of actively platelet repellant factors, such as ADPase, prostacyclin and a plasminogen activator, as

well as being a thin, smooth cellular monolayer, the presence of which should prevent the sequence of events described above leading to platelet activation and ultimate thrombosis.

The reason for the consistent failure of prosthetic grafts to endothelialise in human subjects remains elusive, but there exists a widespread belief that, if endothelium could in some way be encouraged to grow into and along the surface, then the performance of small calibre, low flow grafts would be substantially improved, perhaps with the additional benefit that anastomotic neo-intimal hyperplasia may be reduced.

1.9 Summary

The present day situation is, therefore, that standard knitted or woven Dacron or Teflon, with or without an internal or external velour surface, give excellent patency rates when applied to large, high flow arteries, such as the aorta or iliacs, but in the case of smaller vessels, such as the femoropopliteal segment, these materials give poor results.

Despite extensive research, extending over many years, no completely satisfactory small vessel synthetic substitute has been discovered. Of those assessed to date, expanded polytetrafluoroethylene has given the most acceptable long term patency rates, although most series have found these significantly inferior to those of autogenous vein.

One of the main reasons for the consistently poor performance of small diameter prosthetic grafts may be the absence of significant endothelial coverage of synthetic materials in humans, irrespective of time since implantation. Thus the blood in the graft, which may be flowing at a very much reduced rate if distal run-off is poor, is constantly being presented with a surface which retains a degree of inherent thrombogenicity indefinitely.

CHAPTER 2

ENDOTHELIAL AND MESOTHELIAL CELL SEEDING OF PROSTHETIC VASCULAR GRAFTS

2.1 Introduction

Human endothelial cells had always proved resistant to growth in culture until Jaffe, in the early 1970's, developed a technique for isolating and growing human umbilical vein endothelial cells (Jaffe et al 1973). Unfortunately this technique was not successful when applied to cells of adult derivation, and it was only with the identification of an endothelial cell growth factor (ECGF) derived from bovine hypothalamus, together with the demonstration that the growth enhancement endowed by this was potentiated by the presence of heparin, that long term growth of adult human endothelial cells in culture became possible (Maciag et al 1981; Jarrell et al 1984). Recent work, made possible by these improvements in cell culture techniques, has revealed a complexity of structure and function which had never previously been attributed to endothelial cells, whose thromboresistant properties were originally attributed mainly to the smoothness of the endothelial monolayer (Ryan & Ryan 1984). It is now recognised that healthy endothelium maintains a clot-free surface by the active production of thromboresistant secretions, notably ADPase and the arachidonic acid derivative prostacyclin, the most potent platelet aggregation antagonist as yet identified. In addition, these cells have a complex array of surface enzymes, receptors, and transport structures, responsible for a variety of metabolic functions. The theoretical benefit of an endothelial lining within prosthetic grafts is clear.

With this objective in mind, and disappointed with the potential for endothelialisation of all presently available prosthetic graft materials, researchers have in recent years directed their efforts to a new approach to this problem, and have been attempting to induce endothelial cells to grow inside grafts by introducing autogenous endothelium into the graft, either before, or at the time of, implantation.

2.2 Endothelialisation of Prosthetic Graft Surfaces

2.2.1 Endothelialisation by Culture

The idea of "artificially" lining synthetic materials with endothelium was first introduced by Mansfield who, in 1975, reported the successful culture of calf endothelial cells on 2 different artificial surfaces. The cells, harvested enzymatically from carotid artery, were cultured and then seeded into graft segments fashioned from the synthetic materials. Following 7 days of culture, during which the cells formed a confluent monolayer, the segments were implanted as descending aortic replacements in calves. Thromboresistance of the cultured endothelial cells was confirmed by incubating cover-slip preparations of the cultured cells with platelet rich plasma. Adherence of platelets to the cellular monolayer was very much less than to fibroblast controls, confirming the thromboresistance of the endothelial cells.

On examining the grafts following their removal from the animals after one, four and eight weeks, some of the grafts were found to have a uniform endothelial lining at all these time intervals, without adherent thrombus except at the anastomotic margins.

Although giving grounds for some optimism, these studies were not carried on any further, although the concept of "helping" a graft to endothelialise was taken up by other workers. More recently, Foxall and co-workers have successfully grown adult human endothelial cells within segments of ePTFE graft material by incubation for 18 hours, followed by a period of culture (Foxall et al 1986). Interesting as these results may be, the practicality of maintaining grafts in culture on a routine basis prior to implantation is extremely limited. For this reason, most investigators have concentrated on developing a technique of immediate seeding i.e. introducing a suspension of cells into the graft lumen at the time of surgery, in the expectation that the cells will adhere to the graft wall and subsequently proliferate to confluence.

2.2.2 Seeding Immediately Prior to Implantation

In 1978 Herring, considering a technique of immediate seeding of grafts to be of more value, described a method whereby endothelial cells could be harvested rapidly from a segment of autogenous vein and seeded immediately into a prosthetic graft using pre-clot blood as the seeding medium. In a series of experiments using a canine model, he obtained endothelial cells by "scraping" the inside of the previously removed saphenous vein. The cells were suspended in chilled Sack's solution, and mixed with blood used for pre-clotting the Dacron grafts, which were implanted as infra-renal aortic replacements. 12 animals were included in the study, six of which received seeded grafts, and six control, unseeded grafts. The grafts were removed at 2, 4, and 8 weeks after implantation, their surfaces compared morphologically and their thrombogenicity assessed. The seeded grafts were found to be significantly less thrombogenic than the controls, the percentage clot-free surface area was significantly higher in the seeded group, and histology revealed an endothelial like lining within seeded grafts, there being no evidence of this in the control grafts (Herring et al 1978).

Similar results were obtained by a group from Ann Arbor Michigan, using a similar pre-clot seeding technique, but employing an enzymatic method of venous endothelial cell harvesting, and maintaining the cells in culture for 14 days prior to seeding. The endothelial nature of the lining was confirmed by immunofluorescence using factor VIII related antigen (Graham et al 1979; Graham et al 1980a).

This same group of investigators subsequently reported a series of 28 canine thoraco-abdominal Dacron double velour grafts, 14 of which were seeded with enzymatically derived venous endothelial cells, the remainder acting as non-seeded controls. A method of harvesting cells from a length of autogenous jugular vein was developed, the cells then being seeded into the graft immediately

prior to implantation, suspended in the blood used to preclot the graft. The authors found that the seeded grafts exhibited greater than 80% endothelial coverage after 28 days, with 88.5% clot-free surface, compared to 40.1% clot-free area in the control animals (Graham et al 1980b). Histological examination of seeded and control grafts at various time intervals from 1-28 days revealed endothelial cells becoming visible at 4 days, growing into a virtually confluent layer by day 28, representing a coverage of approximately 80%. At this time, control grafts showed only a thick fibrin coagulum, with endothelium only in limited areas of pannus ingrowth. The authors concluded that endothelial cells are relatively resilient, and their seeding onto prosthetic grafts as part of a pre-clot procedure provides a reasonable environment for continued growth.

The first attempt to seed ePTFE grafts was made in 1982 by the Ann Arbor group, again using the canine model, with cultured external jugular venous endothelial cells (Graham et al 1982). 91% endothelial coverage resulted after 4 weeks, compared to 10% in control animals. In contrast to seeded Dacron grafts, ePTFE did not regularly demonstrate a subendothelial layer, and the authors speculated that this may adversely affect the durability of the monolayer. In an attempt to overcome this problem, Kempczinski implanted specially made ePTFE grafts, with an increased porosity of 45 microns, and without the external "wrap" present on standard ePTFE grafts, in the expectation that the increased porosity would allow improved connective issue ingrowth (Kempczinski et al 1985). Those which were seeded with autologous enzymatically harvested cells developed a confluent endothelial lining within 7 weeks, while unseeded controls did not. In addition, endothelialised grafts contained a 75-100 micron subendothelial lining.

All of the above studies confirmed that the concept of endothelial cell seeding of prosthetic grafts was sound, at least in the context of the canine model. Further work confirmed these findings and also demonstrated improved

cumulative patency rates in seeded as opposed to non-seeded canine Dacron thoraco-abdominal grafts (Stanley et al 1982).

This stimulated many other workers to begin investigations into various aspects of the seeding process.

2.3 The Evidence for Reduced Thrombogenicity of Seeded Grafts

Many studies, using a variety of approaches, have demonstrated that the seeding process results in the attainment of a monolayer which is intact functionally, as well as morphologically, at least so far as thromboresistant properties are concerned.

The original studies by Herring's group assessed thrombogenicity by performing clotting tests on the luminal surface of the graft after sacrifice of the animal.

Clagett demonstrated both an accelerated return to normal platelet survival figures (PST), and a normal level of prostacyclin production by the graft lumen, in dogs which received seeded Dacron grafts, compared to non-seeded controls. Examination of removed grafts revealed a close correlation between degree of endothelial coverage and normalisation of PST (Clagett et al 1984).

A further study, utilising controlled flow studies on bilateral canine carotid interposition grafts, one side seeded and the other unseeded, demonstrated increased patency rates and increased thrombus free surface areas in seeded grafts following a period of four hours' exposure to reduced flow rates (Hunter et al 1983).

Graft thrombogenicity after endothelial seeding has also been examined using the technique of radioactive isotope labeled platelet imaging (Whitehouse et al 1983). This study suggested a high correlation between a negative scan and completeness of endothelialisation, and revealed a significant difference in the rapidity with which the scans in the seeded and nonseeded groups became

negative. 100% of those in the seeded group were negative by eight weeks, as opposed to only 50% in the controls, suggesting complete lack of thrombogenicity in seeded grafts by eight weeks.

2.4 Harvesting of Endothelial Cells

One area of doubt which confronted the initial investigators, and is still a matter of some controversy, is the best method of harvesting endothelial cells - in other words, which of the currently available ways of stripping the inner layer of cells ensures the highest possible yield of viable endothelial cells, but at the same time avoids significant contamination with other cell types, such as smooth muscle cells and fibroblasts.

Harvesting techniques can be broadly classified into two groups:-

1. mechanical
2. enzymatic.

2.4.1 Seeding of Mechanically Harvested Cells

Various methods of mechanically dislodging endothelial cells from donor vessels have been used, in various contexts (Herring et al 1982). Herring's original work, seeding canine grafts with cells derived from autogenous saphenous vein, utilised a technique of "scraping" the exposed endothelial surface of the vein with a no. 2 steel wool pledget, this then being rinsed in 2 ml chilled Sack's solution to produce a cell suspension (Herring et al 1978).

A similar technique, which has recently been advocated for obtaining endothelial cells from stripped varicose long saphenous veins, involves lightly drawing a scalpel blade along the inside surface of the vein, and then immersing the blade in culture medium to dislodge the attached cells (Ryan and White 1985). Ryan and White claim that cells harvested in this way are capable of forming confluent monolayers very quickly.

Potential Benefits of Mechanical Harvesting

In selecting a mechanical method of harvesting his cells, Herring considered the potential benefits to be:-

1. No specialised instrumentation or additional technical staff is required.

2. No need to remove the cells from the operating field, since no centrifugation or processing is required.

3. Mechanical harvesting can be completed fairly quickly.

4. Any potentially toxic effects of enzymes are avoided. This may be important, as the long term effect on the cells of exposure to crude collagenase, an enzyme contaminated with many other, as yet unidentified, proteases, is unknown. It is conceivable that these enzymes may alter cell surface proteins sufficiently to interfere with the cell's ability to prevent coagulation.

All of these benefits suggest that mechanical harvesting is potentially more practical than the enzymatic alternative. There are, however, some disadvantages.

Problems with Mechanical Harvesting

The above harvesting methods have the following potential drawbacks:-

1. Cells derived in these ways tend to occur in large clusters, which may not be ideal for promoting an even spread when seeded.

2. Tests of viability of harvested cells have suggested only a 10-20% viability rate in the case of those harvested mechanically, as compared to 90% viability in those which were derived enzymatically (Slater & Sloan 1975). Herring, however, points out that cell viability tests do not always serve as accurate predictors of the subsequent behaviour of the cells in culture, and it may

be that enzymatic treatment is associated with a significant incidence of late cell death.

As a result of work reported in 1980, which involved using various lengths of vein as a source of mechanically derived endothelium, and calculating the minimum surface area of vein to surface area of graft ratio which would result in complete graft healing, Herring concluded that this minimum ratio was 0.45. On repeating these experiments using an enzymatic harvesting technique, Herring obtained identical results, the critical Av/Ag ratio again being 0.45. These workers concluded that mechanically and enzymatically harvested cells can be seeded with the same efficiency (Herring et al 1980).

3. Mechanical techniques are less selective, and may remove smooth muscle cells and fibroblasts in addition to the endothelium.

2.4.2 Seeding of Enzymatically Harvested Cells

Work has also been carried out to develop a suitable enzymatic technique, enabling a satisfactory cell yield, but minimising the duration of exposure to the potentially toxic enzymes, and reducing contamination with unwanted cell types to a minimum.

Graham's group developed a technique whereby the excised vein (in their experiments the jugular vein of the dog) was everted over a stainless steel rod and exposed sequentially to trypsin and collagenase for ten minutes each. The cells were collected by centrifugation of the enzyme solutions, suspended in tissue culture medium and then cultured or used for immediate seeding of grafts (Graham et al 1980). Using this technique, these workers managed to harvest 0.5 to 1.5×10^6 cells per 12 cm length of vein as estimated by haemocytometer counts. When the cells were cultured confluence was achieved rapidly, and contamination with other cell types, specifically smooth muscle cells, was minimal, ie less than 10% in 2 week cultures.

Watkins described an alternative technique for harvesting endothelial cells from a length of vein (Watkins et al 1984). This involved incubating the collagenase-filled vein segment at 37^{o}C for 20 minutes, and then flushing it out with culture medium, collecting both the collagenase and the washings, which were then spun down to form a cell pellet. These workers showed that the cells from a segment of vein of surface area 1.9 cm^2 are capable, under favourable culture conditions, of multiplying to cover an area equivalent to that of an aortic bifurcation graft. Again, contamination with other cell types was minimal (1 smooth muscle cell nucleus per 100 endothelial nuclei as measured by haemocytometer after nuclear staining).

Jarrell has recently described a technique whereby large numbers of endothelial cells may be harvested from the capillaries of adipose tissue (Jarrell et al 1986). These authors claim cell yields of approximately 10^6 per gram of fatty tissue, the cells obtained in this way then growing in vitro, exhibiting the morphological and immunological properties of endothelial cells obtained in a more conventional way. These cells, when seeded into Dacron pre-clotted with platlet rich plasma, attached rapidly and were able to resist shear stress of 0 - 80 dyne/cm^2. If cell yields of this magnitude can be consistently and easily obtained by other workers, then this technique is obviously highly attractive from the point of view of immediate graft seeding. Macrovascular harvesting releases cell numbers which are limited by the length of available vein, whereas the microvascular technique would enable very high yields from relatively small pieces of tissue.

2.5 Immediate Versus Cultured Seeding

The initial experiments of cell seeding utilised cells cultured prior to seeding. While having the obvious advantage of providing a higher seeding cell density, this has the disadvantages of requiring an additional operative procedure

to remove the donor vein, a delay of 2 weeks or so to allow the cells to multiply, and, perhaps an increased risk of septic complications. Furthermore, it is generally not known in advance which patients are going to require a prosthetic graft on account of an unsuitable saphenous vein.

The same problems apply to the idea supported by Foxall, of maintaining graft and cells in culture conditions for some days prior to insertion (Foxall et al 1986). Although these workers succeeded in growing adult human endothelial cells to confluence within ePTFE graft segments which had previously been coated with collagen and fibronectin, a period of 18 hours' incubation was allowed for initial attachment, followed by 1 week's maintenance in culture. The authors did not comment on the rapidity of cell attachment and spreading. It is more appropriate to develop a technique to enable large quantities of viable cells to adhere rapidly to a surface which they find attractive and conducive to rapid spreading, in order that a near confluent monolayer may form before the graft is exposed to flowing blood.

More recently, Williams has suggested that cells in culture develop a high incidence of genetic abnormalities (Williams et al 1987). This obviously raises doubts regarding the advisability of implanting cultured cells into patients. For these reasons, most workers have sought to identify a means of harvesting cells and to develop a means of introducing these into the graft such that sufficient numbers will attach to allow proliferation to confluence, the cell to surface bond being strong enough to withstand the shear stress of blood flowing past at arterial pressures.

2.6 Problems With Immediate Seeding

The obvious question which arises when considering a technique of immediate seeding is how the seeded cells and prosthetic surface are likely to interact - in other words, will the endothelial cells adhere and spread out upon

the prosthetic surface rapidly enough to be able to withstand the arterial blood flow? When one considers that culture vessels have to be specially manufactured to enable cell attachment and growth, it seems unlikely that prosthetic graft materials, with their irregular surfaces and porous walls, will be suitable. Some modification of the surface is almost certain to be required to permit attachment.

In the early seeding studies this problem was overcome by introducing the cells into the graft suspended in blood being used to preclot the graft, such that the cells would become caught up in and attach to the fibrinous clot penetrating through the interstices of the graft material. While this may be appropriate in the case of grafts of Dacron or standard Teflon, which require pre-clotting prior to use, the idea of introducing clot into grafts such as ePTFE, which do not normally require this treatment, may be considered unwise. In addition, the pre-clotting technique has the drawback that many cells will be lost with evacuation of the luminal clot prior to graft anastomosis.

The results obtained by the groups of Herring and Graham would, nevertheless tend to confirm that autologous clot is satisfactory in this respect, although some studies have suggested that one-stage seeding using the pre-clot technique is highly inefficient, more cells being lost than actually remain adherent. Because of this, a period of 4 weeks or so is required before confluence is reached. During this time, the potentially thrombogenic surface of the graft, together with the adherent clot, is in contact with the flowing blood.

Jarrell used clotted platelet rich plasma in their experiments involving the seeding of microvascular endothelium onto Dacron graft material (Jarrell et al 1986). The Dacron was treated with the plasma prior to introduction of the cell suspension, rather than using the plasma as the seeding medium.

The importance of different graft materials in determining the adherence of seeded cells is illustrated by the finding of a 70% attachment efficiency for Dacron grafts as opposed to an efficiency of only 12% for ePTFE grafts, using

pre-clotting blood as the seeding medium in each case (Sharefkin at al 1982). Despite this, these investigators found that surface coverage at four weeks was similar in the two types of graft. This may suggest that, although Dacron may take up more cells than ePTFE, those attaching to the latter material may be closer to the surface, and therefore in a more favourable position to multiply and eventually line the graft surface.

There remains a belief, however, that if cell adherence could be maximised, then the time taken for a confluent lining to develop would be greatly shortened. This may be of importance, in view of the findings of Campbell that seeding of ePTFE grafts does not protect against thrombosis, and, indeed, may increase thrombogenic potential until healing occurs (Campbell et al 1984). The ideal situation would obviously be a graft on which cells, seeded at confluent densities, would form a monolayer rapidly, such that it was complete by the time circulation is restored. This would also avoid the dependence on multiple cell replications, which may be associated with subsequent genetic damage to the cells (Williams et al 1987).

2.7 Extracellular Matrix Proteins as Substrates for Cell Seeding

Recent advances in the understanding of the structure of basement membranes, and the ability to isolate many of the components thereof, have led to the investigation of many of the "extracellular matrix proteins" with regard to their ability to enhance cellular adhesion and growth in vitro (Makarak & Howard 1983). It is conceivable that a graft material coated or impregnated with one of these proteins may be a more suitable surface for cell attachment than the same material simply pre-clotted. The most widely investigated and freely available of these is fibronectin, a glycoprotein of molecular weight 440,000, found in many situations in vivo, in addition to being a normal component of basement membrane.

2.7.1 Fibronectin

Fibronectin exists in the body in two forms:- soluble, found in plasma, and insoluble, found in basement membranes and in loose connective tissue. In addition, it is produced by some cells in culture, such as endothelium, fibroblasts and smooth muscle cells. The major function of the insoluble, basement membrane variety, is thought to be as a substrate for cell attachment, and to act as a scaffold for cell movement and migration (Stenman et al 1980).

Several studies have demonstrated that fibronectin coating of cell culture vessels and collagenous surfaces significantly increases the number of cells which attach to the growth surface thereby decreasing the time required for the culture to reach confluence (Martin & Kleinman 1981; Macarak & Howard 1983). Fibronectin has also been noted to induce a flattened morphology, as well as having an effect on differentiation and migration of the cultured cells (Kleinman 1980). The fibronectin used for cell culture is generally obtained from human or bovine plasma by gelatin-Sepharose chromatography, and both forms are now available commercially.

The ability of fibronectin to increase the initial uptake of seeded cells by ePTFE prostheses has been assessed in the canine model. Rosenman carried out a series of ePTFE carotid replacement grafts in dogs, seeded with endothelial cells using autogenous blood clot as the seeding medium (Rosenman et al 1985). Using indium-111-oxine labelling of the seeded cells to indicate cell loss, they found a disappointing percentage of cells attached, with only 19.8% adhering initially after seeding, 30.8% of these then being dislodged after 30 minutes of exposure to the circulation. Ramalanjaona and co-workers, again using isotopic labelling of seeded autogenous jugular vein endothelial cells, found that, in canine carotid interposition grafts, fibronectin coating increased initial endothelial attachment from 19.8% to 46.75% and increased the percentage of seeded cells

remaining after 24 hours by a factor of 6, from 3.4% to 21.3% (Ramalanjaona et al 1986).

Sentissi recently demonstrated that ePTFE grafts, pre-coated with collagen and fibronectin, were capable of supporting the growth to confluence of bovine aortic endothelial cells. By examining the endothelialised grafts by light and scanning electron microscopy after exposure to low and high flow rates for one hour in an artificial circulation, they showed that the adherent cells were capable of withstanding shear stress with minimal loss. These findings were corroborated by using the technique of indium-111-oxine labelling of the attached endothelial cells, which demonstrated an endothelial cell loss rate of 7% at low, and 11% at high rates of flow (Sentissi et al 1985). In their discussion, these authors indicated that previous studies from the same centre demonstrated a less than 50% attachment rate for endothelial cells to untreated ePTFE, with a subsequent decreased amount of DNA at 48 hours compared to 1 hour, indicating that the cells did not replicate on untreated ePTFE.

Similar conclusions were reached by Kesler's group who, using endothelial cells derived from human umbilical vein, were also able to demonstrate an improved strength of adherence of endothelial cells to graft surfaces when coated with fibronectin substrate (Kesler et al 1986). 84.9% of inoculated cells adhered to fibronectin coated ePTFE grafts, 61.1% remaining attached after 1 hour's exposure to an in vitro circulation. Corresponding figures for uncoated ePTFE grafts were 52.9% initially, and 1.8% after in vitro circulation. These authors found however, that the enhancement of attachment was greater when polyester elastomer was the graft material, the percentage retention after 1 hour's exposure to an in vitro circulation being 92.1% for these grafts.

In an attempt to estimate the effect of more prolonged exposure to blood flow, the same group of workers, using indium-111-oxine labelling of the seeded cells, estimated the retention of canine endothelial cells by ePTFE and polyester

elastomer grafts after 1 and 24 hours implantation as carotid replacements (Kesler et al 1986). Although retention prior to perfusion was the same with both types of graft, after perfusion for both these time intervals retention was significantly better in the polyester elastomer grafts. 93.3% of cells seeded into polyester grafts adhered initially, the equivalent figure for ePTFE grafts being 92.2%. After 1 and 24 hour's in vivo perfusion, 93.8% and 78% respectively of initially adhering cells remained attached in the polyester group, corresponding figures for the ePTFE grafts being 54.5% and 24.5% respectively. Despite this, patency rates were significantly higher in the ePTFE group. On the basis of their finding of a greater number of cells attached to occluded, rather than patent ePTFE grafts, the authors concluded that, although short term patency appeared to be determined by degree of endothelial coverage in the polyester elastomer group, the same did not apply to ePTFE grafts.

Even with the improvement in adherence which occurs with fibronectin coating, it is obvious that the seeding process is still highly inefficient when considering continuing adherence after restoration of blood flow. Although this appears to be less true for polyester elastomer than for ePTFE, the latter is in wide clinical use, whereas the former remains in an experimental stage of development. There is therefore a need to investigate alternatives to fibronectin.

It is noteworthy that all the above studies have been carried out using cells of a species or type other than adult human endothelial cells. The latter cell type has always proved more resistant to satisfactory growth in culture than have bovine, canine or umbilical vein cell types. It therefore remains to be established whether the conclusions made from these studies are also applicable to the adult endothelial cell.

2.7.2 Laminin and Type 4 Collagen

Laminin is another glycoprotein, of molecular weight 800,000 - 1000,000, which, in contrast to fibronectin, is found exclusively in basement membranes. Its function appears to be an attachment factor for endothelial cells, binding them to the equally basement membrane specific type 4 collagen, which makes up the connective tissue component of the membrane. It seems that, for all cells which normally adhere to a collagenous matrix, there is a specific type of collagen (type 1,2,3 or 4), and a specific protein responsible for binding the cells onto that collagen, this specific protein in the case of vascular endothelium being laminin (Kleinman 1982). The proposed mechanism is that the attachment protein (fibronectin or laminin) initially binds to the substrate (collagen) thereby forming a protein-substrate complex onto which cell receptors bind.

Techniques have been developed for isolating basement membrane type collagen and laminin from the EHS mouse sarcoma, and both are now commercially available (Kleinman et al 1982).

2.7.3 Basement Membrane Gel

A "basement membrane gel" has also been isolated from the same source. This contains many of the components of normal vascular basement membrane, and has been shown to support the growth in culture of many cell types, namely melanocytes, thyroid cells, sertoli cells, hair follicles and retinal pigmented cells (Kleinman, personal communication). As the gel has properties of normal basement membrane, it may prove to be a suitable substrate for the growth and differentiation of endothelial cells within prosthetic grafts.

2.8 Effect of flow on attached cells

Many of the above studies have addressed the question of the newly attached endothelial cells' ability to withstand the shear stress of fluid circulating

through the graft. Methods employed include implantation of seeded grafts into living animals (Rosenman et al 1985, Ramalangaona et al 1986), or their insertion into specially constructed artificial circulation devices (Sentissi et al 1985, Kesler et al 1986). Results have been variable, some studies showing excessive cell loss (Rosenman et al 1985) while others were more optimistic (Kesler et al 1986).

More recently, encouraging results were reported from a group in California, using human umbilical vein endothelial cells, and seeding these at supra-confluent densities onto ePTFE graft segments which had been pre-treated with type 1 collagen (Schneider et al 1988). These workers found that, if seeding cell density was sufficiently high, then a confluent monolayer was obtainable within a period of two hours. By inserting this endothelialised graft into an artificial circulation circuit, these workers demonstrated that the monolayer had the durability to remain completely intact after perfusion for 1 hour at a flow rate of 100 ml/minute. However, if flow rate was increased to 200 ml/minute or greater, scanning electron microscopy revealed areas of graft exposure between attached cells, although significant proportions of the cells remained attached.

2.9 "Pre-wetting" of grafts

The non wettable and impermeable nature of untreated ePTFE causes practical difficulties, in that any cellular attachment will be to the inner surface only, with no penetration into the interstices. Drying out of the surface occurs very readily, this obviously leading to the death of any cells on its surface. Whether permeation into the interstices is necessary is unknown, but it does seem probable that cells trapped within the graft substance are more likely to remain attached than those adhering tenuously to the surface.

ePTFE can be rendered permeable by brief immersion in alcohol, a process which removes the large quantity of air which constitutes the "void space" of the material. It has been previously demonstrated that endothelial cells can

adhere to ePTFE treated in this way, and then proliferate to confluence (McCall et al 1981), although no seeding studies, to date, have used this method of graft preparation, most preferring the pre-clot technique.

2.10 Clinical Experience with Endothelial Seeding

Although several animal and in vitro studies have confirmed that endothelial seeding is effective as a means of reducing thrombogenicity and improving patency rates of small calibre prosthetic vascular grafts, the technique has, to date, received little attention in a clinical context. The only clinical studies so far reported are those of Herring's group (Herring et al 1984; Herring et al 1985). These workers seeded 37 Dacron grafts, using a mechanical harvesting technique and pre-clot seeding medium. Similar numbers of unseeded grafts were implanted for comparison of patency rates. The procedures included extra-anatomical bypasses and femoro-popliteal grafts. Results were rather inconclusive, there being no significant difference in patency rates between seeded and unseeded grafts in the femoro-femoral or femoro-popliteal position, although the seeded axillo-femoral grafts did appear to fair rather better than their unseeded counterparts. When smokers and non-smokers were considered separately, however, there was a significantly better cumulative life patency in seeded than unseeded femoro-popliteal grafts in those patients who did not smoke. Seeding did, however, seem to have the opposite effect in those patients who continued smoking post-operatively.

It is difficult to draw firm conclusions from this study, as numbers were relatively small, and patency was judged by clinical means only. More accurate follow-up is now possible by means of Digital Subtraction Angiography and Indium-111-oxine labelled platelet scanning.

Herring subsequently reported the occurrence of endothelium within a seeded graft which required re-exploration on the 90th post-operative day, due to

the occurence of a distal embolism of unknown source. Seeding on this occasion was carried out with cells obtained enzymatically from a segment of external jugular vein. The actual seeding technique used was a modification of the standard pre-clot technique. Histological examination of a segment of graft removed at embolectomy, taken from a point approximately 10 cm. from the distal anastomosis, revealed that a large part of the surface was covered by an endothelial like monolayer, with an absence of the usual subendothelial smooth muscle cells or fibroblasts.

No other histological examination of seeded human grafts has yet been made.

2.11 The Mesothelial Cell - an Alternative to Endothelium?

In 1984 a group from the Middlesex Hospital in London, realising that mesothelium had many functional, morphological and embryological similarities to endothelium, suggested that mesothelial cells may be a suitable alternative as a non-thrombogenic lining for prosthetic vascular grafts. On the basis of some experiments in which damaged and undamaged segments of rat intestine were exposed to blood in a Baumgartner chamber, these workers concluded that an intact mesothelial layer had thromboresistant properties similar to those of endothelium (Clarke et al 1984).

These workers then developed a means of harvesting mesothelial cells from rat omentum by incubating the omentum in collagenase. The cell suspension was cultured to confluence and passaged into fresh dishes containing Thermanox cover slips. When the cells had attained confluence, the cover slips were placed in a Baumgartner chamber and exposed to blood flowing at 160 ml/min for 10 minutes. On examining the cell monolayer by electron microscopy they found a complete absence of adhering platelets, leading to the conclusion

that mesothelial cells in culture possess the same thromboresistant properties as the intact mesothelium used in their previous studies (Nicholson et al 1984).

In a follow-up to this work, the Middlesex group seeded three canine abdominal aortic "Vascutek" grafts with omentally derived mesothelial cells, using pre-clotting blood as the seeding medium. One control, unseeded graft was inserted in a fourth animal for comparison. On examining the graft surfaces by scanning electron microscopy after 1 month, they found that all the seeded grafts had developed a confluent lining of cells morphologically resembling mesothelium. By comparison, the control graft demonstrated no cellular lining (Clarke et al 1984). The authors concluded that mesothelial cells may be a suitable alternative to endothelial cells for seeding into prosthetic grafts.

In support of this, Bull has recently reported a study which demonstrates in dogs that mesothelial seeded Vaskutek grafts produce significantly more prostacyclin after implantation, than equivalent unseeded grafts (Bull et al 1988).

CHAPTER 3

GENERAL OUTLINE AND AIMS OF STUDY

3.1 Introduction

Before any possible clinical benefit of an endothelialised prosthetic graft lining can be evaluated, it is important first of all to ascertain whether or not this goal can be achieved with currently available graft materials. It is necessary to establish not only that the cells adhere, but that they adhere and spread out rapidly, preferably by the time it takes to fashion the anastomoses at either end of the graft. Only if this occurs is it likely that any attached cells will be able to resist the shear stresses to which they are exposed when blood flow is commenced. The formation of a confluent monolayer within this time should, theoretically, be the best way to prevent platelet activation within the crucial first hours and days of the graft's life.

To date, only one study has been reported which has demonstrated the growth to confluence of adult human saphenous vein endothelial cells within segments of ePTFE graft material. For this to occur the authors found that a pre-coating of the grafts was essential - in their case the pre-coating used was a combination of commercially available collagen and human fibronectin (Foxall et al 1986). No evidence was, however, obtained as to the proportion of seeded cells which actually attached to the prosthesis, and only the one substrate preparation was evaluated. In addition, a period of 18 hours' incubation was allowed for cell attachment. From the point of view of seeding immediately prior to graft implantation this is clearly impractical.

As ePTFE is the most widely used prosthetic material for small vessel bypass, it is relevant to establish whether the findings described in chapter 2 in relation to bovine and human umbilical vein endothelial cells apply also to the adult cell. This is of obvious importance if the process is to become clinically useful. An immediate seeding technique must be shown to result in attachment and spreading of large numbers of cells in as short a time as possible.

In addition, it is important to establish the likely behaviour of attached cells when exposed to the shear stress of blood flowing at rates equivalent to those occurring *in vivo*. An immediate monolayer is valueless if it is washed off immediately blood flow is established through the graft.

3.2 Aims of Study

The aim of the present study was, using an *in vitro* technique, to answer the following questions with regard to endothelial cell seeding of ePTFE grafts, with specific reference to clinical applicability of the technique:

1. Is mechanical harvesting as effective as enzymatic methods, in obtaining sufficient numbers of viable cells to adhere quickly to a prosthetic surface and subsequently proliferate to confluence? This is relevant from the point of view of ease, and time taken to harvest the cells.

2. Will adult saphenous vein endothelial cells adhere to PTFE graft material in sufficient numbers and sufficiently quickly to make the seeding procedure useful in a clinical context, and does pre-coating with any of the previously mentioned substrates improve the seeding efficiency? Are adherent cells capable of rapidly forming a confluent monolayer within the graft lumen?

3. Do mesothelial cells behave similarly to endothelium when used to seed prosthetic grafts, and which substrate is best to use with this cell type? Do these cells have potential as a substitute for endothelial cells for seeding of grafts?

4. Are attached cells sufficiently adherent to be capable of withstanding blood flowing past at flow rates equivalent to those normally pertaining in femoro-distal bypass grafts?

All the studies in the present work were performed using cultured cells. This ensured a regular supply of healthy endothelial and mesothelial cells. Cells in early passages were used in all experiments to reduce the likelihood of artefactual results due to prolonged culture.

CHAPTER 4

MATERIALS AND GENERAL METHODS

RESULTS OF HARVESTING AND CELL CULTURE TECHNIQUES

4.1 Materials

All culture procedures were carried out in a class 2 microbiological safety cabinet (Medical Air Technology Ltd., Manchester).

Sterile tissue culture polystyrene flasks, multiwell plates, test tubes and pipettes were purchased from Sterilin Limited, Middlesex. Balanced salt solutions, culture media and trypsinising solution were obtained from Flow Laboratories, Rickmansworth, Herts., and endothelial cell growth supplement (15 mg), human plasma fibronectin (1 mg), gelatin (2%) and collagenase (crude, type 1) from Sigma Chemical Company Limited, Dorset. Foetal calf serum was purchased from Sera-lab, Sussex. Other media supplements were obtained from Flow Laboratories. Laminin 1 mg/ml in tris saline, type 4 collagen 0.5 mg/ml in 0.5N acetic acid, and basement membrane gel were kindly supplied by Dr. H. Kleinman, National Institute of Dental Research, Bethesda, Maryland, USA. Type 4 collagen was always neutralised with 1N sodium hydroxide prior to use.

Endothelial growth supplement was reconstituted with 5 ml Dulbecco's phosphate buffered saline and stored at -20^{o}C until use. Substrate proteins, enzymes and serum were made into small working aliquots and stored at -20^{o}C. Foetal calf serum was batch tested prior to ordering.

ePTFE grafts were kindly supplied by W.L. Gore & Associates, Flagstaff, Arizona. Indium-111-oxine was purchased from Amersham International plc., Buckinghamshire.

An Olympus CK inverted microscope with phase contrast was used for examination of the cell cultures (Olympus, Tokyo).

4.2 Culture Media

The choice of media was based upon previous reports of successful culture of these cell types.

a. Endothelium

Endothelial cells were grown in Medium 199 with Earle's salts and 20mM HEPES buffer. This was supplemented with 20% foetal calf serum, endothelial cell growth supplement 25ug/ml, heparin 15 IU/ml, sodium bicarbonate 1.4 g/l, L-glutamine 200 mg/ml and Penicillin/Streptomycin solution (5000 IU/ml Penicillin and 5000 mg/ml Streptomycin) in a concentration of 1 ml per 100 ml of complete medium. The pH of the medium was adjusted to 7.2 - 7.4 by the addition of 1N HCl or $NaHCO_3$ as required. Medium was made up in 500 ml quantities and stored at 4^oC until use.

All flasks for endothelial cultures were pre-coated with either fibronectin or 1% gelatin prior to plating. Pre-coating was achieved by incubating the flask at 37^oC for 1 hour with 1 ml fibronectin or gelatin covering the flask floor. Following incubation, excess protein solution was removed, and the remainder allowed to dry onto the surface. Before introducing the cell suspension excess unbound protein was removed by washing with Dulbecco's phosphate buffered saline (DPBS).

Pre-coating was used routinely for culture of endothelium in view of the reported difficulties in growing endothelial cells to confluence on uncoated cell culture surfaces (Ramalanjaona et al 1986).

b. Mesothelium

Medium used for the mesothelial cultures was Ham F12 with 10% foetal calf serum, L-glutamine and Penicillin/Streptomycin, all in the same concentrations as for the endothelial medium. Flasks containing mesothelial cultures were "gassed" with 5% carbon dioxide in air prior to firm tightening of the flask cap.

All primary cultures and subsequent subcultures were grown in 25 cm^2 cell culture flasks by incubation at 37^oC.

4.3 General Methods

4.3.1 Harvesting Techniques

a. Endothelium

Enzymatic

For harvesting of macrovascular endothelial and mesothelial cells, collagenase was reconstituted immediately prior to use with Dulbecco's Phosphate Buffered Saline containing calcium and magnesium (DPBS), such that the final collagenase activity was 630 units/ml. Before use, the collagenase solution was sterilised by injection through a 0.2 micron syringe filter (Sigma).

