A COMPARATIVE STUDY OF ENDOTHELIAL CELLS FROM DIFFERENT SOURCES

FOR USE IN SMALL CALIBRE VASCULAR GRAFT SEEDING.

AIDEEN KM WALSH

MB BCh BAO LRCPI LRCSI

BSc FRCS FRCS(Ed)

A thesis submitted for the degree of

Doctor of Medicine

to the

University of Leicester

1993

UMI Number: U053459

UMI

Dissertation Publishing

ProQuest®

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

The work on which this thesis is based is my own unless
otherwise acknowledged.

Aideen KM Walsh

1993

To JJMcC

SYNOPSIS

The work in this thesis was carried out to examine currently acknowledged
sources of endothelial cells for use in prosthetic graft seeding with the
aim of assessing their practicality and applicability in a human clinical
situation.

Chapter 1 reviews the theories of the aetiology of peripheral vascular
disease and discusses the clinical presentation and natural history of
lower limb ischaemia. Chapter 2 reviews the therapeutic modalities
currently available and Chapter 3 the current status of knowledge
regarding why one such modality - bypass grafting - fails. Chapter 4
reviews our knowledge of endothelial cell function with emphasis on
relevance to graft seeding and experience with seeding since its
introduction in 1976.

The experimental work is based on three cell types from four sources. In
Chapter 5 the isolation of human umbilical vein endothelial cells is
described, with assessment of cell yield and coagulation functions. Data
on the antigenicity of such cells is presented using quantification of
MHC Class II antigens and lymphoproliferative ability. Chapter 6 details
the application of a similar isolation method to human saphenous vein
segments.

Chapter 7 details a new isolation method for obtaining microvascular
endothelial cells from human omentum with assessment of cell yield,
purity, function and ability to adhere to polytetrafluoroethylene. My
experience with the application of the technique to human superficial fat
is described in Chapter 8. Chapter 9 describes the modifications
necessary to apply this technique to canine omentum and Chapter 10

- 4 -

recounts the results of a series of in vivo experiments in a canine model - assessing autologously seeded graft thrombogenicity and patency.

In the last Chapter the results of my experiments are reviewed and an overall assessment of the potential of the various cell sources in a human clinical situation made. Possible avenues of research uncovered by my work are presented.

CONTENTS

CHAPTER ONE

PERIPHERAL VASCULAR DISEASE

Introduction

Atherosclerosis most commonly causes morbidity and mortality in the coronary and cerebral circulations - taking these together it accounts for most deaths in the Western world. However it is with its effects on the lower limb that the Vascular Surgeon has most often to deal.

Few studies have been done to assess the incidence of asymptomatic disease, but one in 1985 from Basle indicated a cumulated figure of 4% in men aged 35-44 years with the figure rising to 18% over 65 years of age (Widmer 1985). Not all of these patients will develop symptoms but the incidence of intermittent claudication in men has been shown to be 2% aged 45-65 years and 1% of women in the same age range (Hughson 1978). Looking at an older population a Finnish study (Heliovaara 1976) found an incidence of intermittent claudication of 7.7% in men aged 55-74 years. Given that the proportion of the population over 65 years of age is increasing, one must recognise that lower limb peripheral vascular disease represents an increasing demand on the resources of any health service.

Aetiology

The term atherosclerosis was coined in 1904 (Marchand) to describe the lesions subsequently detailed as "patchy thickening of the intima....comprising accumulations of fat and layers of collagen-like fibres" (Crawford 1960). The term atheroma, still commonly used today had first been popularised in 1755 by von Haller who in turn had taken it from the ancient Greek medical literature where it described "a cystic space or sac containing a gruel like material". The presence of atherosclerotic lesions had been described for many centuries with description of "degeneration of arteries into bone" coming from 1575 (Fallopius). That the disease antedates modern Western culture was further emphasised by the discovery of lesions in the mummy of King Menephthah - said to be the Pharoah of the Exodus (Shattock 1909). Despite the amount that has been written on the topic and the extent of research, both ancient and modern, much remains unclear regarding the aetiology of one of the major causes of death today.

In 1844 Rokitansky proposed the so-called "thrombotic theory" where he stated "the deposit is an exogenous product derived from the blood and for the most from the fibrin of the arterial blood". This opinion was disregarded, however, in the face of objections from Virchow (1856) who rightly demonstrated the subendothelial nature of the deposits and favoured a model of proliferation of subendothelial layers consequent on an "imbibing" of blood elements.

The recognition of the possible involvement of thrombosis in the process was revived, a century later, by the discovery that beyond an area of organised thrombus there extended along the artery a subendothelial layer of fatty and fibrous tissue (Duguid 1946, 1948). Since then

platelet fragments and /or antigens have been identified within
athersclerotic plaques (Woolf 1981).

The central aetiological role in atherosclerosis would now seem to that
of the endothelial cell, with injury to it setting up a series of
reactions leading to plaque formation (Ross 1986). Platelet adhesion and
aggregation to the injured cell promotes localised thrombus formation
and also proliferation of subendothelial smooth muscle cells. Platelets
produce mitogens such as Platelet Derived Growth Factor(PDGF), but such
factors are also produced by the endothelial cell and possibly in
greater amounts following injury. White blood cells would also seem to
have an important role with activation leading to lymphokine release,
some of which in turn can cause induction of cell adhesion molecules and
expression of pro-coagulant activity by the normally non-thrombogenic
endothelial cell. This immunological side to atherosclerosis has only
recently been documented with advanced laboratory techniques allowing
the isolation of disproportionate numbers of T-lymphocytes from within
plaques (Stemme et al 1990).

The endothelial cell injury hypothesis would tie in with many of the
known epidemiologicalllly associated factors in atherosclerosis. Visible
changes have been documented in the endothelium of smokers, with
evidence of cell and basement membrane oedema (Asmussen & Kjeldsen 1975,
Woolf 1982). Such changes are seen with increased cell permeability in
which state endothelial cells have been shown to take up cholesterol
(Somer & Schwartz 1971). Amongst the other acknowledged risk factors for
atherosclerosis are age, sex, race and positive family history all of
which may represent genetic factors predisposing to endothelial cell
injury in the face of environmental challenges.

Clinical Presentation of Peripheral Vascular Disease

Intermittent Claudication

The pain is associated with exercise - the patient typically complaining of a heavy cramping pain which usually provokes a limp (hence the name), before causing him to stop. Even if the arterial lesion is sited other than in the femoro-popliteal segment the patient usually complains of calf pain, although iliac lesions may also cause thigh pain with internal iliac artery disease provoking buttock pain - when associated with impotence this latter called Leriche syndrome. Typically pulses distal to the diseased segment will be absent or diminished although on occasions an exercise test will be necessary to demonstrate this.

Critical Ischaemia

This represents a progression from intermittent claudication and has been defined(European Working Group on Critical Limb Ischaemia 1989) as:

Persistently recurring rest pain requiring regular analgesia >2 weeks

and/or

ulceration or gangrene of the foot or toes

plus

ankle systolic pressure <50mmHg

The pain is usually experienced in the foot - classically in the area of the metatarsal heads - the "ball" of the foot. In the early stages the patient describes the pain as being worse when he lies down, being relieved by hanging the foot over the side of the bed or by leaving it outside of the covers - where gravity and/or reduced temperature can

make the vital difference in perfusion pressures and reduced tissue metabolic requirements. As the situation worsens the pain becomes continuous and the risk of tissue loss/necrosis increases.

It is only possible to estimate the incidence of critical ischaemia but it is thought that approximately 10% of claudicants progress to this stage. While accepting that not all patients with critical ischaemia have an amputation, the figures for this operation have been used as the basis for the estimated incidence of critical ischaemia of 50-100/million/year by the European Working Group on Critical Limb Ischaemia. (1989)

Natural History of Symptomatic Peripheral Vascular Disease

The Framingham study (Peabody 1974) showed that intermittent claudication underwent spontaneous remission - albeit with some recurrences - in 59% of cases. This study found that 2.5% of claudicants ended up with a major amputation, Bloor (1961) found an amputation rate of 8.2% over 10 years, while Kallero (1981) reported a 5 year cumulative amputation rate of 3.5%. While most claudicants do not succumb as a direct result of their peripheral vascular disease the associated atherosclerosis elsewhere accounts for most deaths in these patients - earlier that their population counterparts. In Bloor's study 45% of the patients had died in 10 years and only 16% of these were not due to some form of vascular disease. Other studies have shown the risk of cardiovascular death to be up to three times that of a matched population control (Kallero 1981).

For patients with critical ischaemia the outlook is bleak. Exact figures

for the outcome of such patients are not readily available as most studies report only on particular sub-groups. It has been estimated, however, that one year after presentation only 55% will still have both legs, 25% will have had a major amputation and 20% will be dead (European Working Group on Critical Limb Ischaemia 1989).

Conclusion

With an ageing population the problem of peripheral vascular disease is likely to increase. 17,000 reconstructive operations were performed in the UK in 1983 (OPCS) and this number is rising. The in-hospital cost of a major amputation is currently estimated at £10,000 (Cheshire 1991), even without reckoning the cost of the community care subsequently required this represents a major drain on health service resources. 200 patients/million/year are referred to limb fitting centres in the UK (European Working Group on Critical Limb Ischaemia 1989) and this represents only approximately 50% of amputations performed for peripheral vascular disease.

Conservative management and non-surgical intervention can help many patients, particularly those with intermittent claudication, but as mentioned above a significant number require surgical procedures. Current therapeutic options will be discussed in the next chapter.

CHAPTER TWO

THERAPEUTIC OPTIONS IN THE MANGEMENT OF

PERIPHERAL VASCULAR DISEASE

Conservative Management

In the claudicant much is to be gained by doing nothing other than
giving sound advice. I have already mentioned that spontaneous
improvement can occur in up to 60% of cases (Peabody 1974). Suitable
advice has been summed up by Housley (1988) as "stop smoking and keep
walking" summarising the essence of avoidance of risk factors and
promotion of collateral flow. Patient walking distance has been shown to
be increased by a regular exercise regime (Skinner & Strandness 1967)
and also by reduction of body weight (Scott et al 1988) - and the
beneficial effect of giving up smoking was demonstrated by Quick &
Cotton (1982).

While not having been definitely shown to have symptomatic benefit,
avoidance of other risk factors is to be advised and hypertension,
diabetes and hyperlipidaemias should be screened for and treated.

General foot care is also vital in these patients as inexpert chiropody
and/or infection can precipitate disaster in tissues with borderline
blood supply, this is even more important in diabetic patients who often
have reduced peripheral sensation.

Drug Treatment

The very range of drugs available for the "treatment" of peripheral vascular disease is testimony to the fact that none have been shown clearly to work. There are many categories of drug available – aiming to act at various stages of the disease process – including antilipidaemics, anti-platelet agents and anti-coagulants. In a comprehensive review in 1982 Boobis & Bell reported the absence of a proven remedy and little has changed since then (Consumers Assoc'n 1990).

One category of pharmacological agent which shows promise is the group of Prostacyclin analogues which have been shown to increase blood flow in the short term following femoro-distal bypass with in-situ vein, (Hickey et al 1991) and which are currently being assessed in critical ischaemia. Their disadvantages at present are that they are only available for intra-venous use and that any encouraging results are in the short term only.

Interventional Radiology

Where the patient has disabling claudication or critical ischaemia intervention is necessary and recent years have shown a veritable explosion in the number of advances made in conjunction with radiologists. Percutaneous transluminal angioplasty(PTA) is now performed by most vascular radiologists although techniques such as advanced laser ablation of occlusions are sometimes conducted by surgeons in radiology departments. The range of options available means

that assessment and management of such patients benefits in many cases from the input of both specialities.

Balloon Angioplasty

This technique involves the dilation of lesions by a balloon catheter introduced over a guidewire. The pressures required can be very high - routinely being several atmospheres.

Stenotic lesions fare better than occlusions having a better initial success rate - 95% vs. 80% in the superficial femoral artery (occlusions <10cm) (Spence et al 1981, Hewes et al 1986). Long term patency rates are higher in iliac lesions (85% to 90% at 3 years) (van Andel et al 1985) than in the femoral segment - 3 year patency 50% (Knight et al 1984). These latter results referring to both stenoses and occlusions.

Longer occlusions - >10cm - are more difficult to deal with and have lower initial success and long term patency results. One of the many reasons for technical failure is difficulty in passing the guidewire through the lesion - and this led to the development of a laser probe to "core out " a passage for the wire and then balloon. Initial experience with the "hot tip" laser revealed a relatively high incidence of complications such as thermal injury and vessel disruption and newer models use more advanced pulsed waves or adapt pre-existing laser sources by the substitution of sapphire tips for metal. While laser assisted PTA can give primary success rates of 50% in patients who have lesions deemed unsuitable for balloon alone or in whom conventional techniques have failed (Cumberland et al 1989) a recent review has reported no overall advantage as yet for laser compared with conventional methods (Mahler 1990).

- 17 -

Other techniques

Atherectomy catheters have been developed which mechanically pare away atheroma and are fitted with devices to store plaque debris to avoid distal embolisation (Hofling et al 1988). Also available are low (Vallbracht et al 1989) and high speed rotational devices, the latter pulverising the plaque into microscopic fragments which pass through the capillary bed (Rees et al 1988, Snyder et al 1988). Such devices are still at relatively preliminary stages and though some hundreds of procedures have been reported (Zeitler 1988, Wholey 1988) with up to 90% initial success rates no comparative studies with conventional balloon PTA have been reported.

Also limited are reports on other advances such as the various types of intravascular stents available – although a review of approximately 100 patients has reported a success rate of 93% at the iliac level. However indications are limited to failed conventional methods of dilatation, post-PTA dissections or incomplete dilatations (Mahler 1990).

Sympathectomy

While never having been shown to improve limb salvage in critical ischaemia, sympathectomy – whether surgical or chemical – can improve symptoms of rest pain. Chemical sympathectomy in particular, which can be easily performed under local anaesthetic (preferably with radiological imaging (Eaton et al 1980)), may be all that the very frail patient with otherwise unsalvageable disease can stand and allows some patients avoid the trauma of major amputation.

Reconstructive Surgery

Despite exciting advances with minimally invasive techniques many patients require vascular reconstruction and grafting is the most common type of surgery performed.

More and more distal bypass procedures are being done now, largely due to improvements in pre-operative assessment which allow smaller run-off vessels to be identified. Advances such as digital subtraction angiography (Pond et al 1982, Turnipseed et al 1982) and pulse generated run-off (Beard et al 1988) being two widely commercially available techniques.

Prolonged vascular procedures are now more possible in patients with severe cardiac or respiratory disease with pre-operative assessments such as dipyridamole-thallium scanning allowing the identification of the patient at risk of cardiac complications, and the application of modern anaesthetic techniques.

Graft Materials and Techniques

A wide variety of graft materials are available for use today although most infra-inguinal procedures are done using one of four - autogenous vein, Polytetrafluoroethylene(PTFE), Dacron and glutaraldehyde treated Human Umbilical Vein (HUV).

Autogenous Vein

This material fulfills most of the criteria considered necessary in a vascular conduit being non-antigenic, lined with endothelium, flexible and capable of withstanding arterial pressures. Saphenous vein is ideal for lower limb bypasses being adjacent to the surgical field. Many

- 19 -

studies over the years since the first vein femoropopliteal bypass (Kunlin 1949) have demonstrated superior patency rates when vein is compared a variety of prosthetic materials (Veith et al 1986, Rutherford et al 1988, Budd et al 1990). This difference is obvious when the distal anastomosis is below the knee but some of these studies (Veith et al 1986, Budd 1990) failed to show a significant difference when above knee grafts were analysed separately.

Saphenous vein was first used in the reversed position (Kunlin 1949) and it was in 1962 that Hall described the in-situ technique, using micro-venotomies to excise the valves.

The introduction of an internal valve stripper (Connolly & Stemmer 1970) further advanced in-situ grafting avoiding the more time comsuming venotomies. Since then further modifications have been described including the micro-scissors passed towards valves via adjacent side branches (Leather et al 1979) and the microvalvulatome (Shah & Buchbinder 1981) - first described for use in coronary artery bypass grafting (Mills & Oschner 1976).

A number of advantages have been claimed for the in-situ technique including avoidance of damage to the endothelium during reversal, maintenance of vasa vasorum and compatability of diameters at anastomoses - although it has not been shown to confer any haemodynamic advantage (Beard et al 1986, Gannon et al 1986). Studies conflict as to whether in-situ grafting offers advantages in terms of patency and limb salvage when compared with reversed vein although it is difficult to accurately assess figures as many studies are not randomised and others have differing periods for follow-up and criteria for assessing patency. Harris et al (1987), in a prospective randomised study found no

- 20 -

difference between the two in below- knee grafts although a year later
Rutherford et al declared there were significant differences in favour
of in-situ vein if the graft was to a calf vessel. In a very recent
publication (1993) Harris et al have again failed to find a difference
in long term follow between the two techniques.

Where the Saphenous vein is unsuitable or unavailable some surgeons
advocate using the cephalic or basilic veins from the arm (Kakkar
1969). By dissecting out the median cubital vein as well it is possible
to get quite good lengths, but there are disadvantages in that the arm
veins are more thin walled, can be of small diameter and may easily be
rendered unusable by phlebitis caused by an i.v. infusion.

Prosthetic Grafts

With the realisation that a good proportion of patients do not have a
suitable vein for use in bypass surgery (assessed as as much as 25%
(Mosley et al 1983)) synthetic materials have been the object of much
research and development since 1949. Vinyon'N', a nylon substitute was
described in clinical use in 1952 (Voorhees et al), but as experience
demonstrated loss of its tensile strength with time other materials were
tested including Orlon and Dacron (Hufnagel & Rabil 1955). Most early
grafts were made of flat pieces of material sewn up to make a tube and
subsequent developments included the production of seamless grafts and
crimping of materials to allow greater flexibility.

In 1976 Dardik described the use of glutaraldehyde treated human
umbilical vein(HUV) for femoropopliteal surgery. Frozen (Hufnagel 1947)
and freeze-dried arteries (Brown et al 1953, Hufnagel et al 1953) had
been tried for aortic and infra-inguinal surgery but time had shown

degenerative changes and Dardik's report represented a revived interest in 'biologic' graft materials.

In the early and mid-1970s came the first reports of the use expanded polytetrafluoroethylene(ePTFE) in vascular surgery (Matsumoto et al 1973, Campbell et al 1976) and this material has gained in popularity ever since.

The prosthetic materials most commonly in use today are ePTFE, HUV and Dacron.

ePTFE

Initial reports on ePTFE demonstrated a tendancy to aneurysm formation (Campbell et al 1976a, Mohr & Smith 1980) but this problem has been largely abolished by the addition of an outer Teflon coating to the graft, the fibrils of which are directed at 90° to those in the graft itself. ePTFE has many useful characteristics including ease of handling and suturing although its lack of elasticity can lead to troublesome bleeding from needle holes - particularly in the heparinised patient.

As mentioned above, in the below-knee position ePTFE (like all prosthetic materials) does not have as good a patency record as autogenous vein - the difference at 3 years at calf level being reported as 10-15% (Cannon 1983) and, more recently in Leicester, ePTFE grafts to the tibial level had only a 7% 5 year patency rate vs 29% for vein (Budd et al 1990). This has led to a number of modifications aiming to improve haemodynamics at the distal anastomosis, involving the interposition (Miller et al 1984) or incorporation (Taylor et al 1987) of a segment of vein, both aiming to ameliorate any compliance mis-match. The potential importance of this phenomenon will be discussed more fully in the next

- 22 -

chapter as will in subsequent chapters the theory and techniques involved in the graft modification with which this thesis is primarily concerned – endothelial seeding.

Human Umbilical Vein

While some studies (Eickhoff et al 1983, Dardik et al 1988) have claimed superior results for HUV compared with other prosthetic materials, others have found no difference (Cranley & Hafner 1981) and it has not gained very widespread popularity. Much of this is due to the still appreciable incidence of aneurysmal degeneration. Dardik himself has reported an incidence of 36% (Dardik 1988) at and beyond 5 years and similar incidences have been reported elsewhere (Sommeling et al 1990). This is despite the addition of an external supporting Dacron mesh. HUV has several technical difficulties associated with it also, application of clamps to the graft can fracture the "intima" and potentially cause dissection, and owing to the variable thickness it can be difficult to be sure one has passed the needle through the intima. The material is also expensive which has been an important consideration, although in the recent past efforts have been made to make it more competitively priced. However limited shelf-life and the problems mentioned above mean it remains the first choice of few vascular surgeons.

Dacron

While continuing as a popular material in large vessel grafting Dacron is used less often for infra-inguinal procedures.

Woven Dacron was manufactured for femoro-popliteal bypass following its success elsewhere, but was found to be thrombogenic in this position

(Hamlin et al 1978) and the knitted form was substituted. This is considerably more porous which has the theoretical advantage of improving graft incorporation into the tissues but necessitates pre-clotting at operation. While some studies have shown cumulative patency rates in small groups of patients in the region of 57% (below knee) at 2 years (Clifford et al 1986) and 50% at 5 years (above and below knee) (Mosley & Marston 1986), others have had less success, particularly to the calf with Sauvage reporting 12% patency at 30 months (also in a small group of patients) - using an externally-supported non-crimped version (Sauvage 1983).

Manufacturers are attemptimg to improve the handling of Dacron with modifications such as Gelatin coating to avoid the need for pre-clotting but all in all ePTFE has gained a considerable foothold and is still probably the commonest material in use for medium sized vessels.

Conclusion

While interventional radiology has a definite role to play in the management of stenoses and short occlusions many patients still require reconstructive surgery. Autogenous vein is the best graft material but is not always available or suitable in which case prosthetic materials must be used. These have a higher rate of attrition and the next chapter will examine the factors thought to be responsible for this.

CHAPTER THREE

GRAFT FAILURE

Apart from a mixed bag of relatively rare causes such as infection or
aneurysm formation (whether true graft dilations or pseudoaneurysms
related to anastomoses) graft occlusion by thrombus is the main feature
of failure.

Virchow's triad of stasis, hypercoagulability and abnormal endothelium
is applicable to this situation as much as to any other site of
thrombosis — for prosthetic grafts the thrombogenic luminal surface
represents a non-endothelialised permanently 'abnormal' flow surface.

The timing of graft failure has come to be divided into three phases,
immediate, early and late, equally applicable to both prosthetic and
vein grafts, although the causative factors differ slightly between the
two groups. At each stage the interplay between the three components of
the triad may vary and this will be discussed in each case.

Immediate

Failure within the first 30 days is described as immediate although in
truth the majority of these occlusions occur within the first 48 hours.
Most are due to technical factors, some of which should be anticipated
or looked for at the time of surgery. Evaluation of inflow and outflow

can be done by a variety of means. Pre-operative angiography and Doppler assessment provide the surgeon with something of a road-map – a plan of campaign for the siting of anastomoses. There is evidence now to support the routine evaluation of peripheral resistance distal to a planned distal anastomosis with or without on-table angiography, to ensure the planned vessel is suitable for bypass (Parvin & Bell 1984). Evaluation of graft blood flow, completion peripheral resistance measurements and/or completion angiography can provide circumstantial and direct evidence of anastomotic problems such as kinking or narrowing which can be remedied at the time. The importance of stasis at this early stage in contributing to graft failure was demonstrated some years ago by Bandyk et al who showed a significant association between graft flow <45ml/minute and failure (1985).

Immediate graft failure may also be related to a reduction in flow due to a central cause – in a proportion of cases a fall in blood pressure due to myocardial infarction or cardiac failure can be pinpointed as the time the graft pulse disappeared.

It is in the immediate phase also that altered blood coagulability may be at its most potent as a contributory factor in graft failure. Reperfusion of an ischaemic limb is known to cause the release of a number of inflammatory mediators – some of which have coagulation effects. Thromboxane A2 is a platelet product of arachadonic acid – produced by the lipoxygenase pathway – which is a powerful agent promoting platelet aggregation. Elevated levels have been observed in animal reperfusion experiments and also in the femoral venous blood of claudicants after a period of exercise, a much less severe reperfusion model (Patterson 1991). In a such a human situation also, neutrophil

activation has been observed — the neutrophil/endothelial cell interaction is complex one and will be discussed in greater detail in the next chapter — save to say here that potentiation of coagulation may occur.

Reduction in plasma levels of anti-thrombin III (McDaniel et al 1984) and elevation in mononuclear cell pro-coagulant activity (Blakowski et al 1986) have been observed as post-operative phenomena in patients undergoing peripheral vascular as well as other forms of surgery — both of these changes increasing blood coagulability.

The surface of a vein graft is not completely endothelialised at the time immediately after surgery. It is now accepted that even minor trauma during dissection — not to mention the fairly major trauma of having a valvulatome passed in an in-situ bypass — results in varying degrees of endothelial cell loss, exposing collagen and fibronectin among many other thrombogenic subendothelial elements (Bush et al 1986, Shiokawa et al 1989). It is known that 'islands' of intact endothelium eventually replicate and come to cover the luminal surface within 2-4 weeks (Shiokawa et al 1989) but it is more than coincidence, surely, that this coincides with the end of the immediate failure period when so many grafts are lost or require intervention to restore patency.

Immediately on insertion the luminal surface of a prosthetic graft is lined by platelets. In a pre-clotted Dacron graft a layer of thrombus is already present but such a proteinaceous layer forms almost immediately on other prosthetic graft types, platelet-fibrin clots of 100mcm thick have been described in both Dacron and ePTFE grafts within one hour of implantation (Herring et al 1984). Such a layer persists, including also erythrocytes and leucocytes, with platelets aggregating and, providing a

good flow is maintained, disaggregating such that a single platelet layer exists in contact with the flowing blood (Baumgartner 1973). A reduction in blood flow disrupts this aggregation/disaggregation cycle and contact activation may occur progressing to further fibrin formation. At ten days after implantation fibroblasts start to infiltrate prosthetic graft walls (with the exception of HUV) and while never penetrating far into the wall in the case of ePTFE, may in the more porous Dacron extend in as far as the inner surface after weeks/months. However the luminal surface remains one of fibrin, even after many years of implantation (Berger et al 1972) and while the compactness of this varies between different graft types depending on the nature of crimps and interstices, the basic properties remain, with the potential for platelet activation and thrombosis ever present.

Unlike most animals humans do not develop a complete endothelial lining on prosthetic materials - limited ingrowth of cells from the arteries either side of the anastomoses, known as pannus, extends for only approximately 1cm down the graft. Many theories have been proposed as to why humans differ in this regard from animals but none have been substantiated.

Early

Failure of grafts in the period 30 days to one year is primarily related to anastomotic narrowing secondary to intimal hyperplasia and, in the case of vein grafts, also to intrinsic graft stenoses - both of which reduce blood flow, predisposing to thrombosis.

Intimal hyperplasia occurs more markedly in relation to prosthetic

grafts although it is seen in vein grafts also. It is thought to be due to haemodynamic factors such as shear stress and compliance mismatch as well as interactions between components of the vessel wall and circulating blood. The area of vessel primarily involved in the lesion is the smooth muscle layer which proliferates in response to an interplay of many factors.

The initial step in the process is one of endothelial damage. Disruption of the endothelium is known to occur in vein grafts during harvesting but no matter how much careful dissection reduces this, there is inevitable damage to the endothelium of the graft and of the artery being grafted especially in the area of the anastomosis, the area affected by the hyperplastic response in most cases.

Laminar flow is disturbed at the site of anastomosis and this in turn can disturb the endothelium, turbulent flow is thought to be capable of inducing altered cell function even when there is no visible damage. Vessel wall shear stress is thought to be the important factor as opposed to actual flow rates and increases or decreases in shear at the anastomosis are common, particularly in the early stages after insertion. Differences in diameter between graft and artery alter shear stresses (Binns et al 1989) as does the angle at which the graft is sutured - an angle of 15° has been shown to produce minimal changes in flow characteristics while one of 45° is capable of causing retrograde flow in the layer of blood immediately adjacent to the vessel wall (Crawshaw et al 1980). An association between low shear stress and intimal hyperplasia has been demonstrated in prosthetic grafts (Binns et al 1989) and high stress is also known to cause endothelial damage - possibly inducing hyperplastic lesions (Fry 1968).

- 29 -

Mismatch of the compliance of the graft and the host artery is also thought to be a factor leading to intimal hyperplasia. Vein compliance is similar to that of artery and this has provoked the advocation of the incorporation or interposition of a portion of vein at the distal prosthetic graft anastomoses. Dacron is almost completely non-compliant, while the value for ePTFE is low. The hypothesis behind the compliance mismatch theory is that the sudden alteration in vessel wall movement alters blood flow characteristics and thus shear stress. That there is also a direct abrupt stretching effect on the cells of the vessel at the anastomosis is also a possibility.

Disturbed endothelium or areas denuded of endothelium attract platelets largely through the release of Thromboxane A2 from the cells which initially adhere. Activation leads to the release of Platelet Derived Growth Factor (PDGF) as well as platelet factor A, thromboglobulin and thrombospondin. PDGF is chemotactic for vascular smooth muscle cells as well as being mitogenic for them, and is thought to be responsible in part for the migration of these cells from the media - across the internal elastic lamina - into the intima, a feature of hyperplastic lesions. PDGF is also produced by human endothelial cells and production is increased when cells are injured (DiCorleto & Chisolm 1986). Likewise injured smooth muscle cells can produce PDGF-like activity (Walker et al 1986), thus further promoting hyperplastic lesions. The importance of platelets in promoting intimal hyperplasia has been demonstrated by the lack of the usual hyperplastic response to balloon catheter endothelial injury in thrombocytopaenic animals (Moore et al 1976, Friedman et al 1977).

While platelets adhere to damaged intima so also do other blood

components, many of which are postulated also to have a role in the
development of intimal hyperplasia. Polymorphonuclear leucocytes and
monocytes adhere to endothelium under normal conditions in a state of
balance - adhering and detaching continuously. When endothelium is
damaged this balance is lost and adhesion increases. PDGF is chemotactic
for monocytes (Duel et al 1982) and once transformed to macrophages
these in turn secrete chemoattractants for further monocyte adhesion.
Macrophages also produce mitogenic agents which act on smooth muscle
cells (macrophage derived growth factor) (Leibovich & Ross 1976) and on
endothelium (fibroblast growth factor) (Martin et al 1981). Polymorphs,
when activated by complement, produce a number of agents including
oxygen-free radicals which in turn can damage endothelial cells to the
point of detachment - thus magnifying the effect of the initial damaging
stimulus.

The regeneration of endothelium in vein grafts inhibits many of these
responses and is considered to be a major reason why the incidence of
anastomotic intimal hyperplasia is lower than in prosthetic grafts.
Endothelium produces factors which inhibit smooth muscle cell
proliferation notably heparin (Castellot et al 1982), and smooth muscle
proliferation has been noted to stop in areas of balloon catheter injury
once continuity of endothelium has been restored (Fishman et al 1975,
Haudenschild et al 1979). The main sources of endothelium in vein
grafts from which complete re-endothelialisation occurs are islands of
cells which have been noted to persist on the back of valve leaflets
(Shiokawa et al 1989). While such regenerated endothelium does not
produce anti-thrombotic factors, such as Prostacyclin, in the same
amounts as native artery (Bush et al 1986) appreciable amounts are

produced and would indicate that some cell function is preserved. While endothelium eventually covers the immediate area of the anastomosis in a prosthetic graft - pannus ingrowth - this extends for only a short distance along the graft. Thus not only is the anastomotic area constantly stressed by compliance mismatch and altering shear stresses which can disturb endothelium, but activation of blood components may also take place on the thrombogenic graft surface nearby possibly endangering the extremely delicate smooth muscle/endothelial cell balance in the area.

Even when smooth muscle cell proliferation has slowed down or stopped hyperplastic lesion can increase in thickness due to the production of connective tissue elements by the cells (Clowes et al 1983).

Intimal hyperplasia has been noted to more usually and more severely affect the distal anastomosis. The reasons for this are not known, but certainly in reversed vein and prosthetic grafts there is more likely to be a diameter discrepancy at this site and it is also conceivable that, having passed sites of possible activation platelets and leucocytes may more easily adhere and act here as outlined above.

Autologous vein grafts also show a high attrition rate in the period 30 days to one year, with up to 77% of failures occuring then (Wolfe & McPherson 1987).

The additional feature seen in the case of vein grafts which accounts for most of this figure is that of intrinsic graft lesions. In 1973 Szilagyi reported on the histological features of grafts removed at a variety of intervals after insertion - graft lesion having been identified on angiography before removal. Apart from graft atherosclerosis, which was consistently a late feature, he described two

main types of lesion - a long diffuse narrowing and a short stenosis. The long lesion histologically showed all the features of intimal hyperplasia while the short lesions - usually only a few millimetres long - were described as fibrotic. Some of these lesions were ascribed to fused fibrotic valves while others were said to have been caused by focal clamp damage at the time of surgery.

With more modern graft surveillance techniques such as Duplex ultrasound scanning we now know that 90% of these short stenoses occur within the first year of surgery and 60% within the first six months, and that up to 46% of vein grafts develop such lesions (Brennan et al 1991). The aetiology is still not clear as studies which prospectively marked sites of valve disruption and side-branch ligation have failed to find any correlation with subsequent sites of stenosis. The probability is that endothelial damage during harvesting and/or insertion is to blame but why these lesions should show different features to the longer intimal hyperplastic lesions is not clear - differences in degree of underlying smooth muscle damage may be a factor. Such short stenoses are highly amenable to dilation by transluminal angioplasty albeit at relatively high pressures of up to 12 atmospheres. While some centres have reported poor medium term results with such a technique, we have recently reported an 87% one year graft patency rate after stenosis dilatation in Leicester. Those failures seen occurred in the longer lesions (London et al 1992) - this difference possibly emphasising a difference in nature.

