ENDOTHELIAL SEEDING OF ANGIOPLASTY SITES

By

Matthew M Thompson

A Thesis Submitted for the Degree of

Doctor of Medicine

From

The Department of Surgery, University of Leicester

April 1993
The work on which this thesis is based is my own independent work except where acknowledged.

MM Thompson
April 1993
Dedicated to Tricia and Luke.

"If I have seen further, it is by standing on the shoulders of giants"
Isaac Newton (1642 - 1727)

"Angioplasty and Restenosis: Endothelium Remains the Sticking Point"
SYNOPSIS

The major causes of mortality and morbidity complicating percutaneous transluminal angioplasty (PTA) are acute arterial re-occlusion and the development of chronic restenosis at the angioplasty site. These complications may affect PTA in 40-50% of cases, and their occurrence is directly attributable to loss of the endothelial cell monolayer during balloon dilatation. This study examined the hypothesis that acute and chronic occlusive complications following balloon angioplasty may be reduced by rapid restoration of the endothelial monolayer, achieved in turn by endothelial seeding.

To define the optimum parameters for endothelial seeding of angioplasty sites, endothelial attachment to an in vitro model of vascular damage was quantified. Maximal endothelial adherence to damaged vascular surfaces was achieved with a seeding time of 30 min and a seeding density exceeding $3 \times 10^5$ cells/cm². Although initial endothelial adherence is a prime determinant of successful endothelial seeding, cell retention in arterial flow and maintenance of endothelial function are equally important. These parameters were investigated in vitro and the results suggested that native vascular surfaces provided an excellent substrate for endothelial seeding.

Following on from the favourable results achieved during in vitro experiments, a method to transluminally seed experimental angioplasty sites was investigated in vivo. This method used a specially designed double balloon catheter to deliver and retain cultured endothelial cells at angioplasty sites. Using this technique, initial endothelial adherence to dilated arterial segments was 49%, with 35% of the initially attached cells remaining attached after 100 min of arterial flow.

Subsequent experiments revealed that transluminal endothelial seeding reduced acute platelet deposition following angioplasty, and in the longer term reduced both the degree of injury induced myointimal hyperplasia and the rate of arterial occlusion. Experimental endothelial seeding of angioplasty sites reduced acute and chronic complications after balloon dilatation and clearly has clinical potential as a therapeutic technique.
ACKNOWLEDGEMENTS

The work described in this thesis was carried out in the Department of Surgery, University of Leicester under the guidance of Professor PRF Bell, to whom I am extremely grateful for his continual support and encouragement. I am similarly indebted to Mr JS Budd whose original idea forms the basis of this work, and to Dr RFL James for his constant interest and guidance. All of the in vivo experimentation was performed in the Department of Biomedical Services, Leicester University, under the guidance of Dr D Forbes, and I would like to thank him and the staff of this Unit for their invaluable assistance.

I am grateful to the Wellcome Trust who provided me with a salary for 18 months of research and to the British Heart Foundation who funded the project with 2 project grants. Similar gratitude is due to Mr P Draycon, Ms S Heap, Meadox, UK Ltd, and Ideas for Medicine, USA, who manufactured the special catheters used in this investigation and who provided much of the angioplasty equipment free of charge.

One of the daunting aspects of embarking on a laboratory based investigation is the plethora of new techniques to be learnt and mastered. I am particularly grateful to Mrs Sarah Eady who provided exemplary technical advice and assistance throughout the project, especially when problems were encountered.

It remains difficult to express my appreciation of everyone who helped me throughout this project, but I am grateful to all those mentioned below for their individual contributions.

All the technicians of the Department of Surgery, and particularly Mr R Chamberlain and Mrs K Allen for their advice and assistance.

Dr H Pringle and the Department of Pathology for the processing and interpretation of light microscopic slides. Mr George McTurk for his hard work and patience when processing the numerous samples for scanning electron microscopy. Dr M James for his assistance in reporting light micrographs.

Messrs Bailey, Leverment, Firmin, Hickey and Spyt at Groby Road Hospital for provision of venous samples. Mr M Early and Ms G Hartley for labelling platelet suspensions and performing the gamma scintillation scans described in chapter 11.

Mr MJ Underwood for acting as a “blinded” observer and measuring the degree of myointimal hyperplasia in chapter 12.

Dr A Bolia for provision of angiograms.

The staff of the Clinical Sciences Library, Leicester Royal Infirmary for their patience in dealing with my many requests over the past two years.

The Maternity Department, Leicester Royal Infirmary for allowing us to collect human umbilical cords.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AEC</td>
<td>Adult endothelial cell</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>b-FGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>C</td>
<td>Complement factor</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanine monophosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium derived relaxing factor</td>
</tr>
<tr>
<td>EEL</td>
<td>External elastic lamina</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ELAM</td>
<td>Endothelial leucocyte adhesion molecule</td>
</tr>
<tr>
<td>ePTFE</td>
<td>Expanded polytetrafluoroethylene</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical venous endothelial cell</td>
</tr>
<tr>
<td>ICAM</td>
<td>Leucocyte specific adhesion molecule</td>
</tr>
<tr>
<td>IEL</td>
<td>Internal elastic lamina</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Gamma interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IH</td>
<td>Myointimal hyperplasia</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin 1</td>
</tr>
<tr>
<td>L-Arg</td>
<td>L-Arginine</td>
</tr>
<tr>
<td>LMWH</td>
<td>Low molecular weight heparin</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>N&lt;sub&gt;R&lt;/sub&gt;</td>
<td>Reynolds number</td>
</tr>
<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Oxygen</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAI</td>
<td>Plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PF</td>
<td>Platelet factor</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PGI₂</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PTA</td>
<td>Percutaneous transluminal angioplasty</td>
</tr>
<tr>
<td>PTCA</td>
<td>Percutaneous transluminal coronary angioplasty</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SFA</td>
<td>Superficial femoral artery</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>t-PA</td>
<td>Tissue type plasminogen activator</td>
</tr>
<tr>
<td>TFA</td>
<td>Thrombus free area</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TxA₂</td>
<td>Thromboxane A₂</td>
</tr>
<tr>
<td>u-PA</td>
<td>Urokinase type plasminogen activator</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
</tbody>
</table>
PUBLICATIONS ARISING FROM THIS THESIS.

Published Papers.

Platelet deposition following angioplasty is abolished by rapid restoration of the endothelial monolayer.
Thompson MM, Budd JS, Eady SL, Hartley J, Early M, James RFL, Bell PRF.

A method to transluminally seed angioplasty sites with endothelial cells.
Thompson MM, Budd JS, Eady SL, James RFL, Bell PRF.

Effect of seeding time and seeding density on endothelial cell attachment to damaged vascular surfaces.
Thompson MM, Budd JS, Eady SL, Allen KE, James M, James RFL, Bell PRF.

Endothelial cell seeding of native vascular surfaces: prostacyclin production.
Thompson MM, Budd JS, Eady SL, Allen KE, James M, James RFL, Bell PRF.

Published Abstracts.

Thrombosis following angioplasty is abolished by rapid restoration of the endothelial cell monolayer.
Thompson MM, Budd JS, Eady SL, James RFL, Bell PRF.

Thrombogenicity following angioplasty is abolished by rapid restoration of the endothelial cell monolayer.
Thompson MM, Eady SL, Budd JS, James RFL, Bell PRF.
Clinical Science 1993 (In Press).

A method for endothelial cell transplantation.
Thompson MM, Eady SL, Budd JS, James RFL, Bell PRF.
Transluminal endothelial seeding of angioplasty sites: initial results using a double balloon catheter.
Thompson MM, Budd JS, Eady SL, James RFL, Bell PRF.
Cell Transplantation 1992: 1(2/3); 216 (Abstract).

Prostacyclin production by endothelial seeded angioplasty sites.
Thompson MM, Budd JS, Allen KE, James M, Bell PRF.

Endothelial seeding of angioplasty sites: prostacyclin production.
Thompson MM, Budd JS, Allen KA, Thurston H, Bell PRF.
Clinical Science 1992; 82 (Suppl 26); 2p (Abstract).

Presentations Arising From This Thesis.

Thrombosis is abolished by endothelial cell transplantation.
Thompson MM, Budd JS, Eady SL, James RFL, Bell PRF.
Association of Surgeons of Great Britain and Ireland, Birmingham, April 1993.

Thrombosis is abolished by restoration of the endothelial monolayer.
Thompson MM, Budd JS, Eady SL, James RFL, Bell PRF.

Thrombogenicity following angioplasty is abolished by rapid restoration of the endothelial cell monolayer.
Thompson MM, Budd JS, Eady SL, James RFL, Bell PRF.

Thrombogenicity following balloon angioplasty is abolished by restoration of the endothelial cell monolayer.
Thompson MM, Budd JS, Eady SL, Bell PRF.

Platelet deposition following angioplasty is reduced by rapid restoration of the endothelial cell monolayer.
Thompson MM.
Thrombogenicity following angioplasty is abolished by rapid restoration of the endothelial cell monolayer.
Thompson MM, Eady SL, Budd JS, James RFL, Bell PRF.

A method to transluminally seed angioplasty sites with endothelial cells.
Thompson MM, Budd JS, Eady SL, James RFL, Bell PRF.

A method for endothelial cell transplantation.
Thompson MM, Eady SL, Budd JS, James RFL, Bell PRF.

Transluminal endothelial seeding of angioplasty sites: initial results using a double balloon catheter.
Thompson MM, Budd JS, Eady SL, James RFL, Bell PRF.

Prostacyclin production by endothelial seeded angioplasty sites.
Thompson MM, Budd JS, Allen KE, James M, Bell PRF.