Macro-vascular endothelial cells were obtained from segments of adult long saphenous vein using the method of Watkins (Watkins et al 1984). Veins were received from patients undergoing varicose vein surgery or femoro-distal bypass in whom the vein was deemed unsuitable for use as the graft. The vein segment was placed, immediately after its removal, in a 50 ml container containing 15-20 ml Hanks balanced salt solution, and transferred to the laboratory as quickly as possible. Following cannulation with a "Venflon" cannula, it was flushed out several times with fresh Hank's balanced salt solution (HBSS) to remove red blood cells as much as possible. One end was clamped with an artery forceps and the vein filled to distension with collagenase solution. The other end was clamped, the vein immersed in 20 ml pre-warmed HBSS in a sterile 50 ml container, and incubated in a water bath held at $37^{o}C$ for 20 minutes.

Following removal of the clamps and evacuation of the contents into a 10 ml sterile test tube, the vein was flushed several times with 5 ml serum free culture medium E199. The cell suspension was centrifuged at 1000 rpm for 5 minutes at $4^{o}C$, and the supernatant discarded. The cell pellet was resuspended in 5 ml serum free medium and centrifuged once more at 1000 rpm for 5 minutes.

Supernatant was discarded and the pellet resuspended in 5ml complete medium warmed to 37^{o}C. A small aliquot of this was taken for cell counting, and the remainder of the cell suspension was seeded into a 25 cm^2 gelatin- or fibronectin-coated culture flask.

After 24 hours the medium was removed and any red cells washed away with fresh medium. The cells were re-fed with 5 ml medium. They were then fed every 2-3 days until confluent. When colonies became confluent, the volume of medium was increased to 8 - 10 ml.

Mechanical

Mechanical harvesting of endothelial cells was by the method described by Ryan & White (Ryan & White 1985).

The vein segment was opened longitudinally and washed with complete medium to remove red cells. The inner surface was gently scraped with a broad - bladed disposable scalpel, the material adhering to the blade then being removed by immersion in 5 ml of complete medium. The resulting suspension was drawn gently up and down into a 10 ml syringe through a 19G needle in an attempt to separate cell clusters as much as possible. A small aliquot was withdrawn for counting, the remainder being seeded into 25 cm^2 flasks as for enzymatically harvested cells.

Microvascular endothelium

Microvascular endothelium was harvested from subcutaneous or intra-abdominal fat using the technique described by Jarrell (Jarrell et al 1986). Portions of fat were minced into small fragments, and incubated in collagenase in Dulbecco's phosphate buffered saline, calcium and magnesium free (DPBS CMF), for 20 minutes. Collagenase was made up in a solution of 4 mg/ml., with a volume to weight ratio of 1 ml/gm of tissue. The minced fat was incubated in the

collagenase in an Ehrlmeyer flask for 20 minutes at 37^{o}C. Constant agitation was effected by the use of a shaking water bath, and a small "spin bar" placed in the bottom of the flask. The resultant "slurry" was centrifuged at 1000 rpm for 5 minutes. All fatty tissue rises to the top during the spinning, leaving a cell pellet consisting of endothelial cells and red blood cells, along with any contaminating stromal cells which may be present.

The cell pellet was washed twice with DPBS CMF with 1% bovine serum albumin (BSA). It was then suspended in 45% Percoll in PBS CMF and 1% BSA, which was centrifuged in a fixed angle centrifuge at 13000 rpm at 4^{o}C for 20 minutes. During this procedure a narrow whitish band, which Jarrel claims to consist of individual microvascular endothelial cells, forms at the top of the suspension. After centrifugation this band was pipetted off and cultured, using the same methods as for macrovascular endothelial cells.

b. Mesothelium

Mesothelial cells were obtained from portions of human omentum from patients undergoing laparotomy.

The omentum was placed, immediately after removal, into a 50 ml sterile container containing 15 ml HBSS without calcium and magnesium at ambient temperatures, and at pH approx. 7.2-7.4. The specimen was transported to the laboratory as quickly as possible, transferred to a container with 10 ml collagenase solution maintained at 37^{o}C in a water bath. Following incubation at this temperature for 20 minutes, the omentum was removed, the collagenase/cell suspension transferred to a 10 ml sterile test tube, and centrifuged at 1000 rpm for 5 minutes at 4^{o}C. The collagenase was removed and the cell pellet washed twice with complete medium by spinning at 1000 rpm for 5 minutes, at 4^{o}C.

The pellet was finally suspended in 5 ml of medium, and plated in an uncoated 25 cm^{2} tissue culture flask.

4.3.2 Confirmation of endothelial identity

Endothelial cells were identified by the characteristic epithelial morphology, the "cobblestone" appearance of confluent monolayers and the presence of factor VIII antigen on the cell surface as revealed by immunofluorescent staining. The presence of a positive factor VIII stain is widely accepted as confirmatory evidence of an endothelial phenotype (Jaffe et al 1973; Wagner & Marder 1984).

Method

Anti-factor VIII immunoperoxidase staining was performed by the Department of Pathology, Ninewells Hospital and Medical School. Briefly, cultures were fixed in acetone, incubated with rabbit anti-human factor VIII antibody followed by fluoroscene-isothiocyanate conjugated goat anti-rabbit globulin. The stained cultures were examined by light and transmission electron microscopy.

4.3.3 Confirmation of mesothelial identity

Mesothelial cells unfortunately have no characteristic markers, but their nature was implied by the presence of an epithelial morphology, monolayer formation, and the presence of microvilli on transmission electron microscopy. Morphologically, these cells are quite different from endothelial cells. Characteristically, mesothelial cells in culture exhibit a varied morphology before reaching confluence, with islands of polygonal cells interspersed with stellate and spindle shaped ones (Whitaker et al 1982).

4.3.4 Subculture of cells

When confluent cell coverage extended virtually completely across the flask floor, they were resuspended by treatment with a solution of 0.05% (w/v) trypsin (1:250) and 0.02% (w/v) EDTA. Culture medium was removed from the flask and replaced with 1 ml Hank's balanced salt solution without calcium and magnesium. This was incubated at 37^{o}C for 1 minute, the HBSS then being removed. 1 ml of trypsin/EDTA was added, incubated at 37^{o}C for one minute, and excess trypsinising solution removed. Incubation was continued until visible separation of the cell monolayer had occurred. This usually took place after 4-5 minutes.

Medium with serum was added, and the monolayer suspended by gentle agitation. The volume of medium added depended on the split ratio required, 5 ml being allowed for each new flask to be used. Once completely suspended, the cell supension was replated into 25 cm^2 flasks, the number of flasks depending on the split ratio required. A small (0.1 ml) aliquot was saved to obtain a cell count.

In order to compare the growth characteristics of each of the cell types on each of the different substrate proteins, a small number of the primary endothelial cultures were subcultured onto flasks coated with laminin or type 4 collagen. Similarly, a small number of primary mesothelial cultures were subcultured onto flasks coated with fibronectin, laminin, or type 4 collagen. Pre-coating with laminin and type 4 collagen was achieved using the same technique as for coating with gelatin or fibronectin (see paragraph 4.2).

4.3.5 Cell Counting

Counts of freshly harvested cells were obtained using a Neubauer counting chamber. For ease of cell recognition, all aliquots used for counting were diluted 1 in 3 with crystal violet (0.1%) in citric acid (0.1M) (Paul 1975). This was

necessary on account of the inevitable presence of red blood cells, even after repeated washing of the specimen.

As chamber depth was 0.1 mm, the total counts obtained from five 1 mm^2 squares in each of the two chambers gave the cell number per mm^3 of cell suspension. Thus, total harvested cell count in the stained suspension = count in 2 chambers x 3 x 1000 x initial volume in ml.

Cells being trypsinised for passaging after initial growth to confluence were counted in a similar manner, except that crystal violet staining was unnecessary.

4.3.6 Estimation of Cell Population Doubling Number

Population doubling number (PDN) for primary cultures was estimated using the formula

$$PDN = \log_2 N_f/N_i$$

where N_f = cell count at confluence

N_i = initial cell count

Doubling number was obtained for subcultures by estimating $\log_2$ of the split ratio used. For example, a confluent culture trypsinised and replated into 2 new flasks (1:2 split) will have undergone one ($\log_2$ 2) population doubling when both the new flasks reach confluence. Similarly, a 1:4 split will require cells to undergo 2 doublings ($\log_2$ 4) to reach confluence.

The cumulative population doubling number was obtained by summing the individual PDN's in each passage. The population doubling time was obtained using the formula PDT = time in days from initial plating until confluence was reached/PDN.

4.3.7. Electron microscopy

Scanning electron microscopy was carried out, using a standard technique, by the Department of Pathology, Ninewells Hospital and Medical School. Briefly, specimens for scanning electron microscopy were fixed in glutaraldehyde, dehydrated in a graded series of acetone, critical point dried and sputter coated with gold-palladium. They were then examined in a Jeol J.S.M. 35 scanning electron microscope.

4.4 Harvesting Technique - Results

a. Endothelial Cells

Enzymatic Harvest

Lengths of vein used for the experiments ranged from 1.5-10.0 cm., with an average available endothelial surface area of 4.5 ± 2.5 cm^2 (Table 1).

Cells were harvested enzymatically from 23 saphenous vein segments. Average cell yield was $2.6 \pm 1.2 \times 10^4$ per cm^2 of vein surface. The majority of the cells obtained in this way were successfully grown to confluence (see below).

Mechanical harvesting

Mechanical harvesting was used in an attempt to culture endothelial cells from 12 saphenous vein segments. Lengths of vein used ranged from 1.5 - 34 cm, with an average endothelial surface area of 6 ± 10.2 cm^2.

Cell counting was attempted, but proved difficult for mechanically harvested cells, as the cells tended to occur in clumps, and excessive debris made cell identification difficult. Coulter counting was also precluded by the occurrence of cell clumps. For these reasons, estimated counts were considered too inaccurate to be of value.

None of the primary cultures attempted in this way was successful in producing a confluent monolayer, the best result being the adherence of one or

TABLE 1

ENDOTHELIAL CULTURES - YIELD AND TIME TO CONFLUENCE

Culture	Vein size (cm)	SA (cm^2)	Yield ($x10^4/cm^2$)	Confluence time (days)
1.	3.0 x 1.0	3	3.7	25
2.	3.0 x 1.0	3	2.3	28
3.	1.5 x 1.0	1.5	1.3	21
4.	2.0 x 1.4	2.8	2.5	28
5.	2.0 x 1.0	2	2.0	21
6.	3.2 x 0.9	2.9	4.1	21
7.	7.0 x 0.6	4.2	1.4	18
8.	7.0 x 0.6	4.2	1.9	20
9.	3.0 x 1.0	3	5.7	17
10.	4.0 x 1.5	6	4.5	19
11.	2.0 x 1.4	2.8	1.4	21
12.	5.5 x 0.7	3.9	2.3	30
13.	7.0 x 1.0	7	1.4	20
14.	4.3 x 1.0	4.3	2.3	14
15.	10.0 x 1.0	10	2.2	8
16.	10.0 x 1.0	10	2.6	21
17.	5.0 x 1.0	5	2.0	28

Mean available surface area = $4.5 \pm 2.5 cm^2$

Mean yield per cm^2 = $2.6 \pm 1.2 \times 10^4/cm^2$

Success rate = 17/23 (74%)

two isolated colonies, these rapidly dying after a day or two. Although many cells were usually visible in the medium, the majority of these failed to attach, even after culture for periods of up to 1 week.

Microvascular harvesting

This technique was used 16 times. In ten the source of cells was subcutaneous fat from the leg, while in the remaining 6 omental fat was used. In the former, large numbers of cells were visible in the primary isolate, occurring both individually and in small clumps. These adhered very rapidly and spread out within some hours. Initially, many of the cells were morphologically similar to the endothelium harvested enzymatically from vein segments, but within 2-3 days a large number of elongated spindly cells resembling fibroblasts became apparent. These proliferated rapidly to the detriment of the endothelial cells, eventually covering the complete flask floor. These cells did not form a confluent monolayer, but overlapped each other, forming multiple superimposed layers. In no case was a confluent monolayer of cells achieved.

The microvascular technique also released large numbers of cells from omental fat. These cells, although epithelial in morphology, resembled the mesothelial cells obtained from omentum when incubated without mincing and without Percoll centrifugation (see below). Factor VIII staining was negative in these cells and transmission electron microscopy revealed surface microvilli, suggesting a mesothelial, rather than an endothelial phenotype.

b. Mesothelial cells

28 specimens of omentum were incubated with collagenase as described. Specimen weights ranged from 2.1 Gm - 35 Gm, with a mean of 10.4 $\pm$ 8.4 Gm (table 2). The average cell yield per gram of tissue ranged from 1.2 to 5.6 x 10^4,

TABLE 2

YIELD AND TIME TO CONFLUENCE FOR MESOTHELIAL CULTURES

Culture No.	Wt. (Gm.)	Yield ($x10^4/cm^2$)	Confluence time (days)
1.	10.4	1.1	21
2.	10.1	1.4	28
3.	18.9	2.3	18
4.	19.1	1.6	21
5.	12.0	1.6	17
6.	8.7	2.8	9
7.	8.2	1.2	14
8.	6.9	1.1	20
9.	2.1	3.6	13
10.	19.3	4.6	7
11.	4.8	4.7	6
12.	2.6	5.2	8
13.	4.4	5.2	21
14.	3.4	4.8	13
15.	5.1	2.8	28
16.	7.2	3.4	21
17.	35.0	4.2	5

Average Weight = 10.4 ± 8.4Gm.

Average Yield = $3.0 \pm 1.5 \times 10^4$ cells/Gm

with a mean of 3.0 $\pm$ 1.5 x 10^4. Cells occurred mainly in small clumps, adhering and spreading within 24 hours.

4.5 Cell culture results

a. Endothelium

All results for endothelium now refer to those cells obtained enzymatically from vein segments.

Cells were obtained on each occasion, occurring in small clusters (figure 1). Without exception the cell clusters adhered within 24 hours, the process of attachment and spreading of some clusters beginning within 15-30 minutes. Thereafter the cells spread out widely and formed small colonies within 1-2 days (Figure 2 & 3). Although adherence and initial spreading was universal, a proportion of the cultures failed to grow to confluence, dying after 3-4 days in culture.

17 of the cultures (74%) grew to visual confluence covering the floor of the flask within 1-4 weeks (table 1), all of these subsequently being subcultured in split ratios of 1:2 up to 1:5. The number of population doublings required to reach confluence in each case, assuming adherence of all of the cells, is illustrated in tables 3 and 4. It should be noted that the split ratio at passage varied, explaining the variation in the ratio of passages to number of doublings. Considerable differences were noted between different batches of foetal calf serum, and this was always batch tested before ordering a new supply. A "learning curve" was also noted, in that a higher proportion of failed cultures occurred in the initial attempts. Of the last 9 attempts, the success rate in achieving confluence was 100%.

Growth rates in primary culture varied somewhat, with population doubling times (PDT) ranging from 2.7-9.5 days (mean 4.7 $\pm$ 1.7). After subculture, however, doubling times were more constant. Those cultures which

Figure 1. Freshly harvested macrovascular endothelial cell isolate, illustrating the cells occurring mainly as small clumps floating in the medium. The multiple darker spherical cells are contaminating red cells (original magnification x 100).

Figure 2. Small colony of endothelial cells after 2 days in culture (original magnification x 100).

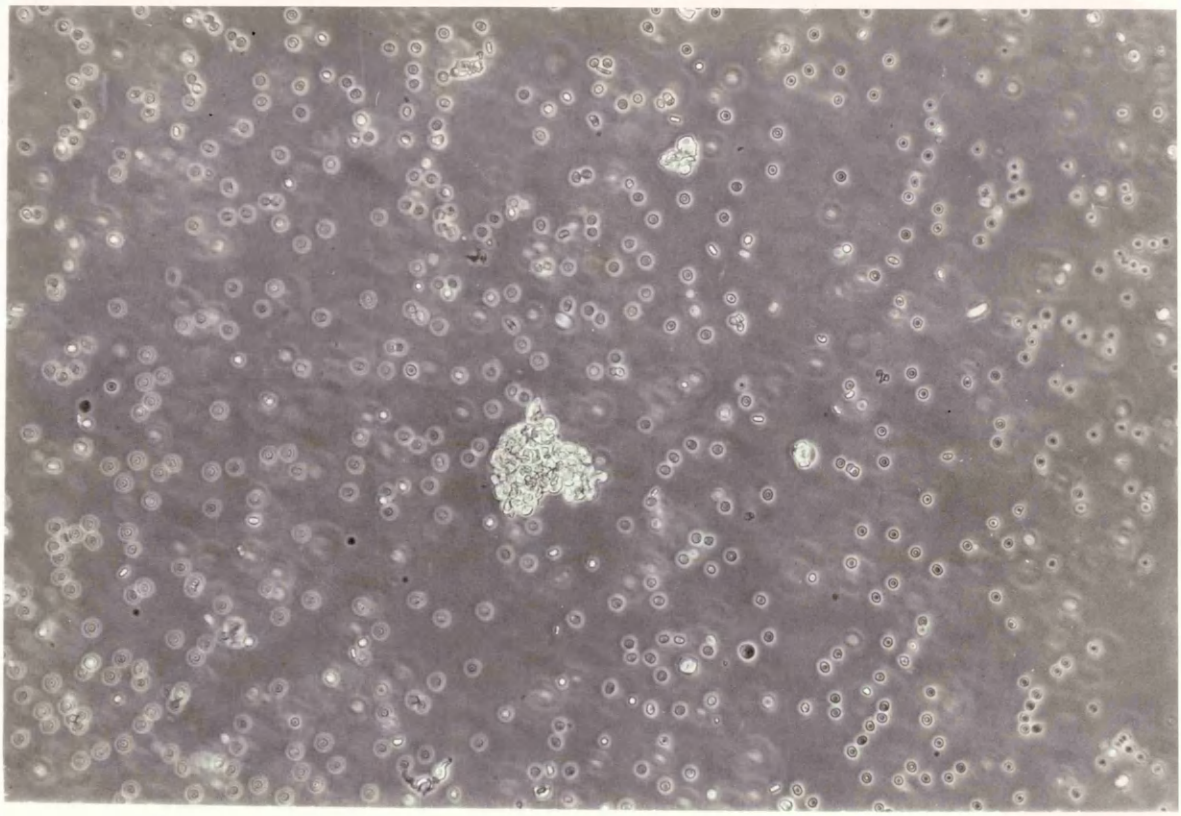


Figure 1

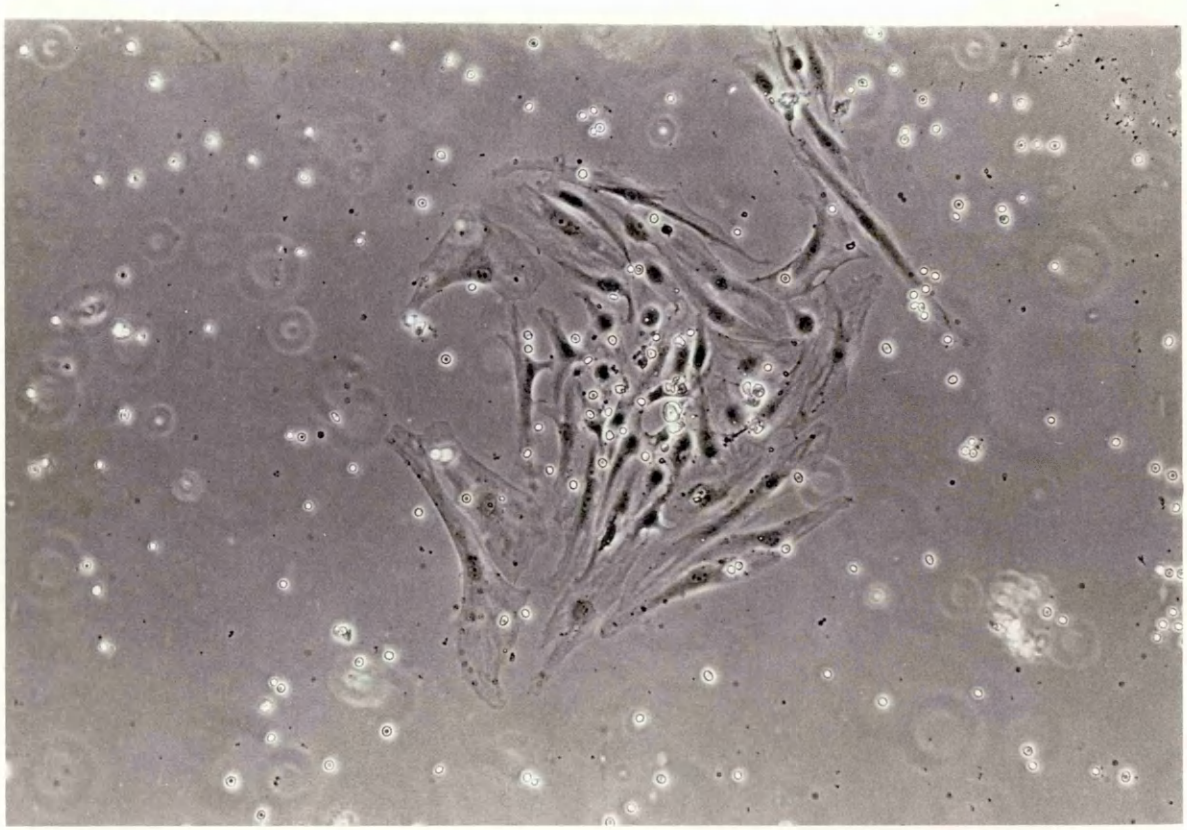


Figure 2

Figure 3. Low power view of multiple colonies of endothelial cells forming after 2 days in culture (original magnification x 40).

Figure 4. Confluent culture of macrovascular endothelial cells showing "cobblestone appearance" (original magnification x 40).

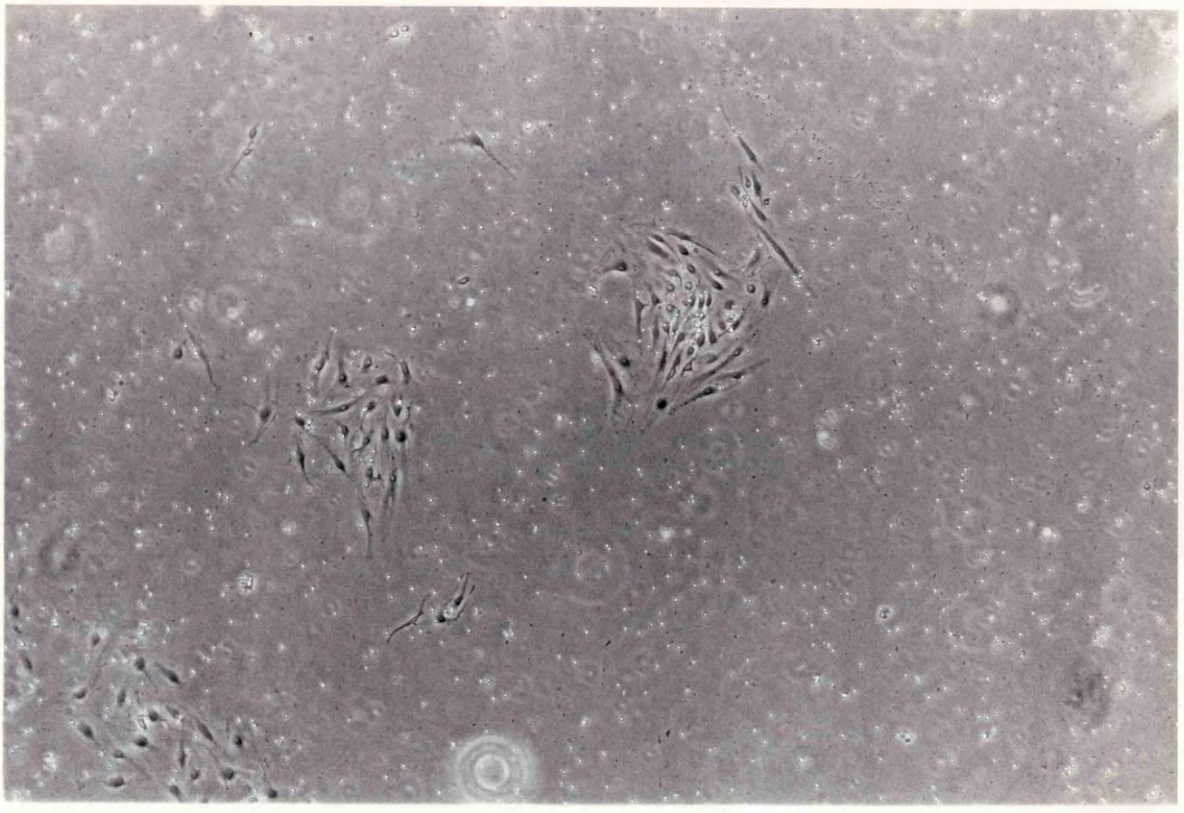


Figure 3

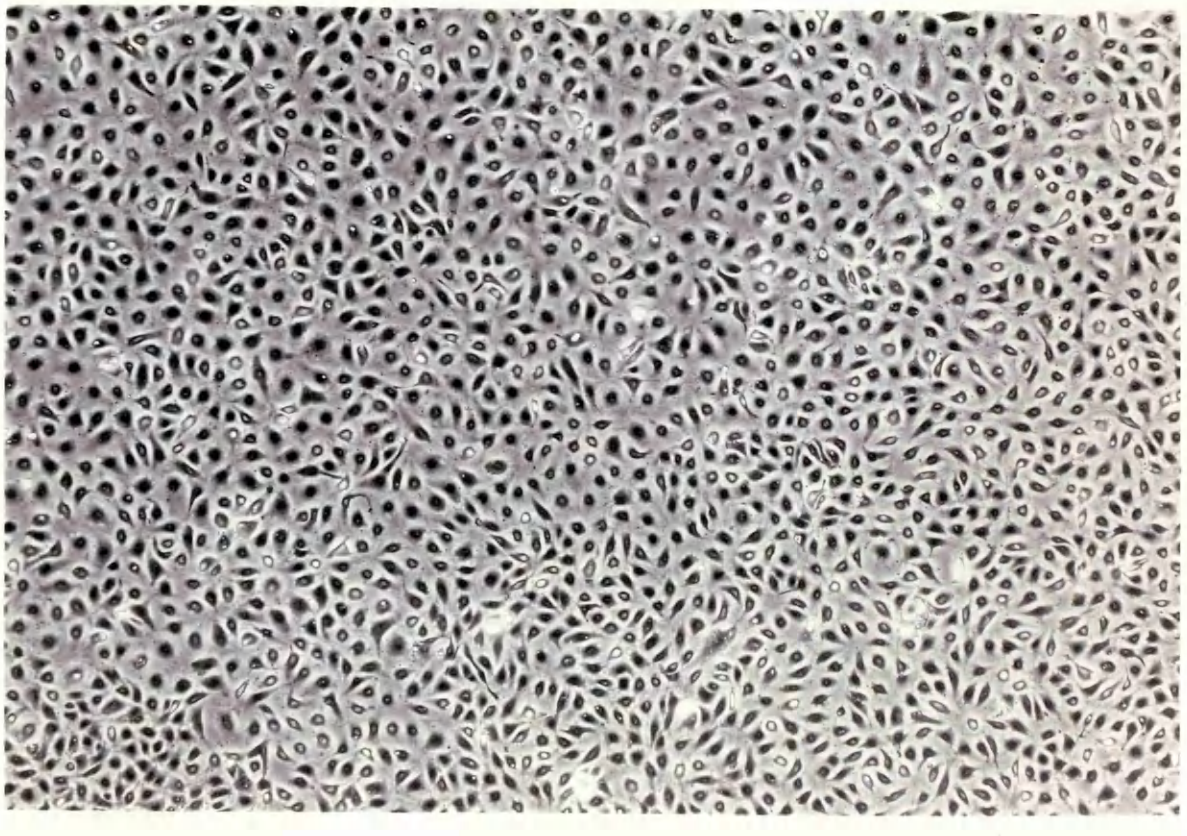


Figure 4

TABLE 3

PDN AND PDT OF PRIMARY ENDOTHELIAL CULTURES

Culture	Ci ($x10^5$)	Cc ($x10^6$)	Cc/Ci	PDN	Tc (days)	PDT (days)
1.	1.1	1.6	14.5	4	25	6
2.	0.7	1.1	15.7	4	28	7
3.	0.2	2.1	105.0	7	21	3
4.	0.7	1.7	24.3	5	28	6
5.	0.4	1.8	45	5	21	4
6.	1.2	2.3	19.2	4	21	5
7.	0.6	1.8	30.0	5	18	4
8.	0.8	1.3	16.3	4	20	5
9.	1.7	1.9	11.2	3	17	6
10.	2.7	1.0	3.7	2	19	10
11.	0.4	1.1	27.5	6	21	4
12.	0.9	1.1	12.2	4	30	8
13.	1.0	2.0	20	4	20	5
14.	1.0	2.0	20	4	14	4
15.	2.2	2.0	9	3	8	3
16.	2.6	2.4	10.8	3	21	7
17.	1.0	1.0	10	3	28	9

Average population doubling time = 5.4 ± 1.9 days

Ci = initial total yield

Cc = count at confluence

PDN/T = population doubling number/time

Tc = time to reach confluence

TABLE 4

CUMULATIVE PDN FOR ENDOTHELIAL CULTURES

Culture No.	No. of Doublings in primary culture	No. of Passages	Total Doublings
1	4	1	5
2	5	3	10
3	7	2	10
4	5	1	6
5	5	1	6
6	4	1	5
7	5	1	6
8	5	5	12
9	3	3	9
10	2	1	4
11	6	1	7
12	4	4	8
13	4	1	5
14	4	1	5
15	2	3	8
16	3	2	6
17	3	1	5

were split in a ratio 1:2 reached confluence in 2-3 days, indicating that this was their PDT. Similarly, cultures split 1:4 reached confluence in 3-4 days, again indicating a PDT of approximately 2 days. Cell yield at confluence ranged from 1 x 10^6 to 2.4 x 10^6 (mean 1.7 $\pm$ 0.5).

The discrepancy between PDTs in primary and subculture may be due to a "lag phase" of some days following initial plating, during which the cells do not divide, although they do attach and spread. Variability in the duration of this from patient to patient may account for the differences in PDTs in primary culture. Another possible explanation is that only a small proportion of freshly harvested endothelial cells are likely to adhere to the culture flask floor. Using the total number of cells harvested as C_i in the calculation of PDN will therefore almost certainly give an underestimate of the number of doublings occurring, and an overestimate of doubling time. At subculture, since only cells with an ability to adhere will have survived thus far, the proportion adhering after replating will obviously be higher, and this error much smaller.

The maximum number of cumulative doublings occurring in each culture is indicated in table 4. Cells were not maintained longer than 12 doublings, since it was considered desirable to use cells in early passages for the seeding experiments to avoid possible alterations in cell behaviour ensuing from prolonged culture and repeated trypsinisations (Watkins et al 1984). By the time cells reached 12 doublings there were many large cells with rather bizarre shapes, and the development of confluence was delayed, suggesting impending senescence. The majority were therefore used for experiments well before this stage was reached.

Two of the initially successful primary endothelial cultures subsequently had to be discarded because of contamination with another cell type, of fibroblastic morphology. These became evident in the first passage, and at second passage outgrew the endothelial cells. The source of these cells was

presumably the adventitia of the donor vein segment. None of the other 15 cultures demonstrated such contamination, even after multiple passages.

All confluent cultures demonstrated the typical "cobblestone" morphology of endothelial cells (figure 4), and representative samples from each donour stained positively for factor VIII antigen (figure 5 & 6). The cobblestone morphology was quite distinct from the elongated "spindly" appearance of fibroblasts in culture (figure 7 & 8).

b. Mesothelium

Of the 28 attempts, cells were successfully grown from 17 (61%). Two of the cultures included as failures grew initially, reaching confluence, but succumbed to bacterial or fungal contamination before subculture was possible. As with endothelium, a learning curve was observed, the last eight successive harvests being successful.

The cells took on a varied morphology when subconfluent (figure 9 & 10), many of those occurring individually being of a fibroblastic appearance, while those in the centre of colonies were small, polygonal and of a more epithelial morphology. When confluence was reached, all cells took on a polygonal morphology, the whole having a "cobblestone" appearance similar, but not identical to, that of confluent endothelial cultures. The latter cell type, on transmission electron microscopy, demonstrated surface microvilli, unlike those obtained from the saphenous vein segments (figure 11). Mesothelial cells appeared of a similar size, but with more easily perceptible cell boundaries and a more polygonal shape than endothelium. There was no contamination with other cell types in any of the mesothelial cultures, either in primary culture or subsequent passages.

The time taken for primary mesothelial cells to attain confluence ranged from 6 - 28 days. Average population doubling time was 5.8 $\pm$ 3 days (table 5).

Figures 5 & 6

Figure 5. Positive immunofluorescent staining for factor VIII antigen in cultured macrovascular endothelial cells (original magnification x 100).

Figure 6. Higher power view of factor VIII staining in endothelial cells, illustrating multiple granules dispersed over the surface of the cells (original magnification x 200).

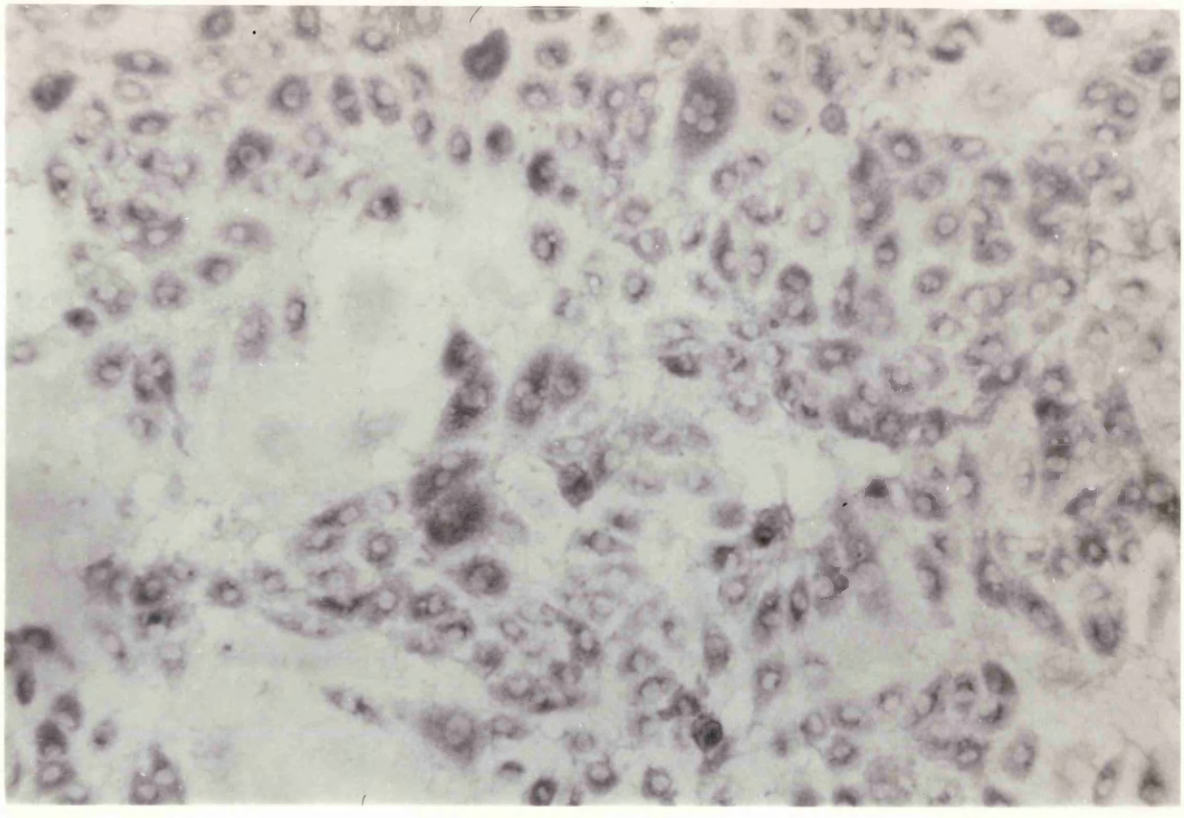


Figure 5

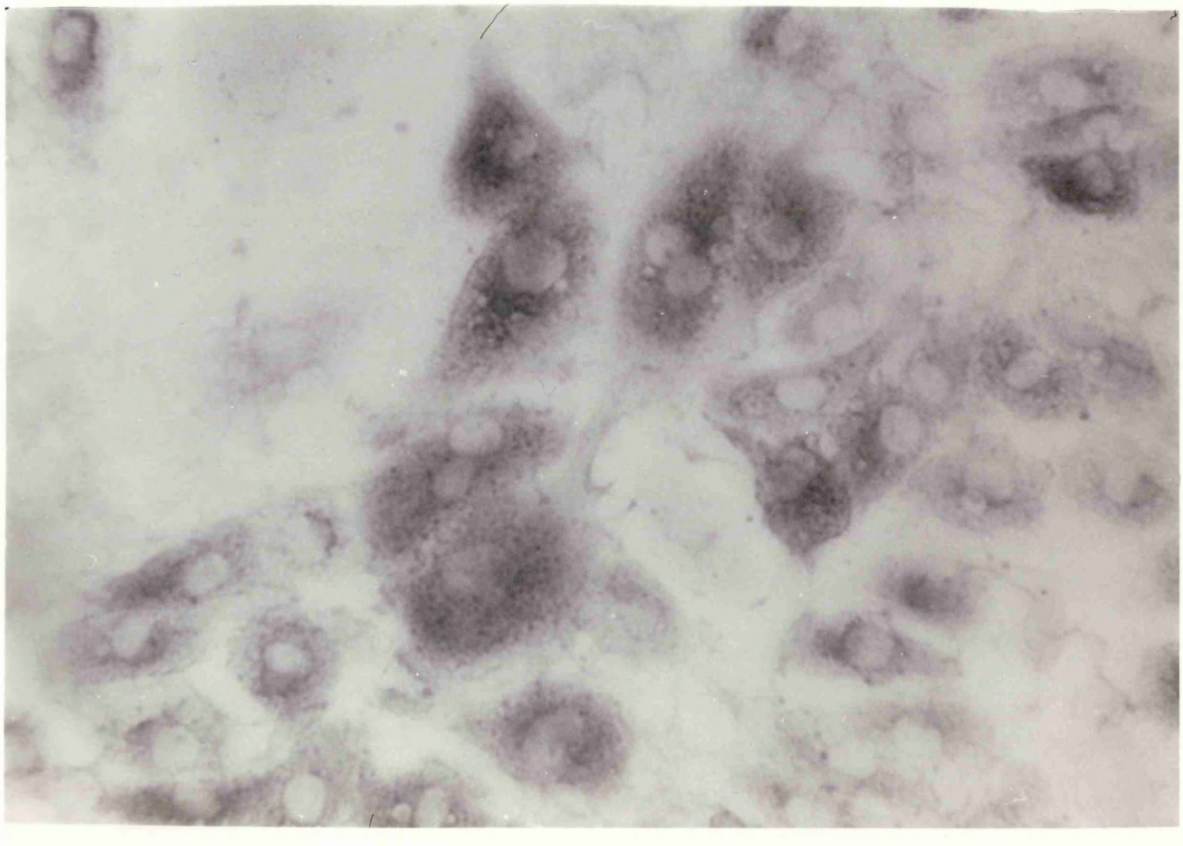


Figure 6

Figures 7 & 8

Figure 7. Confluent endothelial culture (original magnification x 100).