Late

The steady annual attrition rate seen after one year with all graft

types is primarily related to progressive atherosclerotic disease in the proximal and distal vascular tree - although some is probably related to more indolent anastomotic hyperplasia and, in the case of vein grafts, to graft athersclerosis.

That many patients continue to smoke despite encouragement to stop and warnings of the danger from their doctors is known. Even after intervention such as angioplasty or surgery when the 'it will never happen to me' myth has obviously been exploded many patients still smoke - a recent survey in the Vascular Studies Unit in Leicester Royal Infirmary revealed a figure for persistent smokers of 42% (Reid 1990 unpublished observation) and I have no reason to believe the situation is different elsewhere. Greenhalgh et al showed in 1981 that out of a number of serum risk factors including hypercholesterolaemia and raised fibrinogen, only raised carboxyhaemoglobin, an index of smoking behaviour, correlated with infrainguinal bypass failure. This relationship between smoking and graft failure has been reported by others also (Wray et al 1971, Myers et al 1978).

Conclusion

From the above one can see that graft failure is a complex process with a variety of factors acting at different stages. The inherent thrombogenicity of prosthetic materials means they are more susceptible to thrombosis in the case of any fall in graft blood flow, and they are more commonly and aggressively associated with the process of anastomotic intimal hyperplasia - a major cause of flow reduction. The presence of endothelial cells on the surface of vein grafts protects

them from luminal thrombosis and may well also have a relationship with
the lower incidence of anastomotic hyperplasia. The main difference is
the presence/absence of the endothelial cell – and appreciation of this
fact has led to the interest in endothelial cell seeding with which this
thesis is concerned. The following chapter reviews the work done on
endothelial cell seeding since its 'birth' in the mid-1970s and is
prefaced by an overview of our current knowledge of the many and varied
functions of the endothelial cell.

CHAPTER FOUR

ENDOTHELIAL CELL SEEDING.

REASONS AND EXPERIENCE

With the recognition of the problems associated with prosthetic vascular grafts, attention came to be focussed, in the 1970s, on the important difference between them and the vein grafts - the absence/presence of endothelium. It was at this time that scientists started to fully appreciate the functional capacity of endothelial cells which had, until then, been thought to be relatively inert. The discovery of the endothelial synthesis of an anti-platelet agent in 1974 (Saba & Mason), subsequently named Prostacyclin (Moncada et al 1976), was a major landmark in this new field of science and has been followed by a plethora of publications which have contributed to our current understanding of the intricate and diverse functions of the cell. While vascular surgeons have tended to focus on the coagulation associated functions, we now know that endothelium has many other actions and plays a pivotal role in many disease processes. In this chapter I shall review the experience to date with endothelial cell seeding of prosthetic vascular grafts but I shall start with an overview of the current

knowledge of endothelial cell function - emphasising those aspects most

relevant to graft seeding.

ENDOTHELIUM

The phrase "nature's blood container" was coined in 1986 (Gimbrone) and

has become something of a catchphrase - but tends, I feel, to

underestimate the extent of endothelial functions. These are summarised

in Table 1 and one can see at a glance there's a lot more to the cell

than "containing" blood within the circulation.

Table 4.1 Functions of the vascular endothelium

Maintenance of thromboresistance
Maintenance of selective permeability
Integration and trasnduction of blood-borne signals
Modulation of leukocyte interactions with tissues
Regulation of inflammatory and immune reactions
Regulation of vascular tone
Regulation of vascular growth
Synthesis and secretion of peptides
from: Petty RG, Pearson JD.
Endothelium - the axis of vascular health and disease.
J Roy Coll Phys Lond 1989; 23(2): 92-102.

Coagulation

While endothelium maintains a non-thrombotic state on its luminal

surface, this is in fact the result of opposing pro- and anti-coagulant

processes. Vascular thrombosis is not due to the switching OFF of the
anti- and ON of the pro- but rather to the disruption of a delicate
balance between the two. In addition to these properties endothelial
cells are vital for fibrinolysis although this is in turn subject to
inhibition and again a balance exists in vivo.

Pro-coagulant

For many years it was taught that activation of the extrinsic pathway of
the coagulation cascade was dependent on breaching of the tissues and
exposure of the blood to Tissue Factor(TF), also known as
Thromboplastin. However it is now known that endothelial cells are
capable of producing and expressing TF on their intact surface, which by
binding with Factor VII leads to the activation of Factor X. This
expression of TF is not seen in the unperturbed cell but is markedly up-
regulated in the face of a number of stimuli including bacterial
lipopolysaccharide(LPS or endotoxin), interleukin-1(Il-1) and tumour
necrosis factor(TNF), as well as other cytokines (Colucci et al 1983,
Bevilacqua et al 1986, Nawroth & Stern 1986). Of interest also is the
fact that thrombin too can increase TF expression (Brox et al 1984), an
example of how thrombosis exerts a positive feed-back effect on itself -
one of many complex interactions in the process.
Along the intrinsic pathway also, endothelial cells are capable of pro-
coagulant actions having surface receptors for Factors Xa, IXa, Va and
thrombin all situated closely together.

Anti-coagulant

The anti-coagulant functions associated with endothelial cells are

- 38 -

intricate and involve activation of mechanisms which act at a variety of stages along the coagulation cascade.

Expressed on the cell surface are glycosaminoglycans of the heparan sulphate class (Buonassi 1973, Marcum & Rosenberg 1985), these molecules bind the serum protein anti-thrombin III, which complex can then bind and inactivate thrombin (Marcum et al 1984, Rosenberg 1985).

Another thrombin binding protein found on the endothelial cell surface is thrombomodulin. Once bound with thrombin this activates protein C which with protein S as a co-factor inhibits activated Factor V (Esmon NL et al 1982, Esmon CT et al 1982, Stern et al 1986). Protein S is, in turn, an endothelial product (Stern et al 1986a).

Fibrinolysis

Two types of plasminogen activator have been shown to be active - urokinase which acts on the fluid phase, and tissue plasminogen activator(tPA) which must be bound to fibrin to be fully active. While urokinase is produced by endothelium in culture, it ia thought that tPA is the only endothelial derived factor involved in in-vivo situations. By acting only at sites of fibrin formation tPA is more specific than urokinase, or indeed streptokinase both of which are in use clinically. Endothelial cells are also capable of producing a specific tPA inhibitor known simply as Plasminogen Activator Inhibitor(PAI). In certain situations PAI production may exceed that of tPA - identified causes of this include Il-1, TNF and LPS, further demonstrating the pro-thrombotic activities of these agents (Colucci et al 1985, Hekman & Loskutoff 1987).

Platelet Interactions

Closely involved with the cell coagulation mechanisms are the interactions with circulating platelets. As I mentioned in an earlier chapter we know that platelets in-vivo aggregate and disaggregate at the intimal surface of vessels with the numbers remaining constant at any given time, assuming that all other factors eg. blood flow are within normal limits. However in the normal course of events these platelets do not adhere to the endothelium and adhesion of even stimulated platelets does not usually occur. This keeping of platelets "at bay" is largely performed by the arachadonic acid metabolite Prostacyclin(PgI2) - which is secreted in response to a wide variety of stimuli. Amongst these sre thrombin, bradykinin, adenosine di- and tri-phosphate(ADP and ATP) (Weksler et al 1978). Both ADP and ATP are produced by aggregating platelets - which thus have a negative feed-back effect on the generating thrombus - they also cause endothelium to produce Endothelium Derived Relaxing Factor(EDRF) which apart from its vasoactive properties has been shown to synergise with PgI2 to inhibit platelet function (Radomski et al 1987). PgI2 is not the only prostanoid produced by the endothelium, there is evidence to suggest that proportionately more PgE2 is produced by microvascular endothelial cells (Charo et al 1984), and there is also evidence that production mechanisms are different in these cells as responses to stimuli differ - with thrombin failing to induce PgI2 release in microvascular cells in-vitro (Carter et al 1989).

An additional anti-platelet mechanism of endothelium is the ability to de-phosphorylate ADP and ATP to adenosine, which is in turn an inhibitor of platelet aggregation - this ability also varies between cells from different sites and has been noted to occur in human umbilical vein

cells but not in microvascular cells (Petty & Pearson 1989). While endothelium acts indirectly to enable platelets to adhere to the basement membrane by production of some of its components such as fibronectin and collagens, and of von Willebrand factor which acts as co-factor for such binding, the cell itself when intact does not allow platelet adhesion.

Vasoactive Properties

As mentioned above endothelium produces a powerful relaxing agent - EDRF. This was discovered somewhat fortuitously by Furchgott in 1981 when he found strips of aorta in an organ bath failed to relax after acetylcholine(ACh) induced contraction, while rings of vessel, exposed to less trauma in preparation thus maintaining the endothelium, did. Since then EDRF has been identified as Nitric Oxide which is very labile in-vivo, with a half-life of only a few seconds. It is probable that EDRF is not the only endothelium dependent/produced relaxing agent, and other compounds are under evaluation - in particular other nitric compounds. EDRF acts by stimulating smooth muscle guanylate cyclase which increases celluar guanosine monophosphate which in turn inhibits contractility. While EDRF is produced in response to many blood borne mediators it is thought that shear stress at the vessel wall is one of the major factors involved in-vivo - with stress being "sensed" by the endothelial cell which in turn "decides" how much EDRF to release (Vanhoutte 1988). Again there is a site dependent variation in EDRF release with more being produced from arterial than venous endothelium (Vanhoutte 1987).

As I mentioned above EDRF is released with PgI2 in response to platelet ADP and ATP and appears to act synergistically with it against platelet aggregation. In the role of relaxation too the two compounds can act together and PgI2 is quite a potent vasodilating agent.

As with other functions endothelium is capable of producing antagonistic factors – and more recently a potent vasoconstrictor Endothelin has been described (Vanhoutte & Katusic 1988). The main stimulus for its release at the present time would appear to be hypoxia, particularly in the coronary and cerebral circulations.

By a complex interplay of pro- and anti-coagulant properties, platelet interactions and adjustment of vascular tone endothelium is responsible for maintaining a steady state of thrombus free blood vessels. It is with these aspects of function that vascular surgeons involved with graft seeding have been primarily concerned, but endothelial cells function in many other ways and some of these also must be taken into account when embarking onto the field of graft seeding.

Growth Factors

In the discussion on intimal hyperplasia in the last chapter I described at some length how disturbed endothelial cells can produce smooth muscle cell mitogens and also how intact endothelium acts to inhibit underlying smooth muscle proliferation. How this might affect endothelial cell seeding of prosthetic grafts is important. We know that intimal hyperplasia in the area of the graft/artery anastomosis is a major cause of graft attrition – and that hyperplasia to the same degree is not seen

at vein/artery anastomoses. It must then be reasonable to assume that the presence of endothelium on the graft acts to down-regulate hyperplasia at the anastomosis - whether by locally ensuring quicker restoration of the monolayer or by production of factors to act downstream at the anastomosis is not known.

The danger of aggravating intimal hyperplasia by the introduction of cells other than endothelial cells onto the graft has been raised (Sterpetti 1988), this possibly upsetting a mitogen/inhibitor balance. This is one of the problems this thesis aims to address and will be discussed in greater detail in the context of graft seeding later in this chapter.

Leucocyte Interaction

There normally exists a state of dynamic equilibrium between leucocytes adherent to the endothelium and those circulating - at any moment large numbers are adherent. Increase in adhesion is seen in the first stages of inflammation and may be due to alterations in either cell type (Harlan 1985). A variety of stimuli including cytokines and LPS induce an endothelial leucocyte adhesion molecule(ELAM-1) (Bevilacqua et al 1987).

Adhesion of lecocytes to, and migration through the endothelial cells seeded onto prosthetic grafts has been reported (Emerick et al 1987) - the same group reported better retention of endothelial cells on grafts in leucopaenic animals. The ability to attract leucocytes might be of benefit in seeded grafts if it could reduce the incidence of graft infection. The evidence on this point is, at present, contradictory with

- 43 -

Rosenmann et al (1985) showing a reduced adherence of bacteria to seeded PTFE grafts, while Arregui et al in the same year found no reduction in infection in seeded Dacron compared with controls.

However, as the report from Emerick suggested, leucocyte adhesion may be a factor in the loss of cells. Adhesion of leucocytes could also have a deleterious effect on the graft short of cell loss. Activated monocytes are capable of producing mitogens for mesenchymal cells, such mitogens are not produced merely as a result of endothelial cell damage, and primary monocyte activation by a number of factors can initiate secretion (Glenn & Ross 1981).

Immunological Reactions

Expression of host "self" on cells rests in the Major Histocompatability(MHC) Class II antigen - responsible for antigen presentation to T lymphocytes. Recognition of foreign Class II antigens is the trigger for the cellular immune response. It had been considered for many years that only cells of the bone marrow posessed such antigens but we now know that endothelial cells, while not expressing MHC Class II antigens in a resting state show marked up-regulation of expression in the face of activated T lymphocytes and gamma-interferon among other stimuli (Pober & Gimbrone 1982, Pober et al 1983).

Not only that, but certain other stimulators such as TNF or LPS can induce expression of IL-1 genes in endothelial cells, and its secretion (Stern et al 1985). Il-1 is a potent stimulator of T cell proliferation, in turn a major source of gamma-interferon. Thus endothelial cells alone once activated by gamma-interferon are capable of antigen presentation

to T cells ie. are theoretically capable of themselves initiating the rejection response (Hirschberg et al 1980, Pober et al 1983).

As endothelium is the first line of contact between donor and recipient in transplant surgery this has obvious implications. If one were to consider seeding grafts with homologous cells such a rejection possibility must be borne in mind. The volume of transplanted tissue would obviously be much smaller and exposure to lymphocytes different than one would expect in the capillaries of a solid organ but it is theoretically possible that even if the host did not develop a generalised reaction that the local accumulation of lymphocytes with cytokine production etc. might make the graft more thrombogenic.

SEEDING

While not all of the above information was available to the early workers in the field, endothelial cell seeding of vascular grafts was first proposed in the mid-1970s.

Endothelial cell seeding has come a long way since the first report of successful endothelialisation of Dacron aortic grafts in a canine model in 1978 (Herring et al). The ability to harvest endothelial cells and culture them was then newly appreciated and the maintenance of an endothelial lining on a graft, four weeks after insertion promised great things. However as one of the more recent publications on the topic (Zilla et al 1990) was still looking at ways of optimising human vein endothelial harvest it is clear that, despite great promise, problems remain.

The vast majority of work done, particularly in the early years, was in animals and much information has been gained with time. In this section I shall review experience to date under certain headings but will stress those areas which have a bearing on endothelial cell seeding in a clinical context today - ending with a review of human trials of endothelial cell seeding.

Cell Source

Herring's first experiments used cells scraped off vein segments with a steel wool pledget. In subsequent reports he confirmed that the cells found in the lining of his canine grafts were endothelial, identifying factor VIII related antigen and Weibel-Palade bodies (Herring et al 1979). However in view of the obvious hazard of gross smooth muscle cell

contamination such a method carried, Graham et al (1979) described a collagenase harvesting technique for vein segments. She used a method of everting the veins over a steel rod, then immersing this in collagenase solution. Subsequently filling of a non-everted vein segment with collagenase was applied (Watkins et al 1984) and was deemed more successful in terms of cell yield (Burke et al 1986).

Graham's work also involved the use of cells cultured for 14 days before seeding (Graham et al 1980) and she showed they also produced an endothelial lining in a canine Dacron model – this time using thoraco-abdominal bypasses (Graham et al 1980a).

Application of the collagenase technique has also been successful in other animal models including baboon (Callow et al 1984), pig (Hollier et al 1985) and sheep (Ortenwall et al 1988).

Based on his initial cell scraping technique Herring et al (1980) estimated that a vein half the area of a proposed graft would be necessary to provide anything more than 85% cell coverage. The clinical drawbacks of this were obvious and a great deal of work since has been focussed on optimising human vein cell yield. However an even more basic problem has been encountered by some workers with human vein, that is the problem of getting any endothelial cells to grow at all, with Watkins et al (1984) acknowledging no cell growth from 20% of veins studied. Kent et al (1989) in a comparison of canine and bovine vessels found grossly inferior cell yields and growth characteristics in human tissue and another study found cell yields of only 12–15% (Sharefkin et al 1986). Work on adult human vein cells has tended to centre on sequential sub-confluent passaging in an effort to provide adequate numbers from small segments (Watkins et al 1984, Zilla et al 1990).

– 47 –

Perhaps because of such difficulties much of the in-vitro work has been done using human umbilical vein cells - the majority of workers using as a basis the canulation collagenase harvesting technique described by Jaffe et al (1973).

Citing difficulties with veins, adipose tissue has been examined as a possible cell source. Most work has been done on omentum with Jarrell et al (1986) applying a collagenase digestion technique on human tissue in vitro and Pearce et al (1987) applying a similar isolation method successfully in a canine model in-vivo. Superficial and perinephric fats have also been examined as sources (Williams et al 1989, Sharp et al 1989). The main problem with these techniques is that while large cell yields are described - in most of the reports cited above >10^{6} cells/g tissue - there remains considerable doubt as to cell purity. The method described by Sharp et al yields up to 50% comtaminants - this group are using this method in a clinical setting with 17 patients reported as having received grafts seeded with cells from superficial fat (Sharp et al 1989) - the outcome has not been reported as yet. In a canine model omental cell harvests, using current techniques, have been shown to produce greater thickness of the subendothelial cell layer than venous cell seeded or controls (Sterpetti et al 1988) - and at present most centres involved with this source utilise one of a variety of purification methods.

There has been a limited amount of work done on homologous or even heterologous seeding. Zamora et al (1986) described homologous cell seeding of arterio-venous shunts in dogs. When cells were seeded at high density initially there was evidence that endothelial cell linings were present in some cases on removal, although at lower seeding densities

- 48 -

this was not the case. Pennell et al (1986) described heterologous seeding using porcine cells in a canine model - seeding aortic grafts. This paper compared heterologous with autologous seeding and non-seeded controls, and while greater numbers of cells were noted on explanted seeded grafts there was no difference in thrombus free area when compared with controls. The authors raised the possibility that in reality host cells had come to populate the grafts. This suggestion has been examined in greater detail by other groups. Hussain et al (1989) found no difference in endothelial cell coverage of limbs of canine aorto-iliac grafts on removal (approximately 35% each) despite only one having been seeded with autologous cells four weeks earlier. Previously published work involving chromosomal analysis of cross sex allografted cells in a porcine (Hollier et al 1986) or canine (Wakefield et al 1988) model had suggested that it was predominantly host cells on the grafts at removal. How seeding might provoke host cells to produce a lining earlir than one would expect in an animal is not known.

Seeding Techniques

Herring and Graham's work in the early days used as a means of introducing cells into the graft the method - widely adopted since - of mixing the cells with the blood used to pre-clot Dacron. Graham et al (1980) examined grafts seeded this way at 2 and 4 weeks after insertion and found 60-70% cell coverage at 2 weeks and a minimum of 80% coverage at 4 weeks. At this stage unseeded grafts displayed only limited pannus ingrowth. Burkel et al (1981) also examined grafts seeded in this way and found that in the first days after insertion there was no

discernable difference between seeded and un-seeded but that endothelial cells appeared on the luminal surface around day 7.

The widespread clinical use of ePTFE led workers to examine its ability to be seeded. Graham et al (1982) demonstrated endothelial coverage of grafts seeded in a manner similar to Dacron, but when the two materials were compared (Sharefkin et al 1982) only 12% of seeded cells adhered to ePTFE compared with 70% to Dacron. Even poorer figures of 4.4% remaining on ePTFE after 72 hours were reported in 1985 (Rosenman et al). In both these reports complete surface endothelialisation was reported at 4-6 weeks but attention focussed on improving initial adhesion, thus aiming to reduce early thrombogenicity. Much of this work was done using ePTFE as it became the graft of choice for small/medium vessel work, although a proportion of groups still use Dacron in their experiments.

The common techniqe with ePTFE has been to pre-coat the graft before introducing the cells in suspension, and coating materials examined include collagen (Baker et al 1985), laminin (Anderson et al 1987), plasma, amnion (Jarrell et al 1986b) and fibronectin (Williams et al 1985, Lindblad et al 1986, Ramalanjaona et al 1986). Most of these materials are components of the sub-endothelial matrix in-vivo. One of the most commonly used substrates has been fibronectin and marked improvements in adhesion have been reported in the order of 65% adherent to coated compared with 5% to un-coated (Seeger 1985). A more recent paper has shown fibronectin to be superior to laminin or type 4 collagen for increasing initial adhesion to ePTFE, however in the study cells seemed to be better spread out one hour after adhering to a pre-clotted graft - possibly because of patchy coating by the proteins (Thomson et al 1991). An even more recent concept is that of inducing a true extra-

cellular matrix on the surface of the ePTFE by culturing corneal endothelial cells on it long enough for them to produce matrix compounds, then harvesting them and seeding vascular endothelial cells onto the matrix. It is proposed that the presence of growth factors in the matrix will regulate endothelial cell behaviour but to date this technique has only been reported in an in-vitro setting (Schneider et al 1992).

With the development of two methods of introducing cells ie. in blood pre-clot or onto pre-coated graft has come the development of a new addition to the vocabulary - and with it not a little confusion. Most workers still use the term "seeding" to cover any techniques in the field, but with time the term "sodding" has appeared. Rupnick et al (1989) use this latter term for the introduction of large numbers of cells onto a pre-existing matrix (in their case pre-clot) - citing a Dr Joel Williams as the source of the new word. The problem now is that the terms have not been standardised - the potential confusion is great!

Developments in improving efficiency of adhesion have not completely solved the problem. Even when a good proportion of cells initially adhere many are lost if quickly exposed to flow situations. Seeger (1985) reported only 25% remaining after one hours exposure to flow and a similar figure of 21% was reported by Ramalanjaona et al (1986) as remaining after 24 hours. When a coating matrix of sub-endothelial components has been used the question has been raised of possibly aggravating graft thrombogenicity, knowing the affinity of platelets for such materials. Such a potential problem may be overcome by using anti-platelet agents (Ramalanjaona et al 1986), but the alternative suggestion has been the possibility of growing the cells to confluence

on the graft before implantation - this would reduce the area of naked matrix and reduce cell loss in the face of flow, their having had a chance to flatten and spread out.

Sentissi et al (1986) found only 11% of cells pre-cultured on a graft were lost after one hour of flow exposure and retention (rather than loss) figures of 81% (Schneider et al 1988) and 75% (Prendiville et al 1989) have been described in similar circumstances. In a recent study Budd et al (1991) have compared an immediate seeding technique with one involving overnight cell incubation on ePTFE, exposing lengths of of graft to flow rates of 100ml and 200ml/minute. Over two hours cell retention was superior on the incubated grafts (80% vs 40%). No in-vivo studies have reported such a comparison. The concept of seeding the graft at the time of insertion has theoretical advantages in a clinical context - assuming one can harvest adequate numbers of cells at the same time. If culturing of cells between harvest and insertion is to be, then pre-insertion graft incubation may have a role. A theoretical risk is one of a higher incidence of infection, but in the absence of any comparative in-vivo studies one cannot comment.

Functional Effects of Endothelial Cell Seeding

The effect of seeding on graft behaviour has been examined in a number of ways. Thrombus free area was the first parameter measured with Herring et al (1978) reporting an advantage for seeded grafts of 76% vs 22%, and Graham et al (1980) 90% of their seeded graft surfaces as being clot free. Assessment was based on measuring red vs white areas on the luminal surface using photographs taken at the time of removal, although

Graham et al (1980) acknowledged that a red area could be, on occasions, due to erythrocytes from the original pre-clot underlying the endothelial cell layer.

Herring et al (1978) used a ratio of static surface clotting time to Lee-White clotting time to assess increased thromboresistance on seeded grafts. Since then other methods have been employed, Sharefkin et al (1982) demonstrating quicker return to normal of platelet survival in seeded graft subjects and Allen et al (1984) - using an [111]Indium labelling technique - reduced platelet deposition on seeded grafts compared with un-seeded controls. Sharefkin et al (1982) had also demonstrated the ability of seeded grafts to produce Prostacyclin(PgI2) and this was also noted subsequently by Clagett et al (1984). Sicard et al in the same year noted that not only did seeded grafts tend to increase PgI2 production but that they also produced less Thromboxane B2.

Whether or not reduction in platelet adhesion is dependent on anti-platelet medication is still subject to debate. Allen et al had found that seeding increased platelet adhesion in the first two weeks, but that this was abolished if the animals were treated with aspirin. However in an in-vitro model Dekker et al (1989) found non-aspirin treated endothelial cells offered best protection against activated platelets.

The acid test of endothelial cell seeding is its effect on graft patency - in particular on small diameter grafts where occlusion is a clinical (and experimental animal) problem. Many studies have been published showing an improved patency in small calibre ePTFE (Campbell et al 1985, Campbell et al 1988, Hirko et al 1987, Budd et al 1991) and Dacron

(Stanley et al 1982, Schmidt et al 1984). The last study also demonstrated a resistance to thrombosis in seeded grafts when subjected to an experimental reduction in flow just prior to removal.

Clinical Experience

Despite a large volume of material in favour of endothelial cell seeding in animals there has not been a widespread uptake of the technique in a human context. The main reason for this is the fact that human data so far have not been as significantly favourable.

Herring quickly applied his mechanical harvesting method to a human trial, seeding Dacron for femoro-popliteal bypasses (Herring et al 1984a). While this study failed to show a significant advantage for the seeded grafts it did throw up the discovery that smokers with seeded grafts fared worse than if they had unseeded grafts. This group subsequently seeded ePTFE using endothelial cells harvested with collagenase from external jugular vein - introducing the cells onto pre-clotted grafts. This time seeding proved beneficial with a cumulative 2 year patency of 73.9% vs 44.5% for unseeded, and smoking did not have the same effect as on Dacron with seeded smokers maintaining an advantage over unseeded (Herring et al 1987). It is obviously difficult to assess grafts in humans but this group has reported on a few cases where graft material became available for a variety of reasons after insertion. Of three segments available (1Dacron, 2ePTFE) only one ePTFE showed extensive endothelialisation at 90 days. The other two patients were however, smokers (Herring et al 1984, Herring et al 1985). Herring's group have also reported 3 infections in 39 seeded ePTFE

- 54 -

grafts - with 2 of these occurring in 10 patients who had grafts seeded in the operating theatre rather than in a laminar flow hood. While these infections occurred after approximately 3 months ie. possibly not related to seeding, the authors advocated routine use of laminar flow sterile facilities (Herring et al 1987).

In two other studies looking at infra-inguinal ePTFE grafts results to date are either disappointing or insignificant. Zilla et al (1987) seeding autologous vein cells onto fibrin glue coated grafts showed that platelet uptake and survival was at best similar, if not worse, in seeded patients and he concluded on the basis of platelet deformation studies that at three months there was little endothelialisation of the seeded grafts. In a shorter term study using mechanically harvested cells, Walker et al (1987) found an insignificant trend in favour of seeded graft patency at nine months, these grafts had also displayed a favourable trend at three months with reduced platelet uptake, however in the absence of long term follow-up comment is difficult.

Another European group studying human seeding is the one in Goteborg - this group however, seems to have concentrated on larger diameter grafts reporting the seeding of Dacron aorto-iliac grafts. In 1987 (Ortenwal et al) they reported reduced platelet uptake (and hence reduced thrombogenicity index) in seeded limbs of bifurcation grafts - following these up at four months. A more recent publication from this group (Ortenwall et al 1990) reports similar results in the same situation. Of interest are the data this group has reported, or left out, regarding the ability to harvest adult saphenous vein cells. In the 1987 report 15 patients were studied - the authors say it was possible to establish cultures in the 'majority' of these patients cells but then acknowledge

- 55 -

that only four cultures could be passaged - suggesting that establishment of a culture did not necessarily provide cells of good viability or growth ability.

As I mentioned earlier in this chapter Sharp et al (1989) have an ongoing study of impure endothelial cell seeding in femoro-popliteal grafts with no data as to outcome available as yet.

A recent report (Magometschnigg et al 1992) has claimed improved 30 day secondary patency and reduced 18 month amputation rates in 13 patients receiving seeded ePTFE grafts to the crural level compared with 13 controls - all patients having had failed reconstructive procedures in the past. While such patients are probably the ideal candidates for seeded grafts the paper demonstrates many of the possible pitfalls of the technique with up to seven weeks being required for cell culture and seeding using arm vein as a source of cells. Other major problems encountered by the authors were infected or impure cultures (mainly fibroblasts or smooth muscle cells) or simple failure of the endothelial cells to grow in culture. Of 34 patients who had arm vein harvested 18 were lost to such factors and of 16 successful cultures seeding was abandoned in 3 patients as clinical deterioration outpaced cell growth in culture and unseeded ePTFE grafts were inserted.

All in all while there is a trend in favour of seeded grafts in humans the evidence is not as significant as in animal studies. However to be fair there is not the same volume of work in the human situation and follow up poses obvious problems, it is difficult to be sure of the fate of the seeded endothelium when the graft must stay in-situ. It is true that one cannot extrapolate directly from the animal model and to finally pronounce on endothelial cell seeding in humans more clinical

- 56 -

trials are needed. It is with this in mind that this thesis was undertaken, before embarking on a trial of seeding one must be sure the cells are reliably available!

SCOPE OF THIS THESIS

From this review it can be seen that peripheral vascular disease is a major problem in current medical and surgical practice. While many treatment modalities are available most are not without their problems. In the field of bypass surgery, while autogenous vein has proven itself to be overall the best material it is not always suitable or available and on occasions prosthetic materials must be used. In an effort to improve the behaviour of these prosthetic grafts endothelial cell seeding has been proposed, with encouraging results in animal models, although in humans there is not yet the same wealth of evidence. One of the main problems with the application of the technique of endothelial cell seeding to humans is the one of finding a suitable, plentiful source of cells. Autologous cells would obviously be best and most work has been done in animal models on autologous vein cells. In humans however there is already a problem with superficial veins if one is comtemplating seeding a prosthetic graft and adult human cells do not share the same good growth characteristics as animal ones (Kent et al 1989).

Autologous cells from adipose tissue have been examined by some workers but isolation and purification methods to date have yielded contaminated harvests with mixed cells types and the suitability of these for seeding is seriously questioned.

The possibility of using allograft cells for seeding has been proposed on only a few occasions in the past. Using foetal umbilical vein cells might reduce the theoretical problem of antigenicity.

The aim of this thesis, therefore, is to examine a number of endothelial cell sources to see which might be suitable for use in a human trial.

Factors common to all cell types which will be assessed will include ease of harvest and culture, efficiency of harvest with numbers of cells made available, and coagulation functions including Prostacyclin production and pro-coagulant activity. Individual problems which will be examined will include the antigenicity and lymphoproliferative potential of human umbilical vein endothelial cells, and the purity of harvests of microvascular endothelial cells - the ability of these cells to adhere to graft material will also be the subject of study.

The application of autologous endothelial cell seeding in an in-vivo canine model will also be described.

At the end of this thesis I will review the results and discuss their implications for the feasibility of human graft seeding.

CHAPTER FIVE

HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

Introduction

Human umbilical vein endothelial cells(HUVECs) have been used extensively in in-vitro experiments in the last 20 years or so. 1973 saw the reporting of a cannulation with collagenase harvesting technique (Jaffe et al) which has been the basis for many workers since - a good deal of what we know today regarding endothelial cell function is based on studies done using HUVECs.

In the field of endothelial cell seeding also, HUVECs have been used, in particular in studies examining adhesion to graft material pre-coated with tissue matrices (Schneider et al 1988, Budd et al 1990, 1991). The concept of using HUVECs for seeding in-vivo has many attractions. There would be few problems with finding cell source material and, if one were to set up a cell bank, much time could be saved in and around the period of graft insertion. However there is evidence now that endothelial cells posess - or are capable of expressing - MHC Class II antigens, in particular Dr (Moen et al 1980, Pober et al 1986, Ferry et al 1987) - and as such may precipitate a rejection reaction. Such an occurence might not only lead to cell loss but could also lead to derangement of the functions of any remaining cells - coagulation and mitogenic functions being of greatest concern to the vascular surgeon. A good deal

of the in-vitro work on MHC Class II expression has been done on HUVECs,
but on the basis of my initial experiments on the proliferation of
peripheral blood leucocytes(PBLs) which were at variance with the
literature, I decided to perform a series of experiments to assess HUVEC
class II expression and the effect of HUVECs on the proliferation of
PBLs in culture.

The work in this chapter is divided into 3 sections:
In the first the harvesting and culture of HUVECs are described with
data on cell yields and details of identification.
The second recounts experiments performed to assess some of the HUVEC
anti-coagulant and pro-coagulant functions - vital if they are to be
suitable for seeding.
In the third section a series of experiments on the effect of HUVECs on
peripheral blood leucocytes in culture is described including data on
FACS(fluorescence activated cell sorting) analysis of MHC antigen
expression.

SECTION ONE

Harvest and Culture of HUVECs

Materials

Minimum Essential Medium(MEM): This was prepared from MEM10X (Northumbria Biochemicals, UK) by dilution in sterile water with the addition of penicillin (100u/ml), streptomycin (100u/ml), amphotericin (2.5mcg/ml), Hepes buffer (20mmol/ml) and sodium hydroxide to bring the pH to 7.4 (all these reagents coming from Flow Laboratories, UK).

Collagenase: CLS 1 as crude powder was obtained from Worthington Biochemical, NJ, USA and made up to 0.1% in MEM with calcium chloride (15mmol/L). It was subsequently filter sterilised by being passed through a 0.45 micron(mcm) filter (Falcon 7104, Becton Dickenson & Co, NJ, USA). Aliquots were stored at -20°C.

