AN INVESTIGATION INTO HUMAN VEIN
GRAFT INTIMAL HYPERPLASIA

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BSc

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for the degree of Doctor of Philosophy

from

The Department of Surgery, University of Leicester
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STATEMENT OF ORIGINALITY

I certify that the work described in this thesis is my own independent work except where acknowledged, and was performed in the Department of Surgery, University of Leicester, between October 1991 and July 1994.

Karen Elizabeth Porter

January 1995
Dedicated to my family

"I have been trying to point out that in our lives chance may have an astonishing influence and, if I may offer advice to the young laboratory worker, it would be this - never to neglect an extraordinary appearance or happening. It may be - usually is, in fact - a false alarm that leads to nothing, but it may on the other hand be the clue provided by fate to lead you to some important advance."

Alexander Fleming (1881 - 1955)

Lecture at Harvard
SYNOPSIS

The most common cause of vein bypass graft failure in the postoperative period of 1 month to 1 year is stenosis, which occurs in up to 30% of arterial reconstructions. This thesis investigates the intimal hyperplasia underlying such lesions using a laboratory model.

The first chapter reviews the current literature regarding vein graft stenoses and is followed in Chapter 2 by a brief introduction to tissue and organ culture and their usefulness as investigative research tools.

Before embarking on a study of a pathological condition, Chapter 3 studies the structure of the "normal" long saphenous vein in patients undergoing arterial surgery. A degree of intimal thickening was identified in the majority of the veins in this population, the possible causes of which are discussed.

The fourth chapter describes and validates an organ culture of human saphenous vein to study the vascular biology of vein graft intimal hyperplasia. Since smooth muscle cell proliferation is a pivotal event in the development of such lesions, a reliable and reproducible method of assessing proliferation was required and is described in Chapter 5.

Chapter 6 investigates the effect of endothelial denudation on the development of intimal thickening, and an organ coculture study described in Chapter 7 positively identifies a soluble paracrine mediator produced by the endothelium which can promote intimal hyperplasia.

The following chapters utilise variations of the coculture method to further define the precise role played by the endothelium. Chapter 8 demonstrates that isolated, cultured endothelial cells do not promote intimal thickening in denuded veins, suggesting that the normal anatomical location of endothelial cells overlying smooth muscle cells in the vein wall may be important. Chapter 9 therefore describes the development of a method to
reseed endothelial cells onto denuded vein segments in order to observe whether the development of intimal hyperplasia can be restored. This proved not to be the case, possibly because the process of culturing had phenotypically altered the endothelial cells, thereby rendering them incapable of producing their paracrine factor. However, a number of other hypotheses and methods by which they could be investigated, are also discussed.

The main drawback of human saphenous vein organ culture is that it is a no-flow system. There is considerable evidence in the literature to show that haemodynamics modify the normal and pathological structure and function of blood vessels. Chapter 10 therefore describes the development of an in vitro flow model of saphenous vein graft intimal hyperplasia in an attempt to model the in vivo situation more closely.

The final chapter summarises the data presented in this thesis, draws conclusions, and examines prospects for future research in this field.
ACKNOWLEDGEMENTS

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I am grateful for the help of the staff in the Electron Microscope Unit, particularly Mr. George McTurk for the Scanning Electron Microscopy.

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The funding for this work was provided by The British Heart Foundation, to whom I am most grateful.

Finally, and most importantly, I wish to express my gratitude to my husband, Terry, whose patience and support was unfailing, even when events did not always go to plan.
LIST OF ABBREVIATIONS

ACE  Angiotensin converting enzyme
ADP  Adenosine diphosphate
Ang II Angiotensin II
ATP  Adenosine triphosphate
bFGF Basic fibroblast growth factor
BrdU Bromodeoxyuridine
°C  degrees Celsius
CABG Coronary artery bypass graft
CaCl₂ Calcium chloride
cpm  counts per minute
DNA Deoxyribonucleic acid
EC  Endothelial cell(s)
ECGF Endothelial cell growth factor
EDRF Endothelium-derived growth factor
EGF Epidermal growth factor
EVG Elastic Van Gieson
FCS Foetal calf serum
g grams
cGMP cyclic guanosine monophosphate
HSVEC Human saphenous vein endothelial cells
IGF-1 Insulin-like growth factor-1
IH Intimal hyperplasia
KH₂PO₄ Potassium dihydrogen orthophosphate
KV Kilovolts
l litres
LMH Longitudinal muscle hypertrophy
LMWH Low molecular weight heparin
M Molar
mg  milligrams
MgSO₄  Magnesium sulphate
ml  millilitre(s)
mM  millimolar
NaCl  Sodium chloride
NaHCO₃  Sodium hydrogen carbonate
PBS  Phosphate-buffered saline
PCNA  Proliferating Cell Nuclear Antigen
PDGF  Platelet-derived growth factor
PTFE  Polytetrafluoroethylene
RPMI  Roswell Park Memorial Institute
RNA  Ribonucleic acid
SD  Standard Deviation
SEM  Scanning Electron Microscopy
SMA  Smooth muscle actin
SMC  Smooth muscle cell(s)
TEM  Transmission Electron Microscopy
TGF-β  Transforming growth factor-beta
TNF-α  Tissue necrosis factor-alpha
T/S  Transverse section
µg  micrograms
µl  microlitre(s)
µm  micrometres (microns)
µM  micromolar
PUBLICATIONS ARISING FROM THE WORK IN THIS THESIS TO DATE

Articles


Published Abstracts


**PRESENTATIONS**


Poster presentation at the Physiological Society Meeting, Queen Mary and Westfield College, London, 17th-18th December, 1992.


Presented at the Biological Engineering Society, Vascular Disease - Modality, measurement and management. Ninewells Hospital, Dundee, Tayside, 15th-16th April, 1993.


Presented at the Midland Vascular Surgical Society Meeting, Royal Hallamshire Hospital, Sheffield. 18th March, 1994.


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INTRODUCTION TO THE STUDY
Intimal hyperplasia is a smooth muscle cell phenotypic change and proliferative
disorder initiated by vessel injury which occurs as a consequence of a variety of vascular
procedures. However, these procedures have been implicated as the cause of clinically
significant stenoses or restenosis in arterial reconstructions.

Most blood vessels diseased by atherosclerosis can be reconstructed as long as there
is reasonable inflow and outflow distal to the stenotic or occluded segment. All forms of
reconstruction, whether they directly attack the occluding lesion (endarterectomy,
angioplasty), or bypass it (vein or prosthetic bypass), inevitably cause injury and a wound-
healing response. Although long-term function depends on the reparative process after
surgery, this wound healing response may also be the cause of lumenal narrowing and the
ultimate failure of the reconstruction.

Lumenal narrowing as a result of excessive intimal hyperplasia is a particular
problem after carotid endarterectomy, aorto - coronary bypass, superficial femoral
angioplasty or atherectomy, coronary artery balloon angioplasty, arterial bypass using
prosthetic materials and femoro - popliteal bypasses using autogenous vein. This latter
problem is the subject of investigation in this thesis.

It is very difficult to obtain more than a sketchy outline of the arterial injury response
in man since graft excision is rarely employed except when the the new intimal lesions are
very large and patency - threatening. Most of our current knowledge has been derived from
cell culture studies and animal models of intimal hyperplasia. Although cell culture enables
us to study the cellular mechanisms underlying intimal hyperplasia, the results need to be
interpreted with caution, and unfortunately the results of studies in animals, particularly
small animals, have not been reproducible in man. Consequently, their relevance to the
human situation has been questioned.

The long saphenous vein still remains the conduit of choice for infrainguinal bypass
procedures, although as many as 36% of these grafts may develop stenoses in the first
postoperative year due to intimal hyperplasia. Currently available treatments depend upon the use of close graft surveillance and interventional surgery to treat these lesions and prevent occlusion of the graft. Clearly there is a need to obtain a more coherent picture of the events leading up to excessive smooth muscle cell proliferation in saphenous vein grafts, such that future treatments may be able to focus upon prevention rather than treatment of established lesions.

Organ culture of human saphenous vein to study intimal hyperplasia has recently been described which may prove to be a valuable experimental tool in the future in probing the mechanisms involved in the aetiology of vein graft stenoses. The potential advantages that this model offers are that the structural integrity of the vein wall can be maintained and interactions between the endothelium and the smooth muscle cells may be studied.

The major drawback of organ culture is that it represents the response of a vessel to culture in the absence of flow. There is now considerable evidence in the current literature that intimal hyperplasia in vein grafts is influenced by haemodynamic factors, although most of the work has been performed in animal models or isolated cell cultures. Therefore a system in which vein culture could be performed under flow conditions may be beneficial.

This thesis further develops and characterises an in vitro model of intimal hyperplasia in human saphenous vein, and describes the development of a novel “flow rig” whereby vein may be cultured under variable flow conditions.
CHAPTER 1

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INTIMAL HYPERPLASIA AND VEIN GRAFT STENOSES:
A REVIEW

1.1 Introduction.
Intimal hyperplasia (IH) poses a major problem in many forms of medicine and surgery. The essential feature of IH is excessive smooth muscle cell (SMC) proliferation and extracellular matrix production in the intima of the blood vessel wall. This has been observed following vein grafting, at prosthetic graft anastomoses, after balloon angioplasty, organ transplantation, angiography, catheterisation of arteries and atherectomy, and a recent study suggests that it may be the single most important factor affecting long-term patency. In all of these diverse procedures, endothelial cell loss is a prominent and inevitable feature, and any form of endothelial trauma and/or smooth muscle cell injury will initiate smooth muscle cell proliferation and subsequently a thickened neointima as part of the healing process. The sequence of wound-healing responses seen after vessel injury induced by vascular repair procedures can cause clinically significant stenoses with varying frequency for different procedures.

Arterial bypass surgery becomes a necessary procedure for patients in whom advancing atherosclerosis is posing a threat to the coronary or peripheral circulation. Atherosclerotic occlusive disease of the coronary arteries produces symptoms of angina pectoris and acute myocardial infarction. The standard and most common surgical procedure for myocardial revascularisation is the use of the autogenous saphenous vein to bypass the diseased artery. The earliest description of vein grafting was in 1910 when Alexis Carrell successfully transplanted the vena cava into the abdominal aorta of dogs (Carrell 1910). In 1964, Favoloro pursued the vein graft technique for myocardial revascularisation and is credited with having established the coronary artery bypass graft (CABG) (Favoloro 1968). Today, the autologous saphenous vein is the most frequently used graft for aortocoronary bypass (Favoloro 1969; Angelini and Newby 1989) owing to its ready availability and ease of surgical removal.

Autogenous saphenous vein is also the favoured conduit used to reconstruct the femoro-popliteal segment of the lower limbs in patients presenting with critical limb
ischaemia. Vein grafts have been used for the relief of the effects of ischaemia in the lower limbs since the 1950’s, and the femoro-popliteal bypass graft (FPBG) is now widely used. The saphenous vein is generally used for both CABG and FPBG owing to its superior patency rates over prosthetic alternatives. Although autogenous vein provides patencies superior to any other arterial substitute, FPBG and CABG still carry a 30% 5 year failure rate due to the development of stenoses caused by IH. Carrell and Guthrie (1910) first described this wall thickening by noticing that “Within a few days after the operation the stitches placed in making the anastomosis became covered with a glistening substance similar in appearance to the normal endothelium.” It was suggested by Smith et al. (1983) that the substance seen at the anastomosis may be the normal response of injured endothelium. However, it is the abnormal, continued proliferation of SMC in response to endothelial cell injury that is the lesion that we refer to as intimal hyperplasia.

To consider IH as a purely mechanical obstruction is simplistic. When the vein wall has lost the integrity of its endothelial lining, at least transiently, and the SMC’s have undergone phenotypic modulation from a quiescent to a proliferative state, it is not surprising that many of the normal physiological functions of the vein wall are also disturbed because the endothelium performs so many functions (Section 1.5(v)).

1.2. History.

It is now more than forty years since autogenous saphenous vein was first used to bypass the femoropopliteal segment. In 1949, Kunlin introduced the end-to-side anastomotic technique for reversed vein grafts, which was later adopted by Linton and Darling (1962) with an 86% technical success rate. However, numerous reports soon appeared of stenoses occurring in these grafts, associated with a return of ischaemic symptoms or total graft failure (Estrup et al. 1961, Breslau and DeWeese 1965, DeWeese et al. 1966, McCabe et al. 1967, McNamara et al. 1967, DeWeese and Rob 1971, Downs and Morrow 1972). The first group to report a comprehensive study of these changes were Szilagyi and colleagues (1973). Using angiographic follow-up data from 260 grafts, they reported two major types of change: stenotic lesions in 55 grafts (21%), which occurred at a mean interval of 15 months after implantation; and the later development of
atherosclerosis in 20 grafts (8%) at a mean interval of 45 months. The stenotic lesions were further subdivided as follows: diffuse intimal thickening (38%), fibrotic stenosis (20%), fibrotic valve (27%) and suture stenosis (15%). Histology was performed only in a small number of cases, and therefore this classification was based largely on angiographic appearances. Anastomotic stenosis was generally considered to be "traumatic fibrotic stenosis" following clamp injury; mid-graft stenosis was interpreted as valvular, and stenoses occurring at the site of tied branches were thought to be caused by sutures. In a study of human reversed vein grafts (Whitney et al. 1976) 10 out of 11 stenoses were anatomically and histologically confirmed as valvular in origin. However, as angiographic follow-up experience increased, fewer and fewer stenotic lesions could be attributed with certainty to degenerative valves (Sladen and Gilmour 1981, Berkowitz et al. 1981). The precise aetiology of the remaining stenoses was unknown.

At about the same time, in situ vein grafting was becoming increasingly popular following the reports of increased patency rates compared with reversed vein grafting by Leather and colleagues (1979). It was also claimed that in situ bypass reduced the incidence of endothelial and vein wall injury because of reduced dissection, handling and distension (Connolly and Harris 1965, Leather et al. 1981, Cambria et al. 1985). In addition to improving the early patency rate, it was hoped that this would lead to a decreased incidence of the traumatic fibrotic stenoses described by Szilagyi and colleagues. However, this hope has not been fulfilled, illustrated by the fact that the incidence of stenoses in the two types of graft is similar (Table 1.1). It is now known that the in situ technique produces significant endothelial and smooth muscle cell injury (Sayers et al. 1991, Sayers et al. 1992), and that factors other than vein trauma at the time of harvesting may be involved in the aetiology of vein graft stenoses.
1.3. Clinical features.

Approximately 20% of all vein grafts develop a stenosis, (Table 1.1), and nearly 80% of these occur in the first post-operative year. Most of these lesions are short (< 2cm) and localised (Moody et al. 1992, Berkowitz et al. 1992, London et al. 1993), although the occurrence of diffuse lesions (2 - 6cm) has also occasionally been reported (Donaldson et al. 1992, Whittemore et al. 1991).

Recent advances in the ability to investigate vein grafts has aroused interest in the concept of graft failure originally suggested by Szilagyi in 1973. Although he referred to autogenous veins as “the most nearly ideal arterial substitutes” he was also aware of the importance of their histologic behaviour in determining long-term patency. By studying the natural history of venous implants he identified stenoses and emphasised their adverse effects on long-term patency.

The majority of haemodynamically significant stenoses are asymptomatic and not associated with ischaemic symptoms or decreased peripheral pulses (Grigg et al. 1988), thus emphasising the need for some form of graft surveillance to detect these changes before the graft occludes. Secondary patency rate after thrombectomy of an occluded graft is far inferior to the primary assisted patency rate that can be achieved if the stenosis is treated before graft failure.

Vein grafts develop both anastomotic and intragraft stenoses, with just over half of vein graft stenoses being true intragraft lesions and the remainder occurring at the anastomoses (Table 1.1). In contrast, in infrainguinal prosthetic grafts anastomotic intimal hyperplasia is the major cause of graft failure (Taylor et al. 1987) and intragraft stenoses are rarely seen.

Detailed studies of the factors associated with vein graft stenoses are few. A study by Sladen and Gilmour in 1981 found no association between stenoses and patient age, gender, presenting symptoms, hypertension, diabetes, smoking and outflow artery. Berkowitz and colleagues (1989) found a higher incidence of stenoses in more distal grafts, and that the most common site for a stenosis in a reversed vein graft was close to the proximal anastomosis (Berkowitz et al. 1992). In another study of in-situ grafts, stenoses were more common in the distal portion of the graft (Vary et al. 1993). However, these
<table>
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<td></td>
<td></td>
<td>Ana</td>
<td>Intr</td>
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<td>379</td>
<td>26 (7)</td>
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<td>521</td>
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<td>173</td>
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<td></td>
<td>Szilagyi et al. 1973</td>
<td>260</td>
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<td></td>
<td>Wengerer et al. 1991</td>
<td>63</td>
<td>3 (5)</td>
<td>8 (13)</td>
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<td>Moody et al. 1992</td>
<td>40</td>
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<td>MEAN (%)</td>
<td>7</td>
<td>11</td>
<td>19</td>
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<td>In situ</td>
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<td>4 (6)</td>
<td>6 (10)</td>
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<td>Moody et al. 1992</td>
<td>34</td>
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<td>48</td>
<td>0 (0)</td>
<td>7 (15)</td>
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<td>Bergamini et al. 1989</td>
<td>316*</td>
<td>20 (6)</td>
<td>34 (11)</td>
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<td>9 (11)</td>
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Values in parentheses are percentages. * Series of 361 grafts minus early failures, deaths and those lost to follow-up.
two sets of observations are not contradictory since it is the same distal section of the original vein that is developing the lesions. Two other studies have identified a correlation between vein diameter and long-term patency (Buxton et al. 1980, Sasajima and Kubo 1989), and therefore the natural taper of the long saphenous vein explains the observations of Berkowitz and colleagues that the more distal the procedure, the higher the incidence of stenoses.

1.4. Histopathology.

The morphological lesion underlying vein graft stenoses is intimal hyperplasia, defined as an abnormal accumulation of cells and extracellular matrix in the intima of the vessel wall (Haudenschild 1989). This appears to be a uniform response in the vasculature to a wide variety of injurious stimuli and is seen after angioplasty (Essed et al. 1983), endarterectomy (Barker and Cannon 1953), embolectomy (Chidi and Depalma 1978, Greenwood et al. 1984, Bowles et al. 1988), arterial catheterisation (Reidy 1989), vein grafting (Dilley et al. 1988, Spray and Roberts 1976, Fuchs et al. 1988, Jones et al. 1973) and at anastomoses between prosthetic grafts and the native vessel (Sottiurai et al. 1983). Although the lesions at these various sites are morphologically similar, suggesting a common aetiology, this review focuses on the changes occurring in vein grafts because veins possess a number of unique properties that could influence the development of IH (Cox et al. 1991).

It is an important observation that most saphenous vein grafts are not "normal" to begin with. The classical textbook description of the structure of the saphenous vein (Cormack 1987) is of a thin intima comprising the endothelium and supporting connective tissue, a muscular media separated from the intima by a poorly defined internal elastic lamina, and a thick adventitia of loose connective tissue. However, in patients undergoing arterial bypass these features are rarely seen and the majority have some degree of pre-existing intimal thickening and medial fibrosis (Marin et al. 1993a, Dilley et al. 1988, Spray and Roberts 1976, Cheanvechai 1975, Vogt 1978). Although the term phlebosclerosis has been used to describe these changes, it is not entirely a fibrotic process (Leu et al. 1991). The intimal thickening is often highly cellular, containing smooth muscle
cells of both contractile and secretory phenotypes (Milroy et al. 1989, Marin et al. 1991). Animal studies suggest that during the first few weeks after implantation, the changes seen in vein grafts are largely inflammatory and reparative in nature, the extent of such changes depending on the degree of injury sustained during harvesting and implantation. There are areas of endothelial denudation, with deposition of platelets and fibrin and an accumulation of neutrophils and macrophages in the vein wall (Dilley et al. 1988). Medial SMC necrosis is also observed (Brody et al. 1972). The re-endothelialization process is usually very efficient (LoGerfo et al. 1983, Cambria et al. 1985), but persistent gaps between cells have been observed, suggesting that a fully confluent endothelial layer may not always be achieved (Fonkalsrud et al. 1978). The time taken for re-endothelialization is variable, depending on the extent of the initial loss (Dilley et al. 1988). Short defects (1cm) can recover within a week and longer lesions (5cm) in six weeks. Unless there has been severe endothelial denudation, the source of regenerated cells is from adjacent areas of the vein wall. Disrupted vasa vasora are re-established within a week (Wyatt et al. 1964, McGeachie et al. 1981), but re-innervation of the vein wall takes up to three weeks (Meagher et al. 1984).

The mature vein graft develops a thickened intima, whereas the media becomes thinned (Dilley et al. 1988, Spray and Roberts 1976, Cox et al. 1991). This process has been incorrectly referred to as "arterialization", but the thickened intima and thin, non-compliant media of a vein graft bear little resemblance to normal arterial structure. A graft stenosis represents a focal area of excessive IH on top of the background IH seen along the graft. The lesions consist of SMC's contained within an abundant stroma of acid mucopolysaccharide matrix (Cox et al. 1991). As the lesion matures, increasing quantities of elastin and collagen are also observed in the extracellular matrix (Forrester et al. 1991). Electron microscopic evidence has shown that a significant proportion of intimal SMC's undergo a phenotypic change from contractile to secretory phenotype, with reduced numbers of actin filaments and more rough endoplasmic reticulum and Golgi apparatus (Sottiurai et al. 1983, Dilley et al. 1989). These ultrastructural features are similar to those seen in fibroblasts, which were once thought to be a major component of the intimal hyperplastic lesion. This subsequently led to some confusion in nomenclature, with
stenoses being referred to as “fibrotic” lesions. Although extracellular matrix and collagen are important components of the lesion, this term fails to portray the proliferative nature of the problem and the central role of the smooth muscle cell. Fibrosis is a more prominent feature in the media of vein grafts (Cox et al. 1991) following necrosis of SMC’s. The morphological appearances of an anastomotic stenosis is very similar to that of an intragraft stenosis, with IH occurring at the suture line, in the heel and toe of the graft, and in the floor of the recipient artery (Sottiurai et al. 1983).

1.5. Aetiology.

Since William Harvey’s original description of the circulation in which the vasculature was conceived as a passive conduit transporting blood to and from vital organs (Harvey 1628), the concept of vascular biology has evolved dramatically. It is now recognised that the vasculature is a complex organ which can synthesise local mediators that promote structural or functional responses. Vascular cells produce vasoconstrictors as well as vasodilators, procoagulants and anticoagulants, and growth stimulators as well as inhibitors. Any disturbances in this delicate balance undoubtedly play a role in the development of vascular pathology.

The cellular and molecular mechanisms underlying IH involve the interplay of numerous factors including growth-stimulating and growth-inhibiting factors and the haemodynamic action of the blood flow across the lumenal surfaces of the vessel. In vivo investigations into the aetiology of IH demand the use of animal models. Unfortunately the results of such experiments, particularly in small animals, have not been reproducible in humans, therefore their relevance to the human situation is unclear (Ferrell et al. 1992). Cell culture techniques have been useful in investigating the cellular mechanisms involved in IH but again the results need to be interpreted with caution. The use of an organ culture of human saphenous vein was first described in 1990 (Soyombo et al. 1990, Angelini et al. 1991), and this model may prove to be a useful experimental tool as an in vitro model of IH in human saphenous vein grafts. The further development of this model for evaluating IH should increase our elucidation of the mechanisms underlying this intimal lesion.

Information on the sequence of events producing IH has been gleaned primarily from
balloon - injured animal artery studies, and extrapolated to the human vein graft situation (Dilley et al. 1988, Chervu and Moore 1990). Despite the limitations of these experimental techniques, it is still possible to draw a coherent picture of the main precipitating elements of IH.

The vein used as a bypass conduit has inevitably been disturbed by the dissection procedure, handling, distension and cannulation. It is then exposed to pulsatile high pressure and high flow rates, therefore injury and haemodynamic stress have been prime aetiological suspects since stenoses were first described. There is now strong evidence that flow abnormalities are the main causative factor of anastomotic stenoses. A number of other possible factors may be involved, and of these, the pre-existing venous abnormalities described above have received most attention.

(i) Pre-existing venous disease

The classical textbook description of the structure of the long saphenous vein is of a thin intima of endothelial cells overlying a muscular media, separated by a poorly-defined internal elastic lamina, and a thick adventitia of loose connective tissue (Cormack 1987). In most patients undergoing arterial bypass procedures, these features are rare and the majority of veins have some degree of pre-existing intimal thickening (Dilley et al. 1988, Spray and Roberts 1976, Cheanvechai 1975). The intimal thickening is frequently very cellular, containing SMC’s of both the contractile and secretory phenotype (Milroy et al. 1989, Marin et al. 1991). The morphological similarity between these changes and in the IH seen in vein grafts has inevitably led to speculation that they may be related (Marin et al. 1991). Although it is difficult to link the two, the results of two recent studies provide indirect evidence of an association. Firstly, it has been demonstrated by Panetta and colleagues that veins with thickened walls and areas of calcification have inferior patency rates at 30 months (32% vs 73%) when used as a bypass graft (Panetta et al. 1992). Although such gross macroscopic changes would be expected to significantly influence patency, microscopic changes in the vein wall remain a separate issue. In another study, Davies and colleagues have suggested that the microscopic changes of intimal thickening reduce the compliance of the vein wall, an observation also observed by Marin (1993a),
and that these veins with a low compliance were more likely to develop stenoses (Davies et al. 1992).

(ii) Injury

Animal models have been used extensively in examining the role of injury in the pathogenesis of IH; angioplasty of the carotid, aorta or iliac arteries being used as the injurious stimulus. This technique effectively de-endothelializes the artery and induces medial SMC injury (Clowes et al. 1983, Groves et al. 1979, Clowes and Schwartz 1985, Manderson et al. 1989). Platelets are then deposited on the exposed sub-endothelium (Schwartz et al. 1975) and up to 25% of SMCs undergo necrosis (Painter 1991). Of the surviving SMC's, 20-30% start synthesizing DNA (Clowes and Schwartz 1985), undergo phenotypic modulation (Manderson et al. 1989), and migrate into the intima. These SMC's express mRNA for tissue plasminogen activator (Clowes et al. 1990) which suggests that the migratory process may be facilitated by plasmin which is able to degrade a number of matrix molecules. In areas where endothelial regeneration is rapid, intimal SMC proliferation is limited and returns to basal levels within 8 weeks (Clowes et al. 1983). However, intimal thickening may still progress for up to 12 weeks following injury owing to the secretion of extracellular matrix molecules by these cells (Clowes et al. 1983).

Paradoxically, even though delayed re-endothelialization leads to a higher rate of SMC proliferation, this does not necessarily produce more IH. If the endothelium is removed by a fine wire catheter, thus avoiding injury to the medial SMCs, intimal proliferation does not occur (Reidy and Silver 1984). These findings suggest that in the response to injury, the three main cellular participants in order of importance are the SMC, followed by the endothelial cell and lastly the platelet.

(iii) Vascular smooth muscle cells.

In arteries, it has been demonstrated that the degree of SMC proliferation is related to the extent of the initial trauma, suggesting that direct SMC injury is the major determinant of IH (Bjorkerud and Bondjers 1971). An integral part of the response to injury is the phenotypic modulation of these cells from a contractile to a secretory state.
(Clowes and Schwartz 1985). Ultimately, the main bulk of the restenotic lesion is produced by the secretion of glycosaminoglycans, elastin and collagen (Fagin and Forrester 1992). In a canine vein graft model a recent demonstration by Quist and LoGerfo (1992) showed that veins harvested by an "injury" technique resulted in 100% of SMC's undergoing phenotypic modulation to the secretory phenotype, whereas SMC's in optimally prepared veins did not. SMC's are able to release their own mitogens which stimulate proliferation in an autocrine manner. These include platelet-derived growth factor (PDGF) (Winkles and Gay 1991), basic fibroblast growth factor (bFGF) (Lindner et al. 1991), insulin-like growth factor (IGF) (Gianella-Neto 1990) and thrombospondin (Raugi et al. 1990). The stimulus for these changes may also be due in part to platelet and endothelial cell mitogens which will now be discussed further.