Figure 8 Cultured fibroblasts grown from explants of human skin (original magnification x 100).

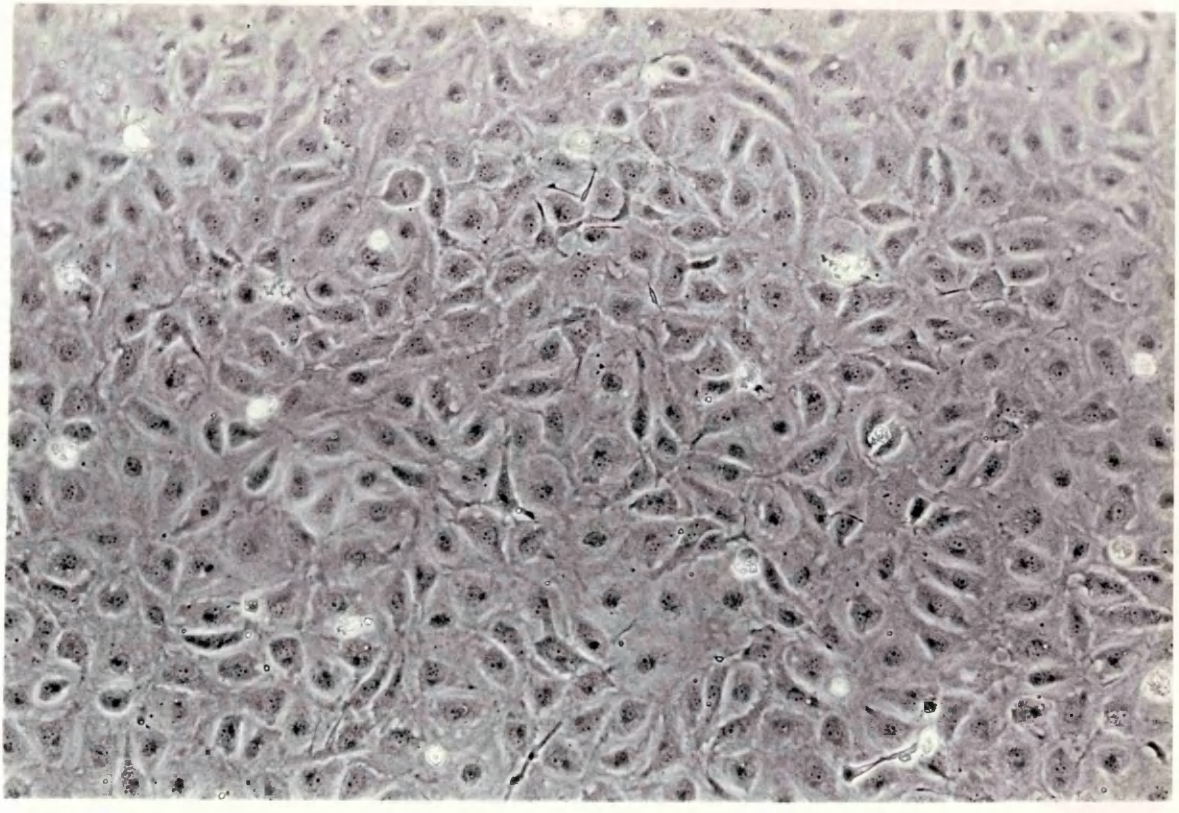


Figure 7

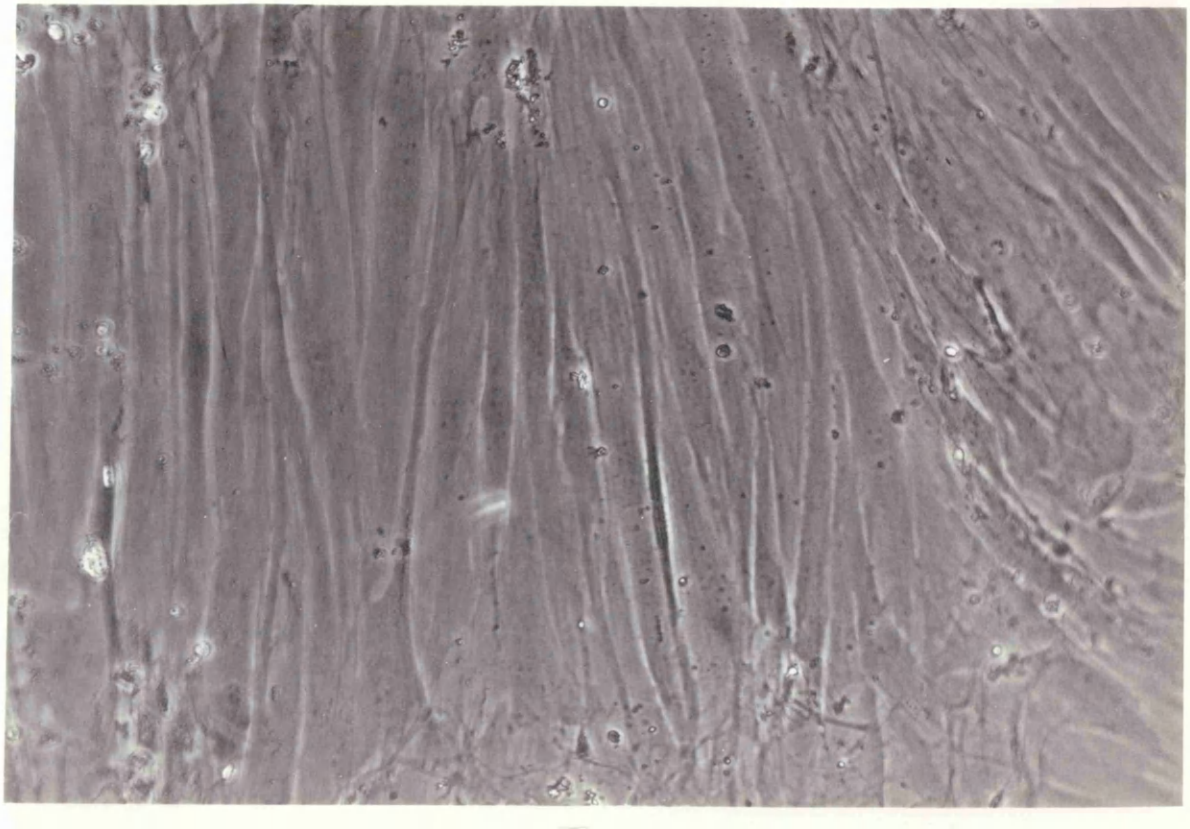


Figure 8

Figures 9 & 10

Figure 9. Subconfluent culture of mesothelial cells illustrating pleomorphic morphology (original magnification x 40).

Figure 10. Higher power view of mesothelial cells in culture (original magnification x 100).

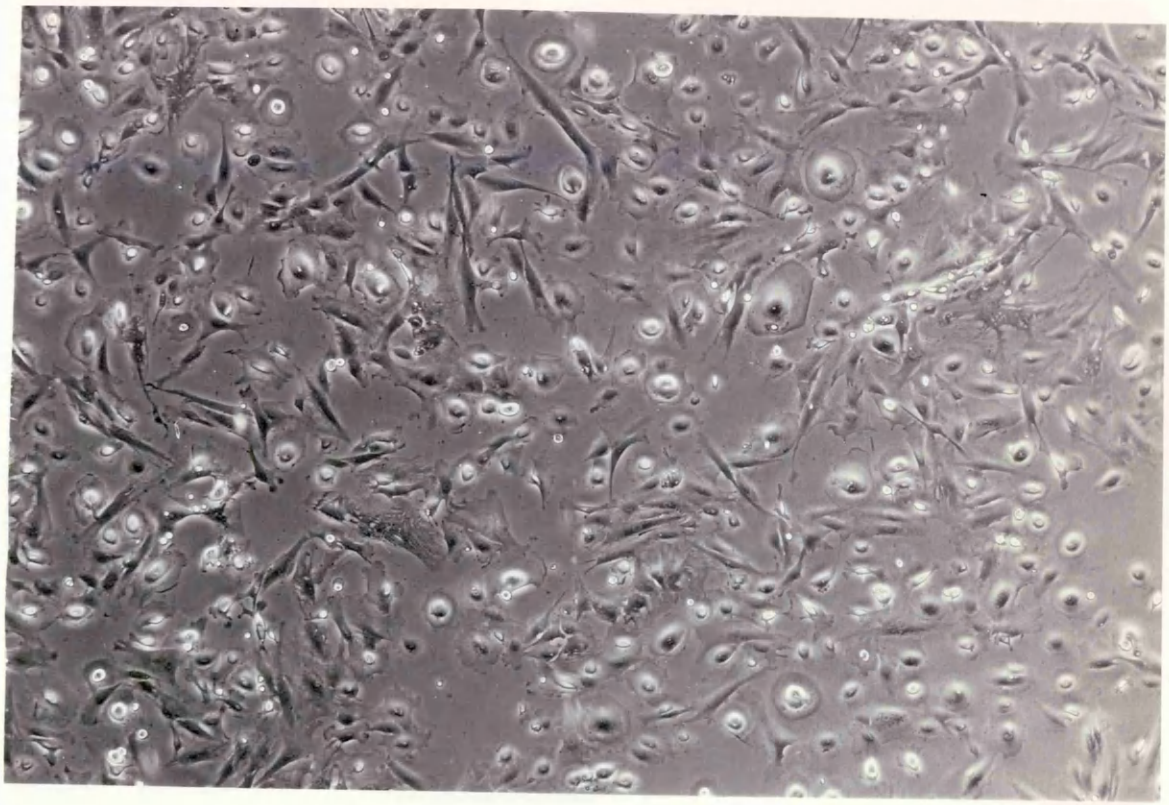


Figure 9

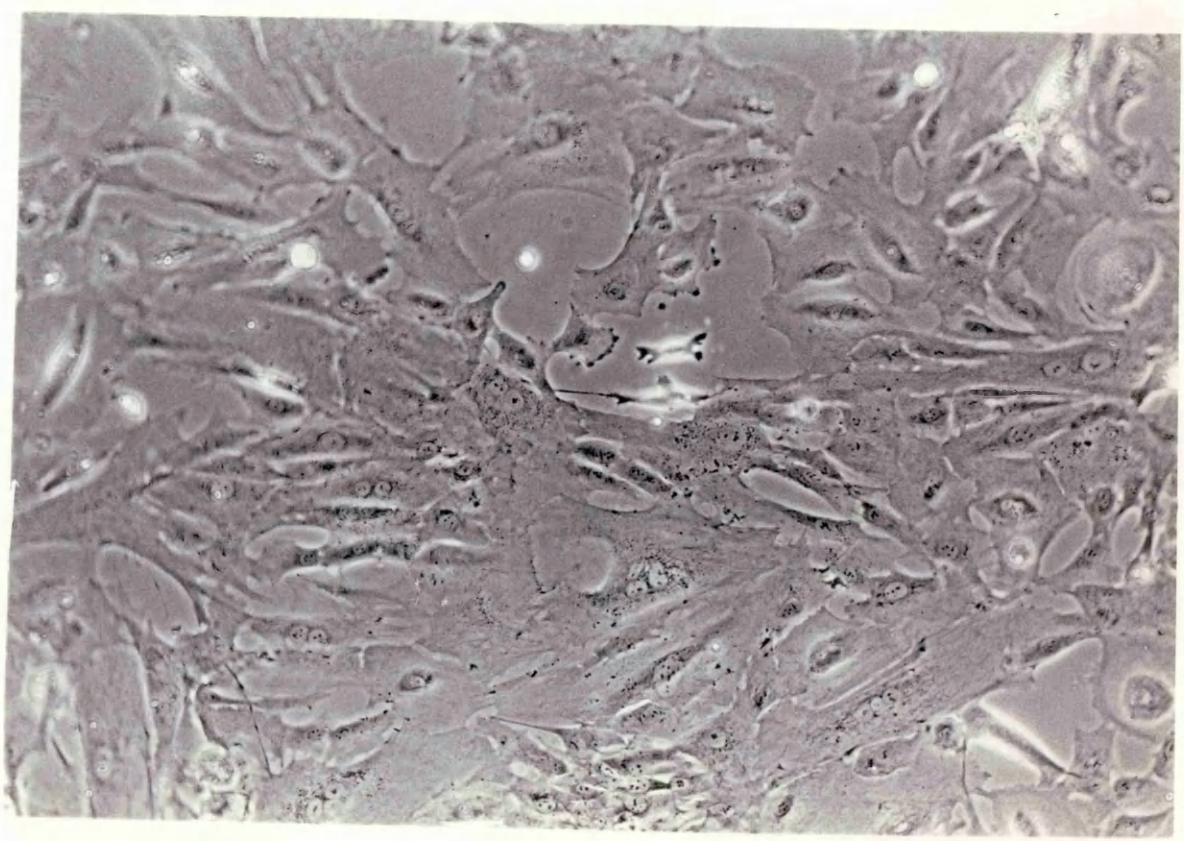


Figure 10

Figure 11

Figure 11. Transmission electron micrograph of mesothelial cell in culture, illustrating surface microvilli (original magnification x 32000).

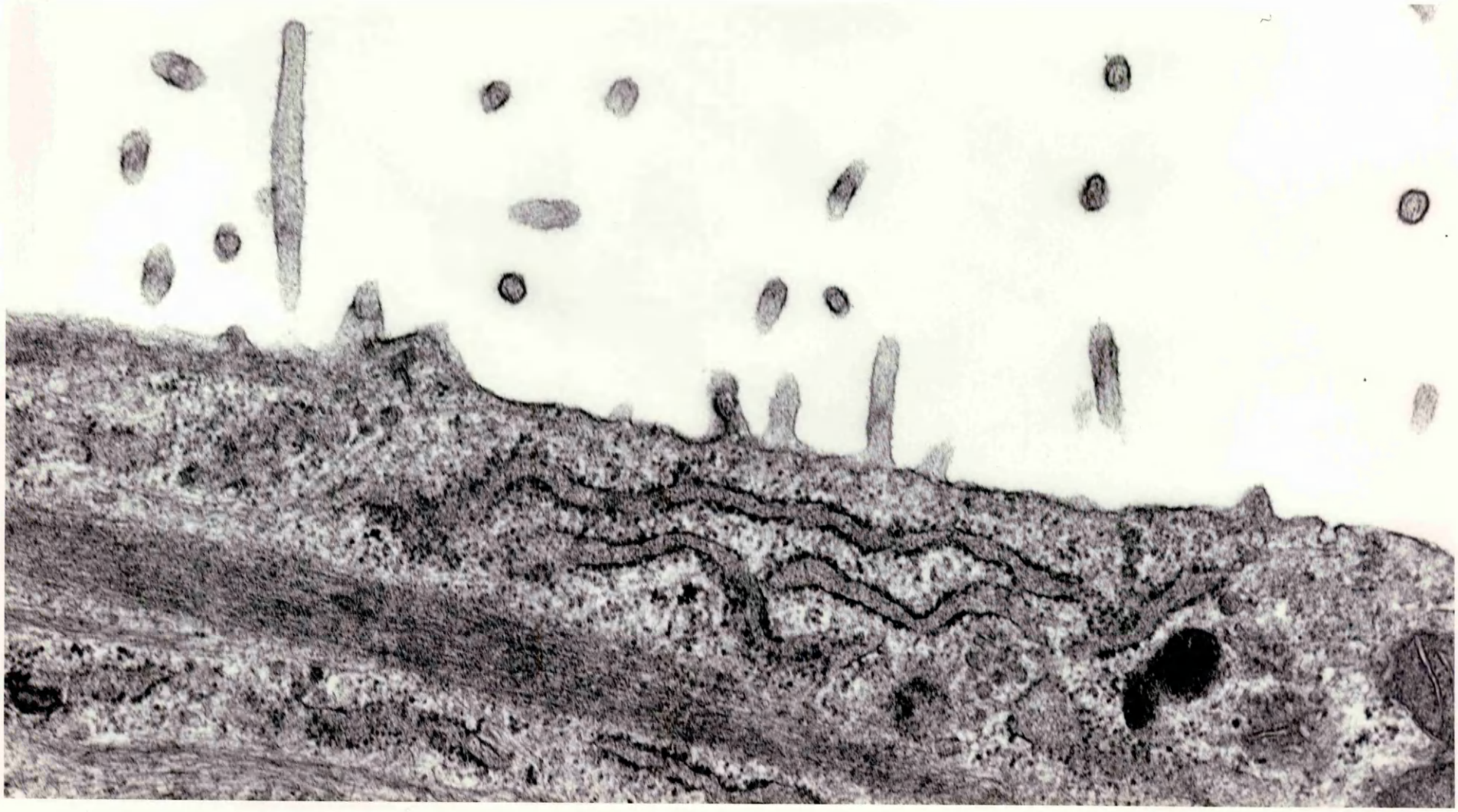


Figure 11

TABLE 5

PDN AND PDT OF PRIMARY MESOTHELIAL CULTURES

Culture	Ci ($x10^5$)	Cc ($x10^6$)	Cc/Ci	PDN	Tc (days)	PDT (days)
1.	1.1	1.3	11.8	3	21	7
2.	1.4	1.2	8.6	3	28	9
3.	4.3	2.3	5.3	2	18	9
4.	3.1	1.2	3.9	2	21	10
5.	1.9	1.0	5.3	2	17	8
6.	2.4	2.9	12.1	3	9	3
7.	1.0	1.5	15.0	4	14	3
8.	0.8	1.0	12.5	3	20	6
9.	0.8	1.6	20.0	5	13	3
10.	8.9	3.5	4.0	2	7	3
11.	2.3	2.5	10.8	3	6	2
12.	1.4	2.3	16.4	4	8	2
13.	2.3	0.8	3.5	2	21	10
14.	1.6	1.3	8.1	3	13	4
15.	1.4	1.3	9.3	3	28	9
16.	2.5	2.2	8.8	3	14	4
17.	14.7	2.1	1.4	1	5	5

Average population doubling time = 5.8 ± 3 days

Ci = initial total yield

Cc = count at confluence

PDN/T = population doubling number/time

Tc = time to reach confluence

TABLE 6

CUMULATIVE PDN FOR MESOTHELIAL CELLS

Culture No.	No. of Doublings in Primary Culture	No. of Passages	Total Doublings
1.	3	1	4
2.	3	1	4
3.	2	1	3
4.	2	1	3
5.	2	1	3
6.	3	2	6
7.	4	3	10
8.	3	2	6
9.	5	1	7
10.	2	2	5
11.	3	2	8
12.	4	5	9
13.	2	2	4
14.	3	2	5
15.	3	2	5
16.	3	1	5
17.	1	1	3

Doubling time in subsequent passages was 3 days, and estimated cell yield at confluence ranged from 0.8 - 3.5 x 10^6 (mean 1.8 $\pm$ 0.8 x 10^6).

As with endothelial cells, the proliferative life of mesothelium was limited. The maximum number of doublings obtained in each of the cultures is shown in table 6. A "learning curve" in the initial stages explains the small number of doublings achieved in the first 4 cultures. Discernable cell enlargement usually occurred after approximately 10 doublings, at which stage cell proliferation slowed down. Cells were used in the seeding experiments and adhesion assays at as early a stage as possible, usually from 2nd to 6th passage.

4.6 Substrate Variation

Both cell types were successfully subcultured on each of the substrates, with the exception of the basement membrane gel. Cells plated onto flasks coated with this attached very rapidly, but quickly assumed an elongated "stringy" morphology. Occasional cells appeared morphologically normal, and seemed to begin to form small colonies. The majority, however, remained in the former condition, and failed to multiply. The appearance was suggestive of the cells "curling up" longitudinally as if to form micro-blood vessels.

There was no difference in the growth capacity or morphology between cells plated onto the other substrates, although the process of adhesion and spreading did appear rather slower on laminin than on the other substrates.

Attachment and spreading of mesothelial cells to all substrates, and to uncoated flasks and wells, appeared slower than endothelium. The former cells maintained a rounded up appearance for 1-2 hours, whereas endothelial cells seemed well spread by 1 hour (figure 12 & 13). The culture well adherence assays confirmed the slower attachment of mesothelium (see below).

Although cell adherence assays for both cell types demonstrated reduced attachment to laminin and collagen 4 coated surfaces compared to those coated

with fibronectin, time taken to reach confluence was the same, whichever substrate was used. This suggests that, although laminin and collagen 4 are not so "sticky" as fibronectin, those cells which do attach proliferate more quickly. Complete confluence was not reached when endothelial cells were plated directly onto uncoated culture flasks, although mesothelial cells did proliferate to confluence in this situation regularly and rapidly. When endothelium was plated onto uncoated flasks, although initial attachment did occur in large numbers, detachment of large numbers occurred before confluence was reached.

Figures 12 & 13

Figure 12. First passage endothelial cells, one hour after trypsinisation and plating onto fibronectin coated flask. Cells are seen spreading and migrating from a large cluster in the lower left corner. The majority of the cells are firmly adherent and fully spread (original magnification x 40).

Figure 13. Endothelial culture in first passage 3 hours after plating, showing a more wide dispersion of the adherent cells (original magnification x 40).

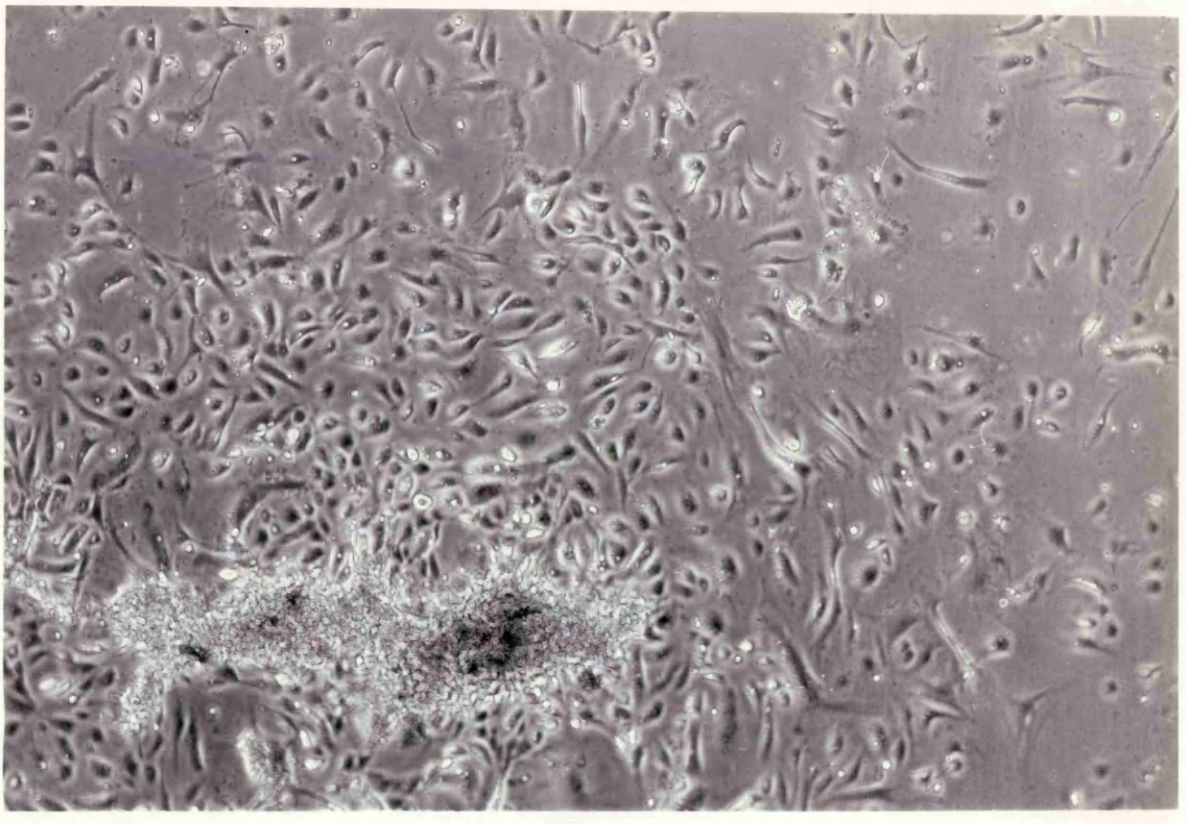


Figure 12

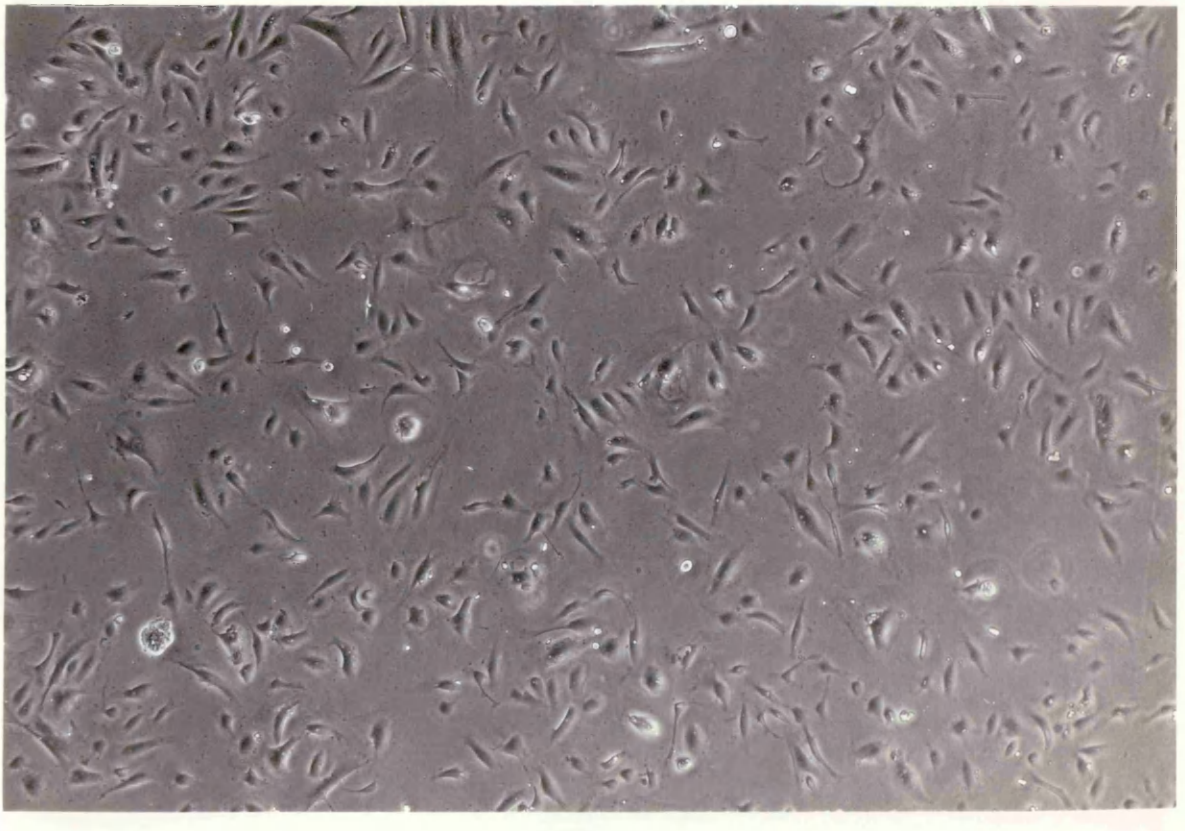


Figure 13

CHAPTER 5

STUDIES OF CELL ATTACHMENT TO VARIOUS PROTEIN COATINGS ON CULTURE WELLS

5.1 Introduction

For the seeding process to become a practical clinical concept, a time interval must be identified which is sufficient to allow firm attachment of significant cell numbers, while at the same time enabling the operation to proceed relatively unhindered. A series of experiments was therefore performed to assess how quickly cells attach to various substrates.

As an indication of the relative abilities of these substrates to enhance attachment of cells to the already favourable surface of tissue culture plastic, the experiments were carried out first of all using multi-well tissue culture plates. Each plate contained 24 wells, 2 cm^2 in diameter. For each assay, 4 wells were coated with each substrate, and four were left uncoated to act as controls (figure 14).

5.2 Coating of wells with substrate

Wells were coated with substrate as follows:- 0.5 ml of the substrate solution was pipetted into the well. This was agitated gently to ensure complete floor coverage. The wells were incubated for 1 hour at 37^{o}C, after which excess protein solution was removed. The substrate was allowed to dry onto the plastic by further incubation at 37^{o}C. Makarak and Howard (1983) found that drying substrate proteins onto tissue culture surfaces in this way resulted in a good coating, capable of supporting cell attachment and growth, even in the absence of serum. Unbound protein was removed by washing with DPBS. Substrates were originally supplied in the following concentrations:-

Fibronectin	0.1 mg/ml
Laminin	1 mg/ml
Collagen	0.5 mg/ml

To obtain a fair comparison, the laminin and collagen were diluted to 0.1 mg/ml prior to use.

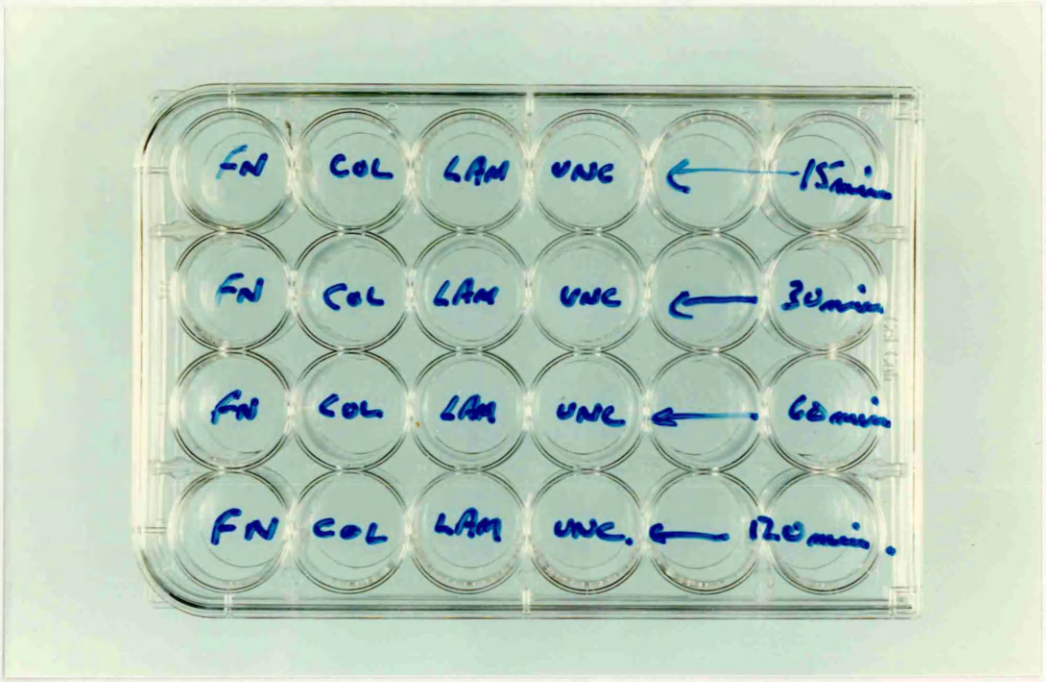


Figure 14. Multiwell plate used for estimating attachment rates to different protein substrates. Confluent cells were trypsinised, suspended in complete medium and pipetted into the wells at subconfluent densities. At the time intervals indicated, overlying medium in each well was removed and the well floor washed twice with HBSS. Medium and washings were pooled and a cell count performed. After carrying out this procedure for the 120 minute wells, all wells were trypsinised and the trypsinised cells counted. Attachment was expressed as a percentage of total cells present.

5.3 Measuring the rate of attachment

The surface area of each well floor was 2 cm^2. As cells for each assay were taken from one 25 cm^2 flask containing, on average, 2 x 10^6 cells, the approximate number of cells seeded into each well was 1.25 x 10^5, giving a seeding density of 6.25 x 10^4 per cm^2.

1 ml of freshly trypsinised cells was pipetted into each well, and the plate incubated at 37°C. At 15, 30, 60 and 120 minutes, the overlying medium from one well coated with each substrate was removed and the well washed with DPBS. The medium and the DPBS were collected and a cell count performed. Fresh medium was added to the well, and incubation continued.

Having counted the non-adherent cells up to the 120 minute time period, all the wells were trypsinised with 0.25 ml trypsin/EDTA, and the cells in each well counted. The proportion of cells adhering in each well was obtained using the formula $C_a = C_t/(C_t + C_{m+w})$, where C_a represents the proportion of initially plated cells adhering, C_t the number of cells released by trypsinisation, and C_{m+w} the number of non-adherent cells in the medium and wash prior to trypsinisation.

To improve the accuracy of the cell counts, the cell suspensions from each of the washes and trypsinisations were centrifuged for 5 minutes at 1000 rpm and resuspended in a very small volume (0.04 ml) of DPBS.

The results enabled a graph to be constructed, illustrating the cell adherence rates for the different substrates over a 2 hour period. These studies were carried out three times for both endothelial and mesothelial cells, and an average obtained.

To avoid any possibility of different media affecting the results, complete endothelial medium with 20% foetal calf serum was used in all the attachment assays.

5.4 Statistical analysis of results

The result obtained for each time period was compared, for each substrate coating, with that obtained for the equivalent uncoated well. The comparison was performed using paired Student's t tests. Each result obtained using endothelial cells was compared to the equivalent value using mesothelial cells, again using separate Student's t tests.

Differences between the various substrate coated wells were analysed using a one way analysis of variance.

5.5 Results

The results together with the statistical analysis of the differences in attachment rates between the uncoated and coated culture wells are given in figures 15 and 16 and Appendix 1. These studies revealed a significant increase in cell attachment to wells coated with fibronectin compared to uncoated wells. This applied to both cell types as far as the 60 minute time interval. At 120 minutes, although endothelial attachment was still better on the fibronectin coated wells, the difference was not significant. Attachment rates to type 4 collagen coated wells were equivalent to those to the uncoated surface throughout. Laminin appeared to reduce the rate of attachment, although only in the case of endothelial cells at the 60 and 120 minute time intervals, did this reach statistical significance. In the case of fibronectin, 86% and 58% of the total endothelial and mesothelial cell counts respectively adhered within one hour of plating.

The general pattern was for endothelium to attach more rapidly than mesothelium. This difference was significant for all time intervals in the case of fibronectin coated wells, but only in the later intervals in the case of the other substrate coated and uncoated wells.

Figures 15 and 16 illustrate the attachment curves obtained for the different substrates over the 120 minute period. Each point represents the average of three experiments. These graphs demonstrate that for all substrates other than laminin, the steepest part of the curve is within the first 60 minutes. For this reason, in all the subsequent studies 1 hour's incubation was allowed for cell attachment. This was considered the optimum time interval, allowing large numbers of cells to attach, while also being the maximum time allowable for attachment to occur in a clinical context.

Figure 15. Time course of attachment of macrovascular endothelial cells to uncoated and substrate coated culture wells.