Complete Culture Medium: The basis for this medium was M199 (Northumbria Biochemical, UK) which was supplemented by a number of additives. Foetal calf serum was added to make up 20% of the medium, while this was consistently obtained from Sera Laboratories UK, different batches were used as available. Other reagents added were:

Endothelial cell growth supplement (35mcg/ml), heparin (90mcg/ml) – Sigma Chemicals, UK. Penicillen (100u/ml), streptomycin (100u/ml), Hepes buffer (20mmol/ml), L-glutamine (2mmol/ml), pyruvate (1mmol/ml) – Flow Laboratories, UK.

Trypsin/EDTA: Diamino ethenetetra-acetic acid(EDTA) (Fison, UK) was made up to 1% in distilled water and sterilised by autoclaving.

Trypsin 2.5% (Flow Laboratories, UK) was diluted in phosphate buffered

saline(PBS) (Northumbria Biochemicals, UK) and the EDTA solution added to give a final concentration of 0.1% Trypsin/0.02% EDTA.

Methods

Isolation and Culture of HUVECs

Umbilical cords were detached from the placenta and placed by labour ward staff into sterile pots containing MEM. These were maintaineed in a 4°C fridge on the labour ward and the staff performed all manipulations while still "scrubbed" for the delivery. The pots were collected within 24 hours and brought to the laboratory where all manipulations were carried out using sterile instruments and gloves in a laminar flow hood. In the laboratory the cord was examined for areas of trauma, needle holes and clamp marks and any such areas were removed. The umbilical vein was identified and cannulated at one end, the grooved metal cannula having been specially designed in the department for this purpose. The vein was secured with a silk tie, the grooves allowing good purchase for the tie. To the cannula was attached a standard 3-way tap (Monoject, USA) and using a syringe the vein was **gently** flushed through with MEM to remove old blood. As soon as the effluent was clear the other end of the vein was cannulated as the first (it soon became clear that cannulating both ends at the start frequently led to one becoming blocked with clot). The vein was gently distended with up to 5ml 0.1% Worthington's collagenase pre-warmed to 37°C, and having closed the 3-way taps, was placed in a sterile plastic bag in an incubator at 37°C for 15 minutes (Galenkamp, UK). At the end of this time the vein was flushed through with 20mls MEM – washing out detached endothelial cells. The cell

suspension was collected in a sterile 50ml conical polypropylene tube (Sterilin, UK) and then pelleted by centrifugation at 300g at 4°C for 7 minutes (Jouan, France).

After decanting off the supernatant the pellet was resuspended in 4mls complete culture medium and plated in a T25 25cm^2 tissue culture plastic flask (Nunc, Denmark). The cell cultures were maintained at 37°C in an atmosphere of 95%air/5%CO_2 - leaving the flask lids a little undone allowed equilibration between the inside and outside of the flask. The medium was changed completely the day after plating and subsequently half changed twice weekly.

Cell growth was assessed daily by examination using a phase contrast microscope (Nikon, Japan). On reaching confluence (typically day 5-7 although on occasions as early as day 3) the cells were passaged 1 to 3. To do this the culture medium was removed and 5mls MEM placed in the flask to wash away any residual foetal calf serum - an antagonist to the action of trypsin. After removing the MEM 2ml of Trypsin/EDTA pre-warmed to 37°C was added to each flask, and after 10-20 seconds 1ml removed. The flask was then examined under the phase contrast microscope until all the cells looked rounded and were starting to detach (approx. 2-3 minutes). A few brisk taps on the flask at this stage hastened the detachment and after checking this was the case under the microscope the reaction was stopped by neutralising the trypsin with 11ml complete culture medium. This was then divided into 3 X 4ml aliqouts and each of these plated in a new T25 flask. The medium was subsequently changed twice weekly as before and cells passaged in the same manner as they reached confluence again. In all experiments (other than counts) on HUVECs cells were between passage 2 and 4.

Cell Identification

The morphology of the cells on phase contrast microscopy was one of the features assessed in confirming identity - the cobblestone monolayer pattern of growth being an endothelial characteristic.

The other method is the identification of endothelial cell specific markers. The marker examined most frequently here was von Willebrand factor (vonWF), which has only certainly been identified in platelets as well as endothelial cells, using a rabbit anti-human vonWF monoclonal antibody (Dakopatts, Denmark). Also for the identification of HUVECs a commerciallly available murine antibody directed against a membrane component of HUVECs was used - QBEnd 40.1 available from Quantum Biosystems Ltd UK. The method employed was a 3 layer Biotin-Avidin staining method on cytospin cell preparations which is described in Appendix 1.

Cell Yield

In a consecutive series of 20 preparations cell yield per cm^2 umbilical vein surface was assessed. I decided that counting cells immediately after harvesting would give misleading results as HUVECs, as all endothelial cells, show a tendency to clump on initial harvest making accurate counting difficult and also cells initially harvested may fail to attach despite apearing to be viable at the end of the end of the harvesting procedure - counting of such cells might falsely elevate the counts.

I had noted that approximately 24 hours after plating the majority of cell clumps had attached and were starting to spread and I decided that counts of viable cells at this stage would give the most reliable

numbers.

At the time of harvesting the vein length and diameter were measured and recorded. 24 hours after plating the medium was removed and the cell layer washed with MEM. It was then treated with trypsin/EDTA as it would for passaging. However once the complete medium was added the cell suspension was centrifuged at 300g at 4°C for 7 minutes, the supernatant was decanted and the pellet resuspended in 4ml complete medium. 50microlitres(mcl) of this was removed and mixed with 50mcl Trypan Blue (Sigma Chemical Co. UK) in an Eppendorf tube. Cell counts were done using a haemocytometer (Neubauer), and viability assessed on the basis of Trypan Blue staining with non-viable cells taking up the blue stain. The remaining cell suspension was replated in a T25 flask and cultured as normal.

Results

Cell Identity

At all stages cells showed the typical appearance of endothelium, at confluence producing the well described cobblestone appearance (Fig 5.1).

They also stained positively for the von Willebrand factor antigen (Fig 5.2)and for the QBEnd 40 antigen.

Fig 5.1

Human umbilical vein endothelial cells in culture.

Phase contrast micrograph (x100).
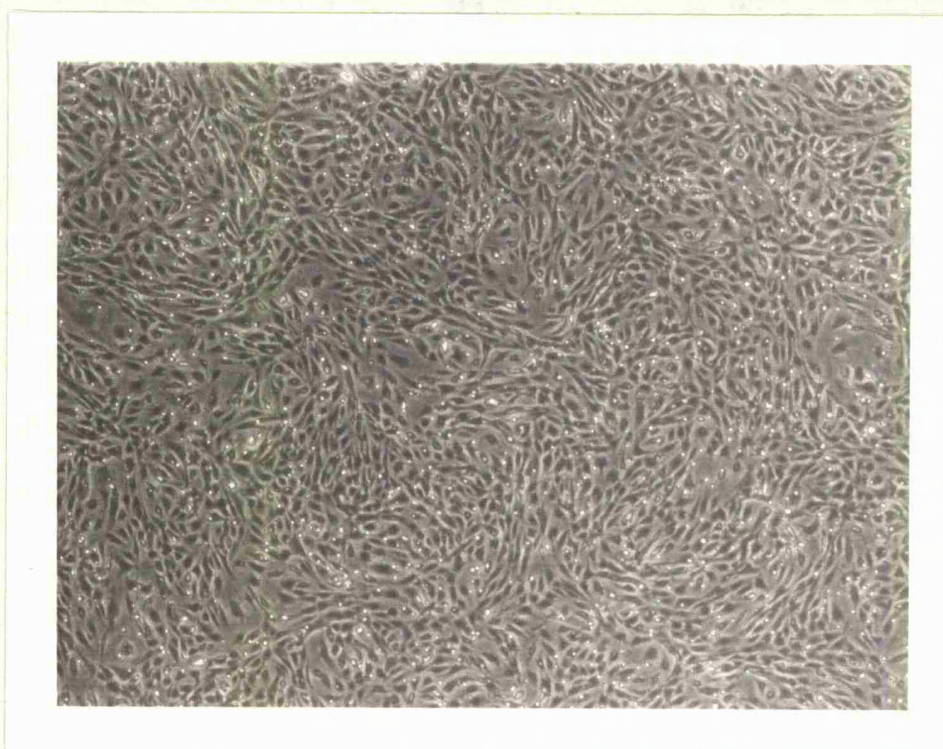
Fig 5.2

Human umbilical vein endothelial cells.

Biotin-Avidin stain for von Willebrand factor,control and positive, the

latter staining red.
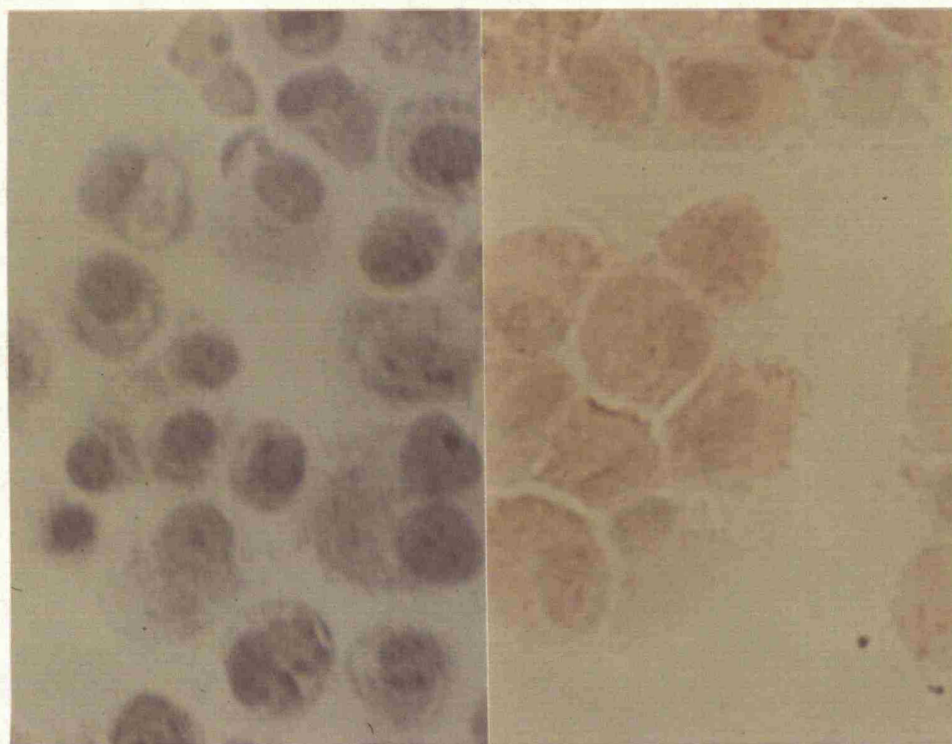
Cell Yield

The mean length of cord when the vein was distended with collagenase was 28.4cm (SD 7.16cm) - while the umbilical vein curves in its course through the cord when distended these curves are minimised and the length of cord when the vein was distended was presumed equal to length of vein. The mean diameter of umbilical vein was 4mm.

The mean cell count at 24 hours was 1.064×10^6 - thus giving a yield per cm^2 vein of 3.11×10^4.

SECTION TWO

Coagulation Function

Prostacyclin Production

Prostacyclin (PgI_2) is a very unstable product of endothelial cells and measurements were made of its more stable metabolite 6-keto PgF_1alpha. (In this and subsequent chapters alpha will be represented as "a".)

Pro-coagulant Activity

Both cell surface and total cell activity was assessed — the cell surface method based on that of Bevilacqua et al (1984), and the total cell method on that described by Lyberg et al in 1983.

Prior to these experiments it was necessary to establish a thromboplastin curve from which future clotting times could be translated into procoagulant(PCA) units.

Materials

Thromboplastin: Thromboplastin was obtained as powder from Sigma Chemicals, UK and diluted by factors of 10 in sterile MEM.

Calcium Chloride: This was obtained from Flow Laboratories and diluted to 0.025Molar in sterile water.

Platelet rich plasma: Venous blood samples were drawn from volunteers into vacuumed tubes already containing Sodium Citrate (Sarstedt). These samples were immediately centrifuged at 4500rpm for 5 minutes. If the plasma was not for use immediately it was stored in 1ml aliqouts at −70°C.

Methods

Prostacyclin Production

HUVECS were plated onto 24-well plates (Costar, USA) at confluent density (10^5 cells/well) in 1ml complete medium on the day prior to each experiment. They were cultured overnight in the same conditions as before. Immediately before each experiment the cells were observed under the phase-contrast microscope to ensure they had attached and were confluent. The medium was then aspirated and 1ml MEM pre-warmed to 37°C was added to each well and this was allowed to incubate at 37°C in air/CO_2 as before for 20 minutes. At the end of this time 100mcl of the MEM was removed and stored in an Eppendorf tube, this sample representing BASAL release levels. Then one of a number of stimulating agents was added to each well to a given concentration leaving 2 wells on each plate unstimulated. Agents added (with final concentration in brackets) were human thrombin (0.5unit/ml), Bradykinin (10nmol/ml) and Calcium Ionophore (10mcmol/ml), (all chemicals from Sigma Chemicals, UK, diluted in MEM).

The plates were then incubated as before for a further 20 minutes at which time another 100mcl sample was taken - representing STIMULATED release - and the cell appearance was once again checked under the phase-contrast microscope. The samples were stored at -70°C until assayed using a radio-immunassay kit (Amersham Radiochemicals, UK). Details of the radio-immunassay method are given in Appendix 3.

Thromboplastin Curve

Immediately before the clotting assay all reagents were warmed to 37°C in a water-bath, 0.1ml each of plasma and thromboplastin were placed in

a clean glass tube and this warmed in the water-bath for a few seconds. To these was then added 0.1ml of the Calcium Chloride starting a stop-watch as it was added. The tube was then held in the water-bath for 8-10 seconds rocking it gently backwards and forwards to ensure mixing of the reagents without too much agitation. At 2 second intervals after this the tube was removed from the water-bath to examine for the formation of fibrin strands, as soon as these were seen the stop-watch was stopped. These assays were performed in duplicate so that results were correct to within one second. By entering these times onto a log-log graph a standard thromboplastin curve was obtained which was linear between 16 and 80 seconds, a measure of its reliability. By using this curve it was possible to convert clotting times in future experiments into units of pro-coagulant activity (PCA). For future comparisons 1000 units of PCA was ascribed to full strength commercial thromboplastin.

Total Cell PCA

HUVECs were plated in 24-well plates as in the prostacyclin experiments and incubated overnight. The medium was then removed and the cell layer washed in 0.5ml MEM. This was then removed and a fresh 0.2ml MEM added - each plate was then examined under the phase contrast microscope to ensure the cell layer was intact and the plates were then sealed with tape and placed in a -70°C freezer and allowed to freeze completely ie. minimum overnight freeze.

Prior to the assay the plates were thawed and the cells further disrupted by scraping the well repeatedly with a sterile pipette tip. 0.1ml of this suspension of disrupted cells was then used in the one-stage clotting assay, in duplicate as above, in place of thromboplastin,

- 72 -

with a thromboplastin control performed alongside.

Cell Surface PCA

In the initial steps of this experiment the cells were treated as above, being plated and incubated in 24-well plates overnight and the cell layer washed in MEM. After this washing step (to remove traces of foetal calf serum), to each well was added 0.2ml MEM to keep the cells moist, this MEM having been pre-warmed to $37^{\circ}C$. From each well in turn this was aspirated and 0.1ml each of pre-warmed pooled plasma and 0.025M Calcium Chloride added, starting the stop-watch as the last reagent was added. Again the watch was stopped when the appearance of fibrin strands indicated that clotting had occured.

To confirm that the cellular pro-coagulant factor being assessed was, indeed, thromboplastin/tissue factor these experiments were repeated using factor VII deficient plasma (Sigma Chemicals, UK) in place of the pooled plasma. Clotting times of commercial thromboplastin were also measured using factor VII deficient plasma.

Results

Prostacyclin production

Unstimulated cells produced low levels of $PgF_{1\alpha}$ in the range 1.56-5.82pcg/min/10^5 cells and this level did not alter without stimulation over a further 20 minutes, in five experiments mean basal and "stimulated" levels (when the stimulant was simply MEM) were 5.74pcg/min/10^5 cells and 2.44pcg/min/10^5 cells respectively ($p>0.5$ T-test, $p>0.8$ Mann Whitney U test).

However in the face of the stimulating agents the cells reacted strongly. The rise was statistically significant in all cases with the actual figures being higher in the case of Thrombin and Calcium Ionophore. The variance was greater in the Thrombin results causing the differences between the two sets of tests.

	Basal	Stimulated		
Control	5.74(4.55)	2.44(9.45)	p>0.5	p>0.8
Bradykinin	3.08(2.77)	19.13(3.89)	p=0.001	p<0.008
Thrombin	1.56(1.16)	82.89(69.60)	p<0.06	p<0.008
Ca Ionophore	5.82(4.42)	80.08(18.45)	p<0.003	p<0.008

Results = $pcg/min/10^5$ cells mean with standard deviation in brackets.

Statistical tests = Paired t-test and Mann Whitney U test. (Performed using Amstat statistical package which was also used for tests elsewhere in this thesis)

Procoagulant Activity

Total Cell PCA

In twelve experiments on cells from different cords the total cell activity was 6.86 (SD 2.56) units, indicative of a low thrombotic potential.

Cell Surface PCA

Here the results were even lower with a mean of 1.62 (SD 0.8) units PCA. In all cases, including experiments using commercial thromboplastin, clotting did not take place within 180 seconds when factor VII deficient plasma was used.

SECTION THREE

Immunogenicity of HUVECs

In any immune reaction lymphocyte proliferation takes place, and in an in-vitro situation such a reaction offers an index of the powers of the stimulator. Endothelial cells are known to be capable of expressing MHC Class II antigens and have been reported in the past to excite lymphocyte proliferation in-vitro (Hirschberg et al 1975), this reportedly being maximal after 5 days of co-culture. After initial attempts on my part failed to to reproduce the same results a series of experiments were performed to clarify in detail the MHC Class II status and lymphoproliferative capacity of the human umbilical vein endothelial cells being isolated.

Materials

Lymphocyte Culture Medium: The basic medium was RPMI 1640 (Northumbria Biochemical, UK) to which was added human serum to 5% concentration. Other additives, with final concentrations in brackets were:
Penicillin (100U/ml), streptomycin (100U/ml), 1-glutamine (2mmol/ml), pyruvate (1mmol/ml), 2-ME (0.1mmol/ml).

Mitomycin C: This was obtained from Sigma Chemicals, UK as a stock solution of 1mg/ml. It was used at a concentration of 25mcg/ml diluted in MEM.

Lymphocyte freezing mix: This was made up by mixing DMSO with RPMI 1640 and Foetal Calf Serum in a ratio of 1:4:5.

Concanavalin A: Obtained from Sigma Chemicals, UK and a solution made of 0.8mg/ml in RPMI 1640. This was filter sterilised prior to storing at 4°C. On the day of experiment it was further diluted to a concentration

of 4mcg/ml, again in RPMI 1640.

[3]H *Thymidine*: Obtained from Amersham, UK. Activity 37MBq in 1ml, specific activity 25Ci/mmol. Used as a 1:20 dilution in RPMI 1640.

Methods

Isolation and Storage of Lymphocytes

Lymphocytes were isolated from the peripheral venous blood (hence the abbreviation PBL - peripheral blood lymphocyte) of volunteers. 40ml of blood was withdrawn into syringes previously loaded with sodium Heparin to a concentration of 10units Heparin/ml blood. The blood was then placed in sterile universal containers in 10ml aliquots and to each was added 10ml MEM - this was mixed by gently inverting the capped container. 10ml Lymphocyte Separation Medium(LSM) (Flow Labs, UK) was then placed into each of a series of fresh universal containers and onto this was layered gently 10ml of the blood/MEM mixture using a pipette with powered pipette-aid. This was repeated until all the blood was layered leaving a total of 8 universal containers. These were then centrifuged at 2000rpm for 30 minutes at 20°C - ensuring the brake on the centrifuge was switched OFF.

At the end of this time the PBLs could be found at the interface now between LSM and MEM - the other blood components having clumped at the bottom of the tube. The cells were aspirated from the interface using a Pasteur glass pipette and placed into a 50ml conical polypropylene tube, it was not possible to adequately aspirate the cells without taking some of the fluid also and on average there was a volume of 20ml at this stage. The tube was topped up with MEM containing 5% FCS - the contents

mixed by gentle inversion and then centrifuged at 1000rpm for 10 minutes
at 20°C. The supernatant was then decanted and the cells resuspended in
50ml MEM (no FCS) before being centrifuged similarly again. After this
second wash the cells were resuspended again in 50ml MEM, but before
being centrifuged two 50mcl aliquots were taken for cell counts and
viability assessement using Trypan Blue. Thus after the third wash one
had accurate assessment of the number of cells in the final pellet.

If the PBLs were not to be used immediately they were frozen in liquid
Nitrogen(LN$_2$). To do this the final cell pellet was resuspended in
lymphocyte freezing mix which was kept at 4°C until immediately before
use. The cells were resuspended at a concentration of no greater than
7.5X10^6 cells/ml, and placed in 1ml aliquots into 1.5ml polypropylene
freezing vials (Sterilin, UK). To ensure as steady a rate of freezing as
possible these vials were placed into a polystyrene box which was then
put into a 4°C fridge for one hour, before being transferred to a -20°C
freezer for a further four hours. It was placed in a -70°C freezer
overnight (minimum 16 hours) before the vials being plunged separately
into LN$_2$ -196°C.

On the day of use the vials were removed from the LN$_2$ and rapidly thawed
by placing them in a 37°C water-bath. The cells were then washed as
before, once in MEM + FCS and twice in MEM alone with a pair of cell
counts and viability assessment at the last step. For the experiments
the final pellet was resuspended in lymphocyte culture medium at a
concentration of 10^6/ml.

Mixed PBL/HUVEC culture
For mixed lymphocyte/endothelial cultures HUVECs passage 2-4 were

harvested from tissue culture flasks where they had grown to confluence. They were harvested and counted as before and resuspended in 2ml MEM at concentration of no more than 2 X 10^7/ml. PBLs from one donor were likewise resuspended in MEM, these being known as stimulator PBLs. To each of these cell suspensions was added Mitomycin C (to inhibit stimulator replication) at concentration 25mcg/ml and this was allowed to incubate in a 37°C waterbath for 30 minutes. At the end of this time the cells were washed three times in MEM with a cell count/viability assessment before the last wash. The final resuspension being in endothelial cell medium for the HUVECs and in lymphocyte medium for the PBLs at 10^6/ml.

For each experiment responder PBLs were set up in culture in 96-well plates at 10^5/well against a number of agents:

Culture medium only to give an assessment of background proliferation.

Stimulator PBL (also 10^5/well) — this being a mixed PBL reaction provided an index of PBL activity and an assurance that responder PBLs were indeed capable of a proliferative reaction.

Concanavalin A — another positive control/assurance that responder PBL could and would react.

Mitomycin C treated HUVECs — the mixed HUVEC/PBL reaction. The Mitomycin C treatment aiming to reduce/abolish HUVEC background proliferation without altering cell surface characteristics.

Each reaction was set up in quadruplicate and each reagent was also incubated in conjunction with medium only to minimise any error that could possibly arise related to background activity.

The plates were incubated in 95%air/5%CO_2 at 37°C in a humidified incubator.

On the day prior to harvest each well was "pulsed" with ^{3}H Thymidine by adding 10mcl solution, and returned to the incubator. On the following day the cells were harvested onto paper discs (Titertek harvester filters, Flow Labs) using a cell harvester, wells being washed with 50:50 distilled water and Methanol. After drying the discs in a dry 37°C oven each one was placed in a scintillation vial to which was added 3ml Optiscint 'Safe' scintillation fluid (LKB Scintillation Products). The beta radioactivity was then measured in a LKB 1217 Rackbeta liquid scintillation counter (LKB Ltd.) each vial going through for five minutes. Disintegrations per minute(DPMs) were calculated in the counter and used in all comparisons.

Results

In an initial series of five experiments where cells were pulsed on day 5, PBLs failed to show a proliferative response in co-culture with HUVECs, despite showing a significant response in culture with allogeneic lymphocytes (Table 5.1).

Table 5.1

PBL vs Medium   1.6 +/- 0.45
PBL vs HUVEC   1.6 +/- 0.61
PBL vs PBL   7.03 +/- 0.87
(DPM X 10^3 mean +/- SD)

The failure of marked proliferation PBL vs HUVEC at day 5 was repeated in a separate series - this time of 4 experiments - where each set of plates was assessed daily over 7 days. Again PBL vs PBL response was

most marked at day 5 but it was clear that the maximal PBL vs HUVEC response was at day 3, fading at day 4. Unlike the first series there was a small rallying of the response at day 5 but this never reached the earlier peak.

Direct comparison of the DPM figures is not possible because of the span of days therefore results are expressed (in Table 5.2) as a means (+/- sem) of the multiple of the control activity (ie PBL vs Medium only) on the day specified. It can be seen that the response to Concanavalin A (Con A) was maximal on day 3 as would be expected.

Table 5.2
Maximal Response as multiple of control
PBL vs HUVEC   day 3   8.7 +/- 3.3
PBL vs PBL   day 5   29.1 +/- 18.25
PBL vs Con A   day 3   25.5 +/- 5.42

MHC Class II expression of HUVECs

In parallel with these experiments another series of experiments was conducted to assess using FACS analysis the MHC Class II antigen expression of the cultured HUVECs.. These were examined using HUVECs in straightforward culture and also in HUVECs whose Class II expression was stimulated with gamma interferon (IFN).

Materials

Gamma Interferon (IFN): obtained in recombinant form from Sigma Chemicals.

Control Antibody: Anti HLA A human 131 A3 (Dakopatts)

MHC Class - Anti Dr Antibody: HB 55 (Dakopatts)

Fixative for FACS analysis: Paraformaldehyde (BDH Chemicals) 1g in 100ml particle free saline (sheath fluid) (Becton Dickinson Ltd.)

Fluorescent Medium: A solution made up with MEM with 5% foetal calf serum and 0.2% sodium azide.

Serum: Rabbit serum and anti-mouse FITC obtained from Sigma Chemicals and diluted in MEM.

Methods

HUVECs were harvested and cultured as before. 72 hours prior to preparation for FACS analysis the cell culture medium was changed completely and to one flask was added gamma interferon to a concentration of 1000U/ml. The flask in parallel had normal medium exchanged. The flasks were then left undisturbed in the usual incubator for 72 hours.

On the day prior to the experiment the endothelial cells were harvested from the flasks with Trypsin/EDTA as usual and left in the incubator overnight in non-tissue culture Petrie dishes (2.5 x 10^6 cell/90mm dish). On the day of experiment the cells were centrifuged at 1500rpm for 10 minutes and then resuspended in fluorescent medium at 5 x 10^6/ml. 100mcl of this suspension was placed in each of a series of 2052 tubes (Sterilin). 100mcl of 1:20 rabbit serum was added to each tube and left on ice for 30 minutes. After this the tubes were centrifuged at 1500rpm for 10 minutes and the supernatant decanted off. To each tube was then added one of the antisera or fluoroescent medium as control. These were mixed gently on a vortex and left on ice for one hour. To each was then

added 4ml cold MEM, this was gently mixed and then centrifuged at 2000rpm for 5 minutes.

The supernatant was then decanted off carefully and the pellet resuspended in the remaining droplet. The cells were again washed in 4ml MEM and the resuspension in the droplet repeated.

A second layer of anti- mouse serum was then added 100mcl of 1:20 dilution this being fluoroscein labelled (FITC), this was left on ice for 40 minutes. After this the cells were washed in fluorescent medium three times. After the last wash the cells were resuspended in the remaining droplet and to this was then added 1ml FACS fixative. Cells were then passed through the cell sorter.

Results

Results of FACS analysis are expressed as a percentage positive cells of the total number of cells to which the machine is exposed. Results are expressed as mean +/- SD., of seven analyses.

HUVEC No IFN   0.86% +/- 1.7%

HUVEC + IFN   66.06% +/- 19.49%

p=0.0002 Paired T test.

Fig 5.3

Effect of IFN pre-treatment on MHC Class II expression of HUVECs.

CLASS II WITHOUT/WITH IFN
Histogram parameter (H) Fluorescence 1
Vertical scale 256
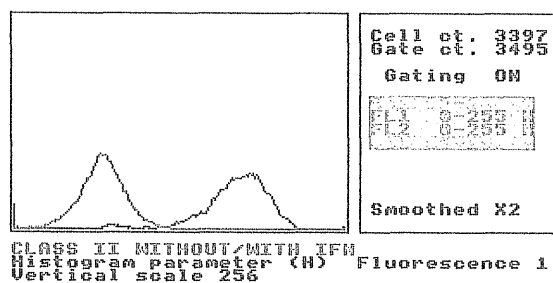
Effect of IFN pre-treatment of HUVECs on PBL proliferation in co-culture.

Bearing the results of the above sets of experiments in mind I set up a further series of experiments to look at the effect of pre-treatment of HUVECs by IFN, and thus induction of Class II antigen expression, on PBL proliferation. In view of the results of the initial proliferation data I reviewed plates at 1,2 3,5 and 7 days. Pre-treatment with IFN was for 72 hours at 1000U/ml as before. On each plate PBLs were set up in culture against treated and non-treated HUVECs – otherwise all details were as before.

Results

In a series of 3 experiments there was a significant difference between treated and non-treated HUVECs in their effect on PBLs. (cHUVECs = conditioned/pre-treated HUVECs).

The results are represented graphically in Fig. 5.4. One can see that cHUVECs excite a significantly greater response at day 2 and this is also the case at day 5 – at both these times the difference between control and cHUVECs is $p=0.02$ while control vs HUVECs fails to reach significance at $p=0.5$ on both days.

At day 2 the difference between the reaction to the different HUVECs reaches significance $p=0.01$ but at day 5 the figure is 0.08.

At day 3 both sets of HUVECs excite a significant proliferation and by day 7 neither reaction is obvious.

(Paired T-test).

Fig 5.4

Effect of IFN pre-treatment of HUVECs on subsequent PBL proliferation.
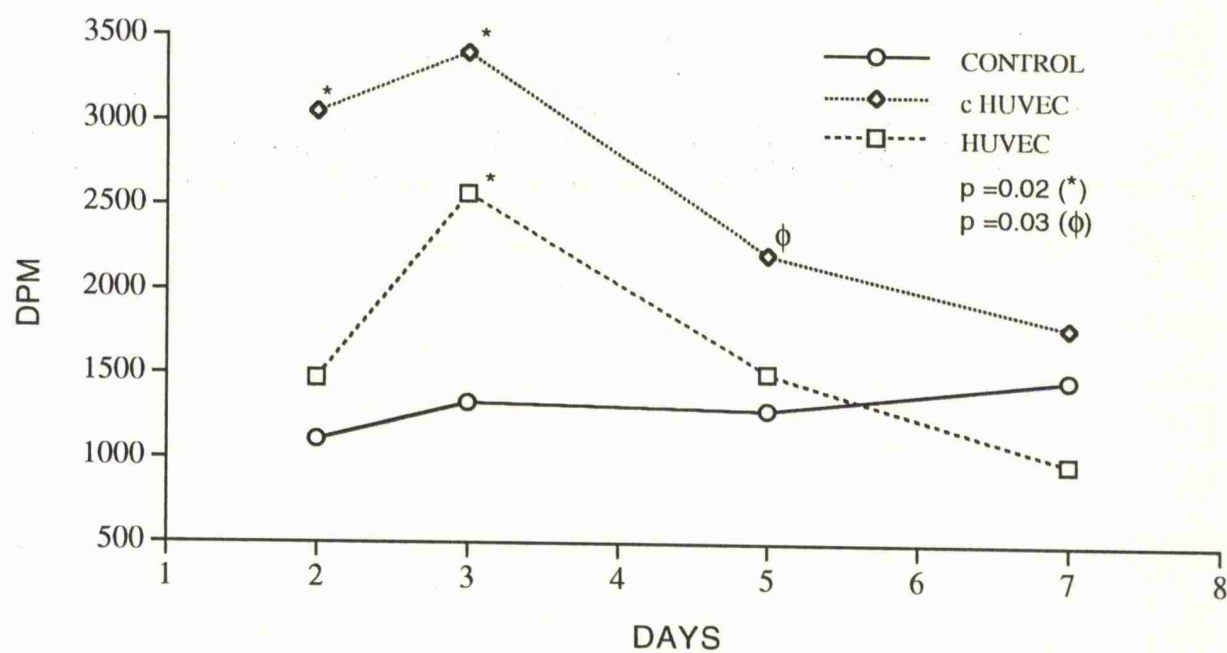
Discussion

The concept of using non-autologous cells for endothelial cell seeding is not a new one and has many theoretical advantages. If one could build a cell bank - whether it be of cells in continuous culture or of cryopreserved cells - one would be in a position to answer urgent clinical demands such the situation of critical ischaemia, while avoiding the time consuming and potentially variable measures of harvesting and isolating autologous cells.

HUVECs proved to be easy to harvest and, given the basic culture environment, to culture and passage. The cells are reproducibly positive for endothelial cell markers and even in sequential sub-culture display endothelial cobblestone morphology. Using widely accepted techniques these cells produce appreciable levels of the powerful anti-platelet agent prostacyclin, although in this study the basal levels were not as high as had been suggested in the literature. A significant response to all the stimulating agents studied was noted. The cells failed to demonstrate any significant pro-coagulant activity in culture, both of these features in favour of their suitability for use in graft seeding.

However the theoretical disadvantage of using allogeneic cells is that of exciting a host rejection reaction. It has been shown in the past that white blood cells play a role in the loss of cells from seeded grafts - with more cells remaining attached when tissue culture medium rather than blood was used in a perfusion circuit in vitro (Gourevitch et al 1988) and in vivo leucopaenia being associated with improved cell retention in a canine model (Emerick et al 1987). Short of cell detachment several products of white cells including interleukins are capable of exciting endothelial pro-coagulant activity (Bevilacqua et al

1986) which could endanger graft survival. It has not been proven that endothelial cells under "attack" by white blood cells are definitely associated with mitogens for smooth muscle - but pro-coagulant function will attract/excite platelets which are.

