CLINICAL ASPECTS OF VEIN GRAFT STENOSIS AND THE ROLE OF ENDOTHELIN AND ITS INHIBITORS IN INTIMAL HYPERPLASIA

BY

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A Thesis Submitted to the University of Leicester

for the Degree of

Doctor of Medicine

From the Department of Surgery University of Leicester

April 2000

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The work on which this thesis is based is my own independent work except where acknowledged

Deji H Olojugba

April 2000

Dedicated to Joshua

A man "must have one great ideal to aim at, to a certain extent excluding all else..... and his convictions must be very strong."

William Beveridge (1879-1964)

ACKNOWLEDGEMENTS

The work described in this thesis was carried out in the Department of Surgery, University of Leicester under the supervision of Professor N.J.M London. Completion of this work would have been impossible without his strong support and encouragement. I am totally indebted to him. I am especially grateful to Professor P.R.F. Bell for his advice and support and providing me with the opportunity to conduct this research in his department.

Dr Karen Porter deserves special mention, I thank her for the immense support she gave me in all aspects of the laboratory work and for sticking with me even when every thing was going wrong! I acknowledge her contribution to the work on ECE inhibitors.

My acknowledgements are extended to all the members of the Vascular Studies Unit for performing the duplex scans on the patients and their assistance in the prospective clinical studies in particular to Ann Reid for setting me up on the departmental database.

I acknowledge the assistance of the technical staff in the histopathology laboratories of the Leicester Royal Infirmary in preparing and staining the numerous histological slides.

I thank all my medical and non medical colleagues in the department of surgery for making the working place home away from home.

I thank Mike Jackson along with Mrs Jane Allen for sorting out all types of issues during my period in the department. I also thank Sandie Smith for typing the abstracts for my presentations.

I made new friends in Leicester. I am grateful for their collective and individual support on which I had to rely on so often. In particular I thank Miss Manon Kiers who did so much for so little.

I thank the Nuffield hospital for part funding my research. I also thank all the nursing staff for making the on call shifts bearable. I thank my parents who saw me through this, I hope I have made you proud.

Lastly but by far the most important, I thank God by whom all things are made possible

ABSTRACT

For over 25 years there has been intense research into vein graft stenoses. Despite the vast amount of current information, there is still no effective method of preventing them. This thesis looks at clinical and biological aspects of vein graft stenoses in order to improve on existing management strategies and to explore the possibility for a new pharmacological therapy using antagonists of the endothelin system.

After an overview of peripheral vascular disease, the introductory chapters discuss vein graft surveillance, intimal hyperplasia and properties of the vasoactive peptide, endothelin.

The work described consists of clinical and laboratory based research. In the clinical chapters a retrospective study analysed the influence of patient factors on the outcome of lower limb vein grafting in the current era of postoperative vein graft surveillance. Following this, two prospective studies examined specific aspects of graft surveillance. Firstly, the predictive value of pre-discharge duplex vein graft scans was determined. The second study validated the criteria for intervention in duplex detected vein graft flow abnormalities.

The first laboratory experiments set out to determine the effect of endothelin and endothelin receptor antagonists on proliferation in isolated venous smooth muscle cells. Following this, an organ culture system, a more representative model of intimal hyperplasia, was used to demonstrate the association between endothelin production and development of intimal hyperplasia. Using the same model, a series of experiments were then performed to determine the effect of endothelin inhibition. Endothelin was inhibited at the level of its synthesis, and by none selective and then selective receptor blockade.

The final chapter summarises and concludes the main findings and discusses areas of future work that could arise from this thesis.

Publications and presentations arising from this thesis

Published papers

Olojugba D H, McCarthy M, Naylor A R, Bell P R F, London N J M. 1998. At what peak velocity ratio should duplex detected infrainguinal vein graft stenoses be revised? *European Journal Of Vascular And Endovascular Surgery* 15 (3): 258 - 260.

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Published abstracts

Olojugba D H, Porter K E, London N J M 1997. ET_B receptor mediates intimal hyperplasia in human long saphenous vein. *British Journal of Surgery*

Olojugba D H, K E Porter, P R F. Bell, N J M. London. 1998. Both ET_A and ET_B receptors mediate proliferation in isolated human saphenous vein smooth muscle cells. *British Journal of Surgery* (85) 691

Oral presentations

Both ET_A and ET_B receptors mediate proliferation in isolated human saphenous vein smooth muscle cells.

Presented at the Surgical Research Society meeting London, January 1998.

Saphenous vein intimal hyperplasia is mediated via the ET_B receptor.

Presented at the East Midlands surgical society meeting, Doncaster October 1997.

Human saphenous vein intimal hyperplasia is mediated via endothelin B receptor.

Presented at the Surgical Research Society meeting July Nottingham 1997.

Poster presentation

Human saphenous vein intimal hyperplasia is mediated by ET_B receptors

Porter K E, Olojugba D H, Bell P R F, London N J M. Fifth international conference on endothelins, Kyoto Japan 1997.

List of abbreviations

ABPI	Ankle Brachial Pressure Index
ANP	Atrial Natriuretic Peptide
bFGF	Basic Fibroblastic Growth Factor
big ET	Big Endothelin
cAMP	Cyclic Adenosine Monophosphate
CDS	Colour Duplex Scan
CLI	Critical Limb Ischaemia
DAG	Diacyl Glycerol
DMSO	Dimethyl Sulphoxide
EDV	End Diastolic Velocity
EC(s)	Endothelial Cell(s)
ECE	Endothelin Converting Enzyme
ELISA	Enzyme Linked Immunosorbent Assay
ET-1	Endothelin - 1
ET(s)	Endothelin(s)
ET_A	Endothelin Receptor A
ET_B	Endothelin Receptor B
FCS	Foetal Calf Serum
GS	Graft Surveillance
HUV	Human Umbilical Vein
IC	Intermittent Claudication
IH	Intimal Hyperplasia
IP4	Inositol Tetrakisphosphate
ISVG	In Situ Vein Graft
MEM	Minimal Essential Medium
NEP	Neutral Endopeptidase
NGS	Normal Goat Serum

NI	Neointima
PDGF	Platelet Derived Growth Factor
PKC	Protein Kinase C
PLD	Phospho Lipase D
PSV	Peak Systolic Velocity
PTA	Percutaneous Transluminal Angioplasty
PVD	Peripheral Vascular Disease
PVR	Peak Velocity Ratio
QOL	Quality Of Life
RVG	Reversed Vein Graft
SMA	Smooth Muscle Actin
SMC(s)	Smooth Muscle Cell(s)
STX	Sarafotoxin
TGF-ß	Transforming Growth Factor Beta
VCM	Vein Culture Medium
VEGF	Vascular Endothelial Growth Factor
VSMC(s)	Vascular Smooth Muscle Cell(s)

Contents

Overview and scope of this thesis

Using the long saphenous vein as a conduit to bypass segments of occluded lower limb arteries is an effective method of revascularisation. However, an average of 30% of these grafts develop stenotic lesions that can lead to graft failure. Unsuccessful salvage of a failing or failed graft would mean the return of limb threatening ischaemia. Thus great efforts have been made to understand these lesions. Despite this, there is no current method of preventing their occurrence. The work described in this thesis has examined specific clinical and laboratory aspects of vein graft stenosis.

The first three chapters introduces various subjects which are of relevance to this thesis.

Chapter 1 discusses peripheral vascular disease and explains the symptomatology and management strategies. Long saphenous vein grafts are introduced as the conduit of choice for surgical revascularisation. The clinical implications of developing stenosis within these grafts are then discussed. Graft patency has improved with the advent of postoperative surveillance. The various techniques for detecting, monitoring and treating stenosis are included in this chapter.

Chapter 2 describes the biological processes that veins undergo once they have been grafted into the arterial circulation and how this relates to the development of graft stenosis. The roles of the endothelium, growth factors, and haemodynamic factors in intimal hyperplasia are discussed.

In Chapter 3, endothelin is introduced as a polyfunctional peptide. Its structure, synthesis, types of receptors and functions are discussed. The use of endothelin inhibitors as experimental tools and possible therapeutic drugs becomes evident. Specifically, its actions as a mitogen with a role in intimal hyperplasia are highlighted.

Graft patency has been improved by attention to surgical techniques and postoperative surveillance. With the failure of pharmacotherapy to prevent graft stenosis, the aim of the study in Chapter 4 was to determine if other clinical factors significantly affected the outcome of vein graft revascularisation. Such factors could be modified to further improve the outcome of these procedures.

There is a lot of controversy concerning the principles and practice of graft surveillance. It does not prevent graft stenosis, however, most authors accept that it provides a significant improvement in graft patency. The prospective studies described in Chapter 5 examine two aspects of graft surveillance. The first study looks at the benefits of starting graft surveillance prior to discharging patients from hospital, i.e. within the first 2 postoperative weeks. The hypothesis is that such early scans can detect the flow abnormalities that eventually progress into graft stenosis. If this was true, then high risk grafts can be identified at an early stage. The second study in Chapter 5 looks at the criteria used to distinguish significant duplex detected graft stenosis from non significant flow abnormalities. This issue is of importance as various centres use different criteria to decide on which flow abnormalities require correction. Thus there is a danger of correcting either too many or too few lesions.

The subsequent experimental chapters describes the laboratory work aimed at using endothelin antagonists to prevent intimal hyperplasia which is the underlying cause of stenosis. Smooth muscle cell proliferation is central to the formation of intimal hyperplastic lesions, thus Chapter 6 examined the effect of endothelin receptor blockade on proliferation in isolated saphenous vein smooth muscle cells.

Chapter 7 describes a validated in vitro model of saphenous vein intimal hyperplasia. In the same chapter, experiments that aimed to establish endothelin peptide production and expression in the model are described.

The effects of endothelin can be inhibited at the level of synthesis or at the level of its receptors. In Chapter 8a, the effects of inhibiting the conversion of endothelin to its active form is examined in respect to experimental intimal hyperplasia. A similar study is undertaken in Chapter 8b which describes the effect of dual receptor blockade. Finally, in 8c, selective antagonists are used to determine which of the receptors play the significant role in the formation of intimal hyperplasia in this human experimental model.

The work in this thesis answers some important question but at the same time it raises issues that deserve further attention. The last chapter summarises the main conclusions and discusses the prospects for future work.

Chapter 1

PERIPHERAL VASCULAR DISEASE AND VEIN GRAFT REVASCULARISATION

Ia *Peripheral Vascular Disease*

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Ia

PERIPHERAL VASCULAR DISEASE

1a.1 INTRODUCTION

Atherosclerosis is the leading cause of arterial disease in the lower limbs. It is a disease process that affects medium to large-sized arteries and results in the deposition of fibrous plaques in the intima which cause a thickening that encroaches on the vessel lumen. The widespread occurrence of atherosclerosis in the coronary, cerebral and peripheral circulation accounts for the spectrum of arterial occlusive diseases which constitute the commonest cause of morbidity and mortality in the Western population. Atherosclerosis in the iliac arteries and vessels distal to it in the lower limbs is referred to as Peripheral Vascular Disease (PVD). However, there are other much less common conditions such as thromboangiitis obliterans (Buerger's disease - an inflammatory vasculopathy) and cystic adventitial disease of the popliteal artery which can give rise to lower limb arteriopathy.

The first part of this chapter will review the prevalence of PVD, its symptomatology and treatment options. The next part will focus on vein grafts in the management of lower limb arterial disease. The last part will discuss the clinical aspects of vein graft stenosis.

1a.2 PREVALENCE

The prevalence of PVD in adults over 40 ranges between 0.5% to 4.5% for symptomatic disease and 4.2% to 28% for asymptomatic disease (Tables 1a.1 and 1b.2). Any quoted prevalence of PVD has to be interpreted in context of the population that the data is taken from as well as the method of measurement. This is because prevalence varies with age, population of study and gender. Furthermore, the majority of patients with PVD are asymptomatic *(Dormandy and Mahir 1992)* and some reports have only measured symptomatic disease.

Diagnosis of peripheral vascular disease for epidemiological purposes is either in the form of standard questionnaires such as the Rose questionnaire *(Leng and Fowkes 1992; Rose 1962)* which measure symptomatic disease only, or in the form of non invasive tests which measure asymptomatic disease as well. The most commonly used non invasive test is the

Ankle Brachial Pressure Index (ABPI) which has been shown to have a sensitivity and specificity above 95% *(Fowkes 1988)*. Thus studies have shown that the prevalence of PVD measured with the ABPI is 3-4 times that measured by questionnaires alone *(Fowkes et al. 1991; Schroll and Munck 1981)*.

Table 1a.1 Prevalence of peripheral vascular disease using the Rose questionnaire

Author/year	Population studied	Number in study	Age (yrs)	Sex Distribution	Prevalence (%)
Gofin, 1987	Israel	1,592	35-64	1,036 men 556 women	1.3 1.8
Davey-smith, 1990	England	18,388	40-64	All men	0.8
Fowkes, 1991	Scotland	1,592	55-74	men and women	4.5
Smith, 1991	Scotland	10,042	40-59	men and women	1.1 0.7
Stoffers, 1991	Netherlands	3,654	40-79	men and women	0.5
Newman, 1993	U.S.A	5,084	>65	2214 men 2870 women	2.0

Table 1a.2 Prevalence of peripheral vascular disease using the ABPI

Author/Year	Population studied	Number in study	Age (yrs)	Men/ Women	Prevalence (%)
Gofin, 1987	Israel	1,592	35-64	1036 men 556 women	4.2 5.4
Fowkes, 1991	Scotland	1,582	55-74	men and women	18
Newman, 1991	U.S.A	187	> 60	82 men 105 women	26 28
Stoffers, 1991	Netherlands	3,654	40-79	men and women	6.7 5.6
Postiglione, 1992	Italy	124	> 80	37 men 87 women	35 33

1a.3 SYMPTOMS

The majority of Patients with PVD are asymptomatic, with only between 7% to 9% of patients having symptoms *(Criqui et al. 1985; Newman et al. 1993)*. However the incidence of symptomatic disease increases with age *(Kannel et al. 1985)* and the presence of risk factors *(Hale et al. 1988)*.

Symptomatic PVD presents as varying degrees of limb ischaemia. The majority complain of intermittent claudication (IC) which is described as a cramp like discomfort in the calf that develops on walking, is relieved by rest and reproducible on further exertion. The site of the

discomfort can give an indication of the vessel that is diseased. Thus, pain in the hips and thighs is indicative of aorto iliac disease, whilst pain in the back of the calves suggests disease in the femoropopliteal vessels.

Critical limb ischaemia (CLI) describes a more severe form of the disease. It presents as pain at rest usually in the foot and may be associated with tissue loss in the form of ulceration or gangrene such as is seen in figure 1a.1.

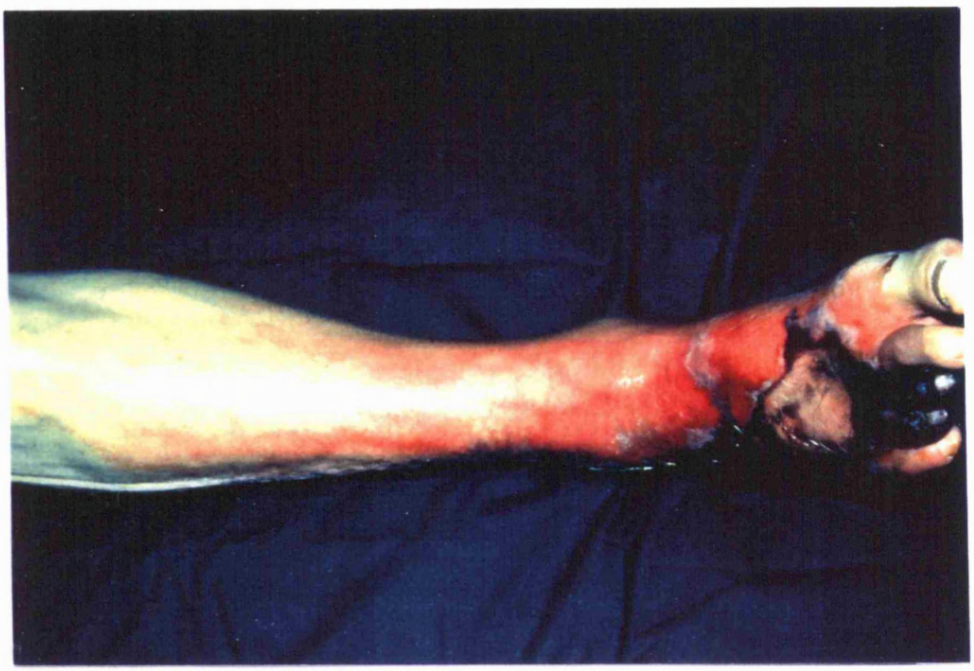

Figure 1a.1. Limb of a patient with critical limb ischaemia. There is established gangrene of the toes.This patient would require urgent revascularisation if major amputation is to be avoided.

Patients with CLI represent a population who risk death as well as limb loss *(Hoofwijk 1991; Wolfe 1986)* if not promptly identified and treated. Thus there should be clear cut criteria for defining these patients. Recently, there have been several definitions of CLI such as that of the international vascular symposium working party, *(Bell et al. 1982; Tyrrell and Wolfe 1993)* and the European working group definition *(European Working Group On Critical Limb Ischaemia 1989)*. The current European consensus defines critical ischaemia as "a persistently recurring rest pain requiring regular analgesia for >2 weeks, and / or ulceration of the foot or toes, plus ankle systolic pressure ≤50 mmHg, or a toe systolic pressure of ≤ 30 mmHg" *(European Working Group On Critical Limb Ischaemia 1991)*. Though these definitions seem

adequate for comparative purposes of clinical trials from different centres, they have been shown to be inadequate in the clinical setting *(Thompson et al. 1993)*. This problem has been illustrated in a recent Italian epidemiological study of 574 patients with critical limb ischaemia. They found that between 20 to 80% of patients would have been excluded if they adhered strictly to the criterion in the European consensus *(The I.C.A.I Group 1996)*.

The current definitions of CLI do not cater for the different grades of severity of CLI. In a review of 6118 patients with CLI pooled from 20 different publications, Wolfe et al. found that it was possible to regroup these patients into low or high risk groups. Interestingly in that study, the 1 year mortality in both groups was similar, though the surviving high risk patients with CLI seemed to benefit more from revascularisation in terms of limb salvage *(Wolfe and Wyatt 1997)*. From the foregoing, it is clear that a redefinition which can also grade the severity of CLI may be more useful in the clinical setting.

1a.4 MANAGEMENT

Traditionally, the management of symptomatic PVD has been based on knowledge of its natural history. About 20%-25% of patients with IC will progress and develop gangrene or rest pain *(Dormandy et al. 1989; Imparto et al. 1975; McAllister 1976)*. The other 80% will either stabilise or resolve with only a 1.6% amputation rate. Thus it is reasonable to adopt conservative measures for the majority of patients. Recently however, there has been renewed interest in the quality of life (QOL) of patients with symptomatic peripheral disease *(Currie et al. 1995; Khaira et al. 1996; Ponte and Cattinelli 1996)*. These suggest that patients with otherwise mild cases of PVD have a reduced quality of life *(Pell 1995)* and that this can be improved by early intervention *(Currie et al. 1995; Ponte and Cattinelli 1996)*. With the availability of minimally invasive techniques to treat PVD, inclusion of the QOL as a parameter for assessing patients with PVD may significantly influence the future management of this disease.

Conservative Measures

This is the first line treatment for most patients presenting with mild to moderate IC. It is in the form of exercise programmes, risk factor modification and drug therapy. Exercise programmes entail walking or exercise for periods ranging from 30 minutes to an hour a day *(Hiatt et al. 1990; Larsen and Lassen 1991; Mannarino et al. 1989)*. It was previously thought that such measures increased blood flow through the collateral circulation *(Ekroth et al. 1978)*. However more recent studies suggest that exercise is more likely to improve metabolic efficiency, muscle oxygen utilisation *(Terjung et al. 1988)* and encourage micro vascular growth *(Lash et al. 1995)*. Though there have been concerns that exercise in patients with PVD can induce a harmful ischaemia-reperfusion type injury *(Khaira et al. 1995; Tisi and Shearman 1998)* the implications of these findings to current practice is not clear. Several clinical trials have shown the ability of exercise programmes to improve patients symptoms and walking performance *(Ernst and Matrai 1987; Hiatt et al. 1990; Larsen and Lassen 1991)*. Recent meta-analysis of such trials have found that exercise can improve walking distance by 179% to 210% *(Gardner and Poehlman 1995; Robeer et al. 1998)*. However, claudication distance

should not be the only clinical end point used to asses the effectiveness of such programmes. Thus, Hiat et al *(Hiatt et al. 1995)* have proposed that QOL questionnaire should be included in future trials. An on going debate in exercise therapy concerns the degree of supervision required to achieve clinical improvement. Recent randomised studies suggest that patients benefit more from hospital based supervised programmes *(Patterson et al. 1997; Regensteiner et al. 1997).*

Risk factor modification entails the cessation of smoking and control of hypertension. The Framingham study demonstrated a two fold risk of developing PVD in smokers and a 2 to 4 fold risk amongst hypertensive patients *(Kannel et al. 1985).* Previous studies have shown that cessation of smoking in patients with IC is associated with a reduced risk of developing critical ischaemia *(Hughson et al. 1978; Jonason and Bergstrom 1987).* However, only about 30% of patients will actually stop smoking on a clinicians advice *(Smith et al. 1996).* Diabetics are at an increased risk of developing PVD and its complications *(Orchard and Strandness 1993).* However, even though tight glycaemic control has been shown to improve the micro vascular complications of diabetes, *(Shamoon et al. 1993)* there is no evidence that it would improve the outcome of macro vascular complications such as PVD.

Three main classes of drugs have been used for the treatment of PVD. These are vasodilator drugs, anti platelet drugs and hemorrheologic agents. However the effectiveness of any of these drug has been difficult to analyse because of inconsistent study designs *(Cameron et al. 1988; Duprez and Clement 1992).*

Though aspirin has had no effect in treating PVD, it has been shown to reduce mortality *(Anti-platelet trialist 1994b).* The hemorrheologic agent pentoxyfylline is licensed for the treatment of PVD in the united states. Though this drug has been shown to improve tread mill exercise time in claudicants, *(Porter et al. 1982)* other studies have suggested that its effects may not persist in the long-term *(Ernst et al. 1992).* In Europe, guidelines have been recently published in regard to studies of drug therapies in PVD *(CPMP Efficacy working party 1994)* . The results of a recent randomised study of a prostaglandin pro drug that has adhered to these guidelines are encouraging *(Belch et al. 1997).* Clearly more trials of this sort are needed.

Endovascular interventions

This minimally invasive form of revascularisation is rapidly becoming an option for the management of all stages of symptomatic PVD. *(London et al. 1995; Sayers et al. 1993c; Tunis et al. 1991).*

(1) Percutaneous Transluminal Angioplasty (PTA) : The first intentional percutaneous angioplasty was performed by Dotter and Judkins in 1964. The basic principle involved percutaneous vessel puncture and passage of a guide wire to negotiate the lumen of the vessel and its stenosis. Angioplasty at that time was performed by the serial passage of dilators over the guide wire. Nowadays, following the introduction of the double lumen catheter by Gruntzig and Hopff *(Gruntzig and Hopff 1974)*, angioplasty is achieved by inflation of a balloon that has been passed over a guide-wire and positioned in the lumen of the narrowed segment of artery. The mechanism of the procedure is to stretch the vessel wall enough to cause localised fractures of the atheromatous plaque. This has been confirmed both in post-mortem specimens *(Block et al. 1981)* and in vivo by using serial intravascular ultrasound *(Losordo et al. 1992).* Apart from the balloon catheters, other endovascular devices have been used for angioplasty. These include laser based probes and mechanical athrectomy rotational devices. However randomised trials of these devices have shown no advantage over conventional angioplasty *(Jeans et al. 1990b; Lammer et al. 1992; Tobis et al. 1991; Vroegindeweij et al. 1992).*

The technical success of PTA in the lower limb can be as high as 80% *(Capek et al. 1991; Johnston 1992; Krepel et al. 1985).* However, in general, technical success in PTA is dependent on the experience of the operator and is more difficult to attain in occluded compared to stenosed vessels *(Capek et al. 1991).* As with any other vascular procedure, initial technical success does not translate into long-term patency. Long-term success of PTA is dependent on several factors. These include the, length, site, location of the lesion and degree of ischaemia *(Becquemin et al. 1994; Jeans et al. 1990a; Krepel et al. 1985).* Occluded vessels are more difficult to treat than stenotic ones. Thus long-term patency can be predicted in the short stenotic lesion located in the iliac vessel in a patient with intermittent claudication.

Reports of long-term success can be detailed according to site of angioplasty. Thus, in iliac angioplasty, patency at 5 years is between 50%-87% *(Becker et al. 1989; Johnston 1993).* The data from 667 procedures presented by Johnston et al. is worthy of note as they used objective clinical and haemodynamic criteria to define long-term success. They reported an overall success rate of 96% and a 3 year success ranging between 30% to 73% depending on the characteristics of the stenotic lesion. They also found poorer results in occlusions and tandem lesions *(Johnston 1993).*

Long-term patency following angioplasty of the femoropopliteal segment averages about 53% to 67% in the larger series *(Becker et al. 1989; Johnston 1992).* The most important determinant of success is the morphological characteristic of the lesion. However, several studies have shown that successfully dilated occlusions have identical long-term patencies to similarly treated stenoses *(Capek et al. 1991; Matsi et al. 1994).* With the introduction of steerable guide wires, low profile balloons and catheters as small as 3.5 French and 2.5 French, tibial and peroneal angioplasty is possible with acceptable results in experienced hands. In selected cases, one can expect a 2 to 3 year clinical success rate of between 76% and 83% in limbs with isolated disease with fewer than five stenoses *(Bull et al. 1992; Schwarten 1991).*

There have been studies comparing PTA with other forms of treatment. The initial benefits of angioplasty versus conservative treatment in intermittent claudication are conflicting. The comparative study by Whyman et al. found that after 6 months, angioplasty resulted in reduced pain on walking when compared to a similar group on medical treatment alone *(Whyman et al. 1996).* The study of 36 patients by Creasy et al. of exercise versus angioplasty showed a progressive increase in maximum walking distance in the exercise group compared to the angioplasty group even though the APBI improved in the latter group only *(Creasy and Fletcher 1991).* Perhaps more importantly, the six year follow up of the same group of patients showed that exercise produced a significantly greater improvement in claudication and walking distance than PTA *(Perkins et al. 1996).*

Angioplasty seems to have results comparable to surgery. In a prospective study of 263 patients with moderate to severe IC, Wolfe et al. found no difference in terms of patency and

limb salvage after 4 years between patients treated with angioplasty or by bypass grafts *(Wolfe et al. 1993)*. Reports such as these may justify the increase use of PTA rather than surgery in the management of patients with IC.

(II) Intravascular stents: There is an appreciable failure rate following PTA. Early failure is due to complications such as elastic recoil, intimal dissection flap and acute thromboses. Late failure is as a consequence of restenosis. Intravascular stents were first introduced to counteract elastic recoil. They are now used to treat PTA induced arterial dissection and raised intimal flaps. Two randomised controlled trials have shown the benefits of primary intravascular stents in the coronary vessels *(Fischman et al. 1994; Serruys et al. 1994)*. There are no randomised studies of stenting in the lower limb vessels and stenting in PVD is currently restricted to complicated iliac and femoropopliteal stenoses and occlusions *(Strecker et al. 1993)*. Nevertheless, iliac stenting is associated with a high technical success rate *(Gunther et al. 1991)* and long-term patency which may be between 69% and 86% at 4 years *(Murphy et al. 1995; Palmaz et al. 1992)*. Recent evidence suggests that primary stenting of illiac vessels does not offer any advantages over angioplasty and selective stenting *(Tetteroo et al. 1998)*. Thus there is insufficient data to warrant primary stenting of uncomplicated iliac and femoropopliteal segments, though some centres have practised this with reasonable clinical success *(Sullivan et al. 1997)*. There is no current evidence to support the deployment of stents in the tibio-peroneal vessels.

(III) Endovascular grafts: Drawing from the experience of using intravascualr stents and grafts to treat abdominal aortic aneurysms, it is now possible to use endovascular techniques of place intraluminal grafts in segments of occluded femoropopliteal arteries. This involves the endoluminal placement of a prosthetic graft in a dilated or recanalized artery. some authors also perform a pre implantation endarterectomy *(Bergeron et al. 1995)* The short term results have been encouraging with one year primary patency of about 75% *(Cragg and Dake 1997; Marin et al. 1995; Spoelstra et al. 1996)*. However, reports on this technique are still too few and procedures tend to be performed on selected patients.

Surgical Revascularisation

The surgical restoration of blood supply distal to an area of infrainguinal occlusive disease is most often achieved by a bypass procedure. This remains the gold standard by which other techniques are compared. The purpose of the graft is to divert blood beyond the stenosed or occluded segment of the vessel. With the routine use of magnification loupes and modern perioperative assessment it is now routine to perform distal anastomoses to the small crural foot vessels *(Bell 1985)*,a feat that was rarely practised 20 years ago.

The ideal essentials of a suitable conduit include biocompatibility, a non-thrombogenic surface, an elastic behaviour that can accommodate the pulsatile arterial pressure and suitable luminal dimensions. Three main types of conduits are currently available for bypass procedures (Table 1a.2).

Autologus vein is the conduit of choice for infrainguinal revascularisation procedures and these will be discussed in detail in the next section. However, the other types of commonly used conduits will be discussed here.

Table 1a.2 Types of grafts used for infrainguinal bypass

Type of grafts	Description	Examples
Homografts	Grafts taken from the patients own blood vessels	Long saphenous, lesser saphenous, cephalic, basilic vein
Allografts	Grafts taken from other patients	Cryopreserved graft (not routinely)
Biological grafts	Grafts obtained from processed living tissue	Human umbilical vein
Synthetic grafts	Grafts manufactured from biocompatible synthetic material	PTFE, Dacron

(I) Prosthetic conduits: The two types of prosthetic grafts commonly used are the textile grafts and expanded polytetrafluoroethylene (ePTFE) grafts. The textile graft is made of Polyethylene tetrephthalate polyester (Dacron) and the multifilaments can be either woven or knitted. The knitted dacron graft has the advantage over the woven graft of being more compliant and not fraying at ends. Expanded polytetrafluoroethylene is a fluorocarbon polymer which by nature of its electronegative surface is relatively none thrombogenic.

In clinical practice, ePTFE is the most commonly used prosthetic graft for infrainguinal revascularisation procedures, though a recent American multicentre randomised prospective trial found no significant difference in the performance of ePTFE over Dacron grafts over three years. *(Abbott et al. 1997).*

Following the introduction of PTFE, Campbell et al. reported encouraging early patency rates *(Campbell et al. 1976).* However these results were not matched in the longer-term studies *(Bennion et al. 1985; Budd et al. 1990; Charlesworth et al. 1985; Oriordain et al. 1992).* Direct comparisons of the patency rates from various studies using veins or PTFE grafts is difficult as often there are discrepancies in the indication for surgery and level of anastomosis. However from a meta-analysis by Michaels, it seems that PTFE grafts have never been shown to be superior to autologous vein *(Michaels 1989).* Infrainguinal PTFE grafts are prone to early occlusion *(Veith et al. 1980)* but beyond that time period, late graft failure is usually as a result of the progression of atherosclerosis in the native inflow and runoff vessels *(Veith et al. 1980)* and the development of intimal hyperplasia at the anastomoses *(Taylor et al. 1987a; Veith et al. 1980).* The 5 year primary patency rates of PTFE grafts to the above knee popliteal ranges between 43% to 60% *(Budd et al. 1990; Michaels 1989; Quinones-Baldrich et al. 1992; Wilson et al. 1995a).* Patency of PTFE grafts to the below knee popliteal segment average at 40% after 4 years *(Dalman and Taylor 1990).* The patency following PTFE grafting to distal vessels is poor, ranging between 7- 23% at 4-5 years *(Budd et al. 1990; Dalman and Taylor 1990; Londrey et al. 1991).*

(II) *Operative adjuvants to improve prosthetic distal bypass:* The poor patency rates that result from using prosthetic grafts in distal infrainguinal bypasses precludes their routine use in this situation. However between 10% to 30% of patients requiring lower limb revascularisation do not have adequate veins *(Myhre et al. 1995; Wolfe and Tyrrell 1991).* Therefore a sub population of patients would require some form of prosthetic bypass. In order to improve the results of PTFE grafts to the distal vessels, adjuvant operative techniques are often employed. The idea of an interposition cuff of vein between the prosthetic graft and the artery was first

described by Siegman in 1979. The cuff anastomoses he suggested was too cumbersome to construct as it required 4 separate anastomoses *(Siegman 1979)* and since then there have been several modifications such as the Linton patch, the Miller cuff *(Miller et al. 1984)* and the Wolfe Hood *(Tyrrell and Wolfe 1991)* Currently the most widely used technique is the Miller cuff, introduced by Miller and colleagues in Australia in 1984 *(Miller et al. 1984)*. Vein cuffs have been shown to reduce the formation of intimal hyperplasia at the anastomoses *(Suggs et al. 1988)* and its encroachment into the recipient artery *(Tyrrell and Wolfe 1997)* both of which account for up to 30% of prosthetic graft failures *(Taylor et al. 1987b)*. Thus, they were shown to improve patency rates in experimental animals *(Tyrrell et al. 1990)* and the early results of some of the uncontrolled clinical studies were also encouraging; 69% to 78% at 12 months *(Miller et al. 1984; Tyrrell and Wolfe 1991)*. Recently, a trial conducted by the Joint Vascular Research Group comparing PTFE with and without the miller cuff reported a significant benefit in distal PTFE grafts with a distal collar *(Stonebridge et al. 1995)*.

Creation of arteriovenous fistulae is another type of operative adjuvant used to improve graft patency in distal prosthetic grafts. Such fistulae are thought to increase graft blood flow. The results of the retrospective studies on its benefits are conflicting *(Dardik et al. 1991; Paty et al. 1990; Snyder et al. 1985)* and there has been no prospective study to validate its use as an adjuvant procedure thus so far there is little evidence that such fistulae affect long-term results *(Harris and Campbell 1983)*.

(III) Human umbilical vein grafts: Human umbilical veins (HUV) undergo several processing procedures before being used as conduits. They are subjected to glutaraldehyde tanning and multiple ethanol extractions and are then externally reinforced with a dacron mesh to provide strength. The performance of this graft depends on proper attention to technical handling *(Dardik 1984)* as they can be difficult to implant. However, in good hands grafting to the femoropopliteal segment provides a patency rates of 70% and 50% at 1 and 5 years respectively *(Dardik et al. 1988)*. The patency in the femorotibial segments is less impressive; 50% and 25% at 1 and 5 years respectively *(Dardik et al. 1988)*. HUV are prone to

degenerative changes and aneurysmal changes in these conduits have discouraged many surgeons. The incidence of reported aneurysmal changes varies. It has been found to be as low as 3.5% on clinical assessment alone *(Boontje 1985)* and Dardik et al. reported an incidence of 7.7% on an angiographic 6 year follow up study *(Dardik et al. 1988)*. However, reported findings from other studies using angiograms or duplex scans have been consistently higher, ranging between 33% to 65% after 5 years *(Boontje 1986; Hasson et al. 1986; Karkow et al. 1986)*. The HUV has been modified in an attempt to reduce its susceptibility to degradation, and the proponents of its use report improved patency rates and lower incidence of aneurysmal dilatation *(Dardik 1995)*. However despite an improvement in patency rates, the modified graft is still associated with an unacceptably high incidence of aneurysmal changes that precludes it future routine use *(Sato et al. 1995; Strobel et al. 1996)*.

(IV) Other surgical procedures: Less extensive procedures can assist in revascularisation. Endarterectomy involves developing a dissection plane in the arterial wall. This plane is between the media and the overlying diseased layer of the artery which can then be removed, therefore disobliterating the lumen. This procedure has largely been abandoned by surgeons. However it seems that its abandonment may have been unjustified *(Inahara and Scott 1981; Ouriel et al. 1986; Vansterkenburg et al. 1995)*. Some surgeons have continued with this procedure for femoropopliteal disease and their long-term results have reported 5 year primary patency rates of up to 70% *(Vanderheijden et al. 1993)*.

1b

VEIN GRAFTS

1b.1 HISTORY

The first report of the successful use of a patients own vein to act as a conduit is from the work of Goyanes in 1906 *(Goyanes 1906)* when he excised a popliteal aneurysm and replaced the defect with a segment of adjacent popliteal vein. In 1907, Lexer is reported to have used a segment of greater saphenous vein to bridge a defect in a patients axillary artery *(Lexer 1907)*. These precede the animal work of Carrel in 1908 *(Carrel 1908)* who grafted a preserved segment of dogs venae cava into the carotid artery. Apart from a handful of other reports using vein grafts further progress in this field was halted by the two world wars. Shortly after the second world war, investigators worked on both arterial and vein grafts. In 1948, Kunlin, a French surgeon, introduced the first vein by pass procedure by joining the common femoral artery to the popliteal artery by a 26-cm length of autogenous long saphenous vein *(Kunlin 1948)*. This was quickly adopted in Europe and North America. In the mid 1950s the results of arterial homografts was increasingly disappointing and this increased the popularity of vein grafts. However because suitable veins were in short supply the search for an alternative synthetic graft continued.

After Kunlin introduced the reversed technique of arterial bypass vein grafting, Hall et al. introduced the in situ technique in 1962 *(Hall et al. 1962)* and this enjoyed a lot of interest because several of the authors at that time suggested that the in situ technique resulted in a better patency rates than the reversed technique *(Leather et al. 1979)*. By this time the principle of vein bypass procedures for peripheral vascular disease was universally accepted.

In current practice, the patients own vein is the preferred conduit for lower limb revascularisation *(Michaels 1989; Veith et al. 1986)*. This is certainly true for infragenicular reconstructions. However, the routine use of the vein for above knee bypass procedures remains controversial, as various studies have shown no difference in patency rates between vein and synthetic grafts in the above knee situation *(Budd et al. 1990; Sterpetti et al. 1985)*.

Some centres use prosthetic grafts in the above knee situation in order to preserve the vein for subsequent secondary procedures or coronary artery bypass procedures *(Quinones-Baldrich et al. 1988; Rosenthal et al. 1994)*. However, a recent study by Wilson et al. showed that the demand for veins for secondary procedures is low, less than 4% *(Wilson et al. 1995b)* and other studies have previously shown that very few cardiac patients have inadequate vein as a result of previous peripheral bypass procedures *(Sterpetti et al. 1985)*.

The long saphenous vein is the commonest source of autologus vein. However autologous vein has been harvested from other sites when the long saphenous is inadequate and prosthetic materials are not suitable *(Taylor et al. 1987a)*. The arm veins *(Andros et al. 1986)* and the lesser saphenous vein *(Weaver et al. 1987)* have been used with results comparable to the long saphenous vein bypass *(Graham and Lusby 1982; Harris et al. 1984)*.

1b.2 VEIN BYPASS GRAFTING TECHNIQUES

The guiding principles of the vein bypass techniques as introduced by the earlier surgeons are still applicable today. However there have been modifications aimed at improving the performance and longevity of the grafts.

Depending on availability of adequate vein and the operating surgeons practice, the in situ, reversed and none reversed grafts taken from the patients own long saphenous vein are the main types of grafting techniques currently used. When the long saphenous vein is inadequate or in short supply alternative sources of vein can be used from the short saphenous or the arm veins *(Andros et al. 1986; Weaver et al. 1987)*.

Reversed and none reversed vein grafts

These techniques involve the removal of a length of vein from its native site, ligating its side branches and then re implanting it in a tunnel deep to the subcutaneous tissue or muscles in either the reversed or the none reversed position. Proximal and distal anastomoses to the artery are created to divert the blood flow. When the vein is reversed, the valves do not impede blood flow however the valves have to be rendered incompetent in the none reversed graft. During its removal, the vein has to be dissected free and as a consequence suffers varying

degrees of injury. Furthermore, it is often necessary to distend the vein in order to assess its suitability for grafting. Distension pressures above 100 mmHg may cause significant injury to the vein *(Bush et al. 1984)*. These observations are important because injury may be an important etiologic factor in the subsequent development of vein graft intimal hyperplasia.

In situ vein grafts

The technique is performed using the long saphenous vein in its native position. The proximal and distal ends are mobilised to create the anastomoses diverting blood from the proximal part of the artery through the in situ vein and then back into the artery distal to the diseased segment. The valves have to be destroyed or rendered incompetent and the tributaries ligated or occluded. The valves can be destroyed by passing a valvutome up and down the graft and the tributaries can be ligated directly after exposure via either a whole length incision or multiple short incisions.

With the advent of the angioscopically assisted techniques it is now possible to embolise the tributaries and remove the valves intraluminally *(Maini et al. 1993; Matsumoto et al. 1987)* and therefore obviate the need for multiple or long skin incisions or blind passage of the valvutome. This should translate to a reduced morbidity and length of hospital stay, however one randomised trial failed to show a clear benefit *(Clair et al. 1994)*, contrasting with the study by Rosenthal et al. that did demonstrate a reduction in the length of hospital stay and incidence of wound complications *(Rosenthal et al. 1994)*. Clearly the adoption of angioscopically assisted in situ grafts requires further evaluation.