Endothelial seeding of angioplasty sites: prostacyclin production.
Thompson MM, Budd JS, Allen KA, Thurston H, Bell PRF.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statement of Originality</td>
<td>i</td>
</tr>
<tr>
<td>Dedication</td>
<td>ii</td>
</tr>
<tr>
<td>Synopsis</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>v</td>
</tr>
<tr>
<td>Publications</td>
<td>vi</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>vii</td>
</tr>
<tr>
<td></td>
<td>viii</td>
</tr>
<tr>
<td></td>
<td>ix</td>
</tr>
<tr>
<td>Chapter 1</td>
<td></td>
</tr>
<tr>
<td>Atherosclerosis and Peripheral Vascular Disease</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Introduction.</td>
<td>2</td>
</tr>
<tr>
<td>1.2 Pathology of Atherosclerosis.</td>
<td>2</td>
</tr>
<tr>
<td>1.3 Theories of Atherogenesis.</td>
<td>3</td>
</tr>
<tr>
<td>1.4 Peripheral Vascular Disease.</td>
<td>5</td>
</tr>
<tr>
<td>1.5 Incidence and Prevalence of Peripheral Vascular Disease.</td>
<td>7</td>
</tr>
<tr>
<td>1.6 Risk Factors for Peripheral Vascular Disease.</td>
<td>9</td>
</tr>
<tr>
<td>1.7 Treatment of Peripheral Vascular Disease.</td>
<td>10</td>
</tr>
<tr>
<td>Chapter 2</td>
<td>13</td>
</tr>
<tr>
<td>Regulatory Functions of the Vascular Endothelium</td>
<td></td>
</tr>
<tr>
<td>2.1 Introduction.</td>
<td>14</td>
</tr>
<tr>
<td>2.2 Vasoactive Mediators and Control of Vascular Tone.</td>
<td>14</td>
</tr>
<tr>
<td>2.3 Regulation of the Coagulation System by Vascular Endothelial Cells.</td>
<td>19</td>
</tr>
<tr>
<td>2.4 Interaction of the Endothelium with Circulating Platelets.</td>
<td>23</td>
</tr>
<tr>
<td>2.5 Endothelial Cell - Leucocyte Interactions.</td>
<td>24</td>
</tr>
<tr>
<td>2.6 Vascular Endothelium and the Immune Response.</td>
<td>25</td>
</tr>
<tr>
<td>Chapter 3</td>
<td></td>
</tr>
<tr>
<td>Endothelial Cell Seeding</td>
<td>27</td>
</tr>
<tr>
<td>3.1 Introduction.</td>
<td>28</td>
</tr>
<tr>
<td>3.2 Prosthetic Graft Failure.</td>
<td>29</td>
</tr>
<tr>
<td>3.3 Sources of Endothelial Cells.</td>
<td>31</td>
</tr>
<tr>
<td>3.4 The Seeding Process.</td>
<td></td>
</tr>
<tr>
<td>3.5 Results of Experimental Endothelial Cell Seeding.</td>
<td>35</td>
</tr>
<tr>
<td>3.6 Clinical Trials of Endothelial Seeding.</td>
<td>39</td>
</tr>
<tr>
<td>Chapter</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>7</td>
<td>Results</td>
</tr>
<tr>
<td>7.4</td>
<td>Discussion</td>
</tr>
<tr>
<td>7.5</td>
<td>Summary</td>
</tr>
<tr>
<td>8</td>
<td>Chapter 8</td>
</tr>
<tr>
<td>8.1</td>
<td>The Effect of Shear Stress on Endothelial Retention to Native Vascular Surfaces</td>
</tr>
<tr>
<td>8.1</td>
<td>Introduction</td>
</tr>
<tr>
<td>8.2</td>
<td>Methods</td>
</tr>
<tr>
<td>8.3</td>
<td>Results</td>
</tr>
<tr>
<td>8.4</td>
<td>Discussion</td>
</tr>
<tr>
<td>8.5</td>
<td>Summary</td>
</tr>
<tr>
<td>9</td>
<td>Chapter 9</td>
</tr>
<tr>
<td>9.1</td>
<td>Prostacyclin Release From Seeded Native Vascular Surfaces</td>
</tr>
<tr>
<td>9.1</td>
<td>Introduction</td>
</tr>
<tr>
<td>9.2</td>
<td>Methods</td>
</tr>
<tr>
<td>9.3</td>
<td>Results</td>
</tr>
<tr>
<td>9.4</td>
<td>Discussion</td>
</tr>
<tr>
<td>9.5</td>
<td>Summary</td>
</tr>
<tr>
<td>10</td>
<td>Chapter 10</td>
</tr>
<tr>
<td>10.1</td>
<td>Transluminal Endothelial Seeding of Angioplasty Sites:</td>
</tr>
<tr>
<td>10.1</td>
<td>Attachment Kinetics and Retention in Arterial Flow</td>
</tr>
<tr>
<td>10.1</td>
<td>Introduction</td>
</tr>
<tr>
<td>10.2</td>
<td>Methods</td>
</tr>
<tr>
<td>10.3</td>
<td>Results - Pilot Experiment</td>
</tr>
<tr>
<td>10.4</td>
<td>Results - Definitive Experiment</td>
</tr>
<tr>
<td>10.5</td>
<td>Discussion</td>
</tr>
<tr>
<td>10.6</td>
<td>Summary</td>
</tr>
<tr>
<td>11</td>
<td>Chapter 11</td>
</tr>
<tr>
<td>11.1</td>
<td>The Effect of Transluminal Endothelial Seeding on Platelet Deposition Following Angioplasty</td>
</tr>
<tr>
<td>11.1</td>
<td>Introduction</td>
</tr>
<tr>
<td>11.2</td>
<td>Methods</td>
</tr>
<tr>
<td>11.3</td>
<td>Results</td>
</tr>
<tr>
<td>11.4</td>
<td>Discussion</td>
</tr>
</tbody>
</table>
Chapter 12
The Effect of Transluminal endothelial Seeding on Myointimal Hyperplasia Following Angioplasty
12.1 Introduction. 174
12.2 Methods. 175
12.3 Results. 179
12.4 Discussion. 190
12.5 Summary. 194

Chapter 13
Conclusions and Prospects for Future Research

Appendix A 200
Appendix B 202
Appendix C 210
Appendix D 213
Bibliography 215
CHAPTER 1
ATHEROSCLEROSIS AND PERIPHERAL VASCULAR DISEASE

1.1 Introduction. 2

1.2 Pathology of Atherosclerosis. 2

1.3 Theories of Atherogenesis. 3

1.4 Peripheral Vascular Disease. 5
- Symptomatology 5
- Intermittent claudication 6
- Ischaemic rest pain 6

1.5 Incidence and Prevalence of Peripheral Vascular Disease. 7
- Progression of disease 7

1.6 Risk Factors for Peripheral Vascular Disease. 9

1.7 Treatment of Peripheral Vascular Disease. 10
- Pharmacologic agents 10
- Percutaneous transluminal angioplasty 11
- Operative surgical procedures 12
ATHEROESCLEROSIS AND PERIPHERAL VASCULAR DISEASE

1.1 Introduction.

Cardiovascular disease is the chief cause of death in the Western world, with atherosclerosis accounting for the majority of these deaths [Report of the working group of atherosclerosis of the National Heart, Lung and Blood Institute, 1981]. Atherosclerosis is a widely prevalent disorder affecting large elastic and muscular arteries, which is present to some extent in virtually all adult members of the community [Walter, Israel, 1987]. In this chapter the pathology and aetiology of atherosclerosis will be discussed, initially in general terms and then as specifically applied to peripheral vascular disease.

1.2 Pathology of Atherosclerosis.

The characteristic lesion of atherosclerosis is the fibro-fatty plaque, which is composed of a fibrous cap overlying an inner yellow core [Ross et al, 1984]. The fibrous cap contains multiple layers of smooth muscle cells (SMCs) and connective tissue elements, beneath which is a highly cellular lesion, containing further SMCs, macrophages and extra-cellular lipid droplets. This lesion rests upon an area of necrotic debris, cholesterol crystals and calcification.

Fibro-fatty plaques represent the end stage of atherosclerosis, which may evolve from precursor lesions (fatty streaks and gelatinous plaques) found in the arteries of children and young adults. Fatty streaks are grossly flat, lipid rich lesions consisting of macrophages and SMCs [Stary, 1983], which occur at the same anatomical sites as atherosclerotic plaques in later life [Leary, 1941; Robertson et al, 1963]. Gelatinous plaques are commonly found in the aorta and contain SMCs in combination with cross-linked fibrin [Smith et al, 1976]. Fatty streaks and gelatinous plaques may both potentially progress to fibro-fatty plaques [Ross, 1986], and may thus be regarded as early atherosclerotic lesions.

Atherosclerotic plaques occur throughout the arterial tree but are characteristically severe in the coronary and cerebral arteries, carotid bifurcation, infra-renal aorta and ilio-femoral vessels. Their clinical manifestations depend upon the effect that the lesion has on the arterial wall and lumen. In general, there are four clinical sequelae; chronic arterial occlusion, acute arterial occlusion, aneurysm formation and distal embolisation. Chronic arterial occlusive disease is caused by protrusion of the fibro-lipid plaque into the arterial lumen, causing stenosis or occlusion of the affected artery. The clinical features associated with chronic arterial occlusion are due to ischaemia, and vary with the site affected.

Plaques may be complicated by calcification, ulceration, haemorrhage or extensive
Peripheral Vascular Disease

necrosis. Plaque rupture with rapid expansion of the atheromatous core may cause sudden vascular occlusion, as may plaque dissection [Friedmen, van Den Bovenlcmp, 1966; Davies, Thomas, 1984]. Carotid plaques seem particularly prone to rupture into the arterial lumen resulting in distal embolisation or thrombosis [Lusby et al. 1982].

1.3 Theories of Atherogenesis.

Two theories dominated early research on the aetiology and pathogenesis of atherosclerosis. The "lipid filtration" hypothesis postulated that the cellular changes in an atherosclerotic plaque were secondary to lipid infiltration [Virchow, 1971; Anitschkow, 1931], whilst the "thrombogenic theory", proposed by Rokitansky [von Rokitansky, 1852], suggested that fibro-fatty plaques developed from blood products incorporated into the vessel wall. Both theories were supported by some experimental evidence, but neither satisfactorily explained the spectrum of changes that occur in atherosclerosis, especially the central role of SMCs [Ross, 1986].

In 1976 Ross and Glomset [Ross, Glomset, 1976a; Ross, Glomset, 1976b] reasoned that atherosclerosis was initiated in response to endothelial cell damage - the "response to injury hypothesis" [Ross, 1981; Ross, 1986]. In a non-damaged vessel the endothelium comprises an anti-thrombogenic interface between the bloodstream and the arterial wall [Reidy, 1985], damage to which may initiate a chain of events ultimately resulting in the formation of a fibro-fatty plaque.

Endothelial injury may be caused by numerous agents, some of which may be recognised as established risk factors for atherosclerosis, e.g. mechanical forces [Fry, 1973; Moore, 1973]; lipoproteins [Nelson et al. 1976; Weger et al. 1974]; immunoglobulins [Hardin et al. 1973]; and toxins [Harker et al. 1974]. The spectrum of endothelial injury ranges from complete desquamation, through single cell detachment [Reidy, Schwartz, 1984], to functional changes that have no morphological manifestation, but still result in physiological dysfunction, e.g. increased permeability and the release of mitogenic growth factors [Ross, 1986].

Secondary to endothelial cell damage, platelets adhere to the sub-endothelial matrix, aggregate and release their contents [Ross, 1981]. Platelets contain several SMC mitogens [Ross et al. 1974; Oka, Orth, 1983], as well as numerous chemotactic and thrombogenic agents. These factors increase the thrombotic tendencies around sites of arterial injury and also act on the medial SMC's to stimulate migration and proliferation. This proliferative SMC response is crucial to the development of atherosclerosis and is reduced if platelets are absent from the circulation [Moore et al. 1976; Friedman et al. 1977].

Recent evidence has suggested that the macrophage / monocyte system plays a
pivotal role in atherogenesis. Sub-endothelial migration and localisation of monocytes initiate the formation of fatty streaks [Stary, 1983]. Monocytes have the capacity to internalise lipoproteins to form foam cells [Brown, Goldstein, 1983] and more importantly are an integral component of the inflammatory reaction, with the facility to damage neighbouring cells by secreting toxic substances, and to stimulate connective tissue growth by secretion of fibroblast growth factors [Leslie et al. 1984; Dohlman et al. 1984]. T lymphocytes are also present at the sites of endothelial injury [Jonasson et al. 1986], and have the capacity to stimulate SMC proliferation [Libby et al. 1988], induce expression of leucocyte adhesion molecules on the endothelium [Luscinias et al. 1991], and regulate lipoprotein uptake [Ross, 1986]. The involvement of immunocompetent cells in atherogenesis [Hansson et al. 1989; Libby, Hansson, 1991] has prompted a modification of the "response to injury" hypothesis, in which it is suggested that initial endothelial cell injury or dysfunction leads to an inflammatory response that then initiates excessive SMC proliferation and migration [Fogleman et al. 1981] (Fig. 1.1).

Smooth muscle cells are found in fatty streaks and in fibro-fatty plaques. Their proliferation may partly determine the size of the plaque, but they are also capable of synthesising large amounts of connective tissue [Burke, Ross, 1979], and accumulating lipid [Chait et al. 1980]. Smooth muscle cells respond to chemotactic and mitogenic signals produced after endothelial injury, but these signals, when produced in isolation, may be insufficient to induce SMC proliferation and formation of a plaque. Reidy and Silver [Reidy, Silver, 1985] demonstrated that endothelial injury in the absence of medial damage did not initiate intimal thickening, and it is probable that damage to both the intima and media is required to initiate atherosclerosis.