The excitation of PBL proliferation in co-culture with HUVECs was first described in 1975 (Hirschberg et al). In their paper a significant proliferation was described at day 5 although they did mention that this was less in magnitude than the PBL/PBL response. It had been the intention here to try to modify endothelial cells in some way eg. gamma irradiation or ultraviolet light, to ameliorate this proliferative response but, despite repeated attempts it proved impossible to reproduce Hirschberg's results. Time and again the most significant response was at day 3 with only a slight proliferation - usually not significant - at day 5.

Having performed - in parallel - a series of experiments which confirmed the previously reported stimulation of MHC Class II (HLA Dr) antigen by gamma interferon (Collins et al 1984) the HUVEC/PBL culture experiments were repeated with a comparison of treated vs non-treated HUVECs. In these were shown that pre-treatment with IFN magnified - and hastened - the early proliferation, and attenuated it such that there was a significant difference in PBL proliferation to be seen with treated HUVECs at 5 days. This again is at variance with published reports which have shown no variation in proliferation when HUVECs were pre-stimulated, assuming there were sufficient T cells in a PBL population to produce stimulatory IFN (Pober et al 1986). Measuring PBL proliferation is an indirect way of assessing endothelial cell antigenicity in that it is a measure of the sum of effects - class II

expression or possibly some other stimulatory molecule, leucocyte responsiveness etc. However given that here gamma IFN has been shown to stimulate HLA Dr expression by several folds - and that such stimulation is capable also of increasing PBL proliferation, the case against IFN would appear strong.

In the context of clinical work such data are of importance. It is widely acknowledged that secretion of a wide variety of lymphokines is increased following the stress of surgery and thus one can assume that any transplanted HUVECs would be stimulated to express HLA Dr antigens - thus potentially exciting rejection. Even without rejection, proliferation of host white cells and subsequent cytokine production could disrupt endothelial cells enough to produce damaging pro-coagulant reactions. Loss of even some endothelial cells could theoretically expose any cell matrix used to increase initial cell adhesion - many of these being thrombogenic.

All in all this work has been of interest - stimulating excitement in the idea of a cell bank when the cell proved easy to harvest and culture - while turning up potential difficulties when the antigenic nature of such cell became more apparent. Why these PBL stimulation data are at some variance with the literature is not clear save to say that they were reproducible. The PBL cultures reacted appropriately to stimulator PBLs and to the stimulating agent Concanavalin A, two quality control steps. The HUVECs showed themselves capable of expressing HLA Dr in response to gamma IFN, and this stimulation altered the kinetics of the PBL response, indicating that this was, in truth, an immune response.

There are obvious avenues for research into endothelial cell antigenicity and its possible modulation. Pre-seeding techniques would

obviously be preferable given the multitude of side-effects associated with anti-rejection chemotherapy.

An added complication to the concept of using HUVECs for graft seeding is the current state of affairs pertaining to donor HIV infection status. The most current advice from the Chief Medical Officer in the UK is "in the case of tissues from living donors which may be stored prior to use, the tissues should not be transplanted until a second negative test at least 90 days later is obtained" (1990). This follows similar advice from the Center for Disease Control in the USA (1988) following a case of HIV being traced to tissue obtained from a bone bank. Obviously endothelial cells for seeding are not currently on the CMO's list, but in the light of their being theoretically capable of being "stored" – either in culture or frozen – an ethical point is raised. The logistical problem of maternal counselling along the lines currently advised is also present.

In the light of the above – while retaining the source as possibility – I turned my attention to autologous/adult endothelial cells.

CHAPTER SIX

ADULT VEIN ENDOTHELIAL CELLS

Introduction

In the face of undoubted antigenic properties associated with umbilical

vein endothelial cells and the theoretical harm these could cause in the

context of graft seeding I focussed attention on autologous sources.

The most widely used source in animal experiments and human clinical

trials has been superficial vein. In animals this was the first source

used with Herring initally scraping the endothelial layer and Graham

subsequently refining the eversion collagenase harvesting method.

Collagenase harvesting (whether by eversion or the more efficient

cannulation technique) has been shown to release large numbers of cells

in animal models - yields of the order of $0.4-1.5 \times 10^6$ cells from 10-

12cm lengths of canine external jugular vein being a widely quoted range

(Graham et al 1982, Stanley et al 1982, Koveker et al 1986).

However the data from the literature examining human veins are much more

varied. Harvesting efficiencies as low as 12-15% have been reported

(Sharefkin et al 1986) and even these same proponents of saphenous vein

as a cell source have described failure in the order of 20% to harvest

any cells at all (Watkins et al 1984). Even if the cell yields were

lower from human veins it would be theoretically feasible to culture

such cells until one had enough to seed a graft - however data so far

suggest that adult human vein cells show considerable lag in growth

curves when compared with animal cells (Kent et al 1989). This lag seems

less in the cells of non-smokers (Zilla et al 1989) but this is not the

population with which the vascular surgeon has to deal!

In the light of such data one set out to assess how successful the

application of the method outlined in the previous chapter would be when

applied to adult saphenous veins.

Materials

Fibronectin: Bovine fibronectin (Sigma Chemicals, UK.) - solution of

20mcg/ml in MEM.

Glutaraldehyde/Formalin: Glutaraldehyde 3%/ paraformaldehyde 3% in 0.1

Molar Cacodylate buffer.

Methods

Segments of saphenous vein were obtained from patients undergoing either

coronary artery bypass grafting (CABG), peripheral vascular grafts or

sapheno-femoral disconnection. In all cases segments were retrieved

which were excess to requirements and/or which would otherwise have been

discarded. In all cases the surgeon used a no-touch technique as much as

as possible as indicated by the operation (CABG/femoro-distal graft) or

because he knew the discarded segment would be examined (S-F

disconnection).

Vein segments were placed immediately into cold MEM solution and

transported to the laboratory (longest time 30 minutes). Where a section was to be examined under the scanning electron microscope(SEM) it was divided with a blade in the laminar flow hood and placed in glutaraldehyde/formalin fixative, processing for SEM is described in Chapter 10.

The differences in harvesting techniques between saphenous and umbilical veins were:

The use of smaller diameter metal cannulae in the saphenous veins

The placing of the collagenase distended saphenous vein in a petrie dish (Nunc, Denmark) containing pre-warmed MEM, rather than plastic bag, in the incubator as in the absence of supporting tissues the thinner walled saphenous vein displayed a tendency to dry out unless kept moist.

Cell pellets were resuspended in 1ml complete medium and plated in 2 x 450mcl aliquots into 2 wells of a 24-well plate (Nunc). These wells had been pre-coated with fibronectin, 0.5ml being left in each well for 30 minutes before being aspirated and the well left to dry before addition of the pellet. 0.05ml of each aliqout was examined for viability with Trypan Blue as before.

The wells were examined immediately after plating under the phase contrast microscope and daily thereafter. Medium was changed the day after harvest and half changed on alternate days subsequently.

Results

A total of 20 specimens was examined. All were segments of long saphenous vein - the majority (15) from CABG patients, while 3 were from patients undergoing femoro-distal grafts and 2 from sapheno-femoral

disconnections.

The mean length of segment cannulated was 3.6cm (range 1.8-6.5cm) and the mean diameter was 3.2mm (range 2.5-4mm).

Viable cells were harvested from 14 of the specimens - the remaining 6 showing only cell fragments and debris on phase contrast microscopy. Cell counts at harvest were not performed as, as with HUVECs, clumping renders counts at this stage unreliable. However under the microscope the best description of the harvest was "moderate". All plates were placed in the incubator whether or not the impression was that there were cells present.

Of the 14 segments initially showing cells 12 displayed evidence of cell adhesion at 24 hours, with the usual endothelial clumps. However in no case did the cells appear to spread or divide despite observation and changing of the medium up to 5 days. Plates were discarded when the cells detached.

Of 6 randomly selected specimens sent for SEM only one showed an intact endothelial cell monolayer (Fig. 6.1) - this was from one of the sapheno-femoral disconnections. All specimens demonstrated the undulating appearance of vessels fixed without intraluminal pressure, but most showed only sub-endothelial elements without cells (Fig. 6.2).

Figs. 6.1 & 6.2

Scanning electron micrographs of long saphenous vein segments with (upper figure) and without intact endothelial cell layers.

Discussion

While the literature, and experienced colleagues, had suggested there might be difficulties with this cell source the results proved to be, to say the least, disappointing.

Photographs from SEM returned after most of my failed cultures and proved to be most illuminating. In all cases care had been taken to handle the vein with gentleness - the specimens from femoro-distal bypasses were taken before passage of a valvulatome - an instrument known to wreak intra-luminal havoc (Scott et al 1988).

Sharefkin et al (1986) found only a 12-15% harvesting efficiency from adult vein - viewed realistically this figure probably reflected the real numbers of cells on the vein available for harvest. In 1987 Ortenwall et al reported the harvesting of $2.7 +/- 1.4 \times 10^4$ cells from long saphenous vein segments area $5.0 +/- 1.3cm^2$. The report then mentions that 80% of this yield (the amount they used for seeding) contained a mean of $1.7 +/- 0.8 \times 10^4$ viable cells - and of the 20% retained for culture such culture was established in "the majority" of cases - with only 4 persisting to passaging, this being a further reflection of impaired growth ability. While this group contented themselves with the term "majority" other groups have been more specific and reports of successful growth span the range 33-81% (Glassberg et al 1982, Watkins et al 1984, Gourevitch et al 1987).

It must be admitted, however, this series of results is worse.

The source of tissue I used was similar to most of the reports cited (with the exception of Glassberg et al who used iliac artery) - most specimens coming from patients having CABG grafts. While dependent on the cardiac surgeons for samples their harvest was observed in most

cases and vein trauma was avoided as much as possible, as cardiac surgeons have a vested interest in preserving endothelial cells also. ·

The cell harvesting technique was similar to that for umbilical vein which, as has been shown in the previous chapter, regularly yielded good numbers of viable cells. The same concentration of collagenase solution was used, such a concentration having been recommended for use on saphenous vein cells also to minimise cell damage (Sharefkin et al 1987). In all cases HUVEC work was progressing successfully in parallel, so faulty batches of collagenase cannot be blamed.

Wwells were pre-coated with fibronectin to maximise cell adhesion and spreading. the concentration of 20mcg/ml has been shown to be optimal in promoting HUVEC adhesion to ePTFE (Budd et al 1990), and is the concentation currently in use in saphenous vein cell in vitro work (Zilla et al 1990). Indeed cell adhesion was not the major problem.

The other potentially important variable was the concentration of endothelial cell growth factor in the culture medium. In the light of HUVEC success the same concentration (20mcg/ml) was utilised in the saphenous vein work. Other workers have used higher concentrations ranging from 75-300mcg/ml (Zilla et al 1990, Watkins et al 1984) and this could be an avenue for further research. However the major problem with these specimens was the failure to yield adequate numbers of cells capable of growth - the variation in growth factor would possibly alter doubling time but could not, one feels, be cited as the sole reason for the poor results encountered here.

The poor harvests are better explained by the SEM pictures demonstrating poor luminal coverage by endothelium. From the randomly selected specimens it was clear that large numbers of cells are lost during even

gentle mobilisation. Application of an in-situ cannulation technique by Zilla et al (1990) – where collagenase is instilled into the isolated vein segment <u>before</u> it is removed – has still yielded low cell numbers – 20.4 +/– 13.8 cells/cm^2 vein although a similar technique applied to baboon external jugular vein yielded a mean of 1.2 x 10^{4}cells/cm^2 – demonstrating graphically the difference between animals and humans. Even given that such human cells grow and divide Zilla et al found it took a mean of 26.2 days (22.3–32.2) to yield sufficient cells to confluently seed a 62cm x 6mm graft from a "small" segment of saphenous vein. Such a delay may well be acceptable if one is operating for claudication but 4 weeks is a long time in critical ischaemia terms – in many cases too long. The real danger of harvesting nothing at all still remains.

Summary

Despite using a technique previously successful with human umbilical vein, culture of endothelial cells from adult saphenous vein virtually completely failed. A random selection of such vein segments displayed largely absent endothelial coverage on SEM – the most likely explanation for the failure to harvest adequate numbers, such cells as were harvested proving unsuitable for in vitro culture.

CHAPTER SEVEN

ISOLATION OF HUMAN MICROVASCULAR ENDOTHELIAL CELLS FROM OMENTUM

Introduction

Attention in more recent years has turned towards adipose tissue as a source of microvascular endothelial cells for seeding. The first description of an isolation method from omentum was in 1983 when Kern, a cell biologist, detailed the use of a collagenase digestion technique with subsequent filtration of the digest to purify the cell yield. Vascular surgeons have investigated the source since the mid-1980s with Jarrell et al (1986) and Pearce et al (1987) using density gradient centrifugation on the polysucrose medium Percoll instead of/ as well as filtration for purification. Numerous other workers have adapted the technique since (Table 7.1). However virtually all authors have acknowledged a considerable degree of contamination by other cell types in the final isolate. Sterpetti et al (1988 and 1990) examined the use of such cell isolates in in-vivo canine models and demonstrated increased subendothelial layer thickness when filtered microvessel cells were compared with autologous vein cells and when filtered cells were compared with Percoll centrifuged cells. Such a result might indeed be expected from implanting smooth muscle cells and fibroblasts in situ on a graft and would eventually be expected to negate the potential benefits of seeding the grafts.

Table 7.1

Year	Author	Tissue	Method
1983	Kern	Human omentum	Mince/collagenase filter x2
1986	Williams	Human omentum	Mince/collagenase Percoll
1987	*Pearce	Canine omentum	Mince/collagenase filter x2
1988	*Sterpetti	Canine omentum	Mince/collagenase filter x2
1989	*Williams	Human omentum Human fat	Mince/collagenase filter
1989	*Sharp	Human fat	Mince/collagenase
1990	*Sterpetti	Canine omentum	Mince/collagenase filter vs Mince/collagenase Percoll
1990	*Wang	Canine omentum	Mince/collagenase Lymphocyte separation medium

*=author acknowledges cell contamination in text.

Bearing this in mind I set out to establish a reliable, reproducible isolation technique which would avoid the result of significant contamination by cells associated with intimal hyperplasia - this is described in Section One. In Section Two details of experiments performed to examine the suitability of such cells for graft seeding, namely coagulation function and graft adhesion ability, will be related.

SECTION ONE

Harvesting and Culture of Microvascular Endothelium.

Materials

Bovine Serum Albumin: This was obtained from APP Ltd, Brierley Hill, UK as a stock 35% solution. This was kept in bulk at -20°C until used. Solutions made up with MEM were kept in 100-200ml aliqouts at 4°C for no longer than 2 weeks during a course of experiments.

All other reagents were as previously described.

Methods

Pieces of omentum were taken from 20 patients undergoing laparotomy for non-malignant disease. All patients had previously given informed consent along guidlines approved by the Leicester Health Authority Ethics Committee.

Tissue was isolated by the operating surgeon using a standard technique of division and ligation, this took no more than 5 minutes. Tissue

samples were placed immediately into cold MEM in a sterile container and taken to the laboratory (100 yards away). The weight of each piece was calculated by subtracting the weight of the pot + MEM (ascertained before going to theatre) from the weight of the pot + MEM + sample. This method ensured the tissue was kept sterile and avoided moving the laboratory balance. All manipulations of the tissue took place in a laminar flow cabinet unless otherwise specified.

The piece of omentum was washed in fresh MEM and immediately placed into 20ml 0.1% Worthington's collagenase pre-warmed to 37°C in a closed sterile pot and incubated on a shaking table (set at 120 movements/minute) in an incubator at 37°C for 15 minutes. The omentum was then removed and washed in 30ml MEM + 5% Foetal Calf Serum(FCS) before being placed on a sterile plastic dish (bottom half of a petrie type plate). The collagenase solution and the 30ml MEM + FCS were then mixed in a 50ml conical tube and centrifuged at 200g for 7 minutes at 4°C. This mesothelial cell pellet was subsequently resuspended in complete culture medium and plated in a 25cm² tissue flask and incubated at 37°C in 95% air/5% CO_2.

The piece of omentum was then minced finely using sterile scissors, until it was of a consistency "liquid" enough to be aspirated into a 20ml pipette (Costar, USA). It was then added in 10ml aliquots to 10ml aliquots of 0.1% Worthington's collagenase pre-warmed in sterile universal containers to 37°C. The containers were then shaken to mix the aliquots and placed on their sides (with the lids further secured with tape) on the shaking table in the incubator at 37°C. They were incubated thus for 30 minutes. At this stage the digest was passed through a 250mcm pore filter into a sterile glass beaker, being washed through

- 101 -

with a further 50mls MEM + 5% FCS. The beaker was then left to stand on crushed ice in an insulated container for 10 minutes - thus facilitating the separation of the lipid layer - the fat rising to the top. The cell suspension was then drawn off from beneath the fat and centrifuged at 200g for 7 minutes at 4°C. This cell pellet was then resuspended in stock 35% BSA - this necessitated repeated mixing by drawing up and down in the pipette because of the very viscid nature of the BSA at this concentration. The suspension was then divided 5ml each between two glass tubes (Dupont, UK), taking care to avoid smearing the suspension along the sides of the tubes. On each was layered 4ml each of 29% and then 26% BSA (densities 1.087 and 1.076g/ml) - again avoiding letting any smear the inside of the tubes. A final layer of 1ml MEM was added to each tube to ensure any cells rising to the top of the BSA would be kept moist. The tubes were then covered with laboratory film (Nescofilm) and transferred to the high speed centrifuge which had been pre-cooled to 4°C. They were spun in a fixed angle rotor at 18,000rpm for 30 minutes - care being taken to turn the centrifuge brake "off". The total spin and slow down time was 45 minutes.

Cells were gently aspirated from each interface using a Pasteur glass pipette and washed separately 3 times in MEM. They were subsequently plated in uncoated 25cm^2 tissue culture flasks and incubated in 95% air/5% CO_2 at 37°C. The cells were observed daily using the phase contrast microscope. When signs of adhesion and early spreading were evident (invariably by day 2 - approximately 36 hours after plating) the cell layers were washed with MEM and the cells harvested using Trypsin/EDTA in the manner described for HUVECs. Cell counts were performed in duplicate on 50mcl aliquots of cell suspension, also as for

HUVECs, and the cells returned to culture flasks and incubated. Cells were subsequently observed and assessed daily until confluent - typically between day 5 and day 7 but in exceptional cases on day 3 or 4.

At confluence cells were released from the flasks, counted and then processed for estimation of prostacyclin production and for immunohistological staining for von Willebrand factor antigen. A specimen of cells was also sent for transmission electron microscopy. In an initial series of 5 preparations cells from all layers were processed thus but subsequently as soon as cells were obviously non-endothelial (on microscopic appearance) they were discarded.

The methods for vonWF staining and estimation of prostacyclin production were as for HUVECs. Percentage positive staining cells was calculated following counting of cells in three fields on each slide. In practice it was easier to pick out the negatively staining cells after counting the total as these were so few. Fields counted contained at least 20 cells each.

Cells not utilised for these investigations were returned to a fresh tissue culture flask for culture and subsequent passaging. They were used in experiments on Indium labelling and adhesion to graft material as will be described later in this chapter.

Results

Cell Culture and Identification

On phase contrst microscopy it became evident that it was from the 35%/29% interface that endothelial cells were collected - the cells grew

- 103 -

to display the cobblestone morphology typical of endothelium in culture. (Fig. 7.1) Some endothelial cells did appear in cultures from the 29%/26% interface but these were invariably heavily contaminated by spindle shaped cells which showed no evidence of contact inhibition and which subsequently came to dominate the cultures.(Fig. 7.2) Such cells - which were probably a mixture of smooth muscle cells and fibroblasts - also appeared at the 26%/MEM interface and occasionally in the pellet beneath the BSA - in neither of these two layers were endothelial cells apparent.

The cells cultured from the initial mesothelial cell harvest failed to reliably resemble endothelial cells and typically presented a mixed cell pattern. (Fig 7.3)

Transmission electron microscopy (TEM) confirmed the mesenchymal nature of the cells, and while Weibel-Pilade bodies (an inconstant feature of endothelium) were not identified, the pattern of intra-cytoplasmic filaments and granules was consistent with endothelium.(Fig. 7.4)

Using the stain for von Willebrand factor antigen it was obvious that most of the cells from the 35%/29% interface stained positively. The staining pattern was a cytoplasmic granular one as has been described for HUVECs.(Fig 7.5) In all cases positively staining cells made up at least 98% of the total. In the initial stages when cells from all layers were stained positively staining cells accounted for no more than 50% of the total and this figure was usually much lower.

Fig 7.1 Human Microvascular Endothelial cells in culture.

Phase contrast Micrograph.
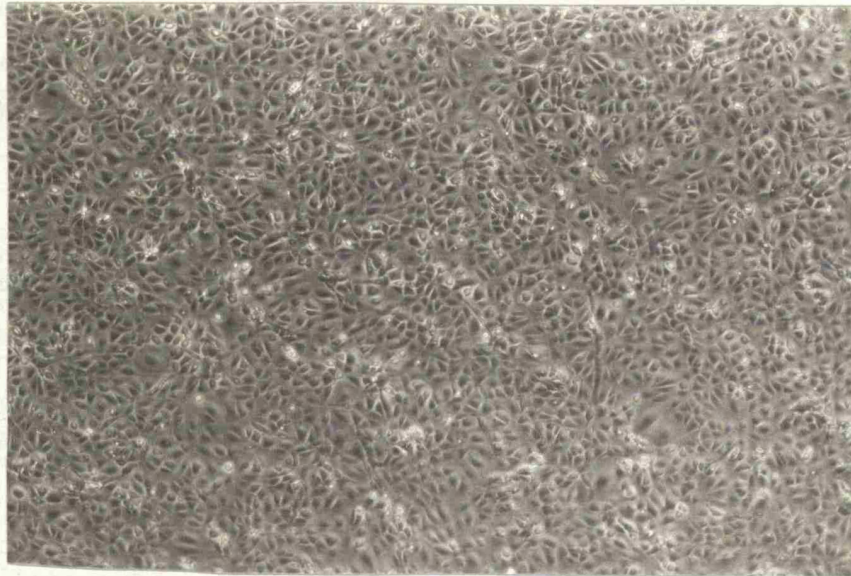
Fig 7.2 Cells from 29%/26% interface in culture.

Phase contrast Micrograph.

Fig 7.3 Mixed cell population in culture - in this case from initial

collagenase digestion.

Phase contrast micrograph.
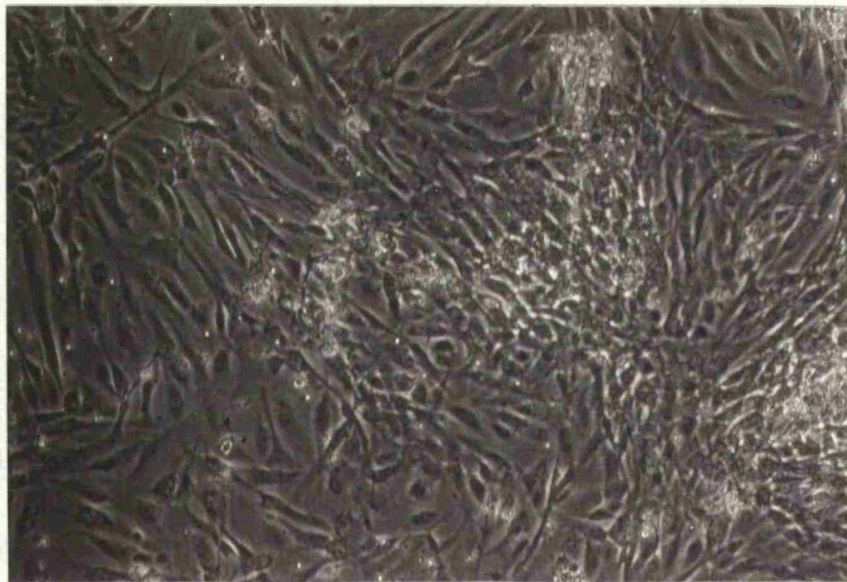
Fig 7.4 Transmission Electron Micrograph of Human Microvascular
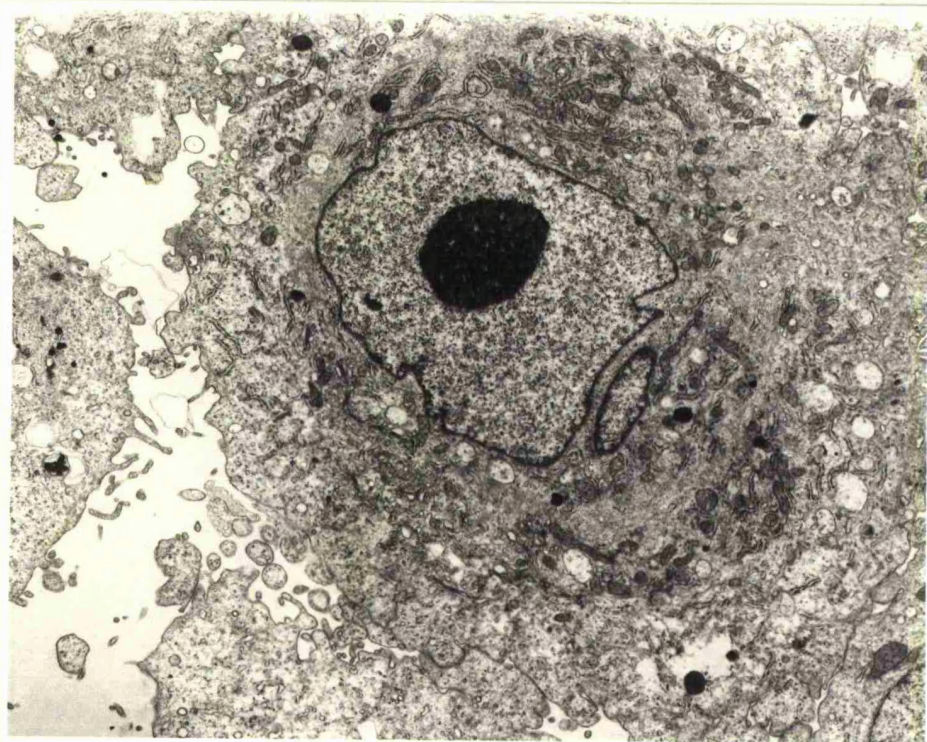
Endothelial Cell.

Fig 7.5 Human Microvascular Endothelial Cells - vonWF stain.

   Control (left) and Positive.
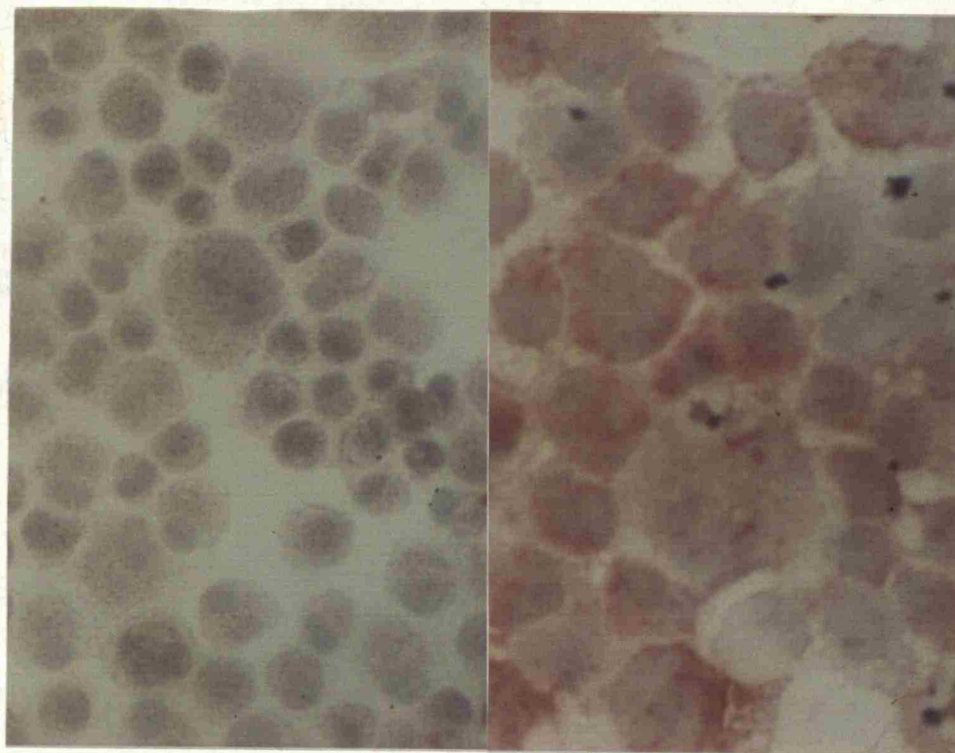
Cell Yield

The mean weight of piece of omentum taken at operation was 32.5g (SD 11.4g). Counts were performed only on the cells from the 35%/29% interface and these are expressed in terms of the weight of the original piece of omentum. The mean yield at 36 hours of plating was 4.45 $x10^4$/g (SD2.6 $x10^4$/g).

SECTION TWO

Suitability for seeding

Materials

Graft Coatings: Bovine fibronectin and poly-l-lysine (Sigma, UK), Collagen S (Boerhinger). Each solution was made up in MEM to the following concentration: fibronection 20mcg/ml, poly-l-lysine 20mcg/ml, collagen (20mcg/ml)

Human plasma was obtained from the venous blood of volunteers collected in citrated comtainers (Sarstedt) and centrifuged at 4500rpm for 5 minutes. Aliquots (1ml) were stored at -70°C until immediately prior to use.

Graft Material: Expanded polytetrafluoroethylene of standard thickness (0.64mm) was the kind gift of WL Gore & Associates Ltd., Livingstone, UK.

Methods

Coagulation Activity

Assessment of microvascular endothelial cell pro-coagulant activity and prostacyclin production was performed in a manner similar to that described for HUVECs in Chapter 5.

Graft Adhesion

As part of assessing the suitability of these microvascular endothelial cells for seeding it was necessary to examine their ability to adhere to the prosthetic material most commonly used for infra-inguinal bypasses − ePTFE. The method chosen was one of measuring the radioactivity present on graft surfaces having earlier radiolabelled the cells. However prior to this it was necessary to ensure that these cells could be labelled with the most commonly used isotope [111]Indium and that the leakage of the isotope from the cells was not so high that experiments over a number of hours might not be possible. Radiolabelling of vein endothelial cells has been described (Sharefkin et al 1983) but details of microvessel cell labelling/leakage characteristics are unavailable in the literature.

Indium Labelling/Leakage

HMVECs of no greater than passage 2 were harvested on reaching confluence from tissue culture flasks using Trypsin/EDTA. The Trypsin/EDTA was neutralised using serum containing medium and the suspension centrifuged as before. The cell pellet was resuspended in 0.5ml of an original 0.6ml solution containing 60mcCi [111]Indium in MEM − thus 50mcCi was added to the cells. The remaining 0.1ml was retained for

− 109 −

measurement of labelling efficiency.

The cell suspension was incubated for 15 minutes at room temperature and the centrifuged at 200g for 7 minutes. The supernatant was removed and saved. The cells were then washed twice - first in 24.5ml and then in 25 ml MEM - these washing being saved with the 0.5ml supernatant.

To determine labelling efficiency the original 0.1ml Indium solution was made up to 10ml by adding MEM - this was called solution A. The 50ml of supernatant plus washings became solution B. The radioactivity of 0.02ml of each was then measured in a LKB-Wallac 1280 Ultro Gamma II counter (LKB-Produkter AB. Sweden). Using the formula below the labelling efficiency was calculated:

$$\text{labelling efficiency}(\%) = \frac{\text{counts sol.A} - \text{counts sol.B}}{\text{counts sol.A}} \times 100$$

The cells having been resuspended in 5ml MEM, 0/05ml were taken for cell counts and viability assessment using Trypan Blue.

For the leakage experiments 0.5ml of the cell suspension was placed in each of 8 Eppendorf vials. Immediately 0.1ml was withdrawn from one of the vials and saved and the cell suspension in that vial spun at 1000rpm for 5 minutes. The remaining Eppendorfs were incubated at 37°C and at intervals 10,20,30,45,60,90 and 120 minutes one was removed as the first - which was ascribed 0 minutes. The cell suspensions were called B and the supernatants A - these were counted in the gamma counter and the percentage Indium leakage calculated using the formula A/B x 125 for each of the time intervals.

In a separate series of 6 experiments adhesion of labelled endothelial cells to ePTFE segments precoated with one of a number of coatings was examined.

Adhesion to graft surfaces

Segments of 6mm internal diameter ePTFE graft were opened longitudinally and mounted in a well made from a modified Eppendorf vial (Budd et al 1990). The luminal surface was thus exposed with an area of $0.5cm^2$ available using a standard size Eppendorf. 0.1ml of a graft coating material - or culture medium as control - was added to each well and allowed to incubate at $37^{\circ}C$ for 1 hour. The solution was then removed and the surface allowed to air dry at room temperature - in practice for 40 minutes.

Onto each well were then placed Indium labelled microvessel endothelial cells - labelled as described above. 0.5ml of cell suspension containing $3X10^4$ cells was added to each well with 7 wells for each coating material ie. seeding density $6X10^4/cm^2$. These wells were incubated at $37^{\circ}C$ in $95\%air/5\%CO_2$ for intervals of 10, 20, 30, 45, 60, 90 and 120 minutes. At each interval a well was removed from the incubator and the supernatant aspirated and saved. The well was then washed gently by dropping on MEM (0.1ml), this was added to the saved supernatant. The graft segment was then carefully removed from the Eppendorf vial and each graft segment and supernatant/washings counted in the gamma counter.