Key

- ● Fibronectin coated
- ■ Collagen type 4 coated
- × Laminin coated
- ▲ Uncoated

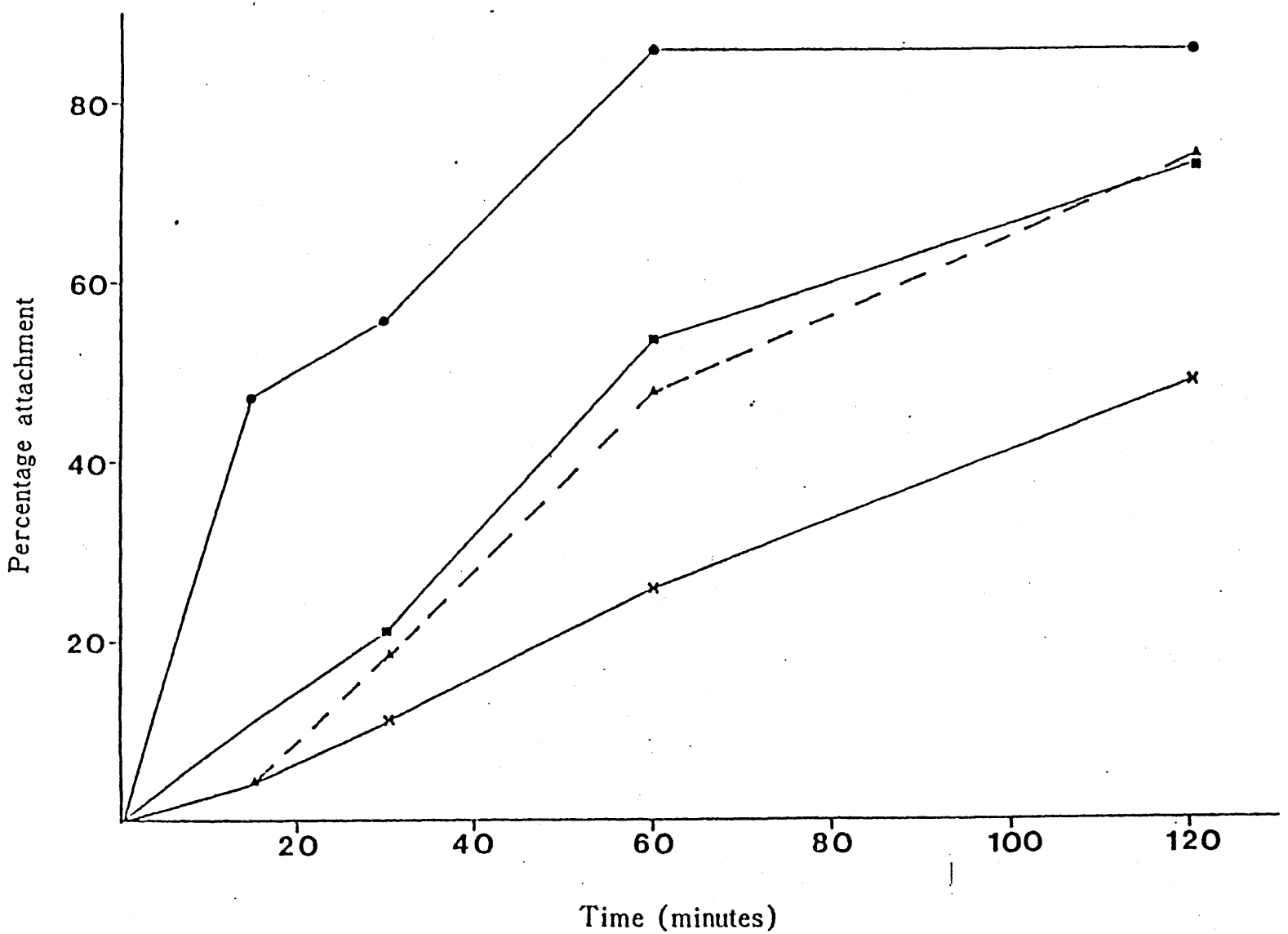
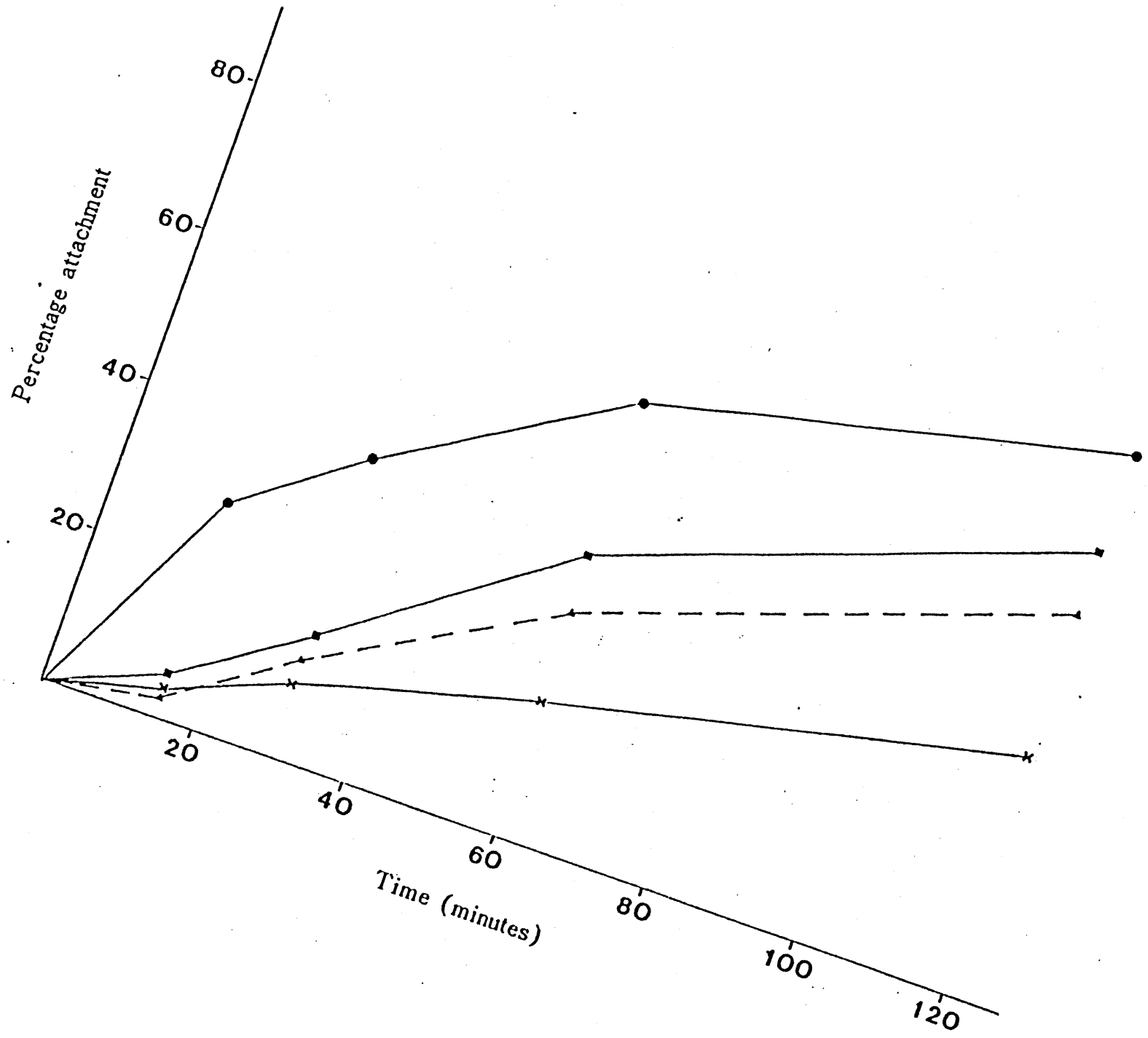

Percentage attachment
80
60
40
20
20
40
60
80
100
120
Time (minutes)

Figure 16. Time course of attachment of omentally derived mesothelial cells to uncoated and substrate coated culture wells.

Key

- ● Fibronectin coated
- ■ Collagen type 4 coated
- × Laminin coated
- ▲ Uncoated


Percentage attachment
80
60
40
20
20
40
60
80
100
120
Time (minutes)

CHAPTER 6

STUDIES OF CELL ATTACHMENT TO UNTREATED AND TREATED ePTFE GRAFT MATERIAL

6.1 Methods

Having identified the minimum time period required for reasonable cell attachment to ordinary tissue culture plastic, a comparison was now made of cell adherence to ePTFE graft material using the same substrate proteins. Standard thin-walled 30 micron ePTFE taken from 60 cm by 6mm ID grafts was used for all the experiments.

Two types of experiment were performed :-

1. Measurement of adherence to small discs of ePTFE immobilised in "seeding chambers" fashioned from Eppendorf plastic reaction vials.

2. Measurement of adherence to tubular ePTFE graft segments, using indium-111-oxine labelling as a measurement of cell attachment.

a. Seeding Chambers

Small cylindrical chambers, 2 cm in length by 1 cm internal diameter, were made by cutting off the conical end of 1.5 ml Eppendorf plastic reaction vials (Sarstedt Laboratory Wares, West Germany). The cap was removed and re-applied over a piece of ePTFE just large enough to stretch across the diameter of the vial. The cap, when fully closed, held the ePTFE firmly in place, this now making up the floor of the chamber (figure 17). In this way, a disc of the material 1 cm in diameter was immobilised, and available for study.

The ePTFE in the chambers was pre-treated with substrate by incubation and drying as described above for the culture wells. Pre-clotted ePTFE was obtained from graft segments pre-clotted as described in section 6.1.b.

Cells were incubated for 1 hour on each substrate, following which overlying medium was removed, the surface washed twice with 0.4 ml DPBS and the cells in medium and wash pooled and counted. The attached cells were then trypsinised with 0.2 ml trypsene/versene and counted. Percentage adherence was

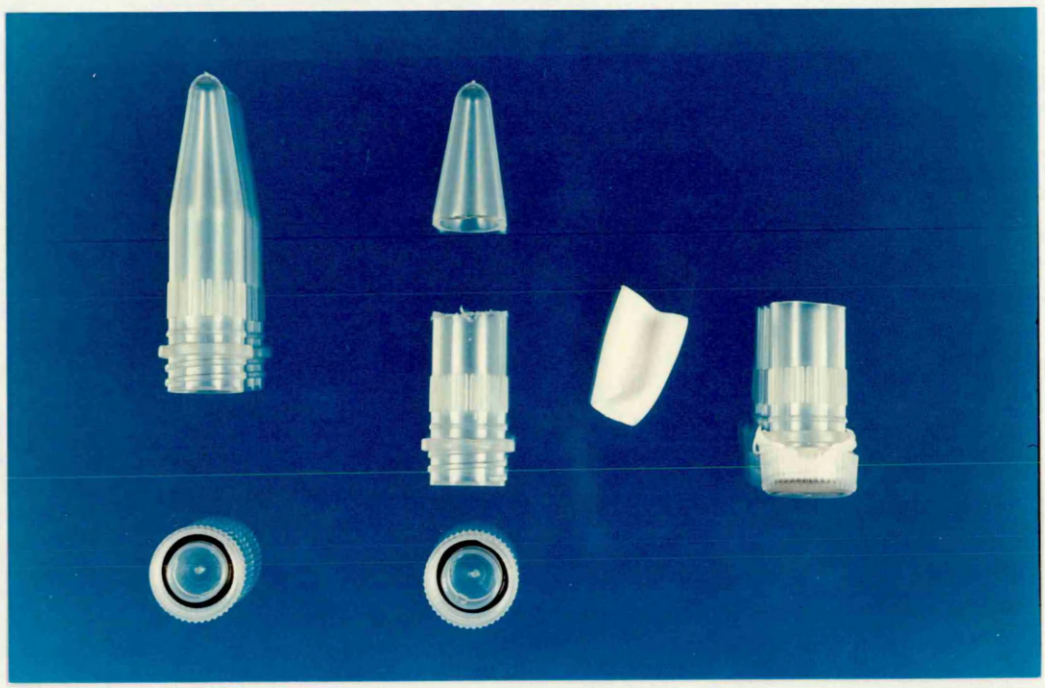


Figure 17. "Seeding chambers" used for assessing adherence of cells to ePTFE. The cap was removed from an Eppendorf plastic reaction vial (a). The conical end was removed with a scalpel blade (b). A piece of ePTFE (c) was inserted between the cap and the body of the vial (d), the ePTFE thus forming the floor of the chamber.

calculated in the same way as described above for the culture well experiments. As the surface area of the ePTFE on the floor of each chamber was small (0.79 cm^2), confluent cells from one flask were used to seed 10 chambers. In this way, ten adhesion assays could be performed at one time, the approximate seeding density in each chamber being $2 \times 10^6/10 = 2.0 \times 10^5$ per chamber (2.53×10^5 per cm^2 of ePTFE). To increase the cell concentration for counting, and thereby improve the accuracy of the counts, all cell suspensions from washes and from trypsinisation were spun down and suspended in a very small volume of medium (0.04 ml) prior to counting. The number of cells in ten 1 mm^2 squares of the counting chamber was always obtained, and this multiplied by 40 to give the count in the total 0.04 ml volume (40 mm^3).

Some discs were sent for examination by light and scanning electron microscopy. Five specimens of each ePTFE preparation (fibronectin coated, collagen type 4 coated, laminin coated, pre-clotted, uncoated) were examined 1 hour after seeding. The five specimens in each substrate group were seeded at different cell densities, ranging from $2.53 \times 10^5/cm^2$ to $6.19 \times 10^5/cm^2$ to assess whether high density seeding may enable the development of a completely confluent monolayer on the surface.

b. Graft Segments

6 cm graft segments were coated with substrate using the method described by Kesler et al (Kesler et al 1986). The segments were clamped at one end, filled with the relevant substrate solution, and clamped at the other end. They were then incubated at 37°C for one hour. Excess substrate was allowed to drain out, the remainder being allowed to dry onto the graft surface. The interior of the graft was washed with culture medium by flushing this through the lumen. The grafts were now considered ready for inoculation with cell suspension.

"Pre-wetting" of Grafts

Cell adherence to ePTFE may be limited by the inherent hydophobicity of the surface rather than the actual chemical structure of the polymer. To assess whether abolishing this property may improve cell adherence, some ePTFE discs and graft segments were pre-treated by immersion in absolute alcohol for one or two minutes. Thereafter they were thoroughly washed with serum free culture medium to remove the alcohol, before being inoculated in the usual way. McCall and colleagues have previously shown that pig aortic endothelial cells will adhere to and proliferate on ePTFE treated in this way (McCall et al 1981).

Pre-clotting of grafts

Rather than adding cells to the pre-clot blood before introducing the blood into the graft, the following procedure was adopted :

The graft segment was placed in a sterile 13 ml test tube, which was filled with 10 ml whole blood. This was left to clot for 2-3 hours.

The graft segment was removed and placed in a sterile test tube containing Dulbecco's phosphate buffered saline (DPBS). Blood clot was broken up by vigorous shaking of the tube. The graft was washed and rinsed several times with fresh DPBS until macroscopically free of red blood clot. This treatment facilitated scanning electron microscopy by removing to a large extent the cellular elements trapped within the pre-clot matrix.

Following this procedure, cells were seeded into the lumen as for the other graft preparations.

Inoculation of Graft Segments

Inoculation was performed by running a recently trypsinised suspension of cells into the lumen of the graft segment, one end being occluded with a "bulldog" vascular clamp. When the segment was filled, the free end was also clamped, the

whole then being placed in an incubator at 37^{o}C. Incubation was for a period of 1 hour. During this time, the segment was turned through 90^{o} at 15 minute intervals to ensure an even distribution of cells.

Assessment of cellular attachment to graft segments

Cell adherence was examined quantitatively by indium-111-oxine labelling, and qualitatively by light and scanning electron microscopy.

Sharefkin demonstrated that adult human endothelial cells could be easily and rapidly labelled with this isotope, with no discernible effect on the cells' subsequent ability to adhere to a prosthetic surface (Sharefkin et al 1983). These authors suggested that this may be a reliable way of quantitating cellular adherence to prosthetic surfaces.

Cell labelling technique

Radioactivity of all specimens was measured using a well collimated 4 cm sodium iodide detector linked to a dual channel spectrometer. The distance between detector and specimen was kept constant at 26 cm.

Newly trypsinised cells were suspended in 5 ml serum free medium E199 and the cell suspension immediately divided into two equal volumes, for seeding into two graft segments (approximately 1 x 10^{6} cells per graft segment). 5 MBq Indium-111-oxine was added to each supension, which were then incubated for 10 minutes at 22^{o}C. Activity of the labelled cell suspensions was measured in counts per minute.

After centrifugation at 1000 rpm for 5 minutes at 4^{o}C, the supernatant was separated from each of the cell pellets, and the pellets resuspended in 1.1ml fresh medium, containing serum. The activity in the new cell suspensions and the supernatant fractions was measured. Labelling efficiency was estimated by dividing the activity of the resuspended cell pellet by the total activity present.

Calculation of cell retention

0.1 ml of the freshly suspended and labelled cell suspension was withdrawn to estimate spontaneous leakage of activity from the cells. The remainder was seeded into the graft segment as described above. Initial total counts were measured prior to incubating the graft segment.

Following incubation for 1 hour, contained medium and unattached cells were drained, the lumen of the graft gently flushed with culture medium, and activity in both graft and washings measured.

To compensate the above results for spontaneous leakage of activity from the cells during the period of incubation, the small aliquot (0.1 ml) of labelled cells removed prior to inoculation of the graft was incubated in a test tube in the same incubator as the graft, for the same time interval. Spontaneous leakage was estimated by dividing counts in supernatant, after centrifugation at 1000 rpm for 5 minutes, by the initial counts of the original aliquot. This result was then expressed as a percentage leak over the appropriate time interval.

Knowing the rate of leakage from cells into medium, it was possible to calculate the total number of counts, at the end of the incubation period, which should be attributable to indium contained within cells. After spinning down the effluent from the post-incubation specimen, the cellular counts in this were measured. This was then subtracted from the total expected post-incubation cellular counts, to give an estimate of cellular counts remaining in the graft. This, taken as a percentage of the total cellular counts, gave an estimate of the percentage of seeded cells remaining attached to the graft segment. Appendix 3 illustrates the calculations for each experiment.

This experiment was repeated five times for each of the following substrates:-

1. Uncoated PTFE

2. Fibronectin coated PTFE

3. Laminin coated PTFE

4. Collagen type 4 coated PTFE

5. PTFE prepared by "pre-clotting" the graft segment using the method described above.

The experiments were carried out using both endothelial and mesothelial cells.

Preparation of specimens for scanning electron microscopy

Specimens were immersed in liquid nitrogen slush and transferred onto specimen holders in liquid nitrogen. Specimen holders were then transferred to an Edwards EPDZ tissue dryer at -70^{o}C. The vacuum and temperature were maintained overnight in the presence of P_2O_5. Specimens were warmed up to 30^{o}C and removed from the freezer dryer.

Specimens were fixed on the specimen holders with silver Dag (Agar Scientific). Once the dag was dry, specimens were coated with Gold in an Edwards S150 sputter coater.

6.2 Statistical Analysis of Results

Differences in one hour attachment rates between uncoated and substrate coated ePTFE (including pre-clotted ePTFE) were compared using Student's t tests for paired data.

Differences between the various substrate coated specimens were analysed using a one way analysis of variance.

6.3 Results - Seeding Chambers

Ten adhesion studies were carried out for each substrate preparation using endothelium, and ten using mesothelium. To assess whether abolishing the

hydrophobicity of the ePTFE may enhance substrate binding and therefore improve cell adherence, the experiments were repeated after immersing the ePTFE discs in alcohol prior to incubation with substrate. These assays were also repeated ten times for each cell type. For comparison, equal numbers of pre-clotted and untreated discs were also seeded, and adhesion quantified. The percentage attachments obtained are illustrated in figure 18. The actual results, together with the data from which these percentages were calculated and the statistical analysis thereof, are tabulated in appendix 2.

a. Endothelial Cells

All substrate coatings greatly improved the one hour cellular attachment as compared to the uncoated controls ($p<0.001$ in each case). There was, however, no significant difference between the different substrates ($F = 1.0703$, one way analysis of variance).

The wide ranges and standard deviations obtained for the matrix protein - coated specimens demonstrate a high variability in attachment rates to ePTFE coated with these substrates. This was particularly apparent for endothelial cells in the case of fibronectin coated graft material. Least variation was observed in those ePTFE discs which were pre-clotted prior to seeding.

Pre-treatment of the graft material with absolute alcohol prior to substrate coating failed to improve attachment efficiency, although when this treatment was applied to uncoated ePTFE, cell retention did improve significantly ($p<0.001$) (figure 19).

b. Mesothelial Cells

Mesothelial cells attached most readily to fibronectin coated graft material, with an average of 54% of cells attaching after 1 hour's incubation (figure 18). This was significantly better than the attachment to collagen type 4

($p<0.01$) or laminin ($p<0.001$) coated specimens, although each of these conferred a benefit over the uncoated material ($p<0.001$). Attachment of mesothelial cells to pre-clotted ePTFE was poor, with an average of only 19.9% after one hour. This was not significantly different from untreated specimens. Pre-treatment of the ePTFE with alcohol did not improve attachment rates of mesothelial cells to pre-coated or uncoated graft material (figure 20).

6.4 Results - Indium Labelling Experiments

Average labelling efficiency for endothelial cells was 37% $\pm$ 14%, with a range of 14% to 63%, while that for mesothelium was 52% $\pm$ 19%, with a range of 13% to 75%. Spontaneous leakage rates of isotope from the endothelial cells after 1 hour's incubation at 37^{o}C averaged 38% $\pm$ 15%, while those of mesothelial cells averaged 32% $\pm$ 13%.

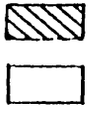
Estimated adherence rates for the different graft preparations were as indicated in figure 21. Again, pre-treatment of the graft substantially improved uptake of both cell types. Although endothelial attachment was improved by all substrate coatings, the result for the laminin coated graft failed to reach statistical significance. Fibronectin coating, and mesothelial seeding gave the best seeding efficiency. Pre-clot was the graft preparation which gave the least variability in results.

There was no difference between the substrates with regard to their ability to potentiate endothelial attachment, but for mesothelium, fibronectin was significantly better than the others ($p<0.001$), and pre-clot was significantly inferior ($p<0.01$).

Figure 18 Adhesion of endothelial and mesothelial cells to substrate coated, preclotted and uncoated ePTFE after 1 hour.

Key

FN	fibronectin coated ePTFE
Col	collagen type 4 coated ePTFE
Lam	laminin coated ePTFE
UC	uncoated ePTFE
PC	ePTFE preclotted with whole blood



endothelial cells

mesothelial cells

Results are expressed as the percentage of initially seeded cells which attach, ± SEM.

P values refer to comparison with the uncoated surface

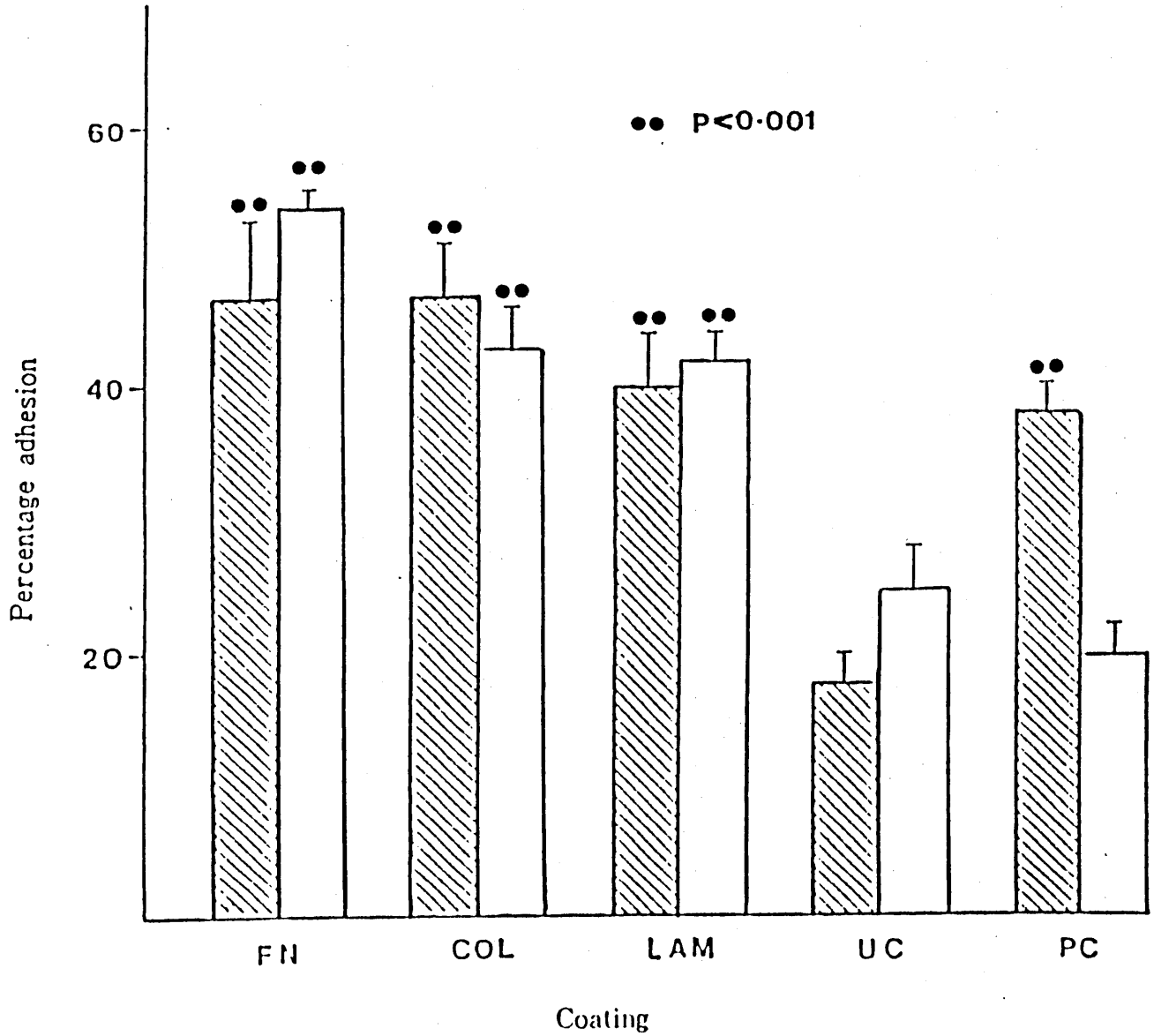

●● P<0·001
60
40
20
Percentage adhesion
FN
COL
LAM
UC
PC
Coating

Figure 19 Effect of "pre-wetting" on endothelial attachment to ePTFE

Key

FN	fibronectin coated ePTFE
Col	collagen type 4 coated ePTFE
Lam	laminin coated ePTFE
UC	uncoated ePTFE
PC	ePTFE preclotted with whole blood


pre-wetted ePTFE


non-wetted ePTFE

Results are expressed as the percentage of initially seeded cells which attach, ± SEM.

P values refer to comparison with the uncoated surface

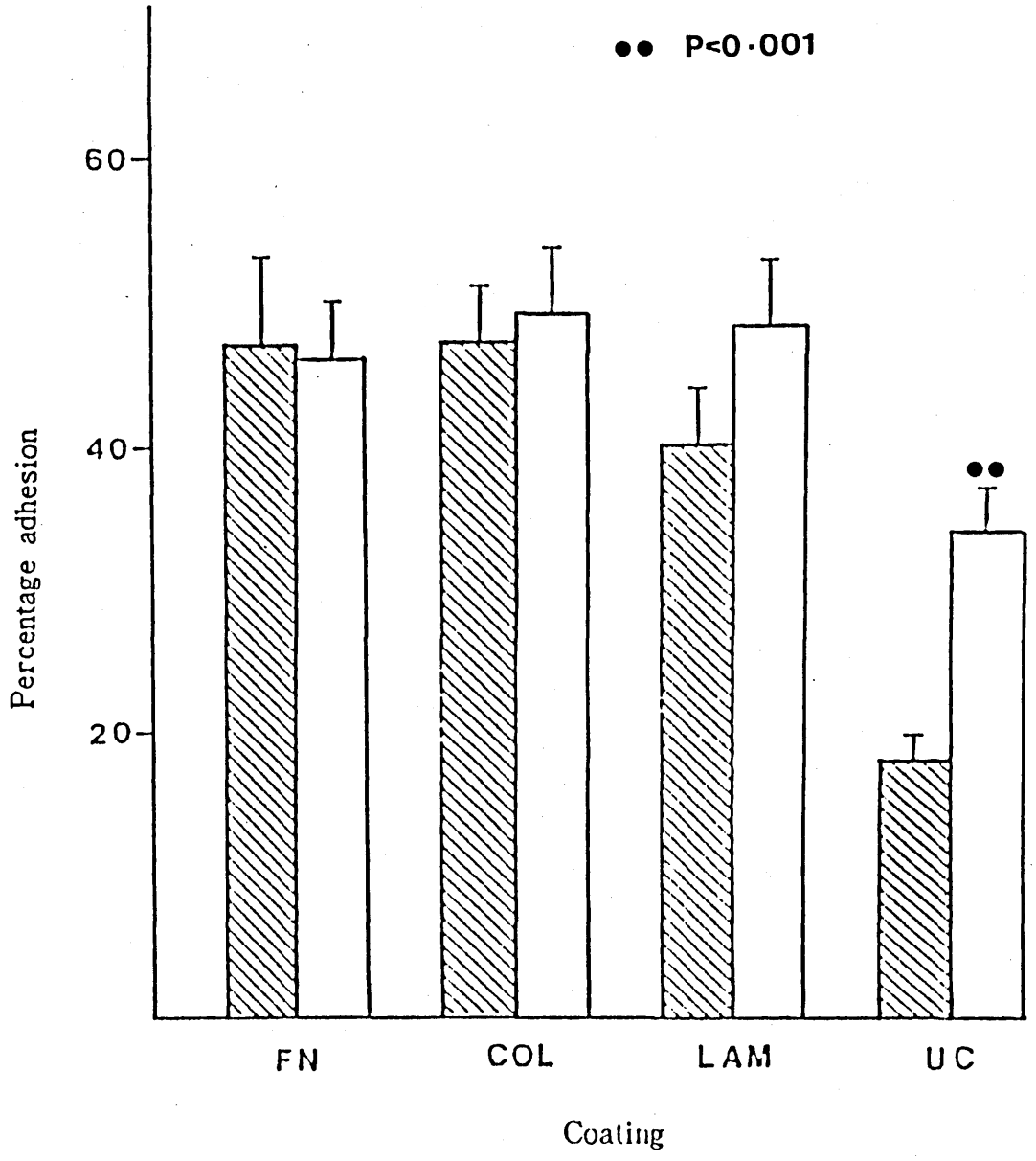

●● P<0·001
60
40
20
Percentage adhesion
FN
COL
LAM
UC
Coating

Figure 20 Effect of "pre-wetting" on mesothelial attachment to ePTFE

Key

FN	fibronectin coated ePTFE
Col	collagen type 4 coated ePTFE
Lam	laminin coated ePTFE
UC	uncoated ePTFE
PC	ePTFE preclotted with whole blood


pre-wetted ePTFE


non-wetted ePTFE

Results are expressed as the percentage of initially seeded cells which attach, ± SEM.

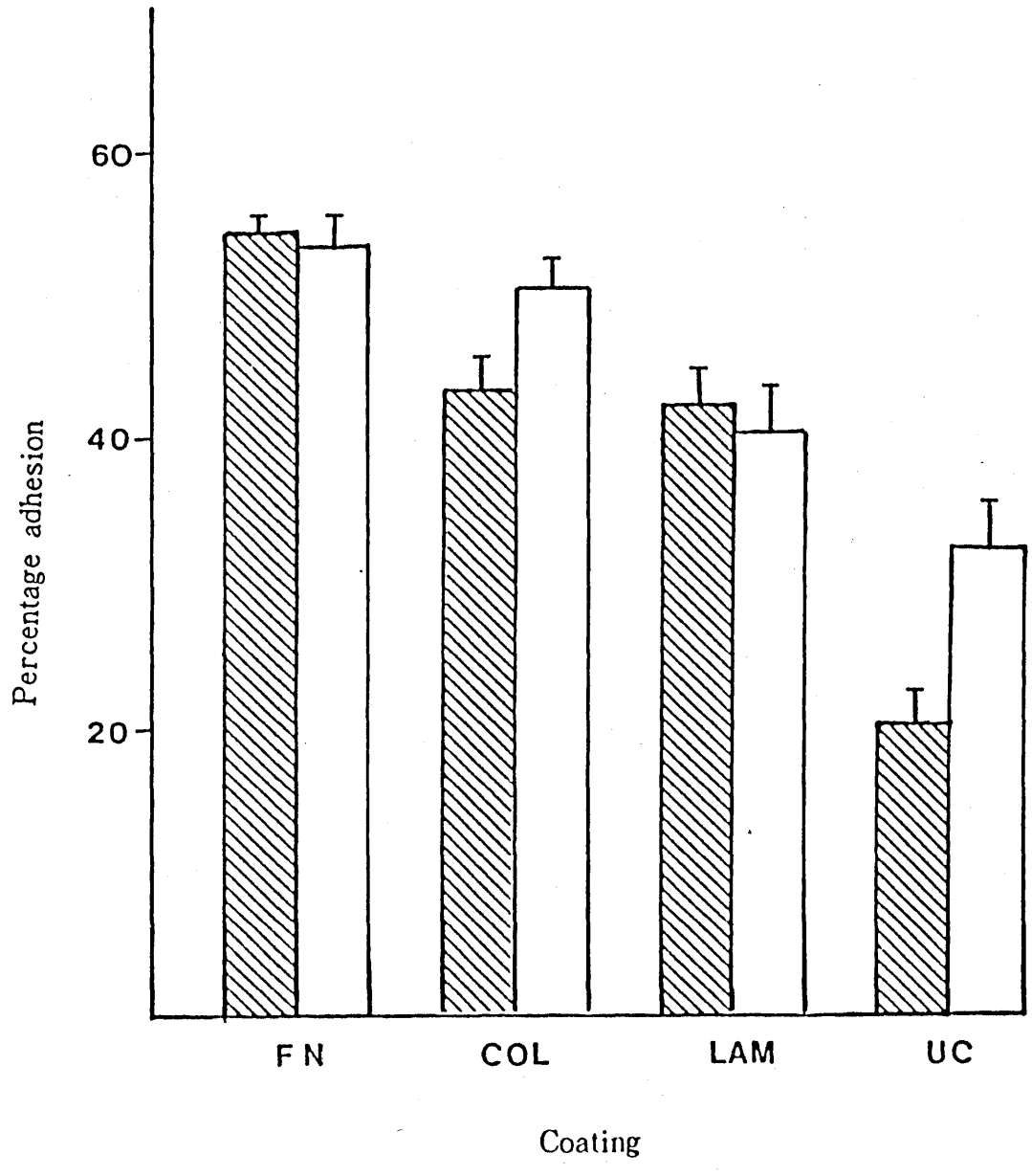
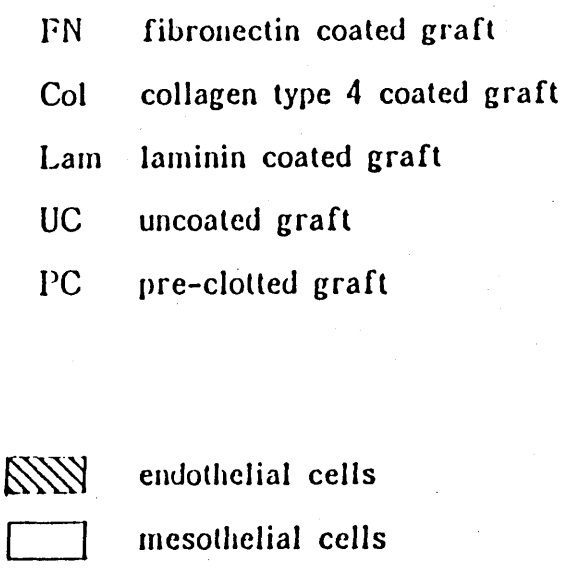

60
40
20
Percentage adhesion
FN
COL
LAM
UC
Coating

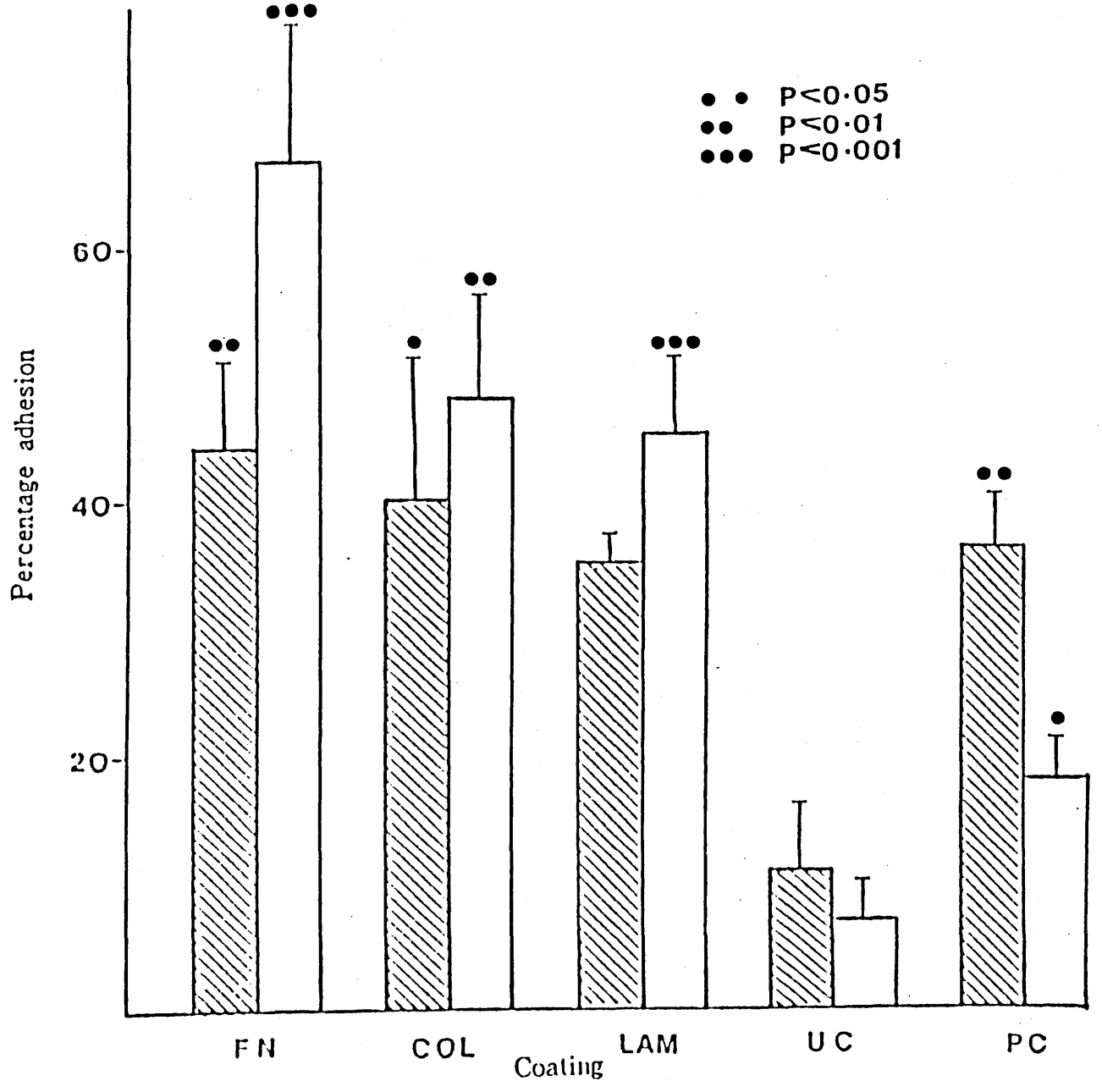
Figure 21

Figure 21 Adhesion of endothelial and mesothelial cells to coated, pre-clotted and uncoated graft segments as measured by indium-111-oxine labelling of cells



Results are expressed as the percentage of initially seeded cells which attach, ± SEM.

P values refer to comparison with the uncoated surface


P≤0·05
P≤0·01
P≤0·001
Percentage adhesion
60
40
20
FN
COL
LAM
UC
PC
Coating

CHAPTER 7

LIGHT AND SCANNING ELECTRON MICROSCOPY STUDIES

7.1 Light microscopy of seeded ePTFE

All specimens examined by light and scanning electron microscopy had been incubated with cells for one hour only.

Ten specimens of each ePTFE preparation (fibronectin, laminin and type 4 collagen coated, pre-clotted and uncoated) were examined, and the features illustrated in the photomicrographs were consistent in all cases. Histological examination of pre-treated (pre-coated or pre-clotted) ePTFE seeded with endothelial cells revealed the presence of sheets of confluent flattened cells adhering to the surface (figure 22 & 23). In some pre-coated graft segments sheets of cells extended round as much as 2/3 of the graft circumference, although in no case was complete confluence achieved round the whole internal surface.