Following either technique it is routine practice to undertake some form of completion study, such as angiography, duplex scan or pressure measurement.

In situ v Reversed grafts

As discussed above, the reversed vein grafting technique was in general use before the in situ technique was introduced. However the in situ graft gained popularity as it was seen to have advantages over the conventional reversed graft. Firstly by definition, it required minimal dissection and disturbance of the vein. This would reduce the chances of damage to the vasa

vasorum. Secondly, the vein would tend to be of similar diameter to the adjacent artery onto which it would be anastomosed, hence improving the haemodynamics at the anastomoses. However, the in situ operation is technically more difficult than the reversed graft; it is important to destroy all valves and avoid leaving significant cusps or tributaries.

These advantages may just be theoretical because in vitro studies have demonstrated equal endothelial and compliance characteristics in both grafts *(Boyd et al. 1987; Cambria et al. 1987)*. Furthermore, the procedure is not as atraumatic as previously thought. Sayers et al. have shown that the valvutome can cause complete loss of endothelium and patchy necrosis of the smooth muscle cells in the media *(Sayers et al. 1991; Sayers et al. 1992)*. Finally, prospective randomised trials have not shown any significant difference in patency between in situ and reversed vein techniques in distal bypasses *(Harris et al. 1993; Wengerter et al. 1991)*. The study by Sasajima et al. found no difference in the results of two skilled surgeons, one who performed only in situ grafts and the other who used only reversed grafts *(Sasajima et al. 1993)*. Thus correcting for any bias that may have arisen as a result of varied surgical skills in other prospective trials.

1b.3 EARLY AND LONGER-TERM PROBLEMS

Shortly after vein bypass grafts became universally accepted, there were several reports indicating subsequent graft failure and return of symptoms in up to 35% of patients *(Deweese and ROB 1971; Erjup et al. 1961; McNamara et al. 1967)*. Later, in a land mark study using mainly angiographic data, Szilagyi found that these failures were due to the development of vein graft lesions *(Szilagyi et al. 1973)*.

Graft failure is currently classified according to specific time intervals in the postoperative period. This classification corresponds to the different causes of graft failure in each time period. Thus early graft failure is defined as failure occurring within 30 postoperative days. It can account for about 30% *(Donaldson et al. 1992; Varty et al. 1993a)* of graft failures, however though the majority of these early failures result from surgical technical errors, there are some other less common causes (Table 1b.1).

Table 1b.1 Causes of early vein graft failures

Technical
Suture line construction
Twisted graft
Graft entrapment in tunnelled grafts
Poor inflow /outflow
Intimal flaps
Missed valves
Missed branches
Others
Poor quality vein
Hypercoagulable states
Low cardiac output

Graft failure after 30 days (late graft failure) is usually as a consequence of various intrinsic or extrinsic factors, as listed in table 1b.2 *(Davies and Hagen 1995)*. In keeping with the original findings of Szilagyi et al. most of the intermediate to late graft failures are as a result of intrinsic vein graft lesions of which the majority are stenotic *(Szilagyi et al. 1973)*. The underlying pathology of these intrinsic lesions is intimal hyperplasia (IH); a proliferative process involving the smooth muscles of the media of the veins *(Sayers et al. 1993a)*. As this is the focus of the experimental chapters in this thesis, the aetiology and pathobiology of IH will be discussed in detail in the next chapter.

In the longer-term, grafts can succumb to degenerative processes. Grafts develop intrinsic atherosclerosis after a long period of implantation. The medial wall can degenerate in areas leading to aneurysmal dilatation with associated risk of spontaneous rupture.

Table 1b.2 Causes of intermediate to long-term graft failure

Intrinsic
Intimal hyperplasia
Aneurysm formation
Atherosclerosis
Extrinsic
Inflow / outflow disease
Graft entrapment
Hypercoaguable states
Graft infection

1b.4 PATENCY RATES

Reporting standards

Though all forms of reports concerning therapeutic intervention require standardisation, it is particularly important in peripheral vascular disease. The current standards recognise that implanted grafts may fail as a primary event. Even more importantly it caters for intervention required to maintain adequate blood flow in grafts. It also allows for comparison between the practice of various groups. The current standard is adopted from the recommendations of the ad hoc committee of the Society of Vascular Surgery *(Rutherford 1991)*. The end point is graft patency which is reported as primary, primary assisted or secondary. Primary patency is defined as uninterrupted graft patency. Primary assisted patency is uninterrupted patency in a graft that has undergone a procedure such as PTA in order to maintain its patency. Secondary patency allows restoration of flow in an occluded graft through most of the graft and at least one of its original anastomoses.

One criticism of the use of graft patency standards amongst surgeons is the overemphasis on patency as the only measure of outcome and the tendency to disregard other important endpoints such as limb salvage and quality of life *(Cheshire and Wolfe 1996)*.

Patency rates

The patency of the vein graft reduces with time. The primary patency for a vein bypass to the femoropopliteal segment ranges from 80-90% at 1 year, 55 to 85% at 5 years and at 10 years it falls to about 38% *(Deweese and Rob 1971; Taylor et al. 1990a; Veith et al. 1986).* Taylor et al. reported overall 5 year patency rate of 79% in their series of 288 femoropopliteal bypasses *(Taylor et al. 1990a).* However, grafts to the above knee popliteal tend to do better than grafts to the below knee popliteal. In the series reported by Budd et al, the 5 year primary patency of infrainguinal vein grafts to the above knee popliteal segment was 67%, whilst in the below knee segment it was 47% *(Budd et al. 1990).* The 5 year primary patency rates of vein bypass, to the distal vessels can range between 29% to about 65% *(Budd et al. 1990; Londrey et al. 1991).* The patency rate in infrapopliteal grafts can vary with the recipient artery. Thus, Shah et al. reported a 50% patency rate for dorsalis pedis grafts, 72% for anterior tibial and 69% for peroneal grafts after 5 years *(Shah et al. 1993).*

There are several risk factors (such as age, sex, diabetes, smoking, hypertension, severity of limb ischaemia) and graft factors (such as material source of graft, level of anastomoses and adjuvant drug therapy) which may influence graft patency. Several studies have sought to analyse and determine which of these factors influence patency *(Budd et al. 1990; Jeans et al. 1990b; Londrey et al. 1991; Ricco et al. 1983; Sayers et al. 1993b; Tobis et al. 1991).* However the reports tend to conflict. One of the studies in this thesis will set out to determine the factors affecting long-term patency in a large series of vein grafts.

1c

VEIN GRAFT STENOSES

1c.1 NATURAL HISTORY

As discussed in the preceding section, intermediate to long-term graft failure is often attributable to the development of stenotic lesions. The incidence of vein graft stenoses can range between 12% and 27% (Table 1c.1) and with the advent of duplex scanners and more recently the colour coded Doppler, it is now possible to study these lesion using easily reproducible none invasive techniques.

Table 1c.1 Reported Incidence of vein graft stenoses

Reference	No. of grafts	Type of graft	No. with stenosis (%)	Location		Method of Detection
				Intragraft	Anastomotic	
Grigg *(1988)*	75	ISVG	19 (25)	10	9	A, DS
Taylor*(1990a)*	301	ISVG	58 (19)			A, DS
Sladen*(1981)*	173	RVG	33 (19)	25	17	CL, A
Sladen*(1989)*	114	ISVG	30 (26)	22	8	A, DS
Moody*(1990)*	63	ISVG	14 (22)	9	9	A, DS
Berkowitz *(1992)*	521	RVG	72 (14)	51	21	A
Bandyk*(1991)*	396	ISVG + RVG	78** (20)	46	32	A, DS
Lundell*(1995)*	56	ISVG + RVG	7 (13)	2	7	A, DS
Mills *(1993)*	231	RVG	28 (12)	6	18	A, DS
London *(1993)*	112	ISVG + RVG	30 (27)	7	26	A, DS

A= angiography, DS = duplex Scans, ISVG =In situ vein graft. RVG = Reversed vein graft,
CL= Clinical Examination, ** Excluding stenosis in adjacent native vessel.

Distribution

From Table 1c.1, it would seem that most stenoses occur within the vein graft (intragraft). However some series have reported a preponderance of anastomotic stenoses *(London et al. 1993; Mills et al. 1993).* The results from these series have to be interpreted according to the type of grafts and the detection method employed. It is thought that anastomotic stenoses are more likely to develop at the end of the graft which has the smaller luminal diameter *(Varty et al. 1993a).* This was supported by studies showing a preponderance of proximally situated stenotic lesions in reversed vein grafts *(Berkowitz et al. 1992; Sladen and Gilmour 1981)* and higher incidence of distal third stenoses in situ grafts *(Moody et al. 1990; Taylor et al. 1990b; Varty et al. 1993b)* However, Mills et al. have suggested from their studies, and following a review of other available data, that it would seem stenosis occur with equal frequency at either end of both in situ and reversed grafts *(Mills 1993).* Thus suggesting that the smaller diameter at the end of these vessels was not a significant factor. They also suggest that discrepancies in the reported incidence may be as a result of the inconsistencies in defining what constitutes an anastomotic lesion.

Outcome of vein graft stenoses

Duplex ultrasonography has allowed the serial monitoring of detected stenoses. It is clear from several studies that detected lesions can resolve *(Caps et al. 1995; Mills et al. 1995a).* In a duplex based follow up study of 98 vein graft stenoses, Caps et al. found that about two thirds of the lesions had regressed after 18 months *(Caps et al. 1995).* Other series of graft stenoses have also noted that not all detected untreated stenoses result in loss of graft patency *(Idu et al. 1992; Mattos et al. 1993; Wilson et al. 1996).* The exact determinants of the outcome of a given stenoses are not known, however, the severity of the lesion on detection is a significant predictor *(Caps et al. 1995; Mattos et al. 1993).*

From the foregoing it is clear that the natural history of vein graft stenosis is not well understood. It is important to gain clearer understanding of these lesions in order to be able to manage them effectively. Thus this continues to be a subject of research in many centres and one of the studies in this thesis will examine the natural history of duplex detected lesions.

1c.2 METHODS OF DETECTION

Angiography

Angiography is considered to be the gold standard technique for detecting vein graft stenoses. This is usually performed by either the intra-arterial digital subtraction angiography (IADSA) or the intra-venous digital subtraction angiography (IVDSA). The IADSA is the preferred choice. However, both techniques are invasive and expensive. Therefore their routine use in the monitoring of detected lesions is not practical. However the use of angiography to confirm stenose detected by non invasive methods is common practice and angiography as an initial screening examination soon after surgery has been advocated.

Magnetic resonance angiography is none invasive and does not involve radiation. It has evolved from being used to measure the size of aortic and thoracic aneurysms *(Dinsmore et al. 1986)* to being used in the detection of failing or failed grafts *(Turnipseed et al. 1992)*. However the expense of this technique would limit its routine use.

duplex scanning

The duplex ultrasound combines B-mode imaging with velocity spectral analysis. It offers several advantages over angiography. It is non invasive, and allows the accurate anatomical and physiological visualisation of vessels. Hence its popularity in detecting and monitoring vein graft stenoses. The grey scale Doppler has been largely superseded by the colour coded duplex where areas of abnormal blood flow can be seen as colour changes (figure 1c.1 and 1c.2), making examinations quicker but not necessarily more accurate *(Killewich et al. 1990)*.

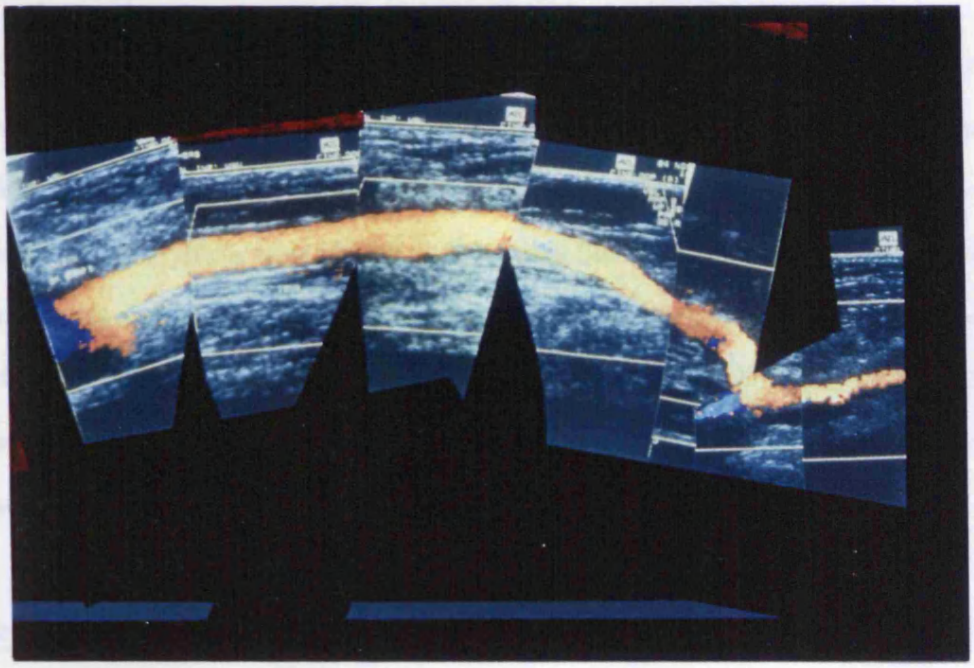

Figure 1c.1. Composite picture of duplex image of a vein graft. There are no areas of flow abnormalities in this graft.

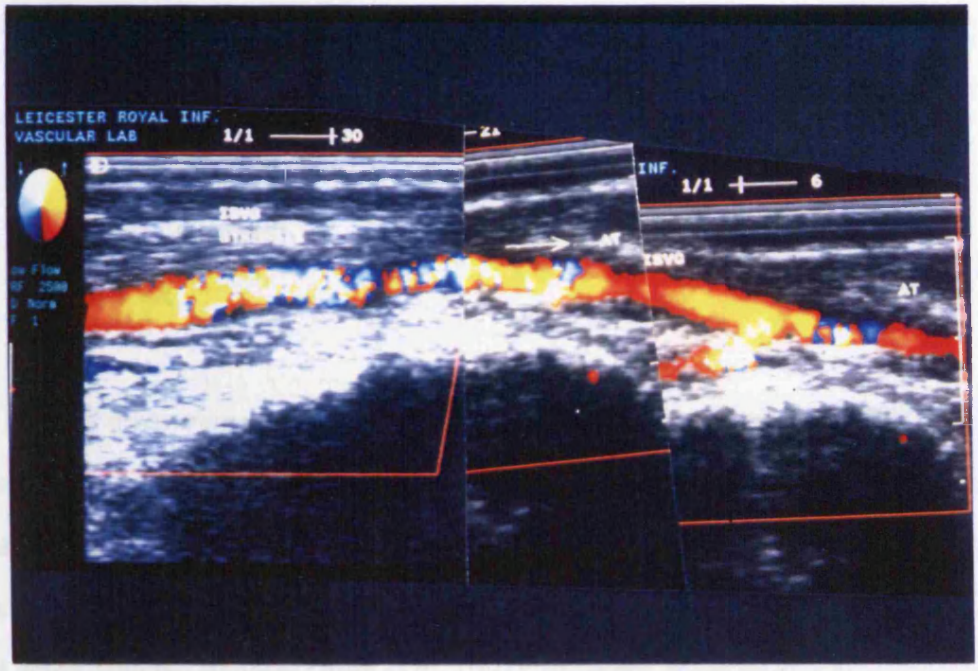

Figure 1c.2. Composite duplex picture of a vein graft. In contrast to Figure.1c.1 there are multiple areas of flow abnormalities indicated by the blue colour coding.

A typical examination would use a 7.5 or 5.0 MHZ transducer, depending on the depth of the graft, insonated and held at 60^{o} to the skin. In colour coded scans, the signal is red during systole and may be dark blue transiently during diastole. The normal graft has a flow velocity associated with a triphasic flow pattern and transient flow reversal at the end of systole. Abnormal colours approaching white, sustained blue or bright red would warrant detailed analysis. Several duplex derived parameters can be used to aid the detection and grading of stenoses (Tables 1c.2). These can be classified as either low velocity or high velocity parameters.

Table 1c.2 Duplex derived parameters

Low velocity parameters
PSV (graft)
Distal graft flow
Hyperaemic/resting distal bypass flow
High velocity parameters
PSV (stenosis)
EDV (stenoses)
PPVR

(I) *Low velocity measurements* : This is essentially the peak systolic velocity (PSV) at a normal mid point of the graft. Bandyk et al. established that a PSV of $\leq$ 45cm/s could detect grafts with stenoses and identify those that are failing *(Bandyk et al. 1985)*. This parameter is easy to measure and is suitable for grey scale scanning. However, it has several draw backs. Firstly it does not grade detected stenoses *(Buth et al. 1991)*, secondly, some studies have reported that this parameter is not sensitive enough *(Buth et al. 1991; Mattos et al. 1993; Sladen et al. 1989; Taylor et al. 1992)*. Thirdly, the predicted PSV for a stenoses can vary with graft diameter, the larger the graft the lower the predicted velocity, a phenomenon that can cause false positive results *(Belkin et al. 1992)*. Lastly, the PSV of a graft normally alters in the postoperative

period, falling by about 29% after 6 months *(Belkin et al. 1992)*. Thus though there have been studies supporting the use of a PSV ≤ 45cm/s as an absolute parameter *(Mills et al. 1990)*, it is more often used in conjunction with other high velocity criteria.
Other low velocity criteria have not been widely adopted. Chang et al. measured the haemodynamic characteristics in 350 in situ bypasses and found that a distal blood flow of < 25ml/min and a hyperaemic /resting blood flow ratio of < 2.5 correlated with graft stenoses *(Chang et al. 1990)*. However these parameters have low specificity.

(II) High velocity criteria: These are parameters derived from high velocity measurements. The absolute increase in velocity in a graft is indicative of a stenosis. Thus Sladden et al. included a PSV of > 300cm/s as a high velocity parameter *(Sladen et al. 1989)* and Passman et al. had used a PSV of ≥ 200 cm/s in their surveillance of vein grafts *(Passman et al. 1995)*. In a study that analysed several colour duplex derived criteria, Buth et al. found that the End Diastolic Velocity (EDV) of more than 20cm/s measured at a stenosis or at the narrowest segment of the graft was highly sensitive in detecting stenoses with a 70 % or more reduction in diameter *(Buth et al. 1991)*. The most commonly used parameter is the peak systolic velocity ratio. This is the ratio of the peak systolic velocity at the site of maximal stenoses to that of the peak systolic velocity measured at an adjacent area of normal graft. This parameter has been correlated to angiographic findings in native lower limb arteries by many authors *(Karacagilet al. 1996; Ranke et al. 1992, Sensier et al. 1998; Sensier et al. 1996; Whelan et al. 1992)* . It lacks the disadvantages of the low velocity criteria and the adjacent normal vessel serves as an internal control. Thus, the Peak Velocity Ratio (PVR) has also been widely adopted to detect and grade vein graft stenosis *(Grigg et al. 1988a; Gupta et al. 1997; Idu et al. 1993; Mattos et al. 1993; Taylor et al. 1992; Westerband et al. 1997a)*. However, the threshold values that were used in native vessels may not necessarily apply to vein grafts. Such discrepancies have implications to the clinician who has to decide when to intervene to correct detected stenosis. Therefore more studies are needed to validate the criteria used when this parameter is used in vein graft surveillance programs. Table 1c.3 shows a suggested clinical correlation of PVR

with reduction in vessel diameter *(Johnson et al. 1989; Moneta and Strandness 1987; Robeer et al. 1998).*

Table 1c.3 Suggested clinical grading of the Peak Velocity Ratio

PVR	Corresponding reduction in diameter (%)
≤ 2.0	<50
2.0 - 3.0	50 -75
> 3.0	>75

Impedance analysis

This relatively new technique involves the measurement of pulsatile pressure and flow signals analysed by computer. The impedance analysis score so derived has been shown to been shown to be better than the low velocity criteria (PSV ≤ 45cm/s) but inferior to the duplex derived PVR *(Davies et al. 1994)*. This technique has not yet been widely adopted.

1c.3 THE CLINICAL PROBLEM

Once a graft develops a stenosis, the risk of subsequent failure increases about three fold *(Mattos et al. 1993; Mills 1993; Sladen and Gilmour 1981)*. In addition, graft stenoses have been shown to account for 80% of graft failures *(Veith et al. 1984)*. Furthermore the majority of these stenoses are asymptomatic *(Bandyk et al. 1991; Grigg et al. 1988b)*. Therefore, a graft harbouring these lesions may fail suddenly with no preceding symptoms. In fact, in the majority of cases, the development or return of ischaemic symptoms is associated with an existing graft occlusion *(Veith et al. 1984)*. Graft failure is to be avoided at all costs as the poor results following attempted revascularisation are well known *(Whittemore et al. 1981)*. Dardik et al. have shown that failure to revascularise a failed graft may convert an otherwise below knee amputation to an above knee loss *(Dardik et al. 1982)*.

Recognition of these consequences spearheaded the need to recognise stenoses early and instigate appropriate management strategies. Crucial to this is a better understanding of the natural history of graft stenosis, which has been a subject of some recent studies *(Caps et al. 1995; Gupta et al. 1997; Mills et al. 1995b; Nielsen 1996)*. The efforts in these areas has seen the emergence of new concepts in the postoperative management of vein bypass grafts *(Harris*

1992). These include the introduction of the term "the failing graft", and the widespread application of postoperative graft surveillance.

The failing graft / The graft at risk

This refers to the graft which is patent but has developed a haemodynamically significant stenoses within the conduit, the outflow or the inflow tracts *(Veith et al. 1984)*.

Graft surveillance

Though postoperative graft surveillance is currently widely practised, its rationale and many aspects of its implementation remain controversial. Graft surveillance (GS) is estimated to improve long-term patency by about 15% *(Moody et al. 1990)*. To date there has been only one randomised study addressing the benefits of GS. This study by Lundell et al. demonstrated a higher primary assisted and secondary patency rates in distal grafts undergoing intensive postoperative surveillance *(Lundell et al. 1995)*. Several other studies have demonstrated the benefits of GS *(Bergamini et al. 1995; Dalman et al. 1990; Dunlop et al. 1995a; Idu et al. 1993; Mills et al. 1990; Moody et al. 1990)*. Despite these reports, not everyone is convinced of its benefits *(Beattie et al. 1997)*. A recent meta-analysis of 17 surveillance and 26 none surveillance graft series could not demonstrate any improvement in limb salvage *(Golledge et al. 1996)*. However this was admittedly not a formal analysis and did not distinguish claudicants from patients with CLI. In another study, Barnes et al. found that a change in ABPI of 0.2 or more did not distinguish between failing or patent grafts *(Barnes et al. 1989)*. Thus they argued against the benefits of non invasive monitoring. That study was based on APBI measurements which have since been shown to be an insensitive tool for detecting graft stenoses *(Davies et al. 1994; Mills et al. 1990)*.

Techniques of graft surveillance

As discussed above, several methods can be used to detect graft stenoses. However for the purposes of GS such a technique must be easily reproducible and preferably none-invasive. The current recommended technique of GS is based largely on serial colour duplex scans (CDS). The CDS has superseded other traditional detection methods as the main detection technique. It has the added advantage of speed over ordinary duplex ultrasonography. The performance of arteriography compares well with duplex scans *(Grigg et al. 1988a)* however because it is invasive and more expensive it is not suitable for repeated surveillance. Arteriography is now used to confirm significant lesions detected by CDS prior to correction. ABPI measurements are unreliable predictors of stenoses *(Mills et al. 1990)*. Despite the individual drawbacks of these techniques, many centres combine them with CDS to formulate criteria for defining and correcting stenoses. Most often serial APBI measurements are taken alongside CDS examinations *(Dunlop et al. 1995a; Lundell et al. 1995; Wilson et al. 1996)*.

Protocols of graft surveillance

The frequency and duration of GS is debatable. This is evident from the variations in practice in different centres. Whilst some advocate commencing surveillance as early as the first postoperative week *(Mills et al. 1995b; Wilson et al. 1995a)* others commence surveillance at 1 month or at 3 months *(Grigg et al. 1988a)* postoperatively. The interval between follow up varies from 3 monthly *(Dunlop et al. 1995a; Lundell et al. 1995; Westerband et al. 1997a)* to 6 monthly *(Green et al. 1990; Gupta et al. 1997)*. The length of surveillance also varies. In their retrospective review, Mohan et al. suggested that in situ grafts only required a period of 6 months intensive surveillance *(Mohan et al. 1995)*. There are those that believe that GS is no longer cost effective after 12 months *(Grigg et al. 1988a; Taylor et al. 1990b)* whilst some authors continue for 2 years *(Bandyk 1990)*.

Criteria for intervention

As previously discussed, a plethora of direct and indirect measurements can be derived from duplex scans. In GS there is no consensus as to which measurement to use or which criteria best predicts a significant stenoses. Bandyk used a low velocity criteria of a PSV of < 45 cm/s *(Bandyk et al. 1985)*. This was found to predict 96% of failing grafts. However, more often authors have combined this low velocity criteria with other high velocity criteria. Thus Sladden et al. added a high velocity criteria of PSV > 300 and a PVR of > 3.0 to identify failing grafts *(Sladen et al. 1989)* whilst Taylor et al. used a PSV < 45 cm/s and a PVR of 2.0 or more *(Taylor et al. 1992)*. The EDV of >20 cm /s can also predict severe stenosis *(Buth et al. 1991)*. Though the PVR has been shown to reliably estimate the degree of a given stenoses *(Grigg et al. 1988a)* there is no agreement on which PVR value to intervene. In the literature, authors have intervened at values ranging from 1.5 *(Grigg et al. 1988a; Idu et al. 1993)* to 3.0 or more *(Caps et al. 1995; Mills et al. 1995b; Sladen et al. 1989)*.

Graft surveillance in Leicester

In Leicester, the GS programme is based on serial CDS. All implanted infrainguinal vein grafts are scanned first at one month the at 3 monthly intervals for the first 12 months then 6 monthly indefinitely thereafter *(Dunlop et al. 1995a)*. Until recently, the criteria for correction of a detected stenoses was based on PVR of ≥ 2.0, however as part of this thesis a prospective study has been undertaken to evaluate the effect of intervention at a PVR of 3.0.

1c.4 CURRENT MANAGEMENT

Treatment of vein graft stenoses

The treatment of vein graft stenosis depends on its severity and the options available to the clinician. It can be managed expectantly, or corrected by active intervention using endovascular or operative techniques.

(I) Conservative management: Not all detected stenoses will lead to graft occlusion, therefore an expectant policy can be afforded for some lesions. However, even though various studies have demonstrated that not all stenoses will cause graft occlusion, *(Caps et al. 1995; Mills et*

al. 1995b; Moody et al. 1989) there is still controversy over which lesions to treat and which to observe. It is clear from most studies that a reduction in diameter of less than 50% does not increase the risk of graft thromboses *(Buth et al. 1991; Mills et al. 1995b; Passman et al. 1995; Sladen and Gilmour 1981; Taylor et al. 1992)* and therefore such lesions do not require correction. It is also clear that lesions with a 75% or more reduction in diameter are associated with a high risk of graft occlusion. *(Bandyk 1993; Mattos et al. 1993).* The problem lies with the "intermediate lesion" with a diameter reduction between 50% and 75%. In a study of 98 vein graft lesions, by Caps et al, 30 were identified as having a diameter reduction between 50% and 75%. Of these only six required revision after 18 months follow up and all six were associated with significant drop in ABPI. Thus they concluded that stenoses with a diameter reduction of less than 75% could be observed as long as they remained asymptomatic with no reduction in ABPI *(Caps et al. 1995).* The implication of that study is that clinicians may be able to treat more stenoses conservatively. However further studies are required to determine if this policy can be safely adopted.

(II) Endovascular intervention : PTA offers the least invasive form of intervention for correcting vein graft stenoses. Furthermore, it is repeatable with minimal risk to the patient. However there are concerns over its restenosis rate and long-term success *(Perler et al. 1990; Whittemore et al. 1991).* The restenosis rate can vary from 25% to 35% at two years *(Berkowitz et al. 1992; Taylor et al. 1991)* and 42% to 50% after 4 years *(Dunlop et al. 1995b; Favre et al. 1996).* The series of 54 infrainguinal grafts dilated by Whitmore et al. reported an overall 4 year patency of 18%. Though, 85% of the lesions in that study were located around the anastomosis and the patients in the series were symptomatic with up to 20% presenting as occlusions *(Whittemore et al. 1991).* In comparison, London et al. demonstrated that early detection of stenotic lesions by an aggressive surveillance program resulted in much improved patency rates with up to 70% of lesions patent after a single PTA in a 44 month follow up period *(London et al. 1993).* The results of the longer term follow up of these grafts showed that 58% remained patent after a single PTA but 42% of the PTA treated lesions had recurred, most of which were located in the distal graft *(Dunlop et al. 1995b).* The distal anastomoses

has been shown to be associated with a higher incidence of post angioplasty restenosis in other studies *(Berkowitz et al. 1992; Whittemore et al. 1991).*

The controversy over the durability of PTA compared to operative revision of vein graft stenoses continues *(Bandyk et al. 1991; Sanchez et al. 1994).* However there seems to be a place for PTA of short focal lesions i.e. less than 2cm, which form the majority of early detected lesions *(London et al. 1993),* and also for those lesions not accessible by open surgery *(Sanchez et al. 1994).*

Stenting in infrainguinal vein grafts has received limited attention. Davies et al. stented two infrainguinal vein grafts because of recurrent stenoses. These grafts remained patent at 6 months. Though this report is encouraging, there are too few grafts and the long-term outcome is unknown. This is in contrast to coronary vein grafts were stents are used more routinely with acceptable short term success *(Brener et al. 1997).*

(III) Operative correction: This has been shown to produce durable long-term results *(Bandyk et al. 1991).* There are various operative options for dealing with a vein graft stenotic lesion. These are vein patch angioplasty, excision of the stenosis with primary anastomosis, excision with insertion of an interposition graft or sequential / jump grafting. Grafts with long stenoses (more than 2cm) or with multiple lesions benefit from operative correction particularly interposition or jump grafting *(Thompson et al. 1989; Whittemore et al. 1991).*

Strategies for preventing vein graft stenoses

There is no known effective method of preventing the development of graft stenoses. Current strategies of reducing the incidence of these lesions are aimed at the causative factors of intimal hyperplasia.

(I) Preoperative strategies: The most important preoperative strategy is selection of a suitable vein. There is indirect evidence linking pre existing vein abnormalities with the development of intimal hyperplasia and stenoses which will be discussed in the next chapter. Unsuitable veins can sometimes be identified on the basis of a history of phlebitis or clinical findings of varicosities. However, a number of morphologically normal veins still have histological lesions that can predispose to graft stenoses *(Davies et al. 1993)*. Marin et al. promoted vein biopsy at the time of grafting *(Marin et al. 1993)*. This has not been widely adopted. Preoperative duplex scanning is another method of identifying suitable veins*(Bagi et al. 1989; Sayers et al. 1993b)*. This seems useful in determining the calibre of the lumen of venous conduits *(Panetta et al. 1992)* but is of limited value in identifying pre-existing fibrotic changes in the vein wall *(Giannoukas et al. 1997)*. Angioscopy is also of limited value in the preoperative assessment of diseased vein walls as it cannot differentiate thickened walls *(Sales et al. 1993)*.

(II) Operative care: Though the basic surgical principles have not changed, surgeons have recognised the need to minimise unnecessary vein trauma such as clamp injury and suture narrowing that may result from inadequate side branch ligature during grafting procedures *(Davies and Hagen 1995; Varty et al. 1993a)*. The in situ technique does not involve the trauma of vein harvesting, however, traditional valvutomes used to destroy valves can elicit a significant degree of graft wall injury *(Sayers et al. 1991; Sayers et al. 1992)*. Angioscopically assisted in situ techniques have been used to perform valvulotomy under direct vision *(Stierli and Aeberhard 1992)* and it seems that these techniques may reduce the incidence of valvutome induced trauma and avoid leaving residual competent valves, possible precursors of future stenotic lesions.

(III) Postoperative: Drug therapy would be the ideal adjuvant to prevent stenoses following implantation. A large number of drugs have been tried (Table 1c.4). However despite encouraging results in animal models, the results of clinical trials have been disappointing. In fact no drug has been shown to prevent clinical graft stenoses *(Chan 1997; Davies and Hagen 1995; Kraiss and Johansen 1995; Varty et al. 1993a).* Unfortunately, most of the clinical and experimental research on pharmacotherapy and stenosis has been directed at coronary artery restenosis using animal arterial balloon angioplasty as models *(Chan 1997; Kraiss et al. 1991).* Thus few of the trial drugs have been evaluated in infrainguinal bypass grafts.

The meta-analysis of recent randomised anti-platelet trials has shown that aspirin reduces the rate of peripheral prosthetic graft occlusion *(Anti-platelet trialist 1994a; Anti-platelet trialist 1994b).* The randomised trial of low molecular weight heparin versus anti platelet therapy by Edmodson et al. *(Edmodson 1994)* showed that heparin improved primary patency rates, and because heparin was administered for 3 months, they concluded that their results were due to an effect on intimal hyperplasia. However their methodology and conclusions have been heavily criticised *(Kraiss et al. 1991; London et al. 1994).*

(IV) Gene therapy:

Experimental studies have shown that local delivery of antisense oligonucleotides targeted at proto oncogene *c-myb* or *c-myc* inhibited smooth muscle proliferation in vivo and in vitro *(Bennett et al. 1994a; Simons et al. 1992).* It has also been possible to transfer genetic information into vascular tissue *(Steg et al. 1994)* and investigators have demonstrated a reduction in IH in injured rat artery using this method *(Von der Leyen et al. 1994).*

Table 1c.4 Experimental and clinical studies on drugs used to prevent intimal hyperplasia

Type of drug	Drug, model and (effect on IH)	Drug, clinical trial and (effect on restenoses)
Platelet antagonists	Aspirin, Dipyrimadole, Sulfinpyrazone on rabbit IH-(RE)1	Aspirin, Dipyridamole on CPTA -(NE)2
	Iloprost, daltroban on rat IH-(NE)3	Ciprostene on CPTA- (NE)4
	Aspirin, dipyridamole on primate IH (NE)5	Ketanserin on CPTA (NE)6
		Aspirin, Dipyridamole on LLVG -(NE)7
Anticoagulants	Heparin on rat artery IH -(RE)8,9	Short term LMW heparin on CPTA -(NE)10
	Heparin on rabbit artery IH -(RE)11	Long term LMW heparin on CPTA-(NE)12
	Heparin on rabbit vein grafts-(ME)13,14	r-Hirudin on CPTA (NE)15
	r- Hirudin on rabbit femoral arteries (RE)16	
Angiotensin converting enzyme (ACE) inhibitors	Cilazapril on rat artery IH -(RE)17	Low and high dose Cilazapril on CPTA -(NE)18,19
	Cilazapril on baboon and pig artery IH (NE)20-22	
	Captopril on rabbit vein graft (RE)23	
Lipid lowering drugs	Lovastatin on rat artery IH -(RE)24	Lovastatin on CPTA (NE)25
	Fish oils on canine vein graft IH -(RE)26	Fish oils on CPTA (ME)27
Calcium antagonists	Verapamil on rabbit vein graft IH - (RE)28	Nifedipine , Diltiazem on CPTA (NE)29,30
Steroids	Dexamethasone on rabbit artery IH -(RE)31	Steroid on CPTA (NE)32
Growth factor inhibitors	Trapidil on rabbit artery IH -(RE)33	Trapidil on CPTA ?(RE)34,35
	Angiopeptin on rat and porcine artery IH -(RE)36,37	Angiopeptin on CPTA (NE)38,39

NE; no effect, RE; reduced effect, ME; minimal effect, IH; intimal hyperplasia, CPTA; coronary artery angioplasty, LMW; low molecular weight, LLVG; lower limb vein graft. Numbered references are listed in appendix 1

Id

SUMMARY

Peripheral vascular disease in the lower limb is an important condition which has a wide range of management options. Operative revascularisation remains an important form of treatment. To this end, the infrainguinal bypass procedures using autologous vein as the conduit offers the best long-term benefits. However this is associated with significant early and long-term problems. Furthermore the cause, natural history and management of the stenotic lesions attributable to these failures is not fully understood. However, the research in this field continues to provide useful information. Thus even though there is no known method of preventing vein graft stenoses, strategies are evolving that might prevent these lesions and hence prolong the patency of these grafts.

CHAPTER 2

SAPHENOUS VEIN INTIMAL HYPERPLASIA

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2.1 INTRODUCTION

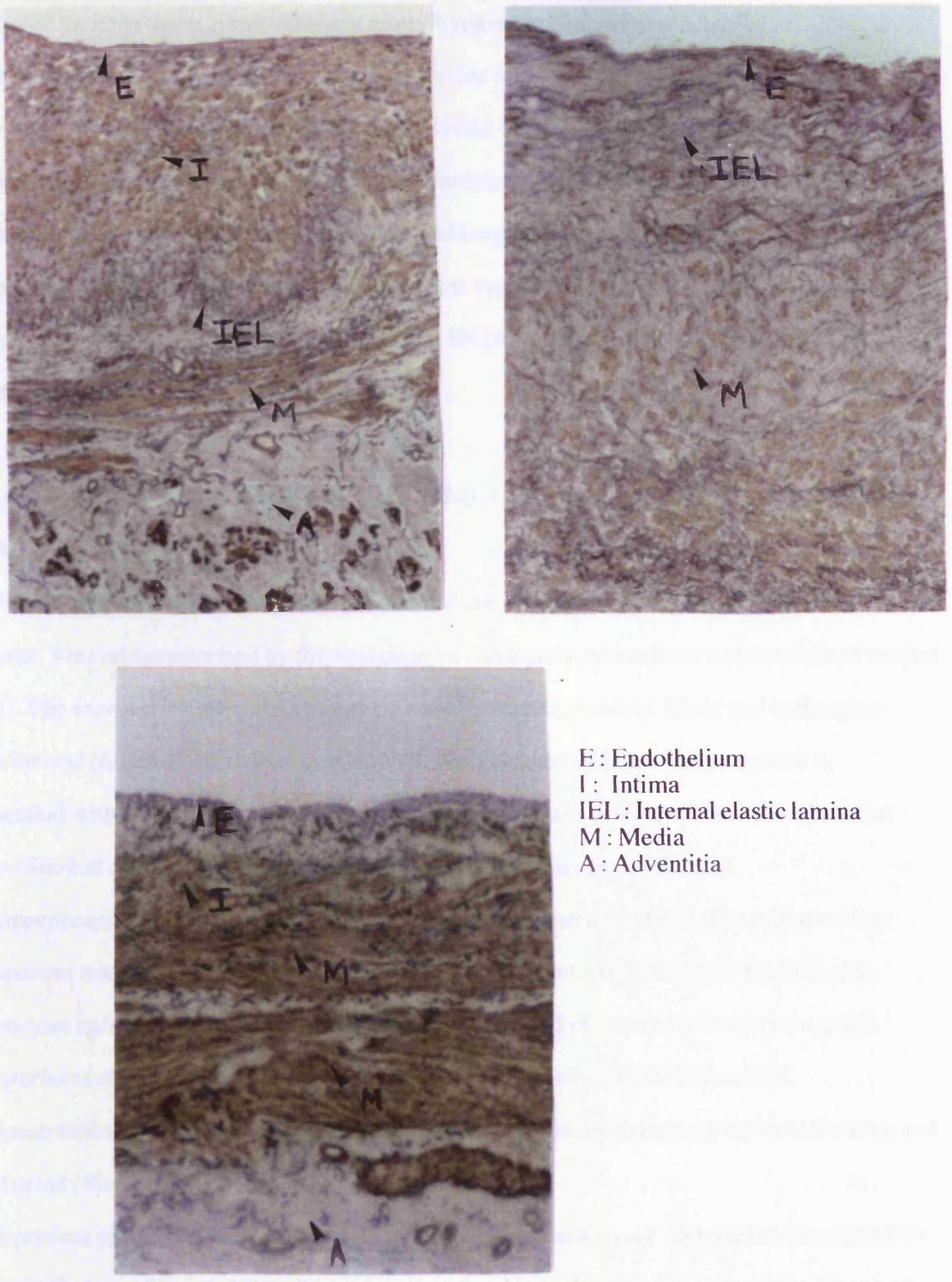
The clinical aspects of vein graft stenosis were discussed in the previous chapter. This chapter will discuss the biological changes that occur in veins that have been grafted into the arterial circulation and how this relates to the subsequent development of stenotic lesions. The biological process that underlies the remodelling seen in grafted veins is termed intimal hyperplasia (IH), it is also the underlying cause of graft stenosis *(Davies and Hagen 1994; Sayers et al. 1993a; Szilagyi et al. 1973).* There is a growing understanding of the mechanisms involved in IH. However, its biology is best described in the context of the multiple physical, humoral and cellular events that have been found to play a significant contributory role in its formation.