Cell attachment as a percentage was calculated using the formula:

$$\text{Cell Attachment \%} = \frac{\text{counts on graft}}{\text{counts in sup/wash + counts on graft}} \times 100$$

Results

Prostacyclin Production

Basal prostacyclin levels were quite considerable in a series of five experiments with an overall mean of 35pcg/min/10^5 cells. There was a good deal of variation in the response of the cells to the stimulating agents however. Mean levels altered (with SD in brackets) from 17(32) to 114.5(101) with Bradykinin, from 35(32.5) to 139.1(191) with thrombin, and from 36(36) to 31.4(25.8) following the addition of Calcium Ionophore - all values are of pcg/min/10^5 cells. As can be readily observed there was a good deal of variation between experiments (although little within each assay) and therefore the data was examined using both parametric and non-parametric tests. The nearest to a significant difference following stimulation was found with Bradykinin, with p=0.074 on Paired T-test but p=0.1 on Mann-Whitney U test.

Procoagulant Activity

In all cases total and cell surface was extremely low with less than 2units PCA/well in all assays.

Labelling Efficiency and Leakage

The overall labelling efficiency - for cells used in leakage and adhesion experiments was 38.7% (SD 7.44%). This is in keeping with the binding efficiency rates described by others for HUVECs (Budd et al 1990).

The ^{111}Indium leakage is best demonstrated graphically (Fig 7.6). At time 0 there was already a mean loss of 8% but this figure at 120

minutes was only 13.8%. This represents a loss of 3% per hour and is of

a low enough level not to significantly alter figures when interprets

the graft adhesion data.

Fig 7.6 ¹¹¹Indium leakage from Human Microvascular Endothelial cells.
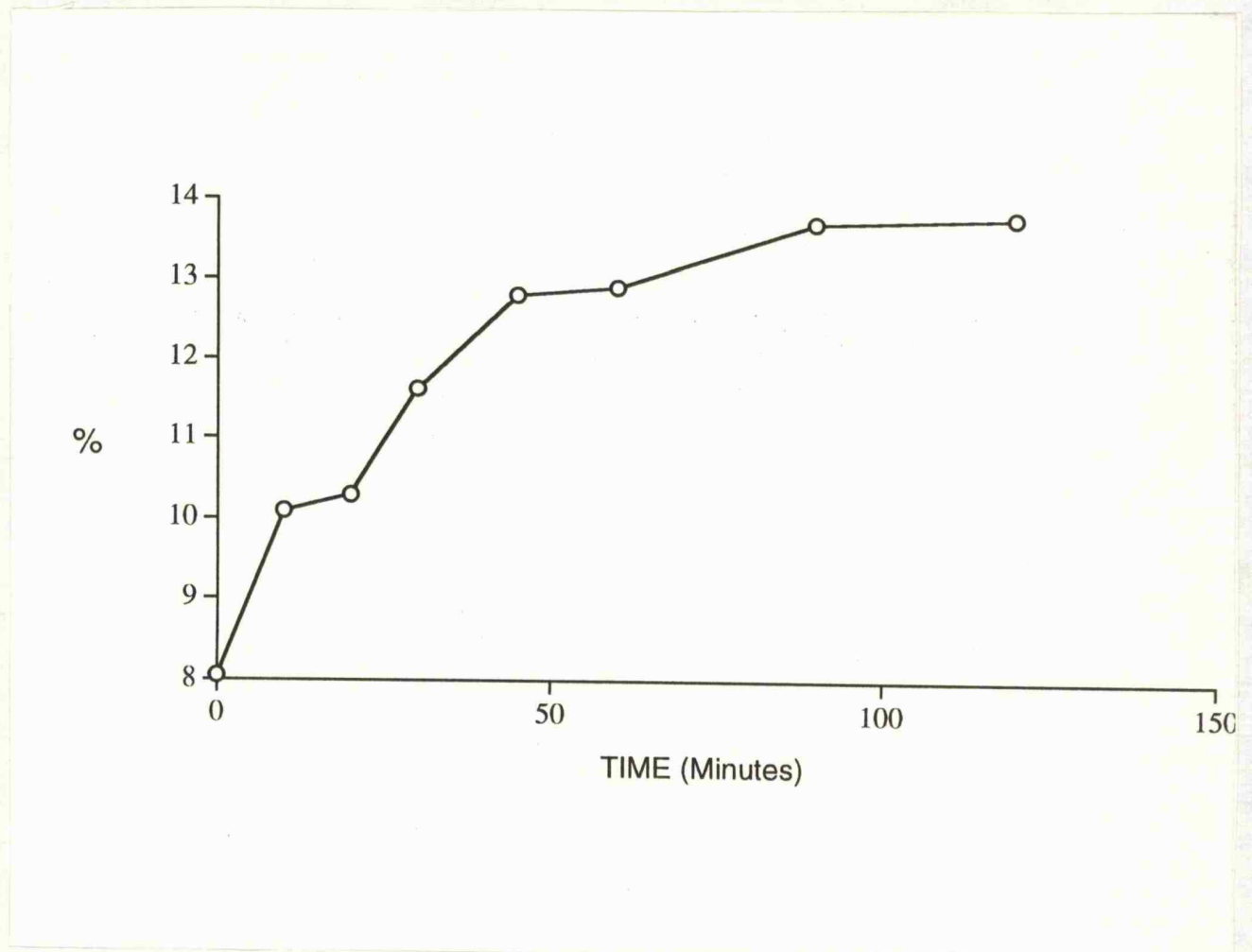
Adhesion to graft surfaces

The results of the adhesion experiments are represented in Table 7.2. As
one can see fibronectin proved to be the best of the three matrices
examined showing highly significant improvement in cell binding at 10
minutes and maintaining this improvement to 120 minutes with 70% of the
cells retained at this time. In keeping with the observation in the
laboratory that these cells adhere well to uncoated flasks is the fact
that 44% of the cells are adherent to uncoated ePTFE at two hours.

Table 7.2

Cell Adhesion to Graft Coatings.

Matrix	10min	30min	60min	90min	120min
Control	4.7(3)	15.5(6.5)	32(7.9)	43(8)	43.8(11)
P-1-L	8.3 (3.5)*	18(5.3)	31.8(7.3)	40(8.3)	45.6(10)
Collagen	10.4(3)*	20.6(9.5)	24.3(10)	34.6(8)	45.8(8)
Fibronectin	32.5(11)"	51.3(12)"	63.6(4.3)"	69.5(8)'	71 (6.5)'

% Mean (95% confidence interval) Paired T-test

* $p<0.05$ ' $p<0.01$ " $p<0.001$.   P-1-L = Poly-1-Lysine.

Discussion

The finding that endothelial cells settled at one of the interfaces on the first choice of discontinuous gradients was a bonus - although the discovery was not as easy as it may first appear. Attempts to predict the BSA densities required had been assisted by a researcher in the department, Mr James Wilson, who had some experience of working with BSA gradients before. In an early experiment he had placed cultured HUVECs on a continuous BSA gradient and had found them to congregate around the density 1.055-1.060g/ml. It proved a great disappointment when discontinuous gradients around these densities yielded no endothelial cells when work started on omentum. As a screening measure therefore discontinuous gradients using 35%, 29%, 26% 23% and 10% BSA were set up, the aim being to see around which range one might have to set up more exact continuous gradients to isolate the cells. The relief at finding the pure population without recourse to the latter more time consuming step was considerable.

The method proved to be very dependent on temperature, this initially posed a bit of a puzzle but was eventually simply remedied by keeping all reagents in the fridge until immediately before use and remembering to pre-cool the centrifuge. The method, once the initial teething problems were ironed out, proved itself reliable and the purity of the resultant cell population reproducible.

Fig 7.7 Discontinuous BSA gradient - here without cell pellet.
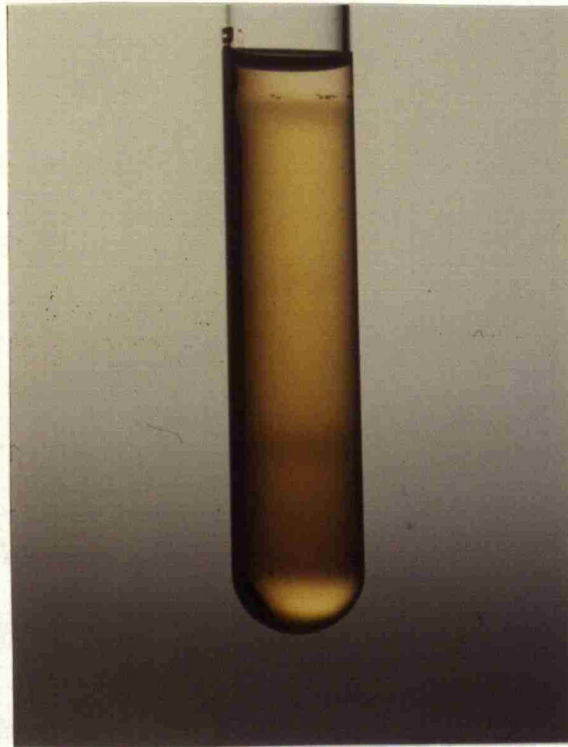
This ability to produce such pure populations would appear to be important – it is of note that endothelial cell morphology was retained as far out as passage 5 – at which stage one might expect contaminants to supervene particularly in view of their sturdy growth characteristics. Authors examining other isolation techniques have almost all reported significant levels (10%+) of contamination – although not all have quantified this accurately. While some have not noted gross contamination in initial cultures they have found the appearance of other cell types/loss of endothelial morphology in early passages (Sterpetti – personal communication 1991).

Bovine Serum Albumin has been used as a density gradient medium by workers in other fields, notably dendritic cell and pancreatic islet isolation (Lake et al 1987). It offers many advantages over other media such as Ficoll and Percoll, here being iso-osmolar, and less prone to producing clumping of the cells at the interfaces. While cells were routinely washed three times after isolation some of these steps could feasibly have been omitted as it known not to be toxic to other cell types.

The ability to produce Prostacyclin is an important marker of endothelial cells. It has been noted before that the response to stimulation is different in microvessel cells than it is in HUVECs (Carter et al 1989) and this has been our experience also, with Thrombin and Calcium Ionophore failing to reproduce the stimulating effects seen in the other experiments. The significance level achieved with Bradykinin is not as great as that seen in Chapter 5 in the case of HUVECs but there is little doubt that it would be maintained. It is possible that Thrombin could produce a significant difference in a

larger series (with p=0.48 Mann Whitney U test here) but it is unlikely

that this would be ever possible in the case of Calcium Ionophore with

p>0.8 in both types of test. The production of significant basal levels

from this cell type is the most important fact and as the dynamics of

Prostacyclin production in vivo are not completely clear it is difficult

to comment on the relevance of the stimulation differences. It may well

be that physiologically different receptors and/or stimulation dynamics

exist and it is interesting that these seem to persist in vitro.

It was reassuring to note these cells had no significant pro-coagulant

activity in culture. A possible avenue for future research would be the

responses of these cells to agents known to excite PCA in endothelial

cells from other sites but this was without the bounds of my brief here.

Having noted the adhesion properties of the cells to the tissue culture

flasks it was not unexpected to find that this pertained on ePTFE also.

The concentrations of binding matrices used were based on recent reports

using HUVECs and adult saphenous vein cells in the literature (Budd et

al 1991, Thompson et al 1991) - and the binding achieved with 20mcg/ml

fibronectin was of the order reported by these other workers. The

binding kinetics would appear to be somewhat different however in that

at two hours none of the other matrices examined showed a significant

advantage over uncoated graft, unlike the reports which demonstrated a

lesser though still important advantage. Fibronectin is relatively

expensive so the use of as low a concentration as possible is to be

favoured when one contemplates seeding larger areas.

The cell yield from this technique is lower than that described by

authors using other methods. However the aim here was to improve isolate

purity in view of the evidence that increased subendothelial thickness

has been the price to pay for impure innocula. The cells grow quickly in culture and it may be possible to harvest larger pieces of tissue initially. Reports in the literature of yields in the order of one million endothelial cells per gram of omentum (Jarrell & Williams 1991) appear somewhat extravagent when one has seen how much of the tissue is made up of lipid which is lost in the isolation procedure. With counts of 1.5-2 $X10^5$ cells on a $25cm^2$ tissue culture flask it is not difficult to see that one would have sufficient cells for a 60cm x 6mm graft (area $113cm^2$) in approximately three weeks from 20gm omentum at a passage rate of 1:3 allowing 7 days for confluence using my technique. Taking larger pieces of tissue would hasten the process as might be required in the clinical situation of critical ischaemia.

Harvesting omentum during a laparotomy is a relatively easy procedure, it is very rarely absent and most people have several hundred grams available. However in the context of graft seeding it is not ideal. Mini-laparotomy or laparoscopy are possible alternatives as means of obtaining tissue but both would entail a general anaesthetic in a patient population which is rarely ASA grade I! Other workers have looked at superficial fat and my experience with this source is the topic of the next chapter.

CHAPTER EIGHT

EXPERIENCE WITH THE ISOLATION OF MICROVASCULAR ENDOTHELIAL CELLS FROM

HUMAN SUPERFICIAL FAT

Introduction

The concept of isolating microvascular endothelial cells from superficial fat is an attractive one. Whichever method of seeding one were to espouse it would be an undoubted advantage to have a source of cells only a local anaesthetic away! Superficial fat as a cell source has its strong advocates - claims of high cell yields are made (Jarrell & Williams 1991) but doubts regarding isolate purity undoubtedly persist. It would appear from the literature that the very large step from initial description of cell isolation to in-vivo and even human clinical experiments has been taken by some workers with little known about the behaviour of the cells.

Having successfully developed an isolation technique for use with human omentum I sought to assess its efficacy on human superficial fat.

Materials

Gelatin: Obtained from BDH Chemicals (Poole, UK), 1% solution made up in MEM. Sterilised after reconstitution by autoclaving.

All other reagents used were as described in the previous chapter.

- 120 -

Methods

Superficial fat was obtained using sharp dissection with scissors from patients who had previously given informed consent. The same procedure as for omental samples was used with approval from Leicester Health Authority Ethics Committee as before.

In all 21 samples were processed - the majority (17) coming from laparotomy wound edges, while 4 came from the edges of inguinal hernia repair wounds. In all cases the operating surgeon took care to avoid any subsequent dimpling of the skin or other cosmetic problems.

The tissue was processed as omentum (with the obvious omission of the mesothelial cell harvest) and with the further modification that the last 15 samples were plated not in T25 flasks but in gelatin coated 24-well plates (Nunc, Denmark). The final cell pellet in these cases was resuspended in 1ml complete medium with 0.5ml being plated in each of two "inside" wells on the plate. These wells had 2 hours previously had 1ml gelatin solution placed in them and left for 1 hour at 37°C. The gelatin had then been aspirated and the plate returned to the incubator until the cells were ready. After plating the cells 1ml MEM was placed in each of two "outside" wells on the plate to ensure adequate humidity under the plate lid.

Results

The mean weight of tissue processed was 17.7g (SD 11.45 range 4-44g). While it was obvious that the superficial fat samples contained capillaries, overall there was less bleeding at harvesting than one would have expected from omentum. The transport medium also, was never

as heavily blood stained after removing the superficial fat samples. At all stages the cell pellet was smaller than one would expect with an equivalent weight of omentum.

In no case did cells grow to confluence either in a T25 flask or in the smaller (2cm^2) wells. In six cases there were obvious islands of spreading endothelial cells at 36-48 hours (Fig.8.1) but these did not manage to sustain growth and showed evidence of death after 5-7 days. In 3 cases there were no cells evident on microscopy at the time of plating - in the remaining 12 isolates there were what appeared to be clumps of rounded endothelial cells on plating. These were always observed carefully and appeared to have adhered at 24 hours but when they had shown no signs of spreading or growth at 7 days the plates were discarded.

Fig 8.1

Island of endothelial cells isolated from superficial fat in culture.

Phase contrast (x100)

Discussion

These results were a great disappointment — running in the face of the previous success with omentum. The reasons for failure are probably many but the main one was the stress laid on cell purity which almost certainly led to loss of some cells.

The mode of tissue harvest was similar to that used in the initial descriptions of superficial fat (and perirenal fat) harvest (Jarrell et al 1987) although in more recent reports liposuction methods are used for superficial fat, and perirenal fat has faded in popularity, presumably in keeping with its inaccessable site in practice (Williams et al 1989, Jarrell & Williams 1991).

What was most notable about the cells which were harvested was their failure to propagate in culture conditions. After the first six experiments the plating technique was adjusted such that the cells were plated at higher density on 24-well plates, as low density plating is held to reduce the concentrations of growth factors in the medium. The step of coating the wells with gelatin prior to adding the cells was added in an effort to optimise cell adhesion.

Why the superficial fat microvessel cells failed to respond to these measures is a matter of conjecture but may well be related to the difference in tissue source. In vivo omentum has a number of largely unexplained characteristics including its ability to "migrate" to sites of infection. What is known, and widely utilised by surgeons, is its ability to adhere and anchor itself to other tissues with noted new vessel formation — it would appear the capillaries of omentum have greater growth potential than their more superficially placed colleagues, where fat necrosis is a not uncommon feature of trauma

and/or surgical dissection.

At all steps in this series of experiments omentum was being processed uneventfully in the laboratory, so one can be sure the method itself did not fall down. Indeed in seven cases omentum and superficial fat were processed from the patient at the same time, with 100% success in the case of the omentum.

At the end the question must be asked as to whether it would have been better to have discarded the density gradient purification step. This would be difficult in the face of the evidence that impure innocula are associated with increased subendothelial thickness in vivo. Despite this evidence, avoidance of an additional purification step has been the strategy of the group from Akron, Ohio who have described the use of microvascular endothelial cells for seeding ePTFE peripheral grafts in 17 patients (Sharp et al 1989). This group, however, acknowledge that in excess of 50% of the cells they place on the graft are not endothelial. The report makes no mention of any graft follow-up and no subsequent reports on these patients are known at the time of writing.

More recent experience with microvessel endothelial seeding has been reported in a review by Jarrell and Williams from Philadelphia (1991), perhaps the main centre involved in graft seeding at this time. These authors quote experience of over 400 human isolations with in excess of 10^6 cells/gram fat being the norm. They state that superficial fat is "predominantly" made up of adipocytes and endothelial cells - somewhat at variance with the Ohio experience. The review is quite dismissive of the danger of contamination, claiming that in vivo performance is the only true guide to the value of the technique. The paper quotes their experience of four patients who have had haemodialysis grafts seeded

with microvascular endothelial cells with a prolonged time to thrombosis compared with previous unseeded grafts in the same patients, the difference being significant (p<0.013). All these grafts failed due to neo-intimal hyperplasia at the venous end. The authors have used the method in six patients with lower limb grafts with "inconclusive" results. What remains a little confusing is the fact that the studies referenced in this review use two approaches, with one utilising a Percoll purification step (Jarrell et al 1986) and the other not (Williams et al 1989), No attempt is made in the review to compare the results before and after the adjustment in method and it is not clear which method was used for the clinical work. Similarities in cell yields between papers also clouds the issue.

Save to say that the experience here of the use of BSA density gradient centrifugation showed a yield of insufficient cells with poor growth characteristics from adult human superficial fat. To abandon purification steps in the quest for higher numbers of a what is possibly a mixed population has not, at this stage, been proven to be wise.

USE OF BOVINE SERUM ALBUMIN DENSITY GRADIENTS IN THE ISOLATION OF

MICROVASCULAR ENDOTHELIAL CELLS FROM CANINE OMENTUM.

Introduction

Despite the lack of success with human superficial fat it was now the

aim to see if microvascular endothelial cells could be harvested from

canine omentum, as if so it would be possible to proceed with a series

of in vivo experiments.

The canine model is the most widely used in experiments on graft seeding

as it is known that endothelialisation of prosthetic grafts by pannus

ingrowth in dogs proceeds as slowly as 0.07-0.13mm/day (Haudenschild &

Schwartz 1979, Hanel et al 1982) — thus leaving a valuable interval of

up to several months, depending on the length of the graft, within which

the effects of seeding can be examined. This, combined with the fact

that small calibre grafts occlude readily in dogs (Sanders et al 1980)

means any advantage associated with seeding can be rapidly seen and one

can be confident that changes are due to the experimental process.

Before proceeding to seed grafts in dogs however it was necessary to

validate the microvascular cell isolation method in canine tissue. BSA

density gradient centrifugation is a very sensitive method, with minor

variations in cell density being reflected accurately. Interspecies

variation in cell density is common and it would not have been safe to

proceed using the same densities in use on human tissue. It was also an

aim to assess canine microvascular endothelial cell coagulation function

in a manner similar to the earlier human work.

Materials

Bovine Serum Albumin: obtained as before, diluted to 10%, 23%, 26% and

29% with isotonic MEM giving densities of 1.032/ml, 1.069g/ml, 1.077g/ml

and 1.086g/ml respectively.

All other reagents as described before.

Methods

Omentum was taken from 10 beagle dogs immediately after humane

sacrifice. All animals had been used in other experiments but in no case

had they had a previous laparotomy, nor had they had any drugs

administered - other than anaesthetic agents - within the previous year.

In all cases a midline approach was used and the tissue removed with

full aseptic technique, before being placed in cold MEM in a sterile

container.

Once in the laboratory the tissue was treated as described in Chapter 7

(including the harvesting of mesothelium) - up to the step of making the

the BSA gradient. The cell pellet at this stage was resuspended in 6ml

35% BSA and 3ml put into each of two sterile Dupont glass tubes. On each

was layered in succession 2ml each of 29%, 26%, 23% and 10% BSA - with a

final layer of 1ml MEM.

The subsequent centrifugation, aspiration, washing and plating

procedures were as for human tissue - with cells from each layer being

plated separately in 25cm^2 tissue culture flasks.

Cells were assessed daily using phase contrast microscopy and notes

taken of cell morphology. In cultures from some layers it became quickly

obvious that non-endothelial cells abounded, and after the first five

cases these were discarded within the first week. As soon as confluence

was reached the cells were passaged using Trypsin/EDTA and cells from

the first passage were used for experiments as detailed below.

Methods of staining for von Willebrand factor, 6-ketoPgF$_1$a assay

(Prostacyclin production) and procoagulant function assessment were as

described elsewhere in this thesis.

Results

The mean weight of tissue retrieved was 38g (range 32-45).

Cell Identity

Cells were seen to grow in all layers except that between MEM and 10%

BSA (called L1). Cell growth from the pellet ie. below 35% was variable

and only scanty spindle shaped cells were seen.

On microscopy the cultures which appeared to be richest in endothelial

cells were L2 and L3 ie. 10%-23% and 23%-26% - judging on the basis of

seeing a monolayer of cells without obvious contamination. (Fig. 9.1) In

layers L4 and L5 ie 26%-29% and 29%-35% some islands of endothelial-like

cells were seen, but even early in culture spindle shaped overgrowing

cells were obvious, with overgrowth of several cells deep seen in

places. (Fig. 9.2)

Fig 9.1 Canine microvascular endothelial cells in culture.

Fig 9.2 Cells from L5 interface in culture displaying overgrowth.
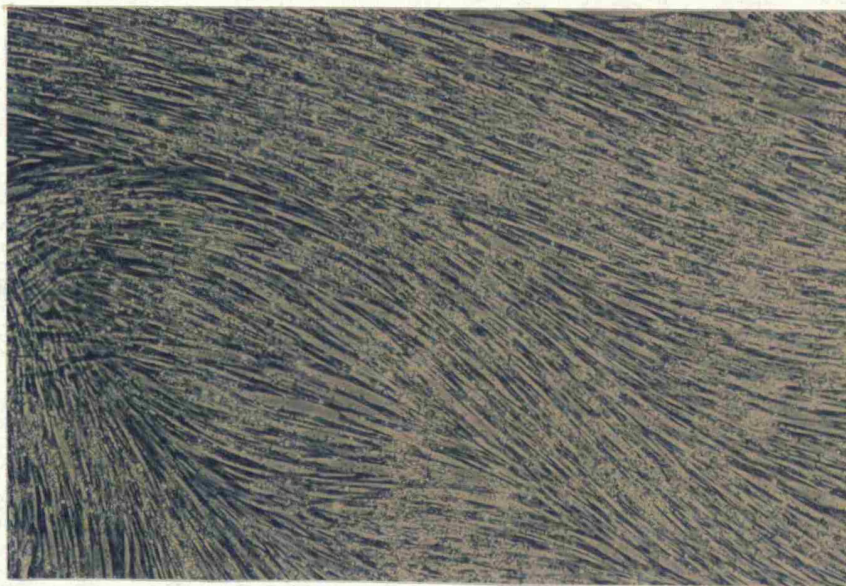
The cells grew rapidly and it was not unusual to have full flasks from all layers by day 4 or 5. What was of note was the variation between cells from different layers in their readiness to detach from the flasks in the face of Trypsin/EDTA - the higher the perceived number of non-endothelial cells the more resistant they seemed. Not infrequently up to 30 minutes incubation with the Trypsin/EDTA was necessary as compared with 1-2 minutes for human endothelial cells and 3-5 minutes for the canine microvascular endothelial cells.

Cells were stained for von Willebrand factor as described in Appendix 1 and counted on high power fields as had been human cells. (Fig 9.3)

The proportion of positively staining cells shown in Table 9.1 - with the figures of L4 and L5 representing a mean of five results - as once these figures were available and in conjunction with the morphology these flasks were discarded after a few days in the last five cases. It can be seen that canine microvascular endothelial cells congregated around the 23% BSA with roughly equal separation above and below this layer.

Table 9.1

Separation of Canine Cells on BSA

Layer on BSA	L2	L3	L4	L5
% +ve cells(SD)	90.7(6.6)	90.3(5.9)	27.4(17.8)	21.4(17.3)

Fig 9.3 VonWF stain of canine endothelial cells control and positive.
While human antibody cross reacts with canine tissue the
reaction (and thus colour) is not as marked.
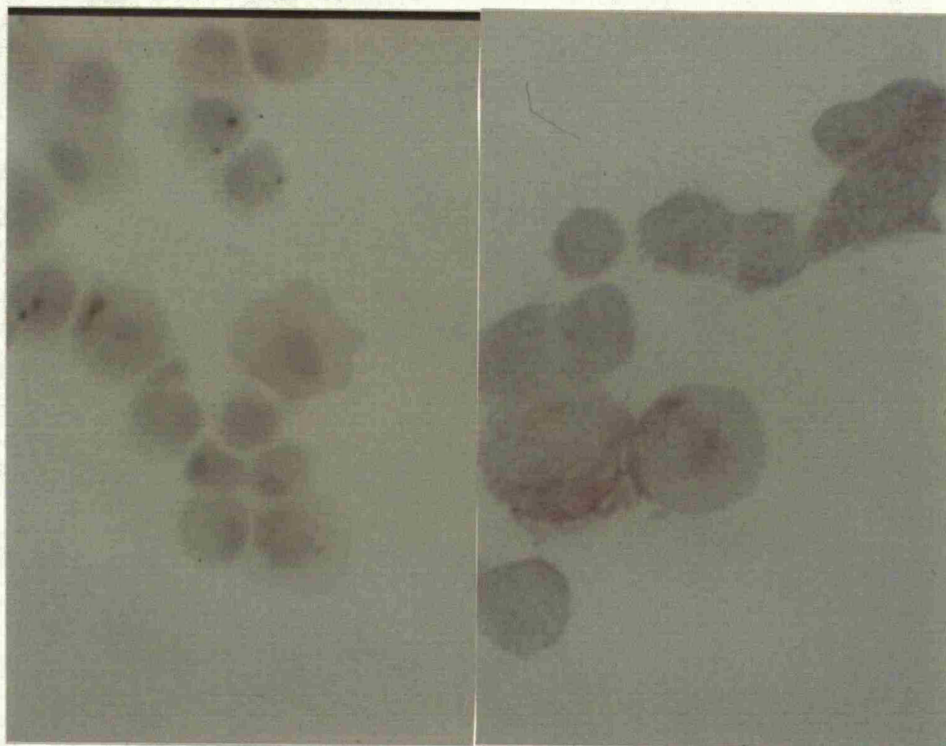
Prostacyclin Production

Canine endothelial cells produced significant amounts of Prostacyclin

(as measured by 6-keto PgF_1a) with basal levels (assessed at 20 minutes)

of $204.8pcg/min/10^5$ cells. However no increase in production was found

following application of human thrombin (1U/ml) or Bradykinin (20ng/ml)

- 180.74 and 177.1 $pcg/min/10^5$cells respectively.

Procoagulant Activity

Neither in the test for cell surface or total cell PCA did human plasma

clot within 300 seconds indicating absence of significant activity.

Discussion

The first description of the use of canine omental microvascular

endothelial cells for graft seeding was by Pearce et al in 1987 in nine

mongrel dogs. He used a double filtration method for cell purification

after collagenase digestion - rather similar to that described for human

tissue by Kern in 1983.

In a series of six dogs Pearce et al used a method based on that of

Clarke et al (1984) to harvest mesothelial cells - and found that these

failed to stain positively for factor VIII while the endothelial cells

harvested in parallel did exhibit staining. He made no mention as to

percentage or proportions of positively staining cells however.

In 1988 Schmidt et al applied a simpler method to canine omentum. Using

very small pieces of tissue they simply diluted the collagenase/fat

mixture afetr digestion and centrifuged it - assuming the pellet to be

- 133 -

composed of microvascular endothelial cells. While grafts seeded with this mixture showed evidence of endothelial cells coverage after five weeks in vivo none of the cell isolates were cultured or stained to clarify the identity of the cells therein. The authors acknowledged in the discussion that the cell population was probably mixed and used this to account for the remarkable thickness of organised subendothelium seen in their seeded grafts.

Also in 1988 Sterpetti et al compared aortic grafts in a canine model seeded with either venous endothelial cells or with omentally derived microvascular cells, the latter isolated using a 25mcm filtration step after collagenase digestion. The authors noted contamination in omental harvests examined under the microscope prior to seeding and attributed the statistically significant increase in subendothelial layer thickness seen in the omentally seeded grafts to this contamination. In a subsequent study Sterpetti et al (1990) found that the addition of a Percoll density gradient centrifugation step to the cell isolation process, while not abolishing contamination did improve the purity of isolates and reduced significantly the thickness of the subendothelial layer after a period of implantation.

Canine omentally derived microvascular endothelial cells have been used to seed inferior vena cave grafts (Wang et al 1990). This group used polysucrose lymphocyte separation medium(LSM) for a density gradient centrifugation step. The density of the LSM they used was adjusted to 1.065 - on top of which they claimed to find a 90% pure endothelial isolate - the authors did not explain how they came to use this particular density. This group is the first to try to assess the purity of their cell isolates, but in the paper the figure of "less than 10%"

contaminants was "estimated" after microscopic examination of cells in
culture. The comtaminant with which these authors seem most concerned in
their discussion is mesothelium - they suggest it may be discounted as
mesothelium is known to be capable of prostanoid production but seem to
have overlooked other cell types which are not!

At the end of these experiments one was satisfied that the BSA density
gradient method could be applied to canine omentum although the order of
10% contaminants was a disappointment having achieved better purity with
human tissue. However as the cells were obviously levelling out around
the one density the gradient was not further adjusted. Splitting the
steps between 1.032 and 1.077 might have improved purity a little in a
given layer but with the cost of loss of a large number of cells. The
limited number of animal subjects available also proved a deterrent.
A study of cell yield per gram of tissue was not undertaken as it
quickly became obvious that the tissue samples taken provided confluent
25cm² layers in culture within one week. Knowing that this yielded 1 to
1.5 million cells meant sufficient cells would be available to seed
canine infrainguinal grafts as will be described in the next chapter.

CHAPTER TEN

SEEDING ePTFE GRAFTS WITH AUTOLOGOUS MICROVASCULAR

ENDOTHELIAL CELLS IN A CANINE MODEL

Introduction

In the previous chapter some of the reasons have been outlined why a
canine model was appropriate for use in vivo experiments, and
adaptations to the BSA density gradient described for this purpose.

It was now time to use these microvascular endothelial cells to seed
prosthetic grafts and to measure the effects. The method for seeding
chosen was that of Budd et al (1991) which was perfected in the
Department of Surgery in Leicester. This has been shown to produce
confluent inner linings of endothelial cells over a 24 hour period -
this lining being resistant to the shear stresses expected in vivo.

In the course of the in vivo experiments graft patency and platelet
uptake were assessed as well as morphology and coagulation function on
removal.

Materials

Experimental animals: 9 Beagle dogs were used in this series of
experiments. The animals were housed in appropriate pens with runs in

the Biomedical Services Department in the University of Leicester where
they were allowed to settle for a minimum of two weeks before
experiments. They were fed twice daily with SDS Diet A and had free
access to drinking water. 6 were female and 3 male with a mean weight of
10.4kg (range 8-13kg).

Most other reagents were as described in previous chapters - where
additional agents were used, as in anaesthesia for example, these are
detailed in the text.

Methods

Harvesting of Microvascular Endothelium

Omentum was taken from each animal via a midline incision laparotomy
using full aseptic technique. For this each animal was under full
general anaesthesia - having fasted overnight and having had a pre-
medication of acetyl promazine (0.1mg/kg) and atropine (0.3mg) 30 to 60
minutes beforehand. Induction was carried out with sodium thiopentone
0.5-1mg/kg iv - the animal was then intubated with a cuffed endotracheal
tube and anaesthesia maintained with halothane, nitrous oxide and oxygen
via a mechanical ventilator. All animals had ECG monitoring per-
operatively.