Cells seeded onto untreated ePTFE failed to attach, there being only occasional rounded up cells visible on the surface.

Examination of specimens seeded with mesothelial cells demonstrated many rounded up, plump-looking cells, with relatively few showing signs of flattening and spreading (figure 24).

7.2 Scanning Electron Microscopy of Seeded ePTFE

This examination was carried out on ten specimens coated with each protein substrate, ten pre-clotted with whole blood and ten uncoated. The features illustrated were typical of each specimen examined.

Scanning electron microscopy of ePTFE incubated with endothelial cell suspensions for 1 hour revealed large confluent areas of flattened cells covering a large percentage of the surface (figures 25, 26, 27). In areas not covered with cells the nodes and fibrils of exposed ePTFE were visible, with numerous rounded up cells. High power views enabled visualisation of the attached cells in more detail, their nuclear elevations being clearly seen, together with cell junctions and

Figures 22a & 22b

Figure 22a. Light micrograph of ePTFE graft segment pre-treated with type 4 collagen, seeded with macrovascular endothelial cells, and allowed to incubate for 1 hour. Flattened cells are seen lining the graft (original magnification x 40).

Figure 22b. High power view of endothelial cells lining a collagen-coated ePTFE graft (original magnification x 100)

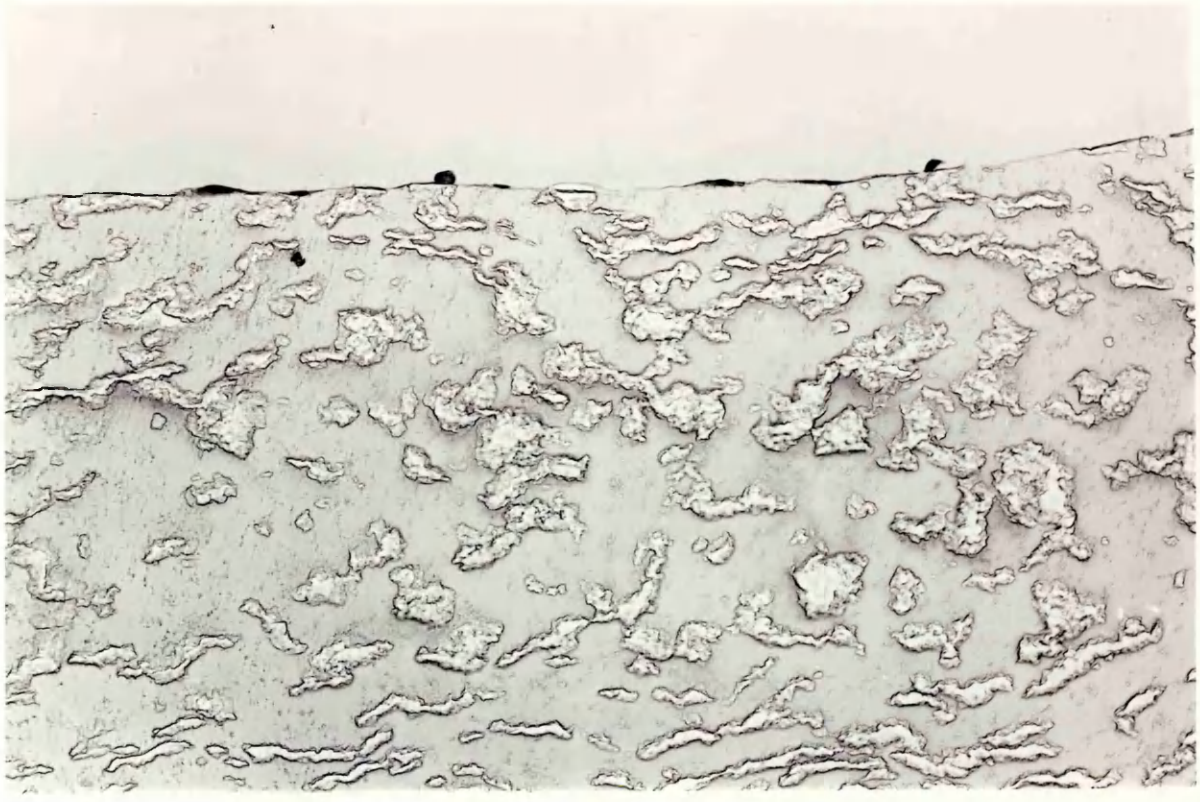


Figure 22a

Figure 22b

cells actually in the process of spreading (figures 28 to 32). Again, these appearances were common to all the substrate preparations and to pre-clotted whole blood.

The appearance of "patchy" endothelialisation and large areas of bare polymer was seen in the pre-coated grafts irrespective of the cell seeding density, whereas cells seeded onto pre-clotted ePTFE in numbers greatly exceeding confluent density formed a virtually confluent lining within an hour (figure 35 and 36).

Specimens seeded with mesothelial cells confirmed the light microscopic appearance of many rounded up cells, with only a small number of flattened, fully spread cells (figure 33, 34, 37, 38). These appearances were common to all substrate graft preparations.

Both cell types, when seeded onto uncoated ePTFE, failed to show evidence of flattening or spreading (figure 39).

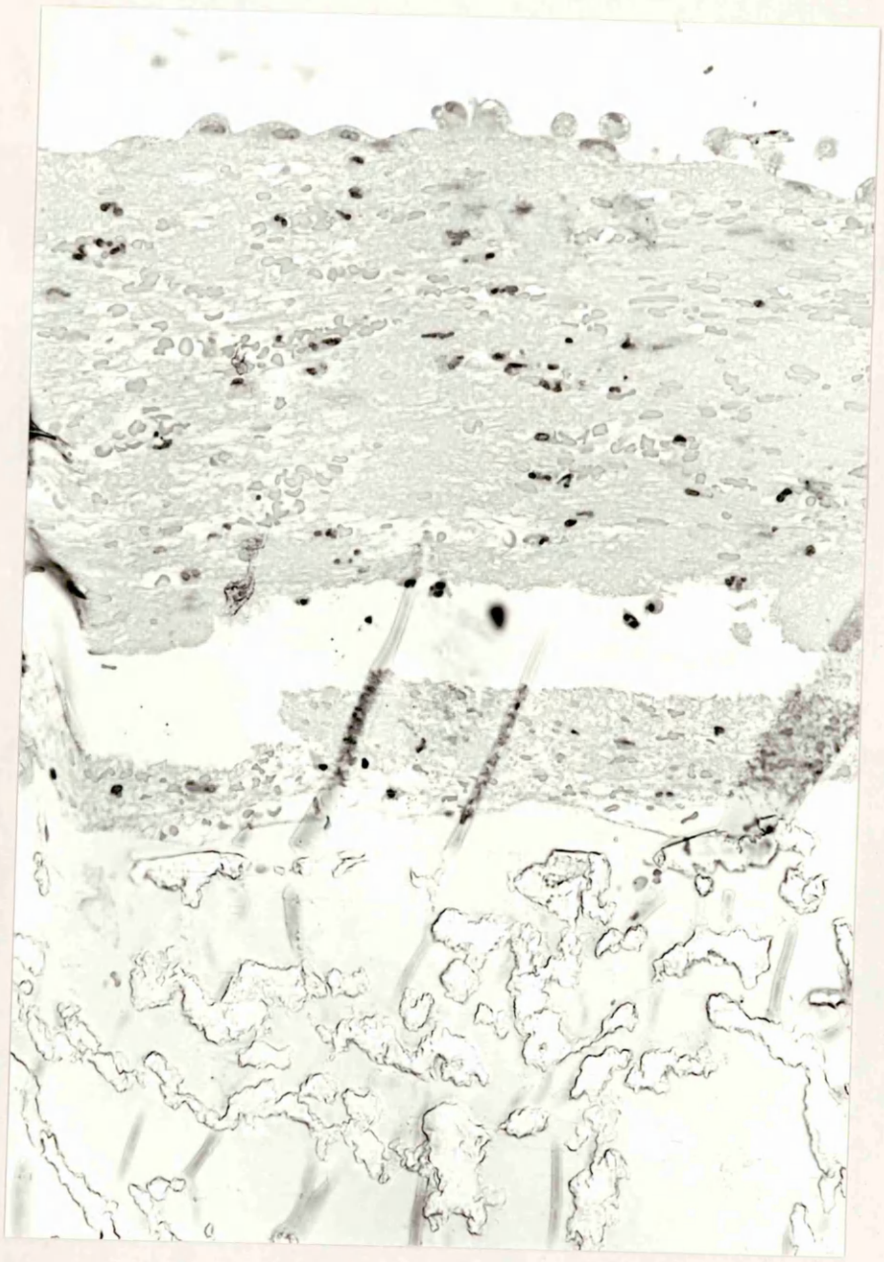


Figure 23 Endothelial cells seeded onto ePTFE which had previously been "pre-clotted" by immersion in whole blood for 3 hours.

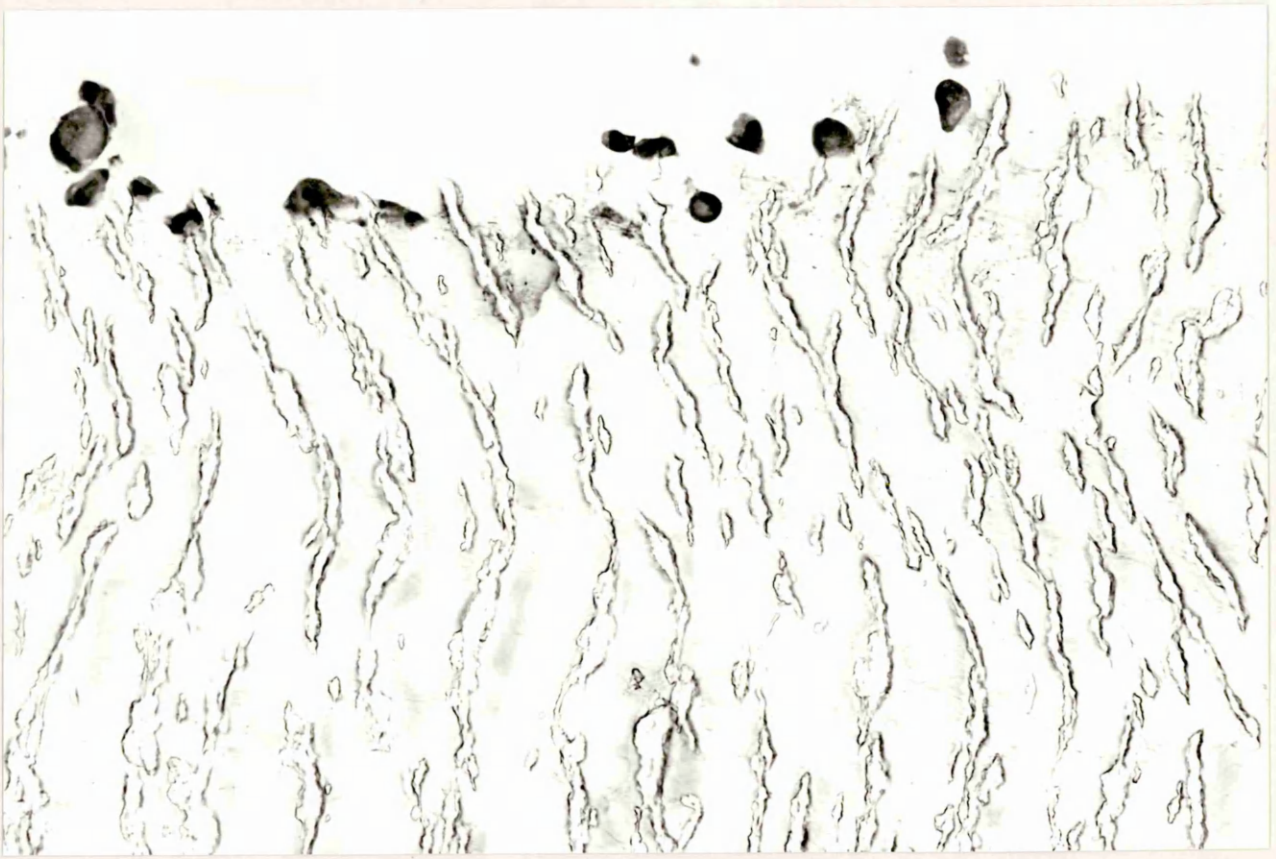


Figure 24. ePTFE graft material pre-treated with collagen and seeded with mesothelial cells. After incubation for a period of 1 hour, numerous "plump" cells line the graft lumen, representing cells which remain in a rounded up state.

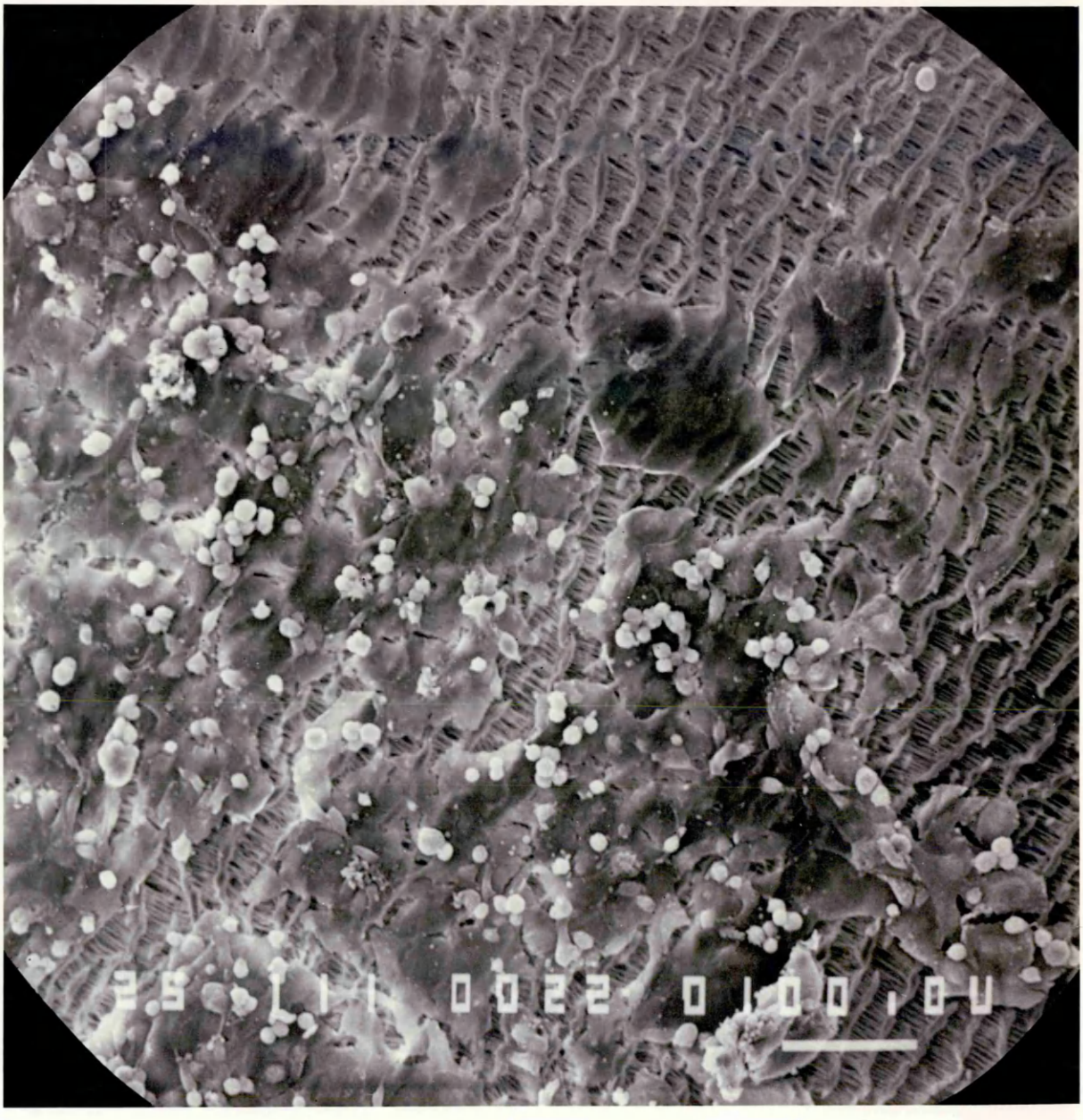


Figure 25. Scanning electron micrograph (SEM) of ePTFE pre-treated with fibronectin and incubated with macrovascular endothelial cells for 1 hour. Large areas of confluent cells are seen attached to the surface of the material (Bar = 100 microns).

Figure 26. SEM of ePTFE graft material pre-treated with type 4 collagen prior to endothelial cell seeding. Large areas of flattened, confluent cells are visualised (bar = 100 microns).

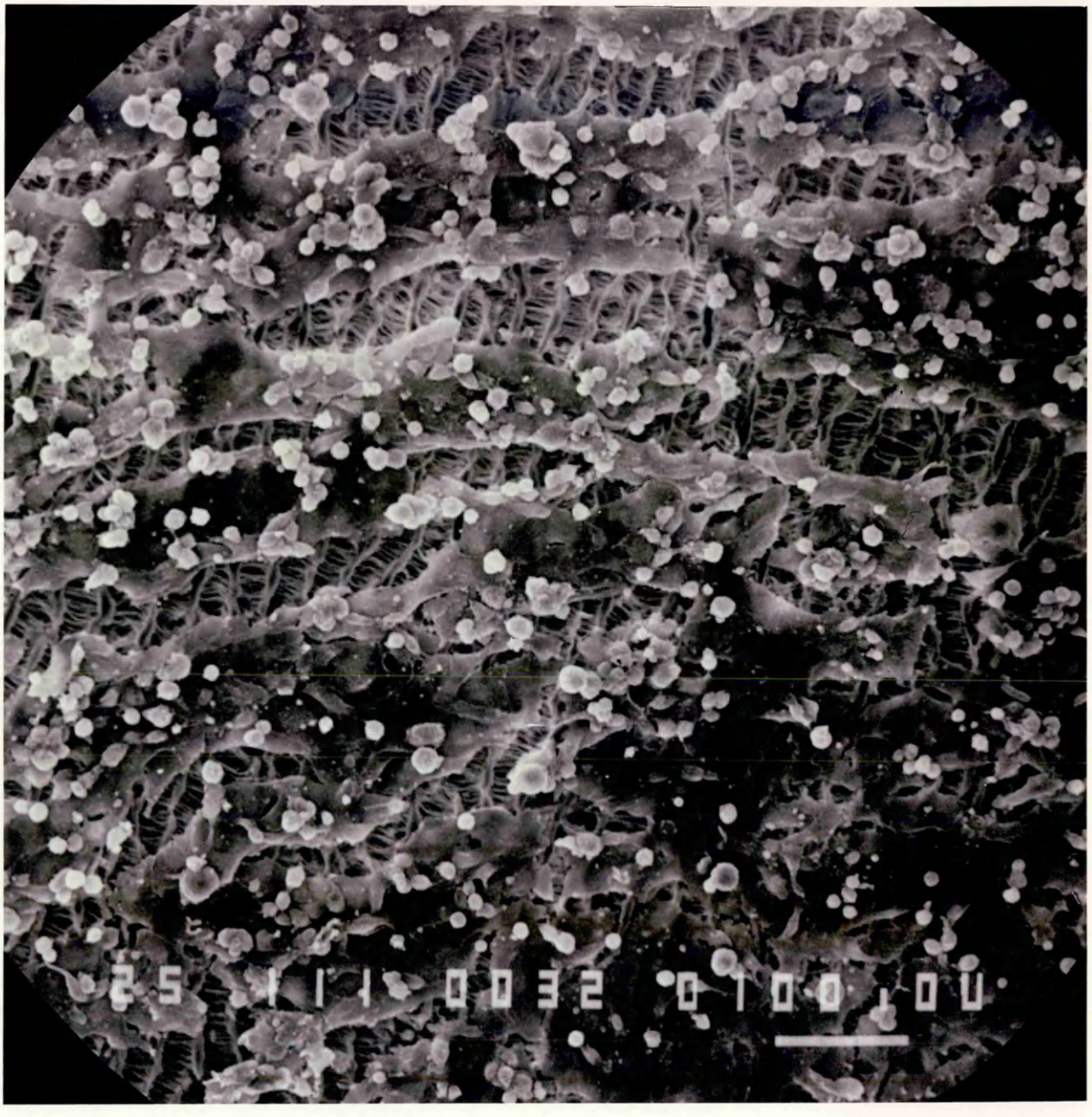


Figure 27. SEM of laminin-treated ePTFE seeded with endothelial cells. Multiple confluent areas of endothelial cells are visualised, as in figures 25 & 26. Rounded up, unattached cells cells lie over the attached cells, rather than the exposed fabric (bar = 100 microns).

Figure 28. Fibronectin pre-treated endothelial seeded graft illustrating fully spread cells with nuclear elevations and close cell to cell junctions. Some areas of exposed ePTFE fabric are seen, along with many rounded up cells, and cells in the process of spreading (bar = 100 microns).

Figure 29. Higher power view of endothelial seeded fibronectin pre-treated graft, showing actively spreading cells along with some fully flattened and some completely rounded up ones (bar = 10 microns).

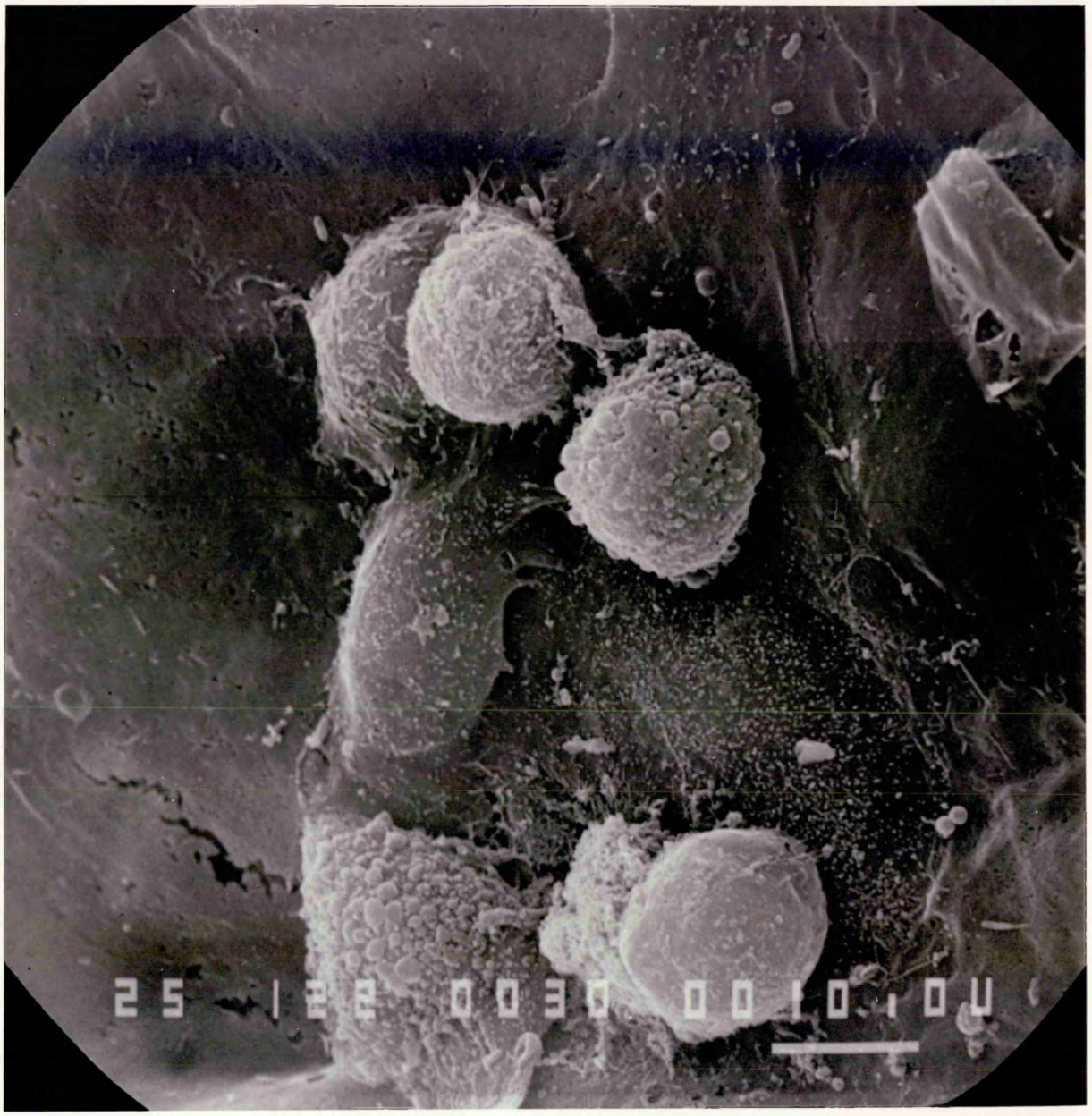


Figure 30. High power view of collagen pre-treated ePTFE, incubated with endothelial cells for one hour (bar = 10 microns).

Figure 31. ePTFE graft "pre-clotted" prior to incubation with cultured endothelial cells for 1 hour. A fibrinous network is visualised, with numerous cells in various stages of adhesion and spreading, ranging from completely rounded up to completely flattened (bar = 10 microns).

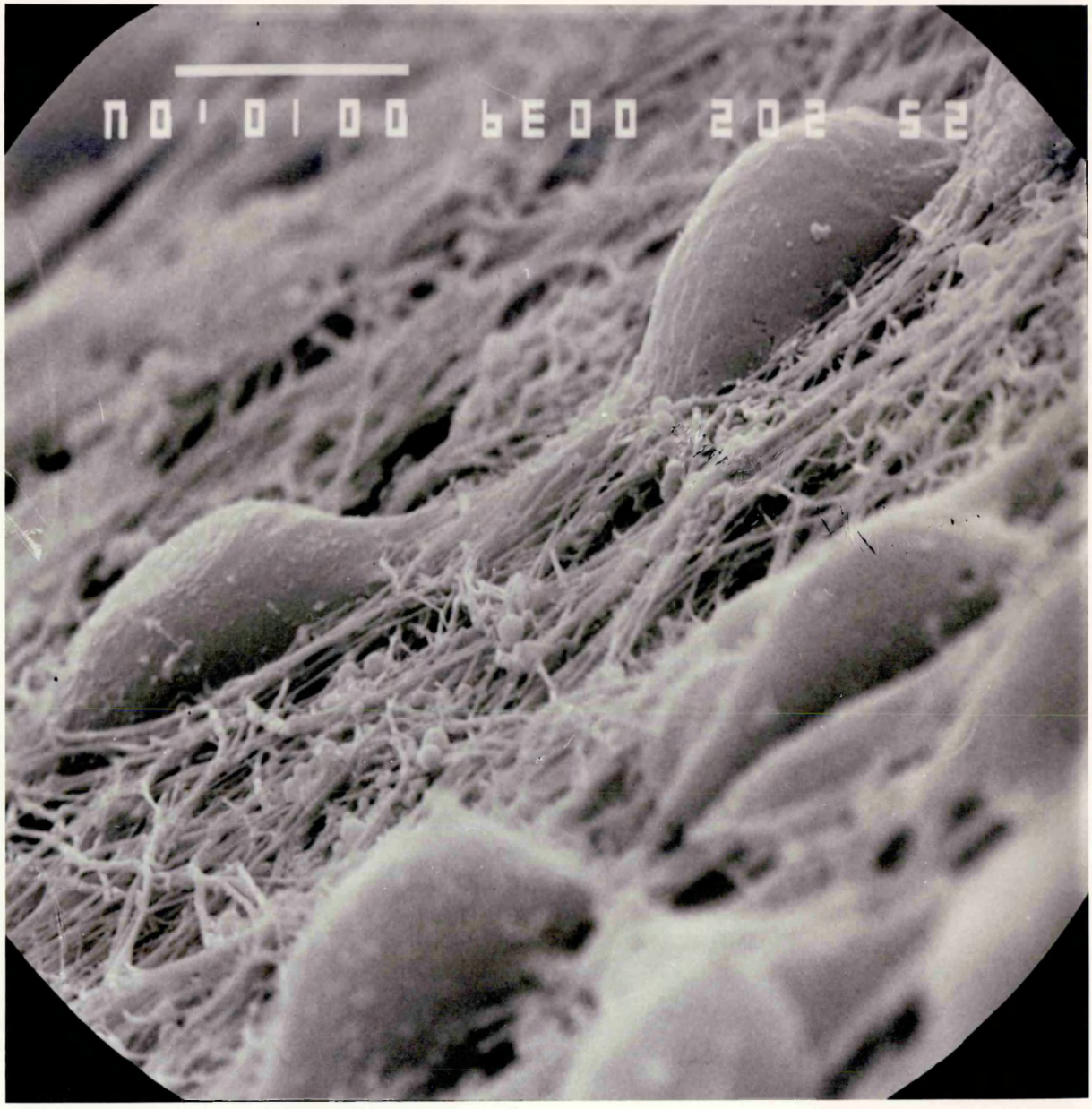


Figure 32. Higher power view of pre-clotted endothelial seeded graft (bar = 10 microns).

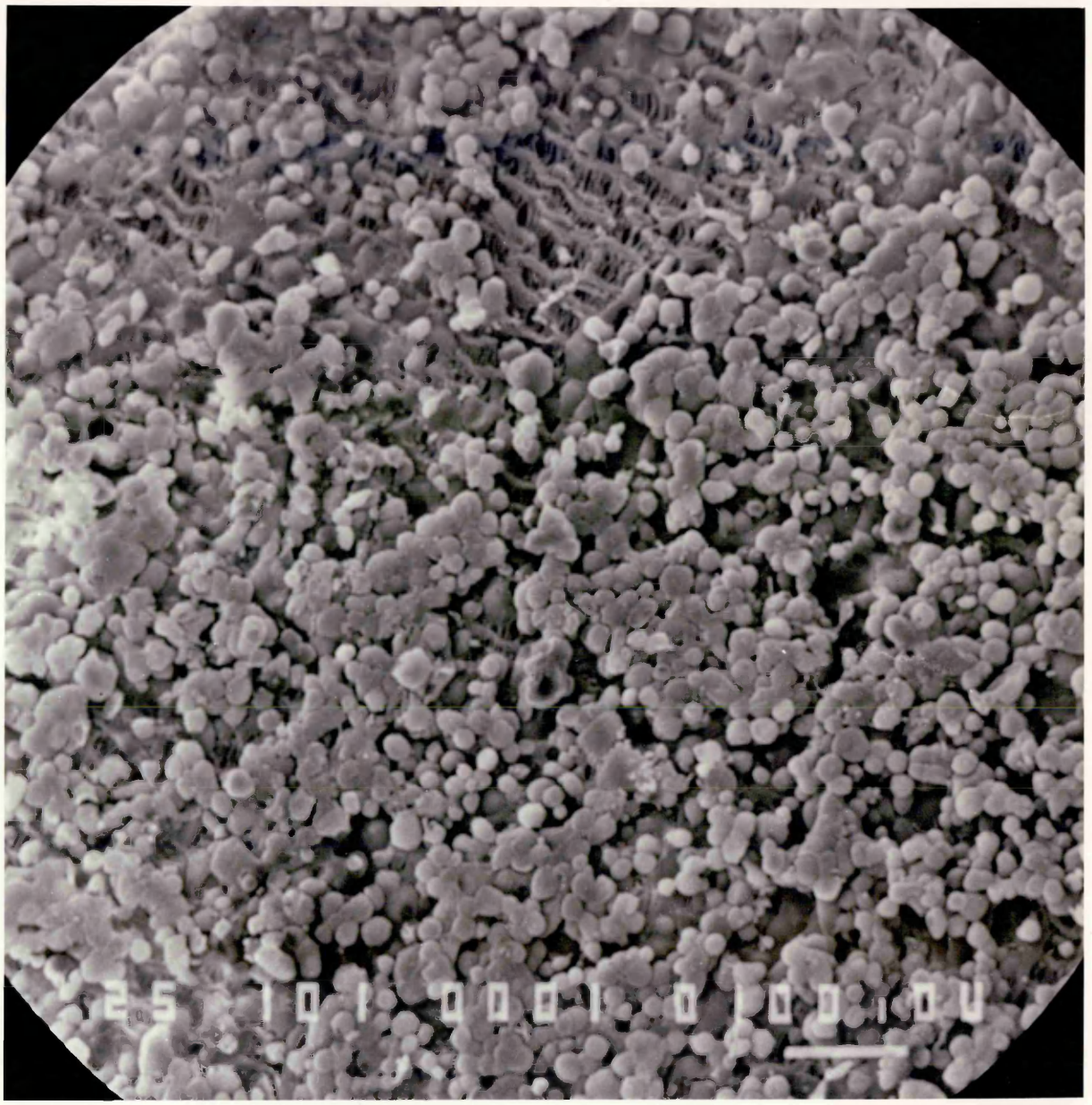


Figure 33. ePTFE pre-treated with fibronectin and incubated with mesothelial cells for one hour. Many rounded up cells are seen, with only a few apparent areas of flattened cells (bar = 100 microns).

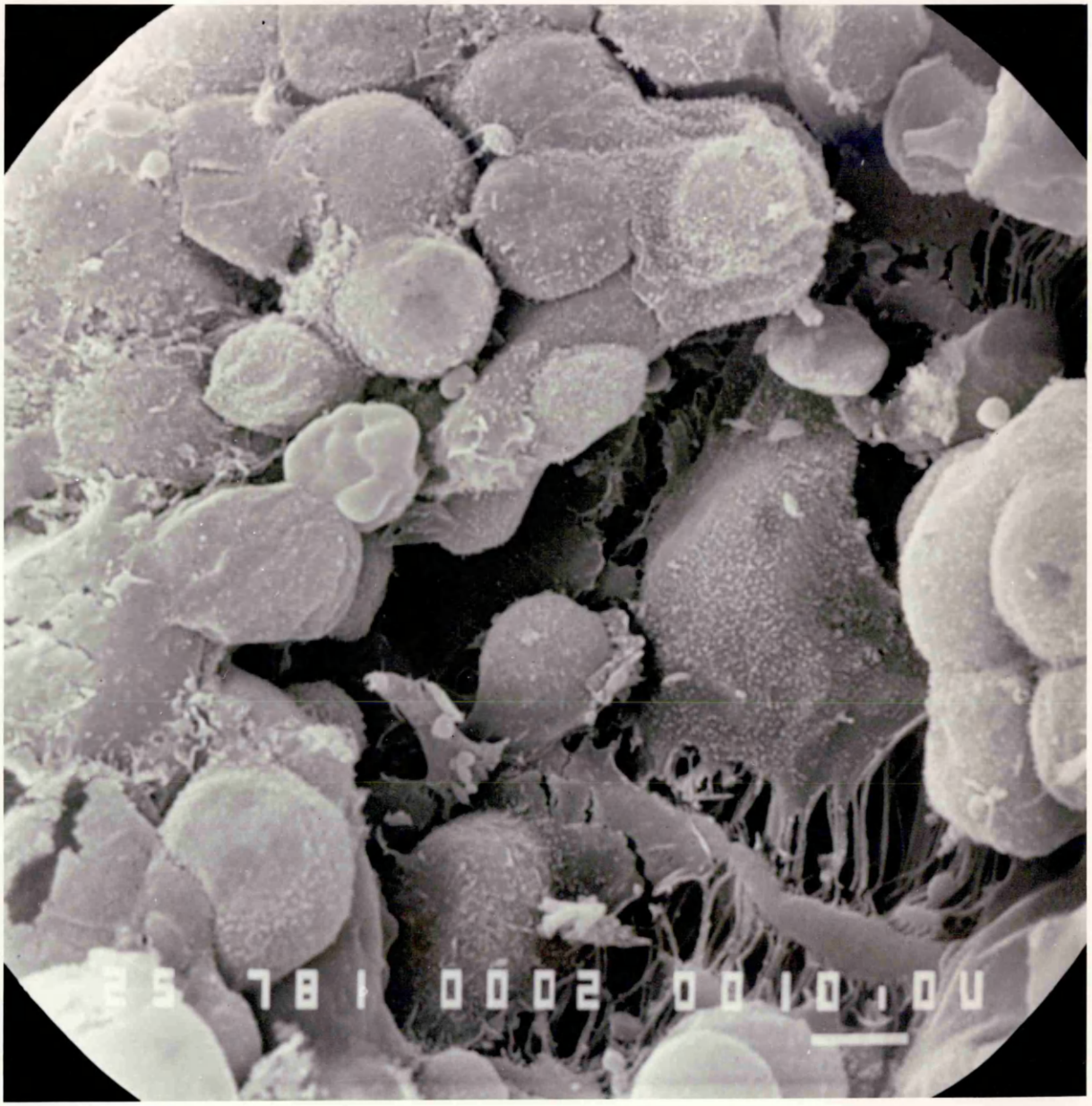


Figure 34. High power view of mesothelial seeded fibronectin-coated graft material. Some flattened and spread cells can be seen, but the majority remain in a rounded-up state (bar = 10 microns).