(iv) Platelets

The suggestion that platelets play a major part in the development of IH is supported by the demonstration that inducing thrombocytopenia in rabbits dramatically reduces IH following angioplasty (Friedman et al. 1976). Platelet granules release PDGF, epidermal growth factor and thrombospondin which act as direct mitogens or facilitate mitogenesis in SMC's (Assoin et al. 1984, Majack et al. 1988). A more recent study in rats made thrombocytopenic for a short time by treatment with a polyclonal antibody, showed no reduction in SMC proliferation following carotid artery angioplasty (Fingerle et al. 1989). However, a reduction in intimal thickness was seen in the first 7 days, this was attributed to a possible reduction in SMC migration from the media to the intima. Migration may not be such an important step in vein graft IH as SMC's are already present in the intima of the saphenous vein before grafting, and therefore platelets may play only a minor role in its aetiology. This view is supported by the observation that antiplatelet agents fail to influence the development of IH in experimental vein grafts or influence patency rates in clinical studies (Chervu and Moore 1990, Landymore and MacAulay 1991, McCollum 1991). Most of the growth factors released by the platelet can also be produced by the endothelium and the SMC (Kaufman et al. 1992, Walker et al. 1986, Winkles and Gay 1991, DiCorleto and Bowen-Pope 1983, Raugi et al. 1990, Jaffe 1987), demonstrating that
these cells are capable of taking over the role of the platelet after the initial brief period of platelet deposition at the site of injury (Painter 1991).

(v) The endothelium

In arteries, localised injury to the endothelium alone does not cause IH (Reidy and Silver 1984), but it is not known whether this is the case in vein grafts. During the grafting procedure injury to the venous endothelium is inevitable and often substantial. The process of re-endothelialization then takes place under arterial conditions of increased flow and pressure to which the venous endothelium is not adapted. Defects may then persist in the endothelium, which may be morphologically intact, but functionally impaired (Jaffe 1987, Henderson et al. 1986, Cross et al. 1988). Under these circumstances the endothelium may act as a promoter of IH rather than an inhibitor (Reidy 1985). Under normal homeostatic conditions a confluent endothelial layer inhibits the development of IH by creating a physical barrier between serum mitogens and the vein wall, and by the production of inhibitors of SMC growth such as heparin (Clowes and Karnowsky 1977, Castellot et al. 1981, Castellot et al. 1987), and endothelium - derived relaxing factor (EDRF) (Garg and Hassid 1989). On the other hand, injured endothelium can produce a variety of SMC mitogens such as PDGF (DiCorleto and Bowen Pope 1983) and bFGF (Vlodavsky et al. 1987), and no longer fulfills its function as a physical barrier. What then is the nature of these dysfunctional changes in the endothelium? The endothelium plays numerous physiological roles (Gimbrone 1976) including (1) provision of a non-thrombogenic surface; (2) a permeability barrier through which there is exchange and active transport into the vessel wall; (3) maintenance of vascular tone by the release of small molecules such as EDRF, prostacyclin (PGI2), and endothelin (ET), that modulate vasodilation or vasoconstriction, respectively; (4) formation and secretion of growth-regulatory molecules and cytokines; (5) maintenance of the basement membrane collagen and proteoglycans upon which they rest; and (6) provision of a non-adherent surface for leucocytes. Changes in any one or more of these properties may represent the earliest manifestations of endothelial dysfunction. Clearly the role of altered endothelial function in IH demands further investigation (Reidy 1985).
(vi) Inflammatory cells.

The presence of macrophages, neutrophils and lymphocytes in the vein graft wall has led to suggestions that they may influence vessel wall healing and hence IH. This view is supported by the demonstration of a monocyte - derived growth factor similar to PDGF (Shimokado et al. 1988) and the mitogenic action of interleukin - 1 (IL1) for SMC's (Libby et al. 1988). At the present time, the relative importance of these mitogens is unclear, and the results of immunosuppressive regimens to reduce IH have been variable (Brody et al. 1977, Colburn et al. 1992). The absence of functional lymphocytes in athymic nude rats had little effect on the development of IH following angioplasty (Ferns et al. 1991).

(vii) Growth factors.

Our present understanding of the relative importance and specific roles of the growth factors that influence SMC behaviour and the development of IH is incomplete. It would appear that there is a certain amount of duplication in the system, with complex interactions occurring between the various growth factors. It is probable that none of these factors works alone in this process. Through a network of cellular interactions, the release of one molecule can lead to expression of a second molecule in a target cell that can then either stimulate neighbouring cells in a paracrine way, or itself in an autocrine way.

Platelet - derived growth factor

PDGF is composed of two polypeptide chains, A and B, the latter being encoded by the c-sis proto-oncogene (Cerco 1991). It is released by platelets but can also be produced by the endothelium (DiCorleto and Bowen-Pope 1983) and SMC's (Winkles and Gay 1991). The growth factor can therefore exist in three different isoforms: AA, AB and BB. Platelets release the AB and BB isoforms, and as previously mentioned, these principally affect SMC migration (Jawien 1992). Following injury, the SMC produces the AA isoform which stimulates its own proliferation in an autocrine fashion (Fagin and Forrester 1992). A study in nude rats demonstrated a 41% reduction in neointimal thickness by administration of an antibody to PDGF following carotid artery angioplasty (Ferns et al. 1991). PDGF acts as a competence factor by enabling cells to move from $G_0$
to G1 of the cell cycle (Cercek et al. 1991). A further progression factor is then required for SMC's to undergo DNA synthesis, and there is evidence to suggest that IGF and thrombospondin may perform this role. IGF is a polypeptide, also known as somatomedin C, and thrombospondin is a glycoprotein component of the extracellular matrix. PDGF induces mRNA expression for both IGF and thrombospondin in vascular SMC's (Gianella-Neto et al. 1990, Majack et al. 1987), and monoclonal antibodies to these compounds inhibit SMC growth (Jaffe 1987, Majack et al. 1987). Interactions between growth factors are further illustrated by the observation that PDGF is also able to act via bFGF which is produced by SMC's in response to PDGF stimulation (Sato et al. 1991). Additionally, induction of the SMC PDGF-A gene initiates the mitogenic action of IL-1 (Raines et al. 1989).

Basic fibroblast growth factor (bFGF)

It was proposed by Lindner (1991) that bFGF released by injured or dying SMC's was able to stimulate surrounding cells to migrate and proliferate. This theory was based on the observation that the degree of direct SMC injury dictated the extent of subsequent IH. Infusion of bFGF into rats caused increased intimal SMC proliferation in injured carotid arteries whereas undisturbed vessels were unaffected. An antibody to bFGF was then used to reduce SMC proliferation after angioplasty in the rat carotid artery (Lindner and Reidy 1991). Additionally, bFGF has been shown to be a potent stimulator of endothelial cell regrowth (Lindner et al. 1990).

Transforming growth factor - β (TGF-β).

This is a polypeptide molecule which is produced by platelets, the endothelium and SMC's at the site of injury, and its most important function is that of promoting extracellular matrix formation (Cercek et al. 1991). When SMC's are exposed to TGF-β they synthesize proteoglycans, a major component of the early lesion of IH (Chen et al. 1987). TGF-β may also play a role in SMC proliferation by interaction with other growth factors. At low concentrations, TGF-β promotes both SMC proliferation and migration by an interaction with PDGF, but at higher concentrations its action is inhibitory via a direct
response (Battegay et al. 1990, Koyama et al. 1990). When SMC's in culture were exposed to a mixture of growth factors, the net effect of TGF-β on DNA synthesis was inhibitory except at low concentrations (Hwang et al. 1992).

Based on the above observations, one can hypothesize that following injury, the early burst of SMC proliferative and migratory activity may be caused by PDGF from platelets, the endothelium and SMCs facilitated by an initial low concentration of TGF-β. These early events may be influenced by inflammatory cells via IL-1 induction of PDGF. The full response to PDGF would require the action of IGF and thrombospondin produced from proliferating SMCs. The b-FGF released by dying SMCs or injured endothelium may provide an additional stimulus to these events. At a later stage, increased concentrations of TGF-β may reduce PDGF-mediated proliferation in favour of ECM secretion.

Insulin-like growth factor-1 (IGF-1)

IGF-1 circulates in plasma and is found in high concentrations within the platelets. Its secretion has now been demonstrated from a variety of cell types including endothelial cells, vascular smooth muscle cells and macrophages. It has insulin-like metabolic effects as well as regulating growth and differentiation of a wide variety of non-vascular and vascular cells (Clemmons 1989, Rechler and Nissley 1990). Several animal and cell culture studies have addressed the localisation of IGF-1 and mRNA in the intact vascular wall and imply that IGF-1 secretion may be induced in response to injury in both endothelial and vascular smooth muscle cells (Hansson et al. 1989, Khorsandi et al. 1990). The mitogenic effect of IGF-1 alone on smooth muscle cells in vitro is weak compared to PDGF and bFGF (Clemmons 1985a, Clemmons 1985b). It appears that IGF-1 acts primarily as a "progression factor" which allows a mitogenic response to occur after stimulation by a "competence factor" such as PDGF or bFGF.

Specific IGF-1 receptors are present in cultured rat and porcine aortic SMCs (Bornfeldt et al. 1991, King et al. 1985) and cultured bovine endothelial cells (Bar and Boes 1984). Furthermore, expression of IGF-1 receptor mRNA is increased in proliferating vascular SMCs after injury (Bornfeldt et al. 1992). Although there is now a
wealth of evidence that IGF-1 may play a role as an autocrine/paracrine progression factor for vascular SMC proliferation in vascular pathologies, a series of studies in animal models using specific neutralising antibodies would potentially be useful in elucidating its exact role.

**Epidermal growth factor (EGF)**

EGF stimulates the proliferation of vascular SMCs and endothelial cells from several mammalian species including man (Gospodarowicz 1981, Grosenbaugh 1988). As with IGF-1 there is evidence that EGF acts primarily as a progression factor and hence acts synergistically with other growth factors such as PDGF or thrombospondin (Grosenbaugh et al. 1988, Clemmons 1984, Majack et al. 1986). Specific receptors for EGF have been identified on the surface of many cultured cell types including endothelial cells and vascular SMCs (Grosenbaugh et al. 1988). Despite recognition of EGF's proliferative effects on vascular SMCs in vitro, its potential role in pathological states such as in vein graft stenoses in vivo requires further investigation.

**Vasoactive substances**

In addition to the classic peptide growth factors already described, it has become increasingly clear that vasoactive substances are important mediators of long-term changes in vascular structure. Angiotensin II (ang II), serotonin, endothelin and other vasoconstrictors can all stimulate vascular SMC growth (Dzau and Gibbons 1991, Gibbons et al. 1992, Dubin et al. 1989, Araki et al. 1990, Nemecek et al. 1986). Cell culture studies have shown that ang II induced SMC growth is mediated by the induction of autocrine PDGF AA, b-FGF and TGF β (Itoh et al. 1991). In vivo, the infusion of ang II has been shown to potentiate intimal lesion formation (Daemen et al. 1991). Indeed, blockade of local generation of ang II with ACE inhibition or ang II receptor antagonists prevent intimal proliferation in rodent models of balloon injury (Dzau et al. 1991, Powell et al. 1989). However, a recent study on the effect of ACE inhibition on the development of restenosis after angioplasty in humans has failed to confirm animal model studies (Serruys and Hermans 1992). In a recent study in this laboratory using an organ culture of human
saphenous vein to model vein graft IH, incubation with the ang II receptor antagonist Losartan resulted in a significant reduction in neointimal thickening (33%), paralleled by a similar reduction in proliferation index (Varty et al. 1993).

Endothelial cell-derived endothelin may also contribute to IH in vein grafts. A potent vasoconstrictor both in vivo and in vitro (Luscher 1991), endothelin is also a SMC mitogen synergistic with PDGF and appears to interact with specific cell surface receptors present on vascular smooth muscle cells (Hirata et al. 1988, Arai et al. 1990). A study by Sharefkin et al. (1991) showed that a period of 24 hours of shear stress on endothelial cells in vitro reduced levels of mRNA for endothelin precursor. Under conditions of normal blood flow the shear stresses exerted on the endothelium keep an inhibitory influence on endothelin release, but under conditions of poor flow this inhibition is reduced, leading to a greater production of endothelin and subsequently more IH.

Serotonin is a well recognised direct vasoconstrictor of smooth muscle and a principal role of serotonin is in acute vasoconstriction during haemostasis. More recently, however, it has been shown that serotonin is a weak mitogen for cultured bovine and rabbit aortic smooth muscle cells on its own and acts synergistically with PDGF (Araki et al. 1990, Nemecek et al. 1986). Additionally, it has also been shown that the mitogenic response of rabbit vascular smooth muscle cells to foetal bovine serum can be markedly reduced by ketanserin, a serotonin receptor antagonist (Uehara et al. 1991, Araki et al. 1990).

In conclusion, the vasoconstrictor agents discussed above all have mitogenic or co-mitogenic actions on vascular smooth muscle cells in vivo and in vitro. Whether this reflects a direct mitogenic action or is mediated through induction of other growth regulators remains an uncertainty at the present time; likewise their role in the aetiology of vein graft stenoses has yet to be determined.

How do the "response to injury" theories of the aetiology of IH fit in with our present understanding of vein graft stenoses? Based on the above proposals, there is a need to propose a localised form of injury to explain the relatively localised nature of most stenoses. Most obvious is possibly a clamp or valvulotome injury at the site of valves or
tributaries (Leather et al. 1984). Although a very attractive theory, it appears to be erroneous.

More recently, a prospective study by Moody and colleagues (1992) found no association between the sites of valves, tributaries, clamps and venotomies and the sites of subsequent stenoses. A further problem is the similar incidence of stenoses in reversed and in situ grafts: one would expect two very different techniques to produce different patterns of injury, but this is not the case. In animal models of IH the significance of injury as the sole cause of graft stenoses is questionable. Two separate studies in animals demonstrated an increase in total wall thickness in injured veins (Quist and LoGerfo 1992, Ramos et al. 1976), but in two other studies no difference in wall thickness was found between distended (Angelini et al. 1992) or stripped (Storm et al. 1975) veins and paired controls implanted using an "optimal preparation" technique. It would therefore appear that despite a wealth of evidence that injury can lead to IH, other factors appear to be involved in the development of vein graft stenoses.

(viii) Haemodynamics

Considerable experimental evidence exists to support the theory that IH in vein grafts is influenced by haemodynamic factors. Dobrin and colleagues (1989) demonstrated an inverse correlation between graft flow and intimal thickness in canine vein grafts. During laminar flow in the blood vessels, the interaction between the blood and vessel wall occurs in the outermost layer, and the force existing between this "boundary layer" and the vessel wall is known as the shear stress. It has been shown that low shear stress, and particularly low variation in shear stress associated with low flow is responsible for the development of IH (Kamiya and Togawa 1980, Morinaga et al. 1985). The endothelium plays a vital role in the detection of these shear stresses and transforms them into vessel wall responses (Sumpio et al. 1987, Kohler et al. 1991). High shear stress, at the other extreme, can also cause injury to the endothelium (Fry 1968). It is therefore possible to propose that this injury might promote IH via some of the mechanisms already discussed. Exposure of the vessel to arterial pressures causes an increase in wall tension, and this has
also been suggested as a factor governing vein graft wall thickening, although this is largely as a result of medial thickening and not IH (Dobrin et al. 1989).

Two different patterns of intimal thickening have been reported at vein graft anastomoses (Bassiouny et al. 1992). A healing response occurs at the suture line leading to IH as a result of a compliance mismatch between the vessels, producing a deformation stress. This problem is most frequently reported when prosthetic conduits are used, since the compliance mismatch is much greater than in vein grafts (LoGerfo et al. 1979). In end-to-side anastomoses IH also develops in the heel and toe of the graft and in the floor of the recipient artery and these coincide with the sites of boundary layer separation caused by turbulent flow (LoGerfo et al. 1979, Crawshaw et al. 1979). A wide inlet angle and reduced distal outflow promote this turbulent flow and further enhance IH. Recent studies in an *in vitro* model suggest that the long term structural adaptation to changes in flow may be mediated by the induction of autocrine/paracrine growth factors such as PDGF and TGFβ (Hsieh et al. 1991, Ohno et al. 1992). Indeed, preliminary studies suggest that shear stress induces endothelial cell gene transcription of PDGF via novel *cis* and *trans* regulatory elements (Resnick et al. 1992). These studies therefore suggest that flow-induced changes in endothelial cell derived growth factors may modulate vascular structure and pathological lesion formation.

The haemodynamic factors described here adequately provide an explanation for the development of anastomotic but not intragraft stenoses. For a focal intragraft stenosis, one must propose on the above theory, a localised flow abnormality within the graft, producing boundary layer separation, possibly at the sites of valves or tributaries. Unfortunately there is little evidence to support such a theory. However, the background level of IH on which a stenosis develops may be determined by haemodynamic factors.

(ix) Other contributing factors.

Although it remains to be proven, in theory, lipids may be involved in the development of vein graft stenoses. Vein grafts accumulate lipid in their wall (Boerboom et al. 1985) and lipids are able to alter endothelial permeability and function (Ferns et al. 1992). However, in a primate vein graft model the induction of hypercholesterolaemia did
not potentiate IH (McCann et al. 1978). Raised lipoprotein levels have been associated with vein graft stenoses occurring 5 years after coronary artery bypass (Hoff et al. 1988), but at this time such a lesion is likely to be due to progressive atherosclerosis, and this is already known to be associated with raised lipid concentrations. As yet no-one has described an association between raised lipid levels and lower limb vein graft stenoses. Vein wall ischaemia has been suggested as an important stimulant of IH, and in a rat vein graft model the degree of IH was inversely related to the number of capillaries in the wall of the graft (McGeachie et al. 1981). Vessel wall ischaemia is most pronounced in the outer media, which relies on the vasa vasorum, and SMC necrosis occurs here. As discussed earlier, this may initiate the release of bFGF, a potent SMC mitogen.

(x) A multifactorial hypothesis.

Our present knowledge of the aetiology of vein graft stenoses is incomplete; it therefore warrants further study. However, it would appear that the aetiology is multifactorial and that there is no single stimulus. There is convincing evidence that for anastomotic stenoses, haemodynamic factors play a major role in promoting IH. In the case of intragraft stenoses, pre-existing intimal thickening may contribute to subsequent IH (Davies et al. 1992) and, although stenoses do occur at the site of valves, this is not as frequent as was once believed (Moody et al. 1992). Injury to the vein during harvesting and implantation is inevitable, and leads to a healing response involving platelets, the endothelium, SMC's, inflammatory cells and a host of growth factors. Additionally, wall ischaemia and SMC necrosis may provide additional mitogens. When the vein is implanted into an arterial environment to which it is not adapted, the endothelium may fail to reach confluence and lipids may accumulate in the graft. If a morphologically intact endothelium is restored this may, however, be functionally abnormal under conditions of arterial flow and pressure. Pulsatile shear stresses also act via the endothelium in the regulation of wall thickness. All these and other unknown factors may lead to areas in the vein wall where the healing response is excessive, resulting in vein graft stenoses.
1.6. Treatment and prevention.

Early detection and surgical intervention before failure forms the basis of the current management of vein graft stenoses. Some stenoses remain stable and do not progress to occlusion, therefore a selective policy is usually adopted (Moody et al. 1989). Haemodynamically significant (fall in ankle: brachial arterial pressure index > 0.2), symptomatic and severe stenoses (> 70%), together with those showing progression on serial surveillance scans all warrant intervention (Grigg et al. 1988, Idu et al. 1992). There is retrospective evidence that this strategy does lead to a significant improvement in patency rates (Sottiurai et al. 1983, Moody et al. 1990), although the benefits of such a policy have not been studied in a randomised trial of subsequent graft patency rates. The majority of short stenoses can adequately be treated by percutaneous angioplasty (Berkowitz et al. 1992, London et al. 1993), but for multiple lesions or longer stenoses (>2cm), better results are obtained with an interposition or jump graft (Thompson et al. 1989, Whittemore et al. 1991). In order to adopt strategies that will prevent vein graft stenosis, more information on their aetiology is required. Hopefully, as we increase our understanding of this subject, and we move towards the prevention rather than treatment of established lesions, the current debate on how to optimally treat graft stenoses will become irrelevant. Prevention is currently aimed at minimising injury to the vein at the time of surgery; present experience with pharmacological agents has been disappointing in the prevention of IH although several agents may prove to be of benefit in the future.

(i) Prevention of injury.

Most of the procedures employed in the preparation of the saphenous vein for use as a bypass conduit inevitably cause injury to the vessel wall. Dissection alone causing spasm of the vein is sufficient to produce endothelial cell loss (Baumann et al. 1981). For reversed grafts, distension under high, uncontrolled pressure is harmful, as is storage for prolonged periods (Bonchek 1980, Angelini et al. 1985). Distension with cold saline solution rather than warm blood is particularly harmful (LoGerfo et al. 1981). The valvulotome is the main cause of vein wall injury for in situ grafts (Sayers et al. 1991, Sayers et al. 1992). Although in theory injury may be minimised by the use of angioscopy
to perform controlled valvulotomy (Stonebridge et al. 1992, Stierli and Aeberhard 1992),
this procedure requires the vein to be distended and therefore whether this will reduce the
incidence of subsequent stenoses will be determined only by randomised comparative
studies.

(ii) Pharmacological agents.

Numerous drugs have been shown to influence the development of IH and several
have now been evaluated in human studies. However, to date none have shown any clear
benefit on the inhibition of IH. In a wide variety of animal models, results have been
conflicting and, more importantly, have not been reproducible in clinical studies, the
majority of which have been involved with the prevention of restenosis after coronary
angioplasty. Table 1.2 summarises these results. The initial lack of success in these studies
is disappointing, but several avenues for future research still remain. A combination of
agents (heparin combined with angiotensin converting enzyme inhibitor) has been shown to
have a synergistic effect (Clowes et al. 1991, Powell et al. 1991), and warrants further
investigation. The dose of unfractionated heparin that may be administered is limited by the
bleeding complications it produces, but non-anticoagulant fractions that still retain their
anti-proliferative action have a potential use. Growth factor antagonists are currently under
evaluation and initial results in animals are encouraging (Liu et al. 1990). Another
possibility is topical treatment of the vein at the time of surgery, recently reported by
Simons and colleagues (1992) who showed that the proto-oncogene c-myb can be
inhibited in vivo by a gel containing antisense oligonucleotides applied to the wall of the
vessel; this led to a dramatic reduction in IH in a rat balloon injury model. This strategy can
also be used to selectively inhibit the expression of growth factors, (Itoh et al. 1990,
Gibbons et al. 1992) adhesion molecules and cell cycle regulatory genes necessary for cell
migration and/or proliferation. In this context, the explosive growth in vascular biology
has provided the foundation to formulate therapeutic strategies that include inhibition of
thrombus formation, cellular migration and proliferation. Finally, it has recently become
possible to introduce recombinant DNA into endothelial cells in order to induce them to
produce large quantities of antithrombotic agents such as tissue plasminogen activator
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<td>ACE Inhibitors</td>
<td>Rat artery IH reduced (Powell 1989, Fish 1992)</td>
<td></td>
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<tr>
<td></td>
<td>Baboon and pig artery IH unchanged (Lam 1992, Hassen 1991)</td>
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<tr>
<td></td>
<td>Rabbit vein graft IH reduced (Nitobe 1991)</td>
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<tr>
<td>Cyclosporin</td>
<td>Reduced medial thickening in rat artery (Tang 1989)</td>
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<tr>
<td>Prazosin</td>
<td>Rabbit artery IH reduced (O'Day 1989)</td>
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<tr>
<td></td>
<td>Rat vein graft IH unchanged (Nisnoff 1992)</td>
<td></td>
</tr>
<tr>
<td>Steroid</td>
<td>Rabbit artery IH reduced (Collins 1992)</td>
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</tr>
<tr>
<td></td>
<td>Canine vein graft IH unchanged (Study 1977)</td>
<td></td>
</tr>
</tbody>
</table>

ASA, Acetylsalicylic acid; CABG, coronary artery bypass graft; ACE, angiotensin converting enzyme; IH, intimal hyperplasia; LMWH, low molecular weight heparin
(Callow 1990). Such "gene therapy" has great therapeutic potential, since the genetically modified endothelial cells may be seeded onto sites of injury and denudation, providing an ideal drug delivery system. This may also translate into improved treatment of vascular disease by improving the efficacy of prosthetic grafts, as future developments may entail genetically engineered endothelial cells (Wilson et al. 1989) or endovascular drug delivery from the prosthesis.

1.7. Summary.

Although knowledge of the biological processes involved in the development of IH has evolved dramatically in recent years, the precise aetiology of infrainguinal vein graft stenoses remains unclear. Current therapy is therefore directed at treatment of the established lesion rather than its prevention. However, recent advances in our understanding of the vascular biology of the normal and pathological saphenous vein will undoubtedly lead eventually to specific targeted therapy that will prevent the development of vein graft stenoses.

Given the complexity of this pathological process combined with the difficulty of avoiding adverse effects of treatment, it seems unlikely that a "magic bullet" will be discovered that selectively blocks a single mediator to prevent stenosis. Further research is necessary to identify a set of final common pathways essential to the development of vein graft stenoses. A combination therapeutic strategy directed at the specific cellular pathways necessary for cell adhesion, proliferation, migration and extracellular matrix production may be necessary for improving the efficacy of revascularisation techniques.
CHAPTER 2

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TISSUE AND ORGAN CULTURE

2.1 Background to tissue culture

Although tissue culture was first developed during the latter years of the 19th century, the early experiments were very short-lived, principally due to the lack of satisfactory media and conditions. As a result there was some doubt as to whether these experiments represented genuine survival of healthy tissues or merely a delayed death of the cells. It was not until 1907 that Harrison's experiment demonstrated unequivocal continuation of normal function in vitro and offered a reproducible technique, that it was generally accepted as the true beginning of tissue culture. As the name implies, the technique was elaborated first with undisaggregated fragments of tissue, and growth was restricted to the migration of cells from the tissue fragment. Harrison chose the frog as his source of tissue presumably because it was a cold-blooded animal and consequently incubation was not required. Furthermore, since tissue regeneration is more common in lower vertebrates, he probably felt that growth was more likely to occur than with mammalian tissue. The greatest difficulty in performing tissue culture during these early years was the avoidance of bacterial contamination. Alexis Carrel, already a Nobel prize-winner for his work in experimental surgery, was largely responsible for the development of aseptic methods over the next few years. One of Carrel's main achievements was the continuous cultivation of rapidly growing and dividing cells over long periods of time. Since the inception of tissue culture as a viable technique, over the years culture conditions have been adapted to suit two major requirements, these being 1) the production of cells by continuous proliferation, and 2) preservation of specialised functions of the cells under study. Although the study of cellular activity in tissue culture clearly offers many advantages, emphasis must also be placed on its limitations.
2.2 Advantages of cell culture

i) Control of the environment

The two major advantages are the ability to precisely control the environment of the cells (pH, temperature, O₂ and CO₂ tension); and the physiological conditions which may also be kept relatively constant.

ii) Homogeneity of the cell sample

Samples of tissue are invariably heterogeneous, but after one or two passages the selectivity of the culture conditions tends to produce a homogeneous culture of the most vigorous cell type. Hence the characterisation of the line may be perpetuated over several generations.