Greater omentum was taken by ligating and dividing across the sheet of
tissue using 2/0 braided nylon ligatures. The tissue was placed in cold
MEM in a sterile container while the wound was closed using 0/0 Prolene
mass closure technique, with an additional layer of 2/0 catgut to the
subcutaneous tissue and 2/0 nylon to skin. At the time of insertion of
the intravenous cannula samples of blood had been taken for full blood

- 137 -

count and clotting studies.

All animals recovered uneventfully and were drinking within two hours.
Full diet was resumed the following day. The procedure took no longer
than 30 minutes.

Back in the laboratory the omentum was processed as described in Chapter
Nine.

Seeding of Grafts

6 days later after daily assessment of culture flasks cells were
released from either L2 or L3 flask - this decision depending on which
was the more full/objectively free of other cell types.

2 X 15cm lengths of 4mm internal diameter ePTFE were cut. One was doubly
packed in autoclave bags and autoclaved while the other was secured
inside a seeding chamber. The chamber has been described (Budd et al
1990), it consisted of a cylinder of polymethylpentane on either end of
which was a stainless steel cap which screwed onto threads on a steel
ring mounted on the cylinder. In each cap were two port holes into one
of which a steel cannula was inserted - and onto this the graft was
mounted. Thus the graft was secured inside the chamber while access to
its lumen was assured via the cannula which was sealable with a cap. The
chamber with graft in situ was sterilsed by autoclaving.

The luminal surface of the graft was coated by inserting 4ml of 20
mcg/ml Fibronectin via one of the cannulae - this was left in situ for
one hour, then removed and the graft left in the 37°C incubator for a
further hour.

The microvascular endothelial cell pellet was suspended in 8ml complete
medium with 50mcl being taken for a cell count.

- 138 -

Cell were introduced into the graft lumen in 2ml aliquots at 30 minute intervals with a 90° turn of the chamber each time and prior aspiration of any residual fluid. The final 2ml was left inside the graft which was then placed, still inside the chamber, on a roller table in a 37°C incubator. This was left overnight rotating at 10rph until the following day when grafts were inserted.

Insertion of Grafts

Each dog received one graft in the femoral region bilaterally – autologously seeded on one side with control (unseeded) on the other.

Each animal was fasted overnight as for omental harvest but having had Aspirin 75mg with its evening meal on the day prior to graft insertion. Anaesthesia was induced as before with each animal also receiving Cefuroxime 15mg/kg (Glaxo, Greenford, UK) on induction. Peroperatively each animal was given 200-300ml 4%Dextrose/0.18%Saline as an intravenous(iv) infusion.

The animals were placed supine on the operating table with the hind limbs being secured to ensure full extension of the joints. Vertical incisions were made over the femoral vessels extending from 2cm above the inguinal ligament to 2cm below the bifurcation. The vessels and femoral nerve were identified and dissected free on each side. At this point Heparin 100U/kg was given iv and allowed to circulate for three minutes. The profunda femoris artery on each side was then ligated in continuity (using braided nylon) – thus diverting flow into the superficial femoral artery. The superficial femoral artery was then occluded 1cm below the profunda origin and above the bifurcation using small vascular clamps and the intervening segment of vessel excised

(length 6-8cm). Grafts were then inserted end to end using 6/0 Prolene
sutures. The order of the graft insertion was randomly chosen as was the
side in which either graft type was put. Segments of unused seeded graft
were kept in complete medium for subsequent examination of the lining
with Toludine Blue dye.

Vascular clamps were removed as soon as each graft was inserted and the
wounds closed when full haemostasis was achieved. Wounds were closed
with 2/0 catgut to the muscle and fascia and 2/0 nylon to skin. Where it
had been necessary to divide fibres of the inguinal ligament to achieve
access to the profunda artery these were repaired with 2/0 catgut. The
midpoint of the graft was marked externally using a blue Prolene suture.
The average time for bilateral graft insertion was 2 1/2 hours. The
animals were allowed to recover and were drinking within 1-2 hours, they
were all standing within 4 hours. Buprenorphine 0.2mg was prescribed as
analgesia but in no case did the animals seem in distress. All were
given an additional dose of Cefuroxime intramuscularly 8 hours after the
first, and all had daily Aspirin 75mg postoperatively.

In the laboratory graft segments were washed in tap water and then
immersed in Toludine Blue for one minute after which uptake of the dye
by cell nuclei present could be assessed.

Graft Patency

Each animal was seen on a daily basis for the first two weeks and then
twice weekly. As care had been taken to cover each graft with muscle it
proved impossible to palpate them. Patency was assessed using a Doppler
flow meter (Doppler Flowmeter Model 810, Parks Electronics Laboratory,
Oregon, USA). For this animals were held by an assistant and by using

headphones with the instrument accuracy was assured. All animals
tolerated this examination well and most became accustomed to their
regular visitor! Doppler signals were assessed along the length of the
graft and their presence and character were recorded.

Graft Thrombogenicity

Assessment of graft thrombogenicity was made using the method described
in 1981 by McCollum which entails the measurement of the uptake by the
grafts of radiolabelled platelets. At one week postoperatively blood was
taken from each dog. In the laboratory it was processed as described in
Appendix 2 - and later that day the labelled platelets were given back
intravenously to the same dog. On a daily basis for the next week gamma
activity was measured over the midpoint of each graft and over a
reference point in the neck using a gamma counter (Isotope Localisation
Monitor 235, DA Pitman Ltd, Weighbridge, UK) with the collimator set at
1cm. Three readings were taken from each point and a mean calculated.
The McCollum method expresses a Thrombogenocity Index (TI). This is
arrived at by plotting the daily ratios of graft/reference counts and
then drawing a best fit regression line - the slope of this line being
the TI.

Removal of Grafts

Grafts were removed 10 weeks after insertion. The dogs were
anaesthetised as before and the incisions reopened. The inguinal
ligament was divided and the iliac artery controlled after giving the
animal Heparin 100U/kg. The grafts and anastomotic areas were dissected
carefully and removed with a cuff of artery at either end. A segment of

iliac artery at least 2cm proximal to the upper anastomosis was also excised. The animal was the sacrificed with a lethal dose of thiopentone.

The graft was the divided with specimens from each anastomotic and mid-graft being fixed for light and electron microscopy. Iliac artery and mid-graft segments were also placed in complete medium for functional assessment in the laboratory.

Preparation for Microscopy

For scanning electron microscopy each segment was divided longitudinally in half and fixed in glutaraldehyde 3%/cacodylate buffer 0.1M solution. After fixing for at least two hours the segments were washed in 0.1M cacodylate buffer for one hour before being transferred to 1% osmium tetraoxide (Sigma Chemicals) in 0.1M cacodylate buffer for one hour. They were then dehydrated through graded acetone (50% for 30 minutes, 70% for 60 minutes, 90% for 15 minutes) with storage in 100% acetone until further processing (at least 15 minutes). They were then dried using a Polaron E3000 critical point drier (BioRad Microscience Ltd, Hemel Hempstead, UK) and sputter coated with gold (Polaron E5150 coater). Viewing was with a ISI-DS 130 dual stage scanning electron microscope.

For light microscopy segments were initially fixed in 10% formol saline. They were dehydrated in graded alcohol (20%-100% in 10% steps for one hour each). The segments were then transferred to xylene in which they were left for 90 minutes - with a further 90 minutes in fresh xylene. Specimens were immobilised in wax over 4 hours. 4mm sections were taken

and stained with Harmatoxylin & Eosin. An Olympus microscope was used
for viewing.

Coagulation Function

Immediately after graft removal, back in the laboratory, four punch
biopsies were taken from the iliac artery segment and each midgraft
segment. This was done using a cork borer and each full-thickness punch
measuered 0.5cm diameter.

Each biopsy was placed luminal side uppermost in a well of a 24-well
plate in which there was 1ml of pre-warmed MEM at 37°C.

For prostacyclin assessment these were left to incubate at 37°C for 20
minutes at which time a 100mcl sample was taken and stored. To each well
was then added Bradykinin 10nmol, this was incubated for a further 20
minutes after which another 100mcl sample was taken. Unseeded sterile
ePTFE was used as control.

Cell surface PCA was measured, again with MEM alone as control, by
measuring the time to clotting of human plasma. For this punches were
transferred to a well containing 200mcl MEM. After a 30 minute period to
equilabrate to 37°C, to this was added 200mcl each pre-warmed human
plasma and 0.25M Calcium Chloride, with the stop-watch starting on
addition of the last reagent.

Results

Microvascular Endothelial Cells

The mean weight of omentum removed from each animal was 32.3g (SD 5.7g).
In all cases there were full 25cm² tissue culture flasks from L2 and L3

at 5 days and the selection of which was used was based on qualitative assessment of maximal endothelial cell purity using the phase contrast microscope. In 7 cases cells were from L3 and in 2 from L2.

Graft Seeding

The mean number of cells used to seed each graft was 1.69×10^5 – which equaled $8.9 \times 10^4/cm^2$ for the 15 cm length.

Seeding efficacy was assessed using Toludine Blue, and in all cases grafts exhibited cell staining in a confluent layer. An example is illustrated in Figure 10.1.

Fig 10.1
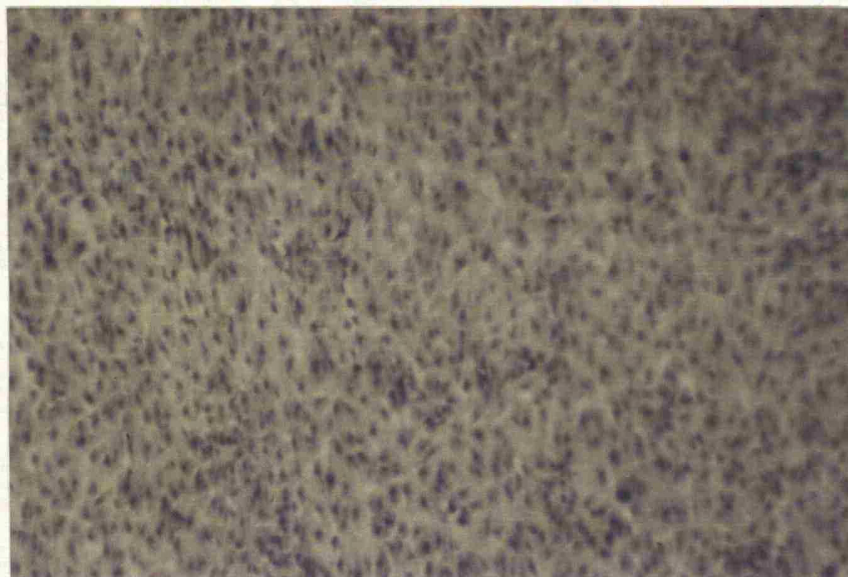
Dog Haematology

The detailed results are shown in Table 10.1. In all cases haemoglobin was satisfactory. Canine blood is known to show a thrombotic tendancy when compared with human and this is reflected in the consistent result of a Prothrombin ratio (INR) of <1 with human plasma as control. The PTTK times were also less than human control.

Table 10.1 HAEMATOLOGY RESULTS

Dog No.	Hb.	Hct.	Plt.	INR	PTTK
1.	10.6	31.6	301	<1	17
2.	12.2	36.0	287	<1	14
3.	12.6	36.8	376	<1	18
4.	11.1	34.3	323	<1	18.5
5.	11.5	33.5	405	<1	16
6.	13.1	36.3	291	<1	16
7.	10.9	30.4	219	<1	14
8.	12.9	36.3	330	<1	16
9.	12.1	35.6	288	<1	15

Hb = Haemoglobin(g), Hct. = Haematocrit(%), Plt. = Platlet count (x10^9), INR = International Ratio, PTTK = Partial thromboplastin time.

Surgery and Wounds

As mentioned above graft insertion took no longer than 2 1/2 hours and all animals recovered well. As is not uncommon in human patients some of the animals developed seromas in the area of the wounds between 3 and 7 days post operatively (5 in 5 animals, 3 controls and 2 seeded). In two cases these discharged spontaneously through the wound and in the others they resolved spontaneously over two weeks.

In one animal one graft (seeded) showed signs of infection, which developed suddenly over a day, at 14 days postoperatively. The limb became acutely swollen and hot and the wound began to break down. At emergency re-exploration there was a good deal of pus around the graft and also evidence that this had spread into the muscle groups in the area. There was obvious thrombus around the proximal anastomosis although the graft was patent. In conjunction with the staff of the Biomedical Services Unit it was deemed necessary to put the animal down as even with removal of the graft primary closure of the wound would be doomed to failure. Unfortunately it proved impossible to culture a causative organism. This animal is represented by the number 9 in the table above.

Graft Thrombogenicity

Given the difficulties encountered later with the infected graft the platelet data associated with it have been excluded, although the TI was lower than its contralateral control. In two further cases it proved impossible to label platelets at the appropriate time when the supplier of prostaglandin containing diluent failed to deliver at minimal notice - thus there are six sets of results for analysis.

Labelling efficiency of Indium was good at 77% (SD 3.2%).

There proved to be a significant difference between the TI for the seeded versus the control grafts with mean TI seed (with SD in brackets) being 0.06(0.03) and TI control being higher at 0.12(0.07) p=0.02 paired T test.

Graft Patency

Grafts were deemed to have occluded when the doppler signal had disappeared or where it became difficult to find a clear signal – with excellent collateral flow in the limb one had to be sure one was listening to the graft itself. All grafts had occluded by 8 weeks.

While control grafts showed a tendency to occlude earlier (2.5-8 weeks) versus (5-8 weeks), the mean values were similar at 6 and 6.6 weeks respectively and this difference was not significant.

Graft Removal

At operation the striking feature of most of the grafts was the way the midportion had collapsed rather than being full of thrombus as one might have expected. In fact only in 2 cases – both control grafts – was the lumen full of thrombus.

The other feature of note in all cases was the solid feeling of the area at each anastomosis which was incompressible.

Graft Microscopy

The obvious feature associated with all grafts was the striking occlusive neointimal hyperplasia found at each anastomosis – both proximal and distal. (Figs. 10.2, 10.3) The midportions of grafts

demonstrated varying amounts of fibrinous material loosely adherent to the graft wall. On scanning electron microscopy this proved to contain trapped blood cells in varying proportions. (Figs 10.4, 10.5)

On one seeded graft on light microscopy it was possible to detect an area of endothelium (Fig. 10.6) and areas of endothelium were also seen on another seeded graft on SEM (Fig. 10.7) but this was not the usual case and no other areas of endothelial lining of any size could be found despite extensive scanning EM.

Fig 10.2 Near obliteration of the lumen at proximal anastomosis.
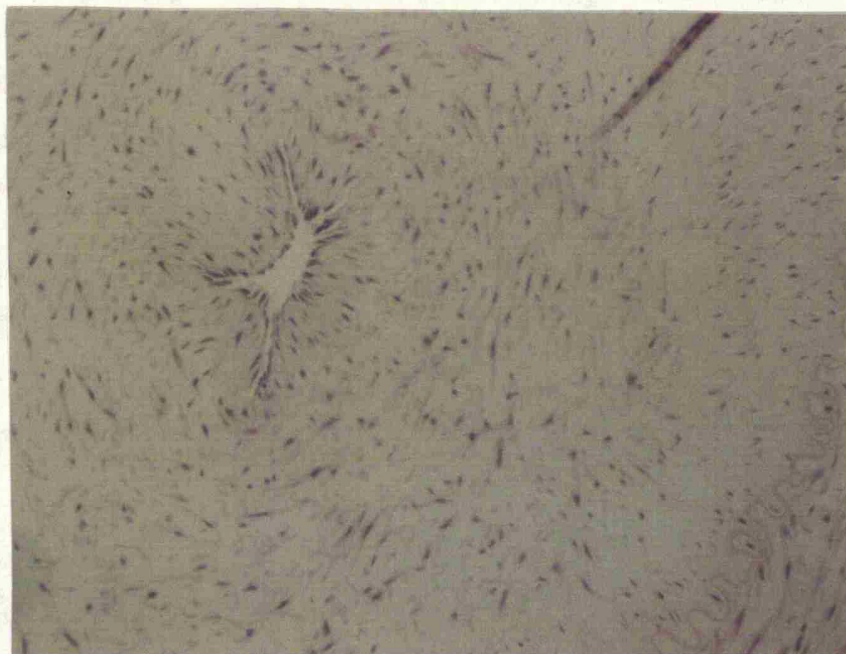
Fig 10.3 Similar appearance at distal anastomosis.
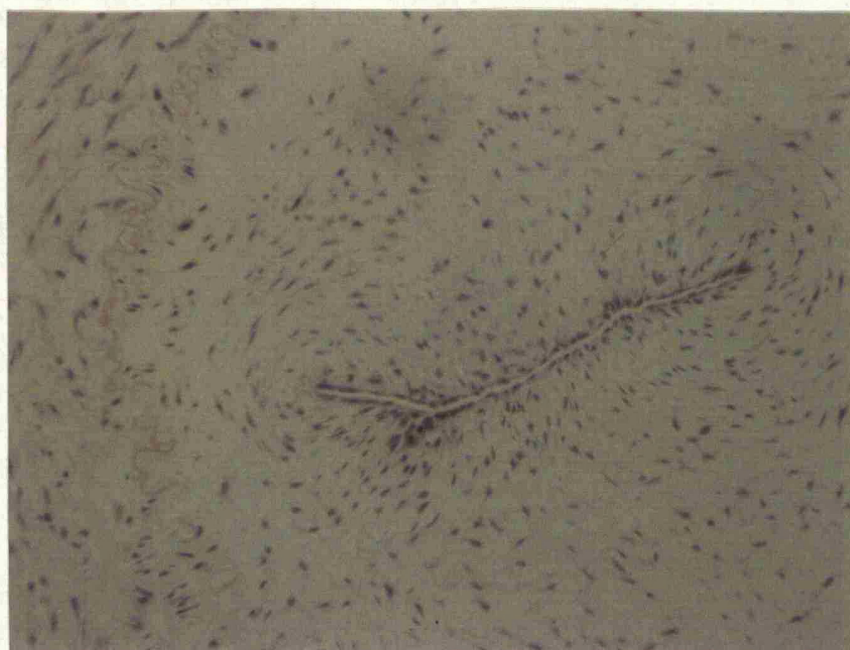
Fig. 10.4 and 10.5

Different degrees of covering of the luminal surface of a graft by fibrin and blood cells.
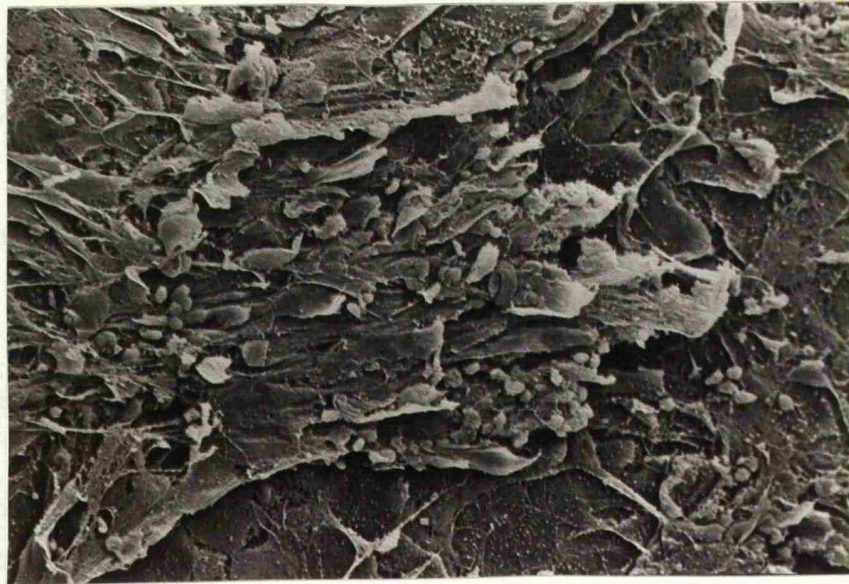
Fig. 10.6 H&E stain of a seeded graft demonstrating luminal endothelial coverage.
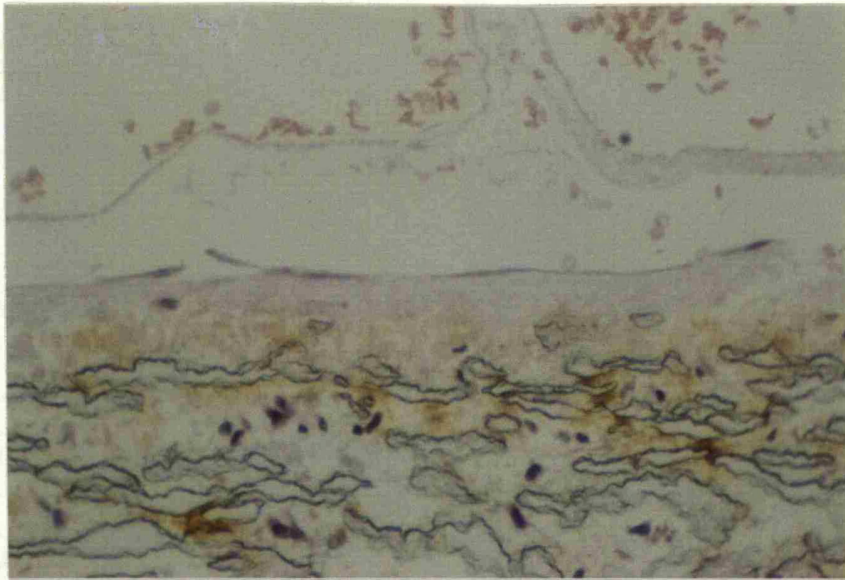
Fig. 10.7 SEM of luminal surface of a seeded graft showing a good number of adherent blood cells but an area of relatively uncovered endothelial lining towards the centre of the picture.
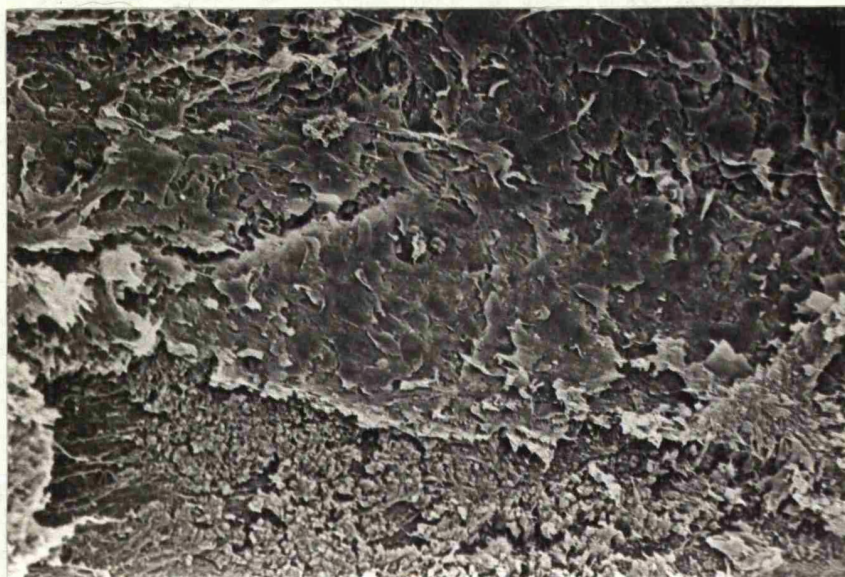
Coagulation Function

Prostacyclin: Not unsurprisingly, in view of the above, Prostacyclin levels for both seeded and control grafts were extremely low although there was a significant difference between the two (control 0.54 pcg/min(SD 0.1) - seeded 3.8pcg/min(SD 3.4). p=0.031). Iliac artery produced appreciable levels of 142.6pcg/min (SD 72.4) and responded weakly to stimulation (175.9pcg/min) while neither seeded, unseeded or control showed any response.

Procoagulant activity: In all cases sterilsed graft showed no procoagulant activity with no clotting observed to 5 minutes. Despite the fact that both seeded and unseeded grafts had occluded there was a significant difference between the two with clotting occurring significantly earlier on the unseeded grafts (time to clotting in seconds seeded= 104.5 (SD 25), unseeded= 79.8 (SD 9.7) p=0.024) . Native artery displayed less activity than either graft (time to clotting 140.7 seconds (SD 12.8)). (Paired t test) (Results expressed as seconds instead of Units PCA as cells here are not human)

Discussion

In this study microvascular endothelial cells have been shown to have a beneficial effect on early graft thrombogenicity. The significant reduction in TI was an encouraging sign that the cells were functioning and this function was maintained out to two weeks. It is not surprising that there was little microscopic evidence of cells persisting on the graft at 10 weeks as in all cases the grafts had occluded and without nutrition from passing blood one would expect the cells to die. While

neither set of data is conclusive the results of both the prostacyclin
and pro-coagulant experiments might lead one to suspect that some cells
remained functional even at this stage after occlusion.

The absence of thrombus in the the grafts leads one to suppose that the
occlusive neointimal hyperplasia was the primary cause of graft failure
rather than a feature that progressed after intraluminal thrombosis. In
contrast to other studies suggesting that the distal anastomosis is the
more severely effected by intimal hyperplasia my study would, based on
the absence of intra-luminal clot, point to a more equal effect on the
two anastomoses.

The occurence of neointimal hyperplasia associated with grafts seeded
with microvascular cells derived from adipose tissue has been reported
in the past with Pearce et al (1987) and Sterpetti et al (1988) noting
greater subendothelial thickness in such grafts when comparing them with
those seeded with vein cells, and Sterpetti et al (1990) when comparing
two methods for purifying omentally derived cells. Indeed as in the
latter paper Sterpetti et al had noted that extra attempts to purify the
cell population improved his results insofar as he found a reduced
subendothelial layer using a Percoll density centrifugation method (as
described by Jarrell et al 1986), impetus was given to my attempts to
further improve purity with BSA.

While the failure to maintain patency in this study is disappointing
these grafts were observed for considerably longer than other published
in vivo studies. In both their reports Sterpetti et al examined grafts
after 5 weeks, and this interval was also used by Schmidt et al (1988)
again in a canine model. Pearce et al (1987), in one of the first papers
on the the topic had only left grafts in situ for 4 weeks prior to

removal and assessment. These authors reported varying patency from Pearce's 58% to Sterpetti's 100% - but at 4-5 weeks most of the grafts in this study were patent too!

Assessment has been made in the past of thrombus free area on seeded grafts - in this study TI has been used - but the major cause of prosthetic graft failure after the immediate period is anastomotic hyperplasia and 4 to 5 weeks in a canine model is probably too short a period in which to observe this adequately.

A note must be made of the problem of graft infection which was encountered in one out of 18 grafts inserted. To find an infection with false aneurysm formation at 14 days leads one to suspect that the problem was primarily with the graft and related to the time of insertion. At all stages every effort was made to ensure sterility but the strictures imposed by the experimental situation meant that grafts had to be transported out of doors to the operating theatre. It is frustrating that cultures failed to isolate the causative organism, but this is too often the case in clinical work also, as the bacteria involved can be notoriously difficult to culture.

This study demonstrated the feasibility of using microvascular endothelial cells for seeding ePTFE grafts in a canine model and that such seeding reduced the early graft thrombogenicity. Long term patency however, was not improved although seeding did not appear to accelerate anastomotic hyperplasia.

CHAPTER ELEVEN

SUMMARY AND DISCUSSION

A FUTURE FOR ENDOTHELIAL CELL SEEDING?

Endothelial seeding has been a topic for study and debate among vascular

surgeons since the early 1980s - much of the debate concentrating on the

difficulties encountered when trying to reproduce successful animal data

in human clinical situations.

The early descriptions of graft seeding used the technique of placing

the cells on the graft in the pre-clotting stage with very early

exposure to blood flow and naturally consequent losses of large numbers

of cells. While pre-coating graft materials with matrix components

improved cell retention the concept of allowing the cell a longer period

of time to adhere and spread on the graft has captured the greatest

attention - fostered by improved cell culture techniques.

One cannot apply seeding techniques at all however unless one has

sufficient cells and my brief when starting this work was to perfect a

reliable method for the harvest and culture of human endothelial cells

suitable for seeding small calibre prosthetic grafts.

Much of the in-vitro work on endothelial cell function and seeding has

been done on human umbilical vein cells and I looked into them as a

possible source for clinical graft seeding. I too found they were

relatively easy to culture and that cell yield from a given length of

vein was good. However I also found them to posess marked
lymphoproliferative capabilities which were up-graded after stimulation
with interferon which I also showed induced MHC class II expression by
the cells. In-vivo this could be expected to lead to cell loss and
possible increase in graft thrombogenicity - both by exposing sub-
endothelial matrix and by inducing cell procoagulant mechanisms - and
so I looked instead at possible autologous sources.

Superficial vein has been a good source of endothelial cells in canine
and other animal models but its success in the human situation has not
been as notable and I too had little success in culturing adult
saphenous cells. One possible cause was the degree of endothelial cells
loss I found on scanning electron microscopy of randomly selected vein
segments although the poor growth characteristics of the cell even when
initial harvest has been successful has been noted by other authors.
Even if my results had improved with time I had proven to my own
satisfaction that a more reliable cell source was required.

Microvascular endothelial cells, from adipose tissue, had been proposed
in the literature as being suitable for graft seeding but there was a
deal of disquiet and some early evidence to suggest that significant
contamination by other cell types such as smooth muscle cells, as
accepted techniques produced, could actually endanger graft survival by
increasing intimal hyperplasia. In this work I set out to develop a
better isolation method and succeeded in reliably producing human
endothelial cell cultures which were 98%+ pure. Cell yield per gram
tissue in the initial harvest was good and growth in culture quick and
reliable. In a series of experiments on these microvascular endothelial
cells I assessed their suitability for use in graft seeding looking at

- 156 -

prostacyclin production, procoagulant activity and ability to adhere to ePTFE graft material. It was some disappointment to me that the cell yield was poor when I applied the technique to superficial fat in a quest for more easily accessible cells. However when one is mindful of the ready bleeding one encounters when working with omentum and its diverse "walling off" activities within the peritoneal cavity the more adaptable nature of its endothelium should not be such a surprise.

When applying my technique to canine omentum I was forced to sacrifice such a high degree of cell purity to adequate numbers of cells for graft seeding. Financial and ethical considerations meant I did not have access to the same number of tissue specimens I had been afforded by human volunteers only too anxious to lose adipose tissue. However I did succeed in producing in excess of 90% pure endothelial cells which is certainly as good as the best reported isolation techniques in the literature. I used canine cells to autologously seed 4mm internal diameter interposition femoropopliteal grafts with each animal having a contralateral unseeded control. Early results were very encouraging with seeded grafts showing reduced platelet uptake and thrombogenicity index but failing to achieve better patency rates in the longer term, falling prey to the same anastomotic hyperplasia seen in controls. This process was not accelerated despite my fears regarding the presence of other cell types.

All in all I have shown that it is possible to produce good numbers of functioning microvascular endothelial cells from human tissue – based on my in-vivo work it is reasonable to assume that if inserted on a graft they would reduce early graft thrombogenicity. It is the longer term that the problem of intimal hyperplasia presents itself and here I have

been unable, based on a canine model, to demonstrate an advantage for seeding. It remains a matter for debate whether the improved purity I achieved in human omental harvests might have a beneficial effect on anastomotic hyperplasia, however I feel it is fair to propose that given that a comtminant level of 10% in dogs did not accelerate occlusion compared with control my isolation method might be applied to humans without long term fears and with the hope of short term advantage.

Realistically there are few patients who will require seeded prosthetic grafts for vascular reconstruction. Above knee bypasses have been repeatedly shown to have results with prosthetic materials on a par with vein. For more distally placed anastomoses there is no doubt that autologous vein is the conduit of choice and experience is demonstrating that even when it is of relatively poor quality the advantage in patency is maintained with techniques of plicating varicosed segments and of anastomosing several segments together to produce enough length being now more frequently described, in some cases with enviable results (Hickey et al 1991).

For those patients who have reached the end of the road — with critically ischaemic limbs and absolutely no vein — endothelial cell seeding offers a possible gleam of encouragement. Such patients will almost always have poor run-off and may well benefit most from the reduced early thrombogenicity that seeding confers. However it is vital that any seeding technique can be applied quickly as this is the population which cannot afford to wait several weeks for cells to grow in the laboratory only to find they've become infected or are not going to be suitable. Here my work with an isolation technique offering good numbers of virtually pure cells capable of anti-platelet function may

well represent an advance. Using a laparoscope it would be quite possible to harvest 100g+ of tissue which could easily provide adequate numbers of cells for a distal graft within 7-10 days - it is unlikely that the clinical condition of many of these patients would allow much longer in practice. The problem of anastomotic hyperplasia which I have been unable to improve may well be helped by an interposition vein cuff/patch both of which have been shown to be of benefit in clinical practice already.

One field of practice not immediately evident to the vascular surgeon but worthy of mention in that of gene therapy, here the ability to seed lengths of prosthetic graft material is definitely of benefit. Workers in some centres are now reporting the insertion of viral particles into cells bearing the DNA codes for enzymes - deficiencies of which cause disease states in man, a similar case in Britain recently featured in the tabloid press. The insertion of such particles into endothelial cells has been described (Ryan 1990) and if one were to use these cells to seed lengths of prosthetic graft one would be in a position to insert, survey and remove if necessary enzyme sources, leaving one in control of the situation. This might not be the case if they were left to seed denuded native vessel or placed in marrow sources as has been suggested. Widespread application of such therapy is some years away yet but represents an exciting prospect - surgeons may well end up inserting seeded grafts more often than they now consider likely!

IMMUNOHISTOLOGICAL STAINING FOR ENDOTHELIAL CELLS

Materials

Chromogenic Substrate: Made up by the addition of 10mg naphthol AS-B1 phosphate (Sigma Chemical Co) to 9.8ml Tris buffer (TBS pH 8.2). To this was added 10mg Fast Red (Sigma Chemical Co) and then 10mcl 0.1M levamisole (Sigma Chemical Co. UK) this was then filtered through Whatman paper to remove any precipitate and used within 15 minutes.