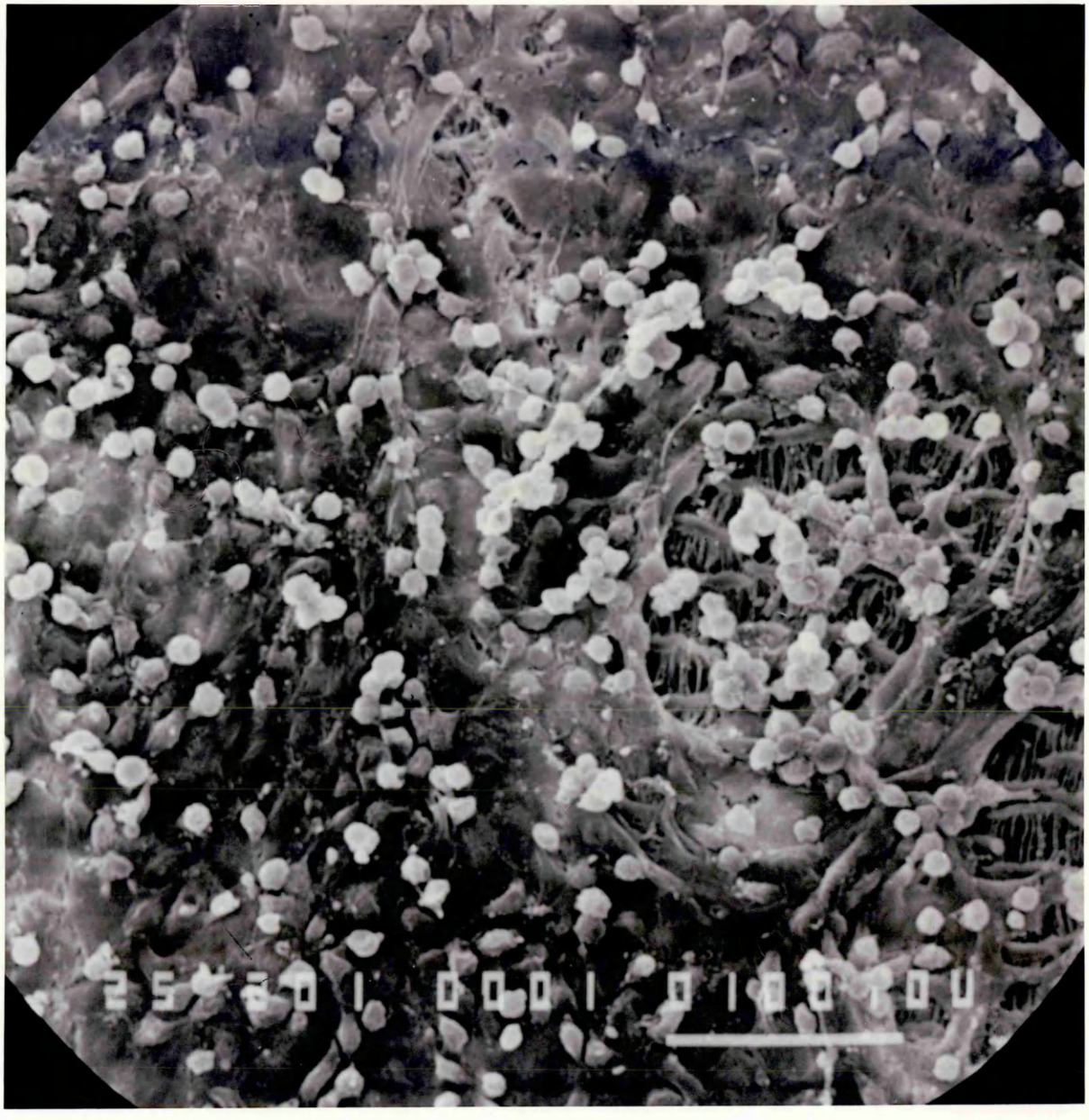


Figure 35. ePTFE pre-clotted and seeded with endothelial cells at very high density ($6.19 \times 10^5/cm^2$) and incubated for 1 hour. Virtual confluence has been reached (bar = 100 microns).

Figure 36 High power view of ePTFE pre-clotted and seeded with endothelial cells at high density, illustrating cells at various stages of attachment, but covering most of the surface (bar = 10 microns).

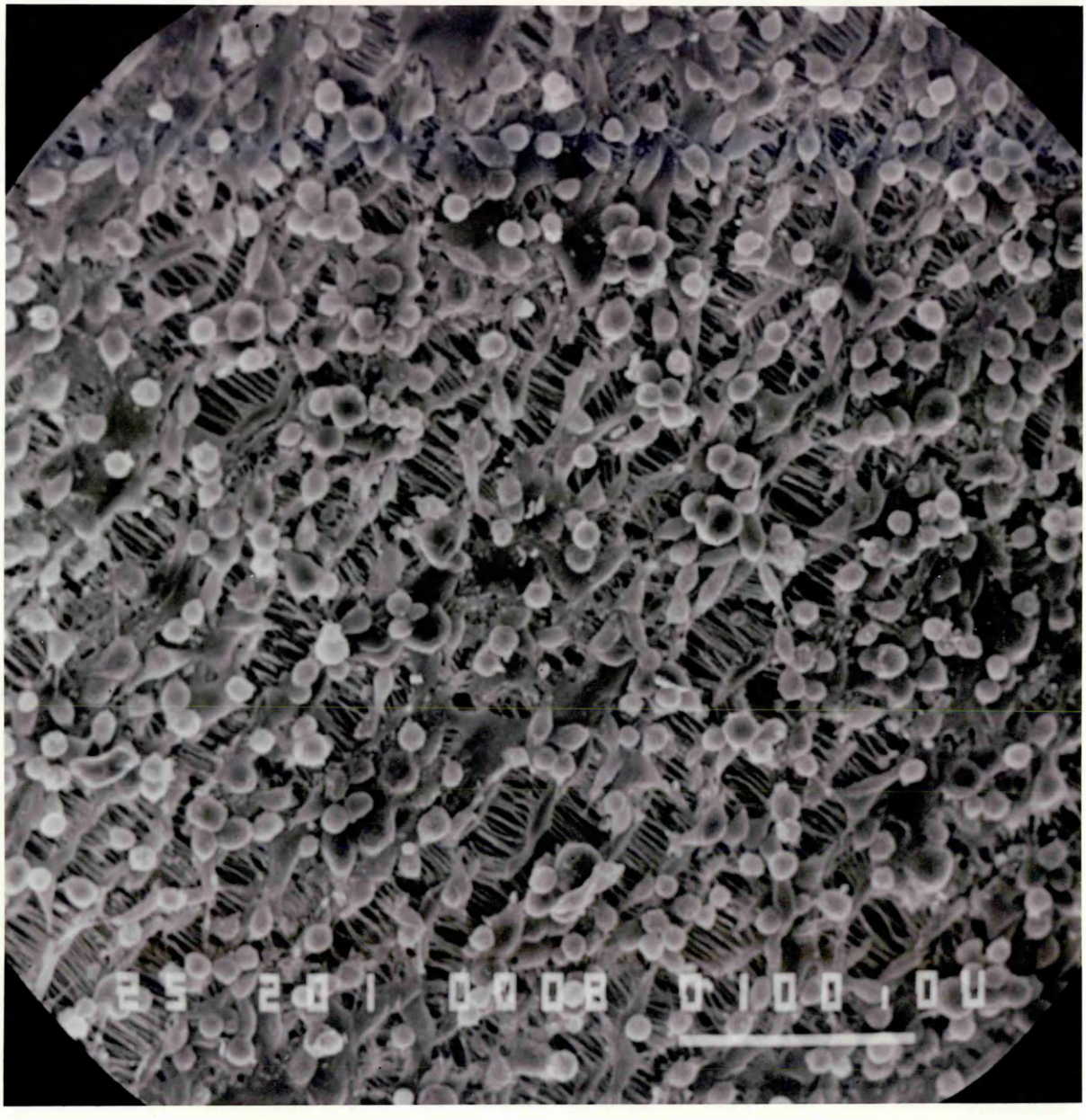


Figure 37 ePTFE graft material pre-clotted and seeded with mesothelial cells at high density. Some attached and flattened cells are visualised, but the majority remain in a rounded up state. Many areas of exposed ePTFE are seen (bar = 100 microns).

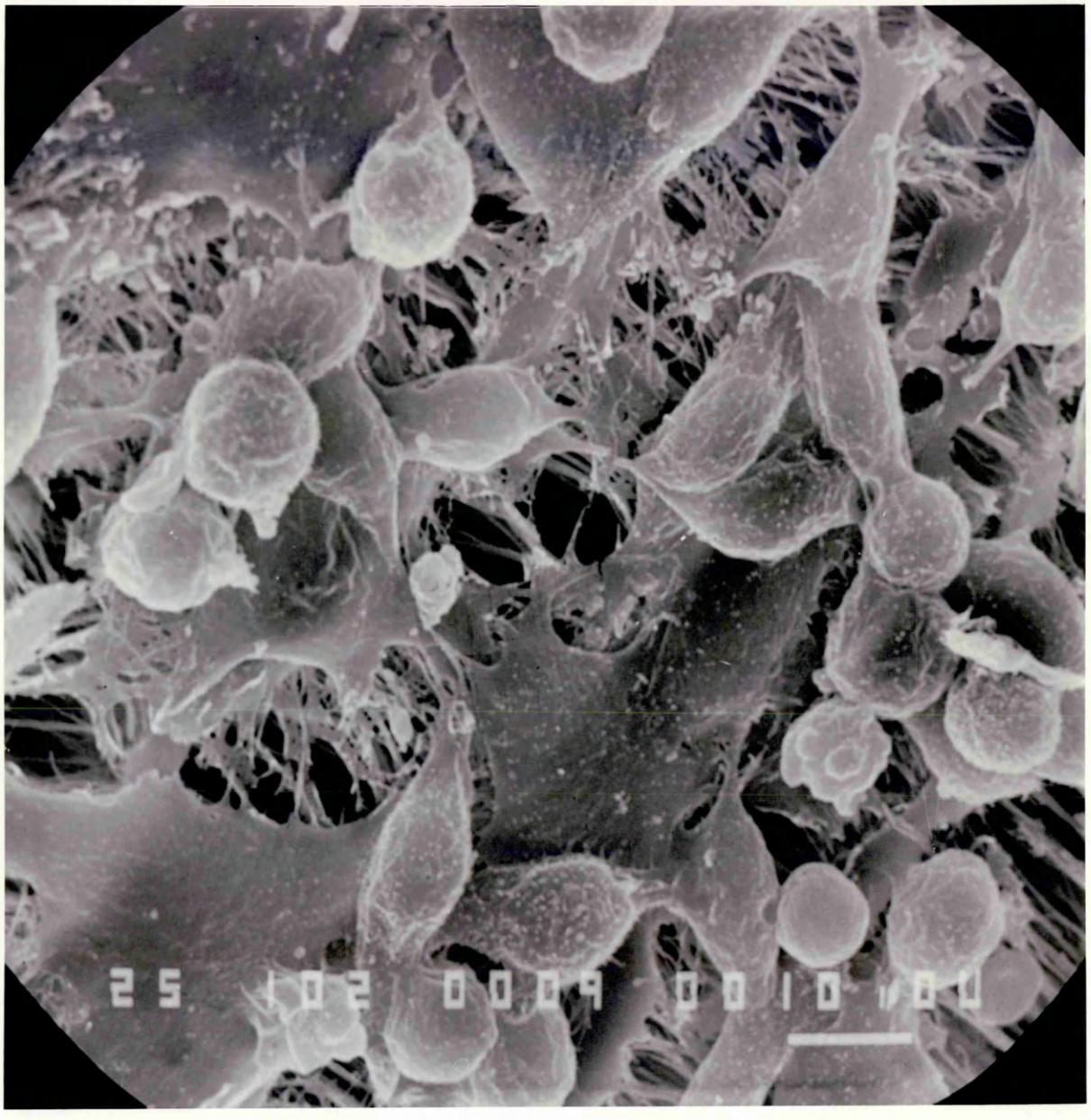


Figure 38 High power view of ePTFE pre-clotted and seeded with mesothelial cells (bar = 10 microns).

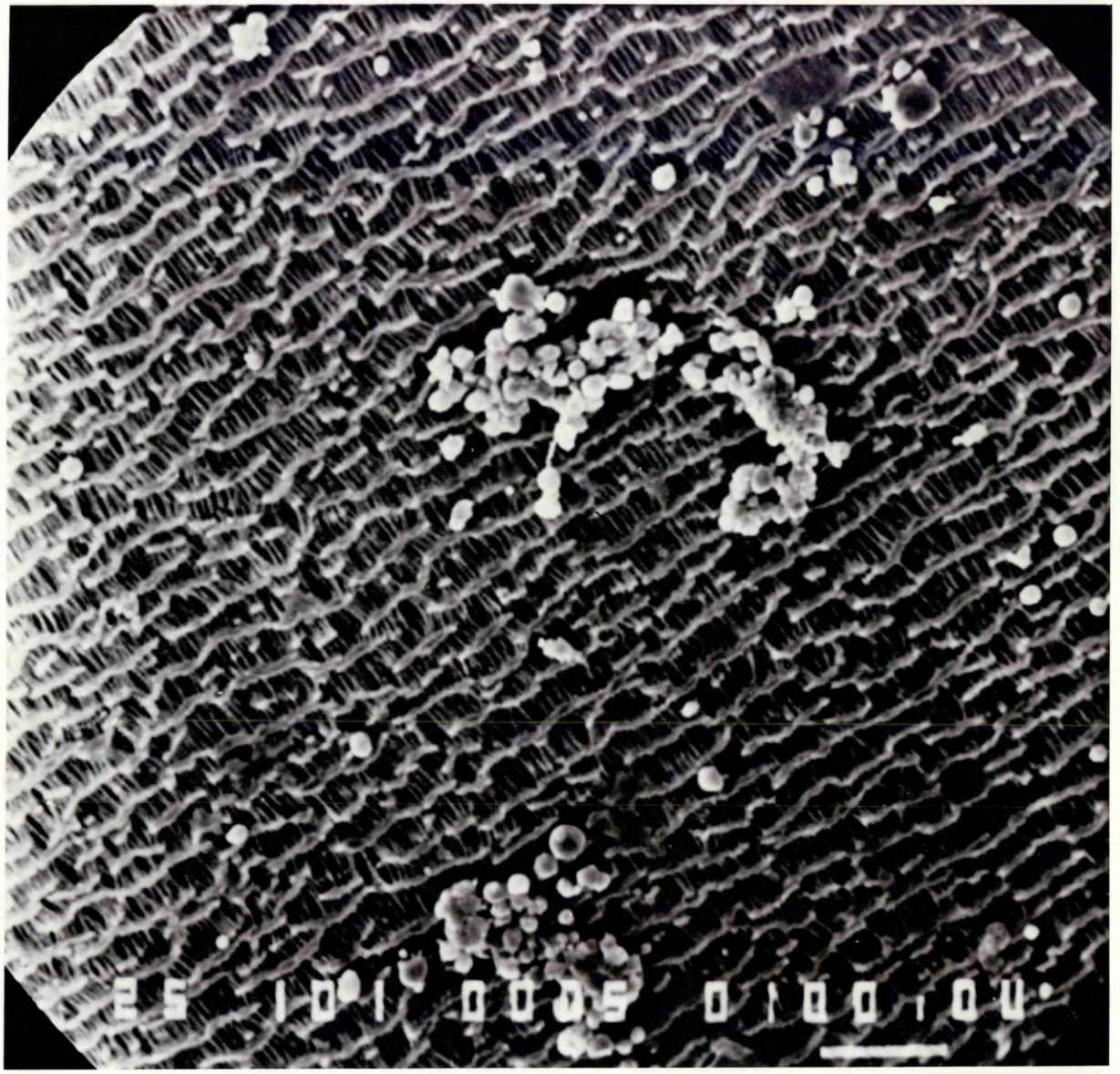


Figure 39 Uncoated ePTFE seeded with endothelial cells. Only a few rounded up cells are visualised, with no attempt at flattening or spreading (bar = 100 microns).

CHAPTER 8

FLOW STUDIES

8.1 Introduction

Having confirmed that it is possible to achieve a confluent endothelial lining in an ePTFE graft within a very short time, it is important to demonstrate whether or not such a monolayer will remain intact, or if the cells are likely to be washed away immediately blood flow is established through the graft.

In order to answer this question, a series of experiments was performed using the best seeding technique as established earlier, implanting seeded graft segments into an artificial circulation device, designed to simulate as closely as possible the conditions likely to be encountered by such a graft *in vivo*. Cells were pre-labelled with indium-111-oxine prior to seeding, the amount of radioactivity retained after flow enabling the durability of attached cells to be established.

8.2 Artificial circulation circuit

a. Design

The basic design of the circuit is illustrated diagrammatically in figure 40, and the actual system is shown in figure 41.

Each end of a 6 cm length of ePTFE graft material (previously seeded with labelled cells) was tied to a length of 6 mm internal diameter plastic tubing, through which culture medium was circulated by a roller pump (Watson Marlow Limited, Falmouth, Cornwall) providing variable rates of pulsatile flow. Any cells which detatched were prevented from recirculating by incorporating a membrane filter into the circuit (Sartorius Membranfilter GmBh Gottingen, FDR) and the temperature of the circulating medium was maintained at $37^{o}C$ by passage through a water bath.

Any air bubbles within the system were removed by a "bubble trap", consisting of a partially filled glass cylinder through which the medium circulated. An air-filled syringe inserted to the top of the "bubble trap" enabled fine adjustments of the pressure within the system. A manometer graduated in mm of

water was attached to the lower part of the bubble trap. The pressure during the experiments was kept constant at 15-20 mm water.

Radio-active counts within the graft segment were measured continuously by a probe connected to a counter ratemeter (J & P Engineering, Reading, England). Probe-graft distance was kept constant during each experiment.

The system was "primed" with approximately 300 ml of medium containing 20% foetal calf serum, L-glutamine and Penicillin/Streptomycin, in the same concentrations as in the medium used for culturing the cells. The system was filled by the use of two Y-connectors as illustrated in the diagram (figure 40). With the free ends open and submerged in medium, operation of the pump resulted in medium being drawn into the circuit. Once full, the free ends were clamped, producing a closed system. The circuit was run for a period of 1 hour before placement of the seeded graft, in order to allow the temperature of the circulating medium to reach $37^{o}C$. During this "warming up time" a segment of ordinary unseeded ePTFE replaced the experimental seeded grafts.

For the actual flow experiments, culture medium was used in preference to whole blood, in order to facilitate microscopic examination of the graft after flow.

b. Estimation of flow rates

Before carrying out the experiments, the system was callibrated to correlate different pump settings with the corresponding flow rates. This was done by disconnecting the distal end of the graft segment, and allowing the medium passing through the graft to drain into a measuring cylinder. By measuring the volume collected over a period of 1 minute, the rate of flow through the graft was obtained in ml/minute. For the experiments, pump settings correlating with flow rates of 25, 100 and 200 ml/minute were selected.

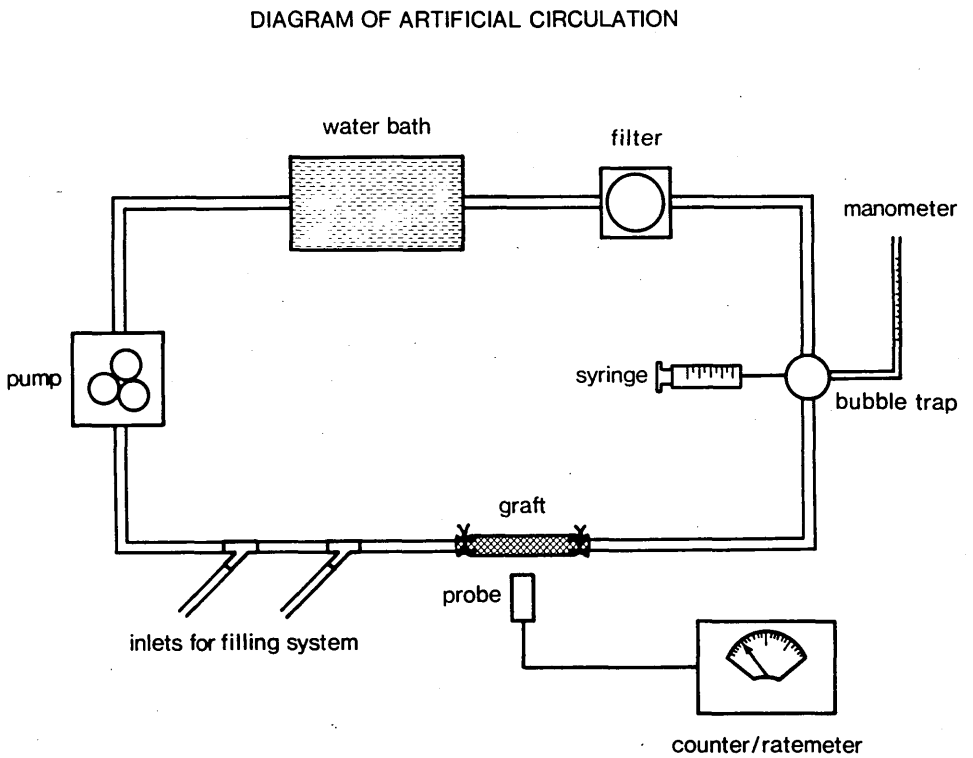


Figure 40 Diagram of the artificial circulation used for flow experiments.

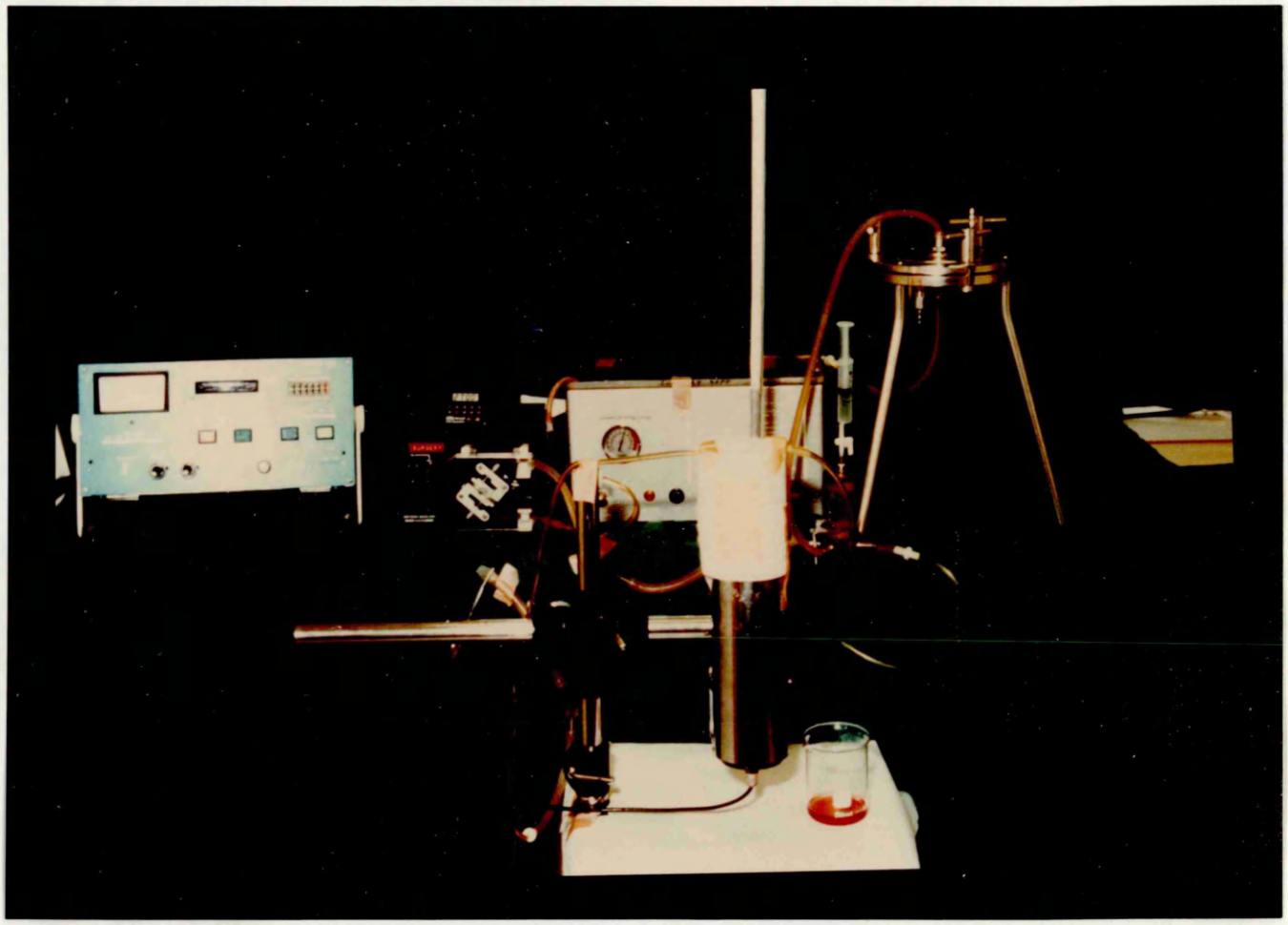


Figure 41 Photograph of artificial circulation circuit.

c. Labelling of cells and seeding of grafts

Cells were labelled with indium-111-oxine as described earlier (see section 6.1). In order to simplify the calculation of cell retention, the problem of spontaneous leakage of isotope from the cells was minimised by holding the newly labelled cells in suspension at 37°C for a period of 60 minutes. After this time, the cells were centrifuged at 1000 rpm for 5 minutes and resuspended in fresh medium prior to seeding. Since most leakage occurs within the first 60 minutes or so of labelling (Sharefkin et al 1984), this procedure allowed spontaneous leakage to be disregarded in the subsequent calculations.

Since pre-clotting of grafts and seeding with macrovascular endothelial cells gave the most satisfactory results for the experiments performed under static conditions, these methods were used for the flow experiments. Pre-clotting and inoculation of the graft segments were achieved using techniques identical to those described in section 6.1. In all cases, cells were seeded at supra-confluent densities. Following incubation of the cells in the graft for a period of 90 minutes, the graft segment was tied into the artificial circulation device as quickly as possible. 90 minutes, rather than 60 minutes, was chosen as the incubation time in order to maximise initial cell attachment.

d. Calculation of cell retention

Initial radioactive counts were measured immediately after placing the graft segment in the circuit (count 0_1), and immediately after medium began circulating through the segment (count 0_2). Count 0_1 was taken as 100% in each case. The difference between 0_1 and 0_2 was an indication of the initial cell detachment on commencement of flow through the graft.

Counts were taken every 5 minutes thereafter for a period of 1 hour. Each reading was converted to a percentage of the original count (0_1).

e. Calculation of shear stress

The shear stress applying under the various flow conditions was calculated using the formula:

shear stress = $4Q\eta/\pi(ri)^3$

where Q = flow in ml/second

ri = internal radius in cm

η = viscosity of medium in poise

The viscosity of the culture medium used was obtained using an Ozwaltz viscometer, giving a value of 0.0108 poise.

f. Morphological examination of cells after flow

The morphological appearance of attached cells after exposure to the various rates of flow was examined by light microscopy and scanning electron microscopy. Representative sections were taken from both ends, and from the centre portion of each graft.

8.3 Results

Six experiments were carried out for each flow rate, giving shear stress values of 0.21, 0.84 and 1.68 dynes/cm^2 for flow rates of 25, 100 and 200 ml/ minute respectively.

The cell number used in each of the eighteen experiments, together with the labelling efficiency and the initial cell attachment achieved, is illustrated in appendix 4. The mean ($\pm$ SD) initial percentage cell attachment was 72.6 $\pm$ 8.7.

Average cell retention during one hour at these flow rates is shown in figure 42, and the actual results obtained are tabulated in appendix 4. Average retention after one hour's flow was 85.7 $\pm$ 7.2 at 25 ml/minute, 82.9 $\pm$ 8.5 at 100 ml/minute and 79.6 $\pm$ 8.9 at 200 ml/minute.

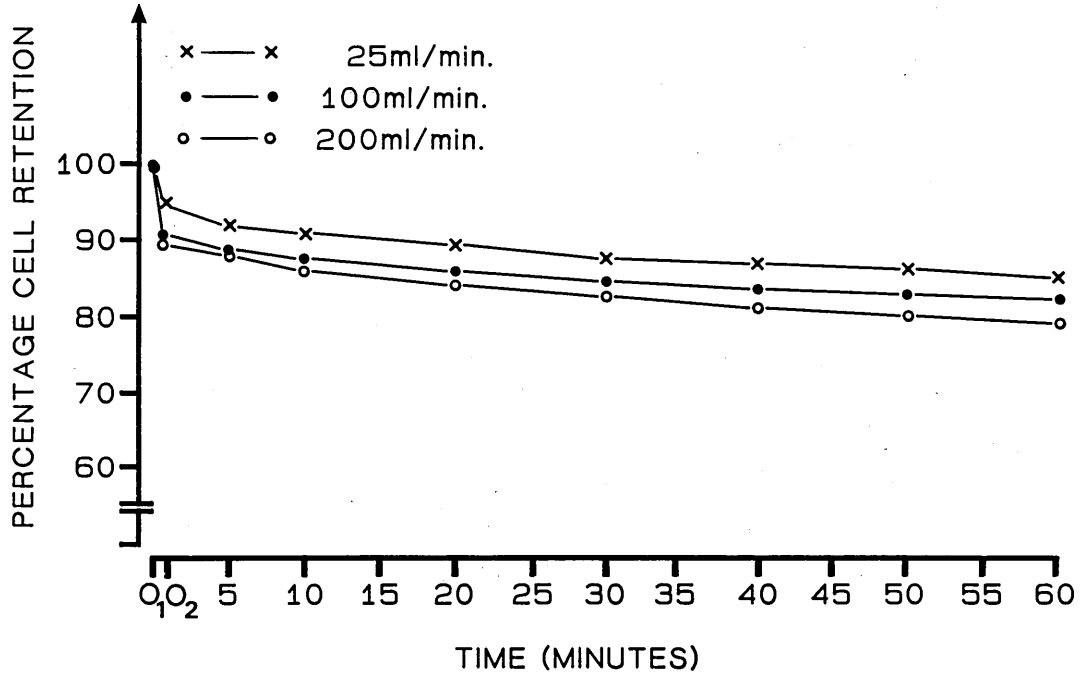


Figure 42 Results of flow studies. Cell retention at the various time intervals is expressed as a percentage of the cells attached before commencement of flow (i.e. at time 0_1).

Light and scanning electron microscopy of post-flow specimens revealed persistence of the virtually confluent monolayer over the majority of the graft surface, even at the highest rate of flow (figure 43, 44), although in those grafts exposed to flow rates of 200 ml/minute some "gaps" in the monolayer are beginning to appear (figure 45).

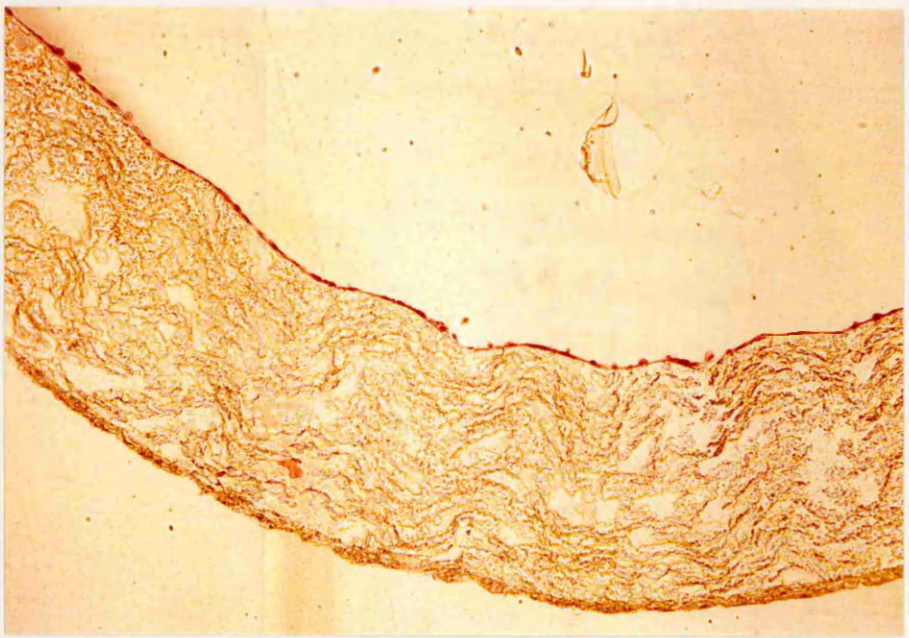


Figure 43 Light micrograph of pre-clotted ePTFE graft segment, seeded with endothelial cells and subjected to flow at 25 ml/minute for 1 hour. An intact endothelial lining is visualised (original magnification x 40).

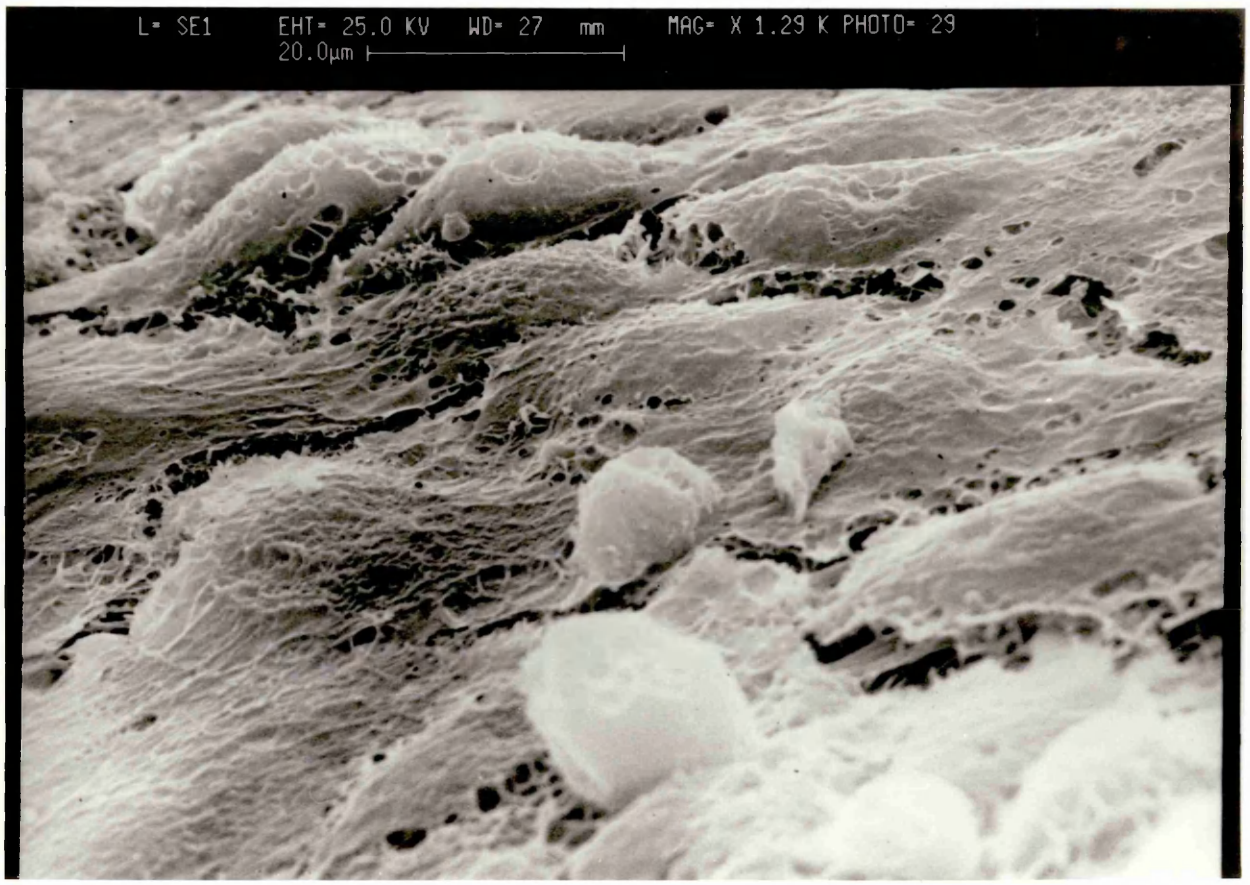


Figure 44 SEM appearance of cell monolayer after flow at 100 ml/minute for 1 hour. The monolayer remains virtually intact.

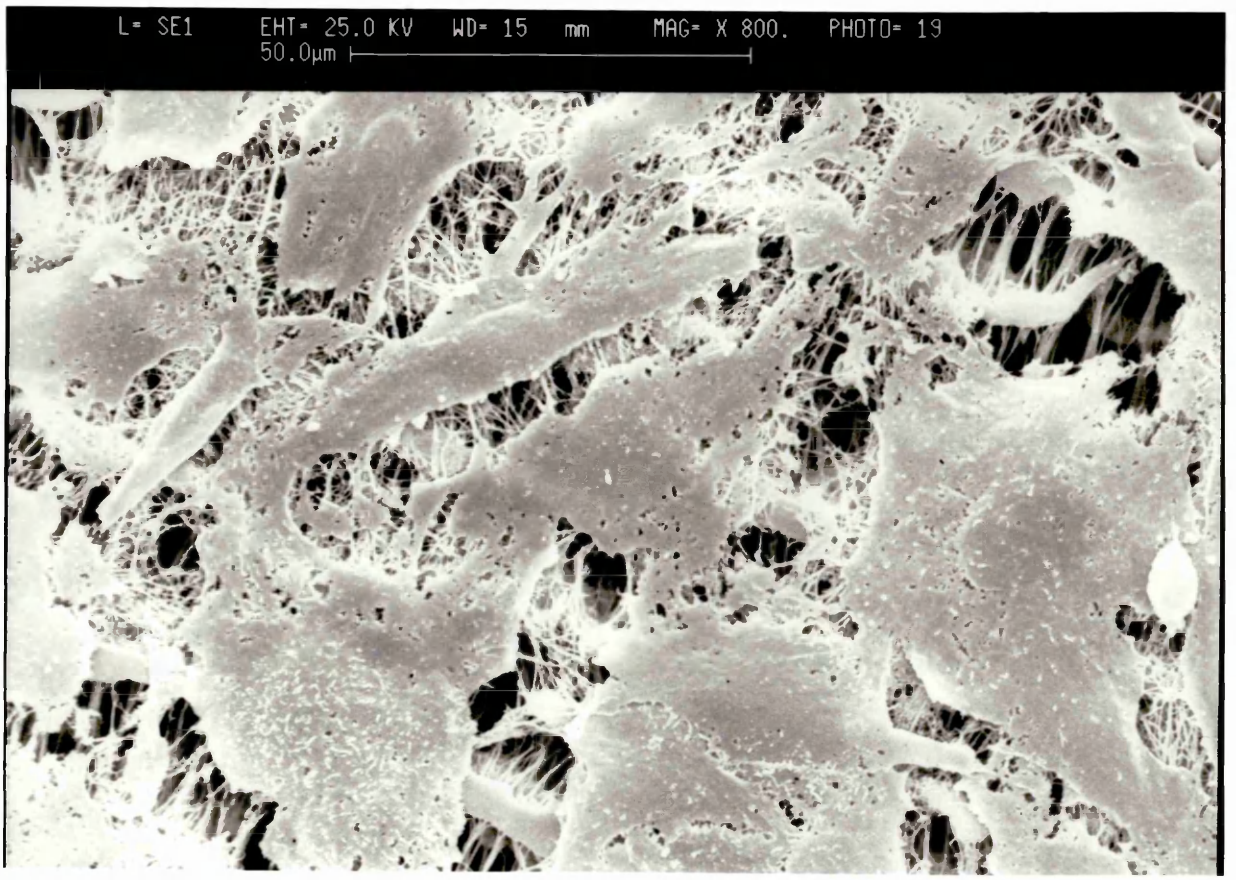


Figure 45 SEM appearance of endothelial monolayer after flow at 200 ml/minute. Gaps are beginning to appear between attached cells, but the majority of the surface is still covered by cells which remain fully spread, indicating strong attachment.