2.3 Disadvantages of cell culture

i) Expertise

Culture techniques must be carried out under strict aseptic techniques, unfortunately animal cells grow much less rapidly than many of the common contaminants such as bacteria, yeasts and moulds. Therefore it is of major importance that a meticulous approach is always made in order to minimise such problems.

ii) Loss of specific cell interactions

Many of the differences in cell behaviour between cultured cells and their counterparts *in vivo* arise as a result of the loss of the three dimensional arrangement in the tissue, and hence specific cell interactions characteristic of the histology of the tissue are lost. Under these circumstances organ culture, because of the retention of cell interactions, may offer an advantage over isolated cell culture. The environment of cultured cells also lacks several systemic components involved in homeostatic regulation *in vivo*, principally those of the nervous and endocrine systems. Despite the differences in environmental conditions between cells *in vivo* and *in vitro* tissue culture can be a most useful experimental tool as long as the limitations of the model are appreciated.
2.4 Organ culture

Although cell culture techniques have provided much understanding of endothelial and smooth muscle cell function under normal and pathophysiological conditions, the interactions of the cell types are probably better studied in an organ culture system. Organ culture implies a three-dimensional culture of intact tissue retaining some or all of its histological features in vivo (Freshney 1987). In organ culture, whole organs, or representative parts, are maintained as small fragments in culture, thus retaining the spatial distribution of the participating cells, without which it may be difficult to reproduce the characteristic cell behaviour of the tissue.

The first report of vascular organ culture was in 1959 when Trowell maintained lengths of rat mesenteric artery for 9 days in culture without any evidence of necrosis. He noted that the flat, thin walls of the opened out vessels made them ideal for study in an organ culture because the distance for the adequate diffusion of nutrients was small. In 1967 Wexler and Thomas described an intimal outgrowth of cells from cultured rat aorta, and this intimal proliferation of smooth muscle cells was subsequently observed in cultured human aorta (Barrett 1979) and in porcine aorta (Gotlieb and Boden 1984). In 1990 Soyombo and colleagues first described the use of an organ culture of human saphenous vein, showing that it remained viable for 14 days in culture, and developed a neointima as a result of the migration and proliferation of smooth muscle cells. Vein culture is therefore a potentially useful model for the study of vein bypass graft intimal hyperplasia. The use of human tissue in such studies avoids the problems of interpreting results from animal tissues which cannot always be related to human studies (Ferrell et al. 1992). Additionally, the preservation of the vein wall architecture maintains components of the extracellular matrix, for example heparan sulphates and thrombospondin, which are known to influence smooth muscle cell proliferation and are lost in isolated cell culture techniques. Finally, by the use of the vein culture model, the interaction between the different cell types can be studied, principally those between the endothelium and the smooth muscle cells. Although the basic techniques of vascular organ culture are well established, vein culture essentially represents a new development within this field.
2.5 Culture techniques used in this study

i) Organ culture of human saphenous vein

This technique, originally described by Soyombo and colleagues (1990), is the principal experimental model used throughout this study. The aim of this thesis was to investigate the role played by the endothelium in the promotion of smooth muscle cell proliferation in the aetiology of intimal hyperplasia. Logically therefore, organ culture of the human saphenous vein seems the ideal candidate suited to this purpose, because both the endothelium and the smooth muscle cells are retained in their native state.

In the studies described in this thesis, further modifications to the basic organ culture were also developed, namely:

ii) Human saphenous vein endothelial cell (HSVEC) culture

These cultures were initiated by enzymatic dispersion of the endothelial cells from a segment of intact human saphenous vein. Incubation with a solution of 0.1% Worthington collagenase enzyme (Lorne Laboratories, Reading, Berks.) followed by gentle scraping of the luminal surface, released the endothelial cells into a suspension and then allowed the establishment of a primary culture by plating onto tissue culture flasks and culturing the endothelial cells as an adherent monolayer in serum-supplemented medium (see Chapter 8). The resulting endothelial cell lines were then used in further coculture studies (Chapter 8) and to "reconstitute" endothelium-denuded veins (Chapter 9).

iii) Organ Coculture and Cellular/Organ Coculture

In vivo studies of vascular wall disease are limited by difficulty in controlling numerous variables in a complex environment. This has therefore led to the study of cellular processes in vitro, where more precise control can be obtained. Unfortunately, as already mentioned, the biology of isolated cells in culture may be quite different from cells in vivo. As a consequence, a number of laboratories are now using techniques in which endothelial cells and smooth muscle cells are cultured in close proximity (Davies and Kerr 1982, Jones 1979, Van Buul-Wortelboer et al. 1986). Several coculture systems have been designed, but one that most closely mimics the in vivo structure is a model in which
endothelial cells and smooth muscle cells are grown on opposite sides of a thin semi-permeable membrane (D'Amore 1992). Another study by Fillinger et al. in 1993 used a modification of this technique to examine the effect of endothelial cells on the growth kinetics and cellular structure of smooth muscle cells. Although this effect is fundamental to understanding endothelial/smooth muscle cell interaction, reports are contradictory, indicating both stimulation (Davies and Kerr 1982, Gajdusek et al. 1980) and inhibition (Castellot et al. 1981, Chamley-Campbell and Campbell 1981). Two different modifications of such coculture techniques are described in Chapters 7 and 8. Initially, segments of intact saphenous vein were cocultured with segments denuded of endothelium. This study was followed up by one in which denuded saphenous vein segments were cocultured with endothelial cells isolated from human saphenous vein. The two were separated by a semi-permeable membrane of an appropriate size, thereby allowing the free passage of biochemical molecules but not cellular elements.

2.6 Summary

This chapter has given a brief outline of cell and organ culture with their relative merits and limitations. The studies described in the following chapters utilise all of these techniques with various modifications in an attempt to simulate the intimal hyperplasia underlying in vivo vein graft stenoses in a laboratory environment in vitro.
# CHAPTER 3

**STRUCTURAL VARIATION IN THE LONG SAPHENOUS VEIN: A QUANTITATIVE STUDY**

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3.1 Introduction

Several studies have identified structural changes in the long saphenous vein at the time of bypass surgery. In 1992, Panetta and colleagues demonstrated that macroscopic abnormalities such as palpable thickening and calcification in vein used as a bypass graft led to a significantly poorer 30 month patency rate (32% vs 73%) in infrainguinal procedures. In this study, pre-existing disease was estimated at 12%, but since histology was only performed in 21 patients, this could represent a serious underestimation. In 1992, Davies and co-workers reported an increased incidence of stenoses in veins of low compliance; it was suggested that low compliance was associated with focal or diffuse intimal hyperplasia in the vein wall. In a more recent histological study by the same workers (1993), the incidence and extent of certain pathological features in vein samples taken prior to bypass surgery were recorded. In agreement with Marin et al. (1993a), it was found that intimal hyperplasia was commonly observed in veins thought to be normal, with these changes being more evident in the distal portion of the vein (87% versus 73% in the proximal segments). Furthermore, muscle hypertrophy (longitudinal and/or circular) occurred in 68% of proximal segments compared to 88% in distal segments. It has therefore been suggested that these pre-existing morphological abnormalities may well influence the patency of the graft in terms of the development of stenoses and ultimate failure.

However, the precise nature of such structural abnormalities, or indeed the "normal" appearance of the long saphenous vein remains poorly defined since most of the previous studies are only descriptive or semi-quantitative in nature. The true incidence and extent of structural changes in the vein wall, which may prejudice graft performance, is still unknown.

The "textbook" description of the normal long saphenous vein is of a thin intima covered by endothelium and separated from a thick muscular media by a poorly defined internal elastic lamina. The media is composed of two distinct layers of muscle fibres;
an inconspicuous inner longitudinal layer and a more prominent outer circular layer (Ham's Histology 1987). In patients undergoing arterial bypass surgery however, the architecture of the vein wall may be considerably disturbed. In 1976 Spray and Roberts gave a descriptive account of a number of abnormalities present in the saphenous vein of patients undergoing coronary artery bypass grafting, and in 1975 Cheanvechai and colleagues used a crude classification system to identify "normal" from "abnormal" veins. More recently in 1989 Milroy gave a more detailed description of these abnormalities and produced a semi-quantitative report of their incidence and extent. Four major structural changes were described and the severity of each was scored on a scale of i-iv. These were i) intimal fibrosis, consisting of collagen and longitudinally arranged smooth muscle cells; ii) medial longitudinal muscle hypertrophy; iii) medial circular muscle hypertrophy; iv) the presence of an additional outer longitudinal muscle layer. Moderate intimal fibrosis was seen in 36% of veins and severe fibrosis in 4%. Moderate longitudinal muscle hypertrophy was observed in 44%, with severe changes in 14% of cases.

A semi-quantitative study performed by Leu and colleagues (1991) analysed the structure of the long saphenous vein in 52 post-mortem specimens from normal subjects with no clinical evidence of arterial disease. Overall, moderate to severe changes were seen in the intima of 60% of the veins and in the media of 33% irrespective of the subject gender. Although the incidence of these changes increased with age, the severity did not, as advanced changes were seen in some young subjects. Indeed, a study of neonates conducted by Lev and Saphir (1951) reported the incidence of focal proliferations of varying amounts of elastic, muscle and collagen fibres with accompanying glycoprotein components of the basement membrane. This process, termed as "endophlebohypertrophy" was found to begin from birth and to increase progressively up to the age of two or three years both in the intima and media of the vein, being localised particularly at regions of mechanical stress.

Based on the above studies, it would appear that "disease" of the long saphenous vein is common. However, one should exercise care in the interpretation of these studies for the following reasons. Firstly, all of these studies were performed on
collapsed veins. In a study of human arteries, Stary (1992) demonstrated that if the vessels were not fixed under some form of tension, then lesions in the vessel wall could be interpreted as being significant, when in fact they may have occurred as a result of artefactual distortion due to contraction and collapse. Secondly, all of the above studies were based on the use of semi-quantitative scoring systems, thus emphasising the need for a quantitative study of veins fixed under tension. This may provide clues to unanswered questions such as what is the exact nature, distribution, incidence and extent of these changes in the vein wall and how might they influence the development of vein graft stenoses?

The aim of this study therefore was to analyse and quantify the structure of the long saphenous vein in patients undergoing arterial surgery in order to answer some of the above questions.

3.2 Patients, Materials and Methods

1) Vein preparation

Biopsies of the long saphenous vein were collected at the time of surgery from 60 patients (median age 62 years, range 44 - 83, 77% male) undergoing the following arterial procedures: coronary artery bypass (33), femorodistal bypass (15) and carotid endarterectomy (12). In each of these procedures the vein was being used as the bypass conduit or as a patch. Twenty segments were taken from the proximal thigh and forty from the calf. Macroscopically abnormal and varicose veins were excluded from the study. The vein was dissected with minimal handling and was not distended. In order to counteract any spasm, veins were transported to the laboratory in calcium-free Krebs physiological saline solution (millimolar composition; NaCl 118, KCl 4.7, MgSO4

Two different methods were used for fixing the veins under tension, this being dependent on the length of vein available. Ten of the vein samples were distended with 10% formal saline at normal venous pressure (40mm Hg) as shown in Figure 3.1. The vein and tubing were filled with 10% formal saline to a height of 52cm, equivalent to
40mm Hg. The vein was then totally immersed in a bath of 10% formal saline for twelve hours to ensure uniform and complete fixation. However, this method of fixation required a minimum length of 3-4 cm of vein, and as this amount could not normally be spared at operation, a second method was developed. Smaller segments of vein (0.5 - 1cm) were opened up longitudinally and pinned out under tension using A1 minuten pins (Watkins & Doncaster, Cranbrook, Kent), lumenal surface uppermost, onto a preformed layer of Sylgard resin (Dow Corning, Seneffe, Belgium) in a glass petri dish (Corning Ltd. U.K.). They were then completely covered with 10% formal saline for 12 hours. Using both of these fixation techniques care was taken to overcome venous spasm, and occasionally the use of papaverine (Martindale Pharmaceuticals, Chesham, Romford) was required to achieve this. For 10 of the vein specimens both the distension and pinning methods of fixation were used in order to validate the latter method. Thereafter all specimens were fixed using the pinning method.

**ii) Staining methods**

Following fixation, all veins were paraffin-embedded and serial sections of 4μm thickness were prepared for the application of the following stains:

i) Haematoxylin and Eosin (H&E)

ii) Alcian Blue / PAS stain for acid and neutral mucopolysaccharides

iii) A combined Millers elastin and monoclonal α-smooth muscle actin stain

This latter stain is a novel combination developed during the course of this study using an indirect immunoperoxidase method for the localisation of smooth muscle actin. Mouse anti-human α-smooth muscle actin antibody (DAKO, High Wycombe, Bucks.) was applied at a dilution of 1:400 and diaminobenzidine used as a final reaction product. Following this, a Millers elastin stain was applied (Miller 1971).
Tubing with Cannula attached

Column of fluid = 52 cm. (40 mm Hg)

FIGURE 3.1 Diagrammatic representation of the apparatus used for perfusion-fixing veins at venous pressure (40 mm Hg).
iii) Measurements and observations

All of the 60 veins in this study were examined microscopically in order to measure intimal and medial thickness. This was achieved by the use of a computerised image analysis system (Kontron Videoplan, Munich, Germany). A mean of 10 measurements were made on each of three consecutive sections evenly distributed across each vein by two independent observers. The reproducibility of the method was assessed in the first 20 veins, which were independently measured by two observers and the results compared (inter-observer error). Additionally, the reproducibility of the measurements was assessed in 10 veins where all measurements were made and later repeated by a single observer and the two sets of measurements compared (intra-observer error).

In order to compare the pinning method of fixation with the perfusion technique described above, measurements of intimal and medial thickness were measured by the two independent observers in both sets of vein in order to directly compare the results (inter-method variation). The veins were coded to prevent the observers pairing the veins fixed by the two different techniques.

Intimal thickness was defined as the distance between the endothelium and the inner boundary of the internal elastic lamina. Medial thickness was defined as the distance between the internal elastic lamina and the outer border of the circular smooth muscle layer where the muscle bundles blended into the adventitia. It was occasionally possible to identify an external elastic lamina in this location also. The morphology of the vein wall was also recorded for each vein. These observations were based on the system described by Milroy et al. (1989), where longitudinal muscle hypertrophy (LMH) was defined as the presence of prominent longitudinal smooth muscle bundles in the media immediately below the internal elastic lamina. Circular muscle hypertrophy (CMH) was defined as any focal enlargement of the circular muscle layer with increased intervening connective tissue. This was more difficult to define since a prominent circular muscle layer is a feature of the normal saphenous vein.
iv) Electron microscopy

Six representative vein sections were prepared for transmission electron microscopy TEM. The veins were fixed in 4% buffered gluteraldehyde for 12 hours, then rinsed in cacodylate buffer for 1 hour and post fixed in Osmium Tetroxide for a further hour. This was followed by dehydration through graded alcohols and embedding in Emix resin. The polymerised blocks were sectioned on a Reichert-Jung Ultracut-E. Sections of 0.5μm thickness were stained with alkaline 1% toluidine blue and examined under the light microscope. Subsequently thin sections of 90nm were cut, mounted on a 200μm mesh copper grid and then double stained with uranyl acetate and lead citrate. The grids were screened and photographed using a Jeol 100CX transmission electron microscope at 80KV.

v) Analysis of data

In order to validate the methods of measurement used in this study, the inter-method, inter-observer and intra-observer agreements were plotted as Bland Altman charts, from which the mean difference and 95% limits of agreement were calculated. (Bland and Altman 1986). The correlation between vein wall dimensions and patient age was assessed by calculating Spearman's rank correlation coefficient and 95% confidence intervals.

3.3 Results

i) Observations

The superiority of the smooth muscle actin / Millers elastin stain (Figure 3.2b) over the basic H&E stain (Figure 3.2a) in delineating the internal elastic lamina enabled a clear distinction to be made between the intima and media of the vein wall. Additionally, the distribution of smooth muscle cells in each of the layers could be visualised. This stain was therefore used preferentially in all quantifications. The commonest structural change in this population was a degree of intimal thickening combined with medial longitudinal muscle hypertrophy (Figure 3.3). In these veins the intima was frequently very cellular, and stained positively for smooth muscle actin.
Intimal morphology varied considerably, the commonest appearance being of longitudinally arranged smooth muscle cells between laminae of elastic fibres (Figures 3.3 & 3.4). Another common observation was of a cellular intima with very few elastic laminae (Figure 3.5).

Circular muscle hypertrophy was rare (4 veins) and was not a feature in veins affected by severe intimal thickening and LMH. Alcian blue staining of the extracellular matrix was most intense in thickened intimas (Figure 3.6). A different pattern of changes was observed in 3 veins in which a highly cellular thickened intima with no underlying LMH was present (Figure 3.5).

Electron microscopy of the intimal smooth muscle cells in the vein wall demonstrated that the majority were of the contractile phenotype (Figure 3.7a) with a thin elongated body and a dense arrangement of contractile filaments. In veins with a thickened intima, approximately 1-2% of the intimal smooth muscle cells were of the secretory phenotype (Figure 3.7b), with large amounts of rough endoplasmic reticulum and Golgi apparatus, and only sparse filaments. Cells of this type were not observed in veins with thin intimas.
FIGURE 3.2 a) Haematoxylin and Eosin (H&E) stain on saphenous vein section. There is poor definition between intima and media. (Mag. x 160).

FIGURE 3.2 b) SMA/Millers elastin stain on the same vein segment. There is a clear distinction between the intima and media facilitated by precise localisation of the internal elastic lamina (arrowed). (Mag. x 160).
FIGURE 3.3
The most commonly observed structural change was of a thickened intima combined with longitudinal muscle hypertrophy (LMH). (Mag. x 160).

FIGURE 3.4
Intimal morphology was frequently observed as longitudinally arranged smooth muscle cells between laminae of elastic fibres. (Mag. x 160).
FIGURE 3.5
Veins with a cellular intima and very few elastic laminae were also commonly observed. (Mag. x 160)

FIGURE 3.6
Alcian blue staining of the extracellular matrix was most intense in veins with thickened intimas. (Mag. x 160)
FIGURE 3.7 a) Transmission electron micrograph of intimal smooth muscle cell of contractile phenotype, characterised by contractile filaments and focal densities. (Mag. x 6,600).

FIGURE 3.7 b) Intimal smooth muscle cell of secretory phenotype, showing large amounts of rough endoplasmic reticulum and Golgi apparatus. Cells of this type were occasionally observed in veins with thickened intimas. (Mag. x 6,600).
**ii) Validation of measurements**

The inter-method variation and the inter and intra observer error data are summarised in Figures 3.8 and 3.9, and the 95% limits of agreement are shown in Table 3.1.
TABLE 3.1 Summary of data from the measurement validation studies.
Mean differences with 95% limits of agreement.

<table>
<thead>
<tr>
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<th>INTIMA</th>
<th>MEDIA</th>
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</thead>
<tbody>
<tr>
<td>Inter Observer Error</td>
<td>1.2 (+/- 14)</td>
<td>7.0 (+/- 59)</td>
</tr>
<tr>
<td>Intra Observer Error</td>
<td>0.5 (+/- 10)</td>
<td>1.5 (+/- 108)</td>
</tr>
<tr>
<td>Inter Method Error</td>
<td>1.1 (+/- 8.2)</td>
<td>0.7 (+/- 137)</td>
</tr>
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</table>

### iii) Frequency distribution
The distributions of intimai and medial thickness in this population of patients undergoing arterial surgery are shown in Figures 3.10 and 3.11. The median intimai thickness was 35.5μm, range 8 - 381, and the corresponding figures for the medial thickness measurements were 314μm, range 135 - 459.

### iv) Correlation
There was a poor correlation between intimai and medial thickness ($r_8 = 0.48$, 95% confidence interval 0.2 - 0.7), Figure 3.12; and no correlation between intimai thickness and patient age ($r_8 = 0.02$, 95% confidence interval -0.3 - 0.25), Figure 3.13.
FIGURE 3.10  Frequency distribution of intimal thickness.
Median (range) = 35.5 µm (8 - 381).

FIGURE 3.11  Frequency distribution of medial thickness.
Median (range) = 314 µm (135 - 459).
FIGURE 3.12 Plot of intimal thickness versus medial thickness showing a weak correlation between them.

FIGURE 3.13 Plot of intimal thickness versus patient age, showing no correlation between them.
3.4 Discussion

The main purpose of this study was to identify changes in the structure of the saphenous vein in a population of patients with arterial disease and devise a basis on which structural abnormalities could be quantified. Owing to the relatively large quantity of vein required to perfusion fix at normal pressure, the pinning method of fixation was adopted which showed no apparent bias for measurements of intimal thickness. However, medial thickness measurements were more difficult to make due to poor definition of the outer border of the media in many veins. When the two fixation methods were directly compared, there was no demonstrable bias although the limits of agreement were wide (137μm). Nevertheless, these medial measurements still represent an improvement on the semi-quantitative methods used in earlier studies.

The development of the combined smooth muscle actin and Millers elastin stain for this study greatly facilitated and improved the accuracy of intimal thickness measurements, as reflected in the inter observer error of 14μm and intra observer error of 10μm.

The necessity for standardised techniques of perfusion fixation have been demonstrated in arterial studies (Wolinsky and Glagov 1964,) but this has not been evaluated in veins. It is well known that the elasticity and distensibility of veins differs markedly from arteries (Dobrin 1989, Schmitz-Rixen 1991). Veins are compliant at low pressures up to 40mm Hg, but stiff and non-distensible at higher pressures, therefore changes in wall tension at high pressures produce relatively little change in vessel diameter and wall thickness. These characteristics should enable reproducible results to be obtained provided that venospasm is overcome at the time the vein is pinned out under tension. Overall, the results of the validation studies suggest that the accuracy of these methods is to within 10-15μm for measurements of intimal thickness.

In agreement with previous studies, the results of this study demonstrate that the classical description of saphenous vein structure is seen in only a minority of patients (13%) with arterial disease. The most common change observed in these patients was a non-uniform thickening of the intima associated with hypertrophy of the longitudinal muscle of the media. The two major components of the intimal thickening
were longitudinally arranged smooth muscle cells and an extracellular matrix containing prominent elastic bundles. The non-uniform nature of the intimal thickening in many of these veins could be directly comparable to that seen in arteries, which has been referred to as "adaptive intimal thickening" (Stary et al. 1992). This is thought to be a response of the arterial wall to altered mechanical stresses occurring at sites where wall shear stress is reduced or tensile stress elevated, the pattern of thickening appearing in relation to the sites of arterial branching or bifurcation. A similar adaptive response of the saphenous vein to haemodynamic forces may therefore offer an explanation for the eccentric nature of the intimal thickening seen in the veins in this study. The observation that in a small proportion of the veins longitudinal muscle hypertrophy was seen in the absence of intimal thickening suggests that LMH may precede intimal thickening in response to these forces. Evidence for a further link between these two processes is provided by the observation that in thickened intimas the smooth muscle cells are orientated in a longitudinal direction.

The prominence of elastic laminae in the thickened vein intima is of a similar nature to that in vein graft intimal hyperplasia, which is, in part, an adaptive response of the vein wall to the haemodynamic stresses of arterial flow and pressure (Dobrin 1989).

In arteries, the advanced lesions of atherosclerosis develop first in regions with adaptive intimal thickening (Stary 1992). It is reasonable to speculate therefore, that vein graft lesions may develop first in regions of the saphenous vein affected by pre-existing intimal thickening. In support of this theory, two recent studies have reported an increased incidence of stenoses in veins with intimal thickening (Marin et al. 1993, Davies et al. 1992).

There are, however, some observations that cannot be explained on the above simple theory. In many veins focal plaques of intimal thickening were observed. The aetiology of such lesions is less clear since localised complicated flow abnormalities seem unlikely. Also, in a small number of veins a different pattern of focal changes was seen (cellular intima with no LMH, Figure 3.5), suggesting an entirely different aetiology. It is possible that these changes are the result of an earlier thrombotic episode.
with re-organisation of the thrombus leading to intimal thickening. It is also interesting to note that some veins show virtually no intimal thickening at all. The reason for this may lie in the fact that these veins had been spared from exposure to a certain "threshold" haemodynamic stress owing to competent valves and deep venous system. This observation suggests that the histological structure of the normal vein wall is controlled by more than just direct venous hypertension, possibly an interplay between mechanical forces, thrombotic events and the strength of the vein wall itself.

The present study has shown that in a population of 60 macroscopically normal veins, 95% had an intimal thickness of less than 200 μm, this figure thus representing the upper limit of the normal range. A prospective study is currently in progress involving a full, quantitative histological analysis of saphenous vein from patients at the time of bypass surgery, with subsequent follow-up of the graft. This is intended to answer the question of whether pre-existing intimal changes significantly increase the risk of stenosis and whether this is directly related to the severity of the observed changes.

Since the completion of this particular study, Davies et al. (1994), using a semi-quantitative classification of vein histology, have shown a correlation between pre-existing "abnormality" and subsequent stenosis. Furthermore, consistent with our own observations, Marin et al. (1994), in a study of 30 subjects, concluded that variations in the structure of the saphenous vein in patients undergoing bypass surgery are common. Clearly, the relationship between vein morphology and subsequent stenosis requires more precise definition, in the light of the present study which demonstrates that veins with an intimal thickness of up to 200 μm are normal and not diseased.

It is apparent that vein quality plays a role in the outcome of bypass surgery. Detection of vein abnormalities pre or intra-operatively by the use of intravascular ultrasound and/or measurements of vein wall compliance are useful, but each technique has limitations and requires further study (Varty 1993).
CHAPTER 4

HUMAN SAPHENOUS VEIN ORGAN CULTURE:
FUNDAMENTAL EXPERIMENTAL METHODS AND
VALIDATION OF THE MODEL

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4.7 Summary 92
4.1 Introduction

Historically, the first report of vascular organ culture was in 1959 when Trowell maintained lengths of rat mesenteric artery for 9 days in culture, which, upon subsequent histological examination, showed no evidence of necrosis. He observed that the flat, thin-walled vessels when opened out made them ideally suited to study under conditions of organ culture because the distance for the diffusion of nutrients was small.

In 1967, Wexler and Thomas described an intimal outgrowth of cells from cultured rat aorta, and some years later further reports of intimal smooth muscle cell proliferation were described in human aorta (Barrett 1979), and in porcine aorta (Gotlieb and Boden 1984). Animal vascular organ culture techniques are therefore well established, although human saphenous vein culture essentially represents a more recent development in this field (Soyombo et al. 1990). This model was developed for the study of vein bypass graft intimal hyperplasia, and it was demonstrated that the vein remained viable for 14 days in culture and developed a neointima as a result of the proliferation of smooth muscle cells. Vein culture is therefore a potentially useful in vitro model of intimal hyperplasia, offering several advantages over animal and cell culture studies as already discussed in Chapter 2.4.

The aims of this study were to establish and validate a human vein culture system as an experimental model of vein graft intimal hyperplasia. This required the study of cultured vein viability, the cellular changes occurring during culture and a comparison of these changes with the morphological appearances of lower limb vein graft stenoses.

The work described in this chapter describes the fundamental experimental methods employed throughout the study, and validates their use in the organ culture model of human vein graft intimal hyperplasia.
4.2 Experimental Methods

i) Source of saphenous vein and collection procedure

Segments of saphenous vein were obtained from patients who were undergoing carotid endarterectomy, lower limb bypass grafting or coronary artery bypass grafting, where the vein was being used as a patch or graft. The length of vein that could be spared was dictated by clinical requirements, but was usually 2-3 centimetres. The vein was exposed using a no-touch technique, distension was avoided and handling kept to a minimum. The vein for culture was placed immediately in the transport medium on ice for transport to the laboratory. LoGerfo (1981) recommended storage at 4°C in saline with the addition of papaverine to reduce vein spasm. As a modification of this medium, a calcium-free Krebs physiological saline (Chapter 3.2i) was used in order to counteract any spasm, such that any drug effects in the cultures were minimised. Previous assessment of calcium-free Krebs in this laboratory has demonstrated good preservation of endothelial morphology and, to a lesser extent, smooth muscle cell function (Sayers 1991).