2.2 FEATURES OF THE NORMAL SAPHENOUS VEIN

Like all veins, the wall of the saphenous vein consists of an inner intima, middle media and an outer adventitial layer. The lumen of the vein is lined by a single layer of endothelial cells (ECs). Underneath this is the intima which consists of varying amounts of supporting connective tissue and smooth muscle cells. An ill defined internal elastic lamina separates the intima from the media. In comparison to other medium sized veins, the long saphenous vein has a well developed media with its muscular fibres arranged as inner longitudinal and outer circular layers. The adventitial layer is thick and consists of a loose network of connective tissue through which the vasa vasorum penetrate to supply the vein.

The above account refers to a textbook description of a normal vein *(Ham 1987).* However, veins intended for grafting tend to have varying degrees of macroscopic and microscopic features. The thickness of the intimal layer in otherwise normal veins has been found to vary between 2-208μm *(Marin et al. 1993; Varty et al. 1996).* Such variations in the relative

proportions of the layers that constitute the wall of the saphenous vein can be seen in the transverse section of veins taken from different patients in figure 2.1.



E : Endothelium
I : Intima
IEL : Internal elastic lamina
M : Media
A : Adventitia

Figure 2.1. Transverse section of long saphenous veins taken from different patients illustrating variations normal in structural features. SMA/Miller elastin stain, magnification X 200

Thus, Waller and Roberts identified varying degrees of pre-existing thickening in unused vein from 402 patients undergoing coronary artery bypass grafting *(Waller and Roberts 1985)*. Panetta et al. found that up to 12% of 513 veins that had been used for infrainguinal grafting had macroscopic abnormalities *(Panetta et al. 1992)*. Not surprisingly, studies based on microscopic observations have found a higher incidence of pre-existing structural variations *(Cheanvechai et al. 1975; Thiene et al. 1980)*. Milroy et al. found microscopic abnormalities in remnants of all of the veins used for femorodistal bypass *(Milroy et al. 1989)*. Similarly in a recent histological study of vein segments from 89 different patients only 8 were entirely normal *(Giannoukas et al. 1997)*.

2.3. CHANGES THAT OCCUR IN VEINS GRAFTED INTO THE ARTERIAL CIRCULATION

Following grafting into the arterial circulation, the vein graft undergoes an initial reparative process. This is characterised by the sloughing of damaged endothelium and necrosis of medial SMC. The exposed intima and damaged ECs acts to attract platelets, fibrin and leukocytes *(Davies and Hagen 1994)* to the vascular wall. A significant inflammatory reaction is associated with this initial phase *(Dilley et al. 1988)*. There is oedema of the subendothelial layer *(Stark et al. 1997)* and an inflammatory infiltrate consisting initially of polymorphonuclear leukocytes and then monocytes *(Hoch et al. 1994a)*. The inflammatory component may be of more significance in vein IH than previously thought. Proliferating monocytes and macrophages have been identified in the NI of excised stenotic vein grafts *(Westerband et al. 1997b)*. Furthermore, in a vein graft model of IH, Faries et al. demonstrated a correlation between cytokine expression and inflammatory cell proliferation and infiltration *(Faries et al. 1996)*.

A process of endothelial regeneration occurs to cover the areas of endothelial loss *(Cambria et al. 1985; Logerfo et al. 1983)*. The time taken for this to develop depends on the degree of injury and has been found to vary between 1 to 6 weeks *(Dilley et al. 1988)*. Denuded areas around the anastomoses are regenerated from ECs from the intima of the adjacent artery.

Within the grafts, regeneration is probably from remaining islands of ECs. *(Dilley et al. 1988).* This regenerated layer is morphologically different from native endothelium *(Stark et al. 1997).*

SMC proliferation has been found to increase as early as 48 hours following injury in arterial models of angioplasty. This early SMC activation is evident from the detection of nuclear oncogenes within 30 minutes of injury *(Bauters et al. 1992).* Rat models of vein graft intimal hyperplasia have shown that there is a high degree of SMC proliferation after 5 days *(Dilley et al. 1992a).* This process has also been observed in organ culture models of saphenous vein IH *(Porter et al. 1996b).* The activated SMC undergo phenotypic changes from contractile to synthetic types *(Schwartz et al. 1986; Thyberg and Blomgren 1990)* and it is these type of cells that are found in the NI. Synthetic SMC are characterised by a well developed Golgi apparatus and a reduced number of contractile elements *(Chamley-Campbell et al. 1981).* Thus these cells have an increased replication and secretory capacity *(Schwartz et al. 1986; Thyberg and Blomgren 1990).* The proliferation and migration seen in the SMC are largely in response to growth factors produced either by the endothelium or by the SMCs themselves. Proteins of the extra cellular matrix can also induce SMC migration *(Nelson et al. 1996).* The cells migrate by cyclic attachment and detachment of the cell membrane to the extra cellular matrix. This process is mediated by cell adhesion molecules and their receptors. There is current interest in integrins which are a major class of such receptors. Itoh et al have recently demonstrated that certain integrin subunits are necessary for growth factor induced migration in isolated SMCs *(Itoh et al. 1997).*

The source of SMC found to populate the intima remains controversial. SMCs have been shown to migrate from the medial layer in arterial models of IH *(Clowes and Clowes 1985).* Thus in such models, proliferating SMC can be seen to have breached the internal elastic laminae and migrate into the sub endothelium after about 8 days *(Davies and Hagen 1994).* Dilley and colleagues reported that the intimal SMC at the anastomoses of vein grafts was morphologically similar to that of the media in the adjacent artery and proposed a contribution from the host vessel. However, this process may differ in the rest of the graft. Some studies have demonstrated extensive destruction of the medial layer of the vein in the first few days following grafting *(Dilley et al. 1988; Stark et al. 1997).* Thus, it is not likely that this layer

would be the principle source of migrating SMC in vein grafts. It may be that the pre-existing SMCs of the intimal layer of the veins may turn out to be the most important source of proliferating cells. However, proliferation of these pre-existing SMCs alone does not account for the large number of cells found in the neointima as some studies have found that up to 50% of these SMCs will not have been dividing *(Chervu and Moore 1990; Itoh et al. 1994).* This implies that migration may explain the presence of some of the SMCs in the neointima.

As the graft matures, the intimal cells continue to proliferate, synthesise and deposit extra cellular material. This leads to the gradual increase in intimal thickness which has been shown to be maximal at about 4 weeks *(Clowes and Clowes 1985).* In this process, there is a balance between replicating and dying cells. There has been recent interest in the role of apoptosis or programmed cell death in vascular neointima. This follows on from the work by Bennet et al. demonstrating that apoptosis occurred in populations of proliferating SMCs in vitro *(Bennett et al. 1994b).* Indeed, apoptotic cells have been found in both the media and the intima of experimental vein grafts *(Hoch et al. 1995; Kockx et al. 1996).* It may be that regulation of SMC apoptosis may play a crucial role in the development of vein graft stenosis, though this remains highly speculative at the present time.

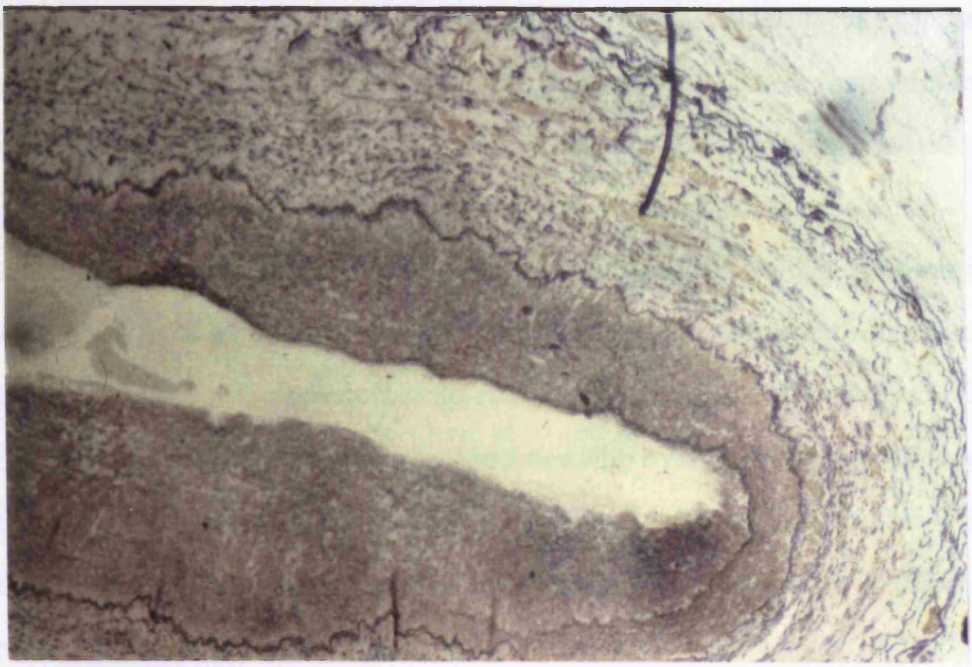


Figure. 2.2. Transverse section of a stenosed vein graft.

2.4. VEIN GRAFT STENOSIS

The preceding section has described the adaptive process seen in vein grafts. This is intimal hyperplasia and it has often been referred to as a process of "arterialisation". However the term arterialisation is misleading as it only refers to compensatory thickening of the media whereas IH is characterised by the proliferation of smooth muscle cells and accumulation of extra cellular material in the sub endothelium. Figure 2.2 illustrates the new layer or "neointima" that develops as a result of this process. This neointima can encroach on and compromise the lumen of the vein

It is this way that vein grafts develop stenotic. There is no way of predicting the circumstances or sites at which this excessive reaction will occur. The causative factors in the development of stenosis is invariably linked to the multifactorial aetiology of IH discussed below. Thus, the strategy for its prevention has been to suppress the process of vein IH as much as possible.

2.5. ROLE OF AETIOLOGICAL FACTORS IN INTIMAL HYPERPLASIA

Pre-existing vein disease

It is commonly hypothesised that certain pre-existing structural features may predispose some vein grafts to excessive IH. The morphological variations that can exist in veins prior to grafting has been described in the preceding section. Some authors have examined the relationship between such variations and the outcome following grafting. Marin et al. demonstrated that abnormal thickening and cellularity was associated with graft failure *(Marin et al. 1993)*. Davies et al. also showed that pre-existing thickening correlated with compliance which in turn correlated with stenosis *(Davies et al. 1992)*. Panneta et al. also found that using veins with areas of pre-existing disease was associated with a reduction in graft patency *(Panetta et al. 1992)*. In further support of this, in vitro studies have shown that pre-existing thickening correlates with the development of IH in culture *(Wilson et al. 1997)*. However, there are other studies that do not support these observations *(Cheanvechai et al. 1975; Leu et al. 1991)*. The study by Cheanvechai et al. reported a 27% incidence of pre-existing abnormalities which had no effect on subsequent patency rates. Another study has found no correlation between pre-existing histological findings and either clinical stenoses or in vitro IH *(Varty et al. 1996)*.

There is also the hypothesis that veins with gross abnormalities such as clamp injuries, tributaries, valve remnants, ligature related strictures and calcified areas may be more susceptible to excessive IH. These abnormalities can be identified by direct examination as well as angioscopically *(Sales et al. 1993)*. Using intra-operative markers and angiographic follow up, Moody et al found no correlation between these areas and the sites that subsequently developed stenosis. Mills and colleagues came to different conclusions. In their duplex scan based study, they found that most graft flow abnormalities developed at the site of pre-existing vein abnormalities or unrepaired defects occurring at the time of implantation. However their conclusions were largely based on the low incidence of "de novo" flow abnormalities in previously normal grafts after 3 months of duplex surveillance *(Mills et al. 1995a)* whereas the conclusions of Moody et al. was based on more objective anatomical data.

Injury

The response to injury hypothesis is that the proliferative changes seen following vascular injury is as a result of a wound healing process. There is little doubt that vascular injury triggers a cascade of cellular and subcellular events. Veins are damaged from the ischaemia resulting from dissection and handling, injury from clamps, fluid distension and the passage of the valvutome. Such surgical preparation results in varying degrees of damage to the endothelium and the underling media. Experimental studies have shown that injury resulting in endothelial denudation alone causes minimal IH in arteries *(Fingerle et al. 1990; Reidy and Silver 1985)*. Marked intimal proliferation is observed when this injury extends into the media *(Reidy and Silver 1985)*. However, in spite of the associations between injury and IH, the response to injury hypothesis has to remain only part of a multi factorial aetiology. This is because injuries sustained by veins during preparation are often diffuse, whereas subsequent stenoses develop in localised areas of the graft.

The endothelium

The endothelium was thought to be merely a none thrombogenic vascular monolayer. It is now known to play a significant role in vascular physiology (Table 2.1).

Normal endothelial function is as result of a balance of effects from the various protagonists and antagonists it produces. Examples of these are seen in the pro coagulant / anticoagulant properties of the endothelium *(Stern et al. 1988)* and the balance between the production of the vasodilatory actions of nitric oxide and the vasoconstrictive effects of endothelin.

Loss of endothelial coverage is related to the time taken for re-endothelialiasation to occur. Rapid re-endothelialization is associated with minimal neointima formation. The loss of the balance in normal EC function may account for events leading up to NI formation. The fact that the regenerated layer is morphologically altered has been pointed out in the last section. Acute endothelial dysfunction is evident from the inflammatory infiltrate seen penetrating the sub endothelium following grafting

Table 2.1. Functions of the vascular endothelium

Function	Mechanisms
Maintenance of vascular tone	Release of vasoactive peptides such as ET, nitric oxide and Prostacyclin
Regulation of coagulation system	Provides a non thrombogenic surface, produces fibrinolytic enzymes such as plasminogen and their inhibitors
Regulation of the inflammatory and immune system	Expression of major histocompatibility antigens; Stimulates T cell proliferation
Vascular remodelling	Release of growth factors such as PDGF, bFGF, ET
Semipermeable barrier	

(Hoch et al. 1994a). Further dysfunction is evident from studies on excised vein grafts that have shown decreased endothelium mediated relaxation in response to agonists such as nitric oxide *(Cross et al. 1988; Park et al. 1993)* and prostacyclin *(Luscher 1992).* In addition endothelium in these veins have demonstrated reduced fibrinolytic activity *(Risberg 1978).* The proliferation and migration of SMC towards the endothelium suggests that this dysfunctional monolayer promotes IH by unregulated secretion of growth factors and chemotactants. In support of this, Koo and Gotlieb have demonstrated that conditioned media from endothelial cells induces SMC proliferation*(Koo and Gotlieb 1989).*

Growth factors

Because SMC proliferation and growth is central to the development of IH, growth factors play a significant role in the aetiology of this condition. Smooth muscle cells respond to a number of growth factors. As a result there are complex interactions between different growth

factors which are ill understood. A list of the growth factors implicated in IH is listed in Table 2.2.

Platelet derived growth factor (PDGF) is a well characterised growth factor secreted by many cell types including platelets *(Ross et al. 1974)*, ECs *(Dicorleto and Bowenpope 1983)* and SMCs. *(Winkles and Gay 1991)*. SMC production of PDGF is an example of autocrine and paracrine activity where the PDGF acts on the producing cell as well as other SMCs nearby.

Table 2.2. Growth factors and cytokines implicated in IH

Growth factor	Source
Platelet-Derived Growth Factor	Platelets, ECs SMCs
Basic Fibroblast Growth Factor	ECs, SMCs, macrophages
Transforming Growth factor Beta	Platelets, Macrophages, ECs, SMCs
Vascular Endothelial Growth Factor	ECs, SMCs
Endothelin	ECs, SMCs
Epidermal Growth Factor	Platelets
Insulin Like Growth Factor	ECs, SMCs, Platelets, Macrophages
Tumour Necrosis Factor alpha	Macrophages, SMCs
Interleukin-1 alpha	ECs, SMCs, Platelets, Macrophages

It exists as three different isoforms and mRNA to the AA isoform is upregulated shortly after arterial balloon injury and continues to be expressed by proliferating SMC up to 6 weeks after *(Majesky et al. 1990)*. PDGF production has been found to correlate with IH formation in models of vein and arterial injury *(Clowes et al. 1983; Faries et al. 1996)*. However observations from some studies have suggested that PDGF may be more important for SMC migration than proliferation *(Ferns et al. 1991; Fingerle et al. 1989)*. Using an antibody to

PDGF, Ferns et al. were able to reduce neointima formation without affecting the mitogenic activity of the SMC *(Ferns et al. 1991).*

Basic fibroblast growth factor (bFGF) is a multifunctional peptide. As well as stimulating mitogenesis in vascular SMC and ECs it possesses chemotactic and cell modulating activity *(Bobik and Campbell 1993; Sato and Rifkin 1988).* The evidence supporting the role of bFGF in IH comes from several studies showing that exogenous bFGF accelerates IH in both normal *(Cuevas et al. 1991)* and balloon injured rat arteries *(Edelman et al. 1992).* It seems that the initial phase of SMC proliferation in IH is driven by bFGF released from damaged cells *(Lindner and Reidy 1991)* Thus, the systemic administration of neutralising antibody to bFGF reduced the first cycle of SMC proliferation by a about 80% but had no effect on the resulting neointimal thickening *(Lindner and Reidy 1991).*

Transforming growth factor ß (TGFß) was originally isolated from platelets but other cells such as SMC and endothelial cells have been shown to produce it *(Antonelliorlidge et al. 1989; Assoian and Sporn 1984).* It has a bimodal effect on SMC proliferation and migration. At low doses it promotes SMC proliferation and migration whilst at higher does it has an inhibitory effect *(Battegay et al. 1990; Koyama et al. 1990).* Its in vitro stimulatory effect is also dependent on culture conditions *(Majack 1987).* In vivo, TGFß mRNA can be seen shortly after balloon arterial injury and continue to be expressed for up to 2 weeks *(Majesky et al. 1991).* Furthermore Reidy and colleagues demonstrated that infusion of this growth factor into denuded carotid arteries can cause a three to four fold increase in intimal SMC proliferation *(Reidy et al. 1992).* TGFß is primarily involved in the regulation and repair of tissue following injury. In this role it is known to stimulate the synthesis of many extra cellular proteins that determine the composition of the extracellular matrix. This includes proteins such as collagens, fibronectins *(Ignotz and Massague 1986)* and proteoglycans *(Bassols and Massague 1988).* This effect is seen in models of arterial injury where direct transfer of TGFß gene into the acutely injured arterial wall results in a NI composed mainly of extra cellular material *(Pompili et al. 1993).*

Vascular endothelial growth factor (VEGF) has structural similarities to PDGF. Though it is essentially a endothelial cell specific growth factor, SMC are known to express VEGF mRNA *(Tischer et al. 1991)* especially under hypoxic conditions *(Brogi et al. 1994)*. VEGF may play a protective role in IH. Exogenous VEGF has been shown to promote re-endothelialization and attenuate IH in balloon injured arteries. In a recent study using a canine vein-artery model, Hamdan et al. found that VEGF was upregulated in the vein graft 48 hours after implantation but fell to baseline levels after 4 weeks *(Hamdan et al. 1997)*. The authors suggest that the elevation of VEGF correlates to the period of active re-endothelialization. However though this may support the theory that VEGF plays a protective role in IH formation found following arterial injury, the interactions between VEGF and other growth factors make this explanation too simplistic.

Endothelin will be discussed in detail in the next chapter. However, suffice to say at this point that there is a growing body of evidence from in vitro and in vivo studies that support the role of endothelins in IH.

The term cytokine and growth factor are often used interchangeably. This may be because of the overlap that exists in their biological effects. The cytokines are involved in the systemic and local reaction to injury. So it is not surprising that increased expression is seen in the inflammatory infiltrate that follows vein grafting *(Hoch et al. 1994b)*. Thus these cytokines mediate the inflammatory reactions immediately following injury. However, some cytokines are still expressed in the latter periods of IH formation *(Faries et al. 1996)*. The mechanism by which Interleukin 1 beta (IL-1ß) promotes SMC proliferation has been elucidated. It indirectly induces the expression of PDGF *(Raines et al. 1989)* and bFGF *(Gay and Winkles 1991)* from SMC. It also stimulates the production of interleukin -6 (IL-6) from endothelial cells and SMC *(Loppnow and Libby 1990)*. IL-6 in turn has a direct effect on cell DNA synthesis as well as an indirect promoter of PDGF production *(Raines et al. 1989)*.

Haemodynamic factors

The remodelling process that occurs in veins implanted into the arterial circulation is as a result of an adaptive response to the changes in the haemodynamic environment. It has been difficult to simulate the in vivo environment effectively in order to study its influence on vein grafts. However it is recognised that several forces acting in this environment can modulate other mechanisms involved in IH. Shear stress and pulsatile cyclic strain are the main features of the arterial haemodynamic circulation. Dobrin et al. further subdivided this into nine different haemodynamic factors *(Dobrin et al. 1989)*. By systematically exposing vein grafts in canine models to each of these factors they found that low flow velocity was an independent factor associated with IH. They also found that medial thickening was associated with circumferential deformation *(Dobrin et al. 1989)*. The flow velocity identified by Dobrin et al. correlates with shear stress which is defined as the tractive force applied longitudinally along the vessel wall as a result of blood flow. In a similar study Schwartz and colleagues found that myointimal thickening correlated most with increased wall tension *(Schwartz et al. 1992)*. Though this latter study did not distinguish between myointimal thickness in the sub endothelium and the media, it is plausible from both studies to conclude that cyclic deformation and tension contribute to overall vessel remodelling and thickening whilst low shear stress promotes intimal hyperplasia in vein grafts. The mechanisms of remodelling in arteries and veins may be different. This is supported by a recent study Galt et al. These authors compared the effects of reduced flow in arteries and grafted veins. They found that even though both vessels responded by wall thickening, arteries underwent medial remodelling whilst most of the changes seen in the vein were in the intima *(Galt et al. 1993)*. Furthermore it seemed that the veins responded more to tangential forces than shear stress. Though this study cannot be seen as a direct comparison since the arteries were not exposed to the consequences of surgical harvesting and implantation. In contrast to the above, a recent clinical study based on duplex scan of infrainguinal vein bypass grafts by Fillinger et al. demonstrated that vein grafts changed diameter in order to normalise to a uniform shear stress regardless of their initial diameter *(Fillinger et al. 1994)*. This suggests that it may be possible to predict the degree of adaptive changes based on a "target shear stress".

The remodelling seen in autogenous vein grafts is reversible if the grafts are re implanted into the venous circulation before the lesions mature *(Fann et al. 1990).*

The qualitative aspects of flow have also been shown to play a significant role in the development of IH. Disturbances of flow occur at suture sites, valve sites and anastomoses. Hence the argument that stenotic lesions are more likely to develop at these sites. Ojha and colleagues have reported a series of studies on flow in models of both proximal and distal anastomoses. They found that flow in this area was associated with variations in shear stress and patterns of flow. Very high wall shear stresses were seen in the toe and heel of the graft model, whilst fluctuating low shear stress predominated in the floor. *(Ojha 1993; Ojha 1994; Ojha et al. 1993).* These areas correspond to the areas that most often develop IH in the anastomoses *(Sottiurai 1990)* .

Haemodynamic parameters also modulate the release of growth factors such as PDGF *(Sterpetti et al. 1992),* endothelin *(Malek et al. 1993)* vasoactive peptides such as nitric oxide *(Rubanyi et al. 1986)* and prostacyclin *(Frangos et al. 1985),* all of which play significant roles in vascular biology.

Systemic factors

The role of co-morbid factors and associated systemic abnormalties in vein graft failure has generated a lot of interest. Factors such as hyperlipedemia, diabetes and smoking have been studied in both experimental and clinical situations. Some animal studies suggest that hyperlipidemia is associated with the development of IH *(Landymore et al. 1985; Klyachkin et al. 1993).* Indeed the vein wall can accummulate lipids *(Fuchs et al. 1972).* However, these associations may be more important for the formation of atherosclerosis in the longer term. There is experimental evidence to show that smoking can induce mechanisms that promotes the development of IH. It can impair graft endothelial function *(Higman et al. 1996)* and has been associated with ultrastructural changes in vein walls. Carty et al. have recently demonstrated that nicotine and its stable metabolite, cotinine promote proliferation and influence matrix metalloproteinase expression in human smooth muscle cells *(Carty et al.1996; Carty et al. 1997).* Law et al. have demonstrated that cigeratte smoke can directly promote IH in vivo *(Law*

et al. 1996). Clinically, smoking has demonstrable adverse effects on graft patency and thrombosis *(Wiseman et al. 1989; Cheshire et al. 1996).* Fibrinogen is chemotactant to smooth muscle cells and lipoproteins are harmful to the vascular endothelium *(Ferns et al. 1992a; Ferns et al. 1992b).* Thus several studies have associated various serological markers with vein graft stenosis *(Woodburn et al. 1996; Cheshire et al. 1996; Wiseman et al. 1989; Irvine et al. 1996; Gentile et al. 1997).* From these studies, smoking markers, elvated fibrinogen and lipoprotein levels levels seem to be consistent risk factors.

2.6 SUMMARY

The biological process that underlies IH has been described in this chapter. It is a complex re-structuring process that involves multiple factors. It is initiated by injury which sets of a cascade of interrelated processes involving growth factor and cytokine release, which activate smooth muscle cells to proliferate, migrate and change their phenotypic characteristics. The extent of this reaction would seem to be modulated by the invivo milieu such as the stresses of haemodynamic flow. Furthermore, structural differences or abnormalities in individual veins may predispose them to excessive intimal hyperplasia and thus stenoses. However, the reason IH becomes excessive in localised areas enough to compromise the lumen of the graft is not clear. The multiple factors that contribute to its formation open up a plethora of therapeutic approaches. Unfortunately, as discussed in Chapter 1, none of these strategies have worked in the clinical setting.

CHAPTER 3

ENDOTHELINS

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CHAPTER 3

ENDOTHELINS

3.1. INTRODUCTION

The roles of various growth factors in the aetiology of Intimal hyperplasia (IH) and graft stenosis were discussed in Chapter 2. Growth factors are of interest because it is believed that if they can be antagonised then IH could be suppressed. Endothelin is a vasoactive peptide with growth factor properties, and the laboratory aspects of this thesis will examine the effects of inhibiting this peptide on vein graft intimal hyperplasia. The aim of this chapter is to provide a detailed overview of the diverse properties of this peptide.

Endothelins (ETs) are a family of peptides that were first discovered by accident in 1984 in an experiment initially designed to look at the release of vasodilator substances from cultured bovine aortic endothelial cells *(Hickey et al. 1985)*. What followed was the discovery of the most potent vasoconstrictive peptide currently known. It was subsequently isolated and purified by Masaki and his colleagues *(Yanagisawa et al. 1988b)*. Since their discovery, ETs have been intensely investigated and apart from their vasoconstrictive properties they are now considered to be multifunctional cytokines.

3.2. Structure

ETs are 21 amino acid peptides (Figure 3.1). They exist as three closely related isomers, endothelin-1 endothelin-2 and endothelin-3 (ET-1, ET-2, and ET-3 respectively) *(Inoue et al. 1989a)*. The ET family have been shown to share sequence and functional homology with the sarafotoxins (STX) a toxin contained in venom of the burrowing asp *(Kloog et al. 1988)*.

The structural features common to the endothelin isoforms include 4 cysteine residues at positions 1, 3, 11, and 15 and the 6 amino acids at the carboxyl terminal. The cysteine residues participate in intra-chain disulphide bonding (Figure 3.1) and are thought to be important in ensuring high affinity binding at receptor sites *(Kitazumi et al. 1990)*. The ET isoforms and the STX differ in the sequences of amino acid residues at the amino terminal. Thus, ET-1 differs from ET-2 and ET-3 by 2 and 4 amino acids respectively. Most of the work done on

endothelins has elucidated the role of the ET-1 isomer resulting in a paucity of comparative information on the other isomers. Thus unless otherwise stated, Endothelin 1 (ET-1) will be used as the representative isoform of the endothelins in this thesis.

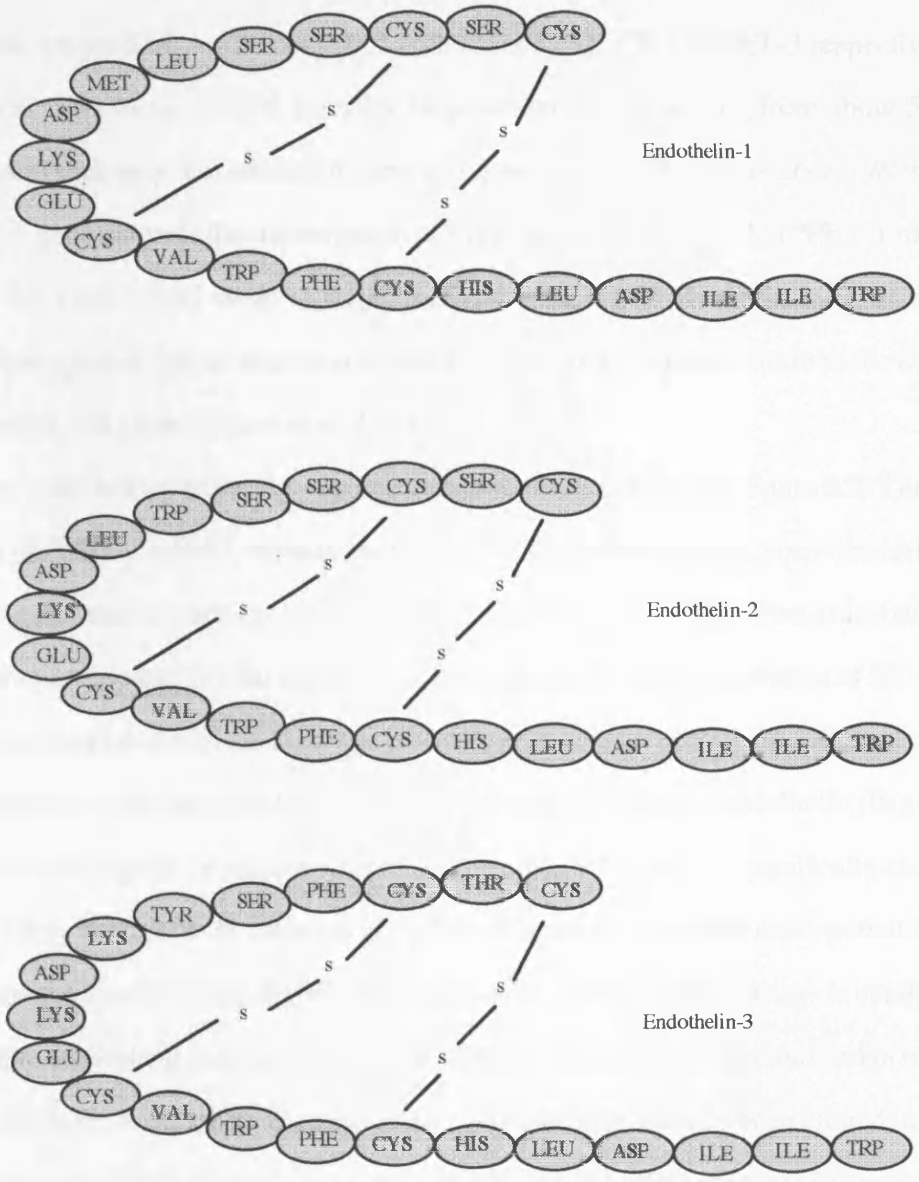


Figure 3.1. Structure of the endothelins

3.3. Synthesis

Endothelin genes

Screening of genomic DNA libraries has revealed that the ET-1s are encoded by three distinct genes in mammalian species *(Inoue et al. 1989a; Inoue et al. 1989b).* In humans, these genes have been mapped to chromosome 6, 1 and 20 for ET-1, ET-2 and ET-3 respectively *(Arinami et al. 1991; Bloch et al. 1989).* They are large structures, expanding from about 5.5 to 7KB of the DNA comprising 5 exons and 4 introns *(Inoue et al. 1989a; Inoue et al. 1989b).*

The ET-1 gene controls the transcription of Preproendothelin mRNA; (PPET-1 mRNA). This mRNA has a half life of about 15 minutes *(Inoue et al. 1989b; Yanagisawa et al. 1988a)* and it has been suggested that its relative instability may be an important feature in the regulation of ET-1 production *(Yanagisawa et al. 1988a).*

The post translation processing of pre-pro endothelin is outlined in figure 3.2. The direct product of PPET-1 mRNA translation is the ET-1 precursor peptide, preproendothelin PPET-1. In humans, this is made up of 212 amino acids *(Itoh et al. 1988).* Post-translational processing is essential for the synthesis of the biologically active isoforms of ET-1. (Figure 3.2). This involves the initial cleavage of the large precursor protein by a dibasic amino acid endopeptidase at the Lys_{51}-Arg_{52} and Arg_{91}-Arg_{92} to yield big endothelin (Big ET-1), a 38-41 amino acid peptide *(Yanagisawa et al. 1988a).* Big ET-1 is then specifically cleaved at the Try_{21}-Val_{22} bond. The 21 amino acid so formed is up to 140 times more potent in its vasoconstrictor actions than big ET-1 *(Kimura et al. 1989).* This cleavage is catalysed by endothelin converting enzyme (ECE). This enzyme has recently been purified in mammalian tissues *(Ohnaka et al. 1993; Waxman et al. 1994)* and has recently been cloned in humans *(Shimada et al. 1995).* Xu et al. have elucidated that it is a 758 amino acid transmembrane metalloprotein *(Xu et al. 1994).* Another form of ECE, ECE-2 has recently been isolated which in contrast to ECE is an intracellular enzyme that acts in a more acidic medium *(Emoto and Yanagisawa 1995).*

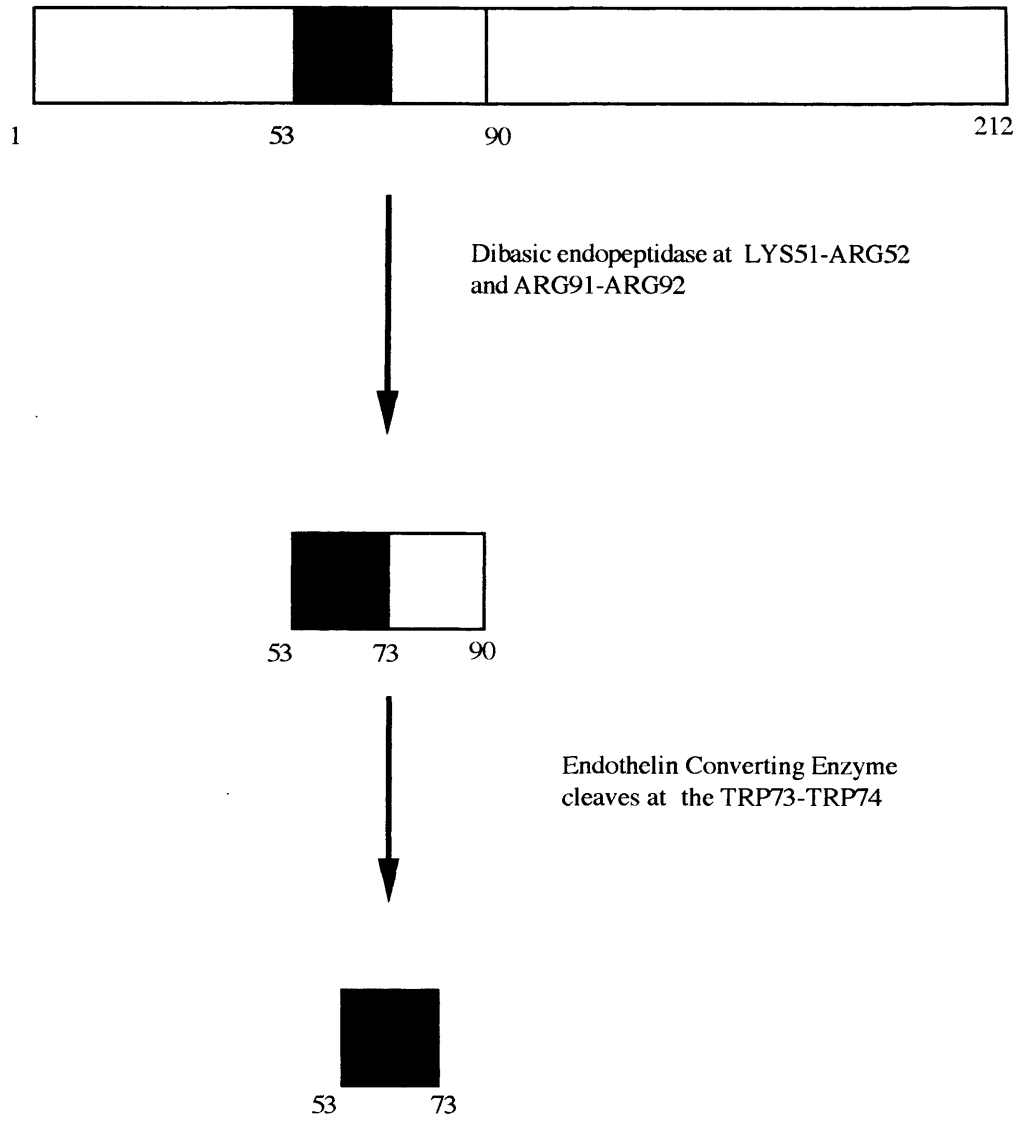


Figure 3.2. Post translational processing of pre-pro endothelin

The action of ECE is seen as an important control point in the regulation of ET-1 production. Thus inhibitors of ECE, based on the structure of the potent metalloprotease inhibitor Phosophoramidon, have been developed and used as pharmacological tools *(Bertenshaw et al. 1993).*

Regulation of ET-1 synthesis

ET-1 synthesis is regulated at the level of gene transcription. The paucity of endothelin storage vesicles in cell cytoplasms and presence of PPET-1 mRNA supports this view *(Yanagisawa et al. 1988a)*. The promoter area of the ET-1 gene has been extensively studied. A primary regulatory site is located upstream of the ET-1 coding region *(Inoue et al. 1989b)* and consists of two functional regions *(Lee et al. 1990)*. An alternate regulatory site has been discovered further upstream of the primary regulatory site *(Benatti et al. 1993)*.

Various regulatory sequences have been identified in these promoter sites. An AP-1 and a GATA motif sequence are found at the primary promoter site *(Lee et al. 1991A)*. The AP-1 motif recognises the nuclear proteins, c-Fos and c-Jun *(Lee et al. 1991B)*. The complimentary DNA of the protein that binds to the GATA motif has been cloned in humans *(Dorfman et al. 1992; Lee et al. 1991B)*. This protein, the human transcription factor GATA-2, has been found to interact with the GATA sequence on the promoter site to increase transcription of reporter genes in endothelial cells. However, this GATA-2 interaction was found to be ineffective in kidney epithelial cell lines *(Dorfman et al. 1992)*, suggesting the existence of other regulatory processes in certain cells.

Other regulatory motifs have been identified. The nuclear -factor -1 (NF-1) found in the 5' region and in the intervening sequence between exon 4 and 5 *(Bloch et al. 1989; Bloch et al. 1991; Inoue et al. 1989b)* mediates the upregulation of ET-1 mRNA induced by the growth factor transforming growth factor-ß (TGF-ß). Motifs for acute phase reactants are also located at the 5' region and the intervening sequence between exons 1 and 2 *(Inoue et al. 1989b)* and are associated with the increased production of ET-1 after acute stress such as myocardial injury or surgery *(Miyauchi et al. 1989)*. Recently, new regulatory elements called Shear Stress Response Elements (SSRE) have been found in the promoter genes of several cytokines including the ET-1 gene *(Malek et al. 1993)* The SSRE may be involved in shear stress induced gene expression.

The structure of the 3' untranslated region of the ET-1 gene consists of a sequence of 250 base pairs which has been conserved between species *(Inoue et al. 1989a; Inoue et al. 1989b)*. These sequences are known to mediate mRNA degradation *(Shaw and Kamen 1986)* and may

account for the short half life of the PPET-1 mRNA and thus play a role in gene regulation at the post transcriptional level *(Inoue et al. 1989b).*

Transcription of ET-1 is influenced by various factors (Table 3.1). It is upregulated by cytokines such as TGF-ß, interferon in concert with tumour necrosis factor-alpha (TNF-a), and interleukin-1; vasoactive substances such as angiotensin II and vasopressin; and other circulatory substances such as thrombin, insulin, prolactin, and calcium ionophores. Stress situations such as hypoxia, myocardial injury surgery and fluid shear stress also stimulate ET-1 production. Various vasodilators including Prostacyclin, nitric oxide and nitroglycerine have been shown to inhibit ET-1 production. Atrial natriuretic peptide has also been shown to reduce ET-1 release from endothelial cells.

Table 3.1. Factors that influence the release of endothelin

Factors that increase endothelin release
Cytokines: Tumour necrosis factor-alpha, thrombin, interluekins.
Arginine vasopressin
Hypoxia
Glucose
Cyclosporin
Growth factors: Transforming growth factor, insulin like growth factor
Low shear stress
Surgery
Cortisol
Low-density lipoproteins

Factors that decrease endothelin release
Nitric oxide
Prostacyclin
Atrial natriuretic peptide
Heparin
Increased shear stress.

Elimination and degredation

Endothelin is stable in blood and plasma, however it has a short half life of a few minutes when administered intravenously ranging from 60 seconds in the rat *(Sirvio et al. 1990)* to 120 seconds in the pig *(Hemsen et al. 1991)*. This is due to a first pass uptake elimination by the lungs and kidneys *(Shiba et al. 1989; Sirvio et al. 1990)*. It is mainly metabolised in these tissues where it is rapidly degraded by neutral metallo-endopeptidase (NEP). This widley distributed enzyme degrades ET at multiple cleavage sites *(Vijayaraghavan et al. 1990)*.