The quantitative increase in SMC number within an atherosclerotic plaque may originate from migration of medial cells or from rapid division of intimal stem cells. Most cells in an individual plaque are genetically monotypic in heterotypic individuals [Benditt, Benditt, 1973], which strongly suggests that plaque development is due to monoclonal proliferation of smooth muscle stem cells. The stimulus for proliferation may be viral [Fabricant et al. 1983], but is more likely to be in response to exogenous noxious stimuli. This scenario is compatible with the "response to injury" hypothesis in which it is envisaged that SMC proliferation occurs due to arterial damage. During the resulting polyclonal proliferation of SMCs, the properties of one clone render them superior for proliferation in the microenvironment of the arterial wall, which would result in clonal selection among the SMC population and the typical monotypism observed in the atherosclerotic lesion [Bondjers et al. 1991]. The cellular events that occur during atherogenesis are almost identical to those described during the formation of myointimal hyperplasia following arterial reconstruction, which will be discussed in much greater detail in chapter 5.
Peripheral Vascular Disease

Atherosclerosis affecting the aorta, ilio-femoral, popliteal, and crural vessels is responsible for the majority of patients who present with lower limb ischaemia [Hertzer, 1991]. Atherosclerotic plaques cause stenosis of an artery by protrusion into the arterial lumen. The stenosis may initially be asymptomatic, but when the arterial diameter is reduced by 50% or the luminal area by 80%, a significant reduction in blood flow occurs [Burger, Hwang, 1974]. At this stage, the stenosis is termed "critical", as any further narrowing will result in a marked decrease in both arterial flow and perfusion pressure, causing symptoms of ischaemia.

Symptomatology.

The clinical presentation of lower limb ischaemia reflects the site and severity of the atherosclerotic disease. Jeurgens et al. [Jeurgens et al.1960] reported that 73% of patients with symptomatic lower extremity ischaemia presented with intermittent claudication, 16% with ischaemic rest pain and 11% with tissue necrosis (ulceration and /
Peripheral Vascular Disease

or gangrene).

**Intermittent Claudication.**

Intermittent claudication is characterised by pain in the thigh or calf which is reproducibly induced by exercise, and resolves with 2-3 minutes of rest. The pain is due to ischaemia of active muscle tissue, the site of which is determined by the location of the disease. Stenosis or occlusion of the aorta or iliac vessels may produce thigh or calf claudication, whilst disease affecting the femoro-popliteal segment causes calf pain alone.

Ischaemia is induced by exercise which causes arteriolar vasodilatation and increases the metabolic requirements of active muscle groups [Ganong, 1987]. Vasodilatation accentuates the distal pressure drop produced by arterial stenoses, and this in combination with increased muscular oxygen (O$_2$) requirements, results in demand exceeding supply. Ischaemic pain thus ensues which will resolve when the O$_2$ demand in the tissues is reduced i.e. when exercise ceases.

**Ischaemic Rest Pain.**

Intermittent claudication is symptomatic of mild to moderate lower limb ischaemia. When blood flow to the most distal part of the limb (i.e. the foot) is reduced to the extent that it fails to meet the nutritive requirements of the skin, ischaemic rest pain ensues [Lowe, 1990]. Rest pain may improve spontaneously as compensatory mechanisms (e.g. arteriolar dilatation and the opening of collateral channels) act to improve distal blood flow. However, in many cases these compensatory mechanisms fail and the limb then becomes critically ischaemic with persistent rest pain or tissue necrosis.

Recent evidence has suggested that critical limb ischaemia is a complex multisystem disorder in which atherosclerotic arterial disease plays a major part. A reduction in cardiac output will in turn reduce arterial perfusion pressure, and may be sufficient to cause peripheral gangrene even in the absence of peripheral arterial occlusive disease [Bird et al.1954]. The microcirculation which also partially determines tissue O$_2$ tension exhibits disordered function in patients with severe limb ischaemia. Maldistribution of microcirculatory blood flow with excessive arterio-venous shunting, has been attributed to microthrombosis [Conrad, 1968], collapse of the pre-capillary arterioles [Fagrell, 1973], abnormalities of vasomotion and rheological occlusion [Lowe, 1990] of the capillaries.

Patients with persistent ischaemic rest pain or tissue necrosis usually require prompt revascularisation to avoid limb loss. In the past it has proved difficult to compare treatment options and prognostic factors in this group of patients as there was no
Peripheral Vascular Disease

The Second European Consensus Document on chronic critical leg ischaemia [1992] defines critical ischaemia as "persistently recurring rest pain requiring regular analgesia for > 2 weeks, and / or ulceration or gangrene of the foot or toes, plus ankle systolic pressure ≤ 50 mmHg, or a toe systolic pressure of ≤ 30 mmHg". This definition allows different treatments from different centres to be accurately evaluated.

1.5 Incidence and Prevalence of Peripheral Vascular Disease.

The prevalence of peripheral arterial disease may be defined in reference to histopathological appearances or clinical presentation. There is a large discrepancy between prevalence determined from histological as opposed to clinical studies, as the majority of atherosclerotic lesions are asymptomatic [Dormandy, Mahir, 1992].

Although histological evidence of peripheral atherosclerosis is almost universal in the adult population [Roberts et al. 1959; Sternby, 1968], it is the clinical prevalence that is of more relevance, as this determines the number of symptomatic patients in the community. Numerous studies (Table 1.1) have reported the incidence and prevalence of intermittent claudication in defined populations. These studies however, are difficult to compare as there are many geographical, racial and chronological variables. A better estimate of symptomatic arterial occlusive disease is obtained from the Framingham [Kanel, McGee, 1985] or Basle [Widmer et al. 1985] studies, which prospectively followed asymptomatic subjects over a number of years. The biennial incidence of intermittent claudication in the Framingham study was 0.4% in men aged 45-54 years and 1.1% in men aged 55-64 years. The reported incidences are higher in the Basle study due to the use of non-clinical investigations. Combining information from all studies Dormandy and Mahir [Dormandy, Mahir, 1992] estimated that the prevalence of arterial disease causing intermittent claudication in men below 50 years was 1%, rising to 5% in older age groups.

Progression of Disease.

Three quarters of patients with intermittent claudication will remain the same or improve symptomatically shortly after the onset of symptoms; 25% will significantly deteriorate to severe claudication or critical ischaemia [Imparato et al. 1975; McAllister, 1976; Kallero, 1981; Jelnes et al. 1986]. The incidence of major lower limb amputation may be used as an indicator of local disease progression, but varies with the study population and with the availability of vascular reconstructive surgery. In the Basle and Framingham studies [Widmer et al. 1985; Kanel et al. 1970] less than 2% of patients who
Peripheral Vascular Disease

developed claudication required a major lower limb amputation.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>No of Subjects</th>
<th>Location</th>
<th>Sex</th>
<th>Age Range</th>
<th>Prevalence-Men</th>
<th>Prevalence-Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Widmer</td>
<td>1964</td>
<td>6400</td>
<td>Basle</td>
<td>Males</td>
<td>15-64</td>
<td>1.1</td>
<td>-</td>
</tr>
<tr>
<td>Reid</td>
<td>1965</td>
<td>676</td>
<td>England</td>
<td>Males</td>
<td>40-59</td>
<td>2.2</td>
<td>-</td>
</tr>
<tr>
<td>Higgins</td>
<td>1967</td>
<td>5140</td>
<td>Michigan</td>
<td>Males</td>
<td>20-79</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Holland</td>
<td>1967</td>
<td>625</td>
<td>USA</td>
<td>Males</td>
<td>40-59</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>Richard</td>
<td>1972</td>
<td>3733</td>
<td>Paris</td>
<td>Males</td>
<td>22-59</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Isacsson</td>
<td>1972</td>
<td>703</td>
<td>Malmo</td>
<td>Males</td>
<td>55</td>
<td>2.8</td>
<td>-</td>
</tr>
<tr>
<td>Reid</td>
<td>1974</td>
<td>18403</td>
<td>London</td>
<td>Males</td>
<td>40-64</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>Bothig</td>
<td>1976</td>
<td>860</td>
<td>Moscow</td>
<td>Males</td>
<td>50-54</td>
<td>6.9</td>
<td>-</td>
</tr>
<tr>
<td>Bothig</td>
<td>1976</td>
<td>582</td>
<td>Berlin</td>
<td>Males</td>
<td>50-54</td>
<td>3.4</td>
<td>-</td>
</tr>
<tr>
<td>Debacker</td>
<td>1977</td>
<td>8252</td>
<td>Belgium</td>
<td>Males</td>
<td>40-59</td>
<td>1.4</td>
<td>-</td>
</tr>
<tr>
<td>Heliovama</td>
<td>1978</td>
<td>1056</td>
<td>Finland</td>
<td>Males</td>
<td>55-74</td>
<td>7.7</td>
<td>-</td>
</tr>
<tr>
<td>Keys</td>
<td>1980</td>
<td>12763</td>
<td>Seven Counties</td>
<td>Males</td>
<td>40-59</td>
<td>0.35</td>
<td>-</td>
</tr>
<tr>
<td>Schroll</td>
<td>1981</td>
<td>666</td>
<td>Glostrop</td>
<td>Males</td>
<td>60</td>
<td>5.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Remanen</td>
<td>1982</td>
<td>10962</td>
<td>Finland</td>
<td>Males</td>
<td>30-59</td>
<td>2.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Kannel</td>
<td>1985</td>
<td>5209</td>
<td>Framingham</td>
<td>Males</td>
<td>35-84</td>
<td>0.7</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Table 1.1: Table showing the prevalence of intermittent claudication in defined study populations [Kannel, McGee, 1985; Widmer et al., 1964; Reid et al., 1974; Higgins, Kjelsberg, 1967; Holland et al., 1967; Richard et al., 1972; Isacson, 1972; Bothig et al., 1976; Debacker et al., 1979; Heliovama et al., 1978; Keys et al., 1980; Schroll, Munck, 1981; Remanen et al., 1982], after Fowkes, 1988.

Despite the low incidence of local disease progression, the overall outlook for patients with intermittent claudication is poor, due to the strong association with coronary artery and cerebrovascular disease. Clinically detectable coronary artery disease is demonstrable in 50% of patients with chronic limb ischaemia, and ischaemic heart disease accounts for 50% of the deaths in these patients [Dormandy et al., 1980; Crawford et al., 1981; Hertzger, 1981]. There is a lesser association with cerebrovascular disease which is still the cause of death of 10% of patients with claudication [Crawford et al., 1981; Hertzger, 1981; Turpinseed et al., 1980].

In most series, the mortality of patients with chronic limb ischaemia is 2-3 times that of an age and sex matched control population, with the mean mortality being 30%, 50% and 70% respectively after 5, 10 and 15 years follow up [Dormandy et al., 1980].
Peripheral Vascular Disease

1.6 Risk Factors for Peripheral Vascular Disease.

Smoking, arterial hypertension and diabetes mellitus are the classic risk factors for peripheral atherosclerosis. Recently, the mechanism of action of these established risk factors has been incorporated into the response to injury hypothesis of atherogenesis. A summary of these cellular events is presented in Table 1.2.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking</td>
<td>- Carbon monoxaemia causes endothelial injury.</td>
</tr>
<tr>
<td></td>
<td>- Increased endothelial cell permeability to LDL's and other proteins.</td>
</tr>
<tr>
<td></td>
<td>- Increased platelet reactivity.</td>
</tr>
<tr>
<td></td>
<td>- Promotes peripheral vasoconstriction</td>
</tr>
<tr>
<td>Hypertension</td>
<td>- Repetitive haemodynamic injury</td>
</tr>
<tr>
<td>Diabetes</td>
<td>- Unclear ? toxic endothelial cell injury</td>
</tr>
<tr>
<td>Lipid Abnormalities</td>
<td>- Increased lipid flux into arterial wall.</td>
</tr>
<tr>
<td>Haemostatic Abnormalities</td>
<td>- Toxic endothelial cell injury</td>
</tr>
<tr>
<td>e.g. elevated fibrinogen</td>
<td>- Plaque thrombosis</td>
</tr>
</tbody>
</table>

Table 1.2: Table showing the cellular actions of atherogenic risk factors.