Vein culture was established as soon as possible after harvesting, and always within one to two hours.

ii) Determination of endothelial integrity

Using fine dissecting scissors and forceps the vein was carefully cleaned of any excess fat and adventitial tissue under sterile conditions whilst still immersed in cold calcium-free Krebs solution. The potentially damaged end pieces of vein were excised with a scalpel blade and discarded. The remaining vein was divided into lengths of approximately 0.5 cm which were then cut open longitudinally. Vein with significant endothelial loss (> 40%) was not used for culture since this would suggest that a significant amount of uncontrolled injury had occurred during harvesting. A method for rapidly assessing endothelial coverage before culture was therefore required and two methods were evaluated:
a) A modification of the silver impregnation method of Lautsch (Poole 1958).

For this method the vein was washed in 5% dextrose for 5 minutes, which was then replaced with an aqueous solution of 0.25% silver nitrate for 30 seconds. A repeat wash with 5% dextrose was followed with a solution of 3% cobalt bromide in 2% ammonium bromide for a further 30 seconds. Exposure to daylight for 10-15 minutes should then expose the dark silver staining of the intercellular cement substance, when viewed under a dissecting microscope.

b) Trypan blue staining (Soyombo 1990, Pederson 1985).

A solution of 0.01% trypan blue (Sigma Chemicals, Poole, Dorset) in Dulbecco's phosphate-buffered saline (pH 7.4), was pipetted over the lumenal surface of the vein. After 1-2 minutes this was washed off with physiological saline. Upon inspection under the dissecting microscope, areas of endothelial loss or structural damage stained blue, whereas intact endothelium excluded the stain.

In evaluating the two methods, the silver staining technique was disappointing with no clear delineation of the intercellular borders. This may well have been due to the short length of time available for the stain to develop: in the original method described by Lautsch the tissue was fixed in formalin overnight.

Assessment of endothelial coverage using the trypan blue technique was clearly superior, and identification of areas of endothelial cell loss was clear. It was therefore routinely used as a quick and simple method to assess endothelial coverage on a representative segment of every vein prior to culture (see Figure 4.3 for example of silver stain versus trypan blue). The reliability between two observers grading endothelial coverage using trypan blue was assessed in 10 vein segments. Each observer independently scored the endothelial coverage as a percentage and the results were compared. The difference between observers did not exceed 10%. (See Results section, Figure 4.4).
iii) Establishment of vein cultures

Providing endothelial coverage was satisfactory the remaining vein sections were transferred into vein culture dishes. These were made using Pyrex 60 x 20 mm dishes (Corning Ltd. U.K.) into which a layer of Sylgard 184 resin (Dow Corning, Seneffe, Belgium) was cast at a depth of approximately 5mm. The vein was opened out, lumenal surface uppermost to approximately its in situ length on a coarse (500mm) polyester mesh, and pinned into the resin using A1 minuten pins (Watkins and Doncaster, Cranbrook, Kent). (Figure 4.1). The vein was then immersed in 6 mls of complete vein culture medium, consisting of RPMI 1640 medium (Northumbria Biologicals, Cramlington, U.K.); 30% v/v Foetal Calf Serum (Seralab, Crawley-Down, Sussex); Penicillin 50U/ml; Streptomycin 50μg/ml; L-glutamine 2mmol/l (All Northumbria Biologicals, Cramlington) and endothelial cell growth factor (Sigma, Poole, Dorset) 15μg/ml. Cultures were maintained at 37°C in a humidified incubator (Queue Systems, West Virginia, USA) gassed with 5% (v/v) CO₂ in air, and the medium was replaced every 2 or 3 days.

FIGURE 4.1 Photograph of vein segment prepared for culture.
At the end of the 14 day culture period, the medium was removed and the vein segments were immersed in 10% formal saline for a minimum of 18 hours whilst still pinned out in the dish. This "in situ" fixation technique was used in order to minimise any shrinkage artefact. The sections were then dehydrated through 70% to absolute alcohol, cleared in chloroform and embedded in paraffin wax.

iv) Light microscopy and Immunohistochemistry

Serial sections of 4µm thickness were taken from the paraffin blocks and mounted onto silane-coated glass slides. A variety of stains were then performed:

i) Haematoxylin and Eosin (H & E).

ii) Elastic Van Gieson trichrome stain (EVG).

iii) A combined Millers elastin and monoclonal smooth muscle actin stain.

iv) Alcian blue/PAS stain (pH 2.5) for mucopolysaccharides.

v) Laminin monoclonal antibody (Europath Ltd. Bude, Cornwall.)

vi) QB.END.IO (Europath Ltd.) and CD 31 (DAKO, High Wycombe, Bucks.), monoclonal endothelial markers, with CD 31 being a specific marker for venous endothelium.

Measurements of intimal and medial thickness were made on 20 cultured vein sections by the methods already described (Chapter 3.2ii) and validated (Chapter 3.3ii). Cultured veins also developed a "neointima" sub-endothelially, on the luminal aspect of the internal elastic lamina. The different morphology of the neointima enabled clear distinction between the original intima and the neointima to be made (See results section Figure 4.6 as a representative example). Neointimal thickness was measured using the same technique as
for intimal and medial measurements, and additionally the interobserver error was assessed in the first 15 veins measured.

v) **Electron Microscopy**

The use of scanning (SEM) and transmission (TEM) electron microscopy facilitated a more precise study of the morphology of the vein, both at the lumenal surface and deeper into the vessel wall. Six cultured veins were processed for both SEM and TEM. These were all prepared by overnight fixation in 4% buffered gluteraldehyde whilst still pinned out in the culture dishes.

For SEM, the veins were washed in 0.2M cacodylate buffer and serially dehydrated through 50% to absolute ethanol, followed by immersion in acetone for storage prior to scanning. Dehydrated specimens were critical-point dried in a Polaron E 3000 critical point dryer with 3 flushes of liquid CO$_2$, and sputter-coated with gold in a sputtering device (Polaron ES150 sputter coater). The coated specimens were mounted on aluminium stubs and examined in a scanning electron microscope (ISI D3 130 bottom stage) at 15 kV.

For TEM, the veins were rinsed in cacodylate buffer and post-fixed in 1% Osmium Tetroxide for 1 hour. This was followed by serial dehydration through graded ethanol and embedding in Emix resin. Sectioning and screening was then performed as described in Chapter 3.2iv.

4.3 **Comparison of cultured vein with vein graft stenoses.**

Histological material was obtained from 5 vein bypass graft stenoses excised during revision procedures. All 5 patients were male with a median age of 67 years (range 49-73). The grafts were all below-knee, femoro-popliteal, 3 *in-situ* and 2 reversed. The median time to development of the stenosis was 14 months (range 7-60). These were all intragraft lesions with no anastomotic stenoses.

The pathological sections were subjected to the same stains as used for cultured veins in order to compare the morphology of the stenoses with that of the cultured veins.
On one occasion, stenotic material was obtained before routine formalin fixation in theatre, enabling half to be fixed in gluteraldehyde for TEM examination.

4.4 Viability of cultured vein

i) Introduction

In order to validate the vein culture model as a true representation of human vein bypass graft stenoses, it is of primary importance to be able to demonstrate viability of the vein during the 14 day culture period. In these studies two criteria were evaluated. Firstly, the cellular morphology of cultured veins was assessed by examination of the SEM and TEM. Endothelial integrity was evaluated on SEM, and TEM used to look for any evidence of smooth muscle cell necrosis.

A more detailed assessment of smooth muscle cell viability was performed using contractility studies, previously described by Mauger (1975), and De-May (1989). In 1987, Fingerle described the use of this technique for assessing the viability of arterial organ cultures, and more recently in this laboratory for quantifying the response of surgically-prepared human saphenous vein to a range of pharmacological agents (Sayers 1991, Sayers et al. 1992). Contraction is an important function of vascular smooth muscle cells, therefore this method gives a good indication of their functional state and viability.

ii) Method

The method used for the contractility studies was that developed by Sayers and colleagues in this laboratory (Sayers 1991). One end of the vein segment was mounted onto a stainless steel plate by means of two fine pins, and the opposite end to a force transducer to measure isometric tension, which was linked to a chart recorder. The prepared vein was then placed into an insulated organ bath containing Krebs physiological saline solution (millimolar composition; NaCl 118, KCl 4.7, CaCl\(_2\) 2.5, MgSO\(_4\).7H\(_2\)O 1.2, KH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 25 and glucose 11.1), at 37°C and pH 7.45, gassed with a mixture of 5% CO\(_2\) and 95% O\(_2\) (Figure 4.2). The veins were maintained at a resting
tension of 3g (mean optimal tension as determined by Sayers et al.) for 1 hour in order to stabilise, with a change of bathing medium during the equilibration period.

FIGURE 4.2 Diagrammatic representation of the organ bath apparatus used in the contractility studies.
At the end of the equilibration period, contractile responses were determined to the following vasoactive agents: noradrenaline (NA) \(10^{-7} - 10^{-5}\) M, high potassium Krebs solution (NaCl replaced by KCl) as a membrane depolarising agent (K+), and prostaglandin F2α (PGF2α) \(10^{-6}\) M.

Assessment of endothelium-dependent relaxation was also performed by attempting to relax veins that had been pre-contracted with noradrenaline by the use of acetylcholine (ACh).

Contractility studies were performed on 5 freshly isolated veins (i.e. no culture), and on veins cultured for 7 (n=5) and 14 (n=5) days. The maximum developed tension for each vein was recorded.

4.5 Results

i) Pre-culture assessment

1. Trypan blue staining.

As already determined in section 4.2ii, the trypan blue stain was preferentially used for assessment of endothelial viability. Figure 4.3 shows a section of vein that had clearly undergone substantial damage and was therefore unsuitable for culturing. Two further examples of endothelial assessment using the trypan blue exclusion technique are shown in Chapter 6, Figures 6.3 and 6.4.

The endothelial coverage scores for the two independent observers grading trypan blue staining are shown as a Bland Altman plot in Figure 4.4. A good agreement between observers was seen using this method, and additionally this validation study demonstrated the efficiency of the "rubbing" technique for gentle endothelial denudation as described in Chapter 6.2ii: the only surviving endothelium after gentle abrasion was adjacent to the pins, an area difficult to reach, and in the base of some of the longitudinal striations.
FIGURE 4.3 Vein segment double stained with trypan blue and silver stain. Trypan blue demonstrates endothelial loss at cut edges and clamped area. However the silver stain fails to delineate intact endothelial cells, and can be observed only as granular, dark grey deposits.

FIGURE 4.4 Bland-Altman plot of inter-observer error in grading endothelial coverage of veins prior to culture. The mean difference between observers was 5%, and the 95% limits of agreement +/- 15%.
2. **Electron Microscopy.**

Scanning electron microscopy revealed the endothelial morphology in more detail. In 5 of the 6 fresh veins, good endothelial coverage was observed. Intercellular gaps were frequently present, and areas of dislodged cells curling at the edges were not uncommon (Figure 4.5). This appearance has previously been observed and reported by Haudenschild (1981).

![Scanning electron micrograph of freshly isolated vein.](image)

**FIGURE 4.5** Scanning electron micrograph of freshly isolated vein. Endothelial preservation was generally good, although cells curling at the edges and intercellular gaps (top panel) were frequently observed. More severe damage (arrowed) was confined to the cut edges (bottom panel). Mag. x 1,000.
ii) Cultured veins

1. Light Microscopic Appearance

After 14 days in culture, the neointimal appearance was of several layers of loosely arranged cells staining positively for smooth muscle actin (Figure 4.6), accompanied by strong staining of the extracellular matrix with the alcian blue/PAS stain (Figure 4.7).

In the EVG stained sections there was no evidence of collagen or elastin (Figure 4.8), but laminin staining was particularly strong in the extracellular matrix of the neointima accompanied by pericellular staining in the media. (Figure 4.9).

Staining with the endothelial markers was variable, suggestive of an alteration in the expression of these antigens during culture, since SEM of cultured veins showed a confluent layer of endothelium over the lumenal surface. The failure of any intact vein to take up trypan blue after culture further supported this observation. In some vein sections endothelial staining within the neointima was observed (Figure 4.10). Such structures have been previously observed and referred to as "heavily stained profiles suggesting capillary microlumeni" (Soyombo et al.1990).
FIGURE 4.6 T/S of cultured vein stained with SMA/Millers elastin stain. Clear definition between the neointima (NI)/intima (I) and intima/media (M) borders can be observed (arrows mark boundaries). The neointima stains strongly for smooth muscle actin. (Mag. x 160).
FIGURE 4.7
T/S Cultured vein stained with Alcian blue/PAS. Strong staining can be observed in the neointima. (Mag. x 320)

FIGURE 4.8
T/S cultured vein, EVG stain. Yellow staining of the neointimal cells demonstrates the absence of any elastin or collagen. (Mag. x 320)
FIGURE 4.9 T/S cultured vein, laminin stain. Strong staining of the extracellular matrix of the neointima, and pericellular staining of the medial cells. (Mag. x 160).

FIGURE 4.10 T/S cultured vein, CD31 endothelial marker showing staining within the neointima, suggesting capillary microlumeni. (Mag. x 640).
2. **Electron Microscopy.**

Scanning electron microscopy of cultured veins showed a smooth, almost confluent surface of polygonal cells with occasional gaps bridged by dendritic-like cell extensions (Figure 4.11). In places some smaller, oval-shaped cells appeared to protrude from beneath this layer and rest between the polygonal cells (Figure 4.12).

Upon TEM examination, the lumenal surface cells were positively identified as endothelial cells by the presence of Wiebel-Palade bodies (Figure 4.13). The underlying neointimal cells had the appearance of secretory smooth muscle cells. Although a peripheral rim of contractile filaments with focal densities could be seen, the centre of the cell was full of rough endoplasmic reticulum. The extracellular space was largely amorphous, although occasional banded filaments were observed, suggestive of some early collagen synthesis (Figure 4.14).

In the original intima of the vein wall, smooth muscle cells of both contractile and secretory phenotypes were observed (defined in Chapter 3.3i). There was, however, a higher proportion of the secretory phenotype than seen in freshly isolated veins. Contractile smooth muscle cells were the predominant cell type in the vein medial layer.
FIGURE 4.11 SEM of cultured vein. Polygonal cells with dendritic-like cell extensions. (Mag. x 1,000).

FIGURE 4.12 SEM of cultured vein, showing protruding oval cells between surface polygonal cells. (Mag. x 1,000).
FIGURE 4.13  TEM of neointimal surface cell of cultured vein. The surface cells can be identified as endothelial cells by the presence of Wiebel-Palade bodies (arrowed), overlying secretory SMC in the superficial layer of the neointima. (Mag. x 6,600).

FIGURE 4.14  TEM of neointimal cell showing features of secretory smooth muscle cell. There are occasional banded filaments in the extracellular space. (Mag. x 16,000).
3. Measurement of neointimal thickness

A Bland-Altman plot of the inter-observer error in measuring neointimal thickness is shown in Figure 4.15. The mean difference between observers was 0.7 μm, with the 95% limits of agreement being +/- 7.3 μm. For the 20 veins in the validation study, the median neointimal thickness of 14 day cultured veins was 25 μm (range 5-81).

FIGURE 4.15 Bland-Altman plot of inter-observer error in the measurement of neointimal thickness. The mean difference between observers was 0.7 μm, and the 95% limits of agreement +/- 7.3 μm.
4. *Intimal and medial changes.*

In order to quantify any intimal and medial changes occurring during culture, these layers were measured before and after culture.

Intimal thickness increased during the culture period from a median thickness of 33.5 μm to 47.5 μm (Median difference 10.0 μm, 95% confidence interval 8, -30, P = 0.2) (Figure 4.16).

Medial thickness also increased from a median of 335 μm to 471 μm (Median difference 119 μm, 95% confidence interval 50, 182, P = 0.002) (Figure 4.17).

![Line graph to show change in intimal thickness during 14 day culture period.](attachment:Figure_4.16)
FIGURE 4.17 Line graph to show change in medial thickness during 14 day culture period.
iii) Morphological similarity between cultured and pathological vein

Intimal hyperplasia was the underlying pathological lesion of all five vein graft stenoses. In each of these, the pattern of staining seen with each of the markers was similar.

The H&E stain clearly demonstrated the presence of a highly cellular intima (Figure 4.18), and furthermore all of these cells stained positively for smooth muscle actin (Figure 4.19).

In 3 of the stenoses, there was a layered appearance to the intima; elastin fibres alternating with smooth muscle cells (Figure 4.20).

Application of the alcian blue/PAS stain showed that these cells were surrounded by an abundance of mucopolysaccharides in the extracellular matrix (Figure 4.21).

TEM was performed on one of the stenoses. This revealed a large number of secretory smooth muscle cells at the lumenal aspect, surrounded by a loose connective tissue matrix (Figure 4.22). A diploid or bilobed nucleus was a frequent observation in many of these cells.

Examination of the cells at a deeper level in the intima showed that most were of a contractile phenotype surrounded by a dense arrangement of collagen fibres (Figure 4.23).
FIGURE 4.18  T/S Graft stenosis, H&E stain showing highly cellular nature of intima and stenotic lumen. (Mag. x 32).

FIGURE 4.19  T/S of same stenosis, SMA/Millers elastin stain, showing abundance of smooth muscle cells. (Mag. x 32).
FIGURE 4.20  T/S Graft stenosis, SMA/Millers elastin stain. Bands of elastin fibres separate layers of smooth muscle cells within the graft intima. (Mag. x 80).

FIGURE 4.21  T/S Graft stenosis, Alcian blue/PAS stain, showing strong staining for acidic mucopolysaccharides (blue) at the lumenal aspect of the stenosis. (Mag. x 80).
FIGURE 4.22 TEM of smooth muscle cell at the lumenal aspect of vein graft stenosis. A diploid or bilobed nucleus was a common observation. (Mag. x 10,000).
FIGURE 4.23 TEM of a deeper layer of vein graft intima. All of these smooth muscle cells were of the contractile phenotype, lying within a dense arrangement of collagen fibres which comprise the bulk of the stenotic lesion. (Mag. x 4,000).
iv) Viability of cultured veins

1. Transmission electron microscopy

A detailed examination of the cells at all levels in the vein wall revealed a predominance of morphologically normal cells. The intima showed no evidence of necrotic cells. However, occasional cells in the inner media showed signs of swelling and vacuolation suggesting that these may be non-viable (Figure 4.24). Necrotic cells were not observed at any other level in the vein wall.

FIGURE 4.24 TEM of smooth muscle cell in the inner media, showing loss of organised structure, swelling and vacuolation. Such cells were an infrequent observation. (Mag. x 6,600).
2. Contractility

Figure 4.25 shows representative ink recorder tracings from freshly isolated and from cultured veins.

All the fresh veins exhibited a contractile response to each of the agents, demonstrating that both receptor-mediated and membrane-depolarising mechanisms were functional. However, there was a large inter-vein variability to any single agent which could, at least in part, be due to varying degrees of SMC damage during harvesting and handling of the veins.

All the cultured veins contracted in response to each of the agents, but the maximum developed tension was generally less than that of fresh veins, suggesting some loss of smooth muscle cell function. However, although functionally impaired, it is clear that the tissue retains cellular viability as indicated by the development of neointimal proliferation.

An interesting phenomenon frequently observed in cultured veins was the development of spontaneous cycles of contraction and relaxation in response to noradrenaline (Figure 4.25). This was not observed in fresh veins.

In fresh veins precontracted with noradrenaline, the addition of acetylcholine at a range of concentrations from $10^{-8}$ to $10^{-5}$ M caused relaxation of the vessels in a dose-dependent manner, a feature which was not observed in cultured veins. As this is an endothelium-dependent phenomenon, the lack of relaxation in cultured veins suggests a possible functional abnormality in the endothelium. However, because acetylcholine indirectly promotes relaxation via stimulation of EDRF (nitric oxide) release, the effector arm of this bioassay depends upon the assumption that vascular SMC function is unimpaired. One way of investigating this further would be by the use of a direct nitric oxide donor such as sodium nitroprusside, which would then be able to distinguish SMC impairment from endothelial dysfunction.
FIGURE 4.25 Representative contractility recordings for fresh and cultured veins.

*Top panel:* Response to noradrenaline (NA), of a) fresh vein and b) 14 day cultured vein.

*Middle panel:* Response to potassium (K+), of a) fresh vein and b) 14 day cultured vein.

*Bottom panel:* showing cycles of contraction and relaxation observed in cultured veins in response to noradrenaline.
FIGURE 4.26 Graph showing median contractile responses of veins cultured for 0 (fresh), 7 and 14 days. (Bars = range). 5 veins per group.
4.6 Discussion

i) Viability

Several conclusions can be drawn from this validation study. Although the veins were harvested with care and minimal handling, examination of intimal surface morphology showed that some endothelial cell disruption occurred during preparation for culture. Dislodged cells, gaps between cells and endothelial loss were all observed, but principally close to the cut edges. The use of the trypan blue technique facilitated rejection of veins from the study where endothelial injury was too severe.

In cultured veins, endothelial morphology was improved, although SEM revealed some cells which were not typical of endothelium; they were oval in shape and more representative of smooth muscle cells. Transverse sections also showed cells at the intimal surface that failed to stain with the endothelial markers CD 31 or QB.END.10. The most likely explanation for these observations is an alteration in the expression of antigens by the endothelium because all veins after culture completely excluded trypan blue. This observation is in full agreement with the observations of Soyombo and colleagues (1990), who reported the presence of cells at the intimal surface of cultured veins which failed to stain with the endothelial marker *Ulex europaeus* lectin. They also observed that upon SEM, some intimal surface cells had a morphology atypical of endothelium.

In a study of canine vein grafts, Fonkalsrud (1978) described defects between cells, and another study by Schwartz et al. (1975) described dedifferentiated and modified smooth muscle cells in the surface layer of healing arterial lesions in the rat. It would appear, therefore, that smooth muscle cells are able to form a pseudo-endothelial layer *in vivo*, and this could be what is being observed *in vitro* in the vein culture model.

In order to maintain cellular metabolism in culture, the vein wall relies on the adequate diffusion of oxygen and nutrients from the surrounding culture medium. The diffusion distance is, therefore, greatest for the cells of the inner media where an occasional necrotic cell was observed. This was not observed at any other location in the vein wall.
Contractility was also used as a measure of vein wall viability. At days 7 and 14 in culture, the median developed tension in response to noradrenaline, potassium and prostaglandin F2α was significantly reduced when compared with fresh veins. There are a number of possible explanations for this reduced responsiveness in cultured veins. Firstly, damage or trauma to some of the smooth muscle cells during culture preparation may have resulted in their inability to survive in the first few days of culture. This would, in turn, reduce the total number of cells with an accompanying reduced contractile capacity. In support of this theory, the contractile response was no worse at 14 days and was, in fact, better than at 7 days suggesting an element of recovery and not that more cells become non-viable as the duration of culture increases up to 14 days. The level of recovery, however, did not reach significance (P = 0.17, day 7 versus day 14 for K⁺; P = 0.14, day 7 versus day 14 for NA).

A further possibility is that reduced contractility is a function of smooth muscle cell phenotypic change from contractile to secretory, with associated changes in membrane receptors. Such a hypothesis has previously been proposed by De Mey (1989) and Mauger (1975) who reported a similar reduction in contractility in cultured rat and rabbit aorta respectively. TEM examination of cultured vein revealed little morphological evidence of a significant increase in the number of secretory SMC's. Nevertheless, it is still possible that functional changes may have occurred in the absence of gross morphological changes. It is important to remember that the vein culture model is one of no-flow, and under such conditions, an impairment of smooth muscle function in the vein wall may have been induced.

A study by Park and colleagues (1993) showed that human vein grafts placed in the arterial circulation for a mean period of 16 months have a reduced capacity to contract to serotonin and noradrenaline when compared with native saphenous vein. They reported that these changes are not endothelium-dependent and appear to reflect alterations in smooth muscle responsiveness to vasoactive agents in the vein grafts. They also showed that vein grafts lose the ability to relax in response to acetylcholine, an action mediated by the
release of endothelial-derived relaxing factor. This phenomenon has also been demonstrated in rabbit vein grafts (Cross et al. 1988, Komori, 1991). Interestingly, the absence of endothelium-dependent relaxation was also a feature of cultured veins in this study, although this might also have been indicative of impaired SMC function, as already discussed above.

For the assessment of tissue viability I decided to use a functional rather than a biochemical assay, and therefore used the measurement of contractility. From the present studies we may conclude that the tissue is alive but functionally abnormal. It is conceivable that contractility could change for example, due to phenotypic modification in the medial smooth muscle cells without a significant reduction in viability of the tissue. These functional observations combined with the observation that neointimal proliferation occurs in cultured veins suggest significant SMC viability. Furthermore, 14 day cultured veins were able to completely exclude trypan blue, and since dye exclusion tests are based on the premise that intact cellular membranes are necessary to maintain the life of a cell, one can also conclude that the endothelium is viable.

An alternative approach used by others (Soyombo et al. 1990, Angelini et al.1991, Holt et al.1992) is the measurement of adenine nucleotides. These authors demonstrated preservation of ATP/ADP ratios in cultured vein and concluded that organ viability can be maintained over a 14 day culture period.

There is no "gold standard" for the prediction of viability, and extensive studies have been performed and reported by Pegg (1985). It is interesting to note that functional assessment of rabbit arteries by the use of contractility has been recently used by the same group as a test of viability following cryopreservation (Song et al. 1994).

ii) Neointimal development

In this in vitro model, human saphenous vein in culture develops a neointima, consisting of several layers of smooth muscle cells, with a predominance of the secretory phenotype. This has previously been demonstrated by Soyombo et al. (1990), and has also been reported in cultures of human (Barrett et al. 1979), rat (Buck 1977) and pig aorta
(Koo and Gotlieb 1989). The smooth muscle cells of the neointima are surrounded by an extracellular matrix rich in mucopolysaccarides and laminin, and TEM examination also reveals some evidence of early collagen secretion as indicated by the specific presence of "banded" filaments.

**iii) Changes occurring in the intima and media of cultured vein**

A small degree of thickening of the original vein intima was measureable after 14 days in culture (Figure 4.16). Although intimai thickening was not significant, a significant degree of medial thickening occurred over this period (Figure 4.17). This thickening in culture, however, was also a feature observed in veins denuded of endothelium (data not shown) and therefore any endothelial-related action could be confidently excluded.

**iv) Comparison of cultured vein with pathological stenoses**

Before attempting to compare the features of the cultured vein with those of the five graft stenoses studied, it is important to note that two morphological types of stenosis were distinguished. Two stenoses showed a disorganised appearance of smooth muscle cells surrounded by amorphous matrix. This sort of appearance was reported by Marin et al. (1993b) in an analysis of 15 vein graft stenoses. In a study of human arteries, Glagov (1989) referred to this non-atherosclerotic thickening as true "intimal hyperplasia".

However, in the other three stenoses studied, the intima consisted of layers of elastin and smooth muscle cells similar in appearance to that described by Glagov as "intimal fibrous hypertrophy". Morphologically, the vein culture neointima more closely represents the former. Soyombo (1990) also commented that the neointima of human saphenous vein grafts stain for elastin, and that saphenous vein organ culture does not mimic this aspect of the intimal changes in arteriovenous bypass grafts. Glagov proposed that the difference between these two types of intimal thickening is the rate at which the lesions develop; intimal hyperplasia being a rapid proliferative response to severe haemodynamic disturbances, and intimal fibrous hypertrophy a gradual adaptation of the vessel wall to altered haemodynamics.
The TEM features of the superficial layers of the vein graft stenoses were of highly secretory SMC's in a loose extracellular matrix (Figure 4.22), similar to those seen in the neointima of cultured veins (Figures 4.13 and 4.14). However, the TEM appearance of the deeper layers of the stenotic intima was of contractile SMC's in a dense collagen matrix (Figure 4.23). The differing morphology of the SMC's within the same stenosis are probably representative of intimal hyperplasia during various stages of remodelling and maturation (Forrester 1991), the most recently developed intimal hyperplasia being composed of SMC's undergoing proliferation and secretion. In this respect, the cultured vein neointima mirrors this phenomenon particularly well.