3.4. ENDOTHELIN RECEPTORS

The various effects of ET-1 are mediated via specific receptors. Prior to the cloning of these receptors by Arai et al. and Sakurai et al. *(Arai et al. 1990; Sakurai et al. 1990)* several pharmacological attributes of ET-1 supported the notion of the existence of at least two receptors. There was a biphasic response of initial vasodilatation then prolonged vasoconstriction following administration of ET-1 *(Spokes et al. 1989)*. Furthermore, the ET-1 isomers were equipotent for the dilatory phase whereas ET-1 was more potent than ET-2 or ET-3 for the pressor phase *(Takayanagi et al. 1991A)*. Lastly, data from binding studies showed that the selective ET-1 bound preferentially to sites on vascular smooth muscle cells whilst the non selective ET-1/ET-1-2/ET-1-3 bound to sites on the endothelium *(Ihara et al. 1991)*. In the work by Maggi and colleagues smooth muscle preparations from various animals were stimulated with ET-1 or ET-1 agonists *(Maggi et al. 1989a; Maggi et al. 1989b)*. They

found that in some preparations this produced little contractile activity and termed the receptors ET_A, (A for aorta). In some preparations these agonists produced significant contractions and the receptors in these preparations were called ET_B (B for bronchus). Though the agonists used in that study have been subsequently shown to have low receptor binding potency *(Doherty 1992)*, the nomenclature is still in use today.

In 1990 the cDNA for the ET_A and ET_Breceptors were cloned for the first time from bovine and rat lung respectively *(Arai et al. 1990; Sakurai et al. 1990)*. Since then, these receptors have been cloned from different human tissues including lung, heart, jejunum, liver, placenta and prostate. An ET_C receptor has been isolated in the Xenopus laevis frog *(Karne et al. 1993)* but has not yet been described in mammalian cells.

Structure of endothelin receptors

The endothelin receptors belong to the rhodopsin superfamily of receptors *(Birnbaumer et al. 1990)*. Other members of this family include the ß- adrenergic (ß1/ß2), vasopressin (v1/v2) and serotonin (5-$HT_{1,2}$). These receptors are characterised by the presence of seven segments that span the cell membrane and are coupled to guanine-nucleotide regulatory proteins (G-proteins). The terminal domains of the receptors extend beyond either side of the membrane. The NH_2-terminal domain is 75 to 100 residues long and is extracellular. The COOH terminal is cytoplasmic and may play a role in anchoring the receptor within the lipid bilayer.

Endothelin receptor gene and its regulation

In humans, the genes encoding the ET_A and ET_B receptors are located on chromosome 4 and 13 respectively *(Arai et al. 1993; Hosoda et al. 1992)*. The expression of these genes is influenced by factors similar to those that influence ET-1 production such as hypoxia and surgery.

Exposure of cells to ET-1 and ET-1 receptor antagonists can modulate receptor expression. ET-1 itself causes a down regulation of its receptors *(Devesly et al. 1991; Hirata et al. 1988; Yu and Davenport 1995)*, whilst selective ET_A receptor antagonists have been shown to cause an upregulation of receptors *(Yu and Davenport 1995)*. These findings have implications for

studies and therapies using ET-1 antagonists. For example, Clozel et al. found that an ECE inhibitor potentiated the release of arachidonic acid from mesangial cells as a result of increased receptor binding of ET-1 *(Clozel et al. 1993).*

The conditions of cell culture and serial passaging can also influence receptor expression. Eguchi and colleagues have demonstrated that the phenotypic change in SMC morphology that follows passaging is also associated with a change in surface ET-1 receptor subtype distribution *(Eguchi et al. 1994).*

Endothelin receptor distribution

Endothelin receptors have been identified in numerous organs and tissues. However, there are species and tissue variations in distribution. This complicates the interpretation of the diverse biological effects of ETs even further. It also mandates a careful appraisal of the use of animals intended to model human conditions. The working knowledge of the distribution of endothelin receptors is based on a combination of data from functional binding *(Masaki et al. 1994)* and in situ hybridisation studies *(Hori et al. 1992).* Using in situ hybridisation techniques Hori and colleagues localised ET_A and ET_B mRNA in a wide range of rat tissues *(Hori et al. 1992).*

Endothelin receptors in the human vasculature

Previous studies on receptor distribution in the vasculature suggested that ET_A receptors were present only on the membranes of smooth muscle cells and mediated contraction *(Lin et al. 1991).* Whilst the ET_B receptors mediated vasodilatation and were restricted to the endothelium *(Takayanagi et al. 1991B).* However subsequent studies have shown that ET_B receptors also exist in smooth muscle cells of human arteries and veins, figure 3.4 *(Davenport et al. 1995; Seo et al. 1994).* The role of these receptors is still not clear. Davenport et al. found that although mRNA for both receptor subtypes was detected in arteries and veins, over 85% of the endothelin receptors were of the ET_A subtype *(Davenport et al. 1995).* A similar distribution was found by Dagassan et al. in coronary arteries *(Dagassan et al. 1996).* Even though some studies have demonstrated that contraction of human smooth muscle cells can be mediated via ET_B as well as ET_A, receptors *(Haynes et al. 1995; Tschudi and Luscher 1994),* the notion

that the ET_B receptor plays a significant role in vasoconstriction has not been supported by other studies where selective ET_A antagonists completely blocked ET-1 induced vasoconstriction in coronary and other vessels *(Maguire and Davenport 1995)*. Thus it is generally accepted that ET_A receptors mediate vasoconstriction in humans.

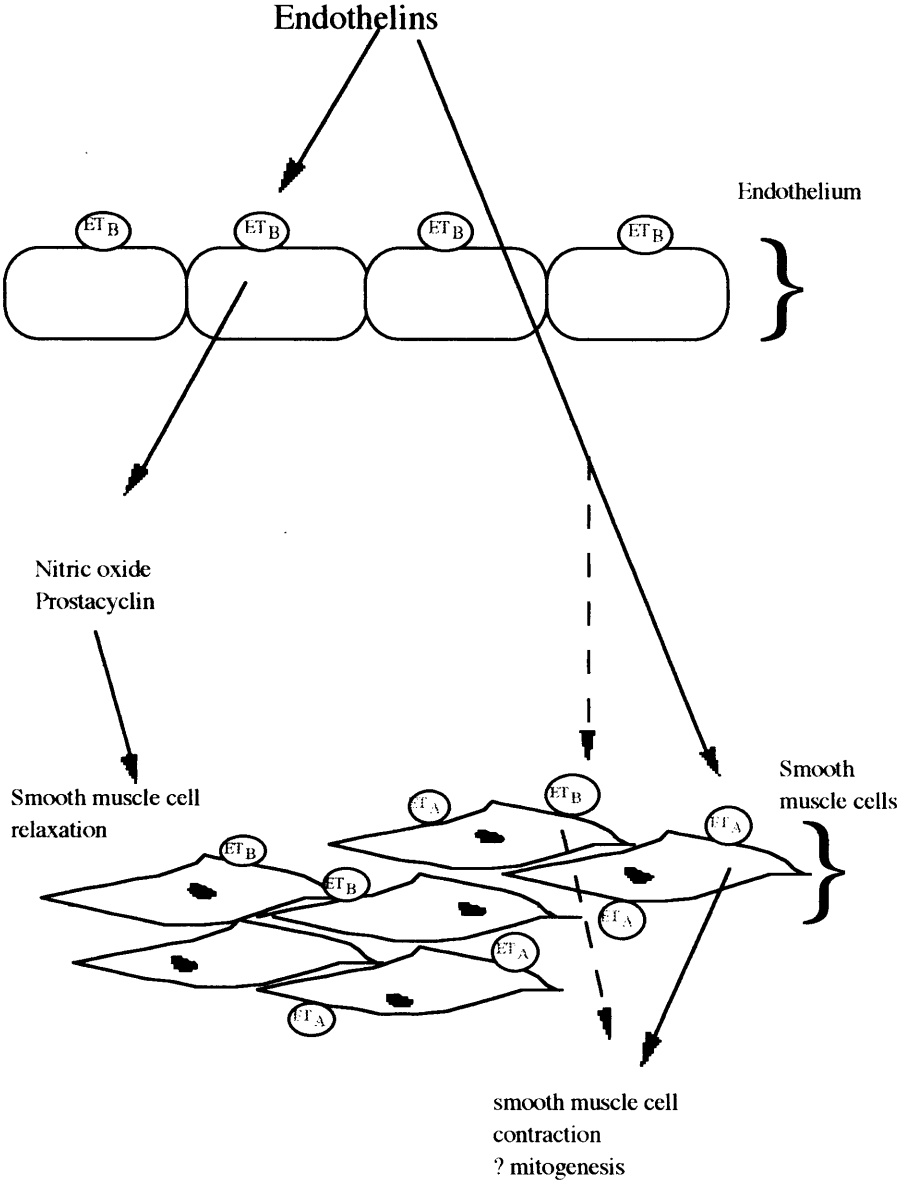


FIG 3.4 Effect of endothelin on vascular endothelium and smooth muscle cells.

The relative paucity of ET_B receptors in the vasculature may account for the overall lack of involvement of these receptors in vasoconstriction *(Maguire and Davenport 1995)*. However,

upregulation of ET_B receptors *(Azuma et al. 1995; Dagassan et al. 1996)*. This suggests that ET_B receptors may play a more significant role in proliferative disease states.

Inhibitors of endothelin receptors

A number of receptor antagonists have been developed in order to investigate the role of ET-1 in various physiological and pathological conditions. Some of these antagonists are currently being evaluated in clinical trials.

ET receptor antagonists can be either selective or non selective. They can be further classified as peptide or non-peptide based compounds. Non-peptide antagonists are generally more useful tools for investigative and clinical purposes as they tend to have better bio-availability than the peptide compounds that are rapidly degraded by proteases. Early attempts at creating these antagonists involved the modification of the parent ET-1 molecule to create a peptide that would bind to the receptor but not induce signal transduction. Amongst the first of these was the cyclic pentapeptide, BE18257B produced from the fermentation of *Streptomyces misakiensis* which paved the way for the identification of BQ123, the highly specific $ET\text{-}1_A$ receptor antagonist. Other receptor antagonists have been derived as a result of either structure-activity studies or the screening of chemical banks (Table 3.2).

Table 3.2. Some endothelin receptor antagonists

Name	Specificity	Structure	Comments
BQ123	ET_A	Cyclic pentapeptide	Highly specific peptide
BQ153	ET_A	Linear tripeptide	peptide
FR-139317	ET_A	Pseudo-tripeptide	peptide
TTA-386	ET_A	Hexapeptide	Synthetic
PD156707	ET_A	Non-peptide	Orally active
BMS-182874	ET_A	Benzene-sulphonamide	Orally active
97-139	ET_A	Caffeoyl ester	Similar potency to BQ123, but binds to plasma proteins
BQ788	ET_B	Tripeptide	
RES-701-1	ET_B	Cyclic peptide	
Tak-044	ET_A and ET_B	Cyclic hexapeptide	
RO-462005	ET_A and ET_B	Sulphonamide	Orally active
Bosentan	ET_A and ET_B	Sulphonamide	Orally active, used in clinical trials.
SB 209670	ET_A and ET_B	Carboxylic acid derivative	Highly potent
SB217242	ET_A and ET_B	Carboxylic acid derivative	Similar to SB 209670 but orally active
PD145065	ET_A and ET_B	Linear-hexapeptide	

3.5 MECHANISMS OF SIGNAL TRANSDUCTION

Upon binding to the appropriate cell surface receptor coupled to a G protein, endothelins can activate any of several signal transduction pathways.

Phospholipase C and phospholipase D pathways

ETs activate the phospholipase C (PLC) pathway by coupling to Gq proteins *(Takuwa et al. 1989)*. Activation of this pathway initiates phosphoatidyl inositol (PI) hydrolysis which leads to the rapid production of second messengers such as inositol 1,4,5-trisphosphate (IP_3), 1,2-diacylglycerol (DAG) and inositol tetrakisphosphate (IP_4). Thus ET-1 has been shown to increase levels of IP_3 in various cell cultures and tissues including vascular smooth muscle cells (VSMC) *(Araki et al. 1989; Huang et al. 1989)* and isolated arteries *(Ohlstein et al. 1989; Rapoport et al. 1990)*.

There is a sustained increase in DAG levels following ET-1 stimulation of SMC *(Griendling et al. 1989; Sunako et al. 1990).* However, other pathways in addition to PI hydrolysis may account for the persistent levels of DAG. Phospholipase D (PLD) pathway hydrolysis of phosphoatidic acid is thought to play a role in ET-1 signalling by generating DAG. This pathway has been demonstrated in VSMC *(Resink et al. 1990)* glioma and fibroblasts *(Ambar and Sokolovsky 1993)* and may be the major source of DAG in some cells *(Pai et al. 1991).*

The second messengers formed as a result of PI hydrolysis play important roles in intracellular signalling. IP_3 causes the release of Ca^{2+} from the sacroplasmic reticulum. This is augmented by IP_4. DAG on the other hand, is known to activate the protein kinase C (PKC) pathway.

Adenylate cyclase and cyclic AMP

The adenyl cyclase (AC) pathway is another signalling pathway that ETs activate. This pathway generates the formation of the second messenger cyclic AMP (cAMP).

The ET-1 receptors have different effects on this pathway. In rat VSMCs which express predominately ET_A receptors, Eguchi and colleagues found that ET-1 caused a dose dependent stimulation of cAMP *(Eguchi et al. 1994).* However, in bovine aortic endothelial cells that express mainly ET_B receptors, they found that ET-3 caused a dose dependent reduction in cAMP formation. Furthermore, this reduction was pertussis toxin sensitive *(Eguchi et al. 1991).* These initial reports suggest that the ET receptors may be coupled to different G proteins in the AC pathway. Specifically, ET_A coupled to Gs whilst ET_B is coupled to Gi *(Aramori and Nakanishi 1992).* However, this has not been a consistent finding in cells from other tissues and species. Studies on guinea pig myocytes showed that ET_A receptors can inhibit adenylyl cyclase activity and this effect is pertussis sensitive consistent with ET_A coupling to Gi proteins *(James et al. 1994; Ono et al. 1994).* Thus, ET-1 modulation of cAMP via G membrane proteins has been found to vary with cell type.

Extracellular calcium

The role of extracellular calcium in ET-1 signal transduction has been clarified. ET-1 elicits a biphasic increase in cytosolic free calcium, the initial transient spike is followed by a sustained elevation of calcium levels. This observation initially led Yanagisawa and colleagues to postulate that ET-1 is a ligand for voltage-operated calcium channels *(Yanagisawa et al. 1988b)*. This view was supported by several studies that found that L-type Ca^{2+} channel antagonists such as verapamil, diltiazem, nifedipine etc. attenuated ET-1 induced vasoconstriction in several isolated animal arterial preparations *(Egashira et al. 1990; Kasuya et al. 1989; Sakata et al. 1989)*. However, other studies reported little or no effect of Ca^{2+} antagonists on ET-1 induced vasoconstriction or Ca^{2+} uptake in cultured VSMCs *(Blackburn and Highsmith 1990; Mitsuhashi et al. 1989)*. Furthermore, the initial transient rise in cytoplasmic Ca^{2+} seen in ET-1 stimulated SMCs is not sensitive to changes in extracellular Ca^{2+} *(Danthuluri and Brock 1990)*. Stasch and Kazda were able to show that dihydropyridine calcium channel antagonists such as nifedipine and nicardipine were non-competitive antagonists of ET-1 induced contractions *(Stasch and Kazda 1989)*, thus confirming that ET-1 does not compete at the same sites and is not a ligand for these L-type Ca^{2+} channels. It is now accepted that the initial rise in cytosolic Ca^{2+} seen after ET-1 stimulation is as a result of the mobilisation of stores in the sacroplasmic reticulum via the PLC pathway as outlined above, whilst the prolonged second phase is due to ET-1 induced influx of extracellular calcium via indirect voltage operated channel modulation *(Rubanyi and Polokoff 1994)*.

Protein kinase C pathway

Several lines of evidence implicate the involvement of the PKC in ET-1 signal transduction. Firstly, DAG which is released as a result of PI hydrolysis is known to activate the PKC pathway *(Nishizuka 1989)*. Secondly, ET-1 treatment of VSMC is associated with an increase in membrane associated PKC activity *(Danthuluri and Brock 1990; Griendling et al. 1989)*. Lastly, studies using PKC inhibitors such as H-7 and staurosporin have been shown to diminish the contractile effects of ET-1 *(Kasuya et al. 1989)*.

PKC activation causes the phosphorylation of various proteins which results in secretory, contractile and proliferative events.

Signalling Pathways for mitogenesis

Calcium mobilisation plays a fundamental role in mitogenesis *(Rasmussen et al. 1984)*, and it represents a common end point for the pathways discussed above.

PKC activation is associated with increased expression of the proto-oncogenes C-fos and c-myc *(Coughlin et al. 1985)*. ET-1 has been shown to increase the expression of these proto-oncogenes in VSMC *(Bobik et al. 1990; Komuro et al. 1988)* and fibroblasts *(Pribnow et al. 1992; Takuwa et al. 1989)*. Depletion of PKC in fibroblasts diminishes ET-1 induced mitogenesis by 60% *(Takuwa et al. 1989)*. Similarly, Bobik et al. investigated the pathways involved in ET-1 induced SMC mitogenesis. They found that ET-1 induced elevation in c-fos mRNA was not totally abolished by PKC depletion and that the mitogenic effect of ET-1 was dependent on both pertussis sensitive and insensitive pathways *(Bobik et al. 1990)*. Indeed, ET-1 has been shown to also activate the pertussis insensitive mitogen activated protein kinase (MAK) in VSMC *(Koide et al. 1992)*.

3.6. PHYSIOLOGICAL ACTIONS OF ENDOTHELINS

Circulatory effects of endothelin

Endothelin is known primarily for its vasoactive effects on the cardiovascular system. Intravenous injection of ET-1 into animals causes an initial transient vasodilatation followed by a sustained vasoconstriction *(Spokes et al. 1989; Yanagisawa et al. 1988b)*. Small doses of ET-1 infused into the brachial artery of male volunteers produced similar effects *(Clarke et al. 1989)*. Systemic administration causes a sustained elevation in blood pressure in humans *(Vierhapper et al. 1990)* whilst receptor blockade causes a reduction in peripheral resistance and blood pressure. Both receptors can mediate the vasoconstrictive response *(Harrison et al. 1992; Haynes et al. 1995; Tschudi and Luscher 1994)*. However, in the human vasculature, vasoconstriction seems to be mediated predominantly by the ET_A receptor and the ET_B

receptor mediates the initial vasodilatory response via an endothelial dependent release of nitric oxide *(Karaki et al. 1993).*

In the heart, local introduction of ET-1 to the coronary arteries of small animals induces vasoconstriction and myocardial ischaemia. *(Kramer et al. 1992).* On the heart muscle ET-1 has a chronotropic and inotropic effect *(Takanashi and Endoh 1991).* It can also induce the secretion of atrial naturetic hormone from cardiac myocytes *(Lew and Baertschi 1989).*

Endothelin and the kidneys

There are numerous studies which have examined the effects of ETs on renal function and haemodynamics *(Harris et al. 1991; Katoh et al. 1990; Miller et al. 1989; Stacy et al. 1990; Uzuner and Banks 1993).* In general, infusion of ET-1 into rats and dogs is associated with a reduction in renal plasma flow (RPF), glomerular filtration rate (GFR) and urine output *(Katoh et al. 1990; Miller et al. 1989; Stacy et al. 1990).* At low doses, infusion of ET-1 has been shown to inhibit the reabsorbtion of sodium. The reduction of GFR and RPF seen following infusion of high doses of ET-1 is due to its direct vasoconstrictive effects on renal cortical vasculature. However there is evidence of tubular endothelin synthesis *(Kohan 1991)* and it has been proposed that ET-1 may have a direct autocrine effect on tubular cells in the regulation of water and salt excretion *(Ong 1996).* The problem with establishing the exact roles of ET-1 receptors in renal physiology is the species difference in receptor distribution. Renal vasoconstriction in rats is mediated by ET_B receptors *(Gellai et al. 1994)* whilst in dogs it is mediated by ET_A receptors *(Brooks et al. 1994).* The distribution of ET-1 receptors in dogs is similar to humans and therefore may be better models for study *(Karet and Davenport 1994).*

Respiratory System

Endothelin receptors are abundant in the lungs *(Cai et al. 1991).* ET-1 induces pulmonary vasoconstriction and bronchoconstriction *(Hay et al. 1993).* Bronchoconstriction is mediated via the ET_B receptor in experimental animals and humans *(Hay et al. 1993).* ETs also promote pulmonary arterial SMC proliferation *(Zamora et al. 1993).*

Developmental biology

ET-1 is important in the development and differentiation of embryonic tissue. Knockout gene experiments of ET-1 peptide and ET-1 receptor genes all cause severe congenital abnormalities *(Kurihara et al. 1994).*

Mitogenic effects of endothelins

The mitogenic effect of ET-1 has been demonstrated in various cells.

ET-1 has been shown to increase DNA synthesis in SMC from different species including rats, *(Bobik et al. 1990; Komuro et al. 1988)* rabbit *(Serradeillegal et al. 1991)* and in the human CRL 1692 cell line *(Bunchman and Brookshire 1991)* and SMC of venous origin *(Masood et al. 1997).* ET-1 has also been shown to increase DNA synthesis in other cells including 3T3 fibroblasts *(Brown and Littlewood 1989; Takuwa et al. 1989)* bovine endothelial cells *(Vigne et al. 1990)* rat glial cells *(Maccumber et al. 1990)* and rat osteoblasts *(Takuwa et al. 1990).* However in some studies, even high concentrations of ET-1 have failed to induce DNA synthesis in rat VSMC *(Chua et al. 1992; Weissberg et al. 1990).* Similarly, ET-1 in the absence of other growth factors failed to stimulate proliferation in rat aortic SMC *(Koide et al. 1992).* Whilst the study by Weissberg and colleagues on rat VSMCs showed that ET-1 had no mitogenic effect on its own, its isopeptides potentiated the mitogenic effect of platelet derived growth factor (PDGF) and calf serum *(Weissberg et al. 1990),* thus suggesting that ET-1 is comitogenic rather than a growth factor. This does not explain the mitogenic effect observed in serum free cultures of 3T3 fibroblast in other studies which could not have resulted from synergism with other growth factors. Furthermore, the rapid increase in proto-oncogene expression observed following exposure to ET-1 *(Bobik et al. 1990; Komuro et al. 1988)* suggests that it acts as direct mitogen rather than a co-mitogen. It is possible that the discrepancies observed in the mitogenic actions of ET-1 may be as a result of the differences in conditions of cell culture *(Serradeillegal et al. 1991).* The synergy between ET-1 and PDGF may be explained in the results of a recent study by Jahan and colleagues *(Jahan et al. 1996).* This study showed that when smooth muscle cells were synchronised in G_0 phase, ET-1 acted

as a progression growth factor that induces mitogenesis after PDGF has acted as a competence factor.

It is not clear which of the receptor subtypes mediate ET-1 induced mitogenesis. In a study by Ohlstein et al., the mitogenic effects of ET-1 on rat VSMC was inhibited by the ET_A receptor antagonist BQ123. Furthermore, in that study, the ET_B receptor agonist sarafotoxin 6c did not significantly increase DNA synthesis. Thus they concluded that the ET_A receptor mediates mitogenesis *(Ohlstein et al. 1992)*. Eguchi and colleagues also confirmed these findings *(Eguchi et al. 1992)*. However, invivo experiments in rat models have shown that ET_A receptor antagonism with BQ123 is insufficient to inhibit neointima formation, a condition that is characterised by SMC proliferation *(Douglas et al. 1995b)*. Thus suggesting a significant role for the ET_B receptor subtype in mitogenesis associated with pathological states. Normal SMC have been shown to possess both ET receptor subtypes *(Davenport et al. 1995; Eguchi et al. 1994; Seo et al. 1994)*. Furthermore, the distribution, and proportion of these receptors changes with phenotype; Eguchi and colleagues have shown that there is increased ET_B receptor expression associated with SMC phenotypic changes *(Eguchi et al. 1994)*. The findings discussed above have been from cells and tissue of arterial origin, little is known about the receptors mediating mitogenesis in human veins. The aim of one of the studies in this thesis was to determine which of the receptor subtypes mediates proliferation in human long saphenous vein SMCs

3.7. PATHOLOGICAL ROLES OF ENDOTHELIN

Since its discovery there has been intense research interest in ET-1. As a result, it has been implicated in a large number of systemic and localised disease conditions.

Cardiovascular diseases

By nature of its vasoconstrictive effects, ET-1 was assumed to play a role in the development of hypertension *(Yanagisawa et al. 1988b)*. However, studies demonstrating normal levels of ET-1 in patients with hypertension have made this controversial *(Miyauchi et al. 1992)* . ET-1 is secreted abluminally *(Wagner et al. 1992)*, and is rapidly cleared from the circulation. Thus

even though ET-1 plasma levels were normal in experimental hypertensive rats, immunoreactive ET-1 levels were increased in the vascular tissue *(Fujita et al. 1995)*. Therefore, ET-1 plasma levels may not be representative of local tissue levels. Plasma levels of big ET-1 or its more stable c-terminal cleavage fragment may be more useful. Despite the problems with plasma levels, there are other findings that may link ET-1 to hypertension. Elevated levels have been demonstrated in pre-eclampsia and malignant hypertension *(Florijn et al. 1991; Widimsky et al. 1991)*. Furthermore, inhibition of the ET-1 peptide or its receptors in rats causes a marked reduction in blood pressure *(Bazil et al. 1992; Hocher et al. 1995)*.

Myocardial ischaemia

Increased levels of ET-1 have been detected in patients *(Miyauchi et al. 1989)* and experimental animals *(Watanabe et al. 1991)* following myocardial infarction. In patients, the levels of ET-1 measured 3 days following infarction has been shown to strongly correlate with the one year mortality *(Omland et al. 1994)*. Inhibition of the endothelin system with ECE inhibitors, selective or non selective receptor antagonists prior to an ischaemic insult reduces the size of the infarct in experimental models *(Grover et al. 1993; Watanabe et al. 1995)*. Similarly, ET-1 levels are elevated in congestive cardiac failure (CCF), and correlate with the severity of the symptoms *(Wei et al. 1994)*. Of immense clinical importance is the study by Kiowski et al. demonstrating that receptor blockade produced a marked improvement in the haemodynamic parameters of patients with CCF. Infusions of intravenous Bosentan (a non selective endothelin receptor antagonist) produced a reduction in systemic and arterial pressures resulting in a reduction in peripheral resistance and a rise in cardiac output *(Kiowski et al. 1995)*. Similar haemodynamic improvements have been noted in CCF patients taking ACE inhibitors after a single dose of the ET_A receptor antagonist BQ123 along with the ECE inhibitor phosphoramidon *(Love et al. 1996)*. With these encouraging results there are plans for major clinical trials using ET-1 antagonists for the treatment of CCF. However it is still debatable whether dual or selective receptor blockade will be beneficial.

Pulmonary hypertension

Several lines of evidence link ET-1 to pulmonary hypertension (PH). Smooth muscle cells isolated from arteries of rats with idiopathic PH produced more ET-1 when compared to control. This is thought to contribute to the enhanced proliferation seen in these cells when cultured in vitro *(Zamora et al. 1996)*. Increased ET-1-like immunoreactivity is seen in endothelial cells from patients with PH. The elevated levels of ET-1 in patients with pulmonary hypertension correlates with the severity of the disease *(Ishikawa et al. 1995b)*. When PH is secondary to congenital heart disease, surgical correction is associated with a fall in these levels *(Ishikawa et al. 1995a)*.

Renal failure

Clinical studies have shown that ET-1 is elevated in both acute *(Tomita et al. 1989)* and chronic *(Saito et al. 1991)* renal failure. Renal injury up regulates both ET-1 receptor subtypes in experimental models of acute renal failure (ARF) *(Roubert et al. 1994)*. ET-1 is linked to various causes of renal injury, including cyclosporin and radiocontrast induced nephrotoxicity *(Kohan 1993)*. It is not clear which, if any, of the ET-1 receptors plays a predominant role in renal injury reperfusion. The situation is complicated by the variation in species distribution of ET receptors. In rats for example, ET_A receptor antagonists have been shown to reduce renal impairment whether it is administrated before or after the ischaemic insult *(Gellai et al. 1994; Mino et al. 1992)*. A similar effect was observed in rats treated with non-selective antagonists *(Kusumoto et al. 1994)*. However ET_A receptor antagonists have had no beneficial effect on renal failure in dogs *(Brooks et al. 1994)*.

In contrast to CCF, there is currently no specific treatment for ARF, thus successful clinical therapy with endothelin antagonists may have a greater impact in the management of renal failure.

Cerebrovascular disease

Elevated ET-1 levels have been demonstrated in the tissue of injured rat neuronal tissue *(Uesugi et al. 1996; Yamada et al. 1995)*. In humans, tissue ET-1 immunoreactivity is elevated

in patients with various forms of viral encephalitis *(Ma et al. 1994)*, and in Alzheimer's disease *(Zhang et al. 1994)*. However the area of most interest has been on the role of ETs in the vasospasm that follows subarachnoid haemorrhage. To this end, elevated levels of ET-1 have been demonstrated in the plasma and cerebrospinal fluid of patients with subarachnoid haemorrhage *(Fujimori et al. 1990)*. Furthermore ET antagonists have been shown to reduce the degree of vasospasm in experimental subarachnoid haemorrhage *(Willette et al. 1994)*.

Atherosclerosis and intimal hyperplasia

The proliferative effects of endothelins on SMC have been described in previous sections. SMC proliferation is central to the development of atherosclerosis and intimal hyperplasia. Thus it is plausible that ETs may play a significant role in these disease processes. There is both circumstantial and direct evidence for this. Elevated ET-1 levels are elevated in patients with atherogenic risk factors such as smoking and hyperlipidaemia *(Haak et al. 1994a; Haak et al. 1994b)*. Oxidised low density lipoprotein induces the release of ET-1 from cultured cells *(Martinnizard et al. 1991)*. Lerman and colleagues demonstrated a correlation between elevated levels of ET-1 and the number of atherosclerotic beds. Furthermore they demonstrated ET immunoreactivity within the SMC and the EC of atherosclerotic plaques *(Lerman et al. 1991)*. ET-1 enhances the formation of IH in animals following coronary PTA *(Douglas and Ohlstein 1993; Trachtenberg et al. 1993)*. This effect has been shown to be ameliorated by non-selective ET receptor blockade but not by selective ET_A receptor antagonists *(Douglas et al. 1995a)*. Thereby suggesting that the ET_B receptor may mediate this process.

3.8. CONCLUSION

It is clear that ET has aroused much interest in a wide range of pathological processes. There is a definite association between endothelin and angioplasty related restenosis. However as can be seen, most studies have defined the role of ET in IH in post angioplasty restenosis in animals and very little is known about the ET system in human vein grafts. The underlying pathology of IH is similar in these disease processes. However, it is clear from previous studies that there is often a marked species and tissue variation in ET receptor and peptide

distribution. This will in turn lead to a variation in tissue response to endothelin. Thus animals cannot be directly correlated to humans, neither should studies on IH in arteries be extrapolated to veins. Clearly the role of endothelin in vein grafts warrants investigation. In order to overcome the limitations of animal models, the experimental chapters in this thesis will use an invitro human model of vein graft IH.

CHAPTER 4

A RETROSPECTIVE STUDY OF THE INFLUENCE OF CLINICAL FACTORS ON THE LONG-TERM OUTCOME OF INFRAINGUINAL VEIN BYPASS GRAFTS

CHAPTER 4

A RETROSPECTIVE STUDY OF THE INFLUENCE OF CLINICAL FACTORS ON THE LONG-TERM OUTCOME OF INFRAINGUINAL VEIN BYPASS GRAFTS

4.1. INTRODUCTION

Current concepts of lower limb revascularisation have been discussed in Chapter 1. Modern practice recognises that meticulous surgical techniques and the implementation of postoperative surveillance *(Bergamini et al. 1995; Dunlop et al. 1995a; Idu et al. 1993; Lundell et al. 1995)* can improve the outcome of autologous vein grafts *(Londrey et al. 1991; Michaels 1989; Veith et al. 1986).* The outcome of these procedures is expressed in terms of patency rates, limb salvage and patient survival. Further improvement may be possible by risk factor modulation. However, the effect of clinical risk factors on these outcomes has been controversial. There is no consensus on the role that factors such as smoking, diabetes, gender or vessel run off play in patency rates *(Rutherford et al. 1988; Sayers et al. 1993b; Shah et al. 1988; Tordoir et al. 1993; Wiseman et al. 1989; Woodburn et al. 1996).* Apart from different study designs, these discrepancies may have been due to case mixing of cohorts of grafts and patients. Thus often in practice published data cannot be appropriately compared. The implementation of postoperative graft surveillance has added a new dimension to infrainguinal surgery. However the role of clinical risk factors in these grafts has not been analysed.

In view of the foregoing, the aim of this study was to examine the association between risk factors and long-term outcomes in a consecutive series of infrainguinal vein grafts that have been performed in the era of graft surveillance. The presence or absence of such relationships could influence modifications in future practice.

4.2. PATIENTS AND METHODS

Between 1988 and 1994 the vascular unit performed 299 consecutive infrainguinal vein graft reconstructions in 275 patients. It was the policy of the unit at that time to use prosthetic grafts for above knee popliteal bypasses. Hence 275 (92%) grafts were for infrageniculate procedures whilst only 24 (8%) were supragenicular. All patients undergoing infrainguinal revascularisation were managed according to a standard protocol. The indication for surgery was Critical Limb ischaemia (CLI) in 258 (87%) of cases and claudication in 40 (13%). All the patients who had CLI fulfilled the European Consensus Document Criteria *(European Working Group On Critical Limb Ischaemia 1991).* All patients underwent detailed preoperative angiography. These studies were used to assess the patency of distal vessels and to determine the number of run of vessels. The vein of first choice was the long saphenous and this was assessed and marked preoperatively using a duplex scanner (Diasonics sonstron, Bedford, UK). If the vein was insufficient or inadequate for reconstruction as a result of calibre (<3mm), previous surgery, or varicosities, then suitable arm veins were marked for use. Both the in situ and the reversed techniques were used. To confirm the suitability of the vessel on to which to perform the distal anastomoses, an intra operative pre-construction angiogram was performed. Intraoperative completion studies consisted of completion arteriography. Postoperatively all the vein grafts underwent long-term clinical and duplex graft surveillance which commenced at the first postoperative month and was then performed at 3 monthly intervals for the first year and 6 monthly thereafter. At each examination, the entire graft as well as inflow and outflow vessels were examined. The vessels were insonated and the peak velocity ratio across a suspected stenoses was calculated. Later in the study a colour duplex scanner (Diasonics ultra mark 9 HDI, Letchworth UK.) enabled rapid examination and detection of stenosed areas. A peak velocity ratio of ≥3.0 was defined as a significant stenoses. Such lesions were corrected by percutaneous transluminal angioplasty.

The data regarding these patients was retrieved from the units database. The variables examined were gender, presence of diabetes, hypertension, ischaemic heart disease, presence of critical ischaemia, level of distal anastomosis, number of run off vessels, use of postoperative warfarin and or anti platelet therapy, technique of vein grafting and early

postoperative (within 30 days) graft thrombosis. Smoking was not analysed in this study because though the history of postoperative smoking was obtained subjectively by directly questioning, this method is unreliable. Thus Wiseman et al. found that about 25% of patients will be untruthful about having stopped smoking *(Wiseman et al. 1989)*. Postoperative warfarin had been prescribed for patients whose grafts had required perioperative thrombectomy and patients were prescribed aspirin as prophylaxis against cerebrovascular or cardiovascular thrombosis. The status of the distal run of vessels was recorded as the number of patent vessels determined by the pre and intraoperative assessments outlined above. Graft patency was determined by duplex examinations during surveillance follow-up.

Data analysis

A database was created using the SPSS for Windows statistical computer program (SPSS, Chicago, Illinois, USA). The details of some patients were incomplete and the numbers analysed for each parameter is indicated in Table 2. Twenty-one patients were lost to follow up, and their details were censored to the last surveillance visit. The 6 year primary, primary assisted and secondary patency rates of the series was determined and reported by constructing a Kaplan Meier life table *(Kaplan and Meier 1958)* as recommended by the ad hoc committee of the society of vascular surgery *(Rutherford 1991)*. Primary patency is defined as uninterrupted patency not requiring additional procedures. Primary assisted patency refers to grafts that have required a procedure in prevent thrombosis. Secondary patency is when patency has been restored with flow in most of the original graft and at least one anastomoses by an additional procedure *(Rutherford 1991)*. Limb salvage refers to the avoidance of major amputation in patients with CLI. The Mantel-Haenszel log rank test *(Mantel and Haenszel 1959)* was used to perform a univariate analysis of the effect of the factors outlined above on outcome. Primary patency was analysed because it examined the factors that influence the patency of the graft without intervention. It is largely accepted that graft failure after 30 days post implantation is as a result stenotic lesions resulting from intimal hyperplasia. With this presumption, all grafts that failed as a primary event after 30 days were subjected to a multifactorial sub-analysis. Secondary patency was analysed in all the grafts because it examined the factors that affected

the patency of the graft taking into account the intervention that may have been necessary to maintain patency. Limb salvage and patient survival were also analysed. Factors that had a significant effect ($p < 0.05$) were entered into a Cox multivariate model *(Cox 1972)* .

4.3 Result

There were 159 (53%) in situ grafts, 115 (38%) reversed grafts and 25 (8%) were arm/saphenous vein composite grafts. The median (range) age of the patients was 71 years (19 to 97). In male patients the median (range) age was 70 (32-97) and in females it was 75 (32-94). The minimum period of follow up of the patients that were alive with patent grafts was 19 months. The 30 day operative mortality was 7.7%. Fifty-five (18%) grafts occluded within the first 30 postoperative days, of which 20 occurred within 24 hours. The 6 year primary, primary assisted and secondary patency rates for all the grafts was 23%, 47% and 57% respectively (Figure.4.1). The 6 year cumulative patient survival and limb salvage was 45% and 68% respectively (Figure.4.2). The incidence of the variables analysed is listed in Table 4.1. The results of univariate and multivariate analysis of the factors affecting patency, limb salvage and patient survival rates are shown in Table 4.3.and 4.4 respectively. There was no difference in results when either forward stepwise entry or backward stepwise elimination methods were used in the Cox model. Primary patency was adversely influenced by the use of composite vein grafts ($p=0.05$). As this was the only significant factor, this outcome was not analysed further in the Cox multivariate model. However, in the univariate analysis, none of the factors was found to have a significant influence on the primary patency of grafts that failed after 30 days. The result of this is shown in Table 4.2. In the univariate analysis, secondary patency was significantly reduced by the presence of critical ischaemia, in composite vein grafts, in patients prescribed postoperative warfarin and in grafts that had required thrombectomy or other additional procedures within 30 days of surgery. The use of aspirin was associated with a significant improvement in secondary patency ($p=0.04$). When these factors were analysed in the multivariate model, postoperative thrombectomy was the only adverse factor affecting secondary patency. Limb salvage was adversely influenced by the presence of diabetes, female sex, poor run off, graft thrombectomy and in composite vein

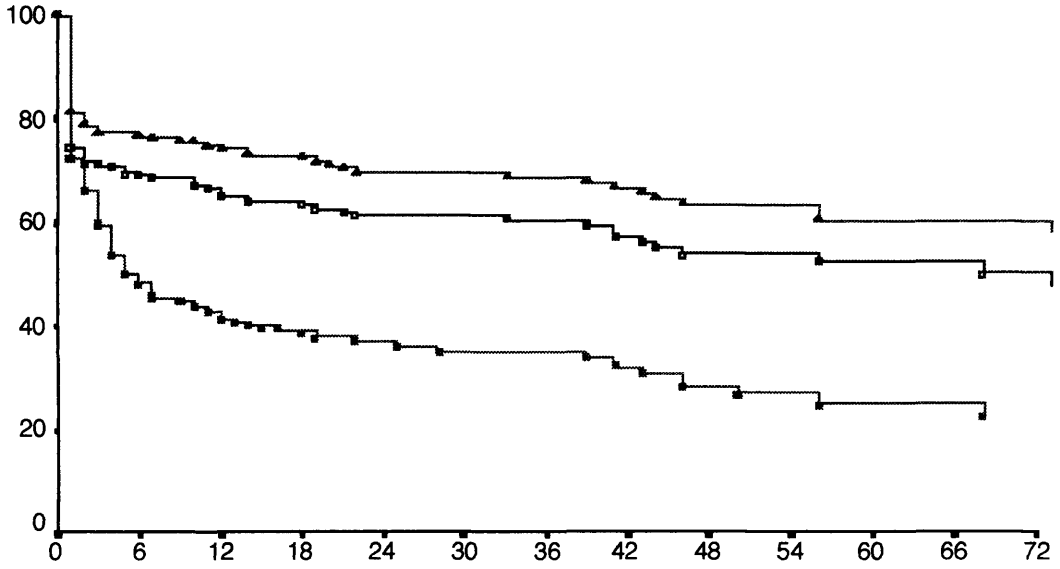
grafts. In the multivariate analysis model, diabetes, female sex and poor run off were independent factors adversely influencing this outcome (Table 4.4).