CHAPTER 9

DISCUSSION

9.1 Harvesting techniques

If cell seeding of vascular grafts is ever to become clinically useful, then the technique of harvesting must be reliable and easy, and must be performed rapidly by personnel with no special expertise in cell biology. It was therefore considered relevant to investigate which of the currently available techniques best fitted these requirements.

a. Mechanical harvesting

Mechanical harvesting of adult human saphenous vein endothelial cells theoretically met these criteria best, and was therefore investigated first. Unfortunately, the technique consistently failed to release cells capable of growing in culture. Although large numbers of cells were released, the majority were non-viable, or unable to attach to the culture surface. The most likely explanation for the failure of the technique is that mechanical scraping is excessively traumatic to cells, and also removes basement membrane along with the endothelium. As cells already attached to basement membrane will have no stimulus to attach to another surface, such cells will fail to adhere to culture flasks or prosthetic grafts. This technique can therefore not be recommended as a means of obtaining cells for seeding of prosthetic vascular grafts.

The reason for the discrepency between these results and those of Ryan and White is unclear, but may lie in the fact that, in the latter study, considerably longer lengths of vein were available as an endothelial source. These authors did not state the average length of vein used, nor did they comment on the cell yields obtained (Ryan & White 1986). They were, however, using long saphenous veins removed by stripping, and it seems likely that the lengths were substantial.

b. Enzymatic harvesting of macrovascular endothelium and omental mesothelium

The enzymatic technique for deriving macrovascular endothelium and omental mesothelium is reliable, although inefficient. As the total number of endothelial cells available per cm^2 of vein lumen is 10^5, the average cell yield of $2.6 \pm 1.2 \times 10^4$ implies a harvesting efficiency in the order of 26%. This agrees with the experience of most authors. It may be that purification of collagenase, or substitution with an alternative enzyme, will result in better cell yields.

Despite the inefficiency, large numbers of viable cells are obtained, which are capable of attaching rapidly to a suitable synthetic surface and going on to proliferate to confluence. Although the overall success rate in these studies was only 73%, if the initial "learning curve" is excluded, the success rate was 100% for the last 8 attempts. Similarly with omental mesothelial cells, enzymatic derivation was consistently successful after an initial learning phase.

This technique proved relatively quick to perform, the complete procedure taking approximately 30-45 minutes (20 minutes incubation with enzyme, 10-15 minutes centrifugation).

c. Enzymatic harvesting of microvascular endothelial cells

The enzymatic technique described by Jarrell for deriving microvascular endothelium from various fat sources has proved disappointing in the present studies. Although large numbers of endothelial-like cells occur in the initial isolate, the subsequent growth pattern suggests overgrowth with contaminant cells in the case of subcutaneous fat, and mesothelial cells in the case of omentally derived fat. In addition, the process proved time consuming. An average time course for the technique as described would be :- 10-15 minutes for mincing and weighing of specimen and making up collagenase in appropriate concentration; 20 minutes for incubation with collagenase; 20-30 minutes for washes and

separation of adipose tissue; another 20 minutes for high speed centrifugation in Percoll; and 10-15 minutes for further washes. The time in total amounts to some 80-100 minutes. Omitting the Percoll stage would save perhaps 30-45 minutes, but, even then, 50-65 minutes are required.

The concept of harvesting endothelial cells from subcutaneous fat is attractive. If, however, it is demanding on time, materials and equipment, as found in the present study, then its clinical applicability is limited.

d. Harvesting techniques - conclusion

These results indicate that the simplest, fastest and most reliable technique for deriving endothelial cells for seeding purposes is enzymatic derivation of macrovascular endothelium from saphenous vein. The method does not require previous experience in cell culture techniques, and could be easily performed in the operating room.

9.2 Adhesion and growth patterns on the various substrates.

With the exception of basement membrane gel, the substrates tested were all equally capable of supporting growth of both endothelial and mesothelial cells. One important difference, however, was that endothelial cells failed to proliferate to confluence in the absence of some form of pre-coating of the flask floor, be it gelatin, or one of the extracellular matrix proteins. Although endothelial cells attached rapidly when plated into uncoated culture flasks, subsequent separation of the cells occurred, precluding their growth to confluence. Mesothelium, on the other hand, regularly proliferated to confluence through multiple passages in the absence of any pre-treatment of the culture surface. In addition, mesothelial cells thrived in the absence of any specific growth supplements, while endothelium required endothelial cell growth factor and heparin. Omission of either of these resulted in a marked retardation of endothelial cell growth.

Under the correct conditions, however, both of these cell types proved capable of multiplying to cover many times their original area. For example, cells harvested enzymatically from a 2 cm length of vein undergoing 10 population doublings will cover 410 cm^2, assuming a harvesting efficiency of 20%. This is more than adequate to cover a 60 cm x 6 mm ID vascular graft.

The time course (culture well) adhesion studies have indicated that the majority of cells, when seeded onto a suitable prosthetic surface at subconfluent densities, attach to the surface within 60 minutes of seeding. This is of importance when considering the feasibility of immediate seeding in the operating theatre context, where the time allowable for cell attachment is limited. On the basis of these results one hour was accepted as the optimum practical time to allow for attachment to ePTFE graft material in the subsequent experiments.

With regard to the relative adherence of the two cell types to substrate coated wells, endothelium attached more rapidly to all the substrates (figures 14 & 15. Appendix 1).

The attachment studies performed on ePTFE have demonstrated that the limited one hour attachment which occurs to the untreated material can be improved by pre-treatment with any of the extracellular matrix proteins, or, in the case of endothelium, by pre-clotting with whole blood. The average attachment rates indicate that the matrix proteins are marginally better in this respect, although the range and standard deviations demonstrate a wider variation in attachment rates to ePTFE when it is protein coated than when pre-clotted with whole blood. SEMs of pre-clotted endothelial cell seeded grafts demonstrated cells in various stages of attachment, while cells attached to matrix protein-coated grafts were in a more advanced stage of spreading. This suggests that cells attach and spread more quickly on the protein coated than the pre-clotted surface.

However, the variability of endothelial attachment to matrix protein-coated graft material suggests an inconsistency in the amount of protein binding

to the ePTFE. This conclusion is supported by the following :- 1. endothelial attachment to pre-clotted ePTFE was more consistent than to matrix protein-coated ePTFE, 2. scanning electron microscopy revealed large "gaps" of exposed ePTFE between the areas of confluent cells on matrix protein-coated material, these presumably representing areas of ePTFE devoid of protein coating, and 3. the one hour attachment rates to pre-coated ePTFE were lower than those to pre-coated tissue culture polystyrene. It is worthy of note that the rounded up, non adherent cells present on protein-coated ePTFE lie over the areas of cellular coverage, those areas of exposed graft material being devoid of cells. This suggests that these areas may be actively hostile to cell attachment.

It is most likely that these gaps exist because protein binding is "patchy", and the ePTFE at these points is not covered with protein substrate. Pre-clotted grafts, on the other hand, have a more consistent and even dispersal of fibrin across the surface. Thus, although the speed of attachment to and spreading upon the fibrin pre-clot is slower than to the purified protein coatings, the more complete covering of fibrin pre-clot compensates for this.

Although Ramalanjaona demonstrated that fibronectin has the ability to form a strong bond with ePTFE (Ramalanjaona et al 1986), the evenness of the dispersion of the protein across the surface of the material has not been examined. The present studies indicate that protein coverage is variable and patchy.

A potential problem with fibronectin coating as a means of promoting attachment is that coated grafts exhibit increased thrombogenicity until cell coverage is complete. The proponents of fibronectin coating do, however, claim that the increased thrombogenicity can be negated by treatment with anti-platelet agents (Ramalangaona et al 1986).

Pre-treatment of ePTFE with alcohol does improve endothelial cell adhesion rates, although to a lesser extent than substrate coating or pre-clotting.

Alcohol treatment prior to substrate coating does not produce any apparent improvement in cell attachment.

There was a significant trend for endothelial adherence to polystyrene to exceed that of mesothelium. Attachment to fibronectin coated ePTFE, on the other hand, was more rapid in the case of mesothelial cells. Cell attachment, however, is not the only requisite for successful cell seeding of vascular grafts. The morphology of the adherent cells is also of importance - large numbers of rounded up cells adhering lightly to the surface are unlikely to be as able to withstand the shear stress of blood flowing past at arterial pressures as flattened out confluent or near confluent cells. Similarly, cells which remain rounded up are unlikely to be functionally normal, even if they do remain attached in the presence of blood flow. Scanning electron microscopy has revealed that, although mesothelial attachment to the fibronectin coated polymer is quantitatively better than that for endothelium, attached cells of the latter type are more spread out, are therefore more likely to be able to resist shear stress, and are more likely to have retained normal functional potential.

The maximum one hour endothelial attachment achieved with any of the preparations of ePTFE was 50%. This being the case, cells would require to be inoculated at approximately twice confluent density (10^5 cells per cm^2) in order to attain confluence after one hour's incubation. For a graft 60 cm by 6 mm ID (surface area 113 cm^2) this would require $113 \times 2 \times 10^5 = 2.26 \times 10^7$ cells. The only practical ways of obtaining such large cell numbers for immediate seeding purposes are by culturing macrovascular cells, or by Jarrell's technique, using microvascular endothelium. The problems with the latter technique have already been discussed. Pre-culture of macrovascular endothelium is feasible, although not without difficulties. The expertise and facilities necessary for long term endothelial culture are not universal, and are expensive. Pre-culture requires a period of some weeks, which is often unacceptable in the patients who are most in

need of an improved vascular prosthesis. Pre-culturing also introduces a potential source of infection. In addition, Jarrell has observed chromosomal abnormalities in cultured endothelial cells, raising questions as to the advisability of introducing these into human subjects (Jarrell et al 1986). It would seem, therefore, that further development of the microvascular harvesting technique is the avenue most likely to solve the problem of obtaining the high cell numbers required.

One other alternative is to use mesothelium, which is available in very large amounts in the peritoneal cavity. The present studies have shown that this cell type is capable of adhering to the treated ePTFE surface in numbers equivalent to, if not better than macrovascular endothelium, although the electron micrographs have shown that the number of fully spread cells is low at one hour.

Although an immediate confluent covering is obviously desirable, many workers feel that this may not be strictly necessary, the firm adherence of only a small number of cells being required. The minimum density of adherent endothelial cells required to proliferate to confluence in an implanted prosthetic graft is unknown, although Stanley claims to have achieved this in dogs with a seeding density of only 8000 cells per cm^2 (Stanley et al 1985). At this density, however, a period of some weeks is essential to allow the small number of cells present to reach confluence, an achievement which requires a very high number of population doublings. The long term effect of multiple doublings on cell function is unknown, but Williams and colleagues are concerned that genetic damage may ensue (Williams et al 1987). It is advisable, therefore, to strive to cover as much of the graft surface as possible with attached cells, in order that excessive proliferation does not occur.

If as much of the surface can be covered with flattened cells as has been demonstrated on the present SEM studies, then sufficient cells may be present to reach confluence very rapidly, and with a small number of population doublings.

It is known that endothelial cells in culture secrete many of the components of vascular basement membrane (Schor et al 1984; Kramer et al 1984), and it may be that if adequate numbers of cells can be encouraged to attach in the first instance, then these will produce sufficient extracellular matrix protein to stimulate subsequent growth, migration and continuing adherence within the graft lumen.

The scanning electron microscopy studies have demonstrated that a large proportion of the surface of ePTFE can be covered by large "islands" of confluent flattened cells. Cells of this morphology when visualised in tissue culture flasks are attached firmly, and are resistant to disruption by flushing fluid over the surface. In addition, all the scanning electron micrographs were taken after the ePTFE surface had been washed by directing a stream of DPBS forcibly onto the material. All the cells visualised had therefore resisted a certain amount of shear stress, although this was admittedly brief and uncontrolled. The appearance of the cells, does nevertheless suggest that they will be capable of resisting the shear stress of flowing blood. Jarrell, after a series of experiments examining the strength of attachment of microvascular endothelial cells to plasma coated Dacron, concluded that "once cell-to-surface interaction and attachment have taken place, the endothelial cells are very firmly adherent".

The indium labelling experiments confirmed the pattern of adherence found in the other adhesion assays, with an improved cell attachment to those graft segments which were pre-treated with protein, or pre-clotted. The pattern of attachment to pre-treated ePTFE immobilised and stretched in a small seeding chamber therefore also applies to tubular grafts.

The results of the indium labelling experiments, taken in conjunction with the SEM appearances, demonstrate a limitation of isotope labelling techniques as an indicator of cell attachment to a prosthetic surface. Although quantitative information is obtained as to how many cells are adhering, the state of adherent

cells remains unknown, i.e. whether they are rounded up or spread out. Only microscopic examination can provide this information.

9.3 Flow Studies

The higher initial cell attachment rates in these experiments indicates a benefit in incubating the cells on the graft for 90 minutes, rather than for 60 minutes as in the earlier experiments. This may, however, be considered less practical in a clinical context.

The flow studies have clearly shown that endothelial cells which have attached to pre-clotted ePTFE over a 90 minute incubation period, have the ability to resist detachment when exposed to a shear stress of similar magnitude to that experienced in a small diameter vascular graft *in vivo*.

Average flow rates encountered in femoro-distal bypass grafts are commonly stated to be in the order of 100-200 ml/minute (Bernhardt et al 1971; Mannick & Jackson 1966, Wilson & McCormick 1988). Although the viscosity of blood is higher than that of the culture medium used in the experiments, the resulting difference in shear stress is small.

At each of the flow rates tested (25, 100 and 200 ml/minute) there was an initial loss of cells immediately flow commenced. However, over the following 60 minutes, when extensive cell detachment may have been anticipated, the actual cell loss was small, even at the highest flow rate. Scanning electron microscopy confirms that the pre-flow confluent monolayer remains largely intact after 1 hour's flow at the lower flow rates (fig. 44), although at rates of 200 ml/minute, gaps in the monolayer are beginning to appear (fig. 45).

It may be argued that cell detachment may progress after the first hour. It is probable, however, that the maximum cell loss will occur within the first few minutes of the cells' exposure to shear stress. Since the loss over this time is small, it is unlikely to be of significance thereafter.

CHAPTER 10

CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

10.1 Conclusions from individual studies.

These studies demonstrate the following:-

1. Enzymatic harvesting of macrovascular endothelial cells from autogenous vein is at present the easiest and most satisfactory technique for harvesting viable endothelial cells.

2. Enzymatic treatment of omentum consistently releases large numbers of viable mesothelial cells.

3. Enzymatic harvesting of microvascular endothelial cells releases large numbers of viable endothelial cells, but also many contaminating cells.

4. Under optimal conditions the growth characteristics of endothelial and mesothelial cells are similar, both proliferating rapidly to form a confluent monolayer of epithelioid cells covering a surface area many times that of the original tissue. Mesothelial cells are the less fastidious of the two, requiring only the addition of 10% foetal calf serum to grow in culture. Endothelium will only thrive in culture if endothelial cell growth factor and heparin are added to the medium.

5. Endothelial cells adhere to and spread more rapidly upon tissue culture polystyrene than mesothelial cells, whether the surface is coated with extracellular matrix protein or not. For continued growth, however, endothelial cells require a protein coating on the culture surface, whereas mesothelial cells do not.

6. Although endothelial cell attachment to untreated ePTFE is low, pre-coating with extracellular matrix protein and pre-clotting with whole blood both result in significant improvement. When seeded at supra-confluent densities, virtual confluence can be achieved on pre-clotted ePTFE within one hour of seeding, although confluence at this time period does not occur on ePTFE coated with extra-cellular matrix protein, irrespective of seeding density used. It is implied, therefore, that matrix protein binding to ePTFE is patchy and irregular.

7. Attachment of mesothelium to fibronectin coated ePTFE is quantitatively better than that of endothelium. Qualitatively, however, mesothelial attachment after one hour is less advanced, with very few of the attached cells having spread to their full extent.

8. Although indium-111-oxine labelling of cells is a reliable way of estimating the number of cells attaching to an artificial surface, it provides no information as to the condition of the attached cells.

9. Once endothelial cells have formed a confluent monolayer within an ePTFE graft, the cells are attached firmly, and are able to withstand the shear stress likely to be encountered when such a graft is implanted *in vivo.*

The optimum seeding technique for clinical use at the present time is therefore enzymatic derivation of large vessel autologous endothelial cells, with a pre-clot preparation of the ePTFE graft prior to introduction of the cell suspension, rather than mixing the cells with the pre-clot blood described in the original seeding trials (Herring et al 1978). This latter technique clearly results in cell loss when excess clot is evacuated.

The present studies have demonstrated that a period of only one hour is sufficient to result in extensive coverage with areas of confluent flattened cells.

Mesothelial cells may be used as an alternative in procedures in which the abdominal cavity is opened, but the early degree of graft coverage will be less.

10.2 Overall conclusion and suggestions for future work

The work described in this thesis has shown that an endothelial cell lining within an ePTFE vascular graft is a practical proposition. Such a lining can be achieved simply, without special facilities, and without the need for time-consuming procedures or expensive modification of the graft surface. ePTFE pre-clotted with whole blood is a suitable surface for rapid endothelial attachment

and spreading, and may form a confluent monolayer within 1 hour if cell density is sufficiently high.

Once endothelial cells are attached and spread upon the graft surface, early detachment by fluid shear stress does not occur to a significant extent under the conditions encountered *in vivo*.

Future studies should address the problem of improving cell yield, possibly by refining the microvascular endothelial harvesting technique of Jarrel and Williams such that purer endothelial populations are obtained. The question of the more long term durability of adult human cells attached to prosthetic graft surfaces should also be explored *in vitro*.

A clinical trial utilising the above methods would then be necessary, to establish whether attached cells will withstand prolonged shear stress *in vivo*, forming a durable functioning endothelial monolayer, and whether or not this will improve the poor long term patency rates of ePTFE grafts.

APPENDICES

APPENDIX 1

Time Course for Adhesion of Endothelial Cells to Uncoated and Substrate Coated Culture Wells over 120 Minutes

	Coating			
Time	Fibronectin	Collagen	Laminin	Uncoated
15	47 (1.7)	11 (4.0)	4 (1.7)	4 (0.6)
30	56 (1.7)	21 (2.3)	11 (1.2)	18 (2.3)
60	86 (1.2)	54 (1.7)	26 (4.0)	48 (3.5)
120	86 (4.0)	73 (1.7)	49 (1.7)	74 (2.9)

Each value represents the mean and standard error of the mean of 3 separate experiments, using cells from different donors. Figures are percentages of the total number of seeded cells which remain adherent at the different time intervals.

Time Course for Adhesion of Mesothelial Cells to Uncoated and Substrate Coated Culture Wells over 120 Minutes

	Coating			
Time	Fibronectin	Collagen	Laminin	Uncoated
15	29 (2.3)	6 (1.7)	4 (0.6)	3 (0.8)
30	40 (3.5)	16 (1.7)	10 (3.5)	13 (0.6)
60	58 (3.5)	38 (3.5)	18 (3.5)	30 (3.5)
120	72 (2.9)	59 (2.3)	32 (1.7)	51 (6.9)

Each value represents the mean and standard error of the mean of 3 separate experiments, using cells from different donors. Figures are percentages of the total number of seeded cells which remain adherent at the different time intervals.

T values for differences in attachment rates between uncoated and substrate coated wells

a. Endothelium

	Fibronectin	Collagen	Laminin
15 minutes	23.552 ($p<0.001$)	1.7146 NS	0 NS
30 minutes	13.164 ($p<0.001$)	0.91856 NS	2.7111 NS
60 minutes	10.407 ($p<0.001$)	1.5492 NS	4.1331 ($p<0.05$)
120 minutes	2.4162 NS	0.29704 NS	7.4261 ($p<0.01$)

b. Mesothelium

	Fibronectin	Collagen	Laminin
15 minutes	9.0067 ($p<0.001$)	1.2247 NS	0.54772 NS
30 minutes	7.6882 ($p<0.01$)	1.6432 NS	0.85424 NS
60 minutes	5.7155 ($p<0.01$)	1.6330 NS	2.4495 NS
120 minutes	2.7979 ($p<0.05$)	1.0954 NS	2.6605 NS

T values for comparison of endothelial and mesothelial attachment to uncoated and substrate coated culture wells

	Fibronectin	Collagen	Laminin	Uncoated
15 minutes	6.2354 ($p<0.01$)	1.1371 NS	0 NS	0.54772 NS
30 minutes	4.1312 ($p<0.05$)	1.7321 NS	0.27386 NS	2.1004 NS
60 minutes	7.6681 ($p<0.01$)	4.1312 ($p<0.05$)	1.5029 NS	5.1255 ($p<0.01$)
120 minutes	2.8189 ($p<0.05$)	4.8497 ($p<0.01$)	6.9402 ($p<0.001$)	49.398 ($p<0.001$)

RAW DATA

TIME COURSE OF ATTACHMENT TO SUBSTRATE COATED AND UNCOATED CULTURE WELLS

Endothelium

Experiment 1 - Cells in 2nd Passage.

Initial count = 1.8×10^6

Counts after trypsinisation ($\times 10^4$) (= a)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	6.0	2.4	1.4	0.6
30	9.2	3.8	1.4	0.4
60	5.2	5.2	2.4	3.2
120	4.4	7.4	3.8	2.8

Counts after wash (before trypsinisation) ($\times 10^4$) (= b)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	8.0	11.8	16.4	14.4
30	7.2	12.4	14.8	2.4
60	0.8	4.8	11.2	4.4
120	1.2	3.0	3.4	1.2

Percentage adherence (a/a+b)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	43	17	8	4
30	56	23	9	14
60	87	52	18	42
120	79	71	53	70

Experiment 2 - Cells in 2nd Passage

Initial count = 1.8×10^6

Counts after trypsinisation ($\times 10^4$) (= a)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	3.4	2.0	0.4	0.4
30	9.0	4.0	1.6	2.2
60	8.2	4.8	2.2	5.4
120	9.8	6.6	4.0	7.4

Counts after wash (before trypsinisation) ($\times 10^4$) (= b)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	3.6	11.8	12.8	8.8
30	6.4	12.4	11.6	8.8
60	1.6	3.6	7.8	4.8
120	1.6	2.0	4.4	2.8

Percentage adherence (a/a+b)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	49	4	3	4
30	58	24	12	20
60	84	57	32	53
120	86	77	48	73

Experiment 3 - Cells in 2nd Passage

Initial count = 2.2×10^6

Counts after trypsinisation ($\times 10^4$) (= a)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	7.6	2.2	0.4	1.0
30	8.4	2.6	1.6	2.6
60	14.4	9.2	4.6	4.0
120	11.2	5.0	2.8	7.8

Counts after wash (before trypsinisation) ($\times 10^4$) (= b)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	8.4	14.4	18.4	20.0
30	7.6	12.4	13.2	9.6
60	2.4	8.0	12.0	4.4
120	0.8	2.0	3.2	2.0

Percentage adherence (a/a+b)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
5	48	13	2	5
30	53	17	11	21
60	86	53	28	48
120	93	71	47	80

b. Mesothelium

Experiment 1 - Cells in 4th Passage

Initial count = 2.26×10^6

Counts after trypsinisation ($\times 10^4$) (= a)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	4.0	1.0	0.6	0.4
30	4.8	2.6	2.0	1.6
60	7.4	3.2	3.0	1.8
120	5.6	4.0	4.4	3.0

Counts after wash (before trypsinisation) ($\times 10^4$) (= b)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	11.6	25.2	19.2	13.2
30	5.4	11.2	22.8	10.0
60	5.6	4.4	23.6	6.0
120	1.6	2.4	10.4	4.4

Percentage adherence (a/a+b)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	26	4	3	3
30	47	19	8	14
60	57	42	11	23
120	78	63	30	41

Experiment 2 - Cells in 5th Passage

Initial count = 1.9×10^6

Counts after trypsinisation ($\times 10^4$) (= a)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	4.8	1.2	0.4	0.1
30	4.4	3.8	1.6	1.8
60	4.6	3.0	2.2	2.4
120	6.4	5.6	2.8	6.2

Counts after wash (before trypsinisation) ($\times 10^4$) (= b)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	11.6	12.6	8.0	14.0
30	7.6	22.0	7.8	10.8
60	4.0	6.8	8.0	4.4
120	3.2	4.0	5.2	6.4

Percentage adherence (a/a+b)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	29	9	5	1
30	37	15	17	14
60	53	31	22	35
120	67	58	35	49

Experiment 3 - Cells in 2nd Passage

Initial Count = 9.2×10^5

Counts after trypsinisation ($\times 10^4$) (= a)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	2.4	0.4	0.4	0.2
30	2.2	1.0	0.4	0.8
60	2.8	1.6	1.2	1.2
120	3.0	3.4	1.6	2.8

Counts after wash (before trypsinisation) ($\times 10^4$) (= b)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	4.8	8.8	7.6	3.0
30	4.0	6.0	6.4	6.0
60	1.6	2.4	4.8	2.4
120	1.2	2.8	3.6	1.6

Percentage adherence (a/a+b)

	Coating			
	Fn	Col	Lam	Uncoated
Time (min)				
15	33	4	5	6
30	35	14	6	12
60	64	40	20	33
120	71	55	31	64

APPENDIX 2

ADHESION OF ENDOTHELIAL CELLS TO ePTFE

	Coating				
Expt. No.	Fn.	Col	Lam	Uncoated	PC
1.	76	56	50	14	32
2.	66	48	46	16	42
3.	69	64	48	21	43
4.	63	62	55	14	36
5.	33	50	55	30	30
6.	27	37	31	30	44
7.	31	50	31	14	46
8.	26	33	32	12	30
9.	28	25	14	14	48
10.	46	46	38	13	30
Mean	46.5	47.1	40.0	17.8	38.1
SD	20.0	12.4	13.1	6.9	6.9
SEM	6.3	3.9	4.1	1.8	2.2

Fn = fibronectin coated ePTFE

Col = collagen coated ePTFE

Lam = Laminin coated ePTFE

PC = pre-clotted ePTFE

Figures are percentages of the total number of seeded cells which remain adherent one hour after seeding.

ADHESION OF MESOTHELIAL CELLS TO ePTFE

	Coating				
Expt. No.	Fn	Col	Lam	Uncoated	PC
1.	56	38	35	19	33
2.	52	41	32	48	14
3.	50	38	35	11	24
4.	60	32	57	16	18
5.	50	42	38	33	20
6.	58	66	45	21	14
7.	50	56	50	25	15
8.	62	32	38	28	23
9.	52	42	43	25	17
10.	54	42	42	27	21
Mean	54.4	42.9	41.5	25.3	19.9
SD	4.4	10.5	7.6	10.1	5.8
SEM	1.4	3.3	2.4	3.2	1.8

Fn = fibronectin coated ePTFE

Col = collagen coated ePTFE

Lam = Laminin coated ePTFE

PC = pre-clotted ePTFE

Figures are percentages of the total number of seeded cells which remain adherent one hour after seeding.

ADHESION OF ENDOTHELIAL CELLS TO ePTFE "PRE-WETTED" PRIOR TO SUBSTRATE COATING

	Coating			
	Fn	Col	Lam	Uncoated
Expt.No.				
1.	31	67	79	43
2.	38	51	54	43
3.	50	54	38	24
4.	79	72	21	35
5.	38	35	55	23
6.	38	19	43	32
7.	43	50	35	27
8.	50	50	38	23
9.	50	38	57	35
10.	38	50	50	43
Mean	46.0	48.8	47.5	33.9
SD	12.7	15.5	15.2	8.3
SEM	4.0	4.9	4.8	2.6

Fn = fibronectin coated ePTFE

Col = collagen coated ePTFE

Lam = Laminin coated ePTFE

PC = pre-clotted ePTFE

Figures are percentages of the total number of seeded cells which remain adherent one hour after seeding.

ADHESION OF MESOTHELIAL CELLS TO ePTFE "PRE-WETTED" PRIOR TO SUBSTRATE COATING

	Coating			
	Fn	Col	Lam	Uncoated
Expt.No.				
1.	52	58	45	38
2.	62	50	42	50
3.	57	62	50	33
4.	56	43	30	28
5.	55	50	38	26
6.	46	50	33	26
7.	63	44	44	31
8.	53	52	42	36
9.	43	48	36	32
10.	40	42	42	26
Mean	52.7	49.9	40.2	32.2
SD	7.7	6.3	5.6	7.8
SEM	2.4	2.0	3.0	2.5

Fn = fibronectin coated ePTFE

Col = collagen coated ePTFE

Lam = Laminin coated ePTFE

PC = pre-clotted ePTFE

Figures are percentages of the total number of seeded cells which remain adherent one hour after seeding.

Raw Data for ePTFE adhesion experiments

Each flask containing approximately 2 x 10^6 cells was used for 10 experiments carried out simultaneously, giving a seeding density of around 2 x 10^5 per chamber. The actual number of cells used is indicated in the table. In order to allow for variation in the adhesion characteristics of cells from different donors, cells from any one donor were used for a maximum of two experiments in each substrate group.

a. Endothelium

Fibronectin

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	1.6	0.5	2.1	76
2.	1.9	1.0	2.9	66
3.	1.8	0.8	2.6	69
4.	1.0	0.6	1.6	63
5.	0.5	1.0	1.5	33
6.	0.3	0.8	1.1	27
7.	0.5	1.1	1.6	31
8.	0.5	1.4	1.9	26
9.	0.8	2.1	2.9	28
10.	1.1	1.3	2.4	46

Collagen type 4

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	1.4	1.1	2.5	56
2.	1.3	1.4	2.7	48
3.	1.4	0.8	2.2	64
4.	0.8	0.5	1.3	62
5.	1.0	1.0	2.0	50
6.	1.1	1.9	3.0	37
7.	1.0	1.0	2.0	50
8.	0.8	1.6	2.4	33
9.	0.6	1.8	2.4	25
10.	1.1	1.3	2.4	46

Laminin

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	1.1	1.1	2.2	50
2.	1.1	1.3	2.4	46
3.	1.0	1.1	2.1	48
4.	0.6	0.5	1.1	55
5.	0.6	0.5	1.1	55
6.	0.5	1.1	1.6	31
7.	0.5	1.1	1.6	31
8.	1.0	2.1	3.1	32
9.	0.3	1.8	2.1	14
10.	0.6	1.0	1.6	38

Uncoated

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	0.3	1.8	2.1	14
2.	0.5	2.6	3.1	16
3.	0.3	1.1	1.4	21
4.	0.3	1.8	2.1	14
5.	0.6	1.4	2.0	30
6.	0.6	1.4	2.0	30
7.	0.3	1.8	2.1	14
8.	0.3	2.2	2.5	12
9.	0.3	1.9	2.1	14
10.	0.3	2.1	2.4	13

Pre-clotted

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	0.6	1.3	1.9	32
2.	1.0	1.4	2.4	42
3.	1.0	1.3	2.3	43
4.	0.8	1.4	2.2	36
5.	0.8	1.9	2.7	30
6.	0.8	1.0	1.8	44
7.	1.1	1.3	2.4	46
8.	0.6	1.4	2.0	30
9.	1.0	1.1	2.1	48
10.	0.6	1.4	2.0	30

b. Mesothelium

Fibronectin

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	1.0	0.8	1.8	56
2.	1.1	1.0	2.1	52
3.	0.8	0.8	1.6	50
4.	1.0	0.6	1.6	60
5.	1.3	1.3	2.6	50
6.	1.8	1.3	3.1	58
7.	1.3	1.3	2.6	50
8.	1.3	0.8	2.1	62
9.	1.1	1.0	2.1	52
10.	1.3	1.1	2.4	54

Collagen type 4

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	1.0	1.6	2.6	38
2.	1.1	1.6	2.7	41
3.	0.8	1.3	2.1	38
4.	0.6	1.3	1.9	32
5.	0.8	1.1	1.9	42
6.	1.9	1.0	2.9	66
7.	1.0	0.8	1.8	56
8.	0.6	1.3	1.9	32
9.	0.8	1.1	1.9	42
10.	1.3	1.8	3.1	42

Laminin

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	0.6	1.1	1.7	35
2.	0.6	1.3	1.9	32
3.	0.6	1.1	1.7	35
4.	0.8	0.6	1.4	57
5.	1.0	1.6	2.6	38
6.	1.3	1.6	2.9	45
7.	1.1	1.1	2.2	50
8.	0.8	1.3	2.1	38
9.	1.0	1.3	2.3	43
10	0.8	1.1	1.9	42

Uncoated

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	0.3	1.3	1.6	19
2.	1.3	1.4	2.7	48
3.	0.2	1.6	1.8	11
4.	0.5	2.7	3.2	16
5.	0.8	1.6	2.4	33
6.	0.6	2.2	2.8	21
7.	0.6	1.8	2.4	25
8.	0.5	1.3	1.8	28
9.	0.6	1.8	2.4	25
10.	0.6	1.6	2.2	27

Pre-clotted

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	0.5	1.0	1.5	33
2.	0.3	1.8	2.1	14
3.	0.5	1.6	2.1	24
4.	0.3	1.4	1.7	18
5.	0.4	1.6	2.0	20
6.	0.3	1.8	2.1	14
7.	0.3	1.7	2.0	15
8.	0.6	2.0	2.6	23
9.	0.2	1.0	1.2	17
10.	0.4	1.5	1.9	21

c. Endothelium - ePTFE pre-wetted prior to coating

Fibronectin

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	0.5	1.1	1.6	31
2.	1.0	1.6	2.6	38
3.	0.8	0.8	1.6	50
4.	1.1	0.3	1.4	79
5.	0.3	0.5	0.8	38
6.	0.5	0.8	1.3	38
7.	1.0	1.3	2.3	43
8.	1.0	1.0	2.0	50
9.	0.6	0.6	1.2	50
10.	0.6	1.0	1.6	38

Type 4 Collagen

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	1.0	0.5	1.5	67
2.	1.9	1.8	3.7	51
3.	1.3	1.1	2.4	54
4.	1.3	0.5	1.8	72
5.	0.6	1.1	1.7	35
6.	0.5	2.2	2.7	19
7.	1.1	1.1	2.2	50
8.	1.0	1.0	2.0	50
9.	0.8	1.3	2.1	38
10.	0.6	0.6	1.2	50

Laminin

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	1.1	0.3	1.4	79
2.	1.3	1.1	2.4	54
3.	1.0	1.6	2.6	38
4.	0.6	2.2	2.8	21
5.	0.6	0.5	1.1	55
6.	0.6	0.8	1.4	43
7.	0.6	1.1	1.7	35
8.	0.6	1.0	1.6	38
9.	1.3	1.0	2.3	57
10.	0.6	0.6	1.2	50

Uncoated

No.	Count after Trypsinisation ($x10^3$)	Count in Wash ($x10^3$)	Total Count ($x10^3$)	Retention (%)
1.	0.6	0.8	1.4	43
2.	0.6	0.8	1.4	43
3.	0.5	1.6	2.1	24
4.	0.6	1.1	1.7	35
5.	0.3	1.0	1.3	23
6.	0.6	1.3	1.9	32
7.	0.6	1.6	2.2	27
8.	0.3	1.0	1.3	23
9.	0.6	1.1	1.7	35
10.	0.6	0.8	1.4	43

d. Mesothelium - ePTFE pre-wetted prior to coating

Fibronectin

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	1.1	1.0	2.1	52
2.	1.6	1.0	2.6	62
3.	1.6	1.2	2.8	57
4.	1.3	1.0	2.3	56
5.	1.1	0.9	2.0	55
6.	1.2	1.4	2.6	46
7.	1.7	1.0	2.7	63
8.	1.0	0.9	1.9	53
9.	0.9	1.1	2.0	43
10.	0.6	0.9	1.5	40

Collagen type 4

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	1.9	1.4	3.3	58
2.	1.4	1.4	2.8	50
3.	1.3	0.8	2.1	62
4.	1.0	1.3	2.3	43
5.	1.0	1.0	2.0	50
6.	0.8	0.8	1.6	50
7.	1.1	1.4	2.5	44
8.	1.1	1.0	2.1	52
9.	1.0	1.1	2.1	48
10.	0.8	1.1	1.9	42

Laminin

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	1.3	1.6	2.9	45
2.	1.0	1.4	2.4	42
3.	1.3	1.3	2.6	50
4.	0.6	1.4	2.0	30
5.	0.6	1.0	1.6	38
6.	0.8	1.6	2.4	33
7.	1.1	1.4	2.5	44
8.	0.8	1.1	1.9	42
9.	0.8	1.4	2.2	36
10.	0.8	1.1	1.9	42

Uncoated

No.	Count after Trypsinisation ($x10^5$)	Count in Wash ($x10^5$)	Total Count ($x10^5$)	Retention (%)
1.	1.3	2.1	3.4	38
2.	0.8	0.8	1.6	50
3.	0.5	1.0	1.5	33
4.	0.5	1.3	1.8	28
5.	0.5	1.4	1.9	26
6.	0.5	1.4	1.9	26
7.	0.5	1.1	1.6	31
8.	0.8	1.4	2.2	36
9.	0.6	1.3	1.9	32
10.	0.5	1.4	1.9	26

T values for ePTFE attachment studies

Uncoated ePTFE versus substrate coated ePTFE

a. Endothelium

Pre-clot	Fibronectin	Collagen	Laminin
6.5786 ($p<0.001$)	4.2898 ($p<0.001$)	6.5294 ($p<0.001$)	4.7414 ($p<0.001$)

b. Mesothelium

Pre-clot	Fibronectin	Collagen	Laminin
1.4662 NS	8.3562 ($p<0.001$)	3.8201 ($p<0.001$)	4.0529 ($p<0.001$)

T values for comparison of endothelial attachment to ePTFE untreated and pre-treated with alcohol

Fibronectin	Collagen	Laminin	Uncoated
0.06674 NS	0.27083 NS	1.1819 NS	4.7170 ($p < 0.001$)

T values for comparison of mesothelial attachment to ePTFE untreated and pre-treated with alcohol

Fibronectin	Collagen	Laminin	Uncoated
0.60618 NS	1.8078 NS	0.43547 NS	1.7098 NS

Comparison of different substrates (one way analysis of variance)

a. Endothelium

Comparison between attachment rates to fibronectin, collagen, laminin coated and pre-clotted ePTFE:

$F = 1.0703$

Not Significant

b. Mesothelium

Comparison between attachment rates to fibronectin, collagen, laminin coated and pre-clotted ePTFE:

$F = 37.501$

$(p<0.001)$

Comparison between attachment rates to fibronectin, collagen, and laminin coated ePTFE:

$F = 8.0221$

$(p<0.01)$

T value for attachment to fibronectin compared to collagen type 4:

3.1943 ($p < 0.01$).