One major difference was observed between the vein culture neointima and the pathological stenoses. This was the presence of endothelium-lined channels or "microlumeni" within the neointima of the cultured veins, consistent with the observations of Soyombo et al. (1990). Although new vessel formation is not a common feature in the intima of vein grafts, they have been described by Sottiurai (1983) as "an unusual observation" in an ultrastructural study of thrombosed human grafts. The formation of endothelial tubules has been reported in some advanced atherosclerotic lesions (Gown 1986), and also in vitro (Feder et al 1983, Folkman and Haudenschild 1980).

In this study, the endothelial markers did not produce any staining within the intima of the graft stenoses. The neointimal "angiogenesis" in the cultured vein is therefore the main morphological dissimilarity from what is seen in most vein graft stenoses, and may be worthy of further study in the light of the proposed importance of endothelium-derived mitogenic factors.

4.7 Summary

To summarise, this validation study has expounded a number of important observations:

i) Although the human saphenous vein remains viable in culture for 14 days as shown by trypan blue exclusion studies and neointimal proliferation, there is clearly an impairment of smooth muscle cell function as indicated by reduced contractility.
ii) The degree of endothelial preservation prior to culture can be adequately and rapidly assessed using the trypan blue exclusion technique, with a good level of agreement between two independent observers.

iii) Endothelial integrity is retained, even improved during culture, but with some gaps and the occasional intervening smooth muscle cell.

iv) Endothelium-dependent relaxation appears to be lost, a phenomenon also observed in vein grafts. However, this requires further investigation using a direct SMC relaxant such as sodium nitroprusside in order to distinguish between endothelial and SMC impairment.

v) A neointima develops during culture. Although its structure is a much looser and more fragile arrangement of cells and matrix, it has many of the morphological features of vein graft intimal hyperplasia observed in the superficial layers of graft stenoses.

vi) Neointimal thickness can be quantified by the use of an image analysis system, with a high level of agreement between two independent observers.

vii) Intimal proliferation is detectable in organ culture within a short time period (14 days), thereby highlighting the usefulness and versatility of the model. It has a further advantage of using human tissue thereby avoiding the use of animal models, results of which may be difficult to reproduce or relate to the human vein graft situation.
CHAPTER 5

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PROLIFERATION STUDIES IN HUMAN SAPHENOUS VEIN
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5.1 Introduction

Cellular proliferation is a fundamental biological process, hence the facility to quantify the extent to which it occurs in both normal and pathological material provides a valuable research tool. In order to localise and quantify the proliferating cells forming the neointima in cultured saphenous vein, a reliable and reproducible method was required.

Although smooth muscle cell proliferation is a pivotal event in the development of vein bypass graft stenoses, the kinetics of this process in human tissue have not previously been described. Cellular proliferation can be assessed by a number of techniques including $[^{3}H]$ thymidine labelling, Ki 67 immunostaining, localisation of Proliferating Cell Nuclear Antigen (PCNA) and labelling with the thymidine analogue 5-Bromo-2-deoxyuridine (BrdU). Each of these methods has advantages and also problems associated with their use (Hall and Levison, 1990), therefore a preliminary study was conducted in order to identify the one best suited to assessing proliferation in cultured human saphenous vein.

SECTION A CHOICE OF PROLIFERATION MARKER

5.2 Methods

i) Vein collection and culture procedure

Segments of human saphenous vein were collected, scored for endothelial coverage and established in culture as described in Chapter 4.2i. Each proliferation marker was initially assessed on 10 veins.
**ii) Preliminary proliferation studies**

Assessment of each of the following markers was performed at the end of the 14 day culture period such that comparisons could be made between them:

(i) **Ki 67.** This antibody recognises a poorly characterised nuclear antigen associated with the cell cycle, being expressed in all phases except G0. Ki 67 is only effective on frozen sections, hence portions of the cultured veins were snap frozen in liquid nitrogen at the end of the 14 day culture period prior to sectioning followed by immunostaining. (Appendix 1).

(ii) **Proliferating Cell Nuclear Antigen (PCNA)** is a cellular protein involved in DNA synthesis which can be detected immunologically. PCNA expression was examined using paraffin-embedded tissue and localised using PC10 monoclonal antibody (DAKO, High Wycombe, Bucks.) (Appendix 1).

(iii) **Tritiated thymidine labelling.** This is considered to be the "gold standard" for cell kinetic studies, the method requiring viable cells to take up [3H] thymidine and for this to be incorporated into DNA. Using scintillation counting, this technique provides a rapid method for analysing thymidine uptake during DNA synthesis.

Vein segments were pulsed with 2μCi/ml [3H] thymidine, Specific Activity 37 MBq/ml, (Amersham, Bucks.) for 18-24 hours. Analysis of thymidine uptake into DNA was a modification of the method of Loeb et al. (1986). Following incubation with the radiolabelled thymidine, the vein was rinsed in ice-cold saline, blotted dry and weighed. It was then homogenised in an heat-resistant glass tube in 12 ml (2 x 6ml) of 0.2N perchloric acid using a Polytron tissue homogeniser (Northern Media Supplies, North Humberside, U.K.) and centrifuged at 1000g and 4°C for 10 minutes. The supernatant was discarded and the pellet washed and centrifuged twice more with 0.2N perchloric acid in order to remove any residual unincorporated thymidine. The pellet was resuspended in 2ml of 0.5N perchloric acid and heated in a water bath at 90°C for 20 minutes in order to hydrolyse and
extract the DNA. This was immediately cooled on ice and recentrifuged for 10 minutes at 1000g to separate the DNA in the supernatant from the protein (pellet).

A 0.5 ml aliquot was taken into a scintillation vial with 10 ml scintillation cocktail (Optiphase, Fisons, Loughborough, U.K.) and counted in a liquid scintillation counter (Wallac 1217 Rackbeta, Finland) in order to measure the amount of [\(^3\)H] thymidine incorporated into DNA. The final result was termed as the "thymidine index" and expressed as counts per minute per microgram of DNA (cpm/\(\mu\)g DNA).

The DNA content of the vein segment was determined by a modification of the colorimetric assay described by Burton (1965). One millilitre of the DNA extract prepared as described above was mixed with 2 ml of a chromogenic reagent comprising 1.5 g diphenylamine (Fisons, Loughborough), 100 ml glacial acetic acid, and 1.5 ml concentrated sulphuric acid. Immediately prior to use, 0.1 ml of a solution of aqueous acetaldehyde (16 mg/ml) was added to each 20 ml of reagent to provide the working reagent. The samples were then incubated in a water bath at 30°C for 16-20 hours for colour development.

A DNA standard curve was prepared at the same time using double stranded calf thymus DNA (Sigma, Poole, Dorset). A working solution was prepared by heating equal volumes of a stock standard DNA solution at a concentration of 0.4 mg/ml in 5 mM sodium hydroxide, and 1 N perchloric acid at 70°C for 15 minutes. This produced a standard working solution of 200 \(\mu\)g/ml which was serially diluted with 0.5 N perchloric acid to achieve a range of DNA concentrations from 0-100 \(\mu\)g/ml. 2 ml of the diphenylamine reagent were added to 1 ml of each of the standards, and incubated alongside the unknowns.

The absorbance of each sample from the standards and extracted venous DNA was read in a Unicam SP1800 ultraviolet spectrophotometer (Cambridge, U.K.) at 600 nm. A DNA standard curve was constructed, from which the DNA concentration of the vein extract could be determined.

In order to measure the efficiency of the DNA extraction procedure, the protein pellet remaining after extraction of the DNA was solubilised in 1 ml of Optisolv tissue
solubiliser (Fisons, Loughborough). The solubilized pellet was then added to 10ml of
scintillation cocktail and counted as described above.

In order to study whether thymidine incorporation was uniform along the wall of
any one vein, tritiated thymidine uptake within a given vein was studied by dividing
individual lengths of vein into segments of equal size (normally 2-4 pieces depending on
the length available) and comparing the thymidine indices between these segments. This
procedure was performed on 4 fresh veins cultured with [3H] thymidine for 24 hours, and
6 veins cultured for 14 days with the addition of [3H] thymidine for the final 24 hours.

Although [3H] thymidine labelling is a widely used and well documented technique
for measuring proliferation, when using the method as described above, histological
analysis and identification of specific cell types is not possible. To some degree, this
problem can be overcome by the use of autoradiography. However, in Chapter 4 the
problem of distinguishing between the different layers of the vein wall using standard
stains such as H&E was demonstrated, owing to a wide variation in the thicknesses of the
intimal and medial layers within normal veins. Although the use of an autoradiographic
technique is able to mark any proliferating cells, it is virtually impossible to confidently
identify their precise location within the vein wall with accuracy. Additionally, the time
required for film exposure amounts to several weeks and with it, the accompanying
problem of radiation containment.

iv) 5-Bromo-2-Deoxyuridine labelling. This is a method of assessing
incorporation of a thymidine analogue into DNA during S phase and localising proliferating
cells with a specific monoclonal antibody. Initial results with a 24 hour labelling period at a
1:1000 dilution were disappointing and failed to produce any labelled cells. In several
subsequent trials in which labelling times and concentrations were varied, strong labelling
of individual cells was seen after a 72 and 96 hour incubation with the labelling agent at a
dilution of 1:1000. These results suggest that the proliferating cells in culture have a long
cell cycle time with a long S phase. It has also been reported that BrdU is potentially toxic.
(Doolittle 1992) and therefore continuous labelling for the culture duration may alter the proliferation profile of the vein. A similar problem was reported by Soyombo et al. (1990) in the use of \(^{3}H\) thymidine, therefore in the preliminary evaluation of BrdU, each vein was cut into three equal portions, cultured under standard conditions (Chapter 4), and incubated with BrdU in the following manner:

a) 14 day culture, continuous labelling.
b) 14 day culture, no labelling.
c) 14 day culture, labelled between days 10 and 14.

At the end of the culture period, veins were immediately formalin-fixed, processed, and 4\(\mu\)m sections were stained with anti-BrdU antibody (DAKO M744, High Wycombe, U.K.) using the avidin binding complex technique (Appendix 1). Sections were also stained with SMA/Millers elastin such that neointimal thickness could be measured.

To investigate the effect of continuous exposure to BrdU, the neointimal thickness of the unlabelled portion of each vein was compared with that of the continuously labelled portion for the 14 day culture period.

Proliferation was quantified by counting the total number of neointimal-labelled and unlabelled cells and calculating a proliferation index by expressing BrdU-labelled cells as a percentage of the total number of neointimal cells along the section length. Counts were made by two independent observers on each high power field (x 320) across the entire transverse section of three consecutive sections and the mean taken as the final result. The inter-observer error for this technique was assessed in the first 12 veins.

The within-vein variation for this technique was also studied by culturing segments (2-4) of the same vein under identical conditions and then comparing the proliferation indices for these segments.
5.3 Results

i) Ki 67. The results obtained with this marker were disappointing; apart from an occasional positively stained cell, the majority were negative (Figure 5.1). The requirement for frozen sections was the main disadvantage of this method because all the other immunohistochemical techniques used in the project were applied to paraffin sections. Loss of morphology through the use of frozen tissue sections emphasized a further problem in the use of Ki 67, because maintenance of the vein wall integrity represented an important part of the study.

ii) PCNA. More staining was seen with this proliferation marker, however the extent was variable and the pattern of staining was difficult to interpret. Both nuclear and cytoplasmic staining was observed, with enhanced staining at the cut edges of the vein (Figure 5.2).

It is widely known that there are inherent problems with PCNA (McCormick and Hall 1992). Firstly, owing to its long half-life in the tissues it will identify cells which are no longer proliferating. There is also a suggestion that PCNA is expressed in response to other stimuli such as trauma, which could explain the enhanced staining at the cut edges of the vessel. Reservations as to the usefulness of this marker are consistent with those of Francis et al. (1992) who reported that immunolocalisation of PCNA in cultured coronary artery gave an overestimation of the growth fraction when compared with autoradiography, and that a significant number of cells positive for PCNA were not labelled with $[^3H]$ thymidine after a 24 hour pulse time. A study by Scott et al. (1991) also emphasized that caution is needed when PCNA is used to estimate cellular proliferation in histological material. For all of these reasons it was concluded that PCNA was not suitable in this particular model, although under the right conditions and in other tissues it is a useful marker of cell proliferation.
FIGURE 5.1
Vein segment stained for Ki 67, showing predominance of negatively stained cells and hence its lack of suitability for the assessment of proliferation in the vein culture model. (Mag. x 160).

FIGURE 5.2
Vein segment stained for PCNA using PC10 monoclonal antibody. There is a large amount of both nuclear and cytoplasmic staining and hence interpretation of the results is difficult. (Mag. x 160).
iii) Tritiated thymidine labelling

The method used for DNA extraction was very efficient, the results of which are detailed in Table 5.1.

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<th>CPM in supernatant</th>
<th>CPM in pellet</th>
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<td>1248</td>
<td>97.1</td>
</tr>
<tr>
<td>2</td>
<td>35048</td>
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<td>900</td>
<td>68</td>
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</table>

**MEAN EFFICIENCY** 95.5%

**TABLE 5.1** Showing figures for efficiency of the DNA extraction technique, mean recovery = 95.5%.
In fresh veins, i.e. cultured for only 18-24 hours in the presence of [3H] thymidine, the mean DNA content was 2.1μg/mg wet weight (SD = +/- 1.2). This was not significantly different from veins cultured for 14 days, mean DNA content 1.9 ( +/- 1.6) μg/mg wet weight. (Mean difference 0.2, 95% confidence interval -0.6, 1.0, Students t-test).

For the fresh veins, the mean thymidine index was 27cpm/μg DNA, range 20-38, with little variability within or between veins (Figure 5.3). However, a different picture was seen in cultured veins; the mean thymidine index was 1412, range 201-2878 showing much greater variability, particularly between veins (Figure 5.4).

Using this method of assessing proliferation, one can only measure total thymidine uptake in the whole vein segment, thus emphasising a need to precisely localise the cells undergoing proliferation. At this stage the use of autoradiography was considered, but it was generally felt that immunohistochemical techniques would be particularly valuable because of their ability to define the different layers in the vein wall and hence yield more information without the hazards accompanying the use of radioisotopes.
FIGURE 5.3 Scatter plot to show within-vein variation in thymidine uptake in fresh veins. The mean thymidine index was 27 cpm/µg DNA (range 20-38).

FIGURE 5.4 Scatter plot to show within-vein variation in thymidine uptake in cultured veins. The mean thymidine index was 1412 cpm/µg DNA (range 201-2878).
iv) 5-Bromo-2-Deoxyuridine labelling

Using a pulsing period of 72-96 hours, this marker provided a wealth of information on both the location and number of proliferating cells. It was therefore decided that this method was most ideally suited to the assessment of proliferation in the vein culture model.

By the use of continuous labelling of the 14 day cultured veins, it was envisaged that the total number of cells that had undergone proliferation might be quantified. However, when the neointimal thicknesses of the two groups (continuously labelled vs unlabelled) were compared, a significant reduction in neointimal thickness was observed in the labelled group (Median difference = 14.0, 95% confidence interval = 7.5, 19.5, P = 0.0002, Wilcoxon paired rank test, Figure 5.5). This observation clearly demonstrated a toxic effect of continuous labelling which did not occur when the veins were pulse labelled. No significant difference in neointimal thickness was observed when comparing 14 day unlabelled veins with 14 day veins exposed to BrdU for only the final 96 hours. (Median difference 2.0, 95% confidence interval = -6.0, 2.5, P = 0.34, Wilcoxon paired rank test, Figure 5.5).
FIGURE 5.5 Scatter plot to show effect of 96 hour pulse labelling and continuous BrdU labelling on neointimal thickness of 14 day cultured veins. Continuously labelled veins developed significantly less neointima than pulse-labelled or unlabelled veins. There was no significant difference in the neointimal thicknesses of the pulse-labelled and unlabelled groups.

Proliferation indices were measured by 2 independent observers and the inter-observer agreement, assessed in the first 12 veins, is shown as a Bland-Altman plot in Figure 5.6. Using this method there was a good agreement between observers, the mean difference between observers was 0.75%, and the 95% limits of agreement +/- 7.5%. The proliferation indices from segments of the same vein are shown in Figure 5.7, showing that there was little variability within veins.
FIGURE 5.6 Bland-Altman plot of inter-observer error in measurement of proliferation index. The mean difference between observers was 0.75%, and the 95% limits of agreement +/- 7.5%.

FIGURE 5.7 Scatter plot to show within-vein variation in BrdU labelling in cultured veins. The mean neointimal proliferation index was 22%.

The BrdU method was therefore chosen for the following study to further define the kinetics of proliferation in cultured human saphenous vein.
SECTION B  KINETICS OF PROLIFERATION IN HUMAN SAPHENOUS VEIN ORGAN CULTURE.

5.4 Methods

For this part of the study a further 10 veins were studied. Each vein was carefully cut into six equal portions, cultured under standard conditions and labelled with BrdU sequentially in the following manner:

(i) 4 day culture, labelled between days 0 and 4.
(ii) 7 day culture, labelled between days 4 and 7.
(iii) 10 day culture, labelled between days 7 and 10.
(iv) 14 day culture, labelled between days 10 and 14.
(v) 18 day culture, labelled between days 14 and 18.
(vi) 21 day culture, labelled between days 18 and 21.

At the end of the designated culture period, each segment was formalin-fixed and processed as described above in order to identify and localise the proliferating cells during each time period. Counts were made by two independent observers and proliferation indices calculated as described above. Sections were also stained with SMA/Millers elastin stain such that measurements of neointimal thickness could also be recorded for each time period.

5.5 Results

The results of the measurements of proliferation indices and neointimal thickness are summarised in Figure 5.8. There was no evidence of any proliferating cells in any of the veins during the first 4 days in culture. The first burst of proliferation was observed in the superficial layers of the vein intima between days 4 and 7 (Figure 5.9), reaching a peak value between days 10 and 14 (P < 0.003, days 4-7 vs days 10-14). Neointimal development initially lagged behind these proliferative events, with a marked increase in neointimal thickness occurring between days 7 and 10, but this finally also reached a maximum by day 14 with many cells throughout the neointima staining positively with


BrdU (Figure 5.10). After 21 days in culture, the proliferation index had dropped dramatically and the majority of proliferating cells were confined to the lumenal surface of the neointima, with negligible proliferation in the deeper layers (Figure 5.11). However, the neointima was maintained with no further significant increase in thickness throughout the period of the study.

FIGURE 5.8 Line graphs to show the relationship between proliferation index and neointimal thickness with time. Bars = 95% confidence intervals.
FIGURE 5.9  BrdU localisation of proliferating cells in the superficial layers of the vein intima between the fourth and seventh days of culture. (Mag. x 320).

FIGURE 5.10  Proliferation reached a peak between the tenth and fourteenth days of culture, with many BrdU-labelled cells throughout the neointima. (Mag. x 320).
FIGURE 5.11 After 21 days in culture there was no further increase in neointimal thickness and the majority of proliferating cells were confined to the superficial layer of the neointima. (Mag. x 320).

FIGURE 5.12 In some veins increased BrdU staining was observed at the cut edges and in the adventitia. (Mag. x 32).
In some veins, increased BrdU staining was observed in the adventitia in association with the vasa vasorum and also at the cut edges of the vein (Figure 5.12). However, there was no visual evidence of any cellular migration, therefore there is no suggestion that cells migrating from the cut edges could contribute to neointimal formation. Labelled cells were first seen in the superficial layers of the vein intima beneath the areas where neointima was initially forming (Figure 5.9), suggesting that it is this population of cells which lead to the production of the neointima.

5.6 Discussion

(i) Choice of proliferation marker

In the current study the relative values of a number of proliferation markers were assessed in the vein culture model. The markers described in this study all measure slightly different variables and although they are comparable they are not identical, each one having its own limitations (Hall and Levison 1990). The main drawback of Ki 67 was the necessity for frozen sections since it required manipulation of the vein segment at the end of the culture period such that half could be formalin-fixed and half frozen, with the accompanying disruption of the vein wall architecture. Although this methodology has successfully been employed in many disease states, particularly neoplasia, it was not considered to be well suited to our own needs principally because it could not be used on formalin-fixed tissue.

The complexity of PCNA has been highlighted by McCormick and Hall (1992) who reported that the use of PCNA antibodies as markers of cell proliferation is not simple and straightforward but requires careful analysis. In our assessments using the PC 10 antibody, staining was erratic which therefore questioned the validity of our results. In view of other cautionary communications (Scott et al. 1991, Francis et al. 1992) it was concluded that PCNA was probably not the best marker for our studies.

Although thymidine labelling was reproducible in fresh veins, in my experience it was very variable in cultured veins, additionally it was difficult to be precise in differentiating the different layers of the vein wall when using autoradiographic techniques. Thymide labelling is a popular and widely used technique in a vast range of conditions, but
I would conclude that bromodeoxyuridine was the best available marker for use in our particular studies. The method was reproducible and also allowed precise localisation and quantification of proliferating cells within any given time period.

In the [3H] thymidine method, the technique of DNA extraction was found to be very efficient, and it was found that the DNA concentration of veins was unchanged after culture. It was demonstrated in Chapter 4.5ii that significant medial thickening along with a little intimal thickening occurred during the culture period. The fact that the DNA (μg/mg tissue) concentration of the vein remained unchanged suggests that these increases were the result of cellular proliferation rather than hypertrophy or matrix accumulation.

All freshly isolated veins had low, reproducible thymidine indices, but in veins after culture not only did the magnitude of uptake increase, but there was greater variability between veins suggesting that they do not all behave similarly in culture. This observation highlighted that in all culture experiments a paired design was imperative in order to exclude the problem of between-vein variability.

A wide variation in thymidine indices was also seen within the same vein after culture. The source of such a variation may be proliferation occurring in the adventitia and at the cut edges of the vein segment which is included in the thymidine index. Proliferation in these areas is undoubtedly contributing to the amount of thymidine uptake and therefore "swamping" any significant neointimal changes which might be occurring. Thus, although [3H] thymidine is successfully employed by others, I have found BrdU to be more reliable in our hands as a marker of cell proliferation.

In support of these proposals, the BrdU technique showed less variability within and between veins, with the additional benefit of being able to exclude proliferative activity in the adventitia and at the cut edges. Although the use of [3H] thymidine labelling is still a sensitive and widely used technique, the reproducibility of this method in cultured veins was subsequently found to be inferior to that of BrdU labelling. A particular advantage of the BrdU technique is that it allows not only the quantification of cellular proliferation, but also its precise localisation, with the additional benefit of being able to exclude background proliferation from the adventitia and cut edges.
ii) Bromodeoxyuridine toxicity

Both \([H] \) thymidine and BrdU are capable of producing toxic effects and can possibly influence cell proliferation in experimental studies (Clowes and Schwartz 1985). In a study by Hanke et al. (1990) the cytotoxic effects of BrdU were minimised by limiting \textit{in vivo} labelling in the rabbit to a time interval of 18 hours prior to excision of the vessels.

The present study has demonstrated that continuous labelling of the vein cultures with BrdU produced toxic effects that could be abolished by the technique of sequential labelling over a period of 72-96 hours. The continuous BrdU labelling showed that only 20-30\% of the neointimal cells remained unlabelled, suggesting that they had not proliferated and presumably had migrated from the intima. Migration from the vein intima is certainly the most likely source of these unlabelled cells because BrdU, once incorporated into a replicating cell, remains confined within the cell and is perpetuated through any subsequent generations (personal communication, J.H. Pringle, Dept. Pathology, Leicester University). The contribution of cellular migration in neointimal formation could be better studied by the use of a fluorochrome such as PKH2 (Zynaxis Cell Science Inc, USA). This is a fluorescent cell linker which can be used to label virtually any cell type either \textit{in vivo} or \textit{in vitro} (Horan et al 1988, Jensen et al. 1990). Potentially, therefore, smooth muscle cells in the vein wall could be labelled prior to culture and detected in histological sections after culture under a microscope equipped with fluorescein filters in order to observe any labelled (i.e. migratory) cells in the neointima. Although this method should give an estimate of the relative contribution of migration in the formation of neointima, it would not determine the original source of the migrated cells (intimal or medial) owing to the inability to selectively label either intimal or medial smooth muscle cells at the start of the study.

In animal studies of arterial intimal hyperplasia, approximately 50\% of the neointimal cells are migrating, non-dividing smooth muscle cells (Clowes 1985). However, it has been suggested by Kohler (1989) that in vein grafts proliferation is more important than migration, in agreement with the present observations made in this study.

It must, however be emphasised that because of the toxic effects of continuous BrdU labelling on neointimal thickness, that the percentage of proliferating cells in the
neointima can only be used as an estimate because the normal proliferation profile may have been affected.

**iii) Proliferation kinetics of the model**

The results of the second part of this study demonstrate a burst of cellular proliferation between the fourth and seventh day in culture. At this time there were only few neointimal cells, but high levels of labelling with BrdU. This observation is consistent with those of Dilley et al (1992) who showed that in a rat vein graft model, five days after implantation there were few neointimal cells, but high [³H] thymidine labelling levels in the graft intima. In these grafts it was shown that high labelling levels were maintained throughout the most active neointimal development (5-14 days after grafting), and declined steadily up to 28 days, by which time they had returned to baseline levels. These observations are mirrored in the vein culture model, with the proliferation index reaching a maximum of 42% by day 14, declining rapidly thereafter to a level of 14% by the 21st day. It was not feasible to extend the study beyond three weeks, owing to progressive loss of tissue viability after this time, smooth muscle cell necrosis and loss of contractile responses to vasoactive agents.

Clowes and Clowes (1985) reported similar findings using a balloon-injured rat carotid artery as their model. Following balloon injury at 7, 14 and 28 days, the intima became progressively thicker as cells accumulated, and intimal thickening was maximal at 28 days. Smooth muscle cell proliferation assessed by [³H] thymidine-labelling indices, was greatest at 7 days in the intima and returned towards baseline by 4 weeks.

Hanke et al. (1990) used angioplasty of the atheromatous rabbit carotid artery as a model for investigating and quantifying intimal proliferative responses using BrdU labelling. Smooth muscle cell DNA synthesis preceded the onset of intimal thickening, with a peak value of DNA synthesis occurring during the first 7 days after injury. The number of SMC's undergoing DNA synthesis decreased between days 7 and 14 after angioplasty, until cell division approached baseline levels between days 21 and 28 after interventional treatment.
Although the results of these animal studies are similar to those of the present study, it must be noted that each of these investigations was performed on balloon-injured arteries. Arterial intimal hyperplasia develops when cell proliferation and migration are significantly increased after trauma and accompanying endothelial denudation (Clowes et al. 1983, Clowes and Clowes 1985). However, the surgical trauma which occurs during harvesting and insertion of a vein graft is also a likely stimulus for increasing cell proliferation. Experimental models of vein grafting have provided us with information on the histogenesis of neointimal hyperplasia, but evidence on the role of cellular proliferation and its quantification is scant, as shown for example by mitotic figures (Brody et al. 1972), or $^{3}$H thymidine uptake (Zwolach et al. 1987). It is therefore an important observation that the kinetics of the in vitro study described here parallel those observed in vivo by Dilley and colleagues.

The control mechanisms of SMC proliferation in the vein wall are as yet unknown, but cells in the vessel wall are capable of producing growth factors and growth inhibitors (Castellot et al. 1981, Schwartz et al. 1986, Luscher 1989, Tennant and McGeachie 1990). In order to develop possible means of controlling the development of intimal hyperplasia in vein grafts, the modulation of autocrine or paracrine control on SMC growth in response to physical stimuli has been suggested (Nilsson et al. 1985, Campbell et al. 1988). With regard to potential pharmacological treatment, from the results of this kinetic study and those referred to above, it would appear to be important that any form of antiproliferative therapy should be commenced at an early stage, either at the time of surgery or immediately following vein grafting.