Smoking is perhaps the most important risk factor for peripheral vascular disease and in the Framingham study accounted for 78% of all cases of intermittent claudication [Kanel, Shurtleff, 1973]. Interestingly, smoking appears to be a more important risk factor for the development of peripheral arterial disease than for coronary arterial disease [Gordon, Kanel, 1972], and has a greater effect than all other independent risk factors [Kanel, Shurtleff, 1973]. Smoking is also directly related to the incidence of amputations performed for chronic limb ischaemia [Jeurgens et al. 1960], and to the late failure of peripheral vascular reconstructions [Wray et al. 1971; Greenhalgh et al. 1981].

Hypertension is a potent risk factor for atherosclerosis but its importance in peripheral vascular disease remains undefined. Numerous studies have shown a strong association between arterial hypertension and chronic limb ischaemia [Jeurgens et al. 1960; Greenhalgh et al. 1971; Vyden et al. 1975], but equally, other longitudinal studies have shown no such association [DaSilva et al. 1979]. It is possible that arterial hypertension
Peripheral Vascular Disease

only exerts its full atherogenic effect in the presence of other risk factors. Diabetes mellitus typically causes diffuse atherosclerosis of the infra-genicular arterial tree, with sparing of the larger vessels [Haimovici, 1961]. Diabetes may combine with other risk factors, especially smoking, to produce a rapid progression of occlusive arterial disease.

Lipid abnormalities predispose to peripheral vascular disease [Steinberg, 1979], although there has been considerable debate on the exact causative mechanisms; recent evidence suggests that apolipoprotein levels may be predictive for atherogenesis [Pilger et al. 1983]. Numerous other risk factors, e.g. obesity, [Barrett-Conner, 1985] and haemostatic abnormalities [Imparato, 1982] have been proposed as risk factors for atherosclerosis, but most remain unproven at this time.

1.7 Treatment of Peripheral Vascular Disease.

The treatment of peripheral arterial occlusive disease involves both invasive and conservative approaches. Intermittent claudication, as discussed earlier, often improves spontaneously, but patients still benefit from cessation of smoking and the institution of regular physical exercise. Stopping smoking improves the prognosis of patients with peripheral vascular disease [Greenhalgh et al. 1981], whilst regular exercise has been shown to decrease symptoms of intermittent claudication [Ruell et al. 1984], an effect which is probably mediated through the development of collateral channels.

Pharmacologic Agents.

The role of pharmacologic agents in the management of peripheral vascular disease remains controversial. Vasodilators e.g. tolazoline and cinnarizine have been postulated to improve blood flow by dilating collateral channels, but may also dilate healthy vascular beds, stealing blood away from ischaemic tissue. In general, clinical trials [Trubestein, 1981; Coffman, Mannick, 1972; Coffman, 1979] have shown no convincing benefit for the use of vasodilators in the treatment of intermittent claudication.

Oxypentifylline, an anti-viscosity agent, acts to increase red cell deformibility and thus improve microcirculatory blood flow. Controlled clinical trials [Reich et al. 1984] have shown a 20% increase in the walking distance of patients treated with this agent, although negative trials have also been reported [Lloyd et al. 1987].

Adhesion and aggregation of platelets to the damaged arterial wall are thought to play a major role in the development and progression of atherosclerosis. Anti-platelet agents have the potential to decrease platelet interaction with the vessel wall and thus minimise platelet induced damage and thrombosis. A recent trial [Hess et al. 1985] suggested that a combination of aspirin and dipyridamole reduced the progression of
atherosclerosis in patients with peripheral vascular disease. Further studies are obviously needed to confirm this effect.

In summary, the short term administration of vasodilator and anti-viscosity agents appears to have a doubtful role in the treatment of intermittent claudication. Long term anti-platelet therapy appears to offer some promise in slowing the progression of disease, but even this effect requires further evaluation.

Invasive treatment of peripheral vascular disease may be broadly divided into percutaneous transluminal angioplasty (PTA) and operative surgical procedures. The dividing line between these two techniques is somewhat artificial as many angioplasties are now performed in the operating theatre as an adjunct to surgical procedures, whilst the interventional radiologist uses lasers and atherectomy devices [Lammer, Kameel, 1988; Parker et al.1990] that were once utilised at open operation. Modern vascular surgical practice relies heavily on both PTA and operative techniques to ensure successful treatment of patients with peripheral vascular disease. The two techniques should be regarded as complementary to each other rather than separate and competing techniques in different disciplines.

**Percutaneous Transluminal Angioplasty.**

Percutaneous transluminal angioplasty involves the enlargement of a narrowed or occluded artery by dilation with an intraluminal catheter. The first non-surgical interventional treatment for atherosclerosis was described by Dotter and Judkins in 1964 [Dotter, Judkins, 1964], who used a series of co-axial catheters to dilate a stenosed artery. This method was subsequently modified by Gruentzig who in 1974 introduced a polyvinyl balloon catheter with a double lumen shaft, for separate guidance and inflation of the angioplasty balloon [Gruentzig, Hopff, 1974].

Percutaneous transluminal angioplasty has become an accepted and widely used treatment in both peripheral and coronary artery occlusive disease. An increase in technical expertise and enhanced equipment design has led to an expansion in the indications for PTA, whose use is consequently increasing rapidly [Tunis et al.1991]. The mechanisms, indications and results of PTA will be further discussed in chapter 4.

The two major complications that accompany PTA are acute occlusion of the dilated vessel [Jorgensen et al.1990] and late restenosis [Fanelli, Aronoff, 1990] at the angioplasty site. These complications may be related to the mechanism of angioplasty itself and may occur in up to 50% of cases. The reduction of both early and late reclosure following angioplasty is a clinical priority. In later chapters, the results of investigations into the effects of locally applied vascular endothelial cells on the prevention of restenosis and reclosure following angioplasty will be presented.
Peripheral Vascular Disease

Operative Surgical Procedures.

The blood flow to a chronically ischaemic limb may be operatively restored by disobliteration of the occluded arterial lumen (i.e. endarterectomy), or by arterial bypass surgery. Endarterectomy aims to remove the diseased atherosclerotic plaque from the underlying arterial wall by dissecting in the intima-media plane. Endarterectomy is most successful when used in large vessels (e.g. iliac arteries), but is almost obsolete in the treatment of chronic lower limb ischaemia. Endarterectomy is nowadays successfully used in the treatment of carotid arterial occlusive disease.

Arterial bypass grafting utilises a vascular conduit to divert blood around an arterial occlusion and thus restore distal blood flow. Reconstructive vascular surgery relies on both natural and synthetic conduits for blood delivery. Synthetic grafts perform well in large diameter, high flow situations (e.g. aorto-iliac reconstruction) [Szilagi et al. 1986], but when the vessel becomes narrower and flow decreases, prosthetic grafts have a high failure rate [Veith et al. 1986]. In these situations, autogenous vein is the conduit of choice but unfortunately is often unavailable [Veith et al. 1979]. This has stimulated research into reducing the thrombogenicity of prosthetic vascular grafts which is thought to account for their high failure rate. Research has developed along two separate lines, incorporating anti-thrombotic substances into the graft matrix or forming a natural endothelial cell monolayer on the graft surface. It has proved possible to line prosthetic vascular grafts with native vascular endothelial cells, and this forms the basis of endothelial cell seeding which will be discussed in chapter 3.
CHAPTER 2
REGULATORY FUNCTIONS OF THE VASCULAR ENDOTHELIUM

2.1 Introduction. 14

2.2 Vasoactive Mediators and Control of Vascular Tone. 14
- Prostacyclin 15
- Endothelium derived relaxing factor 16
- Endothelin 17
- Endothelial control of vascular tone 18

2.3 Regulation of the Coagulation System by Vascular Endothelial Cells. 19
- Anticoagulant potential of endothelial cells 19
- Procoagulant potential of endothelial cells 21
- Endothelial regulation of fibrinolysis 22

2.4 Interaction of the Endothelium with Circulating Platelets. 23

2.5 Endothelial Cell - Leucocyte Interactions. 24

2.6 Vascular Endothelium and the Immune Response. 25
REGULATORY FUNCTIONS OF THE VASCULAR ENDOTHELIUM

2.1 Introduction.

The vascular endothelium envelops the circulation in a continuous monolayer that forms the interface between the bloodstream and the vessel wall. This layer was once regarded as an inert structure that formed a passive mechanical barrier between the blood and the extra-vascular tissues. Knowledge concerning endothelial cell structure and function increased dramatically when endothelial cells were grown and serially passaged in tissue culture. The vascular endothelium is now considered to be the body's most active paracrine organ, occupying a central role in vasomotion, thromboresistance, immunocompetence and the control of vascular growth.

The endothelium may be regarded as the predominant controlling influence over the whole vascular system, affecting and modulating the function of the vessel wall and the luminal constituents, to allow free flow of blood through the circulation. Endothelial cell damage and dysfunction have been implicated in the development of atherosclerosis and also play a crucial role in the failure of vascular reconstructions due to myointimal hyperplasia.

The thromboresistant and anti-coagulatory functions of the vascular endothelium have prompted certain investigators to suggest that endothelial cell transplantation may be used to improve the biological properties of vascular prostheses, and this concept forms the basis of endothelial cell seeding which is further detailed in chapter 3.

In this chapter the role of the endothelium in the control of vascular tone, coagulation, platelet activation and inflammation will be discussed. In all these functions, a recurring theme is that the endothelium has the ability to enhance or downregulate each process as circumstances dictate. Recent evidence has suggested that one of the most important endothelial cell functions is the ability to regulate the proliferative capacity of underlying vascular smooth muscle cells by the secretion of various growth factors. This ability will not be elaborated in this chapter as it will be considered in a discussion of myointimal hyperplasia following percutaneous transluminal angioplasty in chapter 5.

2.2 Vasoactive Mediators and Control of Vascular Tone.

The vascular endothelium is the body's most active paracrine organ [Becker, 1991] secreting numerous mediators that influence both vascular tone and intra-vascular coagulation. Three of the most important mediators are prostacyclin (PGI₂), endothelium-derived relaxing factor (EDRF) and endothelin. In this section the properties of these three substances will be described, followed by an analysis of the factors contributing to the...
maintenance of vascular tone in both healthy and diseased vessels.

**Prostacyclin.**

Prostacyclin was initially described in 1976 [Moncada et al. 1976], and since that time has been implicated as one of the most important local determinants of vascular tone and thromboresistance. Prostacyclin is the most potent naturally occurring inhibitor of platelet aggregation yet discovered [Moncada et al. 1976], and in addition possesses potent vasodilatory functions [Bunting et al. 1983].

Prostacyclin is one of a family of biologically active compounds derived from 20 carbon chain unsaturated fatty acids [Dusting, MacDonald, 1990]. Arachidonic acid is the main precursor of PGI$_2$ and is one of the most common fatty acids found in cellular phospholipids. Endothelial cells synthesise and release PGI$_2$ in response to a number of physical and chemical stimuli including shear stress, thrombin, bradykinin, platelet derived growth factor (PDGF), serotonin and adenine nucleotides [Forsberg et al. 1987]. Prostacyclin synthesis is initiated by phospholipase A$_2$ which releases arachidonic acid from cell membranes. Arachidonic acid may then be metabolised by the cyclooxygenase and prostacyclin synthase pathways to form PGI$_2$ (Fig 2.1). Prostacyclin is not stored by endothelial cells [Piper, Vane, 1971], and must be synthesised de novo when required. It has a short half life which is less than one circulation time, and is metabolised in plasma to form the stable metabolite 6-keto-Prostaglandin F$_1\alpha$.