Diabetes, female sex, and CLI adversely influenced patient survival in the univariate analysis. However, patients on aspirin had a significantly better long-term survival (p= 0.03). In the multivariate analysis, all of these factors except for diabetes remained significant (Table 4.4).

Table 4.1. Prevalence of factors in 299 vein grafts.

Factor	Number(%)
Gender	
Males	204 (68)
Females	95 (32)
Diabetes	
Yes	91 (31)
No	207 (69)
	1 NR
Hypertension	
Yes	112 (38)
No	185 (62)
	2 NR
Ischaemic heart disease	
Yes	76 (26)
No	220 (74)
Aspirin	
Yes	141 (54)
No	122 (46)
	36 NR
Warfarin	
Yes	170 (64)
No	94 (36)
	35 NR
Type of graft	
In situ	159 (53)
Reversed	115 (38)
Composite	25 (8)
Indication	
CLI	258 (87)
Claudication	40 (13)
	1 NR
Run off vessels	
1 or less	158 (53)
2 or more	141 (47)
Distal anastomoses	
Popliteal artery	104 (35)
Tibial and distal arteries	193 (65)
	2 NR
Graft thrombectomy or additional procedures	
Yes	55 (18)
No	244 (82)

NR, Status of factor not recorded.



Grafts at risk

Primary	299	122	92	72	59	40	18	12	6
Assisted	299	168	145	117	96	70	39	28	19
Secondary	299	189	165	138	115	81	50	35	25

Figure 4.1. 6 year cumulative patency. Primary patency ■ , Primary assisted □, Secondary patency ▲.

Number at risk

Limbs with CLI	258	161	138	114	64	39	25	18
Patients	275	204	179	150	92	59	42	29

Figure.4.2. 6 Year Cumulative Limb salvage ■ and Patient survival □.

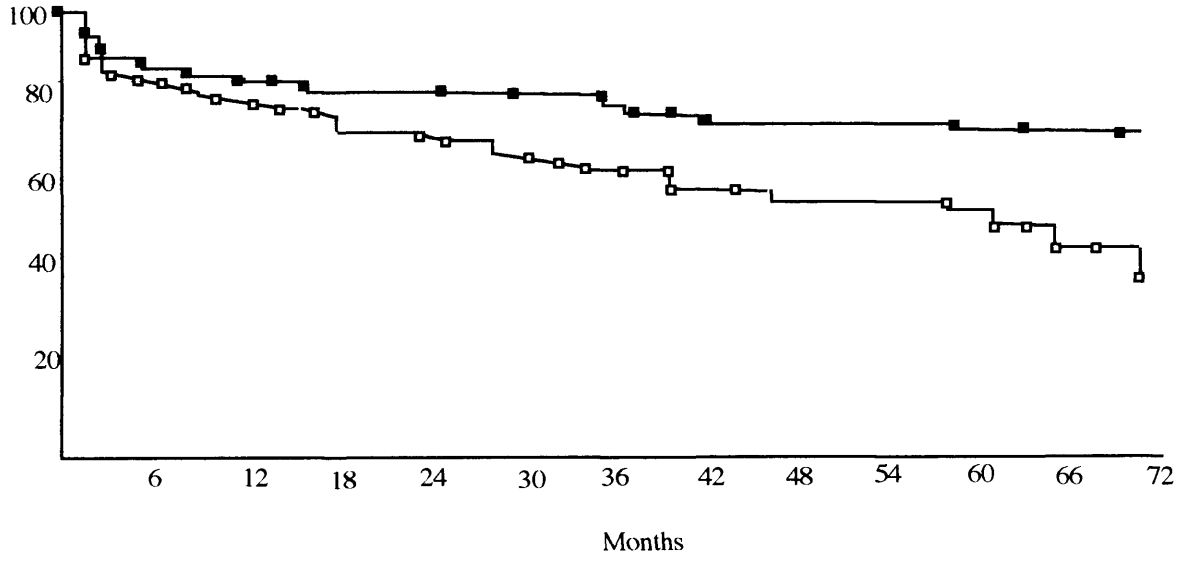


Table 4.2. Univariate analysis of factors affecting primary patency of grafts after 30 postoperative days

Factor	6 year primary patency (%) (excluding graft failing within 30 day)	p value
Gender		
Male	29	0.7
Female	21	
Diabetes		
Yes	27	0.8
No	35	
Hypertension		
Yes	22	0.2
No	45	
Aspirin		
Yes	24	0.5
No	27	
Indication		
CLI	16	0.9
Claudication	36	
Run off		
<I vessel	21	0.4
>1 vessel	32	
Distal anastomoses		0.4
Popliteal	31	
Tibioperoneal	28	
Warfarin		0.4
Yes	23	
No	29	
Type of graft		
Insitu	30	0.5
Reversed	35	
Composite	26	
IHD		0.6
Yes	25	
No	39	

Table 4.3. Log Rank univariate analysis of factors affecting patency, limb salvage and patient survival at 6 years

Factor	Primary Patency%* (P value)	Secondary Patency%* (p value)	Limb Salvage% (p value)	Patient Survival%* (p value)
Gender				
Male	24	60	81	53
Female	26	56	54	37
	(0.7)	(0.1)	**(0.006)**	**(0.02)**
Diabetes				
Present	25	60	59	37
Absent	23	58	80	55
	(0.08)	(0.2)	**(0.0005)**	**(0.01)**
Hypertension				
Present	36	73	75	48
Absent	18	52	73	50
	(0.3)	(0.2)	(0.5)	(0.9)
Ischaemic heart disease				
Present	30			
Absent	20	65	76	50
	(0.5)	55	66	38
		(0.3)	(0.4)	(0.05
Aspirin				
Yes	25	67	75	59
No	18	51	74	47
	(0.3)	**(0.04)**	(0.2)	**(0.03)**
Indication				
CLI	29	58		44
None CLI	15	70		72
	(0.2)	**(0.01)**		**(0.0002)**
Run off vessels				
≤ 1	17	55	59	36
>1	26	62	84	57
	(0.6)	(0.2)	**(0.03)**	(0.06)
Level of Distal Anastomoses				
popliteal/				
Tibio peroneal	24	62	84	60
	25	58	67	41
	(0.2)	(0.06)	(0.08)	(0.08)
Postoperative warfarin				
Yes	19	52	65	43
No	26	69	84	53
	(0.1)	**(0.02)**	(0.6)	(0.5)
Graft type				
Insitu	29	65	82	
reversed	26	63	73	
composite	0	24	35	
	(0.07)	**(0.03)**	(0.1)	
Composite v others**	**(0.05)**	**(0.02)**	**(0.02)**	
Early graft thrombectomy				
Yes				
No		8	0	
		62	67	
		(0.00)	**(0.00)**	

*Cumulative percentages. **Comparison of outcomes of composite grafts to reversed and insitu grafts. CLI, Critical limb ischaemia. Significant analysis with $P < 0.05$ are in bold type.

Table 4.4. Multivariate analysis of significant factors affecting outcome.

Outcome	Factor	Odds ratio	95% CI*	*p* value
Secondary Patency	Thrombectomy	0.2	0.09/0.29	<0.001
Limb Salvage	Diabetes	0.5	0.26/0.82	0.008
	Run of vessels ≤1 vs > 1	0.6	0.42/0.99	0.047
	Thrombectomy	0.2	0.12/0.47	<0.001
Patient Survival	Gender (M/F)	1.7	1.04/2.77	0.020
	Use of Aspirin	1.8	1.12/2.77	0.001
	CLI	0.3	0.15/0.72	0.006

Multivariate analysis performed using the backward stepwise entry method. *95% Confidence interval (CI).

4.4. Discussion

The outcome of lower limb revascularisation has improved with the use of autologous vein grafts, meticulous surgical techniques and postoperative graft surveillance *(Bergamini et al. 1995; Dunlop et al. 1995a; Idu et al. 1993; Londrey et al. 1991; Lundell et al. 1995; Michaels 1989; Veith et al. 1986).* The question remains as whether other factors can be modulated in order to improve outcome even further. The results from this study suggest that risk factor modulation would not have improved long-term primary patency and that only the use of composite vein grafts made a significant difference on this outcome. This is in keeping with the findings from other large studies*(Bergamini et al. 1991; Donaldson et al. 1992).* Composite veins are only used when there are inadequate lengths of good quality long saphenous veins. Thus, the patency of composite or alternate vein grafts can be expected to be inferior to that of long saphenous vein grafts*(Bergamini et al. 1991; Kent et al. 1989; Myers et al. 1993; Taylor et al. 1990a).*

None of the factors examined influenced the patency of grafts that failed after 30 days. The majority of these failures would be as a result of intimal hyperplasia related stenosis

though some late failures may be attributable to progressive atherosclerosis in the inflow and outflow vessels.

The only factor that influenced long-term secondary patency was seen in grafts that underwent either early graft thrombectomy. This is consistent with reports from other modern series *(Nielsen et al. 1997; Robinson et al. 1997).* Neilsen and colleagues found that vein grafts that required thrombectomy within 30 days of surgery were associated with a two fold risk of developing stenoses as well as reduced secondary patency rates *(Nielsen et al. 1997).*

Early graft thrombosis is often due to technical imperfections. However, when Donaldson and colleagues analysed primary graft failure in 455 in situ grafts, they found that a variety of technical and patient specific reasons accounted for the early failures in their series*(Donaldson et al. 1992).* They suggested that a more conservative patient selection may have improved their results. However, the benefits of an aggressive and none selective approach to lower limb revascularisation has been demonstrated in our unit *(Sayers et al. 1993b)* as well as by others *(Hickey et al. 1991; Ouriel et al. 1988)* and this policy may have contributed to the relatively high early failure rate seen in this study.

Prior to the advent of postoperative surveillance, poor run off vessels were reported to significantly influence vein graft patency *(Cutler et al. 1976; Grimley et al. 1979; Miller 1974; Naji et al. 1978; Sonnenfeld and Cronestrand 1980).* Some of these reports were from studies in which most of the vein grafts were inserted for intermittent claudication *(Cutler et al. 1976; Grimley et al. 1979; Miller 1974; Sonnenfeld and Cronestrand 1980).* However, in a previous study from our unit in which the majority of patients had CLI *(Budd et al. 1990),* poor run off was also found to be a significant adverse factor. The lack of influence of run off on long-term secondary patency in the present study may reflect the ability of the surveillance and intervention program to identify and treat graft threatening run-off disease which develops due to the progression of underlying atherosclerosis. None of the co-morbid factors played a significant role in either primary or secondary graft patency. This is in keeping with reports from most modern series *(Bergamini et al. 1991; Myers et al. 1993; Plecha et al. 1993; Tordoir et al. 1993).* However, in the past, there have been many conflicting reports concerning the influence of some of these factors on graft patency*(Cutler et al. 1976; Deweese and ROB 1971;*

Rutherford et al. 1988; Shah et al. 1988). The reason why these factors no longer appear to affect graft patency in more recent reports is not clear. Postoperative surveillance and the ability of currently available drugs to effectively control diseases such as hypertension and diabetes are plausible considerations.

Limb salvage is an important outcome of any revascularisation procedure. It is recognised that a patent graft does not necessarily guarantee limb preservation. Most authors have stated that graft surveillance has resulted in improved limb salvage rates *(Bergamini et al. 1995; Idu et al. 1993; Moody et al. 1990; Sayers et al. 1993b).* However these claims are based on studies that have compared historical data. On the other hand, there are very limited randomised trials on graft surveillance, and the most often quoted randomised study by Lundell et al. did not state the impact of graft surveillance on limb salvage*(Lundell et al. 1995).* The graft surveillance meta-analysis by Golledge et al. concluded that graft surveillance did not improve limb salvage*(Golledge et al. 1996).* In the present study it was found that poor distal run off, presence of diabetes and grafts that required postoperative thrombectomy had significantly worse limb salvage rates. Poor run off in the native vessels would be expected to have an adverse effect on amputation rates. From this study, intense postoperative surveillance and an aggressive intervention policy for both graft and native vessels stenosis has not reduced this effect. The influence of diabetes on limb salvage has been controversial *(Bergamini et al. 1991; Budd et al. 1990; Shah et al. 1988; Taylor et al. 1990a).* In this study, the incidence of limb loss was significantly higher in grafts from diabetic patients even though they represented only 31% of all grafts, though our findings are in keeping with other recent studies *(Luther and Lepantalo 1997; Tordoir et al. 1993).* Thus we can conclude that despite the improvements attainable with graft surveillance in terms of graft patency and limb salvage, a combination of diabetes and poor run off is still associated with poor limb salvage rates. This is not surprising because with the increased tendency of non healing ulcers and gangrene, diabetic patients tend to undergo more amputations in spite of patent grafts. In a recent study of 209 lower limb reconstructions of which 187 were autogenous vein, Luther et al. also found that diabetes had a similar poor influence on limb salvage *(Luther and Lepantalo 1997).* In their analysis they

found that this effect was due to the adverse outcome seen in their population of female patients, though the 46% incidence of female patients in that study was notably higher than most published consecutive series *(Bergamini et al. 1991; Budd et al. 1990; Harris et al. 1993a; Myers et al. 1993; Tordoir et al. 1993).* In our multivariate analysis, female sex was not a significant factor and even though other studies have suggested that diabetic women with critical limb ischaemia tend to have lower graft patency rates than males*(Enzler et al. 1996; Magnant et al. 1993)*, it would seem that there is no gender difference in limb salvage rates *(Harris et al. 1993a; Magnant et al. 1993).* The influence of gender in all these studies is largely academic because it cannot be manipulated.

Clearly more studies are required to clarify the impact of graft surveillance on diabetic patients. Early graft thrombectomy and re-operation has been noted to adversely influence patency rates in this study. Thus a lower limb salvage rate compared to other grafts is expected in this group as the chances of successful revascularisation are low*(Robinson et al. 1997).*

The 6 year patient survival of 45% is in line with other recent reports *(Bergamini et al. 1991; Donaldson et al. 1992; Kalman and Johnston 1997; Robinson et al. 1997; Taylor et al. 1990a).* Graft surveillance is not expected to influence patient survival. The long-term survival of these patients is related to the extent of atherosclerosis in the coronary and carotid vessels rather than graft patency. Long-term survival was better in males, patients without critical ischaemia and in those who took aspirin postoperatively. In a similar analysis by Kalman and Johnston, male gender, diabetes, cerebrovascular disease and chronic renal failure were associated with poor long-term survival *(Kalman and Johnston 1997).* The presence of critical limb ischaemia tends to be associated with a high mortality rate from myocardial infarctions and cerebrovascular events *(Hoofwijk 1991; Wolfe 1986).* Thus the reduced survival of patients with critical ischaemia in this study supports these observations. Men tended to do better than women. The younger median age of the males in this study (70 for males V 75 for females) may explain why they survived longer than the females. Patients on aspirin had a significantly better chance of survival. This is in keeping with the findings of the Anti-platelet trialists *(Anti-platelet trialist 1994a).* Thus aspirin is beneficial in preventing cardiovascular morbidity and mortality and should be prescribed to patients after revascularisation. This is

despite the fact that aspirin has not been shown to improve graft patency in randomised studies *(Anti-platelet trialist 1994b; McCollum et al. 1991).*

In conclusion, this study has analysed the factors that may influence outcome in a series of vein graft bypass that have been followed up using a duplex based graft surveillance program. The only significant factors that influenced long-term patency were the use of composite vein grafts and graft early thrombectomy influencing primary and secondary patency respectively. No co-morbid factors were significant. However, the usage of aspirin postoperatively could significantly increase overall patient survival.

CHAPTER 5

PROSPECTIVE STUDIES ON GRAFT SURVEILLANCE

5a The Predictive Value Of Pre-Discharge Duplex Scans

5a.1 Introduction

5a.2 Patients And Materials

5a.3 Results

5a.4 Discussion

5b *Determination Of The Optimal Peak Velocity Ratio At Which To Correct Duplex-Detected Vein Graft Stenoses*

5b.1 Introduction

5b.2 Patients and Methods

5b.3 Results

5b.4 Discussion

INTRODUCTION TO CHAPTER 5

Though graft surveillance has no influence on the incidence of graft stenoses, it allows the early detection and correction of lesions which in turn reduces the chances of graft failure. However, there are issues in vein graft surveillance that remain controversial and has resulted in variations in the way it is practised. The question as to when to start surveillance, the frequency of examinations and when to stop surveillance has not been resolved. Furthermore, though most centres now agree that the duplex based method of detection is most suitable there is no agreement on which duplex criteria should be used to decide whether a flow abnormality requires correction.

This Chapter describes the results of studies looking at two of these issues. The first is a study of the advantages of starting surveillance prior to discharge from hospital. The second study has looked into the optimal duplex detected velocity ratio at which to correct detected flow abnormalities.

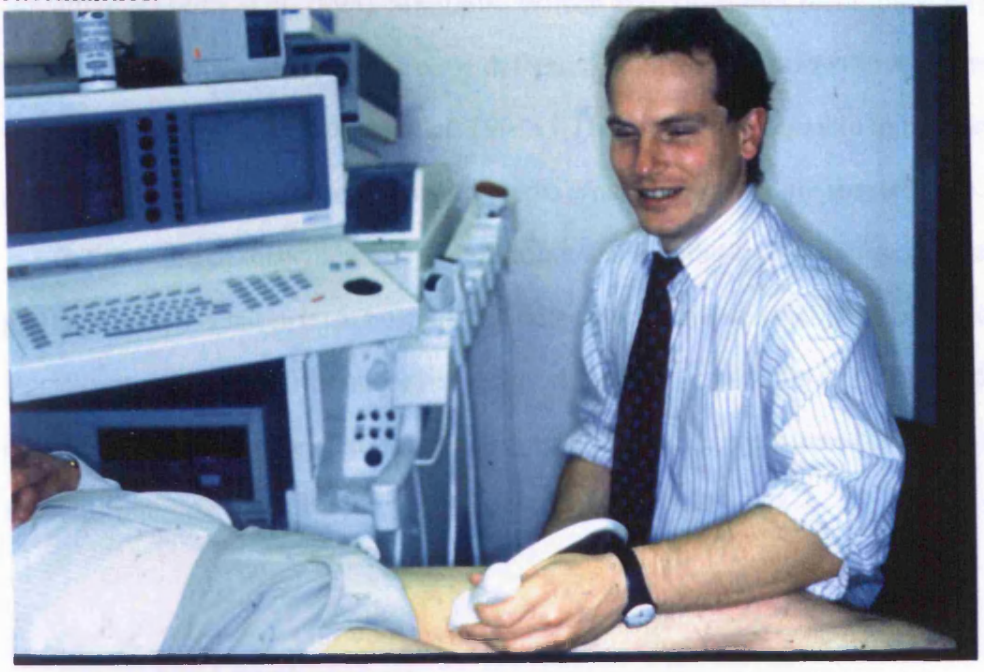


Figure 5a.1. Patient undergoing postoperative graft surveillance using the duplex scanner.

5a

THE PREDICTIVE VALUE OF PRE-DISCHARGE DUPLEX SCANS

5a.1. INTRODUCTION

Several reports have shown that postoperative duplex surveillance of infrainguinal vein grafts can improve long-term patency *(Bergamini et al. 1995; Idu et al. 1993; Lundell et al. 1995; Moody et al. 1990).* As a result, grafts surveillance programmes have been adopted by many vascular units. However, the implementation of these programmes increases the costs and workload required to maintain graft patency *(Loftus et al. 1998).* In theory, not all vein grafts require surveillance as only a proportion will actually fail, thus the early identification of high risk grafts could allow for the running of a selective and hence more cost effective postoperative surveillance programme. In a recent report, Mills et al. suggested that grafts with normal early duplex scans after the first 3 months would subsequently require less intense surveillance *(Mills et al. 1995).* Similarly, Wilson and colleagues recommended pre-discharge duplex scans as a modality for the early detection of intrinsic abnormalities that may develop into significant stenosis *(Wilson et al. 1995a).* Thus it may be possible to use the findings from earlier duplex scans to determine which grafts would require future surveillance.

The purpose of this study was to determine whether duplex scanning of infrainguinal vein grafts prior to discharge from hospital can detect abnormalities that would develop into significant stenoses. Furthermore in view of the report from Mills et al. this study would also examine the ability of the duplex scan performed at the 3rd postoperative month to predict subsequent requirement for long-term surveillance.

5a.2. PATIENTS AND MATERIALS

A prospective study was undertaken of consecutive infrainguinal vein grafts performed between August 1995 and April 1997. All patients who had undergone infrainguinal vein bypass were subjected to colour duplex scan prior to discharge from hospital. These scans were usually performed between the first and third postoperative week. For each pre-discharge scan, the original dressings were taken down and the wounds covered with opsite dressing. The patients were examined supine. Occasionally it was possible to scan around the wounds without disturbing the dressings. In all cases the probe was covered with a probe cover and sterile gel applied to the surface of the opsite. Scans were performed by experienced vascular technologists using one of two colour duplex scanners (Diasonics Masters, Diasonics Sonotron, Bedford UK. or ATL Ultra mark 9 HDI, ATL Letchworth UK). Hand held probes of either 5 or 10MHz were used.

The site and nature of all flow abnormalities (defined as a Peak Velocity Ratio between 1.5 - 2.9, or an area of turbulent blood flow) detected at this first scan were noted. The Peak Velocity Ratio (PVR) was calculated as the ratio of the peak systolic velocity within the stenosis and the peak systolic velocity in an adjacent segment of normal graft *(Sladen et al. 1989).*

Following discharge from hospital, these patients were entered into the routine postoperative surveillance program. This entailed a scan one month after surgery, at the end of the 3rd month, then every three months for the first year and six monthly thereafter. At each visit, the whole graft was scanned and any changes in previously noted lesions were recorded. Lesions were noted to regress, remain stable or to progress to require correction. It is the policy of the unit to correct all lesions with a PVR of 3.0 or above by angioplasty.

In order to determine the predictive value of the pre-discharge scan, the natural history of grafts with abnormal and normal pre-discharge scans was analysed at the end of the study. Similarly, the ability of the 3 month scan to predict subsequent stenosis was determined.

5a.3. RESULTS

Forty-four grafts were performed in 43 patients. They consisted of 21 in situ vein grafts, 17 reversed grafts and 6 composite vein grafts. However two in situ grafts occluded soon after the pre-discharge scan. One of these occluded because of run off disease and the other had required thrombectomy within 48 hours of surgery. Both of them had normal scans prior to discharge. The remaining 42 grafts had each undergone at least 2 postoperative surveillance scans. The minimum folow up period was 9 months, (range 9-29 months). Three deaths occurred during the study and one patient was lost to follow up.

Predictive value of the pre-discharge scan

Sixteen grafts were classed as abnormal based on the detection of lesions on the pre-discharge scan. These pre-discharge abnormalities consisted of lesions with a PVR of 1.5-3.0 in 13 grafts and areas of flow disturbances in 3 grafts. One of these grafts had a lesion with an initial PVR of 3.0 that required immediate correction by angioplasty. Four other abnormal grafts subsequently developed significant stenoses at a median (range) time of 4 (4-6) weeks after surgery. These stenoses had a median (range) PVR of 3 (3-5) and were all located at the site of the original abnormalities. Lesions that regressed either had no residual flow abnormality (n=9) or a persistent PVR < 2.0 (n=2).

Out of the 28 "normal" grafts, 11 developed significant stenotic abnormalities during post-discharge surveillance. Two further grafts were found to have occluded. These occurred at a median (range) of 15 (5-85) weeks after surgery and consisted of stenosis with PVRs of between 3.5 to 7.0. The outcome of the grafts is summarised in Figure 5a.2. The sensitivity and specificity of pre-discharge abnormalities to predict the development of future graft stenoses was 31% and 58% respectively. The positive predictive value and negative predictive value was 31% and 58% respectively. When the two occluded grafts were included in the analysis, the sensitivity and negative predictive values reduced to 28% and 54% respectively.

Predictive value of the 3 month duplex scan

At the end of 3 months 31 out of the original 44 grafts had remained patent and not required angioplasty (Figure 5a.3). The 3 month duplex scan detected flow abnormalities with PVRs of between 1.5-2.5 in 6 of these grafts, 2 of which then developed stenoses with a PVR of 4.0 and 7.0. respectively. The flow abnormalities in the other 4 abnormal grafts resolved completey.

The 3 month duplex scan found no flow abnormalities in 25 of the 31 grafts. Three of these normal grafts subsequently developed significant stenoses at 4, 5 and 28 postoperative months respectively and underwent angioplasty. The sensitivity and specificity of the 3 month duplex scan to to predict future graft stenoses was 40% and 85% respectively. The positive predictive value and negative predictive value was 33% and 88% respectively.

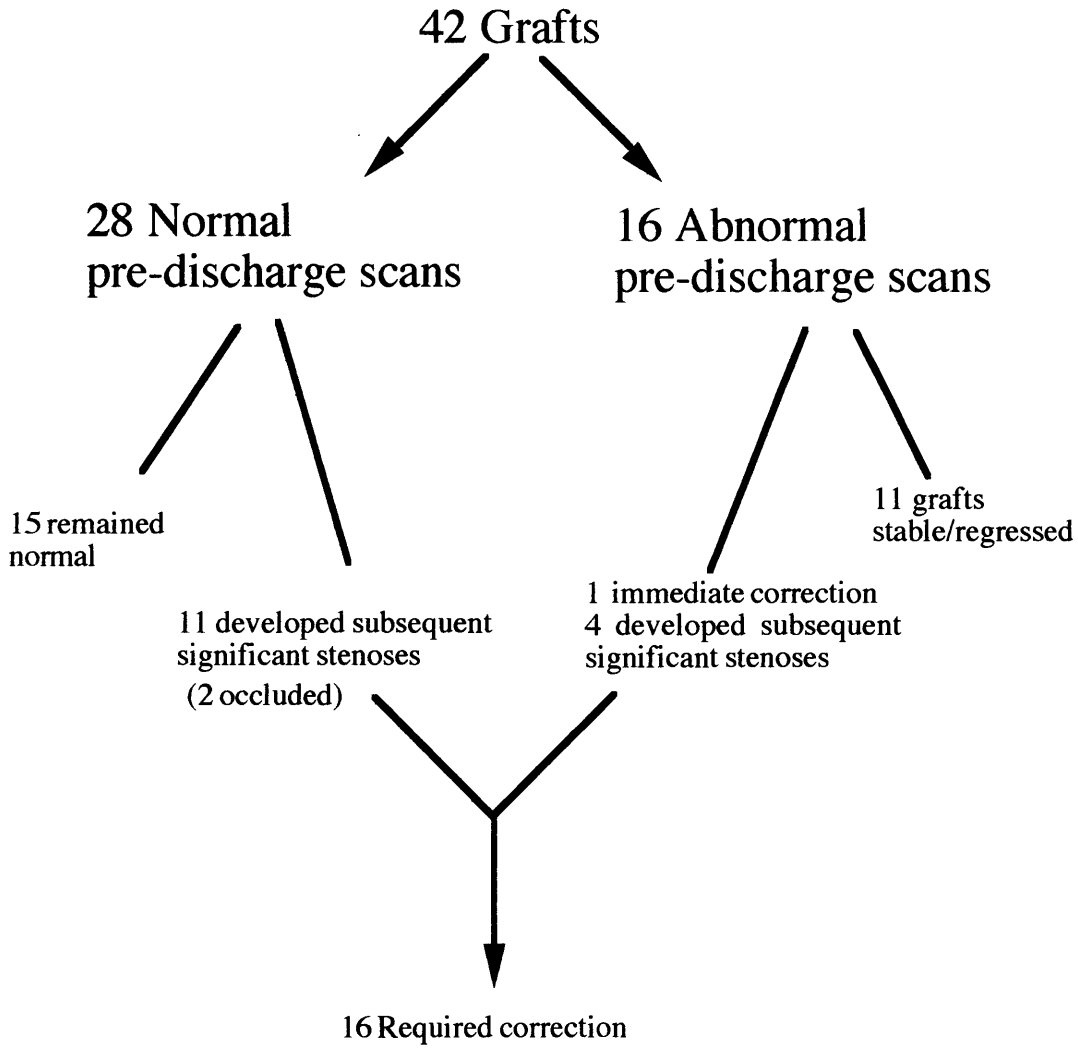


Figure 5a.2. Outcome of normal and abnormal grafts after pre-discharge scans

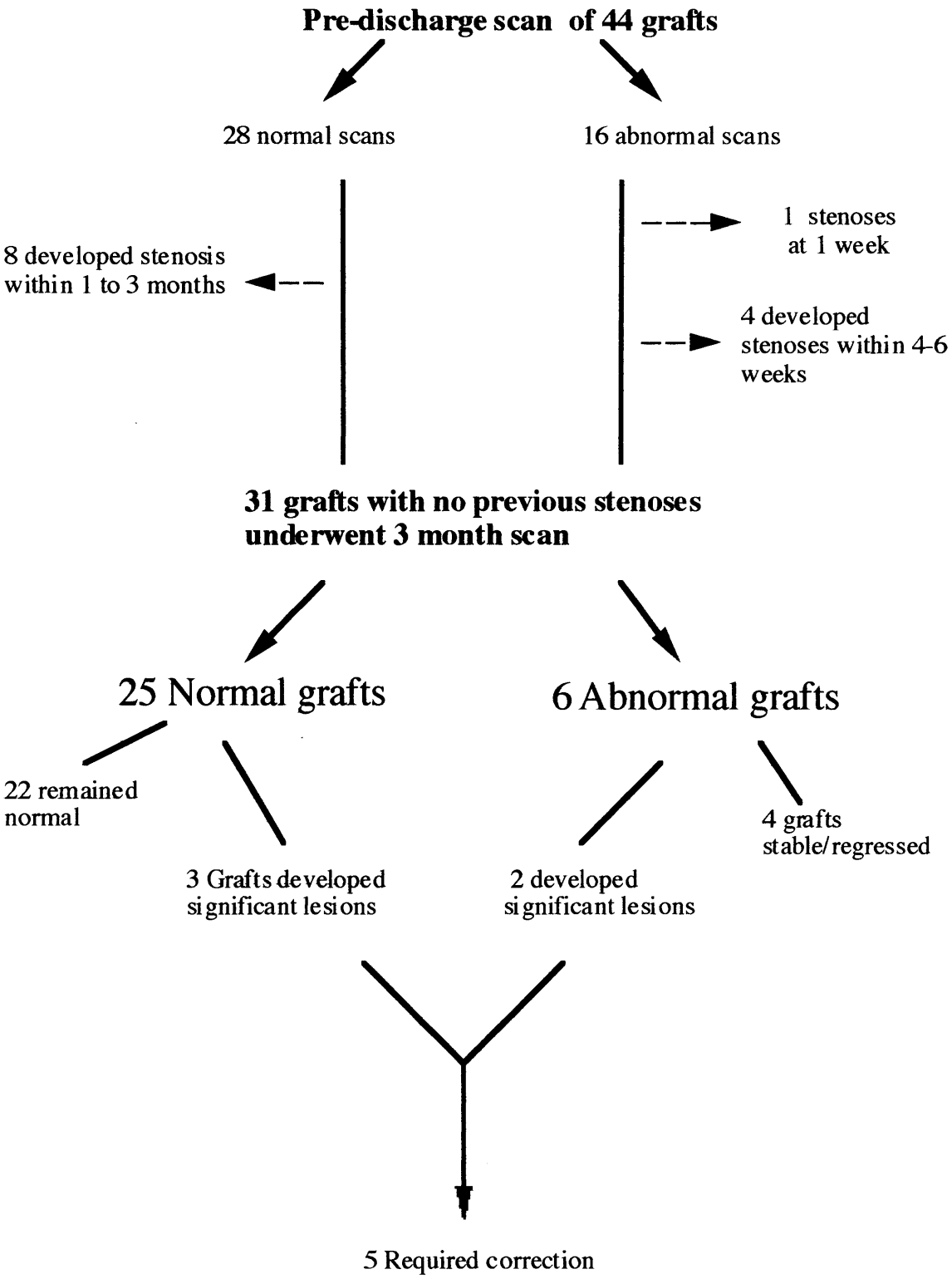


Figure 5a.3. Outcome of grafts found to be normal or abnormal after 3 month duplex scan.

5a.4. DISCUSSION

Though duplex based graft surveillance is widely practised, it is still very much in an evolutionary phase and a great deal of controversy and variation exists in the current practice of graft surveillance. Whilst some authors have addressed the limitations and cost effectiveness of these programmes *(Beattie et al. 1997; Idu and Buth 1997)* others have examined ways of improving its practice *(Bandyk et al. 1994; Mohan et al. 1995; Passman et al. 1995; Westerband et al. 1997a).*

Recent studies have suggested that stenoses develop from intrinsic graft abnormalities and that such abnormalities can be detected within the first few postoperative weeks *(Mills et al. 1995; Wilson et al. 1995a).* Hence, some units prefer to scan their grafts just before discharge from hospital as part of the routine graft surveillance. This study has looked at the usefulness of pre-discharge duplex scans and found that only five of the 16 grafts with pre-discharge abnormalities developed stenoses, whereas 11 of the 26 grafts that were normal at discharge subsequently developed stenosis. Thus from the data, it is clear that though pre-discharge scans can identify a number of grafts with early abnormalities, the findings of these scans is of low predictive value in the context of future stenosis. Other studies found that only 14% of grafts that would eventually develop significant stenoses can be identified within two weeks of surgery *(Passman et al. 1995).*

So does the pre-discharge scan have a role in postoperative graft surveillance? Wilson and colleagues have recently published an audit of their experience with pre-discharge scans following 123 grafts *(Wilson et al. 1995a).* In that study, they found that 30% of grafts with abnormal 1 week scans developed definitive stenoses. However the data on the outcome of grafts with normal pre-discharge scans was not presented and hence the overall value of these scans in their graft surveillance practice was not made clear.

In another study, Mills et al. used the outcome of serial duplex scans performed during the first 3 postoperative months to predict grafts that would develop future stenosis *(Mills et al. 1995).* In that study, grafts were first scanned intraoperatively or prior to discharge and the 3 month watershed effectively increased the predictive value of early duplex scans. Hence the authors found that out of 91 grafts that had been normal through 3 months only 5 subsequently

developed unexpected stenoses. This outcome is not surprising because it is known from previous studies that more than 60% of graft stenoses develop within 3 months of surgery *(Passman et al. 1995)*. Thus a 3 month scan is not truly being used to 'predict' stenosis development because the majority of stenoses would have already developed by this time. For comparative purposes the data in the present was re-analysed using similar criteria and found that (69%) of significant stenoses had developed within the first 3 months (Figure 2). However, even though the 3 month duplex scan had a better predictive value than the pre-discharge scan, it had a low sensitivity. In the first year of routine surveillance in this study, 210 post discharge scans were performed in 42 grafts. If however, after 3 months of surveillance the unit continued to only scan the 11 previously stenosed grafts and the 6 grafts which had flow abnormalities at 3 months as recommended by Mills and colleagues *(Mills et al. 1995)*, then 135 scans would have been performed in the year. This policy would only have saved the cost of 75 scans, yet would have missed stenoses in 3 grafts.

To date there is no method of limiting the number of grafts undergoing postoperative surveillance. A better understanding of the natural history of graft stenoses is needed in this respect. The findings from the present study imply that vein graft stenoses do not develop from intrinsic areas of abnormalities as previously suggested and grafts that are going to develop stenosis in the long-term cannot be easily predicted. The results show that pre-discharge duplex scans cannot be used to group grafts into high and low risk categories for the purposes of future surveillance.

DETERMINATION OF THE OPTIMAL PEAK VELOCITY RATIO AT WICH TO CORRECT DUPLEX-DETECTED VEIN GRAFT STENOSES

5b.1. INTRODUCTION

Postoperative infrainguinal vein graft surveillance using colour duplex is widely practised *(Davies et al. 1994; Green et al. 1990; Idu et al. 1993; Laborde et al. 1992).* Over the years, several parameters have been derived from duplex examinations to estimate the degree of stenosis. Jäger et al. *(Jager et al. 1985)* first demonstrated that the relative increase of velocity across a stenosis could be used as an indicator of disease severity in native vessels and since then, the peak velocity ratio (PVR) has been used to grade stenoses that develop in vein grafts *(Bandyk 1990; Caps et al. 1995; Grigg et al. 1988a).* The threshold value for correction of detected lesions varies from one centre to another. By clincal grading, a PVR of 2.0 corresponds approximately to a 50% or more reduction in vessel diameter (Table 5b.1) and many centres *(Mattos et al. 1993; Mills et al. 1990; Taylor et al. 1992; Wilson et al. 1996)* intervene at this point in order to prevent subsequent occlusion. However, other authors have suggested that intervention is necessary only for those lesions with a PVR above 3.0 *(Sladen et al. 1989)* or even 3.5 *(Bandyk 1993).* The aim of the present study was to determine whether the threshold for intervention could be safely raised from a PVR of 2.0 to 3.0 without increasing the incidence of graft thrombosis.

Table 5b.1. Clinical grading of detected stenoses

V R.	Reduction in diameter
< 2.0	0%-49%%
2.0 -3.0	50%-75%
> 3.0	> 75%

Lesions with a PVR of 3.0 or more underwent angiography and correction.

5b.2. PATIENTS AND METHODS

A prospective study was commenced recruiting from patients attending the vascular studies unit of the Leicester Royal Infirmary for infrainguinal vein graft surveillance. The protocol for postoperative infrainguinal vein graft surveillance in this centre is a colour duplex examination of the graft and its anastomoses at 1, 3, 6, 9, and 12 months after surgery. Thereafter the patients are scanned at 6 monthly intervals. The scans are performed by experienced vascular technicians using one of two colour duplex scanners. (Diasonics Masters, Diasonics sonotron, Bedford UK and ATL Ultramark 9 HDI, ATL Letchworth UK) at probe frequencies of either 5MHz or 10MHz. Detected stenoses are graded according to the calculated PVR. This is determined as the ratio of the peak velocity within the stenosis and the peak velocity of the adjacent segment of normal graft. Grafts were considered occluded if there was no colour on the duplex scan and no pulsatile flow on the pulsed doppler.

Prior to the start of this study the PVR threshold for intervention was ≥ 2.0. However, starting in August 1995 this threshold was raised to ≥ 3.0. For the purposes of this study, grafts that developed a primary stenosis with a PVR of 2.0 - 2.9 were scanned every month. If the stenosis progressed to a PVR of ≥ 3.0 the stenosis was corrected by angioplasty, if however the stenosis regressed or remained stable for 3 months the graft returned to the routine surveillance protocol. A primary lesion was defined as one that was detected in an area of a graft that had no previous abnormalities or endovascular intervention. At the end of the study the data relating to the outcome of these stenoses was analysed.

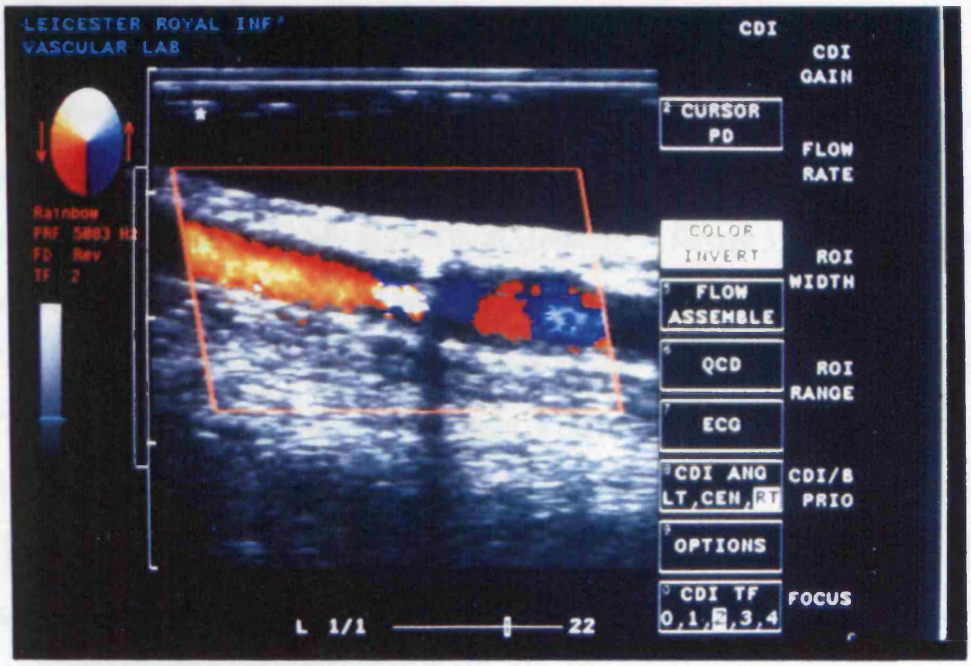


Figure 5b.1 Example of colour coded image of a 3.0 graft stenosis.