5.7 Summary

Having demonstrated that organ culture of the human saphenous vein closely mimics the intimal hyperplasia that underlies vein graft stenoses (Chapter 4), and that the technique of interval pulsing with BrdU allows localisation and quantification of proliferating cells at a given time, this model provides a useful experimental system for further investigation into the aetiology and amelioration of human vein graft stenoses.
CHAPTER 6

THE ROLE OF THE ENDOTHELIUM IN THE PROMOTION OF INTIMAL PROLIFERATION IN HUMAN SAPHENOUS VEIN

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6.1 Introduction

The vein used as a bypass graft is inevitably "disturbed" during the harvesting and implantation procedure. Although the graft is subsequently exposed to a number of potentially injurious stimuli, such as inflammatory mediators and the haemodynamic stresses of the arterial circulation, the aim of this study was to investigate the effect of endothelial denudation on the development of intimal hyperplasia. Intimal hyperplasia has been likened to an aberrant vascular wound healing response (Forrester 1991) and the cellular response to physical injury in organ culture has already been studied by a number of workers (Pederson and Bowyer 1985, Gotlieb and Boden 1984, Fingerle and Kraft 1987), all of whom have reported that cultured vessels can mount a reparative response to injury. The observation that intimal proliferation occurs in an organ culture model of the human long saphenous vein was first reported by Soyombo and colleagues in 1990. Increased cellular proliferation reported by Angelini and colleagues in 1991 directly supports the idea that physical injury can lead to graft hyperplasia with resultant stenosis. To provide a foundation for the following experimental chapters, this study sets out to consolidate the observations that intimal proliferation can be influenced by the endothelium.

6.2 Patients, materials and methods

i) Vein collection and culture procedure

Saphenous vein samples were obtained from 20 patients undergoing arterial surgery. All these samples were macroscopically normal, and dissected with minimal handling before any distension procedure. They were transported to the laboratory with minimum delay in calcium-free Krebs solution and prepared for organ culture. All veins were assessed for endothelial coverage before culture by the use of trypan blue exclusion as described in Chapter 4. Any veins with less than 70% endothelial coverage were rejected from the study. Organ cultures were established and maintained using the technique already described in Chapter 4.
ii) Experimental design

For each of the 20 veins in the study, paired samples were established in culture as shown in Figure 6.1. One of the segments was cultured intact, and the endothelium was removed from the other by gentle abrasion with a moistened sterile cotton wool bud (Figure 6.2). This procedure was very efficient (Figures 6.3 & 6.4) and did not cause any structural damage to the vein as the internal elastic lamina was still easily localised, thereby facilitating measurements of intimal thickness.

At the end of the 14 day culture period the veins were fixed overnight in 10% formalin and processed as described in Chapter 3.2i and ii. Sections were stained with the combined SMA/Millers elastin stain and measurements of neointimal thickness were made on each vein section. The neointima was defined as the new cellular layer developing in culture beneath the endothelium on the luminal side of the internal elastic lamina. All measurements were made by two independent observers as described in Chapter 3.2 iii.

iii) Statistics

Summary data are expressed as median and range. Differences between the groups were analysed using the Wilcoxon paired rank sum test, with 95% confidence intervals.
FIGURE 6.1  Showing experimental design

20 paired segments of vein cultured separately

FIGURE 6.2 Rubbing method for endothelial denudation
FIGURE 6.3  Representative section of intact vein before culture. Viable endothelial cells exclude trypan blue. Endothelial coverage on this vein is good, with staining confined largely to the cut edges.

FIGURE 6.4  Trypan blue staining of vein segment demonstrating efficient removal of the endothelium.
6.3 Results

Histological examination of intact vein segments showed the development of a subendothelial, cellular neointima which was clearly distinguishable from the original vein intima, and staining positively for smooth muscle actin (Figure 6.5). The neointimal thickening was significantly (P = 0.0001) greater in intact veins than in those denuded of endothelium, median thickness 24.5 µm (range 6.5 - 81.0) versus 2.5 µm (range 0 - 27.5) (Figure 6.6). Trypan blue staining of the veins after culture revealed the maintenance of a virtually intact endothelium in the intact veins, with no endothelial regeneration in the denuded veins. This observation was further supported by immunostaining with QB END.10 (Figure 6.7) and by scanning electron microscopy (Figures 6.8 & 6.9). These results therefore suggest that the presence of endothelium normally promotes intimal proliferation.

FIGURE 6.5 Smooth muscle actin / Millers elastin stain on saphenous vein showing development of the neointima. (arrowed)

a) before culture and b) after 14 days culture.
FIGURE 6.6 Effect of endothelial denudation on neointimal thickness in cultured veins
Median (range) thickness of intact veins = 24.5 (6.5 - 81.0) μm
Median (range) thickness of denuded veins = 2.5 (0 - 27.5) μm

FIGURE 6.7 QB END 10 stain on 14 day cultured vein showing presence of intact endothelium on the lumenal surface of the neointima. (Mag. x 160).
FIGURE 6.8 Scanning electron micrograph of a) intact vein before culture, and b) vein denuded of endothelium, exposing sub-endothelial matrix. (Mag. x 1,000).

FIGURE 6.9 SEM of intact vein after culture, demonstrating intact endothelium. There was no evidence of endothelial regeneration in the denuded vessels. (Mag. x 1,000).
6.4 Discussion

This study clearly highlights the role of the *in vitro* endothelium as a promoter of intimal SMC proliferation, supporting the earlier findings of Angelini and colleagues (1991), and Koo and Gotlieb (1989), in human saphenous vein and pig aorta respectively. Such observations provide strong evidence for an endothelium-derived proliferation promoting factor, the nature of which remains undetermined at the present time. The endothelium has a powerful influence on neointimal formation in the vein culture model, thereby suggesting that it may be a major contributory element in the aetiology of intimal hyperplasia. Previous *in vivo* studies using animal models (Haudenschild and Schwartz 1979) have cast the endothelium as an inhibitor of intimal proliferation, and therefore emphasised that maintenance of endothelial integrity during graft preparation is a worthwhile goal. However, although endothelial preservation is important for the prevention of early graft thrombosis, evidence that it prevents intimal hyperplasia is inconclusive (Storm et al. 1975, Angelini et al. 1992).

In conclusion, the endothelium undoubtedly plays a critical role in the formation of neointima in this model of vein graft intimal hyperplasia. The smooth muscle cells in the superficial layers of the vein intima proliferate in order to form the neointima (Chapter 5). The overlying endothelium clearly has a strong influence on these superficial cells, although it could be argued that the high concentration of serum mitogens in the culture medium may be sufficient to induce intimal SMC proliferation. Although this is a possibility, it does not explain the absence of proliferation in the endothelium-denuded vein. The observations made in this study suggest instead that an additional endothelium-derived factor initiates proliferation of the superficial SMC's, which can be readily explained by a concentration gradient of this factor.

Clearly, any approach for improving the long-term patency rates of bypass grafts would benefit substantially from the ability to isolate and characterise this factor such that future therapeutic regimes could be targeted against its action.
CHAPTER 7

HUMAN SAPHENOUS VEIN COCULTURE IDENTIFIES
A PARACRINE ACTION OF THE ENDOTHELIUM

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HUMAN SAPHENOUS VEIN COCULTURE IDENTIFIES A PARACRINE ACTION OF THE ENDOTHELIUM

7.1 Introduction

Advances in cell culture techniques have resulted in a greater understanding of the structure and function of vascular endothelial and smooth muscle cells. Interactions between the two cell types contribute not only to the normal function of the vascular wall, but also the pathogenesis of various disease states including atherosclerosis and intimal hyperplasia. Although it would be ideal to study endothelial/smooth muscle cell interactions in vivo, the complexity of this environment would make the task difficult. By contrast, the study of cells in isolated culture has led to important discoveries but may be a poor model of the in vivo environment. To better simulate the vascular wall in an in vitro environment, a number of laboratories have reported the use of coculture techniques in which endothelial cells and smooth muscle cells are cultured in close proximity (Davies and Kerr, 1982; Jones, 1979; Van Buul-Wortelboer et al. 1986; D'Amore, 1992; Gajdusek et al. 1980). The designs of such techniques are variable, but one that best replicates the in vivo structure is a model in which endothelial cells and smooth muscle cells are grown on opposite sides of a thin semipermeable membrane (D'Amore, 1992).

Although no cell culture method can perfectly mimic the normal endothelial cell/smooth muscle cell environment, coculture can provide an important contribution to extending our knowledge of vascular wall biology. Having already established and maintained an organ culture of human saphenous vein (Chapter 4), the use of this model has demonstrated the important role played by endothelium in the development of a neointima (Chapter 6). The work described in this study utilises a coculture technique to further elucidate the role of the venous endothelium. This method is potentially superior to cellular coculture, as it also retains the normal orientation of the cells of the vessel wall together with the maintenance of the basement membrane and extracellular matrix components.
7.2 Patients, Materials and Methods

i) Vein collection and culture procedure

In this investigation 10 veins were studied. The criteria and conditions of vein collection and culture were identical to those described in the previous chapter (6.2 i).

ii) Experimental design

In this study, four segments were prepared from each vein. One of the segments was cultured intact, and another was denuded of endothelium prior to culture. Additionally a coculture was established, comprising both an intact and a denuded segment (Figure 7.1 & 7.2). All cultures were maintained for 14 days, and 5-bromodeoxyuridine was added to the medium at a 1:1000 dilution for the final 72 hours of culture. Vein segments were then fixed overnight, processed and paraffin-embedded. Sections were cut and stained with SMA /Millers stain and anti-bromodeoxyuridine antibody. Measurements of neointimal thickness were made on each section as previously described (Chapter 6.2 ii), and a proliferation index was calculated for each sample as described in Chapter 5.2iv.

iii) Statistics

Summary data are expressed as median and range. Differences between the groups were analysed using the Wilcoxon paired rank sum test, with 95% confidence intervals.
FIGURE 7.1  Diagrammatic representation of experimental design.
)a) Intact vein alone
b) Denuded vein
c) Denuded vein cocultured alongside intact vein

FIGURE 7.2  Photograph of veins prepared for coculture. Denuded vein segment alongside intact.
7.3 Results

i) Neointimal thickness

In this series of experiments, as observed in the previous chapter, a significantly thicker neointimal layer developed in the intact veins cultured separately from the denuded veins, median 28 μm (range 11 - 53) versus 0 μm (range 0 - 13) (Median difference 28, 95% confidence interval = 16.5, 41.0, P < 0.001). However, the denuded veins cocultured with intact veins developed a thicker neointima than denuded veins alone, median thickness 12 μm (range 0 - 34) (Median difference 12, 95% confidence interval = 4.0, 18.0, P = 0.01). This thickening, however, was less than that observed in individually cultured intact veins. (Median difference 16, 95% confidence interval = 3.0, 31.0, P < 0.01). These results are summarised in Figure 7.3.

In each of the cocultures the vein segments were separated by a distance of approximately 1cm and the paracrine effect of the intact endothelium was observed in all but one of the denuded segments. At the end of the culture period the denuded segments were still devoid of endothelium; this was confirmed by scanning electron microscopy and the absence of any QB.END.IO staining on the paraffin sections.

ii) Proliferation indices

Histological examination of the cocultured vessels demonstrated that the majority of cells undergoing proliferation were located in the superficial layers of the intima and throughout the neointima, similar to that observed in intact vessels.

Proliferation indices followed the same trend as neointimal thickness, that is, intact veins showed significantly more proliferative activity than denuded; 31% (range 23 - 42) versus 10% (range 6 - 16) (Median difference 21, 95% confidence interval = 17.0, 28.0, P < 0.001). The proliferation index of denuded veins in coculture was 22% (range 18 - 28), greater than denuded veins (Median difference 12, 95% confidence interval = 9.5, 17.0, P < 0.001) but still less than the value of intact veins (Median difference 9, 95% confidence interval = 3.5, 13.5, P < 0.01). These results are summarised in Figure 7.4. Proliferation in the vein media, assessed by the bromodeoxyuridine technique, was of the order of 2 - 5% in all veins.
FIGURE 7.3  Scatter plot of neointimal thicknesses of cocultured veins.
Median (range) Intact veins = 28 (11-53) μm
Median (range) Denuded veins = 0 (0-13) μm
Median (range) Denuded-cocultured veins = 12 (0-34) μm

FIGURE 7.4  Scatter plot of proliferation indices of cocultured veins.
Median (range) Intact veins = 31 (23-42)%
Median (range) Denuded veins = 10 (6-16)%
Median (range) Denuded-cocultured veins = 22 (18-28)%
7.4 Discussion

i) A paracrine role for the endothelium

The results of this study highlight a paracrine role for the endothelium in the promotion of human venous intimal smooth muscle cell (SMC) proliferation in vitro. For reasons already discussed in Chapter 6.4, this effect is unlikely to be due to serum mitogens in the culture medium and therefore a soluble endothelium-derived mediator must be involved. This study has shown that when denuded veins are cocultured with intact veins, SMC proliferation is enhanced in the denuded segments. These findings confirm that, in this model, the endothelium is playing an important role in the formation of neointima by secreting a soluble mediator which can stimulate proliferation directly or indirectly.

One of the features of organ culture of the human saphenous vein, is intima-directed SMC proliferation, which has also been observed in aortic organ cultures of the rat (Fingerle and Kraft, 1987), and pig (Gotlieb and Boden, 1984). In agreement with this study, other studies with pig aorta (Koo and Gotlieb, 1989) and human saphenous vein (Angelini et al. 1991) also demonstrated a marked attenuation of the proliferative response when the endothelium was removed. It would appear, therefore, that the endothelial cells play a prominent role in the regulation of SMC in vitro via the secretion of soluble factors that may promote SMC growth.

ii) Endothelial regeneration

In animal models of vein grafting, the initial loss of endothelial cells is replaced by the regeneration of surviving cells, and this process is usually complete within 4-6 weeks (Dilley et al. 1988, Angelini et al. 1992). Although in humans, re-endothelialisation does occur (Sottiurai et al. 1985), the time course is unknown. However, if the time course in humans is similar to that in other species, then a recent study by Idu et al. (1993), showing that 79% of vein graft stenoses develop after the first 12 weeks, suggests that SMC proliferation is continuing beneath an apparently intact endothelium. Interestingly, removal of the endothelium by balloon catheterisation of animal arteries leads to SMC proliferation
which only ceases when the endothelium has regenerated (Reidy and Schwartz, 1981). Such observations emphasise an important difference that exists in the aetiology of intimal hyperplasia between arteries and vein grafts. Although at 12 weeks the endothelium may appear morphologically intact, there is evidence that it is functionally impaired (Angelini and Newby, 1989, Komori et al. 1991).

**iii) Altered endothelial function**

A major contribution to endothelial dysfunction is caused by an alteration in the production of endothelium-derived relaxing factor (EDRF), which is reduced under conditions of endothelial impairment (Komori et al. 1991). The most important physical stimulant of EDRF release is flow rate, and, more importantly, alterations in the rate of flow (Henderson, 1991); and low blood flow is a vital factor influencing intimal hyperplasia in vein grafts. Under normal conditions, EDRF from endothelial cells increases levels of cGMP in platelets, which in turn inhibits platelet adhesion and aggregation. It is during platelet adhesion that platelet-derived growth factor (PDGF) is released, therefore it follows that any reduction in EDRF levels will subsequently elevate PDGF, thus disturbing the normal equilibrium of growth-promoting and growth-inhibiting factors. In the vein culture model flow is entirely absent, and in this situation one would expect the degree of intimal hyperplasia to be further exaggerated. Additionally, endothelial cell regrowth in a vein graft may be followed by impairment of receptor-mediated EDRF responsiveness, reducing levels of cGMP in the vessel wall, and it has been shown by Weidinger and colleagues (1990) that cGMP exerts an anti-proliferative effect in some vascular SMC preparations.

Endothelin is a 21 amino-acid peptide with a potent vasoconstrictive action, and also a smooth muscle mitogen synergistic with PDGF (Sharefkin et al. 1991). The work of these authors suggests that endothelial cell-derived endothelin may also contribute to intimal hyperplasia in vein grafts; they demonstrated that 24 hours of shear stress on endothelial cells *in vitro* reduced levels of mRNA for endothelin precursor. Under conditions of normal blood flow, the shear stresses exerted on the endothelium keep an inhibitory
influence on endothelin release, but under conditions of poor flow, this inhibition is reduced, leading to a greater production of endothelin and subsequently more intimal hyperplasia. Repetitive stretch cycles on endothelial cells in culture also enhances the release of endothelin (Sumpio and Widmann, 1990). Therefore, haemodynamic forces such as heart rate and blood pressure may also regulate cell proliferation \textit{in vivo}.

The role of endothelial cells in controlling cellular activity relating to intimal hyperplasia is further supported by reports that endothelial cells secrete PDGF (DiCorleto and Bowen-Pope, 1983), and basic fibroblast growth factor (bFGF) (Reidy and Schwartz, 1981; Vlodavsky et al. 1987). \textit{In vitro}, DiCorleto (1983) proposed that the endothelium in vitro is analogous to perturbed endothelium \textit{in vivo}. Fox and DiCorleto (1984), showed that PDGF levels produced by \textit{in vitro} endothelium can be significantly elevated by "injuring" the endothelial cells with endotoxin or phorbol esters. This observation suggests that a non-denuding injury \textit{in vivo} may induce the endothelium to produce this mitogen and thereby induce SMC proliferation.

Another possible candidate is bFGF. It is known that endothelial cells synthesise bFGF in vitro, and if their plasma membranes are wounded by a scraping injury, a bFGF-like molecule is released (Vlodavsky et al. 1987). In the same study, these workers also found that conditioned medium from wounded endothelial cells had autocrine activity for endothelial cells which are mitogenically stimulated by bFGF but not PDGF (McNeil et al. 1989). Secondly, Vlodavsky showed that 98% of the growth-promoting activity was destroyed by heat; bFGF is heat-sensitive, but PDGF is not.

The endothelium serves as a bifunctional regulator of SMC proliferation by maintaining a balance between growth-promoting and growth-inhibiting factors, and therefore, any form of injury will undoubtedly disturb the equilibrium between opposing stimuli (Williams, 1991). Both hypertension and hyperlipidaemia are associated with an imbalance of endothelium-derived relaxing and contracting factors, and this may reduce the anti-proliferative properties of the endothelium (Luscher 1990). Thus endothelial
dysfunction may represent a common denominator of vascular injury induced by cardiovascular risk factors.

7.5 Comparison with other studies

In agreement with the porcine arterial studies of Koo and Gotlieb (1989), this study shows direct evidence of the involvement of the endothelium in the regulation of intimal SMC proliferation. The organ culture model provides a means by which one is able to investigate the role of possible mediators responsible for the development of vein graft stenoses in arterial bypass procedures. In interpreting the data presented here, organ culture represents a vessel segment responding to factors in the absence of haemodynamic forces, and therefore the proliferative responses are probably exaggerated in a non-flow situation.

Other studies using an organ culture technique have previously been described (Gotlieb and Boden, 1984; Fingerle and Kraft, 1987; Koo and Gotlieb, 1989; Soyombo et al. 1990; Angelini et al. 1991). However, none of these have utilised coculture or identified the paracrine action of the endothelium. It is also noteworthy that many of these studies have been conducted in animal arteries, and, as discussed earlier, their relevance to the human vein graft situation remains unclear.

7.6 Summary

To summarise, the concept of endothelial injury should not be solely restricted to denudation injury. It is apparent that functional changes can occur in endothelial cells which may be important in the development of vein graft stenoses; that there is no single stimulus involved in their development and that the process is multifactorial. It still remains to be proven how important these changes in endothelial function are in an in vivo flow situation. At the present time, studies are in progress in our laboratory to develop a vein culture model incorporating arterial flow rates and pressures. Although flow is undoubtedly important, the final common pathway leading to smooth muscle proliferation and phenotypic change will be mediated by chemical messengers. It is therefore a worthwhile
goal to accumulate a fuller understanding of the biology of the vasculature which may in the future enable us to modulate intimal proliferation.
CHAPTER 3

HUMAN SAPHENOUS VEIN ENDOTHELIAL CELLS
COCULTURE WITH DENUDED SAPHENOUS VEIN DO NOT
PROMOTE SMOOTH MUSCLE CELL PROLIFERATION

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HUMAN SAPHENOUS VEIN ENDOTHELIAL CELLS COCULTURED
WITH DENUDED SAPHENOUS VEIN DO NOT
PROMOTE SMOOTH MUSCLE CELL PROLIFERATION

8.1. Introduction.
Owing to the complexity of the vein wall structure, it is difficult to study specific
cellular interactions in vivo, hence a variety of in vitro coculture techniques have been
described in an attempt to simulate the vascular wall. Studies on endothelial cell/smooth
muscle cell interaction have been contradictory, reporting both a stimulation (Davies and
Kerr, 1982; Gajdusek et al. 1980) and inhibition (Castellot et al., 1981; Chamley-Campbell
and Campbell, 1981) of smooth muscle cell proliferation by the endothelium. In 1986, Van
Buul-Wortelboer demonstrated an inhibitory action of both confluent and subconfluent
EC's on SMC proliferation in a coculture model using human umbilical cord arterial SMC's
and venous EC's. A more recent study using bovine aortic cells (Fillinger et al. 1993)
studied the effect of confluent EC's on SMC growth over a period of several days, and
showed an initial stimulation followed by a period of quiescence. Such conflicting results
could, in part, stem from species differences, differences in design of the coculture
systems, and the differential function of the cells of the vascular wall in arteries and veins.
The evidence presented in this thesis so far has shown that in an organ culture of human
saphenous vein, the endothelium promotes intimai hyperplasia via the production of a
soluble paracrine mediator. Therefore, using a modification of the coculture model
described in Chapter 7, this study sets out to investigate the effect of coculturing endothelial
cells with denuded vein segments on intimai proliferation.

8.2. Materials and Methods.

i) Saphenous vein endothelial cell culture
Cultures of human saphenous vein endothelial cells (HSVEC) were harvested from
short (1cm) segments of freshly isolated saphenous vein obtained from patients undergoing
arterial bypass surgery. Vein segments were opened up longitudinally and pinned out in a
culture dish, lumenal surface uppermost, and washed twice with Minimal Essential
Medium (MEM). The segments were incubated in a solution of 0.1% Worthington collagenase (Lorne Laboratories, Reading, Berks.), for 15 minutes at 37°C in a CO\textsubscript{2} incubator. At the end of this period, the surface of the vein was scraped gently several times in one direction with a sterile scalpel blade to facilitate detachment of the endothelial cells, and the cell suspension then transferred to a 50ml tube. The surface of the vein was washed twice more with MEM and the washings added to the endothelial cell suspension in the tube. This was then centrifuged at 300g and 4°C for 7 minutes in order to pellet the endothelial cells. The supernatant was discarded and the cells resuspended in 4 ml complete medium (Chapter 4.2iii) and plated onto a T25 tissue culture flask. Cultures were maintained in a humidified atmosphere of 5% CO\textsubscript{2} in air with a half change of medium every 2-3 days, until confluent (10-14 days). At confluence, the cells were released from the flask by trypsinisation (Appendix 2) and replated at a ratio of 1:3. Endothelial cells were identified by their characteristic "cobblestone" morphology (Figure 8.1), and also by immunostaining for Von Willebrand (Factor VIII-related) antigen (Figure 8.2).

In this study all endothelial cells were used at the second passage.

\textit{ii) Establishment of cocultures}

Cocultures were prepared by plating endothelial cells at confluent density into two wells (24.5mm diameter) of a six-well Transwell culture plate (Costar U.K. Ltd, High Wycombe, Bucks) in 2.5 ml of complete culture medium. Medium alone was placed in two further wells for controls, and the plate returned to the incubator overnight (18 hours).

Saphenous vein samples were obtained as described previously from 10 patients undergoing bypass surgery, and four vein segments were prepared from each: 2 intact and 2 denuded of endothelium as described previously (Chapter 6.2ii). Each segment was pinned out as for standard vein culture (Chapter 6.2ii) onto a circular silicone rubber mat of 1.5 cm diameter (Figure 8.3) which was then introduced into a coculture insert (Figure 8.4). The pore size of the insert membrane was 3\textmu m, thereby allowing free diffusion of nutrients and soluble mediators with no cellular transfer between the compartments of the coculture wells. The medium in the control and endothelial-cell seeded wells of the
coculture plate was replaced with 2.5 ml of fresh vein culture medium and the coculture chambers introduced such that one intact vein segment and one denuded segment was cocultured with endothelial cells, and with medium alone (controls). A further 2.5 ml of medium was then introduced into the upper compartment of the coculture assembly, such that the total volume of medium in each well was 5 ml (Figure 8.5). A diagrammatic representation of the complete coculture assembly is shown in Figure 8.6.

Cocultures were maintained for 14 days at 37°C in a humidified CO₂ incubator, and the medium replaced every 2-3 days. At each medium change, the integrity of the endothelial cell monolayer was also examined microscopically. In order to measure cellular proliferation, 5-Bromo-2-deoxyuridine labelling agent was added to the culture medium of 8 of the experiments for the final 72-96 hours as already described in Chapter 5.
FIGURE 8.1 Phase contrast microscopic appearance of saphenous vein endothelial cells showing cobblestone morphology (x40).

FIGURE 8.2 Von Willebrand factor immunostaining of saphenous vein endothelium.
- a) Negative control and b) Positive staining. (x80)
FIGURE 8.3 Photograph showing vein segment pinned out onto silicone rubber mat before introduction into the upper compartment of the coculture chamber.

FIGURE 8.4 Photograph showing the introduction of prepared vein onto the membrane of the upper compartment of the coculture assembly.
FIGURE 8.5 Photograph showing completed coculture assembly. Endothelial cells were seeded in the lower compartment, with vein segment mounted in the upper compartment, separated by a microporous membrane.

FIGURE 8.6 Showing a diagrammatic representation of a complete coculture well assembly.
iii) Immunohistochemistry

At the end of the culture period, veins were fixed overnight in 10% formal saline, processed and paraffin embedded. Serial sections of 4 μm thickness were cut, followed by staining with SMA/Millers elastin stain and anti-bromodeoxyuridine antibody. Measurements of neointimal thickness were made for each set of veins as described in Chapter 6.2i, and a proliferation index was also calculated (Chapter 5.2iv).

iv) Statistics

Neointimal thickness and proliferation measurements are expressed as median and range. Differences between groups were analysed using the Wilcoxon paired rank test, with 95% confidence intervals.

8.3 Results

i) Neointimal thickening

Microscopic examination of the endothelial cells in the seeded wells during and at the end of the culture period revealed the maintenance of a confluent monolayer of cells with a normal morphological appearance.

In agreement with the observations made in Chapters 6 and 7, intact segments of vein developed a significantly thicker neointima than those denuded of endothelium, median thickness 16.5μm (range 10 - 31) versus 0μm (range 0 - 5). (Median difference = 14.0, 95% confidence interval 10.0, 19.0, P = 0.001). However, endothelial cells cocultured with denuded vein segments did not stimulate intimal proliferation in the denuded segments, median neointimal thickness 0μm (range 0 - 0), which was not significantly different from denuded vessels alone (Figure 8.7).

The cocultivation of intact veins with endothelial cells did not further increase neointimal thickening in these segments, median thickness 15.5μm, (range 11 - 22), versus 16.5μm, (range 10 - 31); not significantly different from intact vessels alone (Figure 8.8). Furthermore, an equally important observation to note was that endothelial cells cocultured with intact vein segments did not have any inhibitory action on intimal thickening in this culture system.
FIGURE 8.7 Scatter plot of neointimal thicknesses of intact and denuded veins, and denuded veins cocultured with endothelial cells. (N.S. = not significant.)