Prostacyclin, as with most other mediators synthesised by the endothelium, has its main actions locally. It inhibits both platelet aggregation and vasodilatation by a receptor mediated increase in intracellular cAMP [Tateson et al. 1977; Gorman et al. 1977]. In addition to these two powerful effects, prostacyclin provides further cardiovascular protection by increasing the metabolism of cholesterol esters in SMCs, and preventing accumulation of these esters by macrophages [Vane et al. 1990; Willis et al. 1986].

Abnormalities of prostacyclin production have been implicated in atherogenesis. Vessels from diabetic patients [Dollery et al. 1979] produce less PGI$_2$ than normal vessels, whilst early atherosclerotic lesions exhibit significantly lower PGI$_2$ generative capacity than healthy vessels [Sinzinger et al. 1979]. In addition, it has been suggested that SMC proliferation in atherosclerotic plaques may be attributed to low PGI$_2$ production [Moncada, 1982].
Vascular Endothelium

Fig 2.1: Diagrammatic representation of PGI₂ synthesis from Arachidonic Acid. Enzymes are shown in italics.

The biological properties of PGI₂ make it attractive as a therapeutic tool, but potential applications are limited by the short half life. This difficulty has been overcome by the development of stable synthetic analogs e.g. iloprost. Prostacyclin and its analogs have been used to preserve platelet function during extra-corporeal circulation [Longmore et al. 1981], and current trials have suggested successful use in peripheral vascular disease both as a primary agent [Szczeklik, Gryglewski, 1981] and following bypass surgery [Hickey et al. 1991].

**Endothelium-Derived Relaxing Factor.**

Endothelium-derived relaxing factor is a labile vasodilator that is produced by endothelial cells. EDRF was first discovered by Furchgott and Zawadzki [Furchgott, Zawadzki, 1980] who observed that an intact endothelium was obligatory for acetylcholine to have a vasodilatory effect on arterial rings. The subsequent chemical identification of EDRF was hampered by its very short life, but it has now been suggested that EDRF is nitric oxide (NO) or a chemically related unstable nitroso species [Ignarro,
EDRF is synthesised from arginine in the cytosol of endothelial cells and requires nicotinamide adenine dinucleotide as a co-factor [Palmer, Moncada, 1989]. Endothelial cells constantly maintain high levels of intracellular arginine by generating this from L-citrulline [Vane et al., 1990]. EDRF is continually released from the vascular endothelium of all healthy vessels [Furchgott, 1983], but release may also be stimulated by a number of agents e.g. shear stress, acetylcholine, serotonin, ADP, thrombin [Furchgott, 1983], that interact with the vascular endothelium. It is interesting to note that the majority of these agents also stimulate PGI$_2$ release.

EDRF causes relaxation of vascular smooth muscle which is mediated by an increase in intracellular cGMP levels. The same secondary messenger is responsible for the vasodilatory action of nitrates used therapeutically in the treatment of angina and hypertension. The constant basal release of EDRF maintains the vascular bed in a dilated state. Inhibition of this effect by reversibly inhibiting EDRF synthesis causes a profound and prolonged increase in blood pressure [Rees et al., 1989]. This action has potential clinical applications in treating the profound vasodilatation that accompanies septicamie shock, and early reports of success in this condition are encouraging [Petros et al., 1992].

In marked similarity to PGI$_2$, EDRF has potent effects inhibiting both platelet adhesion [Radomski et al., 1987] and aggregation [Furlong et al., 1987], through an increase in intracellular cGMP. EDRF also protects the vasculature from excessive medial thickening by inhibiting proliferation of fibroblasts and SMC [Barrett, Willis, 1989].

**Endothelin.**

The vascular endothelium produces a number of vasoconstricting agents essential for the maintenance of normal vascular tone. Endothelin is a 21 amino-acid peptide that is the most powerful pressor substance yet identified, with vasoconstricting properties ten times that of angiotensin II [Simonson et al., 1989]. Endothelin is unusual in that it is released slowly following mechanical or chemical stimuli, rather than the almost instantaneous response of most other vasoactive substances [Yanagisawa et al., 1988].

Endothelin acts locally on SMC's to cause contraction by activation of Protein C [Simonson et al., 1989]. The exact physiological role of endothelin remains as yet undefined, but it seems likely that it will have some actions outside the cardiovascular system, as endothelin receptors have been identified in the brain, kidneys, lung and adrenal glands [Davenport et al., 1989].
**Endothelial Control of Vascular Tone.**

Integrated control of vascular tone is achieved through the concerted function of endothelial derived contracting and relaxing factors (Fig 2.2). The endothelium produces two potent vasodilators (PGI$_2$ and EDRF) in response to similar physical and chemical stimuli. Recent evidence has suggested that the release of these two mediators is coupled [Botting, Vane, 1989] and that they are synergistic in their anti-platelet and vasodilatory functions [Moncada et al. 1990]. The constant release of EDRF from the vascular endothelium maintains the normal circulation in a dilated state. In this quiescent state PGI$_2$ contributes little to basal vascular tone [Vane et al. 1990], but when vascular damage occurs PGI$_2$ and EDRF act synergistically to prevent vasospasm and platelet aggregation.

![Diagram illustrating the mediators contributing to control of vascular tone.](image)

Fig 2.2 : Diagram illustrating the mediators contributing to control of vascular tone. AA= arachidonic acid; L-Arg = L-Arginine; TxA$_2$ = thromboxane A$_2$ ; Ang II = angiotensin II, PG = prostaglandin, PGI2 = prostacyclin, EDRF = endothelium derived relaxing factor. After Luscher, 1990b.

Under certain conditions the endothelium can produce and release endothelin and other vasoconstricting mediators to balance the action of PGI$_2$ and EDRF. Activation of the cyclooxygenase pathway (Fig 2.1) may produce thromboxane A$_2$ (TxA$_2$), prostaglandin A$_2$ (PGA$_2$) and superoxide radicals which all stimulate smooth muscle contraction [Luscher, 1990a]. Angiotensin converting enzyme (ACE) is found in vascular endothelium, which is the major synthetic site of the pressor agent angiotensin II [Becker, 1991]. ACE may also act to inactivate bradykinin which is a circulating vasodilator [Vane, 1990].

Vascular tone is controlled by a complex series of interactions between vasoactive
endothelial mediators. It has been speculated that an imbalance between dilating and contracting factors contributes to the pathogenesis of arterial disorders e.g. atherosclerosis and hypertension, but this not proven at the current time.

2.3 Regulation of the Coagulation System by Vascular Endothelial Cells.

One of the primary functions of the vascular endothelium is to present a non-thrombogenic surface to the bloodstream, thus facilitating continuous flow through the vasculature. The relationship between the luminal endothelium and the blood constituents may be broadly divided into interactions with the coagulation system and interactions with circulating platelets. This division is somewhat artificial as both systems are functionally interrelated and contribute to the same physiological haemostatic process. However, the endothelium modulates both processes by differing mechanisms, and as such these will be discussed separately.

The vascular endothelium lines the luminal surface of all blood vessels and is strategically located to regulate blood coagulation. In the quiescent state, anticoagulant mechanisms predominate, but activation of endothelial cells by noxious stimuli can lead to downregulation of anticoagulant properties, with the consequent expression of procoagulant characteristics. The anticoagulant and procoagulant properties of vascular endothelial cells are summarised in Table 2.1.

Anticoagulant Potential of Endothelial cells.

Thrombin is the key enzyme in the haemostatic system (Fig 2.3), and constitutes the most prominent target for the anticoagulatory action of vascular endothelial cells. Antithrombin III, a glycoprotein synthesised in the liver [Downing et al.1978] is the most efficient of all thrombin inhibitors and also inactivates other enzymes of the intrinsic pathway, as well as proteases such as plasmin, trypsin, and complement C1 [Ogston et al.1976]. Endothelial cells express specific proteoglycans which mediate high affinity binding of both antithrombin III and thrombin, which facilitates antithrombin mediated thrombin inactivation on the cell surface [Preissner, 1988]. This reaction is catalysed by heparin sulphate and related glycosaminoglycans which are also expressed on the luminal surface of endothelial cells [Lau, Rosenberg, 1960]. Two other protease inhibitors, heparin cofactor II, and protease nexin I also mediate thrombin inactivation in association with endothelial derived glycoproteins, but these reactions occur predominantly in the extra-cellular tissues following endothelial cell injury [Low et al.1982].
Vascular Endothelium

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>Procoagulant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinolytic system</td>
<td>Expression of tissue factor</td>
</tr>
<tr>
<td>Protein C / Protein S</td>
<td>Binding of factors V, IX, X</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>Surface associated heparin</td>
<td>Synthesis of factor V</td>
</tr>
<tr>
<td>Ectonucleotidases</td>
<td>Synthesis of plasminogen activator inhibitor</td>
</tr>
<tr>
<td>Protease inhibitors</td>
<td>Synthesis of basement membrane</td>
</tr>
<tr>
<td>Vascular anticoagulants</td>
<td></td>
</tr>
<tr>
<td>Extrinsic pathway inhibitor</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1: Table illustrating the anti- and pro-coagulant characteristics of vascular endothelial cells.

Thrombomodulin is an endothelial cell specific membrane receptor for thrombin [Maruyama et al. 1985]. The binding of thrombin to thrombomodulin virtually abolishes the procoagulant action of thrombin with respect to fibrin formation, factor V activation, factor XII conversion and platelet activation [Preissner, 1988]. In addition, thrombomodulin catalyses the thrombin dependent conversion of protein C to activated protein C (C₅₉). Protein C, a Vit K dependent plasma protein [Stenflo, 1976], interacts with protein S, which is synthesised by vascular endothelial cells [Fair, 1986], to inhibit thrombin formation through the inactivation of factors V and VIII, and also to produce fibrinolysis by inactivation of plasminogen activator inhibitor [Marlar, 1982]. Thrombomodulin comprises 50-60% of endothelial associated binding sites for thrombin and constitutes a highly specific anticoagulant mechanism.

Endothelial injury leads to the secretion of vascular anticoagulant protein from intracellular storage pools. Vascular anticoagulant protein exerts a potent anti-thrombotic effect by inhibiting the binding of factor Xa to cell surfaces, and thus inhibits the activation of prothrombin [Reutlingsperger et al. 1987]. In addition, endothelial cells synthesise and secrete an extrinsic pathway inhibitor which interacts with the tissue factor / factor VIIIa complex to inhibit factor X activation [Broze, Miletich, 1987]. Through these various mechanisms, endothelial cells are able to influence coagulation by modification of both intrinsic and extrinsic pathways.
Fig 2.3: Simplified diagrammatic representation of the coagulation cascade. The intrinsic, extrinsic and common pathways are separated by dashed lines. PF3 denotes platelet factor 3.

**Procoagulant Potential of Endothelial Cells.**

In homeostasis the balance of endothelial regulation of the coagulation system is tipped in favour of anticoagulation. However, when perturbed, endothelial cells may participate in procoagulant reactions which are initiated by the expression of tissue factor (thromboplastin, factor II) on the endothelial cell surface [Stern et al. 1988]. Endothelial cells may be induced to express tissue factor by a variety of external agents such as endotoxin, thrombin, interleukin-1, and tumour necrosis factor [Brox et al. 1984; Bevilacqua et al. 1984; Nawroth, Stern, 1986].