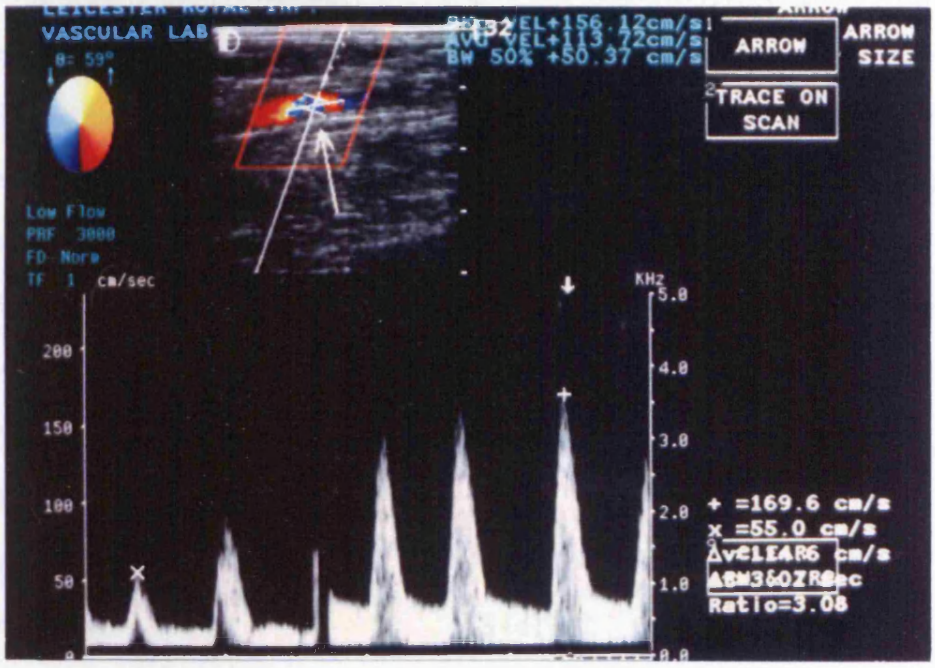


Figure 5b.2 Spectral analysis of the image in Fig.5b.1. The PVR is calculated as the ratio of the velocity in the stenosis (+) to the velocity in adjacent normal graft (x).

5b.3. RESULTS

Two-hundred and ten vein grafts underwent postoperative surveillance between August 1995 and April 1997. During this period 12 stenoses in 11 grafts were detected with an initial PVR ≥ 3.0 and were immediately treated by angioplasty. A further 32 grafts developed 38 primary stenotic lesions with a PVR between 2.0 and 2.9.

The distribution of the stenoses are displayed in table 5b.2 and figure 5b.1. Seventeen of the 32 grafts were in situ, 13 were reversed and two were composite. Twelve grafts were above knee, 20 were below knee. Thirty-two stenoses were located within the graft and 6 were at an anastomosis. Of the 38 stenoses with a PVR between 2.0 and 2.9, 16 (42%) regressed spontaneously, 11 (29%) remained stable and 11 (29%) progressed to a PVR of ≥ 3.0 and underwent angioplasty. There was no significant difference between the proportion of stenoses that progresessed when they were grouped according to location on graft, length of graft or type of graft, p = 0.06, .1 and 0.4 (chi-squared test) respectively. No grafts with a PVR between 2.0 and 2.9 occluded whilst they were being "observed".

The median (range) time taken to develop a stenosis with a PVR of 2.0 -2.9 was 12 (1-100) weeks after surgery. The time of onset of stenoses that progressed (n=11) was 8 (2-100) weeks compared to 18 (1-100) weeks for those that did not (n=27). This trend was not statistically significant (p = 0.46, Mann-Whitney U test). Stenoses that did progress did so at a median (range) time of 6 (4-36) weeks from the time at which they were detected.

Table 5b.2. Graft details — Number (%)

Graft type	
In situ	17 (53%)
Reversed	13 (41%)
Huv/composite	2 (6%)
Distal anastomsis	
Above Knee	12 (38%)
Below knee	20 (62%)

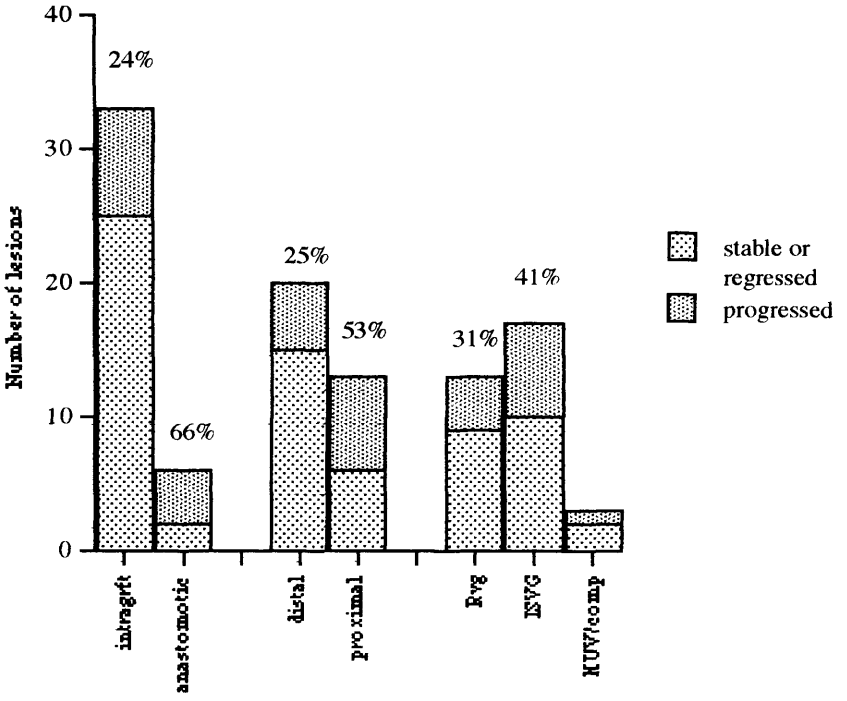


Figure 5b.1 Proportion of stenoses that progressed according to location of lesion, length and type of graft

5b.4. DISCUSSION

There are several duplex derived parameters that can be used to grade the severity of a stenosis. These include the peak mean velocity, the peak systolic velocity index, the end-diastolic velocity and the peak velocity ratio *(Buth et al. 1991; Buth and Idu 1993; Grigg et al. 1988b; Sladen et al. 1989).* In the present study we have used the PVR as the sole parameter to grade detected lesions because it has previously been shown to be highly sensitive for detecting lesions within vein grafts *(Taylor et al. 1992).* Many authors tend to repair all lesions with a PVR of 2.0 which corresponds approximately to a diameter reduction of 50%. One of the strongest arguments supporting the use of a PVR of 2.0 or more as a criteria to correct graft stenoses is evident in the study reported by Mattos et al. *(Mattos et al. 1993)* In their study of 110 vein graft stenoses, 33 grafts harbouring lesions with a PVR of 2.0 or above were not corrected. The three year patency rate in these grafts was 57%. This was significantly worse than the 3 year patency of 83% achieved by correcting lesions with a PVR of 2.0 or more in 24 other grafts. Thus they concluded that lesions with a PVR of 2.0 were at a significantly

increased risk of occlusion and that correction at this stage would significantly improve patency rates. There have been other studies supporting these findings *(Grigg et al. 1988b; Idu et al. 1993; Moody et al. 1990).* The problem with these studies however, is that they have not attempted to determine the natural history of these lesions by observing them until they developed a higher PVR before they were corrected.

In contrast, this study has found that it is safe to observe stenoses that develop in vein grafts if the PVR is between 2 and 2.9. Interestingly, other authors have recently come to similar conclusions. Idu et al. recently presented their findings in a similar prospective study *(Idu et al.1998)* . In their study, analysis of data from 300 patients showed that the PVR provided the best correlation with angiographic detected stenoses and that a threshold level of ≥3.0 was the optimal threshold for predicting grafts that would require revision. Caps et al. *(Caps et al. 1995)* using a cut off PVR of 3.5 reported no graft thromboses in lesions with a PVR of 2.5 or less. However, they experienced 3 thromboses in those grafts with a PVR between 2.5 and 3.5. This may be because of the slightly higher cut off point that they used. Furthermore those three grafts were associated with a significant reduction in the ABPI or return of symptoms.

Bandyk suggested that asymptomatic lesions with a normal flow velocity and an ankle brachial index of more than 0.9 should attain a PVR of 3.5 before correction *(Bandyk 1993).* Westerband et al. have recently completed a prospective study on 101 vein grafts designed to validate a threshold PVR of 3.5 as the criteria for intervention. In that study, Of 43 grafts with stenosis (PVR ≥ 1.5), 20 (46%) remained stable or spontaneously regressed and the remaining 23 (54%) progressed. However out of the 23 lesions that progressed, 3 occluded before intervention *(Westerband et al. 1997a).* The occurrence of 3 occlusions in that study suggests that a threshold PVR of 3.5 may be too high and a PVR of 3.0 may be more appropriate.

Part of the problem in graft surveillance and duplex scanning is the paucity of knowledge on the natural history of detected stenoses. There is a tendency to apply the same criteria used in native vessels to vein grafts *(Taylor et al. 1992).* However, the underlying cause of stenosis is different. The arteriosclerosis seen in the native vessels tends to be progressive whilst intimal hyperplasia has been known to spontaneously resolve *(Mills et al. 1995).* This fact is

supported by these results and there seems to be a consistent pattern emerging from the few studies on the natural history of vein graft stenosis. This study found that most lesions that progress did so within a relatively short period of time (median time of 6 weeks) which is consistent with findings from other studies *(Caps et al. 1995; Mills et al. 1995).*

This study has shown that if vein graft stenoses with a PVR of 2.0 - 2.9 remain stable during the course of 3 months, stenosis with a PVR < 3.0 can be treated expectantly. It can therefore be concluded that with this protocol the threshold PVR for correcting duplex deteced graft stenosis should be ≥ 3.0. This policy should markedly reduce the number of interventions without impairing graft patency.

In conjunction with the study from Chapter 5a, it would be reasonable to propose a modification of the current graft surveillance program. This would advocate commencing surveillance one month after graft implantation. and repeating the scans at 3 monthly intervals for the first year then 6 monthly thereafter. Significant stenosis with a PVR of 3.0 or more should be corrected immediately, however intermediate lesions with a PVR between 2 and 2.9 should be observed closely and scanned monthly for 3 months once detected.

CHAPTER 6

EFFECT OF ENDOTHELIN RECEPTOR BLOCKADE ON ENDOTHELIN INDUCED SMOOTH MUSCLE CELL PROLIFERATION

CHAPTER 6

EFFECT OF ENDOTHELIN RECEPTOR BLOCKADE ON ENDOTHELIN INDUCED SMOOTH MUSCLE CELL PROLIFERATION

6.1 INTRODUCTION

Smooth muscle proliferation and migration is central to the development of intimal hyperplasia. Endothelin (ET) has been shown to be mitogenic for vascular smooth muscle cells *(Bobik et al. 1990; Komuro et al. 1988; Masood et al. 1997)* and it has been shown that ET promotes intimal hyperplasia in animal angioplasty models *(Douglas and Ohlstein 1993; Trachtenberg et al. 1993)*. However it is not clear whether ET acts as a direct or indirect mitogen *(Rubanyi and Polokoff 1994)* and the role of the two ET receptors - ET_A and ET_B, in vascular smooth muscle cell mitogenesis has not yet been clarified.The situation is complicated by the fact that there are variations in endothelin receptor expression in cells from different tissues and different species *(Yanagisawa 1994)*. Thus, the results of studies demonstrating a mitogenic effect and role of specific receptors for ET mediated mitogenesis in rat aortic smooth muscle cells *(Eguchi et al. 1992; Ohlstein et al. 1992)* may not be applicable to human venous smooth muscle cells. Indeed, very little work has been done to establish the nature of endothelin mediated SMC mitogenesis in human saphenous vein. However, this vein is the conduit of choice for peripheral bypass procedures and is associated with a stenosis rate of about 35%.

Therefore the aim of this study was to establish the mitogenic effect of ET on human saphenous vein smooth muscle cells and to determine which receptor type mediates this effect.

6.2 MATERIALS AND METHODS

Method of Cell culture

Human smooth muscle cells were obtained using the explant technique based on the method described by Chamley-Campbell. *(Chamley-Campbell 979).* Ethical approval had been obtained to use segments of human long saphenous vein from patients undergoing either aortocoronary or infrainguinal bypass procedures. Segments taken at these operations were stored in sterile bottles containing calcium-free Krebs solution (Appendix 2) and were transported immediately to the laboratory in an ice container to keep the tissue cooled at 4°C. In the laboratory, each segment was transferred onto a sterile petri-dish within a laminar flow hood. The vein was first cleaned of excess fat and adventitial tissue and then cut open along its length. The exposed endothelial layer was then gently scraped of using the edge of a sterile blade. The vein was washed in minimal essential medium (MEM) (Northumbria Biologicals LTD, Cramlington, Northumberland, UK) and transferred to another sterile petri dish containing 1.8 mls of smooth muscle cell culture medium (Seralab, Crawley Down, UK) (appendix 2). Here the tissue was minced into $1mm^3$ explant pieces with a sterile blade. The explants were transferred to T25 culture flasks (Nunclon, Denmark) and placed in a tissue culture incubator (Queue systems, West Virginia, USA).

Occasionally, a large segment of vein was obtained and this was cultured in a T80 (Nunclon, Denmark) culture flask containing 7 mls of smooth muscle cell culture media. The incubator was maintained at a temperature of 37^0c with humidified air consisting of 95% air and 5% CO_2. The flasks were left for 7-10 days or until a colour change from red to yellow indicated a fall in pH. At this stage half the medium was replaced. Subsequently, medium was changed every 2-3 days.

By 2 weeks, smooth muscle cells were seen to be migrating from the explants and became confluent in 3- 5 weeks, figure 6.1 and 6.2. At this stage, the medium was removed and the cells were washed in MEM and then subcultured into fresh T25 or T80 flasks using the trypsinization technique (Appendix 2) Once these cells had

reached confluencey, they were trypsinized and counted for seeding into multiwell plates. All cells were used at passage 2.

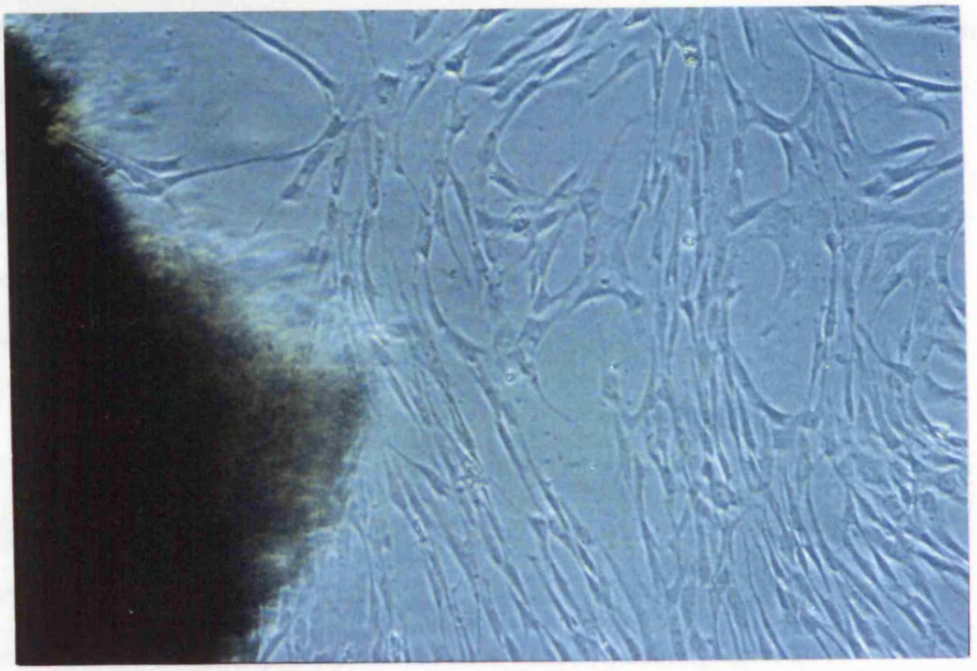


Figure 6.1. Early smooth muscle cell migration from vein explant. Magnification x 25.

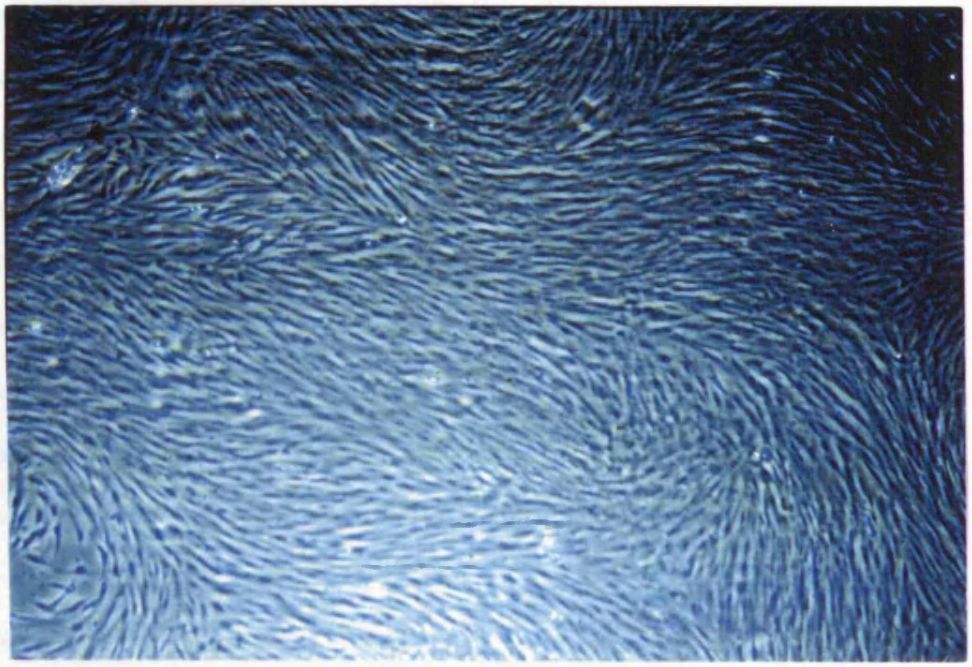


Figure 6.2. After about 3-5 weeks, the smooth muscle cells from the explants become confluent. Magnification x 25

Materials

Endothelin-1 (ET-1) peptide was obtained from Sigma Chemicals (Poole, UK) It was supplied as a lyophilised powder. This was reconstituted in 1mg/ml Bovine Serum Albumin (Sigma Chemicals, Poole, UK) in sterile distilled water to make an effective working concentration of 10nM. This working concentration was based on previous studies on the dose dependent response of SMC cells to ET-1 previously done in our department *(Masood et al. 1997)* and by others *(Ohlstein et al. 1992)*. Aliquots were prepared in Eppendorf vials and stored at -20^0c until required.

The non selective ET_A/ET_B receptor antagonist Bosentan was obtained as gift from Roche products limited. (Wewyn Garden City, UK.) It was in lyophilised form and dissolved in 10% Dimethyl sulphoxide (DMSO) (Sigma Chemicals, Poole, UK) to an effective concentration of 10μM. This was then stored in a refrigerator at 4^oc.

The ET_A receptor antagonist BQ123 and the ET_B receptor antagonist BQ788 was obtained from Calbiochem-Novabiochem Ltd (Nottingham, UK). Each of these antagonists was dissolved in 10% DMSO to achieve a concentration of 3μM. The working concentration BQ123 is higher than the that previously shown to inhibit ET-1 mediated [^{3}H] thymidine incorporation and proliferation in rat aortic SMC *(Kanse et al. 1995; Ohlstein et al. 1992)*. The concentration of BQ788 was based on the manufacturers recommendation

Once reconstituted in 10% DMSO, all the compounds were filter sterilised by passing them through a 0.22μM pore filter (Gelman Sciences Michigan, USA) that had been pre blocked with 1mg/ml albumin. This was to ensure sterility prior to storage or use.

Cell proliferation studies

Saphenous vein smooth muscle cells were seeded into three, 24 well-plates (Nunclon, Roskilde, Denmark) at a concentration of 1x 10^4 cells /ml/well and cultured in the incubator under the conditions specified above. Each experimental set consisted

of twelve wells and one set was used for each antagonist and each control (in duplicate for each time point). The cells were left overnight in 10% SMC media which was then replaced with 0.4% SMC media for 72 hours in order to growth arrest the cells and synchronise them in G_0 of the cell cycle. After this period, the growth arrest media in all the test wells was replaced with 1ml/well of 2.5% SMC media to which was added ET-1 with or without one of the three antagonists. Two sets of 12 wells containing SMC were used as controls. The negative control contained 1ml/well of 2.5% SMC media alone whilst 1ml/well of 15% SMC media was added to the positive controls.

At the end of each 48 hour period the SMC from two wells in each set was harvested and counted, whilst the media and additives in the remaining wells were replaced.

Method of cell harvesting and counting

The medium from the cells to be harvested was removed and then washed twice in MEM to remove any serum that may inhibit the activity of the trypsin. Two hundred microlitres of 0.1% trypsin (Gibco BRL, Paisley, Scotland) with 0.02% EDTA (Fisons, Loughborough, UK) (TE) was added to each well which were then replaced in the incubator for an average of 10 minutes to allow the SMC to detach from the bottom of the plate. Occasional gentle tapping was necessary to achieve this. Once all the cells were detached as visualised under the light microscope, the wells were washed out with 800μl of MEM containing 5% Foetal Calf Serum (FCS) in order to neutralise the activity of the trypsin and the cell suspension was aspirated into a 1ml Eppendorf. The eppendorfs were then centrifuged in a Microcentaur centrifuge (MSE, UK) at 13,000 RPM for 5 minutes. At the end of this, 900μl of the supernatant was aspirated and replaced with 100μl of 0.2% trypan blue (Sigma chemicals, Poole, Dorset UK). Viable cells excluded this stain. The pellet was then uniformly re-suspended by shaking on a vortex (Scientific Industries Inc, New York, USA). A 100μl aliquot of this was transferred onto the counting chamber of a Neuber Haemocytometer, figure 6.3 (Weber Scientific International Limited, Lancing England) which was then covered

with a glass cover slip and placed under a light microscope in order to count the viable cells.

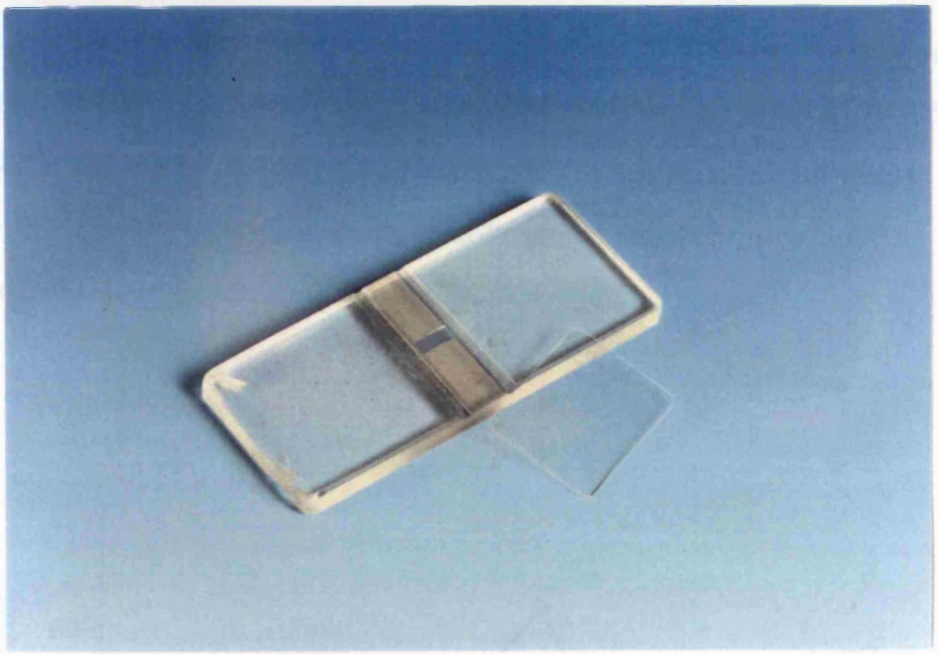


Figure 6.3 The Neuber Haemocytometer used to count the viable smooth muscle cells.

Each experiment lasted 12 days and was repeated with SMC cultured from 8 different patients. At the end of the study a growth curve could be constructed for each set. This was achieved by using the Apple Macintosh Sereplot software package (Scientific Visions, Silver Springs, Maryland USA) to measure the area under the growth curve based on the Simpson rule of least squares (Appendix 2). The growth of each set in an experiment was expressed as a ratio of the growth of the 2.5% control in that experiment. This served to standardise the variable growth from the 8 different SMC isolates. The experimental groups were compared using the Wilcoxon paired rank test.

6.3 RESULTS

Fig. 6.4 shows the median growth curve from each of the experimental groups. The cells cultured in 15% FCS showed maximal stimulation. ET-1 was shown to produce an increase in proliferation over basal. Table 6.1 shows the growth ratio for all the

groups in each of the 8 experiments. After 12 days of incubation, 10 nM of endothelin produced a median 1.5 fold increase in SMC proliferation (range, 0.9-1.9). This effect was reduced by all the receptor antagonists incubated with SMC over the same period of time. The median (range) growth ratio for Bosentan was 0.9 (0.6-1.2), BQ123; 1.0 (0.8-1.3) and BQ788; 0.9 (0.7-1.9). The observed reductions were statistically significant in all groups when compared to the growth ratio of cells cultured in endothelin alone. ($p = 0.02$) Wilcoxon.

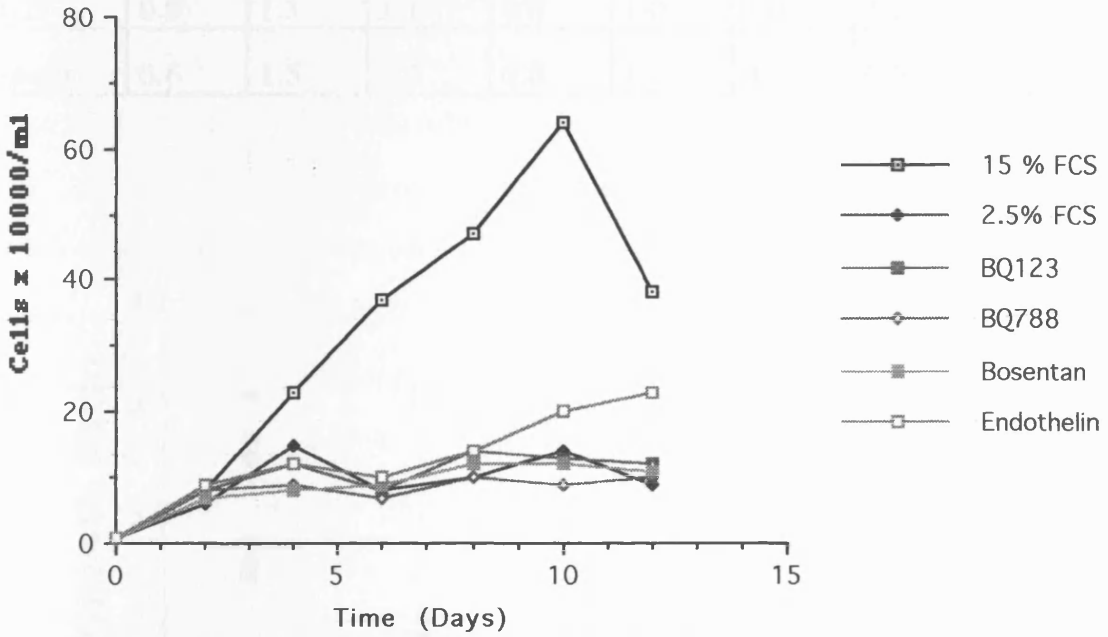


Figure.6.4. Smooth muscle cell Growth curves over 12 days. Each curve represents the median of 8 experiments. Initial seeding concentration was 1x 10000 cells /well

Table 6.1 Growth ratio of smooth muscle cell proliferation. (Growth of each experimental group expressed as a ratio of the growth in the corresponding 2.5% control)

	GROWTH RATIO							
	EXP. 1	EXP. 2	EXP. 3	EXP. 4	EXP. 5	EXP. 6	EXP. 7	EXP. 8
15% FCS	2.3	4.0	3.6	2.4	2.6	2.6	3.4	2.4
2.5% FCS	1	1	1	1	1	1	1	1
ET-1	1.3	1.6	1.9	1.6	1.5	1.3	1.7	0.9
BQ788	0.8	0.7	1.3	1.0	0.9	0.9	1.1	0.7
BQ123	0.9	1.3	1.1	0.8	1.0	1.0	1.2	0.8
Bosentan	0.6	1.5	1.2	0.8	1.0	0.6	0.9	0.8

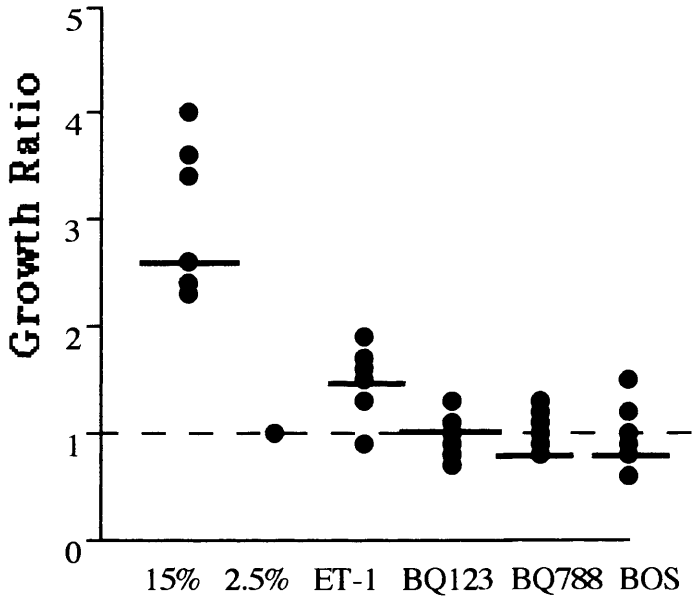


Figure. 6.5. Dot plot of data in Table 6.1 illustrating the median growth ratios in each group.

15% =15 %FCS, 2.5 = 2.5% FCS, BOS = Bosentan, ET-1 = Endothelin-1

6.3 DISCUSSION

This study demonstrates that ET is mitogenic for venous smooth muscle cells. This is in agreement with the reports from other studies *(Bobik et al. 1990; Komuro et al. 1988; Masood et al. 1997).* The mechanism of the mitogenic effects of ET is still debatable. It seems it cannot promote mitogenesis on its own and requires the presence of other factors such as Platelet Derived Growth Factor *(Janakidevi et al. 1992; Weissberg et al. 1990)* or media supplemented with insulin *(Komuro et al. 1988).* Thus in some studies, the endothelin in the culture media has been supplemented with serum *(Hassoun et al. 1992; Masood et al. 1997).* In view of these requirements it is often described as a comitogen *(Weissberg et al. 1990).* In this study it was necessary to use a minimal-growth promoting medium supplemented with 2.5% serum in order to support the cells over the 12 days of culture.

Previous studies have demonstrated that ET-1 can stimulate a 1.4 to 3.6 fold increase in DNA synthesis in 48 hour studies *(Bobik et al. 1990; Kanse et al. 1995; Masood et al. 1997).* In this study ET produced a 1.5 fold increase in cell numbers over basal during a 12 day culture period. Bobik et al found that ET produced a 1.2 fold increase in population after 48 hours *(Bobik et al. 1990).* Kanse et al. studied the proliferative response of isolated arterial and venous smooth muscle cells from 26 patients. In that study, exposure of saphenous vein smooth muscle cells to 100nM endothelin for 8 days effected a 1.6 fold increase in cell numbers. They also found that two thirds of the lines established from these donors did not respond to ET-1 *(Kanse et al. 1995).* In keeping with those observation, we also found a variation in the growth response of SMC obtained from 8 different patients (0.9-1.9).

The data from this study suggests that both ET_A and ET_B receptors are required to mediate ET-1 induced proliferation in isolated venous smooth muscle cells. Both receptors have been found to exist in venous smooth muscle cells *(Moreland et al. 1992; Webb et al. 1993)* and they have been shown to mediate contraction in human vessels including veins. *(Seo et al. 1994).* However the role of these receptors in mitogenesis is not clear. Previous studies on rat aortic smooth muscle cells concluded

that the mitogenic effect was mediated via ET_A receptors *(Ohlstein et al. 1992).* However these cells normally express ET_A receptors only *(Hori et al. 1992)* and therefore their observations cannot be extrapolated to human saphenous vein. In this study dual receptor blockade had a similar effect as ET_B receptor blockade. This suggests that the ET_B receptors may play the more significant role in activation of the mitogenic signalling pathways, though Wang et al. have previously demonstrated that both receptor subtypes can stimulate mitogen activated protein kinase cascade *(Wang et al. 1994).*

In this study we used early passaged cells in order to limit the degree of phenotypic changes that occur following subculture, thus allowing the cells to retain the same proportion of receptors that they normally posses in vivo *(Eguchi et al. 1994).* However certain pathological conditions are associated with a change in receptor expression. Following angioplasty in rabbit carotid artery, Azuma et al. demonstrated an increase in ET_B receptor expression in the neointima *(Azuma et al. 1995).* The ET_B receptor has also been shown to be upregulated in atherosclerotic vessels *(Bacon et al. 1995; Dagassan et al. 1996).* The results from these studies imply that the ET_B receptor plays a more significant role in some vascular pathologies. These reports have studied arterial disease, however, in a recent in vivo study on saphenous vein grafted into the arterial circulation of a rabbit, Eguchi et al. found that functioning ET_B receptor and their mRNA are down regulated without any change in the expression of the ET_A receptors *(Eguchi et al. 1997).* The authors suggest that this is as a consequence of the adaptive response of implanted veins implanted in the arterial circulation.

Studies on isolated cells are not representative of the intact tissue and the phenotypic characteristics may change with the isolation process. However, this study has demonstrated that whilst both ET receptor subtypes are capable of mediating SMC proliferation, early passaged cells require the presence of both these receptors to fully respond to the mitogenic effects of ET. It is possible that certain pathological situations may result in a change in receptor expression and under these conditions, one subtype may play a more significant role in mitogenesis.

CHAPTER 7

THE SAPHENOUS VEIN MODEL OF INTIMAL HYPERPLASIA AND THE ROLE OF ENDOTHELIN PEPTIDE

7a *Methods of Organ Culture*

7a.1 Introduction

7a.2 The Organ Culture Model

7a.3 Method Of Organ Culture

Measurement of neointimal thickness.

7b *Production of Endothelin the model of vein graft intimal hyperplasia*

7b.1 Introduction

7b.2 Materials And Methods

Assay of ET and big ET

Measurement of neointima

7b.3 Results

7c *Localisation of Endothelin peptide in saphenous vein*

7c.1 Introduction

7c.2 Materials And Methods

Tissue culture

Endothelin peptide staining

7c.3 Results

7c.4 Discussion

7a

METHODS OF ORGAN CULTURE

7a.1 INTRODUCTION

The study on the effects of receptor blockade on isolated cells in Chapter 6 has demonstrated that both ET_A and ET_B receptors are capable of mediating venous SMC proliferation in vitro. However, there are draw backs to studies on cultured cells. Isolated cells in culture lose the normal cell to cell and cell to matrix interactions which may play an important role in modulating in vivo responses. Furthermore, procedures such as trypsinization and serial passages may result in phenotypic changes in these cells *(Chamley-Campbell et al. 1981)*. Indeed, such phenotypic changes have been shown to alter ET receptor expression on cultured SMC *(Eguchi et al. 1994)*. These studies would have to be repeated in models that can simulate the in vivo structural and environmental milieu more closely. There have been numerous animal models of in vivo IH. Several of these have been used to demonstrate successful therapeutic strategies for reducing SMC proliferation and IH. However, subsequent clinical trials of these agents have not been successful *(Bauters et al. 1996)*. Perhaps this is as a result of the inherent differences between humans and the animal tissues. In an attempt to simulate human vein graft IH, I have utilised an in vitro organ culture technique. This approach maintains the structural integrity and characteristics of the vein in vivo and has been extensively validated in this department. The first part of this chapter will describe the biology of this model and the methods employed. In the second part of the chapter, this model has been used to examine the relationship between endothelin peptide and saphenous vein IH.

7a.2 THE ORGAN CULTURE MODEL

The major challenge in organ culture is to maintain tissue viability. Trowell demonstrated that diffusion of nutrients from culture medium could maintain the

viability of lengths of rat mesenteric artery *(Trowell 1959)*. When segments of human aorta were maintained in culture, Barret and colleagues noted intimally directed SMC proliferation *(Barrett et al. 1979)*. Similar features were observed in organ cultures of pig aorta which possesses a similar distribution of intimal SMC as humans *(Gotlieb and Boden 1984)*. Further work on this model has demonstrated that this SMC proliferation is dependent on the presence of an endothelial layer *(Holt et al. 1992; Koo and Gotlieb 1991)* which though morphologically intact, may be dysfunctional and hence produce growth factors which are able to stimulate SMC proliferation. Soyombo and colleagues described intimal proliferation in organ cultures of human saphenous veins *(Soyombo et al. 1990)*, which forms the basis of the model of vein graft intimal hyperplasia used in the following chapters. It comprises an opened segment of excised vein maintained in culture medium for a period of 14 days. During this period the segments develop a neointima comprised of several layers of SMC. Soyombo demonstrated that these segments remain viable for this duration and that the characteristics of the SMC in the neointima are similar to those found in the neointima of human vein graft stenoses excised at surgery *(Soyombo et al. 1990)*. There were however some dissimilarities. There is less elastin in the models than in true VGS. Furthermore, the neointima in the vein graft models contain numerous microlumeni, a feature not commonly seen in histological sections of VGS *(Sottiurai et al. 1983)*. Nevertheless it represents a versatile and reproducible model with which to study human IH.

The mechanisms initiating SMC proliferation in this model are not clear. One possibility is the lack of flow, as vein maintained in media flowing at in vivo arterial shear rates develops very little neointima *(Porter et al. 1996a)*. Though it seems that endothelial injury or removal is important for initiating SMC proliferation in vivo *(Clowes et al. 1983; Schwartz et al. 1975)*, in the in vitro model, removal of the endothelium attenuates NI formation *(Angelini et al. 1992)*. The importance of the endothelium in the development of intimal directed SMC proliferation is evident in the study by Koo and colleagues who demonstrated that the conditioned media from

endothelium intact can induce intimal proliferation in arterial segments devoid of endothelium *(Koo and Gotlieb 1989)*. Thus in setting up the organ cultures for my studies, care has been taken to use segments which had not been crushed or distended and that had an intact endothelium. Observations from other studies indicate that surgical preparation does have a significant effect on the development of NI in vitro. *(Soyombo et al. 1995)*.

The kinetics of SMC proliferation in this model have been studied in our laboratories. Cellular proliferation starts between day 4 -7 and peaks between day 10 - 14. Neointimal thickness lags behind this, developing between day 7 and 10 and maximal at day 14. There was no further increase in cell proliferation or intimal thickening after this period *(Porter et al. 1996b)*. Koo and colleagues reported similar findings following 4 weeks of vein organ culture. The SMC population increased up to 14 days after which no further proliferation was noted *(Koo and Gotlieb 1991)*. These observation are similar to previous in vivo findings by Dilley et al. on rat IH *(Dilley et al. 1992)*.

7a.3 METHODS OF ORGAN CULTURE

Segments of long saphenous vein that were surplus to requirement were obtained from patients undergoing either aorto-coronary or infrainguinal vein bypass procedures. Prior consent had been obtained from the local ethical committee to use human tissue for research. They were stored in sterile bottles containing calcium free Krebs solution (Appendix 2) and transported immediately to the laboratory on ice. In the laboratory, excess fat and adventitial tissue was dissected from the vein. Segments that had been obviously traumatised during surgical harvesting were rejected from the study. The vein was then divided into 0.5 cm segments which were then opened longitudinally to expose the lumenal surface. In order to assess the degree of endothelial loss, one representative section was stained with 0.2% trypan blue (Sigma Chemicals, Poole, Dorset) for 30 - 45 seconds. The section was then washed with MEM and the endothelial surface was inspected under a dissecting microscope to determine the

degree of endothelial loss. Areas of endothelial loss or damage take up the dye and stain blue whereas endothelial cells with intact cell membranes exclude the dye. Only veins showing more that 50% endothelial preservation were used in the study. If this assessment was satisfactory, the remaining segments were transferred to culture dishes (Pyrex 60 x 20mm, Corning Ltd UK). The base of each culture dish had been pre-lined with a layer of Sylgard 184 resin (Dow Corning, Seneffe, Belgium) to a depth of about 5mm. The segments were placed lumen uppermost onto a 500μm square mesh and pinned out at each corner to their approximate in situ length by using fine sterile Minuten pins (Watkins and Doncaster, Cranbrook UK). Six millilitres of vein culture medium (VCM) was then added to each dish. This consisted of RPMI 1640 medium (Northumbria Biologicals, Cramlington UK) supplemented with 30% Foetal Calf Serum (Seralab, Crawley Down, UK) (Appendix 3). The dishes were then placed in a cell culture incubator (Queue Systems, West Virginia, USA) maintained at a temperature of 37^0C with 5% CO_2 in air. When required, test compounds were added to the culture medium at the start of the experiments. The compounds and culture medium were changed every 48 hours and each experiment was performed for 14 days. One dish acted as the control and this contained culture medium only. At the end of the 14 day culture period, the medium was replaced with 4% Paraformaldehyde fixative (Appendix 3) overnight. The vein segments were then processed and embedded in paraffin from which 4μm sections were cut. These sections were stained with CD31, (Dako, High Wycombe, UK) a monoclonal venous endothelial cell marker, and with a combined monoclonal anti smooth muscle actin and Millers elastin stain (Dako, High Wycombe, UK) that allowed localisation of the smooth muscle and elastin in the wall of the vein. With the CD31 stain, the endothelial cells stained light brown. The smooth muscle cells stained light brown and the elastin stained black with the actin and elastin stains respectively.