FIGURE 8.8 Scatter plot of neointimal thickness of intact veins, and intact veins cocultured with endothelial cells. There was no significant difference between the two groups.
ii) Proliferation indices

Proliferation indices for the 8 experiments followed the same trend as neointimal thicknesses, that is, proliferation was greatest in intact veins, median 29.5% (range 20-36) versus 9% in denuded veins (range 8-16). (Median difference = 18.5, 95% confidence interval 13.0, 25.0, P < 0.001). In parallel with the measurements of neointimal thickness, there was no significant difference in the proliferative activity of denuded veins when compared with those cocultured with endothelial cells, median proliferation 5.5% (range 0-12) (Figure 8.9).

The median proliferation index of intact vessels cocultured with endothelial cells was 32% (range 16-37), not significantly different from intact vessels alone, median 29.5% (range 20-36). (Figure 8.10).
FIGURE 8.9  Scatter plot of proliferation indices of intact and denuded veins, and denuded veins cocultured with endothelial cells. (N.S. = not significant.)

FIGURE 8.10  Scatter plot of proliferation indices of intact veins, and intact veins cocultured with endothelial cells. There was no significant difference between the groups.
8.4 Discussion

In contrast to the observations made in Chapter 7, this study has demonstrated that in this model, cultured endothelial cells do not stimulate intimal proliferation in denuded vein segments. It is very unlikely that the reason for the absence of proliferation lies in the experimental design, since coculture of vein with vein under virtually identical conditions (Chapter 7) stimulates intimal thickening in the denuded segment. Therefore, a concentration effect of the factor responsible for promoting SMC proliferation can be confidently dismissed.

Instead, the results of this particular study suggest that the close proximity of endothelial cells and smooth muscle cells in their normal anatomical location is a necessary prerequisite for smooth muscle cell proliferation, such that a "cascade" of complex interactions might be initiated between the two cell types.

The endothelial cells used in this series of experiments were harvested from short segments of saphenous vein by a collagenase digestion technique (section 2i), and serially passaged before use in the study. It is well known that cells in culture may behave differently from those retained in their "native" condition (DiCorleto and Bowen-Pope 1983, Fillinger et al 1993), and therefore consideration must be given to the idea that the cultured endothelial cells have undergone phenotypic modulation after exposure to collagenase, resulting in their inability to produce the factor responsible for initiating smooth muscle cell proliferation. The effect of exposure to trypsin during passaging of the endothelial cells may also have an effect on their phenotypic status. It is, therefore, a worthwhile aim to elucidate the exact identity of this endothelium-derived factor, and subsequently to study whether cultured endothelial cells undergo phenotypic modulation and hence lose their capacity to produce it.

The results of the experimental work described in this thesis so far provide strong evidence of the endothelium's involvement in the production of a factor promoting smooth muscle cell proliferation in human saphenous vein. Whether this is a direct or indirect effect
of this factor is unknown at the present time. Although elucidation of the exact nature of this factor is an important issue and work is currently in progress to identify it, the final part of this research study attempts to "reconstitute" denuded vein segments with cultured endothelial cells in order to investigate whether seeding of the vessel wall restores the endothelial stimulation of intimal thickening in cultured veins.
CHAPTER 9

THE EFFECT OF ENDOTHELIAL RECONSTITUTION ON NEOINTIMAL PROLIFERATION IN DENUDED SAPHENOUS VEIN

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THE EFFECT OF ENDOTHELIAL RECONSTITUTION ON NEOINTIMAL PROLIFERATION IN DENUDED SAPHENOUS VEIN

9.1 General Introduction

The results of the coculture studies described in Chapter 8 have raised two possible explanations for the findings that isolated endothelial cells do not stimulate intimal proliferation in denuded vein segments. Firstly, the disruption of the architecture of the vein wall and subsequent loss of close contact between the endothelial cell and the smooth muscle cell, along with the components of the basement membrane and extracellular matrix, may contribute to the absence of the proliferative response. If this is the case, then a "reconstitution" study may, by restoration of the cellular composition of the vein wall, be able to provide information on the relative importance of endothelial/smooth muscle cell interaction in the initiation of intimal proliferation. Secondly, such an investigation will also address the question of whether the venous endothelium has been phenotypically modified in culture, thereby rendering it incapable of producing growth-promoting factors. The work described in this chapter develops a method for reseeding a confluent layer of endothelial cells onto denuded vein segments, and subsequently observes the behaviour of such veins in organ culture.

SECTION A. Development of a method to reseed denuded saphenous vein segments with a confluent layer of endothelial cells.

9.2 Materials and Methods

i) Saphenous vein endothelial cell culture

Human saphenous vein endothelial cells (HSVEC) were harvested and cultured by the method described in Chapter 8.2i. For the vein reseeding procedures, all endothelial cells were used at the second passage.
ii) Determination of seeding time and density for denuded veins

HSVEC's were recovered from tissue culture flasks, counted by the use of a haemocytometer, and resuspended at two different concentrations; \(1\times10^5\) and \(1\times10^6\) cells per millilitre of complete vein culture medium. These concentrations were chosen such that a confluent density of endothelial cells could potentially be achieved on the vein segments.

Saphenous vein samples were collected in the usual manner from 2 patients undergoing arterial surgery, and transported to the laboratory. Each vein was cut into six equal segments of approximately 1cm in length and pinned out in six separate culture dishes as for standard vein culture (Chapter 3.2i). One intact segment was immediately fixed for SEM by immersion in 4% buffered gluteraldehyde at 4°C for 18 hours, and a second segment was first denuded of endothelium before being fixed in an identical manner. The remaining four segments of each vein were all denuded of endothelium and then completely covered with 5mls of the HSVEC suspension, either \(1\times10^5\) or \(1\times10^6\) per millilitre, and incubated at 37°C in a CO\(_2\) incubator. One segment at each incubation concentration was removed at each of the following times: 30, 60 and 120 minutes, and at 18 hours (overnight incubation). The cell suspension was removed, and the segment washed twice with 5ml MEM in order to remove all the unattached HSVEC's, before being fixed for SEM as described above.

iii) Scanning Electron Microscopy (SEM)

After fixation, the veins were processed for scanning by washing each segment in 0.2M cacodylate buffer for 1 hour followed by serial dehydration through graded alcohols (50, 70, 90 and 100%). They were transferred into 100% acetone for storage prior to critical point drying and further preparation for screening as described in Chapter 4.

9.3 Results

SEM examination of each of the intact vein segments revealed a well preserved endothelial layer over much of the lumenal surface (Figure 9.1), with any endothelial cell loss being confined to the cut edges of the vessel. As a comparison, Figure 9.2 shows the
typical appearance of a completely denuded vessel with exposure of the sub-endothelial matrix.

**FIGURE 9.1** SEM appearance of central portion of intact vein segment showing a morphologically intact and almost continuous endothelial monolayer, with occasional gaps.

**FIGURE 9.2** SEM appearance of vein denuded of endothelium, showing exposure of the subendothelial matrix.
i) HSVEC seeding density $1 \times 10^5$ cells/ml

At a concentration of $1 \times 10^5$ cells/ml, and 30 minutes incubation the lumenal surface of the vessel showed exposed subendothelial matrix with no evidence of endothelial cell attachment (Figure 9.3).

After 60 minutes, some endothelial cells had become adherent (Figure 9.4) but coverage was very sparse and cells were mostly rounded in appearance (Figure 9.5).

120 minutes incubation at this cell concentration showed the presence of small clusters of cells beginning to spread (Figure 9.6), but low power scanning of the whole vein surface allowed an estimation of cell coverage to be only of the order of 10-20%.

Following overnight incubation, there was no further improvement in cell attachment as assessed by scanning, although all the adherent cells had spread (Figure 9.7) with gaps being observed between adjacent cells (Figure 9.8).

FIGURE 9.3 At an endothelial cell concentration of $1 \times 10^5$ cells/ml and 30 minutes incubation there is no evidence of any cell attachment. (Mag. x 1,000).

FIGURE 9.4 After 60 minutes some adherent endothelial cells can be observed, although coverage is sparse. (Mag. x 1,200).
FIGURE 9.5  60 minutes incubation at a concentration of 1x10^5 cells/ml. Sparse coverage of rounded endothelial cells. (Mag. x 4,000)

FIGURE 9.6  At 120 minutes small clusters of endothelial cells are beginning to spread, but large areas of matrix are still exposed. (Mag. x 1,000).

FIGURE 9.7  After 18 hours incubation cells have spread but with no further improvement in coverage. (Mag. x 1,000).

FIGURE 9.8  Higher power view of previous figure, showing gaps between adjacent endothelial cells. (Mag. x 4,000).
ii) HSVEC seeding density $1 \times 10^6$ cells/ml

At an incubation concentration of $1 \times 10^6$ cells/ml, attachment of some endothelial cells to the denuded vessels was apparent after 30 minutes, but most of these cells were rounded in appearance (Figure 9.9).

After 60 minutes, the endothelial cells were beginning to spread, although cells could still be observed attaching to the vessel and remained rounded. There were also large areas of vein still devoid of endothelium, with exposed basement membrane (Figure 9.10).

After 120 minutes incubation, good endothelial coverage was observed over much of the lumenal surface with only patchy areas of exposed subendothelial matrix (Figure 9.11), although many cells still had a rounded, rather than flattened appearance.

Following overnight incubation, an intact endothelial "carpet" was observed over the entire surface of the vein, with cells being completely flattened and spread (Figures 9.12 and 9.13), although occasional gaps could be seen between the cells, with intercellular processes being extended between them (Figure 9.14). However, such gaps were also commonly observed in freshly isolated veins (cf. Figure 9.1).

**FIGURE 9.9** Good endothelial cell attachment after 30 minutes at a concentration of $1 \times 10^6$ cells/ml. Most of the attached cells are rounded in appearance. (Mag. x 1,100).

**FIGURE 9.10** After 60 minutes cells are still attaching to the vein and beginning to spread. There are still exposed areas of matrix. (Mag. x 1,100).
FIGURE 9.11 After 120 minutes incubation endothelial cells cover most of the lumenal area. Some cells remain rounded at this stage. (Mag. x 1,100).

FIGURE 9.12 After 18 hours incubation endothelium covers the entire lumenal area, with cells flattened and well spread. Low power view gives landscape coverage of vein surface. (Mag. x 300).

FIGURE 9.13 Higher power view of Figure 9.12, showing "carpet" of adherent endothelial cells. (Mag. x 1,100).

FIGURE 9.14 Another view of same vein segment, showing gaps between endothelial cells. (Mag. x 1,300).
9.4 Conclusion

From the results of the seeding time and density study, it was concluded that an endothelial cell concentration of $1 \times 10^5$ cells/ml was insufficient to adequately cover denuded vein segments at any of the time points studied. However, when the cell concentration was increased tenfold, it was possible to totally restore endothelial coverage on denuded vein segments. An incubation period of 2 hours provided an estimated coverage of 70% of the luminal surface assessed by both SEM and also trypan blue exclusion. However, it was concluded that an overnight incubation gave the additional benefit of providing a well spread, confluent endothelium, able to completely exclude trypan blue. Therefore, a seeding time of 18 hours and a density of $1 \times 10^6$ cells/ml was used in the following study to observe the behaviour of "reconstituted" saphenous vein in organ culture.
SECTION B. A comparison of the behaviour of intact and "reconstituted" human saphenous vein in organ culture.

9.5 Materials and Methods

i) HSVEC culture

Endothelial cells were harvested from five donors and cultured as described previously. All endothelial cells were used at the second passage, with a different population of cells being used for each reseeding procedure. Immediately prior to vein seeding, HSVEC's were recovered from culture flasks by trypsinisation, counted and resuspended in complete vein culture medium at a concentration of $1 \times 10^6$ cells/ml.

ii) Experimental procedure

Segments of the long saphenous vein were obtained in the usual way from 5 patients undergoing arterial surgery. Endothelial integrity was visually scored using trypan blue, and any vein with less than 70% coverage was excluded from the study. Each vein was divided into 3 segments and prepared for culture under standard conditions as follows:

i) 1 intact segment cultured for 14 days.

ii) 1 denuded segment cultured for 14 days.

iii) 1 denuded segment, reseeded for 18 hours at $1 \times 10^6$ HSVEC/ml, washed twice with MEM, then cultured for 14 days.

At the end of the culture period, all segments were fixed overnight in 4% buffered gluteraldehyde, and then carefully cut into two halves with a scalpel blade. One half was processed and paraffin-embedded for the application of SMA/Millers elastin stain, and the other processed for SEM.

Measurements of neointimal thickness were made on each set of veins as described in Chapter 6.2i, and SEM was performed on the corresponding half of each segment in
order to visually assess the presence or absence of endothelium, and the degree of coverage after 14 days in culture.

iii) Statistics

Neointimal thickness measurements are expressed as median and range. Differences between groups were analysed using the Wilcoxon paired rank test with 95% confidence intervals.

9.6 Results

i) SEM appearance of cultured veins

All the intact cultured vessels retained a confluent layer of endothelium with occasional gaps between cells (Figures 9.15 and 9.16).

Denuded cultured vessels generally showed complete exposure of the basement membrane and subendothelial matrix (Figure 9.17), although two of the denuded vessels showed a small area (approx. 10%) of endothelial regeneration (Figure 9.18). This most probably occurred as a result of incomplete endothelial removal before culture, to a degree that a small amount of regeneration took place from the retained cells. However, this was negligible.

Examination of the reseeded vessels showed that endothelial continuity had been retained during the culture period (Figure 9.19), although it was observed that the gaps between cells were, in some places, more apparent than in the intact cultured vessels (Figures 9.20 and 9.21).
FIGURE 9.15 Intact veins retained a confluent endothelial monolayer during culture. (Mag. x 1,200).

FIGURE 9.16 Another example of intact cultured vein showing an area of gaps between the endothelial cells. (Mag. x 1,100).

FIGURE 9.17 Denuded vessel after culture, showing continued absence of endothelium. (Mag. x 1,100).

FIGURE 9.18 Two of the denuded veins showed a small area (approx. 10% of total) where endothelial regeneration had occurred. (Mag. x 1,150).
FIGURE 9.19  Reseeded veins retained endothelial continuity during the culture period. (Mag. x 1,000).

FIGURE 9.20  Reseeded vein showing an area of frequent gaps between the endothelial cells. (Mag. x 1,000).

FIGURE 9.21  Higher power view of a reseeded vein emphasizing gaps between the endothelial cells. (Mag. x 4,000).
**ii) Neointimal thickening**

Neointimal thickening in intact veins was again significantly greater than in denuded segments, median thickness 16μm (range 11-23) versus 0μm (range 0-5). (Median difference = 15.5, 95% confidence interval 10.0, 23.0, P = 0.01).

However, although reseeded veins retained endothelial coverage in culture, the development of neointimal thickening was not restored, median thickness 0μm (range 0-5), versus 16μm (range 11-23) in intact veins. (Median difference = 16.0, 95% confidence interval 10.0, 23.0, P < 0.01). The neointimal thickness of reseeded veins was not significantly different from denuded veins, median difference = 0.0, 95% confidence interval -5.0, 5.0, P = 0.6).(Figure 9.22).

**FIGURE 9.22** Scatter plot of neointimal thicknesses of intact, denuded and reconstituted veins in culture. There was no significant difference between the denuded and reseeded groups.
9.7 Discussion

Having demonstrated that intact veins develop neointimal thickening in culture (Chapter 6), and that a soluble paracrine mediator is responsible for this proliferative response (Chapter 7), a number of noteworthy phenomena have been observed in the two subsequent studies.

In Chapter 8, it was concluded that the endothelium in isolated culture loses the ability to produce the inducing factor necessary for smooth muscle cell proliferation. It was, therefore, reasonable to predict that by restoring endothelial continuity on denuded vein segments, the SMC proliferative response might be reinstated.

In order to test this hypothesis, reseeding of denuded vein segments was established using allogeneic endothelial cells. It would have been preferable to reseed the veins with their own cultured endothelium, but such a procedure poses a problem relating to timing of the events. The time period between initial harvesting and subsequent reseeding would amount to several weeks, during which time the vein could not reasonably be preserved. Although cryopreservation of the denuded veins was considered, this idea was rejected because of the unknown effects of such a procedure on the vessel wall. Consequently, the use of established cultures of allogeneic endothelial cells facilitated immediate reseeding of freshly prepared, denuded veins.

Although a morphologically intact endothelium was reseeded onto denuded vessels and retained during the culture period as shown by SEM, its functional ability in relation to restoring the production of the paracrine factor promoting intimal proliferation was impaired. The present study has shown that the paracrine mediator produced by the endothelium of an intact vein is probably not released by the endothelium reseeded onto denuded veins, owing to an irreversible phenotypic modulation of the endothelial cells during harvesting and culturing (Chapter 8).
It is a point of interest to note that other in vitro studies in this department have examined the behaviour of cultured and subsequently reseeded endothelial cells onto PTFE grafts (Budd et al. 1991), and angioplasty sites of human saphenous vein segments (Thompson et al. 1992). In both of these studies it was shown conclusively that the seeded endothelial cells produced prostacyclin. Therefore, although the process of culturing endothelial cells appears to render them incapable of producing the paracrine SMC growth-stimulating factor identified in this project, other factors are apparently unaffected by such a procedure.

It cannot be totally dismissed that the reason for the inability of the reseeded endothelium to restore intimal proliferation was because the allogeneic cells were rejected. This, however, is unlikely owing to the fact that a morphologically normal, confluent layer of endothelium was still observed on the vessels after the 14 day culture period. This study, as it stands, is also unable to determine whether the cultured and reseeded endothelium has suffered a generalised loss of ability to produce the paracrine factor, or if the SMC's of the vessel wall are not sufficiently sensitive to respond to any factor which may be being produced at a reduced concentration. There are several ways in which this postulation could be investigated:

Firstly, establishment of a vein/vein coculture study as detailed in Chapter 7 using allogeneic in place of isogeneic vessels. That is, intact vessel from one subject cocultured with denuded vessel from another. Such an investigation would determine whether "undisturbed" allogeneic endothelial cells are able to induce intimal proliferation in the denuded vessel, as one would reasonably expect to be the case.

Secondly, in order to test the hypothesis that the cultured endothelial cells may be producing reduced amounts of paracrine factor, coculture of isolated Swiss 3T3 cells with endothelial cells, and isolated SMC's with endothelial cells may be of benefit. 3T3 cells are known to be particularly sensitive to small quantities of cytokines and furthermore, specifically bear receptors for PDGF and bFGF (Sterpetti et al. 1994). If the cultured
endothelial cells were producing reduced quantities of growth factor, such a study may be able to identify a growth-stimulatory action on the 3T3 cells at concentrations under which the SMC's are insufficiently sensitive to respond.

Finally, by using a modification of the transwell coculture method described in Chapter 8, one could coculture 3T3 cells with intact vein, denuded vein and also with reseeded vein in order to examine any differential growth response of the 3T3 cells.

In conclusion, this study has shown that restoration of contact between the endothelium and the smooth muscle cells is not sufficient by itself to initiate production of the endothelial-derived paracrine factor at concentrations which are able to stimulate intimal SMC proliferation. Endothelial cells are usually thought of as a homogeneous cell type lining the vasculature and most studies have not questioned this assumption. A few studies have shown some phenotypic heterogeneity which suggests functional differences as well (Turner et al 1987, Golden et al 1990); it is now well recognised that antigen expression by endothelial cells can be modulated. The most likely explanation for the inability of the endothelium to restore SMC proliferation in reseeded vessels is a phenotypic modulation of the cells during culturing, which cannot be reversed, even when they are replaced into their normal anatomical location.
# CHAPTER 10

DEVELOPMENT OF A LABORATORY FLOW MODEL OF SAPHENOUS VEIN GRAFT INTIMAL HYPERPLASIA

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10.1 Introduction

Although our understanding of the molecular and cellular events underlying the development of vein graft stenosis has increased dramatically over the last decade, there is still no effective treatment to prevent the development of vein graft stenoses. Most cell and organ culture work has been performed under static conditions, whereas blood vessels are continuously subjected to mechanical forces acting upon them. This is of great significance because it is now apparent that mechanical forces play an important role in determining the biological functions and, more specifically, pathological changes within blood vessels.

In this regard, there is now a wealth of information to support the theory that blood flow, and more specifically the resulting shear stress acting on the endothelial cell, plays a central role in determining vascular structure (LaBarbera 1990, Langille and O'Donnell 1986), by a process dependent on an intact endothelium (Langille and O'Donnell 1986). Blood flow is associated with shear stress; the tractive force acting in the direction of flow on the surface of the inner wall of the blood vessel at the endothelial cell. Wall shear stress ($\tau$) is proportional to flow velocity ($Q$) and medium viscosity ($\mu$), and inversely proportional to the third power of the internal radius ($R$) of the vessel and can be calculated according to the Hagen-Poiseuille Formula (Figure 10.1).

The distribution of haemodynamic forces and shear stress has been used to explain the predominance and focal nature of atherosclerotic plaques at arterial bifurcations which are characterised by variable shear stresses of low magnitude (~ 4 dyne/cm$^2$), rather than on segments which are exposed to higher shear stresses (~ 15 dyne/cm$^2$) and with less fluctuation (Zarins et al. 1983, Asakura and Karino 1990).

There is considerable experimental evidence that intimal hyperplasia in vein grafts is also influenced by haemodynamic factors. In 1989 Dobrin et al. demonstrated an inverse
**Hagen - Poiseuille Law**

Wall Shear Stress $= \tau = 4 \mu \frac{Q}{\pi R^3}$

**FIGURE 10.1**  Diagrams to illustrate the Hagen-Poiseuille formula in the calculation of shear stress
correlation between graft flow and intimal thickness in canine vein grafts, and subsequent work has confirmed that it is low shear stress associated with low flow that is responsible for IH. Morinaga et al. (1985) showed that the endothelium plays a central role in the detection of these shear stresses and their subsequent transformation into vessel wall responses. IH is characterised by the abnormal proliferation of SMC’s, the stimuli for which are still not fully defined. Under conditions of shear stress, endothelial cells become elongated and aligned in the direction of flow (Dewey et al. 1981, Langille and Adamson 1981). These morphological changes seem to be caused by reorganisation of actin microfilaments into stress fibres which play a role in endothelial cell adhesion and protection against damage imposed by shear stress (White and Fujiwara 1986, Wechezak et al. 1989). In addition to changes in cellular shape, an increase in flow induces several rapid responses in endothelial cells including potassium channel activation (Oleson et al. 1988), intracellular calcium increase (Shen et al. 1992) and cytosolic acidification (Ziegelstein et al. 1992). These rapid responses are followed by increases in the production of various vasoactive substances such as EDRF (Rubanyi et al. 1986), prostacyclin (Frangos et al. 1985), tissue plasminogen activator (Diamond et al. 1990), PDGF (Hsieh et al. 1991), and a decrease in endothelin production (Yoshizumi et al. 1989, Malek et al. 1993). These substances may therefore all contribute to the shear stress-induced changes in the vascular wall.

Two of the best characterised mitogens for SMC are PDGF and bFGF. It has been shown that PDGF is an important mitogen in both arterial and venous endothelium (Hsieh et al. 1991, Mitsumata et al. 1993), and that PDGF mRNA expression is decreased under conditions of increased shear stress, whilst bFGF mRNA expression is upregulated with increasing shear stress (Malek et al. 1993). Similarly, endothelin mRNA expression by endothelial cells is increased under low shear conditions and inhibited by high shear stress (Shareffkin et al. 1991, Malek and Izumo 1992, Kuchan and Frangos 1993, Morita et al. 1994). Therefore endothelin may also be an important endothelial mediator of IH.

Although it has been shown in this thesis that organ culture of the human saphenous vein can be used as an in vitro model of vein graft IH, the major disadvantage of the model is
that it represents the response of a vein segment to culture in the absence of flow. In view of
the findings of other workers described above, it is possible that the responses observed in
static culture might be modified in a no-flow situation; hence this chapter describes the
development of a laboratory model in which vein segments may be cultured under flow
conditions.

It was concluded in Chapter 3 that there is a marked variation in normal intimal
thickness of the long saphenous vein, therefore all the experiments using cultured vein in the
study so far were designed using a paired analysis. In any one experiment examining the
effect of a stimulus on neointimal thickness, the test group was always compared with a
control vein that came from the same patient. This was a particularly important consideration
when designing an experimental model to simulate flow (hereafter referred to as the "flow
rig"), because the method had to be capable of examining the effect of differing flow
conditions on multiple segments of vein from any one patient, and to make valid comparisons
with vein cultured under conditions of static culture as described throughout previous
chapters. The method described below was therefore developed with these considerations in
mind.

10.2 Materials and Methods

(i) Flow rig development

The method was evolved using modified glass conical flasks as reservoirs containing
500 ml of vein culture medium, such that culture medium outflow and return could be effected
in a closed circulatory loop by the use of sterile silicone rubber tubing of 1 cm diameter. A
further glass inlet tube was linked to a cylinder of 5% CO\textsubscript{2} which was bubbled gently through
the medium at a rate which maintained the pH at 7.4 +/- 0.05 throughout all the studies
performed.

The rig was designed to provide two "channels": one at venous flow, pressure
and shear stress (70 ml/min; 15 mm Hg; 1 dyne/cm\textsuperscript{2}), and the other at arterial flow, pressure
and shear stress (500 ml/min; 85 mm Hg; 9 dyne/cm\textsuperscript{2}), by adjusting the flow rate of the
medium and the height of the reservoir. Circulation of culture medium in each circuit was provided by a Watson-Marlow 505-S pump (Watson-Marlow Ltd, Falmouth, Cornwall) and the circulating medium was maintained at 37°C by the use of an incubator (Pickstone Equipment Ltd, Thetstone, Norfolk) which held the tubing in which the vein segments were pinned, with foam insulating material being used to insulate the reservoirs and tubing exposed to laboratory temperatures. The temperature of the flowing medium was recorded at random times and was found to be maintained at 37°C (+/- 2°C) throughout all experiments.

In order to confirm that the flow circuits were delivering laminar flow, a preliminary observational study was conducted. The reservoirs were filled with water, flow was established in both circuits and any air bubbles eliminated. A quantity of trypan blue dye was then added to each reservoir, and as it travelled along the tubing, a parabolic profile characteristic of laminar flow was observed and maintained along each circuit (Figure 10.2).

FIGURE 10.2 Photograph to demonstrate the parabolic profile of laminar flow in the flow circuits.
**(ii) Experimental design**

Segments of vein (4-5 cm were required for each experiment) were obtained in the usual manner from patients undergoing arterial surgery, scored for endothelial coverage (Chapter 4.2ii), and prepared for culture (Chapter 4.2iii). For each experiment, 2 segments were established in static culture, with the remaining 4 being prepared for flow; 2 at arterial and 2 at venous flow. A 90 cm length of sterile silicone tubing was used to accommodate each pair of vein segments. This was achieved by making a longitudinal slit of approximately 10 cm which facilitated mounting the vein segments on a 500 μm mesh and pinning onto the inside wall of the tubing as for normal vein culture. The veins were totally immersed in complete culture medium whilst the slit was resealed using a proprietary silicone sealant (approx. 45 minutes). Meanwhile the reservoirs were filled with medium, mounted at appropriate heights on a clamp stand and the tubing connected ready for interposition of the 90 cm length in which the vein segments were pinned. This was achieved using 1 cm connectors to complete each circuit, after which flow was initiated and any air was expelled by gentle manipulation of the tubing. The system was allowed to equilibrate at a slow flow rate for 12 hours, during which time any leakage from joints was identified and rectified, after which time arterial and venous conditions were constantly applied for a further 13 days. A diagrammatic representation of the flow circuit is shown in Figure 10.3, and photographs of the complete, functioning flow rig is shown in Figures 10.4 and 10.5.

At the end of the culture period the medium was drained from the tubing, the veins were quickly removed from the flow circuits with a scalpel blade such that they remained pinned into a portion of the tube, and then totally immersed in 10% formalin overnight. One static, one arterial and one venous vein was processed, paraffin-embedded and sectioned for immunohistochemistry. Each was stained with SMA/Millers elastin and CD 31 endothelial stains such that measurements of neointimal thickness could be performed and endothelial integrity observed as described in Chapter 3.2iii.