Tissue factor provides a high affinity receptor for factor VII, which is activated in the presence of calcium ions to initiate the extrinsic pathway [Prydz, Pettersen, 1988]. Propagation of the coagulation cascade is facilitated by the ability of factors V, IX and X to bind to the endothelial cell surface with consequent local production of fibrin [Stern et al. 1988]. The pathophysiological role of procoagulant endothelial cell characteristics have not been fully elucidated. However, it is obvious that endothelial cells possess potent pro-
Vascular Endothelium

and anticoagulatory properties which will modulate local regulation of the coagulation cascade according to the state of the microcirculation.

**Endothelial Cell Regulation of Fibrinolysis.**

Deposition of fibrin occurs during vascular injury in an attempt to minimise blood loss. Removal of the fibrin network is essential for wound healing and to maintain normal vessel patency. Fibrin degradation is accomplished by plasmin, which is formed by the action of plasminogen activators (PA's) on plasminogen (Fig 2.4). Two types of PA have been identified, tissue type PA (t-PA) and urokinase type PA (u-PA). Tissue type PA is only physiologically active when bound to a fibrin substrate whilst u-PA directly activates plasminogen in the circulation [Ranby, 1982].

![Diagram of the fibrinolytic system]

Fig 2.4: Diagrammatic representation of the fibrinolytic system. Inhibitory influences are shown in bold, solid lines. PAI = plasminogen activator inhibitors, t-PA = tissue-type plasminogen activator; u-PA = urokinase type plasminogen activator.

Endothelial cells are the principle source of circulating t-PA [Van Hinsbergh, 1988], and may also produce u-PA when stimulated [Van Hinsbergh, 1987]. The endothelial synthesis of t-PA may be increased by thrombin and histamine [Hanss., Collen, 1987], but fast release of stored t-PA from cellular pools also accompanies physical stimuli such as venous occlusion and physical exercise [Van Hinsbergh, 1988]. This allows local concentrations of PA's to be increased rapidly in times of stress, in addition to providing a mechanism for long term sustained release.

To balance the effect of PA's the vascular endothelium synthesizes and secretes plasminogen activator inhibitors (PAI's) [Loskutoff et al, 1983] which inhibit the activity of t-PA and u-PA. The fibrinolytic potency of endothelial cells in vivo is probably
determined by the balance between PA’s and PAI’s, although this relationship has not been conclusively demonstrated.

2.4 Interaction of the Endothelium with Circulating Platelets.

Under normal conditions, endothelial cells present a non-thrombogenic surface to the flowing blood. However, when the endothelium is damaged platelets adhere and subsequently aggregate on the exposed sub-endothelium initiating the thrombotic process. Platelet deposition at the site of vascular injury is thought to be important in atherogenesis [Friedman et al.1977] and plays a major role in the development of restenosis due to myointimal hyperplasia following arterial reconstruction [Clowes, Reidy, 1991]. The endothelium controls platelet interaction with the vessel wall through intrinsic properties of endothelial cells themselves, as well as through the release of vasoactive mediators.

The anti-thrombogenic nature of the quiescent vascular endothelium has been largely attributed to the anionic charge on the cell surface, which is composed of mixture of glycoproteins and glycosaminoglycans in association with a high proportion of heparin and chondroitin sulphates [Nievelstein, de Groot, 1988]. This surface is unreactive towards platelets, but may lose this property after perturbation [Curwen et al.1980]. Platelet adhesion is further inhibited by the constant release of EDRF [Furchgott, 1983], which is a powerful anti-platelet agent [Radomski et al.1987]. PGI$_2$ is an anti-platelet agent released from the vascular endothelium that has synergistic effects with EDRF [Moncada et al.1990]. Evidence suggests that PGI$_2$ does not play any part in the thromboresistant character of the non-damaged endothelium, [Czervionke et al.1979] but is released in high quantities during vascular damage to prevent massive platelet adhesion. The synthesis and release of both EDRF and PGI$_2$ are stimulated by thrombin and clotting factors [Botting, Vane, 1989], which are present in high concentrations at the site of vascular damage. Endothelial cell injury thus upregulates both EDRF and PGI$_2$ production which act synergistically to limit thrombosis.

Endothelial cells also possess mechanisms to decrease the levels of numerous platelet-active mediators and thus control the degree of platelet aggregation. Thrombin is inhibited by binding to antithrombin III and thrombomodulin on the endothelial cell surface [Preissner, 1988], whilst ADP, a powerful platelet aggregant, is converted by ecto-nucleotidases to adenosine, which has anti-aggregatory properties [Leiberman et al.1977].

Adhesion of platelets to the sub-endothelium may also be promoted by certain functions of the endothelial cell. Disruption of the endothelial cell monolayer allows platelets to adhere to the underlying tissue and subsequently to aggregate and undergo their release reaction. The composition of the sub-endothelial tissue in part determines the
kinetics of platelet adhesion. The sub-endothelium is synthesised by endothelial cells from above and from fibroblasts and smooth muscle cells from below [Nievelstein, de Groot, 1988]. The endothelial produced matrix is primarily composed of collagens, elastic tissue, proteoglycans and non-collagenous proteins. The thrombogenicity of the sub-endothelial matrix may be increased by perturbed endothelial cells which secrete von Willebrand factor (vWF) into the matrix [Loesberg et al. 1983]. Von Willebrand factor is a large plasma glycoprotein synthesised mainly by vascular endothelial cells, that is stored in both endothelial cells and platelets [Sussman, Rand, 1982]. Following stimulation vWF is released from Weibel-Palade bodies within the endothelial cells, and is secreted into the sub-endothelial matrix, where it acts to promote platelet adhesion [Verweij, 1988]. Endothelial cells also release platelet activating factor (PAF) in response to stimulation, which is a potent stimulator of platelet aggregation [Camussi et al. 1983].

2.5 Endothelial Cell - Leucocyte Interactions.

The interaction of vascular endothelial cells with circulating leucocytes is central to the control of localised cellular inflammatory responses that accompany infection, trauma and allogenic rejection. An inflammatory response may be initiated when the surface characteristics of the endothelium are altered by mechanical, microbial or chemical stimuli [Beilke, 1989]. The subsequent development of a cellular infiltrate involves four phases [Pober et al. 1990]. Initially, haemodynamic changes brought about by the release of EDRF and PGI_2 increase delivery of leucocytes to the affected site. Once deposited at the relevant site, leucocytes must bind to the vascular endothelium since this is a mandatory step in the egress of these cells into the extravascular space [Harlan, 1985].

Binding of leucocytes to vascular endothelial cells is receptor specific. Local mediators (e.g. endotoxin, IL-1, IFN-γ, and C5a) cause endothelial cells to express adhesion molecules for leucocytes such as neutrophil-specific endothelial leucocyte adhesion molecule 1 (ELAM-1) [Pober et al. 1986a], and leucocyte specific ICAM-1 [Pober et al. 1986b]. The binding of leucocytes to the vascular endothelium appears to be site as well as receptor specific, as neutrophils and monocytes preferentially bind at the level of the post-capillary venule, where the endothelial cells have a distinct cuboidal morphology [Lewinsohn et al. 1987].

Following binding, leucocytes must migrate into the tissues. Stimulated endothelial cells synthesise a number of low-molecular weight inflammatory cytokines that function as chemokinetic factors and encourage leucocyte migration [Strieter et al. 1989]. Finally, endothelial cells facilitate the passage of migrating leucocytes into the extra-vascular tissues by weakening endothelial cell junctions, and degrading the underlying basement membrane [Stolpen, 1986].
Vascular Endothelium

2.6 **Vascular Endothelium in the Immune Response.**

Vascular endothelial cells are capable of many immune responses that were once thought to be unique to cells of bone marrow origin. Major histocompatibility complex (MHC) class II antigens are expressed on the endothelial cell surface [Pober *et al.* 1986b], and the presence of these antigens may be upregulated by infection or allogenic rejection [Hirschberg, 1981]. Class II expression may be induced in most types of cultured cells, but endothelial cells are unique in the high degree to which antigens are expressed in response to lymphokines [Hayry *et al.* 1987].

The ability to express high levels of class II antigen in association with the ability to synthesise and secrete IL-1 [Miossec, Ziff, 1986] enables vascular endothelial cells to initiate and support a T cell proliferative response, in a fashion similar to monocytes. Endothelial cells may be uniquely important accessory immune cells due to their anatomical position and their ability to mediate a cellular immune response [Pober *et al.* 1990].
### CHAPTER 3
**ENDOTHELIAL CELL SEEDING**

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Introduction.</td>
<td>27</td>
</tr>
<tr>
<td>3.2</td>
<td>Prosthetic Graft Failure.</td>
<td>28</td>
</tr>
<tr>
<td>3.3</td>
<td>Sources of Endothelial Cells.</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>- Isolation and culture of large vessel endothelium</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>- Microvascular endothelial cell isolation</td>
<td>30</td>
</tr>
<tr>
<td>3.4</td>
<td>The Seeding Process.</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>- Kinetics of endothelial cell seeding</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>- Staged seeding procedures</td>
<td>34</td>
</tr>
<tr>
<td>3.5</td>
<td>Results of Experimental Endothelial Cell Seeding.</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>- Healing of prosthetic vascular grafts</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>- Graft patency, thrombogenicity and function</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>- Prosthetic graft infection</td>
<td>39</td>
</tr>
<tr>
<td>3.6</td>
<td>Clinical Trials of Endothelial Seeding.</td>
<td>39</td>
</tr>
<tr>
<td>3.7</td>
<td>Summary.</td>
<td>42</td>
</tr>
</tbody>
</table>
3.1 Introduction.

During the past 30 years reconstructive vascular surgery has become widely established due to advances in anaesthesia, surgical technique and post-operative care, which have developed alongside rapid progress in the manufacture of prosthetic vascular grafts. These advances have led to a significant increase in the rate of arterial bypass surgery [Tunis et al. 1991], which has been quantified within the Leicestershire Area Health Authority (LAHA) for the 17 year period between 1974 and 1990. Patients undergoing arterial reconstruction for chronic lower limb ischaemia were identified from the Hospitals Activity Analysis database of the LAHA between 1974 and 1987 and from the Trent Regional Information System from 1987 to 1990. During the time period studied there were 1095 arterial bypasses. The data was age and sex standardised and expressed graphically as the number of arterial bypasses per 100,000 of the population (Fig 3.1). Throughout the duration of the study, there has been an approximate six-fold increase in vascular reconstructive surgery within the LAHA [Thompson et al. 1992].

---

**Figure 3.1** : Graph illustrating rate of arterial reconstructions for lower limb ischaemia. Points are fitted with the best-fit linear regression line, $R^2 = 0.89$, $p < 0.001$. 

---
Endothelial Seeding

3.2 Prosthetic Graft Failure.

With the expanded indications for vascular reconstructive surgery, the utilisation of prosthetic vascular conduits has escalated. However, whilst dacron and expanded polytetrafluoroethylene (ePTFE) grafts perform well in high flow, low resistance conditions, their failure rates are unacceptable in infragenicular bypass, when the diameter of the graft does not exceed 6 mm [Veith et al. 1986]. In these low flow, high resistance situations an autologous native vessel, specifically the long saphenous vein, is the conduit of choice due to the higher patency rate.

Unfortunately, the long saphenous vein may be unavailable for use as a conduit in up to 30% of infragenicular reconstructions due to previous use in peripheral or coronary revascularisation, or due to pre-existing vein abnormality, disease or small calibre [Veith et al. 1979]. In these cases an alternative venous conduit (e.g., the cephalic or basilic vein) or a prosthetic graft must be used.