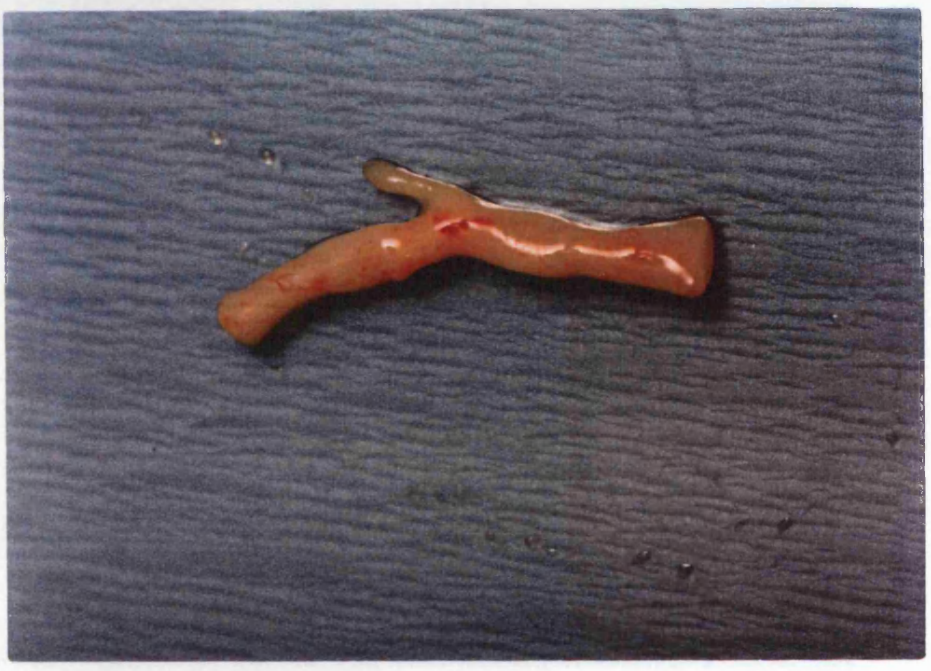


Figure 7a.1 Segment of excised saphenous vein cleared of adventitial tissue.

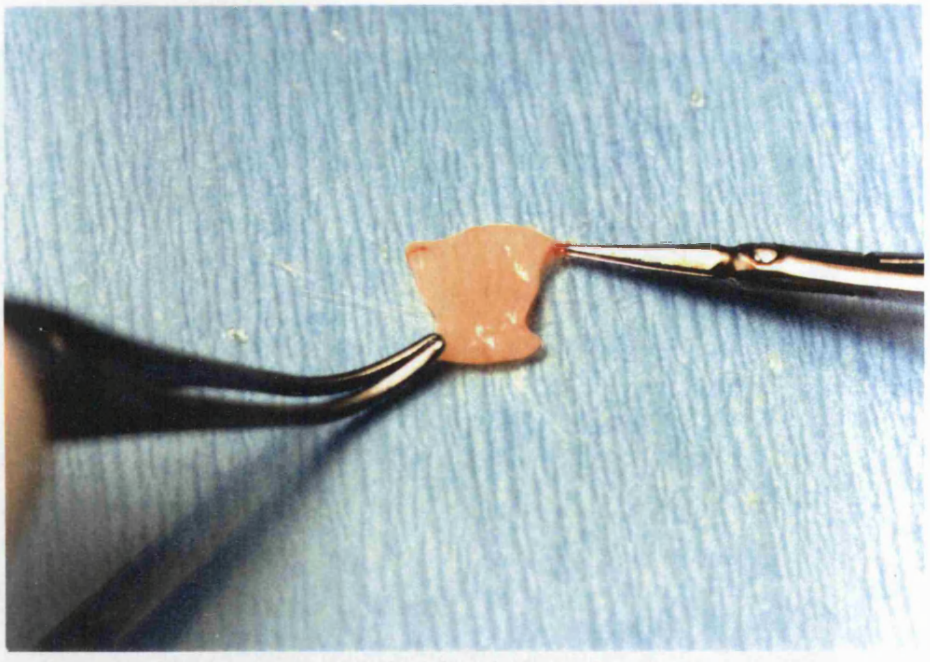


Figure 7a.2 Prepared segment of vein cut along its length exposing the lumenal surface.

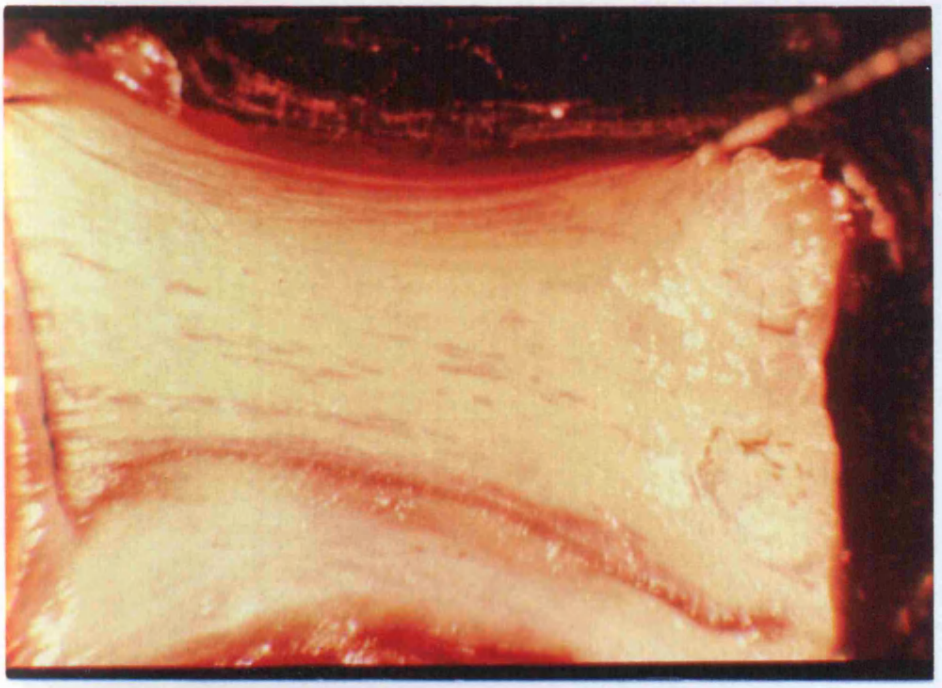


Figure 7a.3. Lumenal surface of segment of vein that has been stained with trypan blue. There is very little uptake of dye by the preserved endothelium.

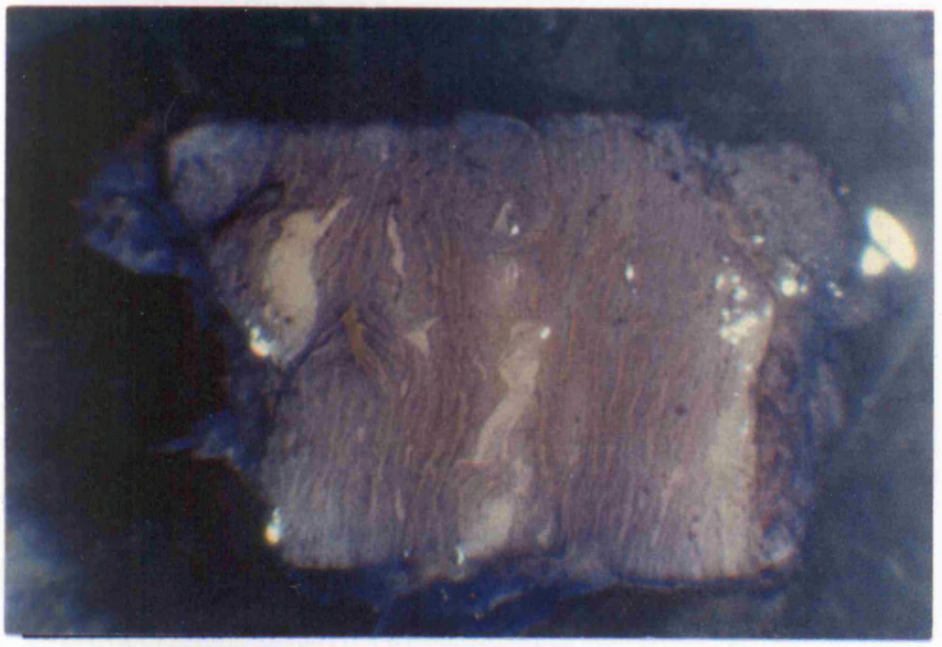


Figure 7a.4. Lumenal surface of vein stained with trypan blue. There is retention of dye in large areas indicating endothelial loss or damage. Such veins would not be used for culture.

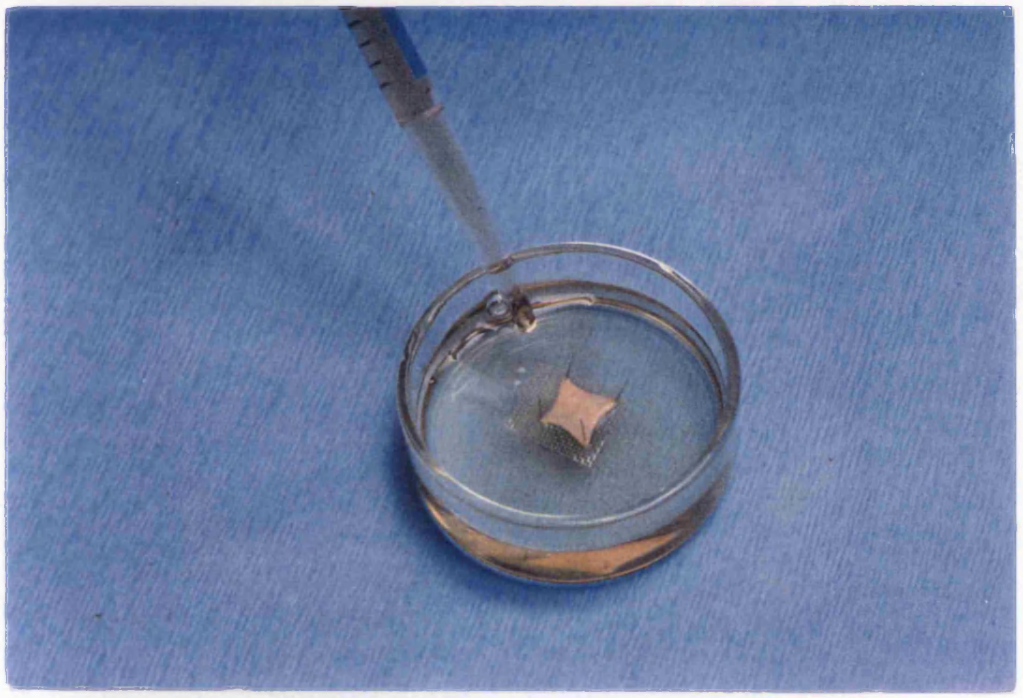


Figure 7a.5. Addition of medium to segment of vein that has been pinned out in culture dish.

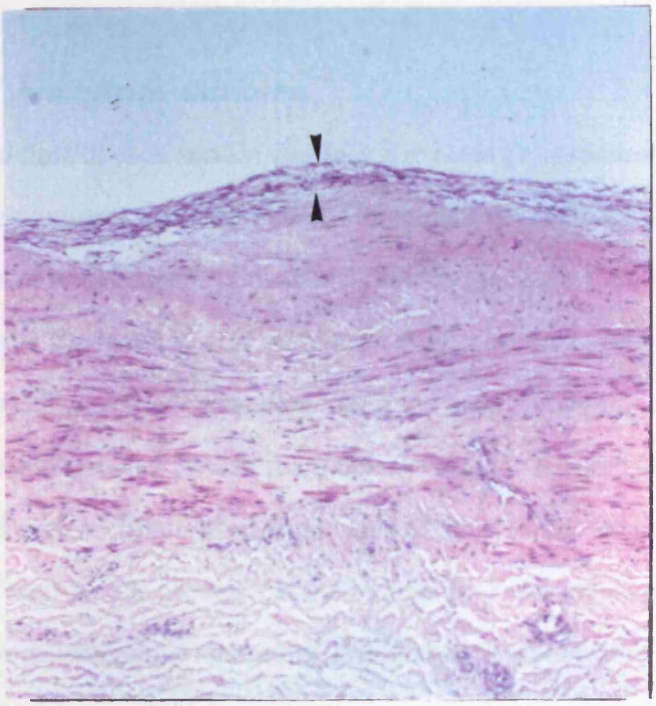


Figure 7a.6. Transverse section of vein segment after 14 days culture.The neointima has developed in the subendothelium. (arrow). H&E stain, magnification x 25.

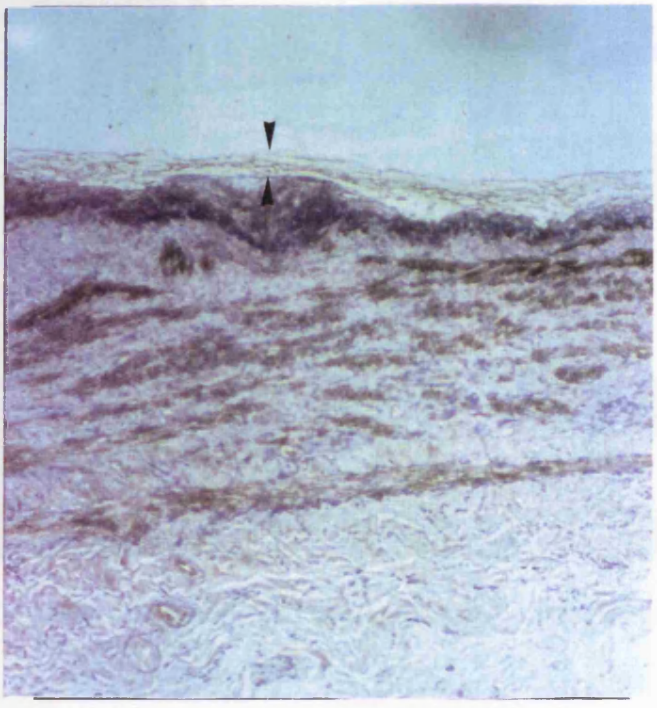


Figure 7a.7. Transverse section of a sgement of vein after 14 days of culture. The neointima can be seen as the light brown layer in the subendothelium. SMA/Miller elastin stain, magnification x 25

Measurement of neointimal thickness

The neointimal thickness of each section was measured using a computerised image analysis system (Improvision Coventry, UK). The system consisted of a light microscope connected to a computer. Features of the vein sections observed under the light microscope were displayed as high resolution images on the computer screen. Calibration of this system enabled measurements of the neointimal thickness in micrometers to be made. At least 30 measurements were made at equal points along the transverse length of each section. The median value of these measurements was calculated for each section. The accuracy of the intimal thickness measurements had been previously determined in this laboratory The inter-observer error of this technique had been found to be 14μm and the intra observer error to be 10μm. *(Allen et al. 1994).*

Figure 7a.9 Light microscope and computer used for morphometric measurements.

7b

PRODUCTION OF ENDOTHELIN IN THE MODEL OF VEIN GRAFT INTIMAL HYPERPLASIA

7b.1 INTRODUCTION

Increased plasma levels of endothelin have been associated with many pathological conditions thus implicating ET in various diseases. So far there is indirect evidence linking ET to IH. For example, increased levels of ET have been found in the coronary sinus following percutaneous angioplasty *(Tahara et al. 1991)*. Exogenous ET has been found to augment restenosis in experimental animals *(Trachtenberg et al. 1993)*. Furthermore, ET and its receptors are upregulated in hyperplastic vascular tissue *(Azuma et al. 1995; Bacon et al. 1996)*. However elevated ET levels have not been directly linked to the formation of vein graft IH. The preceding chapters have established that ET can promote SMC proliferation and that receptor blockade can reduce this effect both in isolated cells and in the organ culture model. However, these findings have not been related to locally released ET peptide. In order to establish a direct association between ET and IH, the aim of this study was firstly to determine if levels of ET and its precursor, big ET are elevated during the formation of neointima in the vein organ culture model and secondly to localise ET peptide in the neointima.

7b.2 MATERIALS AND METHODS

Segments of LSV were obtained as previously described. Each vein was divided into 5 segments. One segment was immediately fixed for immunohistochemical study. The other 4 segments were pinned and set up for culture as has been previously described. One of these was reserved for collecting samples for assay and was cultured for 14 days. The other 3 were cultured for 4, 8 and 12 days respectively. At the start of each experiment, 500μL of the VCM that was to be used was transferred into a freezing vial and immediately stored in the - 80^{0}C freezer. This sample acted as the Day 0 baseline

control. At the end of each 48 hour period, two samples of 500μL each were taken from the reserved dish into freezing vials that were stored at -80^{o}c. At the same time the medium in the dishes were replaced. At the end of the culture period, the vein segments were fixed and sent for immunohistochemical staining. Experiments were repeated 8 times.

Assay for ET and big ET

Enzyme linked immunoassay (ELISA) kits for measuring big ET and ET were obtained from Biomedica (Biomedica Gruppe, Switzerland). Kit Lot numbers 874 and A76 for the big ET and ET respectively. The big ET kit had 100% reactivity with big ET and < 1% cross reactivity with the ET isopeptides (Range 0.05-15.6 fmol/ml). The ET assay kit had 100% reactivity with ET-1 and ET-2 with 5% cross reactivity with ET-3 and <1% with big ET (Range 0.1-15.6 fmol/ml). When required, Samples were taken from the freezer and allowed to thaw at room temperature. They were then kept on ice prior to analysis. Analysis was performed according to the protocol supplied by the kit manufactures.

Big ET-1 assay

Undiluted samples in duplicate, and the appropriate standards and control were incubated for 3 hours with detection antibody in multiwell plates pre-coated with big ET antibodies. The wells were then emptied and washed and incubated with Conjugate for 1 hour. At the end of this the wells were emptied again, washed and substrate was added and incubated for 30 minutes. This reaction was stopped by adding stopping solution and the absorption was read at 450nm and again at 650nm for reference. The values obtained were subtracted from the assay blanks and a calibration curve was constructed to calculate the concentration of big ET expressed as fmol/ml/48 hours.

Assay of ET

Samples (diluted 1 in 10 in assay buffer), standards and assay controls were incubated overnight with detection antibody in multiwell plates pre-coated with endothelin antibodies. The wells were emptied and washed and then incubated for 1 hour with assay conjugate. After this period the wells were again emptied, washed again and incubated for 30 minutes with assay substrate. This reaction was stopped with the stop solution and the absorbance read at 405nm and again at 690nm for reference. The values obtained were subtracted from the assay blanks and a calibration curve was constructed to calculate the concentration of ET expressed as fmol/ml/48 hours.

Measurement of neointima

Using the computerised image analysis system as previously described, Serial measurements of neointimal thickness were made in 5 of the experiments of veins cultured for 0, 4, 8, 12 and 14 days.

7b.3 Results

The peptide produced is expresssed as the net production of ET and big ET . This is calculated for each experiment by substracting the amount of peptide detected at each time point from the amount detected in the day 0 baseline control sample. This was done because small amounts of ET and big ET imunoreactivity were detectable in some day 0 baseline control samples. The detailed results are in Tables 7b.1 and 7b.2 (apppendix 4). The median net production of ET in 6mls of medium rises from 10.2 fmol/48hrs to 27 fmol/48hrs at day 6. Thereafter the levels of ET gradually falls, but remains elevated at 12.6 fmol/ 48 hours (Fig. 7b.1). There was no net production of big ET in the first 48 hours, thereafter the amount detected in 6mls of medium this rises and peaks to 7.8 fmol/48 hours at day 6 and falls sharply thereafter though levels remain elevated 4.2 fmol/48 hours at day 14 (Fig.7b.2).

Neointima was first demonstrable after 8 days of culture, after which it continued to increase till the 14 day of culture. Sections from an index vein taken at day 4, 8, 12 and

14 of organ culture are shown in (Fig 7b.3). This demonstrates the increase in neointimal thickness. The calculated median values of the 5 veins in the study is shown in Figure 7b.4. Detailed data in appendix 4 (Table 7b.4).

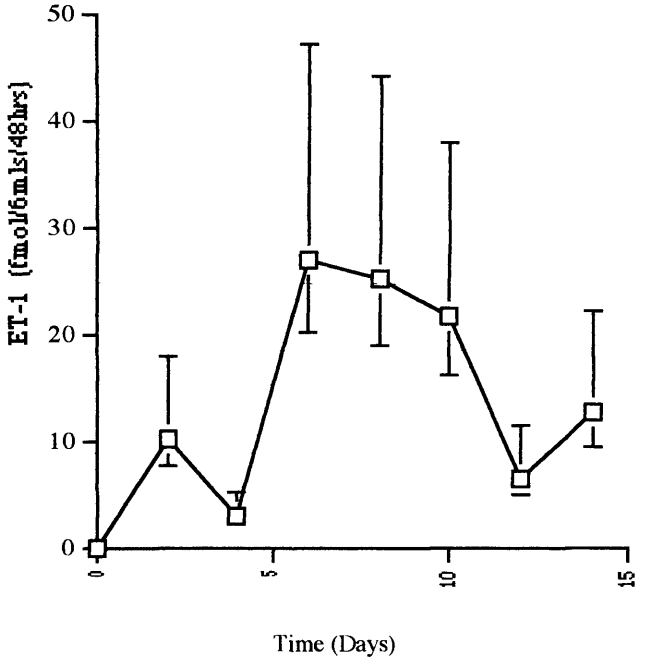


Figure 7b.1. Graph showing the median and interquartile range of net ET produced by human saphenous veins during a 14 day period of organ culture. (n=8).

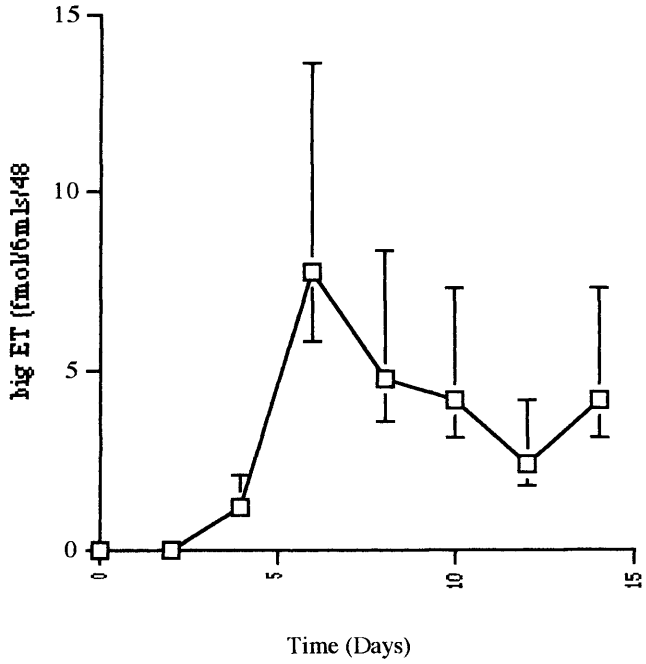
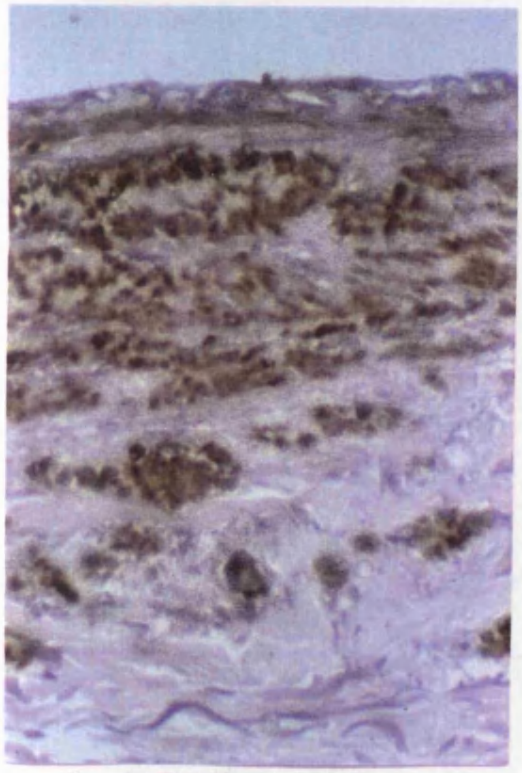
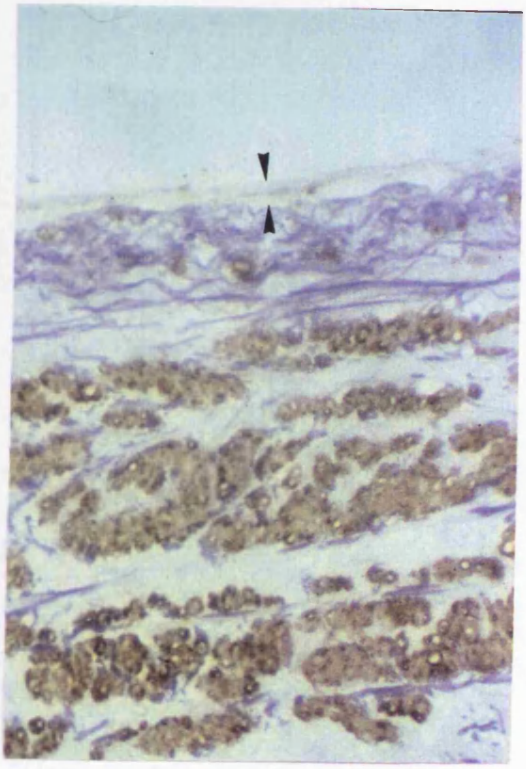


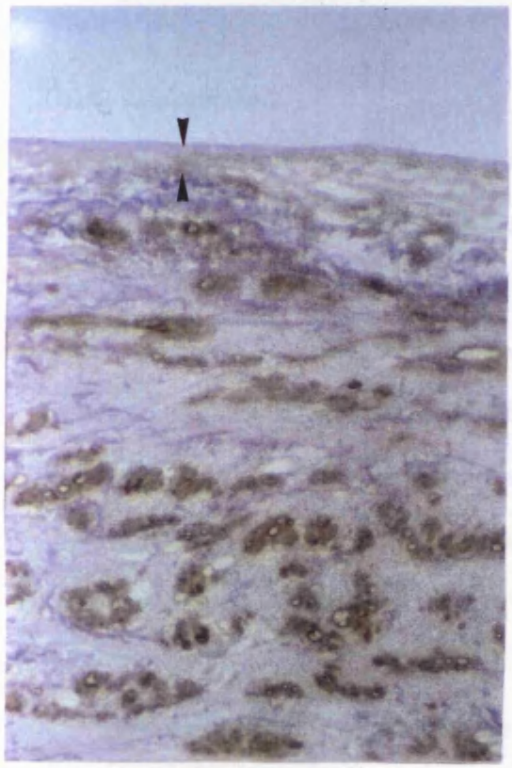
Fig 7b.2. Graph showing the median and interquartile range of net big ET produced by human saphenous veins during a 14 day period of organ culture. (n=8).



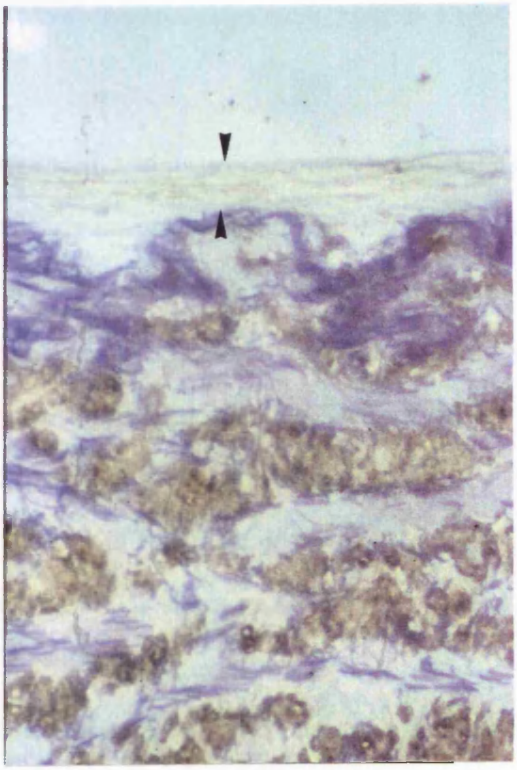
Day 4



Day 8



Day 12



Day 14

Figure 7b.3. Transverse sections of veins after 4, 8,12 and14 days of culture. There is a progressive increase in neointima formation after day 8. SMA/Millers elastin stain. Magnification x 200.

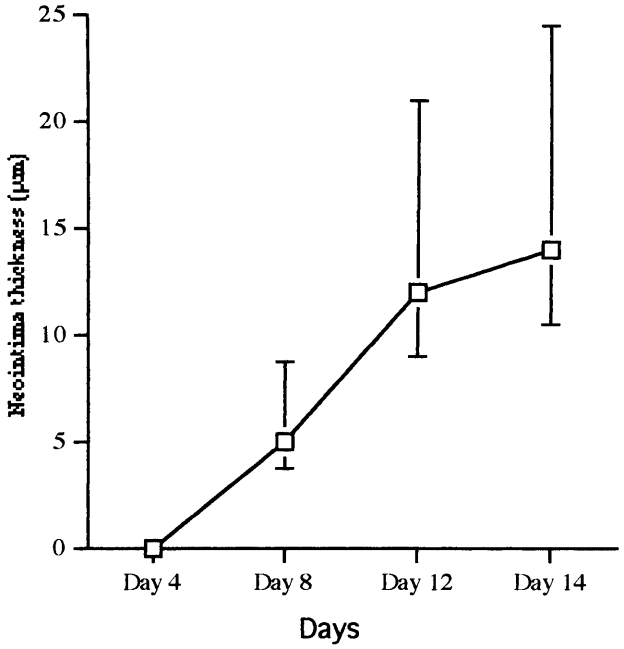


Figure 7b.4. Median and interquartile range human saphenous vein neointimal thickness during 14 days culture. (n=8).

7b.4 Discussion

This study has clearly demonstrated that endothelin and its precursor are produced by saphenous veins whilst in organ culture. There are several mechanisms that may have activated the ET system in this model. The lack of shear stress in the static culture *(Sharefkin et al. 1991)* and the presence of other growth factors in the culture media are known to promote ET synthesis *(Kurihara et al. 1989; Marsden and Brenner 1992; Resink et al. 1990)*. Though the exact site of production of the peptides are not apparent in this study, ECs are an abundant source of ET *(Yanagisawa et al. 1988b)* and SMCs in culture have been shown to be capable of secreting ET *(Resink et al. 1990; Yu and Davenport 1995b)*.

Work done in this department on the kinetics of SMC proliferation in the organ culture model demonstrated a burst of SMC proliferation between day 4 and 7 which peaked on day 14 *(Porter et al. 1996b)*. The pattern of release of ET and big ET in this study corresponds to this period of maximal smooth muscle cell proliferation, thus implying that the SMC activation and proliferation is linked to ET production. In

conjunction with the study in Chapter 6 it would seem that endothelin acts in an autocrine manner.

Compared to ET, there is very little big ET in the media within the first 48 hours. This may be because big ET is being rapidly degraded to active ET This would be prior to the stage of active smooth muscle cell proliferation and thus increased production of both peptides. The levels of big ET and ET peak prior to the development of measurable NI. There may be a feedback regulatory mechanism limiting the expression ET. Thus once there are sufficient proliferating SMC, such high levels of peptide production are no longer required. Nevertheless, after this peak, ET peptide level remained slightly elevated enough to support the reduced level of cell turnover. It may be argued that the decline in peptide levels is as a result of cell death in the model, however, the gradual increase in neointimal thickness observed over the duration of the experiments does not support this.

Possible correlation from the findings of this study are of potential clinical significance. However, to date, no in vivo study has demonstrated a relationship between elevated ET levels and the development of clinical IH. However, the association between elevated plasma levels of ET and some pathological conditions has been controversial. ET is secreted ablumenally and rapidly eliminated from the blood stream *(Wagner et al. 1992)*. Thus, for example even though ET is implicated in the aetiology of essential hypertension, there has been no demonstrable elevation in plasma levels *(Miyauchi et al. 1992)*. It is for these reasons that it may be impossible to find elevated plasma ET levels in patients with vein graft stenosis. At present, the detection of increased ET tissue immunoreactivity in excised stenotic segments may be the only firm method of establishing this association.

7c

LOCALISATION OF ENDOTHELIN IN SAPHENOUS VEIN NEOINTIMA

7c.1 INTRODUCTION

The preceeding section demonstrated that ET levels are elevated in the conditioned medium from the vein intimal hyperplasia organ culture model. However it was not clear which cells are contributing to this. This section describes an immunohistochemical study designed to localise tissue ET immunoreactivity in cultured veins using the previously described organ culture model.

7c.2 MATERIALS AND METHODS

Tissue culture

Segments of vein were obtained and cultured as described in Chapter 7. The veins were assessed for endothelial preservation and each length was divided into segments of about 0.5 cm^2. One segment was immediately fixed in 4% formaldehyde prior to paraffin embedding. Another segment was pinned out and set up for culture in 6 mls VCM as described in Chapter 7. The medium was changed every 2-3 days. At the end of the 14th day of culture the segment was fixed in 4% formaldehyde for immunohistochemical staining.

Endothelin peptide staining

The endothelin antibody had been previously validated *(Lerman et al. 1991)* and used in the department to localise ET in vein graft stenotic lesions *(Masood et al. 1996)*. Primary rabbit anti-endothelin -1 antibody was obtained from Peninsula UK Ltd. This was used at a dilution of 1 in 1000 normal goat serum (NGS). Paraffin embedded sections were cut and mounted on glass slides. These slides were de-paraffinized by soaking in graded concentrations of xylene. The slides were washed in water for 2 minutes and in 6% hydrogen peroxide for 10 minutes. The tissue was then incubated for 25 minutes at 37^0C in trypsin solution (0.3g trypsin + 0.36g calcium

chloride + 300mls water) adjusted to pH 7.8 with weak sodium hydroxide solution. This reaction was stopped by placing the slides in cold water and washing in PBS Solution. Excess fluid was drained of the slides and 200μl of NGS was added directly onto the tissue sections and incubated for 10 minutes. One hundred microlitres of the required primary antibody was then added onto the sections and incubated at 4^{o}C overnight. The following morning, the sections were washed in PBS for 20 minutes and incubated with the goat, rabbit and mouse biotinylated secondary antibody for 30 minutes at room temperature. The sections were washed in PBS again for 20 minutes before incubating with avidin and biotinylated horseradish peroxidase complex (ABC Method, Vector Laboratories) for 30 minutes. In order to visualise the bound antibody, the sections were washed in PBS and incubated with diaminobenzidine and then copper sulphate solutions for 5 minutes each. Counter staining was with haematoxylin for 30 seconds after which the sections were cleared in graded concentrations of xylene and covered with glass cover slips.

Each section had a comparative negative control to which no primary antibody was added.

Prepared sections were analysed by light microscopy and assesed for distribution and intensity of ET-1 immunoreactive staining. Findings were scored as shown in Table 7c.1.

Table 7c.1 Scoring of ET-1 tissue staining.

INTENSITY	SCORE
No stain	-
light brown staining	+
Dark brown staining	++
Very dark brown staining	+++

7c.3 RESULTS

Figures 7c.1 and 7c.2 illustrates the localisation of ET peptide in saphenous vein. ET-1 immuoreactive cells stained brown. Such staining can be seen in both freshly excised veins (Figure 7c.2), and in the veins cultured for 14 days (Fig 7c.3). Analysis and scoring of the intensity of staining showed similar staining intensities in the media and the endothelium of freshly excised veins, though the endothelial stain was more predominant in some veins (Table 7c.2). However after 14 days culture the neoinitimal signals in all the veins were more intense than that of the adjacent media (Table 7c.3).

Table 7c.2 Intensity of ET-1 immunohistochemical staining in fresh vein sections

	Intensity of staining	
Experiment	Media	Endothelium
1	++	++
2	-	+
3	+	+
4	-	+
5	+	+

Table 7c.3 Intensity of ET-1 immunohistochemical staining in 14 day cultured vein sections

	Intensity of staining	
Experiment	Media	Neointima
1	+	+++
2	++	+++
3	+	++
4	+	++
5	++	+++

Figure 7c.1. Negative control. Transverse section of uncultured vein not stained with endothelin antibody. Magnification x 200.

Figure 7c.2. Transverse section of uncultured vein stained with monoclonal endothelin antibody illustrating localisation of endothelin peptid in the endothelium. Magnification x 200

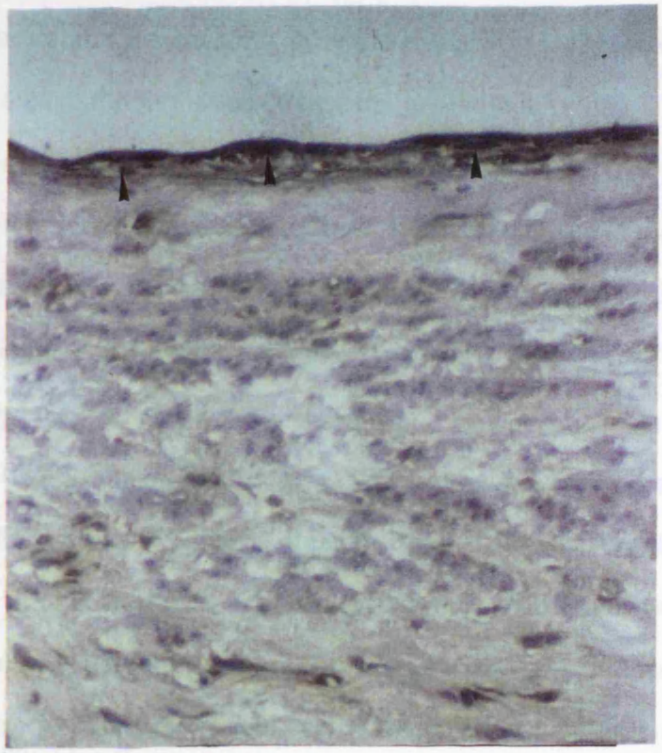


Figure 7c.3. Transverse section of 14 day cultured vein stained with monoclonal anti-endothelin antibody illustrating localisation of endothelin peptide in the neointima. Magnification x 200.

7c.4 DISCUSSION

This study has demonstrated ET-1 immunoreactivity within both fresh and cultured saphenous vein. Furthermore, it has demonstrated that after 14 days of culture, there is more immunoreactivity within the neointimal layer than the media. The findings from this in vitro study are in keeping with studies on excised segments of human vein graft stenosis that have shown extensive ET immunoreactivity in these tissues *(Masood et al. 1996)* as well as observations from other in vivo vein studies *(Dashwood et al. 1995)* and in vivo angioplasty models *(Wang et al. 1996)*. Notably, the differential staining of ET-1 in the neointima and media is consistent with previous studies. Binding studies in hyperplastic rabbit carotid arteries suggested that more binding sites were localised in the neointima (*Azuma et al. 1995*). Since then, Wang et al. have used immunohistochemical techniques to confirm differential ET-1 staining in the neointima that develops in rat carotid artery 14 days after angioplasty *(Wang et al. 1996)* .

Along with other studies, the present study provides conclusive evidence that endothelin is implicated in the formation of vascular neointimal lesions. It seems that the endothelin system is activated during this process and that most of the endothelin is derived from the SMCs within the neointima itself. Further evidence of this comes from the study by Minamino et al. who showed that ECE, the enzyme critical to formation of active ET-1, was also preferentially localised in the SMCs of the neointima in the rat model *(Minamino et al. 1997).*

Since endothelin is normally derived from vascular endothelium, it must be assumed that the SMCs adopt the function of peptide production as a result of the phenotypic changes that occurs during neointimal formation. Particularly as it is mainly the SMC in the neointima that acquire this ability. Since it is known that endothelin acts in an autocrine manner, local production of this mitogen would promote further SMC proliferation and migration. From the foregoing one would expect that inhibition of the endothelin system at any level should be effective at ameliorating IH, however, total abolition of ET-1 activity in vivo may have adverse effects. The following experimental chapters study the effect of such inhibition on the development of human IH using an in vitro model.