The remaining static, arterial and venous segments were assessed for viability at the end of each experiment by measuring smooth muscle contractility as previously described in Chapter 4.4ii.
FIGURE 10.3  Diagrammatic representation of flow rig for culture of vein segments under arterial and venous conditions.
FIGURE 10.4  Photograph to show complete flow rig with \textit{arterial} and \textit{venous} channels.

FIGURE 10.5  Closer view of interior of incubator, showing the two circuits in which the vein segments are pinned.
(iii) Control of infection

Many of the early attempts to establish the flow model were hampered by bacterial infection, resulting in premature termination of the experiment, sometimes within only 3-4 days of the start. Microbiological investigation revealed that all of these infections were due to gram negative organisms, which therefore explained the lack of control by penicillin or streptomycin placed routinely in the culture medium. At this stage it was decided to add gentamicin to the medium in an attempt to control such infections.

Gentamicin, however, is known to have toxic effects in some preparations, for example keratinocytes and fibroblasts (Cooper et al. 1991), and corneal epithelial cells (Medin 1993), therefore an initial study was performed in order to observe the effects of gentamicin, if any, on the development of IH in veins cultured under static conditions.

Saphenous vein was obtained from five patients, each divided into 3 segments and cultured for 14 days as follows:

a) Vein culture medium only.

b) Vein culture medium containing 50 μg/ml gentamicin sulphate (Roussel Ltd. Dublin, Ireland).

c) Vein culture medium containing 5 μg/ml gentamicin sulphate.

At the end of the culture period, veins were fixed overnight in 10% formalin and 4 μm sections were prepared and stained with SMA/Millers stain (Chapter 3).

Neointimal thicknesses were measured as described in Chapter 3.2iii, and the results expressed as median and range. Differences between groups were analysed using the Wilcoxon paired rank test with 95% confidence intervals, and the results shown in Figure 10.6.
Figure 10.6 Scatter plot showing the effect of gentamicin on neointimal thickness of veins in culture. There was no significant difference between the control and low dose gentamicin groups; however 50 µg/ml significantly reduced the proliferative response compared with controls (Median difference = 23.0, 95% confidence interval 17.0, 29.0, P = 0.01).

This small study clearly demonstrates that 50 µg/ml gentamicin is toxic to the development of a neointima in cultured veins but 5 µg/ml does not affect neointimal development. Therefore the lower concentration was added to all media used for the subsequent flow experiments to observe whether it would be capable of controlling gram-negative bacterial infection. Fortunately, this proved to be adequate, hence gentamicin at a concentration of 5 µg/ml is now routinely added to all vein flow media in order to control infection without any toxic effects on the tissue.
10.3 Results

(i) Light microscopic appearance

Histology from one complete flow experiment is shown in Figure 10.7, using SMA/Millers elastin stain to identify the layers of the vein wall and CD 31 to demonstrate that endothelial integrity was maintained over the 14 day period. Interestingly, the endothelium-lined channels observed by ourselves, Soyombo et al. (1990) and Angelini et al. (1991) in the neointimas of static-cultured veins, were not a feature of veins cultured under either venous or arterial flow conditions. It is likely, therefore, that in the absence of flow across the endothelial cells, they form channels which may be purely an artefact of the no-flow conditions.

FIGURE 10.7 SMA/Millers elastin stain on transverse sections of vein cultured for 14 days a) under static conditions, b) under venous conditions and c) under arterial conditions (arrows mark neointima).

Upper panels show corresponding sections stained with CD 31 to show retention of endothelium.
(ii) Neointimal thickness

The neointimal thickness measurements of the first five complete flow experiments are represented in Figure 10.8

![Graph showing neointimal thickness measurements for different types of veins.](image)

**Figure 10.8** Scatter plot of neointimal thicknesses of static and flow veins.

Median (range) static veins = 20 (12-33) µm
Median (range) venous veins = 8 (0-11) µm
Median (range) arterial veins = 0 (0-0) µm

The median neointimal thickness of the static veins was 20 µm (range 12-33), venous 8 µm (range 0-11), with no neointimal proliferation being observed in any of the arterial veins. Although these results represent only 5 preliminary experiments, they demonstrate that in this model, laminar arterial flow prevents the development of IH, whereas venous flow only partly suppresses the response. Because the neointima of cultured veins is a very friable structure, it cannot be ruled out that under the high flow conditions it might have been washed off. It was, however, considered an unlikely possibility because other in vivo studies (see discussion below), where the intimal hyperplastic lesion is a much denser, more robust structure, have also shown that increased shear stress inhibits intimal proliferation. Furthermore, it was observed that the endothelium
was still present at the end of flow culture and this supports the hypothesis that the neointima was unlikely to have been removed by flow.

(iii) Viability

It was found that veins cultured under both venous and arterial conditions exhibited contractile responses of the same order of magnitude as static cultured veins, and as such, it was concluded that SMC function was similar in all groups. However, further viability studies are clearly necessary (see Chapter 4.6i) because contractility is probably not the best parameter to measure.

10.4 Discussion

In this novel *in vitro* flow model of vein graft IH it has been shown that a shear stress of arterial magnitude (~9 dynes/cm^2^) is able to totally suppress intimal proliferation and that a shear stress of venous magnitude (~1 dyne/cm^2^) only partly suppresses the response. Although the possibility exists that the fragile neointima may be washed off by the high flow conditions, the findings in the present study are comparable with those of Kohler et al. (1991) who demonstrated that in a baboon PTFE graft model, increased flow reduced neointimal thickening, whereas a reduction in blood flow led to increased proliferation. The observation that increased blood flow results in decreased neointimal thickness suggests a mechanism for control of SMC growth in response to changes in shear.

Later studies in this primate model by Geary et al. (1993), which showed that endothelium remains present and confluent during manipulation of flow conditions, and that SMC's proliferate in the layer immediately below the endothelium, support the hypothesis that the endothelial cells sense the changes in shear and respond by releasing factors that stimulate or inhibit SMC proliferation. Indeed, the endothelial cell is now believed to be the key mediator of haemodynamic effects, therefore clues as to the prevention of vein graft IH may reside in acquiring a greater understanding of how altered haemodynamics perceived by the endothelium influences intimal SMC proliferation.
The observation that IH does not develop in arterial veins in the flow rig model is not surprising in the light of current literature showing that PDGF (Hsieh et al. 1991, Mitsumata et al. 1993, Malek and Izumo 1994) and endothelin (Sharefkin et al. 1991, Malek and Izumo 1992, Kuchan and Frangos 1993, Morita et al 1994, Malek and Izumo 1994) gene expression is decreased by high shear and increased by low shear conditions.

Although the role of flow in the development of intimal hyperplasia has been investigated in animal models, there have not been any detailed studies using intact human vein because of the difficulties in designing a suitable and reliable flow rig. During the last decade a number of laboratories have used in vitro cell culture systems to investigate the effect of flow on endothelial biology (Eskin et al. 1984, Levesque and Nerem 1985) and SMC biology (Sterpetti et al. 1993, Sterpetti et al. 1994).

The direct contact of the endothelium with the blood means that it is uniquely placed to serve as a mediator of haemodynamic stresses, however it is only one of the participants in the process of intimal proliferation. Consequently, the development of the flow model described here offers future potential as an in vitro system which retains the cellular architecture of the vein wall and the ability to study cellular interactions.

When considering the effects of fluid flow in a vessel, the vessel wall is subjected to two main haemodynamic forces, wall stress, which is proportional to pressure, and shear stress as already defined above. Variations in flow are reflected by changes in shear stress at a fixed viscosity (Figure 1, Hagen-Poiseuille formula), therefore the model described does not separate the two. This could however, be achieved by applying a fixed flow rate and varying the shear stress by altering the viscosity of the medium (Malek and Izumo 1992, Bussolari et al. 1982).
10.5 Future proposals using the flow model

Initially, five more basic experiments as described above will be performed in order to obtain ten evaluations. The following experiments will then be performed:

(i) The effect of injury and arterial flow

The response of an injured vessel may be very different in the presence of flow to that seen in static culture. The aim is to study both endothelial injury alone (produced by a band of gentle denudation), and in combination with SMC injury (by a central crush injury).

(ii) The effect of turbulent flow

It is widely known that the early atherosclerotic lesions develop first at bifurcations, areas characterised by variable shear stress of low magnitude (Ku et al. 1985, Glagov et al. 1988, Asakura and Karino 1990, Kraiss et al. 1991). Turbulence may also be a causative factor in the development of vein graft stenoses, therefore this model will be used to perform a series of experiments to investigate the effect of turbulent flow. Using the trypan blue injection method (10.2i), we have shown that by placing a block of silicone resin inside the vein tubing, turbulent flow is observed distal to the block. Using three segments of vein for each experiment, one will be cultured in static conditions as control, a second segment under arterial laminar flow by placing in the flow rig proximal to the silicone block, and a third segment will be placed at the site of maximal flow disturbance distal to the block.

(iii) Role of growth factors

In all of the studies described above, the effects of the experimental variables on neointimal formation will be measured. We further propose to investigate the expression of the growth factors involved in stimulating SMC proliferation using immunohistochemistry and in situ hybridisation. The growth factor stimuli for intimal proliferation are probably present from the first few hours of culture (Malek and Izumo 1992) and therefore preliminary studies are already underway to perform immunohistochemistry and in situ hybridisation on static and flow veins from 6 hours in culture up to a maximum of 14 days.
It is anticipated that the results of such studies may give important information about the early signalling causing IH, and hence help in the design of strategies to prevent it.

(iv) Future potential

The in vitro flow model developed in this study is unique in that it has devised a system in which intact human vein can be cultured under flow conditions. Although it is still in its infancy, it has the potential for further development and sophistication which will hopefully provide exciting steps forward in the search for effective preventative treatments.
CHAPTER 11

SUMMARY AND FUTURE PROSPECTS
SUMMARY, CONCLUSIONS AND PROSPECTS FOR FUTURE RESEARCH

Despite intensive research over the last decade, our current understanding of the aetiology of vein bypass graft stenoses remains incomplete. A review of the recent literature reveals that as many as 30% of infrainguinal vein grafts develop a stenosis and that 76% of these occur in the first post-operative year (Chapter 1). Recent studies have shown that early detection of such lesions and subsequent correction significantly improves graft patency (Berkowitz 1989, Moody 1990, Idu 1993).

In order to define the microscopic structure of the macroscopically normal long saphenous vein, an ultrastructural and histological study was conducted in patients undergoing arterial surgery (Chapter 3). This study highlighted the problem of defining the vein intima using conventional histological stains such as Haematoxylin and Eosin. The development of a novel stain combining monoclonal anti-smooth muscle actin with Millers elastin facilitated the precise definition between the layers of the vein wall. It was demonstrated that intimal thickening along with medial longitudinal muscle hypertrophy was a common observation. The median (range) intimal thickness of the long saphenous vein in this population of patients was 35.5 μm, (8-381 μm). The median (range) medial thickness was 314 μm, (135-459 μm). Although 95% of the veins in this study had an intimal thickness of less than 200 μm, and therefore were classed as “normal”, one might expect a vein with a thin intima to behave differently from a thickened vein when each is used as an arterial bypass graft. It has now been shown that pre-existing intimal thickening influences the performance of the vein as a bypass conduit, but it is not known what degree will significantly affect graft patency. A study is currently in progress to investigate the correlation between initial intimal thickness and subsequent graft stenosis, since all of these veins were macroscopically normal, despite some marked histological changes.

In order to conduct a detailed study of vein graft intimal hyperplasia, a good experimental model is required. Animal studies are numerous, but relating such studies to
the human vein graft situation remains a problem, and interpretation of the results has been
difficult (Ferrell 1992). Isolated cell cultures have also shed light on the cellular events
involved in graft stenosis, but the limitations of isolated cultures must always be borne in
mind (Chapter 2). The smooth muscle cell and the endothelial cell are fundamental cellular
elements in the development of intimal hyperplasia, and it has been shown that these cells
can be induced to form a neointima under conditions of organ culture of the long saphenous
vein (Soyombo et al. 1990). This is therefore a useful model for further studies, as it not
only avoids the use of animal models, but also retains the architecture of the vein wall and
hence its cellular components. Furthermore, such experimentation involves the use of the
very organ that causes the clinical problem, that is, the human long saphenous vein.

An organ culture of human saphenous vein was therefore established and validated
(Chapter 4). Under culture conditions the intimal smooth muscle cells proliferate
subendothelially and undergo a phenotypic modulation from the contractile to the secretory
phenotype. A comparison of cultured vein with vein graft stenoses was undertaken, and it
was shown that the laboratory model contained all the basic elements of the intimal
hyperplastic lesion: highly proliferative, phenotypically modified smooth muscle cells
interacting with the endothelium within their normal anatomical extracellular matrix
(Chapter 4).

For this study it was important to identify a suitable cell proliferation marker.
Chapter 5 investigated several markers and concluded that in “our hands”
bromodeoxyuridine was the most suited to the vein culture model. SMC proliferation
reached a peak value by 10 days and declined rapidly after 14 days, with neointimal
thickness being maximal by 14 days and being maintained thereafter up to 21 days.

In agreement with Soyombo et al. (1990), it was shown that the endothelium is able
to promote intimal thickening in organ culture (Chapter 6), and a coculture study (Chapter
7) demonstrated that this is facilitated by the production of a soluble paracrine factor which
can promote smooth muscle cell proliferation in a denuded vein segment. Interestingly,
isolated saphenous vein endothelial cells in coculture with denuded vein segments were unable to initiate intimal proliferation (Chapter 8). Furthermore, restoration of an endothelial monolayer on denuded vein segments did not restore the proliferative response of these veins in culture (Chapter 9). A number of possible explanations for such observations were discussed, with phenotypic alteration of the cultured endothelial cells being the most likely.

Many studies have emphasized the importance of blood flow, and more specifically the resulting shear stress it imposes on the cells of the vessel wall. Mechanical forces are constantly present and modifying biological function, but the experiments described so far have all been conducted in static culture. It was therefore a logical progression to try to develop a laboratory model incorporating flow, the results of which are presented in Chapter 10.

**Future Prospects**

Most vein graft stenoses are short, isolated lesions which are difficult to explain, because there is little evidence to suggest that localising factors (injury, valves or tributaries) are causative factors. The possibility exists, however, that areas of turbulent or altered flow may exist in vein grafts, and lead ultimately to a stenosis.

The development of saphenous vein organ culture by Soyombo and colleagues in 1990 was an important landmark in our understanding of IH, and has greatly facilitated the study of the cellular events underlying its development. However, it is limited by the fact that its “static” environment does not reproduce the *in vivo* mechanical forces induced by blood flow which is a potent mediator of vascular cell growth responses.

The observation that IH develops in this no-flow model is not surprising in the light of some other studies showing an inverse correlation between intimal thickness and flow (Dobrin et al. 1989, Morinaga et al. 1985). Further studies are already in progress whereby segments of saphenous vein can be cultured under conditions simulating both arterial and venous flow rates, shear stresses and pressures. Initial results have shown that laminar arterial flow totally prevents the development of IH, whereas venous flow only partially prevents it.
Such a flow model promises to be a useful and versatile experimental tool. It is envisaged that it will be possible to study "injured" veins in this system and also to produce turbulence in the flow circuits.

It is an important objective to identify the endothelial mediator(s) of IH in the static culture. Studies are presently being conducted in our laboratory to observe the effect of a number of recombinant growth factors on the proliferation of isolated saphenous vein SMC's. At the present time, it would appear that PDGF-BB is a likely candidate. It would then be a worthwhile aim to repeat the coculture studies described in Chapter 7 in the presence of blocking antibodies to the growth factor(s) identified in the above studies, and examine their effects on the development of IH.

It can be seen from the results presented in Chapter 5, that intimal cell proliferation is already well established by day 4, and therefore the growth factor stimuli for IH may be present in the vein culture model from the first few hours in culture. This may equally well be the case at the time of arterial bypass grafting. Using in-situ hybridisation studies, the localisation and quantification of mRNA at different time points and different flow conditions, may yield important information about the early signalling causing IH; studies are now underway using these techniques.

Clearly, it would be preferable to prevent the development of stenosis rather than be faced with the problem of treating an established lesion. Currently available treatments have not been shown to be particularly effective: evaluation of a wide range of pharmacological agents (Chapter 1) has shown mixed results, with no single agent having any significant benefit. Furthermore, many such agents have been evaluated in animal models, the results of which have not been reproducible in human studies. Most clinical trials have been involved with the prevention of restenosis after coronary angioplasty, but veins may respond in a very different way to arteries.

The antisense oligonucleotides constitute a new class of therapeutic agents which have been used successfully to investigate single gene function in vascular smooth muscle.
cell proliferation (Simons et al. 1992, Bennett et al. 1994). *In vitro* stimulation of SMC growth has been shown to be followed by an increase in *c-myc* (Bennett et al.) and *c-myb* (Simons et al.) messenger RNA. It may therefore be a future possibility to locally deliver antisense oligonucleotides, for example as a gel applied to the vein graft at the time of insertion, to target and inhibit specific gene products that mediate intimal SMC proliferation.

Vein graft stenoses are the major cause of graft occlusion after the first postoperative month. Graft occlusion carries a significant morbidity, with 3-year limb salvage rates of only 30-40% after repeat bypass surgery (London et al. 1993). Although stenoses can be detected by routine graft surveillance and sometimes treated by percutaneous transluminal angioplasty, the procedure is expensive and long-term results are unknown. To determine the precise aetiology of vein graft IH, and then design strategies to prevent it, would undoubtedly be preferable. Such studies as outlined above, are designed to move towards this goal.
APPENDICES
APPENDIX 1

STAINING METHODS

1. HAEMATOXYLIN AND EOSIN (H & E)
Dewax section in xylene and hydrate in 99% and then 96% methylated spirits. Rinse in water and cover with haematoxylin solution for 5 minutes. Non nuclear staining can be removed by a process of washing with acid alcohol followed by bicarbonate. Then cover with eosin for 2 minutes. Rinse, dehydrate in 96% and then 99% methylated spirits, transfer to xylene and mount in resin. Nuclei are stained blue, muscle dark pink and collagen light pink.

Solutions

Mayer's Haematoxylin

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematoxylin</td>
<td>1g</td>
</tr>
<tr>
<td>Water</td>
<td>1000ml</td>
</tr>
<tr>
<td>Potassium Alum</td>
<td>50g</td>
</tr>
<tr>
<td>Sodium iodate</td>
<td>0.2g</td>
</tr>
<tr>
<td>Citric acid</td>
<td>1g</td>
</tr>
<tr>
<td>Chloral hydrate</td>
<td>50g</td>
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</table>

Acid Alcohol

<table>
<thead>
<tr>
<th>Ingredient</th>
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</thead>
<tbody>
<tr>
<td>99% Methylated spirits</td>
<td>700ml</td>
</tr>
<tr>
<td>Water</td>
<td>300ml</td>
</tr>
<tr>
<td>Conc. Hydrochloric acid</td>
<td>10ml</td>
</tr>
</tbody>
</table>

Eosin solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous Eosin</td>
<td>10g</td>
</tr>
<tr>
<td>Water</td>
<td>1000ml</td>
</tr>
</tbody>
</table>
2. ELASTIC VAN GIESON

Dewax and take sections to water. Rinse in 95% alcohol and place in Millers staining solution for 1 hour. Rinse again with 95% alcohol and wash with deionised water.

Counterstain with Van Giesons solution. Dehydrate through graded alcohols, clear and mount.

<table>
<thead>
<tr>
<th>Elastic fibres</th>
<th>Blue/black</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>Red</td>
</tr>
<tr>
<td>Cytoplasm/muscle</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

Solutions

**Millers staining solution**

- Victoria blue 1g)
- New Fuchsin 1g) Dissolved in 200ml hot deionised water
- Crystal violet 1g)

Add in the following order:

- Resorcinol 4g
- Dextrin 1g
- 30% ferric chloride 50ml

Boil for 5 minutes, filter, discard the filtrate. Dissolve the precipitate in 200 ml of 95% alcohol and boil on electric stirrer for 15 minutes. Filter and make up to 200 ml with 95% alcohol. Add 2ml concentrated hydrochloric acid.

**Van Gieson Stain**

- Saturated Picric acid 50ml
- 1% Acid Fuchsin 9mls
- Distilled water 50ml
3. ALCIAN BLUE/P.A.S. FOR NEUTRAL/ACID MUCINS

Dewax sections, rinse in water and cover with alcian blue for 5 minutes. Wash off with deionised water. Treat with periodic acid for 10 minutes. Repeat wash with deionised water and then treat with Schiff's reagent for 15 minutes. Rinse again with water for 10 minutes and counterstain with Mayers Haematoxylin for 30 seconds. Wash, dehydrate, clear in xylene and mount.

Acid mucins stain blue
Neutral mucins stain magenta

Solutions

1% Periodic acid

50% periodic acid 1ml
Deionised water 50ml

Alcian Blue solution (pH 2.5)

Deionised water 97ml
Acetic acid 3ml
Alcian blue 1g

Schiff's Reagent (BDH Chemicals Ltd. Lutterworth, Leics.)

3. SMOOTH MUSCLE ACTIN/MILLERS ELASTIN

Rehydrate sections as previously described above. Block endogenous peroxidase activity with a solution of 3% hydrogen peroxide for 10 minutes. Wash and incubate in normal rabbit serum (DAKO X902, High Wycombe, Bucks.) diluted 1:20 in tris buffered saline (TBS) for 10 minutes. Drain and then incubate overnight with mouse monoclonal anti-human α actin (DAKO M851) at a 1:400 dilution in TBS at 4°C. Wash in phosphate buffered saline (PBS) for 20 minutes. Incubate in peroxidase conjugated rabbit anti-mouse
immunoglobulins (DAKO P260) diluted 1:50 in TBS for 30 minutes. Wash slides in PBS for 20 minutes. Demonstrate peroxidase using a 5mg/ml solution of diaminobenzidine in PBS for 5 minutes. Wash in water and rinse with 95% methylated spirits followed by 95% alcohol. Cover with Millers staining solution (see above) for 1 hour. Rinse in 95% methylated spirits, dehydrate in alcohols, clear in xylene and mount.

| Muscle fibres | Brown |
| Elastin fibres | Blue/black |

5. AVIDIN BIOTIN COMPLEX METHOD FOR MONOCLONAL MARKERS.

QB-END.10, (Europath NCL-END)
CD 31, (DAKO M823)
Laminin, (Europath PLA)
PCNA, (DAKO M879)

Follow protocol for smooth muscle actin stain as above, with overnight incubation with the relevant primary antibody. For CD 31, the hydrated section should be washed with trypsin at the start of the process. After overnight incubation, wash with PBS and then incubate with rabbit anti-mouse immunoglobulins (1:400) for 30 minutes. Wash again with PBS and incubate with preformed avidin-biotin complex (Vector Elite PK-6100) for 30 minutes. Wash slides in PBS for 20 minutes. Demonstrate peroxidase using a 5mg/ml solution of diaminobenzidine in PBS for 5 minutes. Wash with water and then counterstain with Mayers haematoxylin. Dehydrate through graded alcohols, clear in xylene and mount.

**Solutions**

**Trypsin**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>0.3g</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.36g</td>
</tr>
<tr>
<td>Water</td>
<td>300ml</td>
</tr>
</tbody>
</table>

Adjust to pH 7.8 using weak sodium hydroxide.
Avidin-Biotin complex

- Avidin 100ml
- Biotinylated horse radish peroxidase 100ml
- PBS 5ml

6. BROMODEOXYURIDINE TECHNIQUE

Take sections in 95% alcohol and block endogenous peroxidase with 3% hydrogen peroxide in 50% methanol at room temperature for 30 minutes. Rinse in water and then PBS. Reveal the antigens by incubation with 0.025% pepsin (Sigma, Poole, Dorset) in 0.01N hydrochloric acid for 30 minutes. Rinse in PBS and incubate in 2N hydrochloric acid at 37°C for 30 minutes. After this, cover in Borax (Na$_2$B$_4$O$_7$) for 10 minutes. Rinse with PBS and cover with normal rabbit serum for 10 minutes. Follow by incubation with primary mouse anti-BrdU monoclonal antibody (DAKO M744) for 60 minutes at 37°C. Wash with PBS and incubate with rabbit anti-mouse immunoglobulin (DAKO P161) (1:100) at room temperature for 30 minutes. Wash with PBS and develop colour with a solution comprising diaminobenzidine 5mg, PBS 5ml, 3% hydrogen peroxide 70ml at room temperature for 5 minutes. Counterstain with Mayers haematoxylin, dehydrate, clear and mount. Proliferating cells stain brown.

7. Ki 67 (On frozen sections)

Remove sections from freezer and allow to warm to room temperature. Fix for 10 minutes in acetone, then wash in running water for 2 minutes. Block endogenous peroxidase with 3% hydrogen peroxide in methanol for 30 minutes. Incubate with normal rabbit serum 1:20 for 10 minutes, drain and apply Ki 67 antibody (DAKO M722) at a 1:25 dilution overnight at 4°C. Wash in modified TBS for 20 minutes, then apply rabbit anti-mouse immunoglobulins (DAKO Z259) at a dilution of 1:50 for 30 minutes. Wash again in modified TBS and apply mouse alkaline phosphatase anti alkaline phosphatase (APAAP, DAKO D651) diluted 1:100 for 30 minutes. Wash for 20 minutes in modified TBS and incubate with colour developer for 1 hour. Wash in water for 5 minutes, then counterstain.
with haematoxylin for 30 seconds. Rinse in tap water for 2 minutes and mount in aqueous mountant. Proliferating cells stain brown

Solutions

Colour developer

SOLUTION 1
- Levamisole (Sigma) 24mg
- Fast Red TR (Sigma) 50mg
- Veronal acetate buffer 100ml

SOLUTION 2
- Napthol as B1 phosphate (Sigma) 50mg
- Dimethylformamide Small amount (BDH)

Dissolve napthol in a small quantity of dimethylformamide. Add solution 2 to solution 1 and filter.

Veronal acetate buffer

- Sodium acetate trihydrate 3.886g
- Sodium barbitone 5.886g
- Sodium chloride 5.84g
- Magnesium chloride hexahydrate 10.16g (All BDH)

Take 500 ml distilled water and dissolve each chemical in turn. Adjust to pH 9.2 with 1N hydrochloric acid. Transfer to a 1 litre volumetric flask and make volume up.
APPENDIX 2

TRYPsinisation Technique for Endothelial Cells

At confluence, primary cultures of HSVEC's were released from 25cm² culture flasks by the use of trypsin solution as follows:

1. Aspirate and discard all the cell culture medium from the flasks to be passaged.
2. Wash the cells twice with 5ml MEM. This removes all traces of FCS which inhibits the action of the trypsin.
3. Add 1ml of a solution of working strength trypsin/EDTA solution, tilting the flask several times in order to ensure complete coverage of the cell monolayer. Screw flask up tightly.
4. Incubate at 37°C in CO₂ incubator for 2-3 minutes, agitate flask gently and view under the light microscope to ensure all cells are now rounded in appearance and free-floating.
5. Immediately add 11 ml complete culture medium, mix gently and aliquot 4 ml of the cell suspension into each of 3 new flasks, and return to incubator. Cells will adhere within 1-2 hours and grow to confluence within 5-7 days, when they may be passaged again, frozen down or used in experiments.

Working strength trypsin/EDTA

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5% trypsin (Gibco BRL, Paisley, Scotland)</td>
<td>20ml</td>
</tr>
<tr>
<td>1% EDTA solution (Fisons, Loughborough, Leics.)</td>
<td>10ml</td>
</tr>
<tr>
<td>1M HEPES buffer (Gibco BRL)</td>
<td>10ml</td>
</tr>
<tr>
<td>Phosphate Buffered Saline (PBS)</td>
<td>460ml</td>
</tr>
</tbody>
</table>

Giving a working strength solution of 0.1% trypsin / 0.02% EDTA
BIBLIOGRAPHY
BIBLIOGRAPHY


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