The major difference between patency rates observed for natural venous conduits as compared to synthetic materials, is attributable to the presence of the endothelial cell monolayer that lines the luminal surface of native blood vessels [Williams et al. 1990]. Man appears to be unique, in that of all animals studied, humans are the only species known to be incapable of healing prosthetic vascular grafts with their own natural endothelium [Berger et al. 1972; Wesolowski et al. 1964]. Both dacron and ePTFE grafts show a particular form of healing in humans, with the deposition of proteinaceous materials and fibrin on the luminal surface. Endothelial pannus ingrowth may occur for 1-2 cm at each anastomosis, but never progresses to complete endothelialisation of the graft, which consequently renders the graft surface permanently thrombogenic and prone to occlusion [Eldrup-Jorgenson et al. 1985; Stewart et al. 1977]. Compliance of native grafts may also be important in reducing failure although the effect is likely to be minimal as the compliance of autogenous saphenous vein grafts is lost within several weeks of implantation [Kidson, 1983].

Extensive research has been directed towards producing a durable non-thrombogenic graft. Two general approaches to reducing graft thrombogenicity have been to modify either the graft surface or inhibit the haemostatic system. Pharmacological manipulation of the coagulation system has been advocated to maintain patency in small vessel grafts. Pre-operative use of antiplatelet agents decreases failure rates in coronary artery bypass grafts [Chesebro et al. 1984], whilst iloprost, a prostacyclin analogue has been effective in enhancing blood flow through femorodistal vein grafts [Hickey et al. 1991]. Although pharmacological treatment has been partially successful it would be preferable to produce a graft surface that is intrinsically non-thrombogenic. It has been suggested that this might be achieved through the development of new polymeric materials.
Endothelial Seeding

that evoke no haematologic or cellular response [Yeager, Callow, 1988]. Polyurethanes are relatively non-thrombogenic but are not suitable for use in bypass grafts as they are prone to aneurysm formation [Phua et al. 1987]. Modification of pre-existing graft surfaces by bonding heparin and other anti-thrombotic mediators has been technically successful and the patency of these grafts is now being evaluated in vivo [Nichols et al. 1984].

However, as stated earlier, the prime reason for the failure of prosthetic vascular grafts is their inability to form a complete endothelial lining. It is therefore attractive to hypothesise that lining the luminal surface of a graft with autologous endothelial cells would produce a functioning biologic graft. This process may be achieved by the transplantation of freshly harvested or cultured endothelial cells onto luminal graft surfaces and has been termed endothelial cell seeding. Since the introduction of this technique in 1978 [Herring et al. 1978], endothelial seeding has been the subject of extensive research which is reviewed in the remainder of this chapter.

3.3 Sources of Endothelial Cells.

To achieve successful endothelial cell seeding of prosthetic vascular grafts, viable endothelial cells need to be harvested from a source of functioning vascular endothelium and transplanted to the treatment site where they must attach and replicate to confluence, whilst preserving their antithrombotic functions [Herring, 1991b]. The optimal cells for use in vascular repair are autologous vascular endothelial cells which may be derived from large vessels (i.e. arteries or veins) or capillaries. Some authors have suggested the use of allogeneic [Zamora et al. 1986] or xenogeneic [Pennell et al. 1986] endothelial cells, whilst others have proposed the use of cells derived from pericardial mesothelium [Sugimoto et al. 1989].

Isolation and Culture of Large Vessel Endothelium.

Animal endothelial cells have been studied in culture since the 1920's [Lewis, 1922], but it was not until the 1973 that Jaffe et al. described the successful isolation and culture of human endothelial cells derived from umbilical veins [Jaffe et al. 1973]. These cell lines demonstrated some growth potential, but could not usually be grown beyond ten serial passages. In 1981 Maciag et al. described an endothelial cell growth factor (ECGF) that markedly stimulated proliferation of cultured human umbilical vein endothelial cells [Maciag et al. 1981]. Even so, efforts to culture human adult endothelial cells were hampered by early senescence and it was not until Thornton et al. [Thornton et al. 1983] discovered that the addition of heparin to the growth medium increased reproductive...
capacity, that the serial cultivation of adult endothelial cells (AEC’s) has been possible.

There are two principal methods of harvesting large vessel endothelium, mechanical and enzymatic. In their pioneering work, the University of Indiana seeding group [Herring et al. 1978] used a steel wool pledget to scrape endothelial cells from the surface of the long saphenous vein. The endothelium was removed in clumps but was severely contaminated with smooth muscle cells and fibroblasts [Herring, 1991b]. This form of mechanical harvesting has now been largely superseded by enzymatic methods in which protease enzymes are used to loosen the endothelium from its basilar attachments. Collagenase is the standard protease used for this purpose but other proteases have been investigated. Dispase provides a reasonable alternative, but the use of trypsin has been abandoned as it decreases endothelial viability [Thilo et al. 1980].

To work efficiently the enzyme must be applied to the luminal surface of the vessel. This may achieved either by eversion of the vessel as described by the University of Michigan seeding group [Graham et al. 1980a], or by direct vessel cannulation. Recent evidence suggests that direct cannulation and instillation of collagenase under minimal pressure achieves larger numbers of viable endothelial cells than vessel eversion [Bourke et al. 1986].

Enzymatic methods of cell harvest give an efficiency of approximately 15% [Bourke et al. 1986; Sharefkin et al. 1986]. This relatively low efficiency has led to the concept of a critical threshold for seeding density in human clinical trials of immediate cell seeding. Herring and LeGrand [Herring, LeGrand, 1989] suggested that if the area of the vein used for cell harvest fell below 5.25% of the proposed grafts surface, the cell inoculum would be insufficient for graft endothelialisation.

One potential method of overcoming the low efficiency of endothelial cell harvest is to amplify cell number in tissue culture prior to use. Watkins et al. [Watkins et al. 1984] suggested that human endothelial cells had the in vitro growth capacity to completely line prosthetic vascular grafts, and this was confirmed by Jarrell et al. [Jarrell et al. 1984] who demonstrated that $10^{23}$ adult endothelial cells could be derived from each square centimeter of vascular tissue harvested. The advantages of this vast reproductive capacity must be somewhat tempered by the fact that adequate cell cultures may only be obtained in 50% of cases [Magometschnigg et al. 1992; Glassberg et al. 1982; Watkins et al. 1984].

**Microvascular Endothelial Cell Isolation.**

In an attempt to surmount the inefficiencies associated with the procurement of large vessel endothelium, alternative sources of endothelial cells have been investigated. Microvascular endothelium may be isolated from omental, subcutaneous or perinephric fat [Sharp et al. 1989; Jarrell et al. 1986; Schmidt et al. 1988] by dissociating the adipose tissue
Endothelial Seeding

with collagenase, and then separating the endothelium from the remaining cells by gradient
centrifugation [Jarrell et al.1986]. Using this technique, Williams et al. demonstrated that
large numbers of cells (1x10^6 cells /gm fat) could be isolated in a relatively short period of
time [Williams et al.1989].

Microvascular endothelial cells are suitable for endothelial cell seeding as they
exhibit excellent adhesion to prosthetic vascular grafts [Jarrell et al.1986; Radomski et
al.1987], and release prostacyclin in a similar fashion to large vessel endothelium
[Stansby et al.1991]. Preliminary experimental studies have suggested that the thrombus
free area on both ePTFE and dacron grafts may be improved by microvascular endothelial
cell seeding [Schmidt et al.1988; Sterpetti et al.1988; Pearce et al.1987], which also
increases the secretion of anti-thrombogenic mediators from the graft surface [Bull et
al.1988]. Sharp et al. recently implanted 17 microvascular seeded grafts into patients
requiring limb salvage operations [Sharp et al.1989], but the long term outcome of these
procedures is unknown.

Although the use of microvascular cells offers considerable advantages over large
vessel endothelium in the ease of cell procurement, there are predictable disadvantages.
Microvascular endothelial cell isolates are seldom pure and are often contaminated with
fibroblasts and smooth muscle cells [Sharp et al.1989], which may contribute to late graft
failure through prolific inner capsule formation on the graft surface [Sterpetti et al.1988;
Pearce et al.1987]. The identity of microvascular cells has also been questioned, and it
seems likely that the cell population isolated from microvascular preparations, is
heterogeneous in nature [Latron et al.1991], with the majority of the cells being
mesothelial in origin [Visser et al.1991]. In this thesis mesothelial cells have not been
utilised as a cell source, although this application was investigated, and therefore
subsequent discussion of the seeding process will concentrate on experiments employing
large vessel endothelium.

3.4 The Seeding Process.

The mechanical process of seeding a prosthetic vascular graft with endothelial
cells, entails applying the cells onto a graft surface, and then providing a suitable substrate
onto which the cells can attach. Unfortunately, endothelial cells have a minimal affinity for
synthetic graft surfaces [Williams et al.1985], and although more cells will attach to
dacron than to ePTFE [Stanley et al.1985], the majority of these cells are situated in the
graft interstices and so do not truly line the luminal graft surface. The first successful
method of affixing endothelium to prosthetic graft surfaces was achieved by adding a low
number of immediately procured endothelial cells to the blood used to preclot dacron
grafts [Herring et al.1978]. Since that time indium-111 labelling of endothelial cells

31
Endothelial Seeding

[Sharefkin et al. 1983] has allowed endothelial attachment to be accurately quantified and the optimum conditions for cell attachment to be defined.

Kineties of Endothelial Cell Seeding.

Endothelial cell attachment to prosthetic surfaces is biphasic, the initial phase of rapid adherence is complete within 30 min, which is then followed by a slower second phase [Anderson et al. 1987] during which myofilaments [Toselli et al. 1984], fibronectin and other connective tissue proteins are synthesised and secreted [van Wachem et al. 1988]. The pre-treatment of prosthetic grafts with fibronectin prior to seeding was designed to mimic this physiological process, and has been successful in increasing endothelial attachment to graft surfaces [Budd et al. 1990; Budd et al. 1989; Hasson et al. 1986; Kesler et al. 1986; Ramalanjaona et al. 1986; Vohra et al. 1991; Vohra et al. 1990]. Ramalanjaona et al. [Ramalanjaona et al. 1986] demonstrated a two fold increase in cellular attachment to fibronectin coated ePTFE as compared to untreated controls, and in a similar study Budd et al. [Budd et al. 1989] showed that fibronectin coating increased the seeding efficiency of ePTFE grafts from 5% to 50%.

In addition to fibronectin, several other components of the extracellular matrix, namely collagen [Vohra et al. 1991; Thomson et al. 1991; Hasson et al. 1986], laminin [Hasson et al. 1986; Thomson et al. 1991; Pratt et al. 1988] and gelatin [Budd et al. 1989; Hasson et al. 1986], increase cellular attachment when used to precocat prosthetic grafts prior to seeding. Although precoating of the graft increases seeding efficiency, there has been some concern that the precoating agents may themselves be thrombogenic and thus contribute to early graft failure. This does not appear to be the case as Koveker et al. [Koveker et al. 1991] demonstrated that whilst fibronectin did cause an insignificant increase in platelet deposition on the luminal graft surface, laminin and collagen preparations actually decreased graft thrombogenicity.

Fibronectin is prepared from pooled human serum, and thus a potential disadvantage of fibronectin graft coating is the possibility of viral mediated disease transmission. In direct comparisons, precloetting of the graft with autologous blood appeared to provide an excellent surface for endothelial cell attachment, with higher seeding efficiencies obtained than following precoating with extracellular matrix proteins [Thomson et al. 1991; Lindblad et al. 1986]. Precloetting of the graft offers several advantages over precoating as it uses autologous products and encourages uniform endothelial coverage on a compact fibrin surface [Thomson et al. 1991].