Scotts Tap Water: this was made up by the dissolution of 2g potassium bicarbonate and 20g magnesium sulphate in 1L of distilled water.

Antibodies: Rabbit anti-human von Willebrand factor antibody (Dakopatts, Denmark), QB-END 40.1 (Quantum Biosystems Ltd., UK).

Other reagents in the text from Sigma Chemical Co., UK.

Methods

Cells were harvested from tissue culture flasks using trypsin/EDTA as described and resuspended in culture medium at a maximum concentration of 106/ml. They were then placed in 0.2ml aliquots in a cytospin machine (Shandon Cytospin-2, Southern Products, Runcorn, UK) and spun at 800rpm for 2 minutes such that the cells from each aliquot were spread on a microscope slide. These were then allowed to dry at room temperature and if not being used immediately were frozen at -20°C. On the day of staining cells were allowed to thaw at room temperature and the cells

then fixed by immersing the slides in acetone for 10 minutes. The slides
were then allowed to dry and on each was then placed either 0.1ml
antibody (diluted 1:100 in MEM) or 0.1ml MEM as control. Then slides were
then kept in a humidified atmosphere at 4°C overnight.

The following morning the test solution was carefully tipped off and the
slide washed with 10ml TBS. Then the second layer was added, in this case
0.1ml anti-rabbit or anti-mouse Ig F-ab fragment conjugated to Biotin
diluted 1:100 in MEM. This was incubated at room temperature for 45-60
muinutes before being tipped off and the slides again washed in 10ml TBS.
Onto each slide was then placed 0.1ml 1:400 Extravidin which was allowed
to incubate for 20 minutes at room temperature. Again the slides were
washed and then 0.1ml Chromogenic substrate added to each and incubated
for 15 minutes. The slides were then washed with Scotts Tap Water and
0.1ml acid haematoxylin placed on each to act as a counterstain, this
was left in-situ for 5 minutes before being washed off, again with Scotts
Tap Water.

The slides were then mounted with glycerol gelatin and a coverslip.

APPENDIX TWO

LABELLING OF PLATELETS WITH ^{111}INDIUM

Materials

Sodium Citrate: Made up to a 3.8% solution

Acid Citrate Dextrose (ACD): A solution of anhydrous dextrose in sodium

acid citrate.

Tyrode Buffer: Calcium free with 300ng/ml Prostaglandin E_1.

^{111}Indium Oxine: obtained from Amersham UK.

Methods

From a foreleg vein a total of 26ml blood was withdrawn using a 19 guage

needle, 19ml into a syringe containing 3ml ACD and 9ml into another

syringe pre-loaded with 1ml sodium citrate. In the preparation room with

aseptic technique the blood/ACD was placed in a universal container and

centrifuged at 180g for 10 minutes to yield platelet rich plasma. 5ml of

this plasma were placed in a fresh universal container and to it was

added 5ml of the prostaglandin containing Tyrode buffer. This was then

centrifuged at 640g for 10 minutes as was the blood/citrate, the latter

yielding platelet poor plasma which was reserved. The supernatant from

the plasma/buffer tube was decanted and reserved and a further 2.5ml of

Tyrode buffer added to the tube, without resuspending this was spun for

90 seconds again at 640g. This supernatant was discarded and the pellet resuspended in 2.5ml Tyrode buffer and 10MBq of Indium added. This was allowed to incubate at room temperature for 2 minutes and the volume made up to 10ml by adding some of the reserved plasma/buffer supernatant. This was spun again at 640g for 10 minutes and then the supernatant decanted and the gamma activity in it measured. The platelet pellet was resuspended in the platelet poor plasma and its activity measured before being reinjected.

Labelling efficiency was calculated using the formula:

Efficiency (%) = $\dfrac{\text{platelet activity}}{\text{platelet activity x supernatant avtivity}}$ x100

APPENDIX THREE

ASSAY OF PROSTACYCLIN PRODUCTION

Prostacyclin is very unstable and this assay is actually one of 6-keto PgF$_1$a* the metabolite produced as a result of spontaneous hydrolysis. It is a radio-immunassay based on the competitive binding of labelled and unlabelled 6-keto PgF$_1$a to a protein with high affinity. The assay "kit" was obtained from Amersham International.

Materials

The only reagent not included in the kit was scintillation fluid, I used OptiScint Safe (FSA Laboratory Supplies, Loughborough, UK.) The kit supplies enough reagent to analyse 84 samples, with 16 tubes as standards.

Kit Reagents:Vial 1 contained assay buffer (phosphate buffered saline with gelatein and thimerosal.

Vial 2 contained ^{3}H labelled 6-keto PgF$_1$a (activity approximately 1mcCi)

Vial 3 contained anti-6-keto PgF$_1$a antiserum.

5 standard vials for the assay containing 6-keto PgF$_1$a in the following concentrations: 0.14,0.30, 0.75, 2.0 and 5.0ng/ml.

Dextran-coated charcoal in assay buffer.

Method

The saved specimens of supernatant were defrosted to room temperature.

On day 1 the following parts of the assay were carried out:

a) 200mcl of vial 1 were placed in 2 tubes labelled for total count (TC) and another 2 for non-specific binding (NSB).

b) 100mcl of vial 1 were placed in 2 tubes for standard zero (Bo).

c) 100mcl of vial 2 were placed in all 100 tubes.

d) 100mcl of each standard were placed in the appropriate tubes.

e) 100 mcl of each sample for assay were placed in its own tube.

f) 100mcl of vial 3 were placed in the Bo tubes and all the sample tubes and standards thus leaving 300mcl in all tubes at this stage.

The tubes were vortex mixed and then centrifuged at 1000g for 15 seconds. The contents were again vortexed and then left to incubate at first for one hour at room temperature and then at 4°C for 20 hours.

On day 2:

g) The assay tubes were placed on crushed ice and 1ml of vial 1 was added to the the TC tube. The contents were mixed on the vortex and decanted into 10ml of scintillation fluid

h) 1ml of dextran coated charcoal was added to each tube as they were vortexed.

i) All tubes were then incubated at 0°C for 9 minutes.

j) The tubes were then centrifuged at 1000g at 4°C for 10 minutes.

k) The supernatant from each tube was then decanted into the scintillation fluid and activity counted in a beta counter (1217 RackBeta, LKB Instruments Ltd., South Croydon, UK).

l) The standards were then plotted on the log-logit paper supplied in the kit giving a standard "curve" (in reality a straight line). From

this line the scintillation counts of the samples could be converted to amounts of 6-keto-PgF$_1$a.

* "a" is used here and elsewhere in the text instead of the proper abbreviation of "alpha", a symbol with which I am afraid my keyboard was unable to provide me.

CHAPTER FIVE

HUVECs 24 hour cell counts/yields

Cord No.	Length (cm)	Cell count	cell/cm	cells/cm^2
1	17	1,65x19^6	9,7x10^4	7,7x10^4
2	37,5	6,3x10^5	1,6x10^4	1,28x10^4
3	30	9,3x10^5	3,1x10^4	2,5x10^4
4	27	1,26x10^6	4,6x10^4	3,7x10^4
5	29	2,1x10^6	7,2x10^4	5,7x10^4
6	32	3,3x10^5	1,03x10^4	0,82x10^4
7	24	8,7x10^5	3,62x10^4	2,87x10^4
8	15	4,2x10^5	2,8x10^4	2,2x10^4
9	23,5	2,1x10^5	8,9x10^3	7,1x10^3
10	37,5	6,6x10^5	1,8x10^4	1,43x10^4
11	30	3,0x10^5	1,0x10^4	7,9x10^3
12	21	1,35x10^6	6,4x10^4	5,1x10^4
13	31,5	1,23x10^6	3,9x10^4	3,1x10^4
14	37,5	2,97x10^6	7,9x10^4	6,3x10^4
15	34	1,05x10^6	3,1x10^4	2,5x10^4

Prostacyclin production
pcg/tube read from standard curve drawn for each assay

Control		Bradykinin		Thrombin		Ca Ionophore	
basal	stim	basal	stim	basal	stim	basal	stim
22	<1	13	50	1,24	400	10,1	18
14	41	11,5	62	4	170	22	155
<1	19	<1	40	1,7	51	4,1	225
17,5	12,5	<1	30	<1	115	19	150
<1	5,4	2,4	8,6	6,8	17	<1	11,5

pcg/min/10^6 cells calculated:

11	-10,5	6,5	18,5	0,6	199,7	5,6	68
7	13,5	5,7	25,25	2	84	11	72
1	9	1	20	0,8	24,65	2	110,4
8,7	-2,5	1	15	1	57,5	9,5	65,5
1	2,7	1,2	16,9	3,4	49,1	1	84,5

Procoagulant activity, Time converted to Units PCA using standard curve
Seconds/Units; 56/8,4 56/8,4 60/7,6 56/8,4 46/9,6 44/9,85 73/4,4
            70/5,1 64/6,7 59/7,8 70/5,1 88/0,97

PBL culture, Experiments in quadruplicate, Unit = DPM

Exp, PBL/Medium

Exp					Mean
1	3062	2006	2011	1673	1673
2	1211	849	874	1396	1082
3	1798	1525	1302	1395	1505
4	1837	1419	1634	1711	1650
					1606,25 SD456

PBL/HUVEC

1	1989	3456	3129	870	2361
2	1649	3185	1784	818	1859
3	1438	1216	1426	-----	1360
4	477	1218	952	-----	882
					1615 SD637

PBL/PBL

1	7971	4675	6229	5331	6051
2	4198	4856	12405	-----	7316
3	6161	5735	14256	4747	7725
					7033,3 SD868

Analysis using separate variance in view of PBL/PBL resultsx3
Counts above after deduction of background HUVEC counts

PBL culture, Daily comparison vs control,
Figures represent mean of each experiment in quadruplicate

PBL/HUVEC

Exp	Day1	Day2	Day3	Day4	Day5	Day6	Day7
1	6093	6031	6671	2516	4088	3027	3179
2	6857	5210	9466	3939	2701	1269	649
3	-----	9860	10858	4787	11983	5199	4299
4	-----	6276	8739	2827	6559	6993	2100

PBL/PBL

	Day1	Day2	Day3	Day4	Day5	Day6	Day7
1	911	8401	18835	14626	16550	3935	4197
2	720	2734	9248	12405	21139	14263	10058
3	-----	6346	12577	23606	48760	45056	31180
4	-----	7043	12481	11516	17065	16409	7988

PBL/ConA

	Day1	Day2	Day3	Day4	Day5	Day6	Day7
1	7436	71669	39263	28496	17975	10991	3859
2	4646	38231	29911	26288	15691	11079	17883
3	-----	29871	75020	46253	34124	17231	9762
4	-----	19914	36316	22034	16546	12209	7652

PBL/Medium

	Day1	Day2	Day3	Day4	Day5	Day6	Day7
1	1784	1737	3606	2149	1228	2992	6192
2	584	516	1766	1031	1318	1068	2470
3	-----	1437	2137	1029	585	1935	2162
4	-----	1114	2048	2120	4533	2120	2427

Counts after deduction of HUVEC background counts

FACS analysis, % cells positive for MHC Class II Dr expression

Experiment	Non Treated	IFN Treated
1	0,51	63,41
2	0,24	47,0
3	0,39	92,16
4	0,14	64,39
5	4,7	76,13
6	0,01	82,54
7	0,06	36,85

PBL culture, cHUVEC = treated HUVEC, Figures = DPMs
Each figure represents mean of experiment in quadruplicate

Exp	Day2	Day3	Day5	Day7
PBL/HUVEC				
1	667	2596	738	249
2	1261	2362	1329	1198
3	2488	2739	2447	1482
PBL/cHUVEC				
1	2198	2446	1801	778
2	3178	3295	2114	1248
3	3775	4435	2734	3333
PBL/Medium				
1	723	990	714	1135
2	1357	1237	1535	1918
3	1251	1753	1619	4372

Counts after deduction of background HUVEC activity

CHAPTER SIX

Long Saphenous Vein Segments
Length(cm)/Diameter(mm):
4/3 3,5/3 6,5/3 3,5/3 2,5/3 5,5/3,5 1,8/2,5 2/4 3/4 4/3 4/3 6,5/3
2,5/3 2/3,5 1,6/4 5,5/3 3,8/2,5 3,4/3,5 3,2/3,5 3,8/3,5

CHAPTER SEVEN

Omentum weights (g):
15 15 50 30 28 15 45 34 23 40 32 30 44 42 19 37 28 30 52 41

Cell yield/g tissue (x10⁴):
2,3 3,9 6,8 2,3 7,4 7,5 6,8 2,6 4,5 1,9 1,7 8,6 1,6
counts refer to first 13 samples above

Prostacyclin production:
pcg/tube

Control		Bradykinin		Thrombin		Ca Ionophore	
basal	stim	basal	stim	basal	stim	basal	stim
<1	<1	<1	115	80	110	19	150
<1	13,5	<1	54	<1	32	10,5	30
40	56	<1	240	43	210	94	115
170	250	130	640	155	>1000	165	240

pcg/min/10⁵ cells:

1	1	1	57	40	35	9,5	65,5
1	6,5	1	26,5	1	15,5	5,25	12,4
20	8	1	119,5	21,5	83,5	47	10,5
85	40	65	255	77,5	422,5	82,5	37,5

¹¹¹Indium labelling
Efficiency (%): 47 46 46 44 43 35 35,4 33,7 32 24,9

Leakage
Exp1

Time (mins)	Counts super.	Counts susp.	%
0	48272	591593	10,2
10	56650	567866	12,5
20	57599	583555	12,3
30	62749	568618	13,8
45	61251	517050	14,8
60	68091	624571	13,6
90	69572	579357	15
120	77451	568117	17

Exp2

0	30055	714636	5,2
10	36685	732512	6,3
20	40161	755269	6,6
30	44250	734182	7,5
45	93660	727138	16,1
60	67630	711873	11,9
90	51995	781376	8,3
120	56517	712660	9,9

Exp3

0	44348	642638		8,6
10	50767	698747		9,1
20	52220	668304	9,7	
30	56517	650752		10,8
45	58019	683549		10,6
60	58926	658724		11,2
90	65025	94105		86,4 not incl
120	68708	676017		12,7

Exp4

0	28968	442628	8,2
10	42642	426088	12,5
20	45385	444306	12,7
30	53059	460668	14,4
45	50521	469224	13,4
60	53168	444767	14,9
90	58221	452578	16,1
120	63413	507479	15,6

Cell adhesion to coated grafts (%):

Time	control	fibronectin	p-1-1	collagen
Exp1				
10	3,8	46	7,5	8,5
20	21	50	14	11
30	21	59	18	21
45	26	51	28	29
60	21	67	32	19
90	35	72	33	33
120	39	80	37	21
Exp2				
10	8	41	7,5	8,5
20	7	42	10	14
30	13	60	17	22
45	20	61	34	14
60	34	64	33	21
90	41	54	38	31
120	50	71	53	42
Exp3				
10	5	24	7	14
20	4	48	8	17
30	16	50	12	27
45	22	62	16	27
60	27	57	27	20
90	54	74	36	32
120	29	63	34	45

Exp4	control	fibronectin	p-1-1	collagen
10	8	38	14	14
20	15	45	16	23
30	23	58	27	16
45	35	67	27	27
60	36	65	39	44
90	39	73	36	31
120	43	67	40	45

Exp5				
10	2,5	24	10	13
20	12	46	16	25
30	13	53	19	16
45	40	63	36	27
60	43	68	39	23
90	38	71	42	31
120	40	68	53	33

Exp6				
10	1	22	4,4	7,5
20	8	36	10	20
30	7	28	15	6
45	29	65	30	32
60	31	61	21	19
90	51	73	55	50
120	62	76	57	36

Chapter Eight
Superficial fat Weight (g) of 12 weighed samples:
10 11 18 8 25 19 24 16 4 5 28 44

Chapter Nine
% staining for von Willebrand factor antigen

Animal No	Layer 2	Layer 3	Layer 4	Layer 5
1	76	95	10	50
2	82	95	20	25
3	95	86	40	10
4	95+	78	52	14
5	96	88	15	8
6	92	94		
7	90	92		
8	95+	94		
9	94	96		
10	92	85		

Prostacyclin production from canine microvascular endothelial cells
pcg/min/10^5 cells;

Animal No	Basal	Stim thrombin	Stim Bradykinin
1	199,8	216	
2	198	208	153,3
3	266	250	264
4	124,9	58,6	108
5	235,4	171,7	183

Chapter Ten

Ratios graft/reference (animals indicated using number ascribed to each
on page 145);
Control

No	Day1	Day2	Day3	Day4		Day5	Day6
1	0,75	1,3	1,1	1,4		1,1	1,1
2	1,1	1,2	1,75	1,5	1,6	1,6	1,6
5	1,2	1,7	1,9	2,2		2,2	2,2
6	1,6	2,1	2,4	2,6		2,6	2,7
7	1,3	1,6	1,6	1,5		1,3	1,4
8	1,7	1,7	2,1	2,1		1,8	2,4

Seeded

No	Day1	Day2	Day3	Day4	Day5	Day6
1	0,8	1,4	1,6	1,5	1,2	1,2
2	1,1	0,9	1	1,3	1,2	1,2
5	1,5	1,8	2,0	2,1	2,0	2,0
6	1,9	2,5	2,7	2,6	2,5	2,6
7	2,1	2,2	2,4	2,4	2,2	2,2
8	1,1	1,4	1,7	1,5	1,7	1,8

Time to occlusion (weeks);
Control 8 8 2 7 8 7 4 4
Seeded 8 8 5 8 6 5 5 6

Prostacyclin production (pcg/tube)

No	Iliac basal	stim	Seed basal	stim	Unseed basal	stim
1	360	270	<1	3,4	<1	4,2
	300	420	<1	<1	<1	27
2	250	250	<1	<1	2,5	<1
	270	310	4,4	<1	<1	<1
3	440	280	6,4	8,4	<1	30
	740	500	27	30	<1	26
4	350	440	8,4	35	<1	<1
	170	540	22	44	<1	<1
5	160	440	18	18	<1	<1
	290	420	6	11	<1	<1
6	185	260	<1	<1	<1	9,5
	120	140	<1	<1	<1	<1
7	360	340	<1	<1	<1	<1
	220	410	<1	<1	<1	<1
8	165	190	<1	5,8	<1	<1
	150	150	21	29	<1	<1

Procoagulant activity (seconds, mean of two readings):

	Iliac	Seeded	Unseeded
1	142	107,5	89,5
2	142	149	85
3	147,5	80,5	78
4	162	72	87,5
5	140	128,5	83,5
6	119	87	58,5
7	128	97,5	76
8	145	114	81

APPENDIX FIVE

PRESENTATIONS TO LEARNED SOCIETIES AND SCIENTIFIC PUBLICATIONS

ARISING FROM THE WORK IN THIS THESIS

ISOLATION OF MICROVASCULAR ENDOTHELIAL CELLS FROM HUMAN OMENTUM

Walsh AKM, Budd JS, Allen KE, Rose SH, James RFL, Bell PRF.

European Tissue Culture Society. Endothelial Cell Workshop

Aberdeen December 1990.

IN-VITRO STIMULATION OF ADULT PERIPHERAL BLOOD LEUCOCYTES BY HUMAN

UMBILICAL VEIN ENDOTHELIAL CELLS

Walsh AKM, Budd JS, Allen KE, Bell PRF, James RFL.

British Society for Immunology. London April 1991

ISOLATION OF MICROVASCULAR ENDOTHELIAL CELLS FROM HUMAN OMENTUM - USE OF

BOVINE SERUM ALBUMIN DENSITY GRADIENTS

Walsh AKM, Budd JS, Allen KE, Rose SH, James RFL, Bell PRF.

Tripartite meeting of European Society for Surgical Research, Surgical

Research Society and Society of University Surgeons.

Salzburg May 1991

CHANGE IN LYMPHPROLIFERATIVE EFFECT OF HUMAN UMBILICAL VEIN ENDOTHELIAL

CELLS(HUVECs) BY PRO-TREATMENT WITH GAMMA INTERFERON

Walsh, Budd JS, Allen KE, Bell PRF, James RFL.

British Society for Immunology. Sheffield April 1992.

ISOLATION OF MICROVASCULAR ENDOTHELIAL CELLS FROM HUMAN OMENTUM - USE OF

BOVINE SERUM ALBUMIN DENSITY GRADIENTS

Walsh AKM, Budd JS, Allen KE, Rose SH, James RFL, Bell PRF.

Eur Surg Res 1991; 23(Suppl 1): 72

Allen B, Long Clark R, Sicard G, Hopkins K, Welch M.
    Influence of endothelial seeding on platelet deposition and patency
    in small diameter Dacron arterial grafts.
    J Vasc Surg 1984; 1: 224-233.

Anderson JS, Price TM, Hanson SR, Harker LA.
    In vitro endothelialisation of small caliber vascular grafts.
    Surgery 1987; 101; 577-586.

Arregui M, Dilley R, Herring M, Miller B, Baughman S, Smith J, Glover J.
    The effect of bacteremia on arterial prostheses seeded with
    endothelium.
    Asaio J 1985; 8: 118-121.

Asmussen I, Kjeldsen K.
    Intimal ultrastructure of human umbilical arteries. Observations on
    arteries from newborn children of smoking and non-smoking mothers.
    Circ Res 1975; 36: 579-589.

Baker KS, Williams SK, Jarrell BE, Koolpe EA, Levine E.
    Endothelialisation of human collagen surfaces with human adult
    endothelial cells.
    Am J Surg 1985; 150: 197-200.

Bandyk DF, Cato RF, Towne JB.
    A low-flow velocity predicts failure of femoropopliteal and
    femorotibial bypass grafts.
    Surgery 1985; 98: 799-809.

Baumgartner HR.
    The role of blood flow in platelet adhesion, fibrin deposotion, and
    formation of mural thrombi.
    Microvasc Res 1973; 5: 167-179.

Beard JB, Lee RE, Aldoori MI, Baird RN, Horrocks M.
    Does the insitu technique for autologous vein femoropopliteal

bypass offer any haemodynamic advantage?

J Vasc Surg 1986; 4: 588-594.

Beard JD, Scott DJA, Evans JM, Skidmore R, Horrocks M.

Pulse generated runoff(PGR): a new method of assessing calf vessel

patency.

Br J Surg 1988; 75: 361-363.

Berger K, Sauvage LR, Rao AM, Wood SJ.

Healing of arterial prostheses in man: its incompleteness.

Ann Surg 1972; 175(1): 118-127.

Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone MA.

Interleukin-1 induces biosynthesis and cell surface expression of

procoagulant activity in human vascular endothelial cells.

J Exp Med 1984; 160: 618-623.

Bevilacqua MP, Pober JS, Majeau GR.

Recombinant tumour necrosis factor induces procoagulant activity

in cultured human vascular endothelium: Characterisation and

comparison with the actions of Interleukin-I.

Proc Natl Acad Sci 1986; 83: 4533-4537.

Bevilacqua MP, Pober JS, Mendrick DL.

Identification of an inducible endothelial-leukocyte adhesion

molecule.

Proc Natl Acad Sci USA 1987; 84: 9238-

Binns RL, Ku DN, Stewart MT, Ansley JP, Coyle KA.

Optimal graft diameter: effect of wall shear stress on vascular

healing.

J Vasc Surg 1989; 10: 326-337.

Blakowski SA, Zacharski LR, Beck JR.

Postoperative elevation of human peripheral blood monocyte

tissue factor coagulant activity.
J Lab Clin Med 1986; 108: 117-120.

Bloor K.
Natural history of arteriosclerosis of the lower extremities.
Ann R Coll Surg Eng 1961; 28: 36-52.

Boobis LH, Bell PRF.
Can drugs help patients with lower limb ischaemia?
Br J Surg 1982; 69(Suppl): s17-s23.

Brennan JA, Walsh AKM, Beard JD, Bolia AA, Bell PRF.
The role of simple non-invasive testing in infra-inguinal vein
graft surveillance.
Eur J Vasc Surg 1991; 5: 13-17.

Brown RB, Hufnagel CA, Pate JW.
Freeze dried arterial homografts: Clinical application.
Surg Gynecol Obstet 1953; 97: 657-664.

Brox JH, Osterud B, Bjorklid E, Fenton JW.
Production and availability of thromboplastin in endothelial cells:
the effects of thrombin, endotoxin and platelets.
Br J Haematol 1984; 57: 239-246.

Budd JS, Allen KE, Bell PRF.
Effects of two methods of endothelial seeding on cell retention
during blood flow.
Br J Surg 1991; 78(7): 878-882.

Budd JS, Brennan JA, Beard JB, Warren H, Burton PR, Bell PRF.
Infrainguinal bypass surgery: Factors determining late graft
patency.
Br J Surg 1990; 77: 1382-1387.

Buonassi V.
    Sulfated mucopolysaccharide synthesis and secretion in endothelial
    cell cultures.
    Exp Cell Res 1973; 76: 363-368.

Burkel WE, Vinter DW, Ford JW, Kahn RH, Graham LM, Stanley JC.
    Sequential studies of healing in endothelial seeded vascular
    prostheses. Histologic and ultrastructure charactertics of graft
    incoporation.
    J Surg Res 1981; 30: 305-.

Bush HL, Jakubowski JA, Curl JR, Deykin D, Nasbeth DC.
    The natural history of endothelial structure and function in
    arterialised vein grafts.
    J Vasc Surg 1986; 3: 204-215.

Campbell CD, Brooks DH, Webster MW et al
    The use of expanded microporous polytetrafluoroethylene for limb
    salvage: A preliminary report.
    Surgery 1976; 79: 485-490.

Campbell CD, Brooks DH, Webster et al
    Aneurysm formation in expanded polytetrafluoroehtylene prostheses.
    Surgery 1976; 79: 491-493.

Campbell JB, Glover JL, Herring B.
    The influence of endothelial seeding and platelet inhibition on the
    patency of ePTFE grafts used to replace small arteries: an
    experimental study.
    Eur J Vasc Surg 1988; 2: 365-370.

Cannon JA.
    The expanded reinforced polytetrafluoroethylene prosthetic vascular
    graft.
    In: Wright CB, Hobson RW, Hiratzka LF, Lynch TG, eds.

Vascular Grafting Clinical Application and Techniques
1983. John Wright, Boston.

Carter AJ, Eisert wg, Muller T.
Thrombin stimulates inositol phosphate accumulation and
prostacyclin synthesis in human endothelial cells from umbilical
vein but not from omentum.
Thromb Haemostas 1989;61(1): 122-126.

Castellot JJ, Favreau LV, Karnovsky MJ, Rosenberg RD.
Inhibition of vascular smooth muscle cell growth by endothelial
cell derived heparin: Possible role of a platelet endoglycoside.
J Biol Chem 1982; 257: 11256-11260.

Centre for Disease Control(CDC)
Transmission of HIV through bone transplantation: case report and
public health recommendations.
MMWR 1988; 37: 597-599.

Charo IF, Shak S, Karasek MA.
Prostaglandin I_2 is not a amjor metabolite of arachadonic acid in
cultured endothelial cells from human foreskin microvessels.
J Clin Invest 1984; 74: 914-

Cheshire NJ, Davies L, Noone MA, Tyrell M, Wolfe JHN.
Viability of distal arterial reconstruction.
Br J Surg 1991; 78(6): 752.

Chief Medical Officer.
HIV infection, tissue banks and organ donation.
Letter to all doctors. PL/CMO(90)2.

Clagett GP, Burkel WE, Sharefkin JB, Ford JW, Hufnagel H, Vinter DW,
Kahn RH, Graham LM, Stanley JC, Ramwell PW.
Platelet reactivity in vivo in dogs with arterial prostheses seeded

with endothelial cells.

Circulation 1984; 69: 632-639.

Clowes AW, Reidy MA, Clowes MM.

Mechanisms of stenosis after arterial injury.

Lab Invest 1983; 49: 208-215.

Collins T, Korman AJ, Wake CT.

Immune interferon activates multiple class II major

histocompatability complex genes and the associated invariant

chain gene in human endothelial cells and dermal fibroblasts.

Proc Natl Acad Sci USA 1984; 81: 4917

Colucci M, Balconi G, Lorenzet R, Pietra A, Locati D, Donati MB,

Semeraro N.

Cultured human endothelial cells generate tissue factor in response

to endotoxin.

J Clin Invest 1983; 71: 1893-1896.

Colucci M, Paramo JA, Collen d.

Generation in plasma of a fast-acting inhibitor of plasminogen

activator in response to endotoxin stimulation.

J Clin Invest 1985; 75: 818-824.

Connolly JE, Stemmer EA.

The nonreversed saphenous vein bypass for femoro-popliteal

occlusive disease.

Surgery 1970; 68: 602-609.

Consumer's Association

Do drugs help intermittent claudication?

Drug Ther Bull 1990; 28: 1-2.

Cranley JJ, Hafner CD.

New prosthetic material compared with autogenous saphenous vein for

occlusive arterial disease of the lower extremity.
Surgery 1981; 89: 2-7.

Crawford T.
Some aspects of the pathology of atherosclerosis.
Proc Roy Soc Med 1960; 53: 9-12.

Crawshaw HM, Quist WC, Serrallch E.
Flow disturbance at the distal end-to-side anastomosis.
Arch Surg 1980; 115: 1280-1284.

Cumberland DC, Belli AM.
Laser assisted angioplasty.
In: Zeitler E, Seiforth W eds, Pros and cons in PTA and auxillary
methods. Berlin: Springer 1989: 91-97.

Dardik I, Ibrahim IM, Dardik H.
Femoral popliteal bypass employing modified human umbilical cord
vein:An assessment of early clinical results.
Cardiovasc Dis Bull Texas Heart Inst 1976; 3(3): 34-

Dardik H, Miller N, Dardik A, Ibrahim I, Sussman B, Berry SM, Wolodiger
F, Kahn M, Dardik I.
A decade of experience with the glutaraldehyde tanned umbilical
cord vein graft for revascularisation of the lower limb.
J Vasc Surg 1988; 7: 336-346.

Dekker A, Poot A, Beugeling T, Bantjes A.
The effect of vascular cell seeding on platelet deposition in an
vitro capillary perfusion model.
Thromb Haemostas 1989; 61: 402-408.

DiCorletto PE, Chisholm GM.
Participation of the endothelium in the development of the
athersclerotic plaque
Prog Lipid Res 1986; 25: 365-374.

Duel TF, Senior RM, Huaang JS, Griffin GL.
    Chemotaxis of monocytes and neutrophils to platelet derived growth
    factor.
    J Clin Invest 1982; 69: 1046-1049.

Duguid JB
    Thrombosis as a factor in the pathogenesis of coronary
    athersclerosis.
    J Path Bact 1946; 58: 207-212.

Duguid JB.
    Thrombosis as a factor in the pathogenesis of aortic
    athersclerosis.
    J Path Bact 1948; 60: 57-61.

Eaton AC, Wright M, Callum KG.
    The use of the image intensifier in phenol lumbar sympathetic
    block.
    Radiography 1980; 156(552): 298-300.

Eickhoff JH, Buchardt-Hansen HJ, Bromme A, Ericsson BF Kordt KF,
    Mouritzen C, Myhre HO, Norgren L, Rostad H, Trippesstad A.
    A randomised clinical trial of PTFE versus human umbilical vein for
    femoropopliteal bypass surgery: preliminary results.
    Br J Surg 1983; 70: 85-88.

Emerick S, Herring M, Arnold M, Baughman S, Reilly K, Glover J.
    Leucocyte depletion enhances cultured endothelial retention on
    vascular prostheses.
    J Vasc Surg 1987; 5: 342-347.

Esmon CT, Esmon NL, Harris KW.
    Complex formation between thrombin and thrombomodulin inhibits both
    thrombincatalysed fibrin formation and factor V activation.
    J Biol Chem 1982; 257: 7944-7947.

Esmon NL, Owen WG, Esmon CT.
    Isolation of a membrane bound co-factor for thrombin catalysed
    activation of protein C.
    J Biol Chem 1982; 257: 859-864.

European Working Group on Critical Ischaemia.
    European concensus document on critical limb ischaemia.
    Dormandy J ed. Berlin. Springer-Verlag. 1989.

Fallopius G (1575)
    Lectiones de partibus similaribus humani corporis.
    Cited by Long ER in
    Cowdry's Arteriosclerosis. ed. Blumenthal MT. 1967. Thomas.
    Springfield CT.

Ferry B, Halttunen J, Leszcynski D, Schellekens H, vd Meide PH, Hayry P.
    Impact of Class II major histocompatability complex antigen
    expression
    on the immunogenic potential of isolated rat vascular endothelial
    cells.
    Transplantation 1987; 44(4): 499-503.

Fishmen JA, Ryan GB, Karnovsky MJ.
    Endothelial regeneration in the rat carotid artery and the
    significance of endothelial denudation in the pathogenesis of
    myointimal thickening.
    Lab Invest 1975; 32: 339-351.

Friedman RJ, Stemerman MB, Wenz B.
    The effect of thrombocytopaenia on experimental arteriosclerotic
    lesion formation in rabbits: smooth muscle cell proliferation and
    re-endothelialisation.
    J Clin Invest 1977; 60: 1191-1201.

Fry DL.
    Acute vascular endothelial chnages associated with increased blood

velocity gradients.
Circ Res 1968; 22: 165-167.

Furchgott RF.
The requirement for endothelial cells in the relaxation of arteries
by acetylcholine and some other vasodilators.
Trends in Pharmacological Sciences 1981; Jul: 173-176.

Gannon MX, Simms MH, Goldman M.
Does the in-situ technique improve flow characteristics in femoro
popliteal bypass?
J Vasc Surg 1986; 4: 595-599.