T value for attachment to fibronectin compared to laminin:

4.6452 ($p<0.001$).

T value for attachment to pre-clotted compared to uncoated ePTFE:

1.4662 (N.S.).

APPENDIX 3

ENDOTHELIAL ATTACHMENT TO GRAFT SEGMENTS AS MEASURED BY INDIUM LABELLING OF CELLS

	Coating				
	Fn	Col	Lam	Uncoated	PC
Expt.No.					
1.	43	20	35	0	30
2.	34	13	24	1	38
3.	29	78	53	28	42
4.	69	42	58	9	25
5.	43	47	5	16	45
Mean	43.6	40.0	35	10.8	36.0
SD	15.4	25.6	21.6	11.6	8.3
SEM	6.9	11.4	2.2	5.2	3.7

Fn = fibronectin coated ePTFE

Col = collagen coated ePTFE

Lam = Laminin coated ePTFE

PC = pre-clotted ePTFE

Figures are percentages of the total number of seeded cells which remain adherent one hour after seeding.

MESOTHELIAL ATTACHMENT TO GRAFT SEGMENTS AS MEASURED BY INDIUM LABELLING OF CELLS

	Coating				
	Fn	Col	Lam	Uncoated	PC
Expt.No.					
1.	79	65	31	0	16
2.	68	35	54	16	22
3.	63	33	34	0	28
4.	75	68	59	10	12
5.	50	38	45	8	14
Mean	67.0	47.8	44.6	6.8	18.4
SD	11.3	17.2	12.2	6.9	6.5
SEM	5.1	7.7	5.5	3.1	2.9

Fn = fibronectin coated ePTFE

Col = collagen coated ePTFE

Lam = Laminin coated ePTFE

PC = pre-clotted ePTFE

Figures are percentages of the total number of seeded cells which remain adherent one hour after seeding.

RAW DATA FOR INDIUM LABELLING EXPERIMENTS

Confluent cells from one flask were used for two experiments (giving a seeding density of approximately 10^6 cells per graft segment, assuming 2×10^6 per flask). Each flask of cells was trypsinised, labelled, centrifuged, labelling efficiency measured, and the suspension divided into two, ready for seeding into two graft segments. The initial radioactivity counts in the following tables were measured after the suspension had been divided into two, and immediately prior to seeding. Graft segments were 5cm long by 6mm internal diameter,
giving a surface area of approximately $9.4cm^2$. Seeding density per cm^2 was therefore 1.06×10^5, i.e. just over confluent density.

1. Endothelium

a. Fibronectin coated grafts

Experiment 1

Initial radioactivity counts (cpm)		72612
Counts in cell pellet		45924
Labelling efficiency		**63%**
Counts in graft after 1 hour's incubation		16332
Counts in effluent from graft		23040
		39372
Spontaneous leak over 1 hour		10%
Total cellular counts after 1 hour	=	90% of 39372
	=	**35436**
Counts in cell pellet after centrifugation of effluent	=	20304
Therefore cell counts in graft	=	35436 - 20304
	=	**15132**
Percentage retention	=	15132/35436 x 100
	=	**43%**

Experiment 2

Initial radioactivity counts (cpm)		37560
Counts in cell pellet		4884
Labelling efficiency		**13%**
Counts in graft after 1 hour's incubation		996
Counts in effluent from graft		3492
		4488
Spontaneous leak over 1 hour		62%
Total cellular counts after 1 hour	=	38% of 4488
	=	**1705**
Counts in cell pellet after centrifugation of effluent	=	1128
Therefore cell counts in graft	=	1705 - 1128
	=	577
Percentage retention	=	577/1705 x 100
	=	**34%**

Experiment 3

Initial radioactivity counts (cpm)		64428
Counts in cell pellet		27060
Labelling efficiency		**42%**
Counts in graft after 1 hour's incubation		3924
Counts in effluent from graft		15876
		19800
Spontaneous leak over 1 hour		41%
Total cellular counts after 1 hour	=	59% of 19800
	=	**11688**
Counts in cell pellet after centrifugation of effluent	=	8244
Therefore cell counts in graft	=	11688 - 8244
	=	**3444**
Percentage retention	=	3444/11688 x 100
	=	**29%**

Experiment 4

Initial radioactivity counts (cpm)		36768
Counts in cell pellet		10296
Labelling efficiency		**28%**
Counts in graft after 1 hour's incubation		5268
Counts in effluent from graft		3588
		8856
Spontaneous leak over 1 hour		20%
Total cellular counts after 1 hour	=	80% of 8856
	=	**7084**
Counts in cell pellet after centrifugation of effluent	=	2184
Therefore cell counts in graft	=	7085 - 2184
	=	**4901**
Percentage retention	=	4901/7084 x 100
	=	**69%**

Experiment 5

Initial radioactivity counts (cpm)		44892
Counts in cell pellet		16608
Labelling efficiency		**37%**
Counts in graft after 1 hour's incubation		9120
Counts in effluent from graft		6012
		15132
Spontaneous leak over 1 hour		33%
Total cellular counts after 1 hour	=	67% of 15132
	=	**10138**
Counts in cell pellet after centrifugation of effluent	=	5796
Therefore cell counts in graft	=	10138 - 5796
	=	**4342**
Percentage retention	=	4342/10138 x 100
	=	**43%**

b. Collagen type 4 coated grafts

Experiment 1

Initial radioactivity counts (cpm)		53832
Counts in cell pellet		33912
Labelling efficiency		**63%**
Counts in graft after 1 hour's incubation		5520
Counts in effluent from graft		24024
		29544
Spontaneous leak over 1 hour		10%
Total cellular counts after 1 hour	=	90% of 29544
	=	**26590**
Counts in cell pellet after centrifugation of effluent	=	21384
Therefore cell counts in graft	=	26590 - 21384
	=	**5206**
Percentage retention	=	5206/26590 x 100
	=	**20%**

Experiment 2

Initial radioactivity counts (cpm)		75396
Counts in cell pellet		37116
Labelling efficiency		**49%**
Counts in graft after 1 hour's incubation		10212
Counts in effluent from graft		20244
		30456
Spontaneous leak over 1 hour		41%
Total cellular counts after 1 hour	=	59% of 30456
	=	**17969**
Counts in cell pellet after centrifugation of effluent	=	15552
Therefore cell counts in graft	=	17969 - 15552
	=	**2417**
Percentage retention		2417/17969 x 100
	=	**13%**

Experiment 3

Initial radioactivity counts (cpm)		63168
Counts in cell pellet		38532
Labelling efficiency		**61%**
Counts in graft after 1 hour's incubation		25620
Counts in effluent from graft		8508
		34128
Spontaneous leak over 1 hour		32%
Total cellular counts after 1 hour	=	68% of 34128
	=	**23207**
Counts in cell pellet after centrifugation of effluent	=	5064
Therefore cell counts in graft	=	23207 - 5064
	=	**18143**
Percentage retention	=	18143/23207 x 100
	=	**78%**

Experiment 4

Initial radioactivity counts (cpm)		90144
Counts in cell pellet		26700
Labelling efficiency		**30%**
Counts in graft after 1 hour's incubation		9744
Counts in effluent from graft		11124
		20868
Spontaneous leak over 1 hour		31%
Total cellular counts after 1 hour	=	69% of 20868
	=	**14399**
Counts in cell pellet after centrifugation of effluent	=	8376
Therefore cell counts in graft	=	14399 - 8376
		6023
Percentage retention	=	6023/14399 x 100
	=	**42%**

Experiment 5

Initial radioactivity counts (cpm)		10969
Counts in cell pellet		31092
Labelling efficiency		**28%**
Counts in graft after 1 hour's incubation		18420
Counts in effluent from graft		15828
		34248
Spontaneous leak over 1 hour		43%
Total cellular counts after 1 hour	=	57% of 34248
	=	**19521**
Counts in cell pellet after centrifugation of effluent	=	10296
Therefore cell counts in graft	=	19521 - 10296
	=	**9225**
Percentage retention	=	9225/19521 x 100
	=	**47%**

c. Laminin coated grafts

Initial radioactivity counts (cpm)		37572
Counts in cell pellet		4884
Labelling efficiency		**13%**
Counts in graft after 1 hour's incubation		696
Counts in effluent from graft		2904
		3600
Spontaneous leak over 1 hour		62%
Total cellular counts after 1 hour	=	38% of 3600
	=	**1368**
Counts in cell pellet after centrifugation of effluent	=	888
Therefore cell counts in graft	=	1368 - 888
	=	**480**
Percentage retention	=	480/1368 x 100
	=	**35%**

Experiment 2

Initial radioactivity counts (cpm)		74856
Counts in cell pellet		37128
Labelling efficiency		**50%**
Counts in graft after 1 hour's incubation		12744
Counts in effluent from graft		16200
		28944
Spontaneous leak over 1 hour		41%
Total cellular counts after 1 hour	=	59% of 28944
	=	**17077**
Counts in cell pellet after centrifugation of effluent	=	12900
Therefore cell counts in graft	=	17077 - 12900
	=	**4177**
Percentage retention	=	4177/17077 x 100
	=	**24%**

Experiment 3

Initial radioactivity counts (cpm)		55860
Counts in cell pellet		23460
Labelling efficiency		**42%**
Counts in graft after 1 hour's incubation		5448
Counts in effluent from graft		10920
		16368
Spontaneous leak over 1 hour		41%
Total cellular counts after 1 hour	=	59% of 16368
	=	**9657**
Counts in cell pellet after centrifugation of effluent	=	4584
Therefore cell counts in graft	=	9657 - 4584
	=	**5073**
Percentage retention	=	5073/9657 x 100
	=	**53%**

Experiment 4

Initial radioactivity counts (cpm)		83796
Counts in cell pellet		24300
Labelling efficiency		**29%**
Counts in graft after 1 hour's incubation		11568
Counts in effluent from graft		9564
		21132
Spontaneous leak over 1 hour		31%
Total cellular counts after 1 hour	=	69% of 21132
	=	**14581**
Counts in cell pellet after centrifugation of effluent	=	6192
Therefore cell counts in graft	=	14581 - 6192
	=	**8389**
Percentage retention	=	8389/14581 x 100
	=	**58%**

Experiment 5

Initial radioactivity counts (cpm)		95814
Counts in cell pellet		26829
Labelling efficiency		**28%**
Counts in graft after 1 hour's incubation		7128
Counts in effluent from graft		18126
		25254
Spontaneous leak over 1 hour		43%
Total cellular counts after 1 hour	=	57% of 25254
	=	**14395**
Counts in cell pellet after centrifugation of effluent	=	13680
Therefore cell counts in graft	=	14395 - 13680
	=	**715**
Percentage retention	=	715/14395 x 100
	=	**5%**

d. Uncoated grafts

Experiment 1

Initial radioactivity counts (cpm)		78804
Counts in cell pellet		32448
Labelling efficiency		**41%**
Counts in graft after 1 hour's incubation		2184
Counts in effluent from graft		22524
		24708
Spontaneous leak over 1 hour		47%
Total cellular counts after 1 hour	=	53% of 24708
	=	**13095**
Counts in cell pellet after centrifugation of effluent	=	17988
Therefore cell counts in graft	=	13095 - 17988
	=	**0**
Percentage retention	=	**0%**

Experiment 2

Initial radioactivity counts (cpm)		52416
Counts in cell pellet		17220
Labelling efficiency		**33%**
Counts in graft after 1 hour's incubation		2352
Counts in effluent from graft		10584
		12936
Spontaneous leak over 1 hour		37%
Total cellular counts after 1 hour	=	63% of 12936
	=	**8150**
Counts in cell pellet after centrifugation of effluent	=	8076
Therefore cell counts in graft	=	8150 - 8076
	=	**74**
Percentage retention	=	74/8150 x 100
	=	**1%**

Experiment 3

Initial radioactivity counts (cpm)		57276
Counts in cell pellet		34932
Labelling efficiency		**61%**
Counts in graft after 1 hour's incubation		5268
Counts in effluent from graft		22356
		27624
Spontaneous leak over 1 hour		32%
Total cellular counts after 1 hour	=	68% of 27624
	=	**18784**
Counts in cell pellet after centrifugation of effluent	=	13548
Therefore cell counts in graft	=	18784 - 13548
	=	**5236**
Percentage retention	=	5236/18784 x 100
	=	**28%**

Experiment 4

Initial radioactivity counts (cpm)		65076
Counts in cell pellet		26676
Labelling efficiency		**41%**
Counts in graft after 1 hour's incubation		5184
Counts in effluent from graft		14688
		19872
Spontaneous leak over 1 hour		31%
Total cellular counts after 1 hour	=	69% of 19872
	=	**13712**
Counts in cell pellet after centrifugation of effluent	=	12480
Therefore cell counts in graft	=	13712 - 12480
	=	**1232**
Percentage retention	=	1232/13712 x 100
	=	**9%**

Experiment 5

Initial radioactivity counts (cpm)		51432
Counts in cell pellet		20568
Labelling efficiency		**40%**
Counts in graft after 1 hour's incubation		7260
Counts in effluent from graft		10944
		18204
Spontaneous leak over 1 hour		30%
Total cellular counts after 1 hour	=	70% of 18204
	=	**12742**
Counts in cell pellet after centrifugation of effluent	=	10704
Therefore cell counts in graft	=	12742 - 10704
	=	**2038**
Percentage retention	=	2038/12742 x 100
	=	**16%**

Pre-clotted Grafts

Experiment 1

Initial radioactivity counts (cpm)		56448
Counts in cell pellet		12984
Labelling efficiency		**23%**
Counts in graft after 1 hour's incubation		1944
Counts in effluent from graft		5316
		7260
Spontaneous leak over 1 hour		57%
Total cellular counts after 1 hour	=	43% of 7260
	=	**3122**
Counts in cell pellet after centrifugation of effluent	=	2184
Therefore cell counts in graft	=	3122 - 2184
	=	**938**
Percentage retention	=	938/3122 x 100
	=	**30%**

Experiment 2

Initial radioactivity counts (cpm)		54504
Counts in cell pellet		15096
Labelling efficiency		**28%**
Counts in graft after 1 hour's incubation		5340
Counts in effluent from graft		7584
		12924
Spontaneous leak over 1 hour		20%
Total cellular counts after 1 hour	=	80% of 12924
	=	**10339**
Counts in cell pellet after centrifugation of effluent	=	6408
Therefore cell counts in graft	=	10339 - 6408
	=	**3931**
Percentage retention	=	3931/10339 x 100
	=	**38%**

Experiment 3

Initial radioactivity counts (cpm)		44892
Counts in cell pellet		16608
Labelling efficiency		**37%**
Counts in graft after 1 hour's incubation		6516
Counts in effluent from graft		8664
		15180
Spontaneous leak over 1 hour		33%
Total cellular counts after 1 hour	=	67% of 15180
	=	**10171**
Counts in cell pellet after centrifugation of effluent	=	5904
Therefore cell counts in graft	=	10171 - 5904
	=	**4267**
Percentage retention	=	4267/10171 x 100
	=	**42%**

Experiment 4

Initial radioactivity counts (cpm)		90144
Counts in cell pellet		26700
Labelling efficiency		**30%**
Counts in graft after 1 hour's incubation		5628
Counts in effluent from graft		18780
		24408
Spontaneous leak over 1 hour		31%
Total cellular counts after 1 hour	=	69% of 24408
	=	**16842**
Counts in cell pellet after centrifugation of effluent	=	12624
Therefore cell counts in graft	=	16842 - 12624
	=	**4218**
Percentage retention	=	4218/16842 x 100
	=	**25%**

Experiment 5

Initial radioactivity counts (cpm)		91380
Counts in cell pellet		26700
Labelling efficiency		**29%**
Counts in graft after 1 hour's incubation		9900
Counts in effluent from graft		14604
		24504
Spontaneous leak over 1 hour		31%
Total cellular counts after 1 hour	=	69% of 24504
	=	**16908**
Counts in cell pellet after centrifugation of effluent	=	9216
Therefore cell counts in graft	=	16908 - 9216
	=	**7692**
Percentage retention	=	7692/16908 x 100
	=	**45%**

2. MESOTHELIUM

a Fibronectin coated grafts

Experiment 1

Initial radioactivity counts		73224
Counts in cell pellet		35652
Labelling efficiency		**49%**
Counts in graft after 1 hour's incubation		18456
Counts in effluent from graft		7404
		25860
Spontaneous leak over 1 hour		27%
Total cellular counts after 1 hour	=	73% of 25860
	=	**18878**
Counts in cell pellet after centrifugation of effluent	=	3888
Therefore cell counts in graft	=	18878 - 3888
	=	**14990**
Percentage retention	=	14990/18878 x 100
	=	**79%**

Experiment 2

Initial radioactivity counts		58068
Counts in cell pellet		44712
Labelling efficiency		**77%**
Counts in graft after 1 hour's incubation		25248
Counts in effluent from graft		13464
		38712
Spontaneous leak over 1 hour		24%
Total cellular counts after 1 hour	=	76% of 38712
	=	**29421**
Counts in cell pellet after centrifugation of effluent	=	9312
Therefore cell counts in graft	=	29421 - 9312
	=	**20109**
Percentage retention	=	20109/29421 x 100
	=	**68%**

Experiment 3

Initial radioactivity counts		53832
Counts in cell pellet		19920
Labelling efficiency		**37%**
Counts in graft after 1 hour's incubation		8868
Counts in effluent from graft		6432
		15300
Spontaneous leak over 1 hour		11%
Total cellular counts after 1 hour	=	89% of 15300
	=	**13617**
Counts in cell pellet after centrifugation of effluent	=	4992
Therefore cell counts in graft	=	13617 - 4992
	=	**8625**
Percentage retention	=	8625/13617 x 100
	=	**63%**

Experiment 4

Initial radioactivity counts		32280
Counts in cell pellet		15492
Labelling efficiency		**48%**
Counts in graft after 1 hour's incubation		6576
Counts in effluent from graft		3024
		9600
Spontaneous leak over 1 hour		43%
Total cellular counts after 1 hour	=	57% of 9600
	=	**5472**
Counts in cell pellet after centrifugation of effluent	=	1368
Therefore cell counts in graft	=	5472 - 1368
	=	**4104**
Percentage retention	=	4104/5472 x 100
	=	**75%**

Experiment 5

Initial radioactivity counts		65664
Counts in cell pellet		45312
Labelling efficiency		**69%**
Counts in graft after 1 hour's incubation		22284
Counts in effluent from graft		17364
		39648
Spontaneous leak over 1 hour		36%
Total cellular counts after 1 hour	=	64% of 39648
	=	**25374**
Counts in cell pellet after centrifugation of effluent	=	12660
Therefore cell counts in graft	=	25374 - 12660
	=	**12714**
Percentage retention	=	12714/25374 x 100
	=	**50%**

Collagen coated grafts

Experiment 1

Initial radioactivity counts		106080
Counts in cell pellet		44616
Labelling efficiency		**42%**
Counts in graft after 1 hour's incubation		18000
Counts in effluent from graft		16236
		34236
Spontaneous leak over 1 hour		34%
Total cellular counts after 1 hour	=	66% of 34236
	=	**22596**
Counts in cell pellet after centrifugation of effluent	=	7824
Therefore cell counts in graft	=	22596 - 7824
	=	**14772**
Percentage retention	=	14772/22596 x 100
	=	**65%**

Experiment 2

Initial radioactivity counts		80220
Counts in cell pellet		61212
Labelling efficiency		**76%**
Counts in graft after 1 hour's incubation		18000
Counts in effluent from graft		33120
		51120
Spontaneous leak over 1 hour		45%
Total cellular counts after 1 hour	=	55% of 51120
	=	**28116**
Counts in cell pellet after centrifugation of effluent	=	18228
Therefore cell counts in graft	=	28116 - 18228
	=	**9888**
Percentage retention	=	9888/28116 x 100
	=	**35%**

Experiment 3

Initial radioactivity counts		44400
Counts in cell pellet		21492
Labelling efficiency		**48%**
Counts in graft after 1 hour's incubation		5016
Counts in effluent from graft		8100
		13116
Spontaneous leak over 1 hour		43%
Total cellular counts after 1 hour	=	57% of 13116
	=	**7476**
Counts in cell pellet after centrifugation of effluent	=	5004
Therefore cell counts in graft	=	7476 - 5004
	=	**2472**
Percentage retention	=	2472/7476 x 100
	=	**33%**

Experiment 4

Initial radioactivity counts		59532
Counts in cell pellet		41076
Labelling efficiency		**69%**
Counts in graft after 1 hour's incubation		25716
Counts in effluent from graft		13128
		38844
Spontaneous leak over 1 hour		24%
Total cellular counts after 1 hour	=	76% of 38844
	=	**29521**
Counts in cell pellet after centrifugation of effluent	=	9372
Therefore cell counts in graft	=	29521 - 9372
	=	**20149**
Percentage retention	=	20149/29521 x 100
	=	**68%**

Experiment 5

Initial radioactivity counts		56556
Counts in cell pellet		13008
Labelling efficiency		**23%**
Counts in graft after 1 hour's incubation		2148
Counts in effluent from graft		5412
		7560
Spontaneous leak over 1 hour		39%
Total cellular counts after 1 hour	=	61% of 7560
	=	**4612**
Counts in cell pellet after centrifugation of effluent	=	2856
Therefore cell counts in graft	=	4612 - 2856
	=	**1756**
Percentage retention	=	1756/4612 x 100
	=	**38%**

Laminin coated grafts

Experiment 1

Initial radioactivity counts		80220
Counts in cell pellet		61212
Labelling efficiency		**76%**
Counts in graft after 1 hour's incubation		12084
Counts in effluent from graft		37164
		49248
Spontaneous leak over 1 hour		45%
Total cellular counts after 1 hour	=	55% of 49248
	=	**27086**
Counts in cell pellet after centrifugation of effluent	=	18720
Therefore cell counts in graft	=	27086 - 18720
	=	**8366**
Percentage retention	=	8366/27086 x 100
	=	**31%**

Experiment 2

Initial radioactivity counts		53832
Counts in cell pellet		19920
Labelling efficiency		**37%**
Counts in graft after 1 hour's incubation		7344
Counts in effluent from graft		7680
		15024
Spontaneous leak over 1 hour		11%
Total cellular counts after 1 hour	=	89% of 15024
	=	**13371**
Counts in cell pellet after centrifugation of effluent	=	6084
Therefore cell counts in graft	=	13371 - 6084
	=	**7287**
Percentage retention	=	7287/13371 x 100
	=	**54%**

Experiment 3

Initial radioactivity counts		100236
Counts in cell pellet		83064
Labelling efficiency		69%
Counts in graft after 1 hour's incubation		9060
Counts in effluent from graft		32064
		41124
Spontaneous leak over 1 hour		36%
Total cellular counts after 1 hour	=	64% of 41124
	=	**26319**
Counts in cell pellet after centrifugation of effluent	=	17364
Therefore cell counts in graft	=	26319 - 17364
	=	**8955**
Percentage retention	=	8955/26319 x 100
	=	**34%**

Experiment 4

Initial radioactivity counts		31800
Counts in cell pellet		14628
Labelling efficiency		46%
Counts in graft after 1 hour's incubation		6960
Counts in effluent from graft		3300
		10260
Spontaneous leak over 1 hour		27%
Total cellular counts after 1 hour	=	73% of 10260
	=	7490
Counts in cell pellet after centrifugation of effluent	=	3072
Therefore cell counts in graft	=	7490 - 3072
	=	**4418**
Percentage retention	=	4418/7490 x 100
	=	**59%**

Experiment 5

Initial radioactivity counts		102468
Counts in cell pellet		58404
Labelling efficiency		**57%**
Counts in graft after 1 hour's incubation		17844
Counts in effluent from graft		33588
		51432
Spontaneous leak over 1 hour		36%
Total cellular counts after 1 hour	=	64% of 51432
	=	**32916**
Counts in cell pellet after centrifugation of effluent	=	18108
Therefore cell count in graft	=	32916 - 18108
	=	**14808**
Percentage retention	=	14808/32916 x 100
	=	**45%**

Uncoated grafts

Experiment 1

Initial radioactivity counts		81804
Counts in cell pellet		56448
Labelling efficiency		**69%**
Counts in graft after 1 hour's incubation		5736
Counts in effluent from graft		44052
		49788
Spontaneous leak over 1 hour		36%
Total cellular counts after 1 hour	=	64% of 49788
	=	**31864**
Counts in cell pellet after centrifugation of effluent	=	33528
Therefore cell count in graft	=	31864 - 33528
	=	0
Percentage retention	=	0
	=	**0%**

Experiment 2

Initial radioactivity counts		62748
Counts in cell pellet		48312
Labelling efficiency		**77%**
Counts in graft after 1 hour's incubation		9912
Counts in effluent from graft		31032
		40944
Spontaneous leak over 1 hour		24%
Total cellular counts after 1 hour	=	76% of 40944
	=	**31114**
Counts in cell pellet after centrifugation of effluent	=	26280
Therefore cell count in graft	=	31114 - 26280
	=	**4834**
Percentage retention	=	4834/31114 x 100
	=	**16%**

Experiment 3

Initial radioactivity counts		94824
Counts in cell pellet		65184
Labelling efficiency		**69%**
Counts in graft after 1 hour's incubation		3408
Counts in effluent from graft		46044
		49452
Spontaneous leak over 1 hour		24%
Total cellular counts after 1 hour	=	76% of 49452
	=	37584
Counts in cell pellet after centrifugation of effluent	=	41436
Therefore cell count in graft	=	37584 - 41436
	=	**0**
Percentage retention	=	0
	=	**0%**

Experiment 4

Initial radioactivity counts		81900
Counts in cell pellet		42984
Labelling efficiency		**57%**
Counts in graft after 1 hour's incubation		9144
Counts in effluent from graft		30552
		39696
Spontaneous leak over 1 hour		23%
Total cellular counts after 1 hour	=	67% of 39696
	=	**26596**
Counts in cell pellet after centrifugation of effluent	=	23940
Therefore cell count in graft	=	26596 - 23940
	=	**2656**
Percentage retention	=	2656/26596 x 100
	=	**10%**

Experiment 5

Initial radioactivity counts		89940
Counts in cell pellet		37776
Labelling efficiency		**42%**
Counts in graft after 1 hour's incubation		10368
Counts in effluent from graft		25920
		36288
Spontaneous leak over 1 hour		36%
Total cellular counts after 1 hour	=	67% of 36288
	=	**24313**
Counts in cell pellet after centrifugation of effluent	=	22368
Therefore cell count in graft	=	24313 - 22368
	=	**1945**
Percentage retention	=	1945/24313 x 100
	=	**8%**

Pre-clotted grafts

Experiment 1

Initial radioactivity counts		67656
Counts in cell pellet		15564
Labelling efficiency		**23%**
Counts in graft after 1 hour's incubation		4212
Counts in effluent from graft		8064
		12276
Spontaneous leak over 1 hour		36%
Total cellular counts after 1 hour	=	64% x 12276
	=	**7857**
Counts in cell pellet after centrifugation of effluent	=	6600
Therefore cell count in graft	=	7857 - 6600
	=	**1257**
Percentage retention	=	1257/7857 x 100
	=	**16%**

Experiment 2

Initial radioactivity counts		75564
Counts in cell pellet		56520
Labelling efficiency		**68%**
Counts in graft after 1 hour's incubation		21192
Counts in effluent from graft		33624
		54816
Spontaneous leak over 1 hour		24%
Total cellular counts after 1 hour	=	76% of 54816
	=	**41660**
Counts in cell pellet after centrifugation of effluent	=	32616
Therefore cell count in graft	=	41660 - 32616
	=	**9044**
Percentage retention	=	9044/41660 x 100
	=	**22%**

Experiment 3

Initial radioactivity counts		65784
Counts in cell pellet		39468
Labelling efficiency		**60%**
Counts in graft after 1 hour's incubation		10752
Counts in effluent from graft		25092
		35844
Spontaneous leak over 1 hour		30%
Total cellular counts after 1 hour	=	70% of 35844
	=	**25091**
Counts in cell pellet after centrifugation of effluent	=	18084
Therefore cell count in graft	=	25091 - 18084
	=	**7007**
Percentage retention	=	7007/25091 x 100
	=	**28%**

Experiment 4

Initial radioactivity counts		72144
Counts in cell pellet		43284
Labelling efficiency		**60%**
Counts in graft after 1 hour's incubation		7128
Counts in effluent from graft		28896
		36024
Spontaneous leak over 1 hour		30%
Total cellular counts after 1 hour	=	70% of 36024
	=	**25217**
Counts in cell pellet after centrifugation of effluent	=	22224
Therefore cell count in graft	=	25217 - 22224
	=	**2993**
Percentage retention	=	2993/25217 x 100
	=	**12%**

Experiment 5

Initial radioactivity counts		79032
Counts in cell pellet		48996
Labelling efficiency		**62%**
Counts in graft after 1 hour's incubation		12816
Counts in effluent from graft		33684
		46500
Spontaneous leak over 1 hour		34%
Total cellular counts after 1 hour	=	66% of 46500
	=	**30690**
Counts in cell pellet after centrifugation of effluent	=	26280
Therefore cell count in graft	=	30690 - 26280
	=	**4410**
Percentage retention	=	4410/30690 x 100
	=	**14%**

T values for indium labelling studies. Comparison of uncoated with substrate coated grafts

a. Endothelium

Pre-clotted	Fibronectin	Collagen	Laminin
3.9505 ($p<0.01$)	3.8041 ($p<0.01$)	2.3231 ($p<0.05$)	2.2071 NS

b. Mesothelium

Pre-clotted	Fibronectin	Collagen	Laminin
2.7363 ($p<0.05$)	10.167 ($p<0.001$)	4.9469 ($p<0.01$)	6.0305 ($p<0.001$)

Comparison of different substrates (one way analysis of variance)

a. Endothelium

Comparison between attachment rates to fibronectin, collagen, laminin coated and pre-clotted graft segments:

$F = 0.21789$

Not significant

b. Mesothelium

Comparison between attachment rates to fibronectin, collagen, laminin coated and pre-clotted graft segments:

$F = 12.998$

$(p<0.001)$

Comparison between attachment rates to fibronectin, collagen and laminin coated graft segments:

$F = 3.8456$

(not significant)

Comparison between attachment rates to pre-clotted and collagen of laminin coated graft segments:

$F = 8.0147$

$(p<0.01)$

APPENDIX 4

Cell numbers, labelling efficiency and initial attachment rates for flow experiments (6 experiments)

25 ml/minute

Cell number ($\times 10^6$)	Labelling efficiency (%)	Attachment(%)
2.33	44.2	77.0
1.53	19.7	78.2
3.30	62.3	71.3
3.30	67.1	63.0
2.09	63.6	87.3
2.40	61.0	93.9

100 ml/minute

Cell number ($\times 10^6$)	Labelling efficiency (%)	Attachment (%)
2.29	61.8	75.6
3.00	68.2	85.5
3.67	61.4	71.4
3.97	81.3	67.7
2.13	83.3	94.4
2.23	83.3	80.7

200 ml/minute

Cell number ($\times 10^6$)	Labelling efficiency (%)	Attachment (%)
2.18	70.2	55.9
1.69	75.3	80.0
2.97	68.8	79.5
3.58	64.3	77.3
2.84	71.7	86.5
5.91	81.8	83.3

Cell retention after flow at 25 ml/minute (6 experiments)

0_1	0_2	5	10	20	30	40	50	60
100	87.5	84.3	83.2	82.2	80.8	79.4	78.2	77.4
100	94.7	94.0	93.9	91.2	89.6	87.5	87.7	86.6
100	99.6	99.1	99.3	97.3	94.3	94.5	93.4	91.6
100	94.8	91.6	92.3	90.4	89.7	88.7	88.9	87.9
100	96.7	95.5	95.8	94.6	93.7	94.4	94.2	94.1
100	95.5	85.8	83.7	81.8	79.7	78.8	77.4	76.7
Mean	94.8	91.7	91.4	89.6	88.0	87.2	86.6	85.7
SD	4.0	5.7	6.6	6.4	6.3	6.9	7.3	7.2
SEM	1.6	2.3	2.7	2.4	2.6	2.8	2.4	2.4

Cell retention after flow at 100 ml/minute (6 experiments)

0_1	0_2	5	10	20	30	40	50	60
100	89.2	85.9	84.9	83.0	81.3	81.0	80.0	79.4
100	92.9	91.9	91.2	89.7	88.7	87.7	87.7	86.8
100	83.3	83.0	81.7	79.2	77.0	75.7	74.9	74.6
100	84.5	80.6	78.5	76.9	76.4	76.1	75.5	73.6
100	95.6	95.7	96.0	94.5	93.7	93.5	93.3	93.1
100	96.0	94.5	93.5	93.1	92.2	91.7	91.2	90.2
Mean	90.3	88.6	87.6	86.1	84.9	84.3	83.5	82.9
SD	5.5	6.3	7.0	7.4	7.7	7.8	8.2	8.5
SEM	2.2	2.6	2.8	3.0	3.1	3.2	3.3	3.5

Cell retention after flow at 200 ml/minute (6 experiments)

0_1	0_2	5	10	20	30	40	50	60
100	95.1	96.7	95.3	94.9	93.5	92.4	91.9	90.7
100	83.9	83.6	82.1	80.7	79.1	78.2	76.7	76.5
100	80.1	75.4	71.2	69.3	68.0	66.4	66.0	64.9
100	93.0	88.8	86.7	84.2	82.8	80.6	78.7	78.1
100	94.3	93.2	92.7	91.6	90.9	87.9	87.6	86.5
100	92.4	89.5	88.1	86.7	84.3	83.3	82.1	80.7
Mean	89.8	87.9	86.0	84.6	83.1	81.5	80.5	79.6
SD	6.2	7.5	8.6	9.0	9.1	9.0	9.1	8.9
SEM	2.5	3.1	3.5	2.4	3.7	3.7	2.4	3.7

DATA FOR FLOW EXPERIMENTS

Experiment 1 - 25 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	94785	100
0_2	82944	87.5
5	79995	84.3
10	78893	83.2
20	77967	82.2
30	76571	80.8
40	75243	79.4
50	74129	78.2
60	73318	77.4

Experiment 2 - 25 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	73295	100
0_2	69412	94.7
5	68912	94.0
10	68871	93.9
20	66847	91.1
30	65674	89.6
40	64157	87.5
50	64291	87.7
60	60525	86.6

Experiment 3 - 25 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	79880	100
0_2	79575	99.6
5	79199	99.1
10	79345	99.3
20	77708	97.3
30	75304	94.3
40	75460	94.5
50	74624	93.4
60	73179	91.6

Experiment 4 - 25 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	91962	100
0_2	87215	94.8
5	84207	91.6
10	84908	92.3
20	83102	90.4
30	82499	89.7
40	81641	88.7
50	81771	88.9
60	80902	87.9

Experiment 5 - 25 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	73195	100
0_2	70768	96.7
5	69923	98.8
10	70122	95.8
20	69272	94.6
30	68597	93.7
40	69166	93.5
50	68963	92.8
60	68899	94.1

Experiment 6 - 25 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	88235	100
0_2	84249	95.5
5	75663	85.8
10	73871	83.7
20	72144	81.8
30	70321	79.7
40	69526	78.8
50	68250	77.4
60	67644	76.7

Experiment 7 - 100 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	91176	100
0_2	81370	89.2
5	78347	85.9
10	77422	84.9
20	75712	83.0
30	74115	81.3
40	73879	81.0
50	72936	80.0
60	72432	79.4

Experiment 8 - 100 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	93192	100
0_2	86572	92.9
5	85682	91.9
10	84979	91.2
20	83581	89.7
30	82703	88.7
40	81755	87.7
50	81801	87.7
60	80925	86.8

Experiment 9 - 100 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	73575	100
0_2	61328	83.3
5	61097	83.0
10	60128	81.7
20	58281	79.2
30	56669	77.0
40	55758	75.7
50	55110	74.9
60	54890	74.6

Experiment 10 - 100 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	89076	100
0_2	75273	84.5
5	71850	80.6
10	69915	78.5
20	68531	76.9
30	68055	76.4
40	67777	76.1
50	67291	75.5
60	65631	73.6

Experiment 11 - 100 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	116432	100
0_2	111299	95.6
5	111464	95.7
10	111739	96.0
20	110043	94.5
30	109147	93.7
40	108884	93.5
50	108644	93.3
60	108417	93.1

Experiment 12 - 100 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	103480	100
0_2	99357	96.0
5	97816	94.5
10	96770	93.5
20	96335	92.2
30	95370	92.2
40	94863	91.7
50	94339	91.2
60	93359	90.2

Experiment 13 - 200 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	81514	100
0_2	77558	95.1
5	78852	96.7
10	77717	95.3
20	77334	94.9
30	76210	93.5
40	75268	92.4
50	74892	91.9
60	73918	90.7

Experimnent 14 - 200 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	89848	100
0_2	75375	83.9
5	75094	83.6
10	73786	82.1
20	72506	80.7
30	71058	79.1
40	70241	78.2
50	68952	76.7
60	68764	76.5

Experiment 15 - 200 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	98679	100
0_2	79027	80.1
5	74416	75.4
10	70267	71.2
20	68421	69.3
30	67126	68.0
40	65544	66.4
50	65114	66.0
60	64020	64.9

Experiment 16 - 200 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	98373	100
0_2	91496	93.0
5	87363	88.8
10	85325	86.7
20	82796	84.2
30	81428	82.8
40	80265	81.6
50	77423	78.7
60	76820	78.1

Experiment 17 - 200 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	88319	100
0_2	83368	94.4
5	82334	93.2
10	81941	92.8
20	80885	91.6
30	80241	90.9
40	77673	87.9
50	77395	87.6
60	76361	86.5

Experiment 18 - 200 ml/minute

Time (minutes)	Counts over graft (counts/minute)	Percentage of initial counts
0_1	110584	100
0_2	102135	92.4
5	98941	89.5
10	97394	88.1
20	95862	86.7
30	93259	84.3
40	92170	83.3
50	90810	82.1
60	89256	80.7

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Publications arising from this thesis.

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