CHAPTER 8

EFFECT OF ENDOTHELIN ANTAGONISTS ON INTIMAL HYPERPLASIA

8a

EFFECT OF AN ENDOTHELIN CONVERTING ENZYME INHIBITOR ON INTIMAL HYPERPLASIA

8a.1 INTRODUCTION

The results of Chapter 6 have demonstrated that inhibition of ET at the level of the receptor can reduce proliferation in smooth muscle cells. The effects of ET can also be inhibited at the synthetic level. The potential therapeutic role of endothelin converting enzyme inhibitors was mentioned briefly in Chapter 3. This enzyme prevents the conversion of big ET to active ET isomers. The ET isomers are over 140 times more potent vasoconstrictors than big ET *(Kimura et al. 1989)*. The aim of this study was to determine the effect of the ECE inhibitor, CGS 26303 on IH in the model of vein graft IH.

8a.2 MATERIALS AND METHODS

Undistended segments of long saphenous vein that were surplus to requirements were obtained from 11 different patients undergoing aortocoronary or infrainguinal vein bypass surgery. CGS 26303 was obtained as a kind gift from Knoll AG (Ludwigshafen, Germany). After ensuring adequate endothelial preservation using trypan blue, the veins were divided into two equal segments and set up for organ culture in two separate dishes as described in Chapter 7. Six millilitres of vein culture medium was added to each dish to maintain them in organ culture. One dish acted as control whilst 50μM of CGS 26303 was added to the test segment. This concentration of CGS 26303 has been shown by De lombaert et al. to produce potent inhibition of ECE in vitro (*De lombaert et al. 1994*). The culture medium and the ECE inhibitor were replaced every 48 hours. At the end of 14 days of culture, the medium was replaced with 4% formaldehyde fixative overnight (Appendix 2). The segments were processed and embedded into paraffin wax from which sections could be cut for immunohistochemical staining as previously described. The neointima that had

developed in the cultured veins was then measured from the stained sections using the computerised image analysis system.

8a.3 RESULTS

The median (range) measurements of neointima for the control veins was 23 μm (14-46). The veins cultured with CGS26303 developed a median (range) neointimal thickness of 12μm (6-23). The median difference in neointima between the two groups was 11μm (95% confidence interval 5.4 to 16.6. (Figure 8a.1). Detailed data on these measurements is in table 8a.1 (appendix 3).

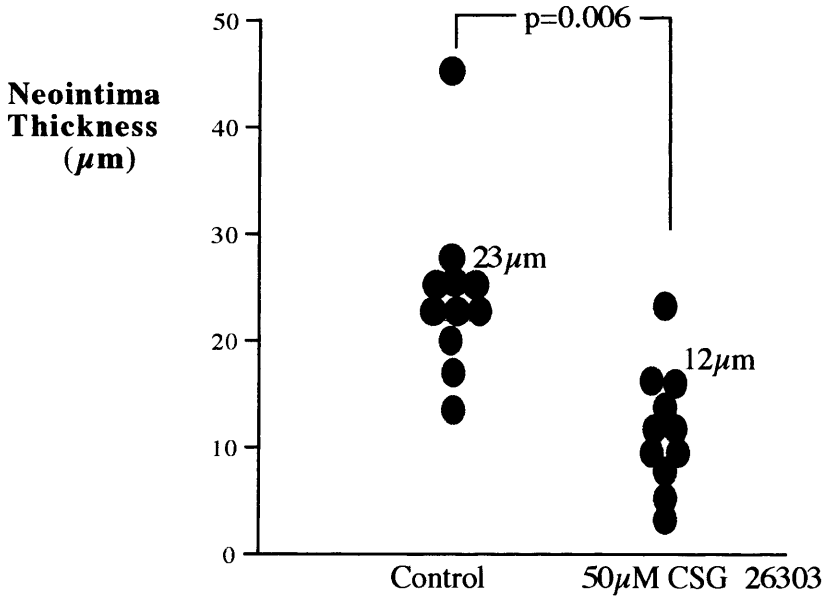


Figure 8a.1 Effect of ECE inhibitor (CGS 26303) on median neointima thickness measurements

8a.4 DISCUSSION

This study has demonstrated that suppression of ET production by ECE inhibitors can limit IH formation. It supports the studies that have shown that exogenous ET promotes IH. Recent studies have demonstrated ECE expression in various vasculoproliferative conditions. In one of the first reports that demonstrated this association, Wang et al. found that ECE mRNA levels were increased at 6 and 24

hours after balloon injury of rat carotid arteries *(Wang et al. 1995)*. Minamino and colleagues localised the ECE expression to the neointimal and intimal layer of smooth muscle cells 14 days after balloon injury *(Minamino et al. 1997)*. This latter study is of interest because it is widely believed that under normal conditions, ECE and indeed ET are predominately localised to the vascular endothelium *(Nunez et al. 1990; Shimada et al. 1994)*. However, it seems that in conditions where the endothelium has been destroyed, the regenerating and migrating smooth muscle cells begin to express these peptides. In the model used in the present study however, the endothelial layer is largely intact. Thus suggesting that endothelial ECE activity is also preserved. Whether the endothelium is the exclusive source of ECE in this model is debatable because some studies have suggested that even normal vascular SMC posses ECE activity *(Hisaki et al. 1993; Maguire et al. 1997)*. It is logical to hypothesise that blockade of the ET-1 synthetic pathway will have a dramatic effect on suppressing ET-1 associated disease conditions. Indeed, the ECE inhibitory activity of various peptides have been widely exploited. However in view of the important role that ET-1 plays in maintenance of vascular and renal physiology, inhibition of ET production at this level may be accompanied by adverse systemic consequences. Selective ECE blockade has not been easy. The problem lies with the specificity of the inhibitors used. As discussed in Chapter 3, ECE is a membrane bound metalloprotease belonging to the same group as and possessing structural similarities and sequence homology with neutral endopeptidase (NEP) *(Schmidt et al. 1994; Xu et al. 1994)*. Thus most of the currently available ECE also inhibit NEP *(Cheng et al. 1997)*. NEP has a broad substrate specificity that enables it to inactivate peptides such as opiates, enkephalins, substance P and atrial natriuretic peptide (ANP). Thus NEP inhibition would potentiate the effects of these peptides. The activities of ANP are relevant to the in vivo correlations of this study. ET-1 has been shown to induce the release of ANP, which in turn causes vasodilatation in vivo *(Goetz 1988)*. Furthermore, experimental studies have shown that ANP inhibits the mitogenic effects of ET-1 and ET-3 in SMCs. Thus the dual ECE and NEP properties of CGS26303 should inhibit vasculoproliferative disorders as has

been demonstrated in this study. Unravelling the contribution of either pathway is not easy. At the time of this study there were no effective selective ECE inhibitors. CGS26303 is a none peptide and is orally active. Thus its pharmacological properties are desirable both clinically and for research purposes. Furthermore, it is feasible that dual ECE and NEP inhibition may be beneficial in the control of some diseases states such as hypertension *(DeLombaert et al. 1997)*. On the other hand suppression of IH and restenosis alone would require a more selective and specific blockade of the endothelin system, avoiding the other possible systemic side effects. Thus the next set of studies examine the effect of blockade of the ET system at the level of its receptors.

8b

EFFECT OF NON-SELECTIVE ENDOTHELIN RECEPTOR ANTAGONISTS ON NEOINTIMA FORMATION IN AN ORGAN CULTURE MODEL

8b.1 INTRODUCTION

The role of ET receptors in isolated SMC proliferation has been described in Chapter 6 and the limitations of that study have been alluded to. It would be interesting if the endothelin receptors played a different role in the context of an intact organ culture. Thus, the following sections in this chapter describe the experiments performed with this model on the effects of ET receptor blockade on human saphenous vein IH.

8b.2 AIM

The aim of this study was to determine the effect of non-selective receptor blockade on the development of intimal hyperplasia in a saphenous vein organ culture model.

8b.3 MATERIALS AND METHODS

Segments of long saphenous vein were obtained and prepared for culture as described in section 7a. The non-selective receptor antagonist LU224332 was obtained as a kind gift from Knoll AG (Ludwigshafen, Germany). Each evaluation consisted of three segments of vein in separate culture dishes. Two of the veins were cultured with the antagonist at either 10^{-6}M or 10^{-7}M concentration. These concentrations were within the manufacturers recommended dose for in vitro use. The third vein had vehicle only and acted as control. Culture medium and antagonist were replaced every 48 -72 hours. After the 14 day culture period, the veins were fixed and stained. The neointimal thickness was measured using the computerised image analysis system. Each experiment was repeated on veins from 10 separate patients.

8b.4 RESULTS

Figure 8b.1 and 8b.2 are representative histological sections of a cultured vein. They demonstrate the effect of LU224332 on the development of intimal hyperplasia. The median intimal thickness of all veins treated with 10^{-6} and 10^{-7}M LU224332 was 11μm and 13μm respectively, resulting in a median difference of 9μm (95% confidence interval of 0.26 to 17.1) and 7μm (95% confidence interval -3.1 to 17.1) respectively.

The details of the median neointimal measurements is in Table 8b.1 (appendix 3).

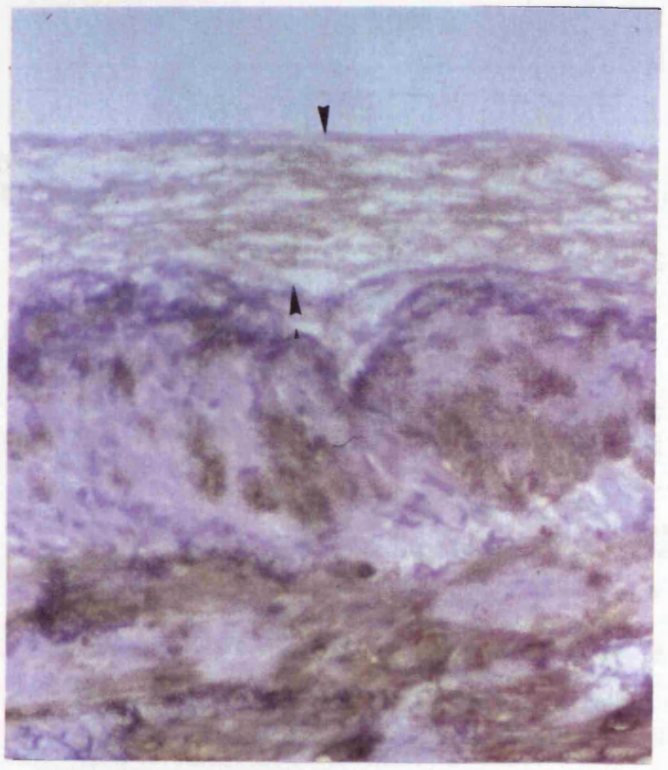


Figure. 8b.1 Transverse section of vein cultured for 14 days without the mixed ET receptor anatgonist, LU224332. SMA/Millers elastin stain, magnification x 200.

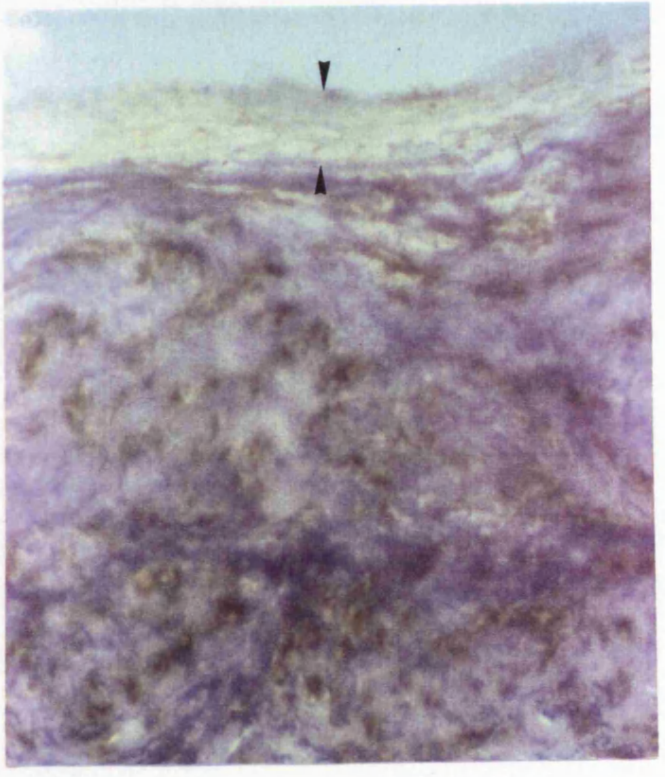


Figure.8b.2 Transverse section of vein cultured with 10-6M of the mixed receptor antagonist, LU 224332 for 14 days. There is a reduction in neointima compared to the control vein in Fig. 8b.1. SMA/Millers stain, magnification x 200.

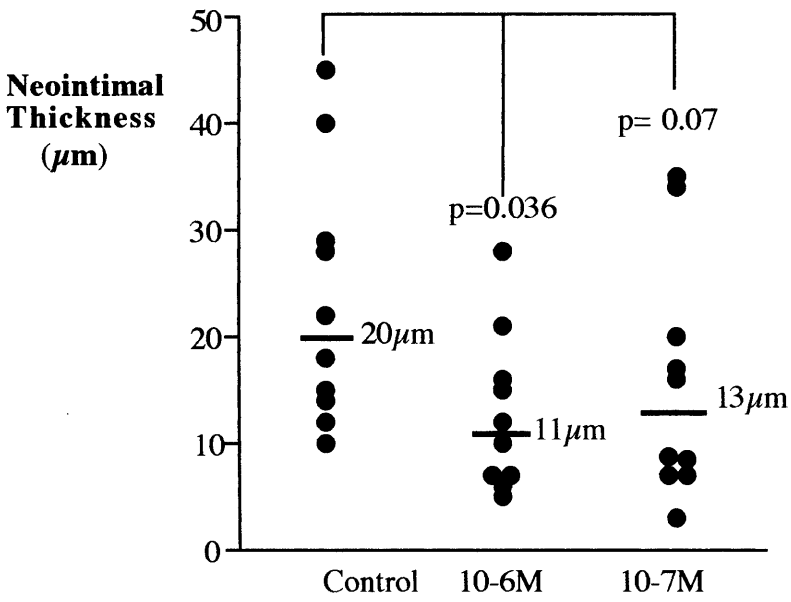


Figure 8b.3. Effect of none selective receptor antagonist. Horizontal bars are the median values.

8c

EFFECT OF SELECTIVE ENDOTHELIN RECEPTOR BLOCKADE ON THE DEVELOPMENT OF INTIMAL HYPERPLASIA

8c.1 INTRODUCTION

In the last section, treatment of the veins with the non-selective receptor antagonist LU 224332 reduced the neointima by about 40%. However, the individual role of the ET_A and ET_B receptors in this effect is not evident. Thus, the following study set out to determine the separate roles of these receptors in IH.

8c.2 AIM

The aim of this study was to examine the effect of selective ET_A receptor blockade, or ET_B receptor blockade on the development of neointimal hyperplasia in an organ culture model.

8c.3 MATERIALS AND METHODS

The experimental groups consisted of 3 segments of vein set up for culture as described in 7a. BQ123 and BQ788 (Calbiochem-Novabiochem Ltd. Nottingham UK.) were used as the ET_A and ET_B receptor antagonists respectively. Each antagonist was studied at two concentrations. Thus in evaluating each antagonist, the veins were cultured with media containing either 1μM or 3μM of BQ123 or BQ788. A third dish had vehicle only and acted as the control. The culture medium and antagonists were replaced every 48 -72 hours for 14 days. The segments were then fixed and stained as described in section 7a. The neointima was measured using the computer image analysis system and the median value calculated. Experiments were repeated on veins from 10 individual patients.

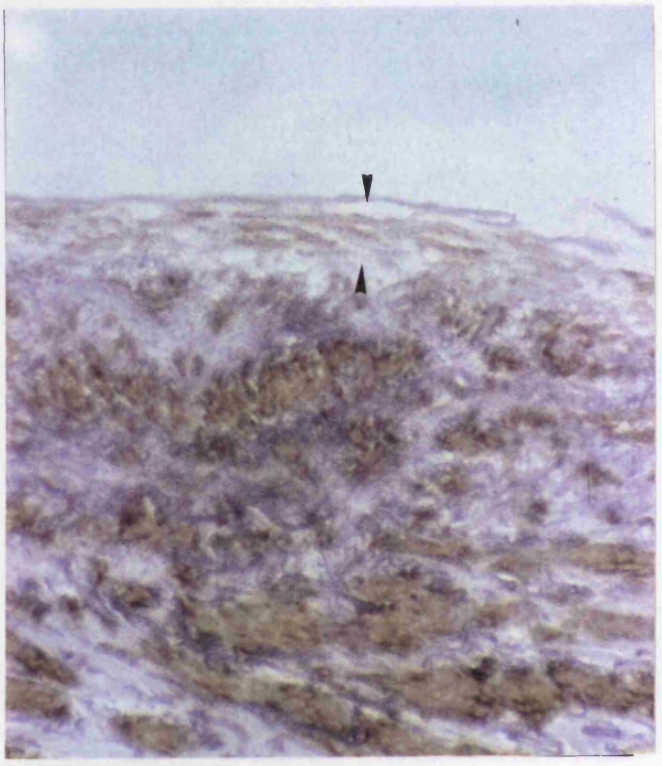


Figure 8c.1. Transverse section of vein cultured for 14 days without the ETA receptor antagonist BQ123. SMA/Millers elastin stain, magnification x 200.

Figure 8c.2. Transverse section of vein cultured for 14 days with 3μM of the E receptor antagonist BQ123. There is no significant difference in th formed neointima when compared to Figure. 8c.1.

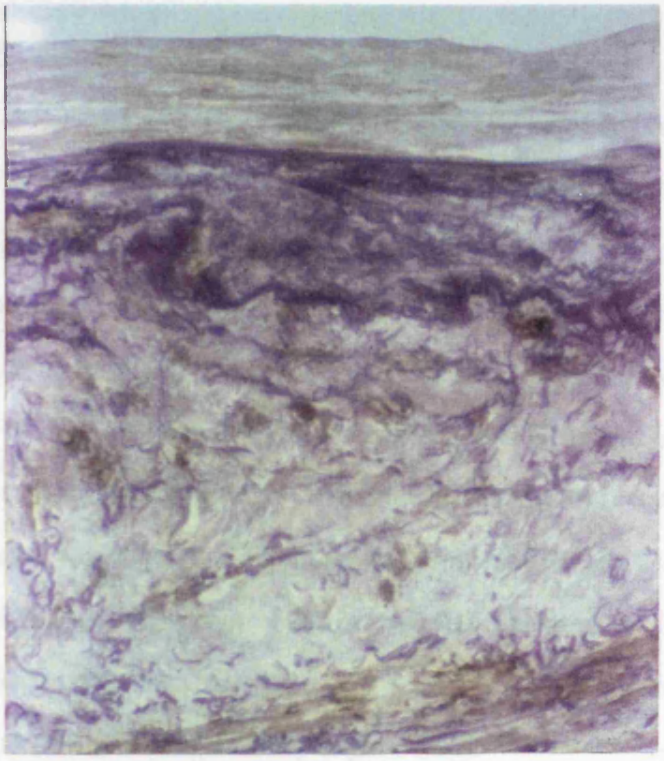


Figure 8c.3. Transverse section of vein cultured for 14 days without the ETB receptor antagonist, BQ788. SMA/Miller elastin stain, magnification x 200.

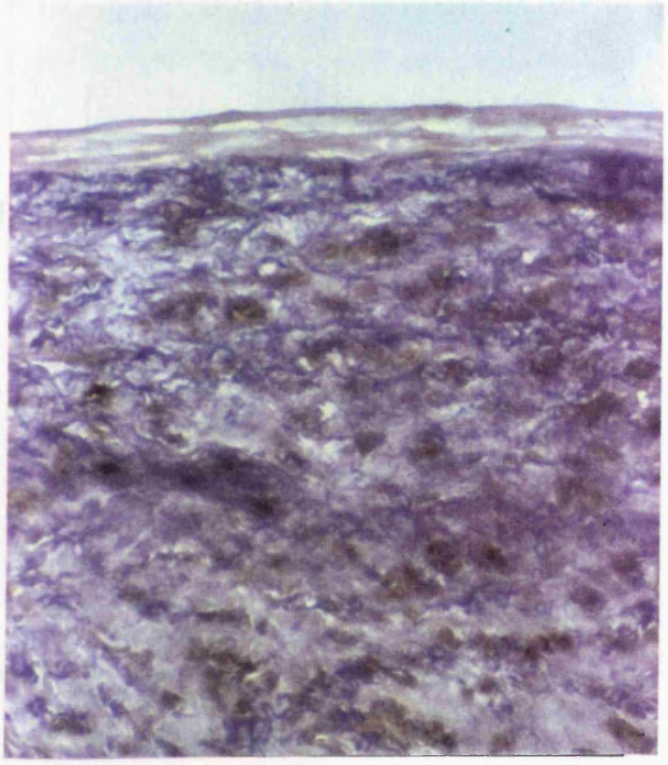


Figure 8c.4. Transverse section of vein cultured for 14 days with 3μM of the ETB receptor antagonist, BQ788. There is a reduction in formed neointima compared to the control in Fig.8C.3. SMA/Miller elastin stain, magnification x 200.

8c.4 RESULTS

Figures 8c.1 to 8c.4 illustrate the effect of the ET receptor antagonists on vein intimal hyperplasia. The median thickness for veins cultured with the ET_A receptor antagonists was 26μm and 25μm for those cultured in 1μM and 3μM BQ123 respectively. This compares with the control group of 26μm. (P=1.00). The median difference was 0 and 1 μm (95% confidence interval -11.6 to 11.6 and -10.2 to 12.2). for the IμM and 3μM concentrations of BQ123 respectively. This result is shown in figure 8c.5. However, there was a significant reduction in the group treated with the ET_B receptor antagonist. The median thickness was 19μm and 16μm for the veins treated with 1μM and 3μM of BQ788 respectively compared to control 28μm (p=0.03). The median difference was 9μm and 12μm (95% confidence interval 1.7 to 15.3 and 2.4 to 21.6) for the 1μM and 3μM concentrations of BQ788 respectively (figure 8c.6).

The median neointimal thickness measurements for each experiment is detailed in Tables 8c.1 and 8c.2 in appendix 3.

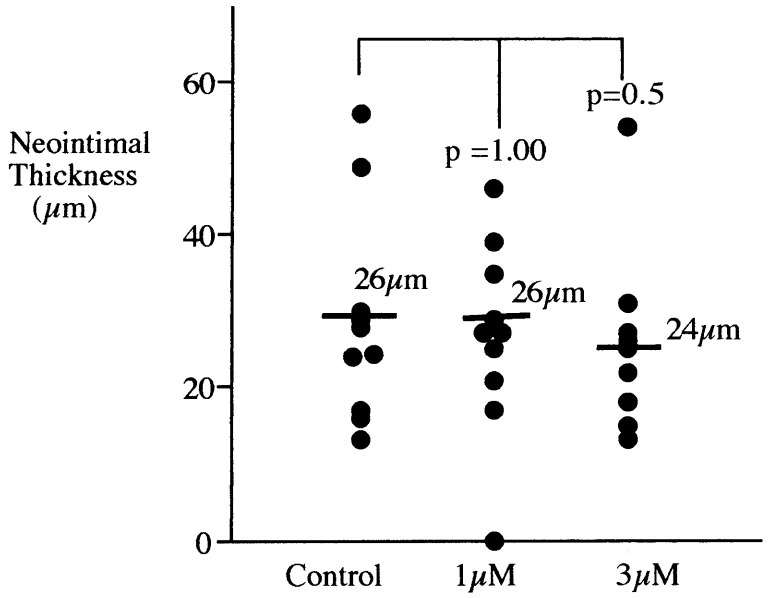


Figure 8c.5 Effect of ETA receptor antagonist. Each bar represents represents median neointma thickness measurements.

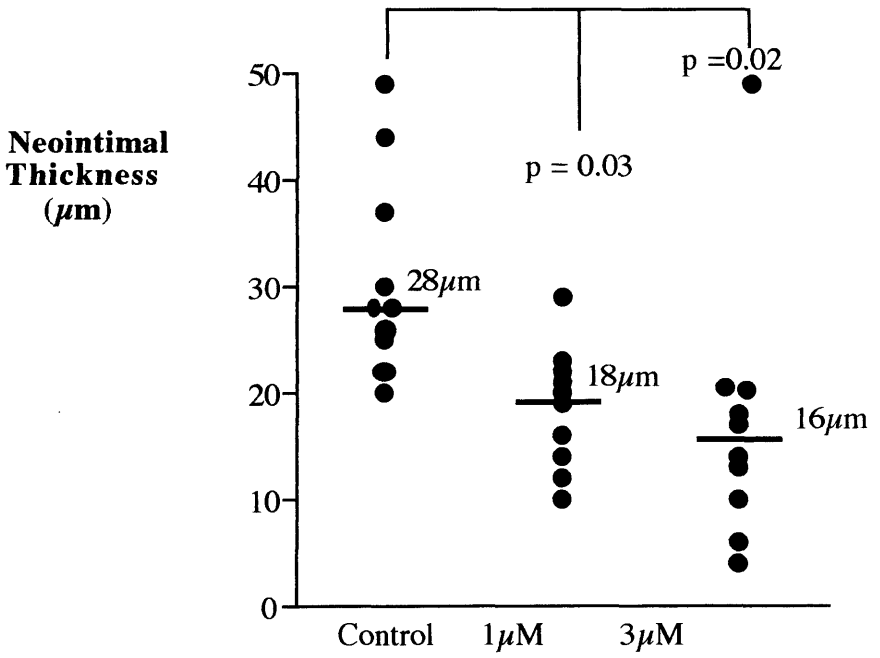


Figure 8c.6. Effect of ETB receptor antagonist
Each bar represents the median neointima thickness measurements.

8d DISCUSSION OF RESULTS OF 8b AND 8c

The experiments in this chapter have described the use of the organ culture model to investigate the role of ET receptors in human vein IH. The first experiment demonstrates that non-selective receptor blockade can reduce intimal hyperplasia by about 40%. The second experiment has shown that the observed reduction in neointima appears to be as a result of ET_B receptor blockade. Blockade of this receptor reduced the neointimal thickness by an average of 40%. Inhibition of the ET_A receptor did not significantly reduce the neointima formation. These observations are in contrast to the results observed in isolated SMCs in chapter 6. This is not necessarily a contradiction of findings, as that study demonstrated that both ET_A and ET_B receptors are required to mediate mitogenesis in early passaged SMCs. An arguable difference between these studies is that the isolated SMCs were stimulated with exogenous endothelin, whereas the organ culture studies were not. On the other hand, the studies in Chapter 7b demonstrated elevated levels of ET in the conditioned media.

Nevertheless the vein organ culture is a more representative model of the features seen in IH taking into account ECM formation. Thus, in the organ culture model where the cell to cell, and cell to matrix interactions are maintained it seems that SMC proliferation and migration is predominantly mediated by the ET_B receptor. Furthermore, it is well recognised that the SMC involved in neointimal formation are phenotypically distinct from SMC found in normal tissue *(Chamley-Campbell et al. 1981)*. This change to the secretory phenotype has been associated with an alteration in receptor expression *(Eguchi et al. 1994; Orlandi et al. 1994)*.

An important consideration in studies using receptor antagonists relates to the properties of the antagonists used. As discussed in Chapter 3, endothelin receptor antagonists can be classified based on their receptor selectivity or on whether they are synthetic or peptide compounds. A number of such antagonists have been developed and used as useful tools in elucidating the role of endothelin and its receptors in various conditions. LU 224332 is a newly developed non peptide and non selective endothelin

receptor antagonist and its role in inhibiting IH has not been previously evaluated. However, its non peptide nature makes it an attractive clinical tool from a pharmacokinetics view point. This is an important step in evaluating the role of ET receptors in IH, as other similar non-selective endothelin antagonists have found a role in clinical trials of systemic diseases. For example, Bosentan and TAK-044 have been entered into phase II clinical trials for clinical conditions such as congestive heart failure and myocardial infarction. Though this in vitro study has demonstrated that non-selective blockade can reduce IH, Patients may not benefit from the systemic consequences such as vasodilatation that has been seen with dual receptor blockade *(Kiowski et al. 1995).*

Other authors have demonstrated that non-selective ET receptor blockade reduces neointimal formation in animal models of post-angioplasty restenosis *(Azuma et al. 1994; Douglas et al. 1995a).* Douglas and colleagues demonstrated a 50% reduction in neointimal thickness following chronic twice daily intraperitoneal injections of the non-selective antagonist SB209670 into rats following carotid artery angioplasty. These same authors failed to demonstrate an effective amelioration of neointima in animals treated with ET_A receptor antagonists *(Azuma et al. 1994; Douglas et al. 1995b).* Thus they proposed that the ET_B receptors may play a more significant role in neointima formation. In support of these observations, a study by Azuma et al demonstrated an upregulation of the ET_B receptor localised mainly in the neointima of rabbit hyperplastic arteries *(Azuma et al. 1995).* In that same study however, a number of receptor sites were not bound by either the ET_A or the ET_B receptor antagonist. The authors proposed the presence of non-ET_A/non-ET_B receptors, However the empty binding sites may have been related to the affinity of the antagonists used. BQ123 and BQ788 are well characterised selective receptor antagonists. The results of this study suggest that for the purposes of amelioration of IH, investigators can now target the mechanisms involved in ET_B receptor activation, and avoid unnecessary ET_A blockade. However there may be other unexplained mechanisms for the effects observed in this study and these take into account the limitations of this study. For

example ET receptor antagonism is known to modulate receptor expression and ET peptide release*(Eguchi et al. 1994)*. The timing and duration of the receptor antagonism may also be critical. prolonged culture beyond 14 days may reveal differing outcomes. In vivo, intimal hyperplasia takes longer than 14 days to develop. A minority of veins did not respond to ET_B receptor inhibition. This suggests that a subpopulation of vein grafts may not respond in the clinical setting.

Indeed, not all studies agree that the ET_B plays an exclusive role in IH. The study by Wang and colleagues on rat carotid intimal hyperplasia, demonstrated an upregulation of the mRNA of both ET_A and ET_B receptors within 24 hours of balloon injury that was demonstrable (albeit at lower levels) after 14 days *(Wang et al. 1996)*. Though the presence of the receptor mRNA or indeed its peptide does not indicate its functional capability. Another consideration is the interaction that exists between ET and its receptors. Whilst Yu and colleagues found that ET_A antagonism increased ET levels in vitro *(Yu and Davenport 1995a)*, some authors believe that in vivo ET_B receptor antagonism is associated with an increase in circulating ET-1 levels *(Fukuroda et al. 1994; Haynes et al. 1996)*. Though it would seem that most reports from other studies are in agreement with the current study, any comparison has to be made with caution. The studies discussed above were performed in living animal arterial models which would also be influenced by neurohumoral factors and the in vivo flow conditions. ET receptor distribution and function can vary greatly from one species to another. On the other hand such similarities in findings also serve to validate the use of the static organ culture models in experimental IH. These studies would form the basis for further in vivo studies using animal models of infrainguinal vein bypass grafts. This would pave the way for clinical trials in the treatment and prevention of vein graft stenosis and indeed restenosis.

CHAPTER 9

SUMMARY, CONCLUSIONS AND FUTURE WORK

SUMMARY, CONCLUSIONS AND FUTURE WORK

SUMMARY

This thesis has examined clinical and laboratory aspects of saphenous vein graft intimal hyperplasia. In the clinical setting, IH manifests to the surgeon as vein graft stenosis. Left untreated, a significant proportion of such lesions will cause the graft to fail. Treatment of IH and the difficulties associated with it have been discussed. This highlighted the extensive research effort that has cumulated in a better understanding of these lesions since they were first documented by Szilagyi. However the diversity in the number of postulated and proven aetiological factors has lead researchers down many strategic pathways. Sadly, to date none of them have produced an effective method of prevention.

The current strategy in the management of graft stenosis is largely based on early detection and intervention. There is a large body of evidence that agrees that this strategy reduces graft failure even though it does nothing to prevent stenosis. Nevertheless the research into producing effective control of IH has to continue as a matter of necessity. In these respects the studies reported in this thesis have attempted to fine tune current clinical practice as well as proposing a future pharmacological strategy to prevent IH.

The retrospective study in Chapter 4 attempted to identify clinical aetiological factors that significantly influenced long-term graft patency. Such factors could be optimised in order to improve patency rates. The finding that none of the factors independently influenced graft stenosis is testimony of the ubiquitous nature of its multifactorial aetiology. Chapter 5 examined graft surveillance. Section 5a concluded that it was unnecessary to scan vein grafts within 2 weeks of implantation or just prior to discharge from hospital. It showed that the findings from such scans were not predictive of future stenosis. More importantly however that study provides evidence that graft surveillance can be safely started from 4 weeks after implantation thus reducing the workload on the vascular unit surveillance programme. Section 5b addressed a controversial issue in graft surveillance and concluded that using a PVR of

3.0 as the threshold for intervention did not impair graft patency. From the findings of these two studies it has been possible to propose a modification to the current graft surveillance program both in Leicester and in other centres.

Proliferating SMC play a central role in IH. Thus Chapter 6 explored the role of endothelin and endothelin receptor antagonists on the proliferation of isolated SMCs. That study demonstrated that endothelin can induce proliferation in saphenous vein SMCs. Furthermore, both endothelin receptors were found to be important in promoting this proliferation. Interestingly, that study also found that some SMC isolates did not respond to endothelin stimulation. However isolated SMCs are not representative of all the features that occur in the biology of IH. Thus the vein organ culture model was used in subsequent studies. The role of endothelin peptide in neointimal formation was established in Chapter 7. Activation of the endothelin system was demonstrated in both the immunosorbent assay and immunohistochemical studies. From these results, the experiments in Chapter 8 set out to determine the effects of inhibition of endothelin at various levels. Using the organ culture, in 8a, endothelin was inhibited at the level of synthesis by the ECE inhibitor. This resulted in just under 50% reduction in neointima. A similar effect was found when a non-selective receptor was used in 8b. However, when selective endothelin receptor antagonists where used in the organ culture model, the study in 8c concluded that the ET_B receptor was responsible for endothelin mediated neointima formation.

CONCLUSIONS AND FURTHER WORK

Endothelin has been shown to play a significant role in the formation of IH in vein grafts. In this thesis, the mechanisms by which it does this has been traced down to the receptor level. This observed that out of the two ET receptors, the ET_B receptor is more specific for human vein graft intimal hyperplasia. Thus inhibition of this receptor subtype could ameliorate vein graft stenosis.

No doubt several important factors have not been evaluated in this study and require further research before firm conclusions can be made regarding these findings. The mechanisms of the observed effect of the ET_B receptor on intimal hyperplasia have not been elucidated. The significance of the vein and SMC isolates that did not respond to endothelin or its antagonists necessitates further research, as it is of potential clinical significance.

The static organ culture model cannot evaluate the mitogenic effects of endothelin under the different flow conditions as seen in vivo. Thus an in vitro organ culture flow model would add valuable information to the findings from this study. Further immunohistochemical studies would localise specific endothelin receptor expression within the neointima. Since the extracellular matrix constitutes a significant proportion of the neointima, the role of endothelin in matrix metalloprotease expression is also worth studying.

The versatility of the organ culture model demonstrated in this thesis can be utilised to asses the potency of future newly developed ET_B receptor antagonists in reducing IH. These newer drugs could have more favourable pharmacological properties than currently available peptides. The use of human veins in this thesis is a clear advantage over previous experimental and animal models that have been used to study the effects of pharmacological agents in IH. Thus, though clinical trials using some form of endothelin antagonism may be several steps away, the encouraging results from this thesis make it feasible in the near future.

APPENDIX

Appendix 1 References to Table 1c.4

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Appendix 2

Vein Transport medium : Calcium-free krebs solution

Sodium chloride	6.9g
Potassium chloride	0.34g
Magnesium sulphate	0.25g
Potassium dihydrophosphate	0.14g
Glucose	1.08g
Sodium carbonate	2.1g
Made up in 1 litre of sterile water	

Smooth muscle cell culture medium

RPMI (Northumbria Biologicals, Cramlington)	176mls
Foetal Calf serum	20mls
L-glutamine 2mmol/L (Northumbria Biologicals, Cramlington)	2mls
Penicillin 50U/ml + Streptomycin 50μg/ml	2ml

Trypsinization technique for smooth muscle cells

1. Aspirate and discard all the cell culture medium from the flask.

2. Wash the cells twice with 5mls MEM in order to remove all traces of Foetal calf serum which inhibits trypsin

3. Add 1ml of working strength trypsin /EDTA (T/E) solution to the flask and tilt several times to ensure distribution over the cell layer. Screw flask top tightly and incubate at 37^0C for up to 5 minutes. Agitate from time to time and view cells under the light microscope

4. Once there is complete detachment of the layer in single cells, add 6-8mls of SMC medium mix gently and aliquot equal volumes of medium into two new T25 flasks.

To make up 0.1% Trypsin/ 0.02% EDTA solution :

2.5% trypsin (Gibco BRL, Paisley, Scotland)	20ml
1% EDTA solution (Fisons, Loughborough, Leicestershire)	10mls
1M HEPES buffer (Gibco BRL)	10mls
Phosphate Buffer Saline (PBS)	460mls

Simpson's rule

A= dx*(y[0]+4*y[1]+y[2])/6

Where A = Area under curve

dx = Equally spaced intervals on the x axis

y = Values of Y at each point

Appendix 3

Vein culture medium (500mls)

RPMI 1640 (Northumbria Biologicals, Cramlington)	280 mls
Foetal calf serum	120 mls
Penicillin 50U/ml + Streptomycin 50μg/ml	4mls
L-glutamine 2mmol (Northumbria Biologicals, Cramlington)	4mls

4% Formaldehyde

PBS	100 mls
Paraformaldehyde	4g

Heat up PBS to 70°C and stir in paraformaldehyde, add 6 drops of 2 molar solution sodium hydroxide to cloudy solution, filter into light protected flask then cool in refrigerator.

Appendix 4

Table 7b.1 Net amount of endothelin found in 6 mls of media after each 48 hour culture period

Production of endothelin in fmol/48 hours

Experiment number	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
EXP. 1	0	8.1	8.2	9.42	3.12	2.1	0.00
EXP. 2	0.06	5.82	15.2	18.8	16.9	6.96	6.36
EXP. 3	0.0	0.00	5.94	8.1	4.14	4.2	28.2
EXP. 4	45.6	7.32	70.8	45	55.8	6.00	5.76
EXP. 5	6.06	0.00	24.5	4.32	0.00	0.00	10.2
EXP. 6	19.2	0.00	51.6	82.8	70.2	70.2	43.8
EXP. 7	14.34	40.26	29.1	31.26	26.5	7.2	15.3
EXP. 8	18.42	19.92	49.2	110.4	56.6	94.2	86.4

Table 7b.2 Net amount of big ET found in 6 mls of medium after each 48 hour culture period

Production of big endothelin in fmol/48 hours

Experiment number	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
EXP. 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
EXP. 2	0.00	0.00	0.96	2.9	8.3	0.00	3.4
EXP. 3	0.00	2.6	7.2	7.7	7.6	5.6	8.8
EXP. 4	0.00	0.9	10.9	0.1	0.2	2.3	9.7
EXP. 5	0.00	0.00	14.2	9.3	9.3	11.2	4.7
EXP. 6	0.00	0.00	0.00	0.00	0.00	0.00	0.00
EXP. 7	4.7	3.3	9.9	6.5	2.8	1.3	1.5
EXP. 8	2.6	8.1	12.1	10.1	5.4	5.4	4.6

Table 7b.3 Development of neointima during 14 days of organ culture.

Neointima thickness in micrometers

Experiment number	Day 4	Day 8	Day 12	Day 14
EXP. 1	0	2	17	18
EXP. 2	0	5	12	14
EXP. 3	0	2	8	10
EXP. 4	0	10	19	19
EXP. 5	0	10	30	32

Table 8a.1 Effect of ECE inhibitor on saphenous vein intimal hyperplasia

Neointimal thickness (μm)

Experiment	Control	CSG26303 (50μM)
1	14	11
2	26	6
3	23	8
4	23	11
5	23	16
6	26	9
7	16	16
8	20	14
9	27	23
10	26	12
11	46	12

Table 8b.1 Effect of non-selective receptor blockade on neointimal hyperplasia.

	Neointimal thickness (μm)		
Experiment	**Control**	**LU224332 10^{-6}M**	**LU224332 10^{-7}M**
1	15	7	17
2	29	10	9
3	10	7	7
4	12	5	7
5	14	6	3
6	28	12	9
7	40	28	16
8	45	15	34
9	22	16	35
10	18	21	20

Table 8c.1 Effect of ET_A receptor blockade on neointimal hyperplasia.

	Neointimal thickness (μm)		
Experiment	**Control**	**BQ123 (1μM)**	**BQ123 (3μM)**
1	24	26	31
2	29	0	22
3	24	26	26
4	49	39	15
5	16	17	13
6	56	46	54
7	28	29	27
8	17	25	18
9	13	21	25
10	30	35	26

Table 8c.2 Effect of ET_B receptor blockade on neointimal hyperplasia

	Neointimal thickness (μm)		
Number	**Control**	**BQ788 (1μM)**	**BQ788 (3μM)**
1	22	23	13
2	28	10	14
3	30	14	49
4	49	12	4
5	44	19	21
6	25	20	18
7	20	29	17
8	26	22	10
9	28	16	6
10	37	21	21

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