Gimbrone MA.
Vascular endothelium: nature's blood container.
in: Vascular endothelium in haemostasis and thrombosis. Gimbrone MA
ed. London Churchill Livingstone. 1-13. 1986.

Glassberg MK, Bern MM, Coughlan SR.
Cultured endothelial cells derived from the human iliac arteries.
In Vitro 1982; 18: 859-866.

Glenn KC, Ross R.
Human monocyte-derived growth factor(s) for mesenchymal cells:
activation of secretion by endotoxin and concanavalin A.
Cell 1981; 25: 603-615.

Gourevitch D, Jones CE, Crocker J, Goldman MD.
An in vitro technique to cover Dacron arterial grafts with human
adult endothelial cells.
Surg Res Comm 1987; 2: 59-64.

Gourevitch D, Jones CE, Crocker J, Goldman M.
Endothelial cell adhesion to vascular prosthetic surfaces.
Biomaterials 1988; 9: 97-100.

Graham LM, Vinter DW, Ford JW, Kahn RH, Burkel WE, Stanley JC.
Cultured autogenous endothelial cell seeding of prosthetic grafts.
Surg Forum 1979; 30: 204-206.

Graham LM, Vinter DW, Ford JW, Kahn RH, Burkel WE, Stanley JC.
Endothelial cell seeding of prosthetic grafts. Early experimental
studies with cultured autologous canine endothelium.
Arch Surg 1980; 115: 929-933.

Graham LM, Burkel WE, Ford JW, Vinter DW, Kahn RH, Stanley JC.
Immediate seeding of enzymatically derived endothelium in Dacron
vascular grafts. Early experimental studies with autologous canine
cells.
Arch Surg 1980(a); 115: 1289-1294.

Graham LM, Burkel WE, Ford JW, Vinter DW, Kahn RH, Stanley JC.
Expanded polytetrafluoroethylene vascular prostheses seeded with
enzymatically derived and cultivated canine endothelial cells.
Surgery 1982; 91: 550-559.

Greenhalgh RM, Laing SP, Cole PV, Taylor GW.
Effect of risk factors on the outcome of arterial reconstruction.
in; Smoking and Arterial Disease Greenhalgh RM ed.
Pitman Medical Bath 1981.

Hall KV.
The great saphenous vein used in situ as an arterial shunt
after extirpation of the vein valves. A preliminary report.
Surgery 1962; 51: 492-495.

Hamlin GW, Rajah SM, Crow M, Kester RC.
Evaluation of the thrombogenic potential of 3 types of arterial
graft studied in a artificial circulation.
Br J Surg 1978; 65: 272-276.

Hanel KC, McCabe C, Abbott WM, Fallon J, Megerman J.
    Current PTFE grafts, a biomechanical, scanning electron and light
    microscopic evaluation.
    Ann Surg 1982; 195: 456-463.

Harlan JM.
    Leukocyte-endothelial interactions.
    Blood 1985; 65: 513-525.

Harris PL, How TV, Jones DR.
    Prospective randomised clinical trial to compare in situ and
    reversed saphenous vein grafts for femoropopliteal bypass.
    Br J Surg 1987; 74: 252-255.

Harris PL, Veith FJ, Shanik GD, Nott D, Wengerter KR, Moore DJ.
    Prospective randomized comparison of in situ and reversed and
    infra-popliteal vein grafts.
    Br J Surg 1993; 80(2): 173-176.

Haudenschild CC, Schwartz SM.
    Endothelial regeneration II. Restitution of endothelial continuity.
    Lab Invest 1979; 41: 407-418.

Hekman CM, Loskutoff DJ.
    Fibrinolytic pathways and the endothelium.
    Seminars in Haemostasis and Thrombosis 1987; 13: 514.

Heliovaara M, Karvonen MJ, Vilhounen R, Punsar S.
    Smoking, carbon monoxide and athersclerotic disease.
    Br Med J 1978; 1: 268-270.

Herring M, Gardner A, Glover J.
    A single-staged technique for seeding vascular grafts with
    autogenous endothelium.
    Surgery 1978; 84(4): 498-504.

Herring MB, Dilley R, Jersild RA, Boxer L, Gardner A, Glover J.
Seeding arterial prostheses with vascular endothelium. The nature of the lining.
Ann Surg 1979; 190(1): 84-90.

Herring M, Dilley R, Cullison T, Gardner A, Glover J.
Seeding endothelium on canine arterial prostheses: the size of the innoculum.
J Surg Res 1980; 28: 35-38.

Herring M, Baughman S, Glover J.
Endothelium develops on seeded human arterial prostheses: A brief clinical note.
J Vasc Surg 1985; 2: 727-730

Herring M, Baughman S, Glover J, Kesler K, Jesseph J, Campbell J, Dilley R, Evan A, Gardner A.
Endothelial seeding of Dacron and polytetrafluoroethylene grafts: The cellular events of healing.
Surgery 1984; 96: 745-754.

Herring M, Gardner A, Glover J.
Seeding human arterial prostheses with mechanically derived endothelium. The detrimental effect of smoking.
J Vasc Surg 1984a; 1: 279-289.

Herring MB, Compton RS, LeGrand DR, Gardner AL, Madison DL, Glover JL.
Endothelial seeding of polytetrafluoroethylene popliteal bypasses; a preliminary report.
J Vasc Surg 1987; 6: 114-118.

Hewes RC, White RI, Murray RR, Kaufman SL, Chang R, Kadir S, Kinnison ML, Mitchell SE, Auster M.
Long term results of superficial femoral artery angioplasty.
AJR 1986; 146: 1025-1029.

Hickey NC, Shearman CP, Crowson MC, Simms MH, Watson HR.
    Iloprost improves femoro-distal graft flow after a single bolus
    injection.
    Eur J Vasc Surg 1991; 5(1): 19-22.

Hickey NC, Thomson IA, Shearman CP, Simms MH.
    Aggressive arterial reconstruction for critical lower limb
    ischaemia.
    Br J Surg 1991; 78(12): 1476-1478.

Hirko MK, Schmidt SP, Hunter TJ, Evancho MM, Sharp WV, Donovan DL.
    Endothelial cell seeding improves 4mm PTFE vascular graft
    performance in antiplatelet medicated dogs.
    Artery 1987; 14: 137-153.

Hirschberg H, Evensen SA, Henriksen T, Thorsby E.
    The human mixed lymphocyte-endothelium culture interaction.
    Transplantation 1975; 19(6): 495-504.

Hirschberg H, Bergh OJ, Thorsby E.
    Antigen-presenting properties of human vascular endothelial cells
    J Exp Med 1980; 152: 249s-255s.

Hofling B, Backa D, Lauterjung L, Polnitz AV, Arnim TV, Jauch KW.
    Percutaneous removal of atheromatous plaques in peripheral
    arteries.
    Lancet 1988; 1: 384-386.

Hollier LH, Fowl RJ, Pennell RC, Heck CF, Winter KAH, Fass DN, Kaye MP.
    Are seeded endothelial cells the origin of neointima on prosthetic
    vascular grafts?
    J Vasc Surg 1986; 3: 65-73.

Housley E.
    Treating claudication in five words.
    Br Med J 1988; 296: 1483-1484.

Hufnagel CA.
    Preserved homologous arterial transplants.
    Bull Am Coll Surg 1947; 32: 321-

Hufnagel CA, Rabil PJ, Reed L.
    A method for preservation of arterial homo and heterografts.
    Surg Forum 1953; 4: 162-168.

Hufnagel CA, Rabil P.
    Replacement of arterial segments utilising flexible Orlon
    prostheses.
    Arch Surg 1955; 70: 105-110.

Hughson WG, Mann JI, Garrod A.
    Intermittent claudication: prevalence and risk factors.
    Br Med J 1978; 1: 1379-1381.

Hussain S, Glover JL, Augelli N, Bendick PJ, Maupin D, McKain M.
    Host response to autologous endothelial seeding.
    J Vasc Surg 1989; 9: 656-664.

Jaffe EA, Nachman RL, Becker CG, Minick CR.
    Culture of human endothelial cells derived from umbilical veins.
    Identification by morphologic and immunologic criteria.
    J Clin Invest 1973; 52: 2745-2756.

Jarrell BE, williams SK, Solomon L, Speicher L, Koolpe E, Radomski J,
    Carabasi RA, Greener D, Rosato FE.
    Use of an endothelial monolayer on a vascular graft prior to
    implantation: temporal dynamics and compatability with the
    operating room.
    Ann Surg 1986; 203(6): 671-678.

Jarrell BE, Williams SK, Carabasi RA, Hubbard FA.
    Immediate vascular graft monolayers using micro-vessel endothelial
    cells.

in: Glover J, Herring M, eds. Endothelial Seeding in Vascular
Surgery.
Grune & Stratton Fl. USA 1987.

Jarrell BE, Williams SK.
Microvessel derived endothelial cell isolation, adherence, and
mono-layer formation for vascular grafts.
J Vasc Surg 1991; 13(5): 733-744.

Kakkar VV.
The cephalic vein as a peripheral vascular graft.
Surg Gynecol Obstet 1969; 128: 551-556.

Kallero KS.
Mortality and morbidity on patients with intermittent claudication
as defined by venous occlusion plethysmography. A ten year study.
J Chronic Dis 1981; 34: 455-462.

Kent KC, Shindo s, Ikemoto t, Whittemore AD.
Species variation and the success of endothelial cell seeding.
J Vasc Surg 1989; 9: 271-276.

Kern PA, Knedler A, Eckel RH.
Isolation and culture of microvascular endothelium from human
adipose tissue.
J Clin Invest 1983; 71: 1822-1829.

Knight RW, Kenney GJ, Lewis EE, Johnston GG.
Percutaneous transluminal angioplasty: results and surgical
implications.
Am J Surg 1984; 147: 578-588.

Koveker GB, Petzke KH, Borg M, Nebendahl K.
Reduction of thrombogenicity in small diameter vascular prostheses
seeded with autologous endothelial cells.
Thorac Cardiovasc Surgeon 1986; 34: 49-51.

Kunlin J.

    Le traitment de l'arterite obliteranre par la greffe veinesse.

    Arch Mal Coeur 1949; 42: 371-372.

Lake SP, Anderson J, Chamberlain J, Bell PRF, James RFL.

    Bovine Serum Albumin density gradient isolation of rat pancreatic

    islets.

    Transplantation 1987; 43(6): 805-808.

Leather RP, Power SRJr,, Karmody AM.

    A reappraisal of the in-situ saphenous vein arterial bypass:

    Its use in limb salvage.

    Surgery 1979; 86: 453-460.

Leibovich SJ, Ross R.

    A macrophage-dependent factor that stimulates the proliferation

    of fibroblasts in vitro.

    Am J Pathol 1976; 84: 501-513.

Lindblad B, Burkel WE, Wakefield TW, Graham LM, Stanley JC,.

    Endothelial cell seeding efficiency onto expanded

    polytetrafluoroethylene grafts with different coatings.

    Acta Chir Scand 1986; 152: 653-656.

London NJM, Sayers RD, Walsh AKM, Brennan JA, Thompson MM, Hartshorne T,

    Bell PRF, Bolia A.

    Percutaneous transluminal angioplasty: an effective treatment for

    infrainguinal vein graft stenoses.

    Br J Surg 1992; 79(4): 367.

Lyberg T, Galdal KS, Evensen SA, Prydz H.

    Cellular co-operation in endothelial cell thromboplastin synthesis.

    Br J Haematol 1983; 53: 85-95.

Magometschnigg H, Kadletz M, Vodrazka M, Dock W, Grimm M, Grabenwoger M,

    Minar E, Staudacher M, Fenzl G, Wolner E.

Prospective clinical study with in vitro endothelial cell lining of expanded polytetrafluoroethylene grafts in crural repeat reconstruction.
J Vasc Surg 1992; 15: 527-535.

Mahler F.
Trends in percutaneous transluminal angioplasty(PTA): application of laser and new mechanical devices.
Vasc Med Review 1990; 1: 35-50.

Marcum JA, McKennedy JB, Rosenberg RD.
Acceleration of thrombin - anti-thrombin complex formation in rat hindquarters via heparin-like molecules bound to the endothelium.
J Clin Invest 1984; 74: 341-350.

Marcum JA, Rosenberg RD.
Heparin-like molecules with anti-coagulant activity are synthesised by cultured endothelial cells.
Biochem Biophys Res Commun 1985; 126: 365-372.

Martin BM, Gimbrone MA, Unanue ER, Cotran RS.
Stimulation of non-lymphoid mesenchymal cell proliferation by a macrophage derived growth factor.
J Immunol 1981; 126: 1510-1515.

Matsumoto H, Hasegawa T, Fuse K.
A new vascular prosthesis for a small caliber artery.
Surgery 1973; 74: 519-523.

McDaniel MD, Pearce WH, Yao JST, Rossi EC, Fahey VA, Green D, Flinn WR, Bergan JJ.
Sequential changes in coagulation and platelet function following femorotibial bypass.
J Vasc Surg 1984; 1: 261-268.

Miller JH, Foreman RK, Ferguson L Faris I.
Interposition vein cuff for anastomosis of prosthesis to small
artery.
Aust NZ J Surg 1984; 54: 283-285.

Mills ML, Oschner JC.
Valvotomy of valves in the saphenous vein graft before coronory
artery surgery.
J Thorac Cardiovasc Surg 1976; 71: 878-

Moen T, Moen M, Thorsby E.
HLA-D region products are expressed in endothelial cells.
Tissue Antigens 1980; 15: 112-122.

Mohr L, Smith L.
Polytetrafluoroethylene graft aneurysms.
Arch Surg 1980; 115: 1467-1470.

Moncada S, Gryglewski R, Bunting S, Vane JR.
An enzyme isolated from arteries transforms prostacyclin
endoperoxidases to an unstable substance that inhibits platelet
aggregation.
Nature 1976; 263: 663-665.

Moore S, Friedman RJ, Singal DP.
Inhibition of injury induced thrombo-athersclerotic lesions by
anti-platelet serum in rabbits.
Thromb Haemostas 1976; 35: 70-81.

Mosley JG, Manhire AR, Raphael M, Marston A.
An assessment of long saphenous venography to evaluate the
saphenous vein for femoro-popliteal bypass.
Br J Surg 1983; 70: 673-674.

Mosley JG, Marston A.
  A 5 year follow-up of Dacron femoropopliteal bypass grafts.
  Br J Surg 1986; 73: 24-27.

Myers KA, King RB, Scott DF, Johnson N, Morris PJ.
  The effect of smoking on the late patency of arterial
  reconstructions in the legs.
  Br J Surg 1978; 65: 267

Nawroth PP, Stern DM.
  Modulation of endothelial hemostatic properties by tumour necrosis
  factor.
  J Exp Med 1986; 163: 740-745.

OPCS + DHSS
  Hospital in-patient enquiry, main tables.
  1983; Series MB4 No.23.

Ortenwall P, Wadenvik H, Kutti J, Risberg B.
  Reduction in deposition of 111-labeled platelets after autologous
  endothelial seeding of Dacron aortic bifurcation grafts in humans;
  a preliminary report.
  J Vasc surg 1987; 6: 17-25.

Ortenwall P, Wadenvik H, Kutti J, Risberg B.
  Endothelial cell seeding reduces thrombogenicity of Dacron grafts
  in humans.
  J Vasc Surg 1990; 11: 403-410.

Parvin S, Bell PRF.
  The use of peripheral resistance measurements in the assessment of
  severe peripheral vascular disease.
  Br J Surg 1984; 71: 311-312.

Paterson IS, Klausner JM, Welbourn CRB, Shepro D, Hechtman HB.
  Reperfusion plasma and microvascular injury.

Br J Surg 1992; 79(5): A441.

Peabody CN, Kannel WB, McNamara PM.
    Intermittent claudication: surgical significance.
    Arch Surg 1974; 109: 693-697.

Pearce WH, Rutherford RB, Whitehill TA, Rosales C, Bell KP, Patt A
    Ramalanjaona G.
    Successful endothelial seeding with omentally derived microvascular
    endothelial cells.
    J Vasc Surg 1987; 5(1): 203-210.

Pennell RC,, Hollier LH, Solis E, Kaye MP.
    Xenograft seeding of Dacron grafts in dogs.
    J Surg Res 1986; 40: 332-339.

Petty RG, Pearson JD.
    Endothelium - the axis of vascular health and disease.
    J Roy Coll Phys 1989; 23(2): 92-102.

Pober JS, Gimbrone MA.
    Expression of Ia-like antigens by human vascular endothelial cells
    is inducible in vitro: Demonstration by monoclonal antibody binding
    and immunoprecipitation.
    Proc Natl Acad Sci USA 1982; 79: 6641-6645.

Pober JS, Gimbrone MA, Cotran RS, Reiss CS, Burakoff SJ, Fiers W, Ault
    KA.
    Ia expression by vascular endothelium is inducible by activated T
    cells and by human gamma interferon.
    J Exp Med 1983; 157: 1339-1353.

Pober JS, Collins T, Gimbrone MA, Libby P, Reiss CS.
    Inducible expression of class II major histocompatability complex
    antigens amd the immunogenicity of vascular endothelium.
    Transplantation 1986; 41(2): 141-146.

Pond GD, Osborne RW, Capp MP
    Digital subtraction angiography of peripheral vascular bypass
    procedures.
    AJR 1982; 138: 279-281.

Prendiville EJ, Gould KE, Connolly RJ, Callow AD.
    Effect of blood flow on retention of endothelial cells cultured on
    fibronectin coated ePTFE vascular grafts.
    Vasc Surgical Society GB & Irel. Dundee 1989. Abst. 24

Quick CRG, Cotton LT.
    The measured effect of stopping smoking on intermittent
    claudication.
    Br J Surg 1982; 69(Suppl): s24-s26.

Radomski MW, Palmer RMS, Moncada S.
    Comparative pharmacology of endothelium dependent relaxing factor
    nitric oxide and prostacyclin in platelets.
    Br J Pharmacol 1987; 92: 181-187.

Ramalanjaona G, Kempczinski RF, Rosenman JE, Douville EC, Silberstein
    EB.
    The effect of fibronectin coating on endothelial cells kinetics in
    polytetrafluoroethylene grafts.
    J Vasc Surg 1986; 3: 264-272.

Rees M, Gehani AA, Richens D.
    Percutaneous dynamic removal of atheroma.
    Lancet 1988; 1: 174

Rokitansky C.
    Hanbuch der Pathologischen Anatomie Vol.2
    Braunmuller + Siedel. 1844. Vienna.

Rosenberg RD.
    Role of heparin and heparinlike molecules in thrombosis and

athersclerosis.
Fed Proc 1985; 44: 404-409.

Rosenman JE, Kempczinski RF, Berlatszky Y, Pearce WH, Ramalanjaona GR
Bjornson HS.
Bacterial adherence to endothelial seeded polytetraflouorethylene
grafts.
Surgery 1985; 98: 816-823.

Rosenman JE, Kempczinski RF, Pearce WH, Silberstein EB.
Kinetics of endothelial cell seeding.
J Vasc Surg 1985(a); 2: 778-784.

Ross R.
The pathogenesis of athersclerosis: an update.
N Eng J Med 1986; 314: 388-400.

Rupnick MA, Hubbard FA, Pratt K, Jarrell BE, Williams SK.
Endothelialisation of vascular prosthetic surfaces after seeding or
sodding with human microvascular endothelial cells.
J Vasc Surg 1989; 9(6): 788-795.

Rutherford RB, Jones DN, Bergentz SE, Bergquist D, Comeroto AJ, Dardik
H, Flinn WH, McIntyre K, Moore WS, Shah DM, Yano T.
Factors affecting the patency of infrainguinal bypass.
J Vasc Surg 1988; 8: 236-246.

Ryan US.
Vascular prostheses and delivery of recombinant endothelial
products.
Presented at VIth International Symposium Biology of Vascular
Cells. Abst PSVII.1 August 1990.

Saba SR, Mason RG.
Studies of an activity from endothelial cells that inhibits

platelet aggregation, serotonin release and clot retraction.
Thrombosis Research 1974; 5: 747-757.

Sanders RJ, Kempczinski RF, Hammond N, DeClementi.
The significance of graft diameter.
Surgery 1980; 88: 856-866.

Sauvage LR.
Externally supported, non-crimped external velour weft knitted
Dacron prosthesis for axillofemoral, femoropopliteal and
femorotibial bypass.
In: Wright CB, Hobson RW, Hiratzka LF, Lynch TG, eds.
Vascular Grafting Clinical Applications and Techniques.
1983. John Wright, Boston.

Schmidt SP, Hunter TJ, Sharp WV, Malindzak GS, Evancho MM.
Endothelial seeded four millimeter Dacron vascular grafts: effects
of blood flow manipulation through the grafts.
J Vasc Surg 1984; 1: 434-441.

Schneider A, Melmed RN, Schwalb H, Karck M, Vlodavsky I, Uretsky G.
An improved method for endothelial cell seeding on
polytetrafluoroehtylene small caliber vascular grafts.
J Vasc Surg 1992; 15: 649-656.

Schneider PA, Hanson SR, Price TM, Harker LA.
Durability of confluent endothelial cell monolayers on small
caliber vascular prostheses in vitro.
Surgery 1988; 103: 456-462.

Scott DJA, McMahon J, Beard JD, Milroy C, Bradfield JWB, Horrock M.
Histology of the long saphenous vein: A cause of femoro-distal
bypass graft failure?
J Cardiovasc Surg 1988; 29: 84.

Scott PMJ, Wyatt M, Scott DJA, Baird RN, Horrocks M, Poskitt KR.
    Does weight influence claudication distance?
    Br J Surg 1988; 75: 1264.

Seeger JM.
    Improved endothelial seeding density after flow exposure in fibro-
    nectin coated grafts.
    Surg Forum 1985; 36: 450-452.

Sentissi JM, Ramberg K, O'Donnell TF, Connolly RJ, Callow AD.
    The effect of flow on vascular endothelial cells grown in tissue
    culture on polytetrafluoroethylene grafts.
    Surgery 1986; 99: 337-343.

Shah DM, Buchbinder D.
    A modified technique to produce valvular incompetence in the
    insitu and reversed vein arterial bypass.
    Arch Surg 1981; 116: 356-358.

Sharefkin JB, Lather C, Smith M, Rich NM.
    Endothelial cell labelling with Indium 111 oxine as a marker of
    cell attachment to bioprosthetic surfaces.
    J Biomed Mater Res 1983; 17: 345-357.

Sharefkin JB, Latker C, Wind G, Cruess D, Rich NM.
    The comparative endothelial seeding efficiency of Dacron velour
    and expanded polytetrafluoroethylene vascular prostheses.
    Presented 16th annual meeting Assoc. Academic Surgery. California
    1982. Cited in:
    Stanley JC, Burkel WE, Graham LM, Lindblad B.
    Endothelial cell seeding of synthetic vascular prostheses.
    Acta Chir Scand 1985; Suppl 529: 17-28.

Sharefkin JB, Van Wart HE, Cruess DF, Albus RA, Levine EM.
    Adult human endothelial cell harvesting.
    J Vasc Surg 1986; 4: 567-577.

Sharefkin JB, Van Wart HE, Williams SK.
    Enzymatic harvesting of adult human endothelial cells for use in
    autogenous endothelial vascular prosthetic seeding.
    in: Herring M, Glover JL. eds. Endothelial seeding in Vascular
    Surgery 1987 Grune & Stratton Inc Fl. USA.

Sharp WV, Schmidt SP, Meerbaum SO, Pippert TR.
    Derivation of human microvascular endothelial cells for prosthetic
    graft seeding.
    Annals Vasc Surg 1989; 3(2): 104-107.

Shattock SG
    A report upon the pathological condition of the aorta of King
    Menephthah, traditionally regarded as the Pharoah of the Exodus.
    Proc Roy Soc Med 1909; 2: 122-127.

Shiokawa Y, Rachman MF, Ishii Y, Surishi K.
    The rate of re-endothelialisatiom correlates inversely with the
    degree of the following thickening in vein grafts: electron
    microscopic and immunohistochemical studies.
    Virchows Archiv A Pathol An 1989; 415: 215-235.

Skinner JS, Strandness DE.
    Exercise and intermittent claudication. Effect of physical
    training.
    Circulation 1967; 36 :23-

Snyder SO, Wheeler JR, Gregory RT, Gayle RG, Mariner DR.
    The Kensey catheter: preliminary results with a transluminal
    atherectomy tool.
    J Vasc Surg 1988; 8: 541-543.

Somer JB, Schwartz CJ.
    Focal ^{3}H-cholesterol uptake in the pig aorta.
    Athersclerosis 1971; 13: 293-303.

Sommeling CA, Buth J, Jakimowicz JJ.
    Long term bahaviour of modified human umbilical vein grafts: Late
    aneurysmal degeneration established by colour Duplea scanning.
    Eur J Vasv Surg 1990; 4: 89-94.

Spence RK, Frieman DB, Gatenby R, Hobbs CL, Barker CF, Berkowitz HD,
    Roberts B, McClean G, Oleaga J, Ring EJ.
    Long term results of transluminal angioplasty of the iliac and
    femoral arteries.
    Arch Surg 1981; 116: 1377-1386.

Stanley JC, Burkel WE, Ford JW, Vinter DW, Kahn RH, Whitehouse WM,
    Graham LM.
    Enhanced patency of small diameter, externally supported Dacron
    iliofemoral grafts seeded with endothelial cells.
    Surgery 1982; 92: 994-1005.

Stemme S, Holm J, Hansson GK
    Phenotype and clonality of T lymphocytes in human atherosclerotic
    plaques.
    Presented at VIth International Symposium Biology of Vascular
    Cells. 28-31 August 1990. Paris. France

Stern DM, Bank I, Nawroth PP, Cassimeris J, Kisiel W, Fenton JW,
    Dinarello C, Chess L, Jaffe EA.
    Self regulation of procoagulant events on the endothelial cell
    surface.
    J Exp Med 1985; 162: 1223-1235.

Stern DM, Nawroth PP, Harris K, Esmon CT.
    Cultured bovine aortic endothelial cells promote activated protein
    C - protein S mediated inactivation of factor Va.
    J Biol Chem 1986; 261: 713-718.

Stern DM, Nawroth PP, Kisiel W, Handley D.
    Participation of endothelial cells in the protein C - protein S

anti-coagulant pathway.
J Cell Biol 1986(a); 102: 1971-1978.

Sterpetti AV, Hunter WJ, Schultz RD, Sugimoto JT, Blair EA, Hacker K,
Chasan P, Valentine J.
Seeding with endothelial cells derived from the microvessels of the
omentum and from the jugular vein; a comparative study.
J Vasc Surg 1988; 7: 677-684.

Sterpetti AV, Schultz RD, Hunter WJ, Cisternino S, Fontaine M.
Comparison of two techniques to isolate microvascular endothelial
cells from the omentum.
J Surg Res 1990; 48: 101-106.

Szilagyi DE, Elliott JP, Hageman JH.
Biologic fate of autogenous vein implants as arterial substitutes:
clinical, angiographic, and histopathologic observations in femoro-
popliteal operations for athersclerosis.
Ann Surg 1973; 178: 232-

Taylor RS, McFarland RJ, Cox MI.
An investigation into the causes of failure of PTFE grafts.
Eur J Vasc Surg 1987; 1: 335-343.

Turnipseed WD, Detmer WE, Berkoff HA
Intra-arterial digital angiography: a new diagnostic method for
determining limb salvage bypass canditates.
Surgery 1982; 92: 322-327.

Thomson GJL, Vohra RK, Carr MH, Walker MG.
Adult human endothelial cell seeding using expanded
polytetrafluoroethylene vascular grafts: A comparison of four
substrates.
Surgery 1991; 109: 20-27.

Vallbracht C, Prignitz I, Beinborn W.
    Low speed rotational angioplasty: clinical results in 53 patients
    with chronic vessel obstructions.
    In: Zeitler E, Seiforth W eds, Pros and cons in PTA and auxillary
    methods. Berlin: Springer 1989: 101-110.

Vanhoutte PM.
    The end of the quest?
    Nature 1987; 327: 459

Vanhoutte PM.
    The endothelium - modulator of vascular smooth muscle tone.
    N Eng J Med 1988; 319: 512-

Vanhoutte PM, Katusic ZS.
    Endothelium derived contracting factor: endothelin and/or
    superoxide anion?
    Trends in Pharmacological Sciences 1988; 9: 229

van Andel GJ, van Erp WFM, Krepel VW, Breleau PJ.
    Percutaneous transluminal dilatation of the iliac artery. Long term
    results.
    Radiology 1985; 156: 321-323.

Veith FJ, Gupta SK, Ascer E, White-Flores S, Samson RH, Scher LA, Towne
    JB, Bernhard VM, Bonier P, Flinn WR, Astelford P, Yao JST, Bergan
    JJ.
    Six year prospective multicenter randomised comparison of
    autologous saphenous vein and expanded polytetrafluoroethylene
    grafts in infra-inguinal reconstructions.
    J Vasc Surg 1986; 3: 104-114.

Virchow R.
    Phlogose und thrombose im Gefass System
    Gesammelte Abhandlungen zur Wissenschaftlichen Median
    1856. Meidinger. Frankfurt. 458-463.

von Haller (1775) cited by Long ER in
    Arteriosclerosis ed EV Cowdry. 1933. Macmillan. New York.

Voorhees AB, Jaretski A, Blakemore AH.
    The use of tubes constructed from Vinyon 'N' cloth in bridging
    arterial defects.
    Ann Surg 1952; 135: 332-336.

Wakefield TW, Earley EM, Brothers TE, Burkel WE, Graham LM, Fessler RD,
    Saenz N, Sell RM, Stanley JC.
    Karyotype analysis of cell sex to determine the source of vascular
    graft luminal linings following autologous and nonautologous
    endothelial cell seeding.
    ASAIO Transactions 1988;34: 864-867.

Walker LN, Bowen-Pope DF, Ross R, Reidy MA.
    Production of platelet-derived growth factor-like molecules by
    cultured arterial smooth muscle cells accompanies proliferation
    after arterial injury.
    Proc Natl Acad Sci USA 1986; 25: 365-374.

Walker MG, Thomson GJL, Shaw JW.
    Endothelial cell seeding versus non-seeded ePTFE in patients with
    severe peripheral vascular disease. Preliminary results.
    In: Endothelialization of vascular grafts. Zilla P, Fasol R,
    Deutsch M (eds). 1987 Karger, Basel. pp245-248.

Wang Z, Du W, Li G, Li-qun P, Sharefkin JB.
    Rapid cellular luminal coverage of Dacron inferior vena cana
    prostheses in dogs by immediate seeding of autogenous endothelial
    cells derived from omental tissue: results of preliminary trial.
    J Vasc Surg 1990; 12: 168-179.

Watkins MT, Sharefkin JB, Zajtchuk R, Maciag TM, D'Amore PA, Ryan US,
    Van Wart H, Rich NM.
    Adult human saphenous endothelial cells: assessment of their

reproductive capacity for use in endothelial seeding of vascular
prostheses.
J Surg Res 1984; 36: 588-596.

Weksler BB, Ley CW, Jaffe EA.
Stimulation of endothelial cell prostacyclin production by
thrombin, trypsin and Ionophore A23187.
J Clin Invest 1978; 62: 923-930.

Wholey MH.
Advances in balloon technology and reperfusion devices for
peripheral circulation.
Am J Cardiol 1988; 61: 87G-95G.

Widmer LK, Biland L, DaSilva A.
Risk profile and occlusive peripheral artery disease (OPAD).
In: Proceedings of 13th International Congress of Angiology. 1985.

Williams SK, Jarrell BE, Friend L, Radomski JS, Carabasi A, Koolpe E,
Mueller SN, Thornton SC, Marinucci T, Levine E.
Adult human endothelial cell compatability with prosthetic graft
material.
J Surg Res 1985; 38: 618-629.

Williams SK, Jarrell BE, Rose DG, Pontell J, Kapelan BA, Park PK,
Carter TL.
Human microvessel endothelial cell isolation and vascular graft
sodding in the operating room.
Annals Vasc Surg 1989; 3(2); 146-152.

Woolf N, Welsen-Holt NJ.
Cigarette smoking and athersclerosis
in: Smoking and Arterial Disease. Greenhalgh RM ed. Pitman Medical.
1981. London.

Woolfe JHM, McPherson GAD.
    The failing femorodistal graft.
    Eur J Vasc Surg 1987; !: 295-296.

Wray R, DePalma RG, Hubay CH.
    Late occlusion of aortofemoral bypass grafts: Influence of
    cigarette smoking.
    Surgery 1971; 70: 969-

Zamora J1, Navarro LT, Ives CL, Weilbaecher DG, Gao ZR, Noon GP.
    Seeding of arteriovenous prostheses with homologous endothelium: a
    preliminary report.
    J Vasc Surg 1986; 3: 860-866.

Zeitler E.
    Dynamische Angioplastie und Atherektomie.
    In: Gunther RW, Thelen M, Interventionelle Radiologie. Stuttgart:
    G Thieme 1988: 117-124.

Zilla P, Diedler S, Fasol R, Sharefkin JB.
    Reduced reproductive capacity of freshly harvested endothelial
    cells in smokers: a possible shortcoming in the success of seeding?
    J Vasc Surg 1989; 10: 143-148.

Zilla P, Fasol R, Deutsch M, Fischlein T, Minar E, Hammerle A, Krupicka
    O, Kadletz M.
    Endothelial cell seeding of polytetrafluoroethylene vascular grafts
    in humans: a preliminary report.
    J Vasc Surg 1987; 6: 535-541.

Zilla P, Fasol R, Dudeck U, Siedler s, Preiss P, Fischlein T,
    Muller-Glauser w, Baitella G, Sanan D, Odell J, Reichart B.
    In situ cannulation, microgrid follow-up and low-density plating
    provide first passage endothelial cell masscultures for in vitro
    lining.
    J Vasc Surg 1990; 12: 180-189.