Research into defining the ideal endothelial attachment substrate continues to the present time. Bowersox and Andersen [Bowersox, Andersen, 1988] investigated endothelial attachment to artificially reconstituted basement membrane. Using this
Endothelial Seeding

substrate, they reported a seeding efficiency superior to fibronectin and in addition the seeded cells had a more mature morphology, analogous to those on native blood vessels. Intuitively, optimal attachment conditions would be provided by the natural endothelial substrate itself, i.e. the basement membrane and extracellular matrix found in native arteries and veins. Part of this thesis will examine endothelial attachment and retention to the damaged vascular surface produced after endothelial denudation of large diameter blood vessels.

Another approach aimed at increasing static seeding efficiency has been to modify the synthetic graft material. Increasing the graft pore size of ePTFE has improved the results of endothelial seeding in several trials [Kempczinski et al. 1985; Boyd et al. 1988], and it has been suggested that an internodal distance of 40 \mu m is optimal. However, Linblad et al. [Linblad et al. 1987] demonstrated that internodal distance alone did not affect static seeding efficiency and it may be that the inner capsule healing of these grafts in animal models, is directly related to pore size [Boyd et al. 1988]. Recent work has illustrated that chemical adaption of ePTFE graft surfaces by pyrolytic carbon coating [Sbarbati et al. 1991] or by anhydrous ammonia gaseous plasma modification [Sipehia, 1990], will support increased endothelial attachment, and these materials may have future clinical applications. Developments of new graft materials also offer promise, with cell affinity to polyester elastomer being superior to that of ePTFE [Kesler et al. 1986; Lundgren et al. 1986].

The ultimate aim of seeding endothelial cells onto synthetic arterial conduits is to enable the graft surface to heal by complete endothelialisation. To achieve this goal, the seeded cells must be able to withstand the shear stresses of the bloodstream when implanted into the arterial circulation. Regrettably, the retention of immediately seeded endothelial cells onto arterialised grafts is poor and represents one of the major failings of endothelial seeding. Rosenman et al. [Rosenman et al. 1985b] demonstrated that endothelial cell loss from seeded carotid interposition vascular grafts occurred in two phases; 70% of the cells were lost within the first 30 min of perfusion, which was then followed by a more gradual cellular loss of 3.7% per hour for the next 24 hours. Overall only 4.4% of the originally harvested cells and 17% of the initially adherent cells were present on the graft surface 24 hours following implantation.

Precocating the graft with fibronectin doubles endothelial cell retention in conditions of shear stress [Kent et al. 1988; Kesler et al. 1986]. Ramalanjava et al. [Ramalanjava et al. 1986] suggested that this ability was not due to improved retention in the initial phase of rapid cell loss, but was due to an overall decrease in chronic cell detachment from 3.7% to 2.2% per hour. Although it is tempting to attribute the loss of seeded cells from vascular grafts to haemodynamic phenomena alone, there is good evidence that many cells are also lost through neutrophil mediated endothelial damage [Emerick et al. 1989] and through
Endothelial Seeding

lysis of the fibronectin substrate by thrombin and plasmin [Herring, 1991b].

**Staged Seeding Procedures.**

The simplest and most expedient technique for seeding prosthetic grafts is to use simultaneous cell harvest and reimplantation - so called "immediate" seeding. Unfortunately, this procedure is limited by inefficient cell harvest and low cell retention, which consequently results in the graft being sparsely covered with endothelial cells shortly after flow is initiated. Logically, the luminal surface would be rendered least thrombogenic when completely covered with autologous endothelial cells at the time of bypass grafting. However, this degree of coverage is patently unattainable by immediate seeding techniques and sporadic graft coverage necessitates endothelial cell replication on the graft surface for confluence to be attained. This process may have serious sequelae as replicating endothelial cells produce potent growth factors which may promote myointimal hyperplasia at the anastomosis sites, leading in turn to graft failure [Herring, 1991a].

One promising alternative to immediate seeding is to utilise a staged or delayed procedure. This involves harvesting endothelial cells and growing them in tissue culture prior to seeding and graft implantation. The obvious advantage of this technique is that large numbers of cells may be procured, which can then be applied onto arterial prostheses in supra-confluent densities to form a confluent monolayer - so called endothelial cell "sodding". In addition, a staged seeding procedure will enable long incubation times to be used which may allow improved endothelial attachment to the graft. Miyata et al. [Miyata et al.1991] have speculated that initial endothelial adherence to graft materials is through transmembrane receptors for extracellular matrix proteins. Prolonged incubation allows these attachment sites to mature with the development of junctional complexes and the secretion of extracellular matrix proteins.

Several investigators have quantified the excellent cell retention achieved by supra-confluent and staged seeding procedures *in vitro* [Sentissi et al.1986; Schneider et al.1988]. In separate studies, Budd et al. [Budd et al.1991a] and Kesler et al. [Kesler et al.1986] demonstrated that cell retention in shear stress was higher with long endothelial incubation on the graft as compared to immediate cell seeding. Prendiville et al. [Prendiville et al.1991] suggested that 72 hours was the optimum incubation time. However, both James et al. [James et al.1990] and Miyata et al. [Miyata et al.1991] proposed that incubation time may not be critical and that maximal cell retention is achieved when cellular confluence is reached on the graft surface. Staged seeding procedures have been employed in several animal experiments [Shindo et al.1987; Tannenbaum et al.1987] with seeded grafts achieving consistently higher patency rates that unseeded controls.
The potential disadvantages of using cultured endothelial cells are that growth in tissue culture may compromise function, encourage genetic mutations and decrease cell adherence [Carabasi et al. 1991]. Seeger and Klingman [Seeger, Klingman, 1988] demonstrated that cells derived following tissue culture had higher seeding efficiencies than freshly harvested cells. However, this finding is at variance with that of Hussain et al. [Hussain et al. 1991] who compared immediate and culture lined grafts in a canine model and found that immediately seeded grafts had increased endothelial coverage 30 days following implantation. Further research is obviously needed to clarify the effects of tissue culture on endothelial cell function. It has been suggested that by using the large number of cells obtained from microvascular harvesting, the use of tissue culture could be avoided. Although theoretically attractive, the use of these particular cells may be complicated by contaminating cell lines and persistent doubts concerning cell origin.

3.5 Results of Experimental Endothelial Cell Seeding.

Healing of Prosthetic Vascular Grafts.

The ability of endothelial seeded grafts to heal with an intact endothelial cell monolayer is central to the strategy of seeding prosthetic vascular grafts. Healing of experimental dacron grafts has been extensively documented by investigators at the University of Michigan [Burkel et al. 1981; Graham et al. 1980b; Graham et al. 1980a], and their observations confirmed by many other centres [Schmidt et al. 1984; Schmidt et al. 1985b; Herring et al. 1984a; Allen et al. 1984; Herring et al. 1978]. In an comprehensive series of experiments, the Michigan group compared healing in endothelial seeded canine thoracoabdominal grafts to the healing observed in similarly placed non-seeded grafts. One to two days following implantation there were no differences between the seeded and control grafts; both were completely infiltrated with fibrin and blood cells and covered by a layer of activated platelets and leucocytes. Differences between the two graft types began to appear from four days onwards, at which time isolated patches of endothelium, with associated lysis of the underlying fibrin coagulum, were noted on the seeded graft surfaces. At 14 days the seeded grafts demonstrated thinning of the platelet layer and continued surface endothelialisation; meanwhile, the control prostheses were completely covered with thrombotic debris. By one month the seeded grafts were 80% covered with a surface endothelium that rested on a layer of smooth muscle cells and fibroblasts, into which vasa vasorum appeared to grow. During the same time period the unseeded prostheses remained uncovered by endothelium apart from 10 mm pannus ingrowth at each anastomosis.

The increased endothelialisation of the seeded prostheses was reflected by the
proportion of the luminal graft surface that remained free of clot. The thrombus free surface area (TFA) of unseeded grafts at 7 and 28 days post-implantation was 81% and 40% respectively, which was significantly inferior to the 95% and 85% TFA on seeded grafts during the same time period. With longer follow up periods all the non-seeded grafts eventually developed an endothelial lining, although thrombus was still present on their surface. The healing of dacron grafts is also reflected in the thickness of the inner capsule that forms on the luminal surface. In most series the inner capsule thickness of seeded grafts is less than that of unseeded grafts [Herring et al.1978; Deutsch et al.1992; Burkel et al.1982; Shepard et al.1986] which may be beneficial in maintaining blood flow through the graft.

Although these initial observations suggested that endothelial seeding conferred some benefit on graft healing, the experimental situation cannot be extrapolated to graft healing in man. In the animal models described, all unseeded grafts developed an endothelial monolayer, which as discussed earlier is not the case for human prostheses. Herring et al. [Herring et al.1984a] suggested that three mechanisms may be responsible for spontaneous endothelialisation of synthetic flow surfaces in animals; transmural ingrowth of multipotential cells, pannus ingrowth and the adherence of circulating precursor cells. In dacron graft healing, the fibroblast layer that forms part of the inner capsule may be multipotential in origin or may stimulate endothelial ingrowth. Fibroblast cell types appear to be critical to graft endothelialisation, as in cases where the fibroblast layer fails to form, surface endothelial cells are also absent.

The initial data suggesting that endothelial seeding resulted in rapid graft healing were obtained using dacron grafts. More recently, seeding research has concentrated on ePTFE grafts which appear to heal in an entirely different manner to dacron. Seeded ePTFE grafts develop a complete endothelial monolayer within one month of implantation [Herring et al.1984a; Campbell et al.1988; Graham et al.1982], whereas unseeded grafts exhibit insignificant pannus ingrowth at the anastomoses only. In these grafts, the endothelial cells were supported by a thin inner capsule consisting of microfibrillar deposits and a sparse number of fibroblasts. Smooth muscle cells were not present as transmural migration appeared to be hindered by the impervious construction of ePTFE. In a direct comparison, Herring et al. [Herring et al.1984a] demonstrated that the inner capsule in seeded ePTFE grafts was significantly less than in dacron grafts.

The time period that elapses between seeded graft implantation and complete endothelial healing, implies that the benefits of seeding are not realised for 4-6 weeks, during which there is a window of increased thrombogenicity when the graft may fail. One potential solution to this problem has been to preform a confluent endothelial monolayer on the graft surface prior to implantation [Shindo et al.1987; Budd et al.1991c]. Budd et al. [Budd et al.1991c] demonstrated that 100% endothelialisation could
be achieved using these techniques with a significant increase in patency compared to unseeded control grafts.

Although the data presented on the endothelialisation of seeded grafts are convincing, several authors have questioned the origin of the endothelial lining that forms on seeded graft surfaces. Both Hollier et al. [Hollier et al. 1986] and Wakefield et al. [Wakefield et al. 1988] studied non-autologous seeding of prosthetic vascular grafts with subsequent karyotype analysis of the cell lining. From these experiments both authors suggested that immediately seeded endothelial cells may not replicate to cover the luminal graft surface but act as a stimulus for growth of host endothelium.

Graft Patency, Thrombogenicity and Function.

The prime determinant of the efficacy of endothelial seeding is the long term failure rate of small calibre prosthetic vascular grafts. The patency of both dacron and ePTFE grafts has been investigated by several groups [Belden et al. 1982; Schmidt et al. 1985b; Allen et al. 1984; Hirko et al. 1987; Kempczinski et al. 1985] who have uniformly reported an improvement in patency attributable to endothelial cell seeding. This improvement is even observed in experimental coronary artery bypass grafts; Hunter et al. [Hunter et al. 1986] reported that 43% of seeded ePTFE aorto-coronary grafts remained patent as compared to a 100% failure rate in unseeded grafts.

In the clinical setting, prosthetic grafts are particular prone to failure in conditions of low flow, where the duration of contact between the blood constituents and the synthetic luminal surface is increased. This was investigated experimentally by Schmidt et al. [Schmidt et al. 1984] who exposed dacron grafts to 4 hours of low arterial flow, 5 weeks after graft implantation. Whilst all of the seeded grafts maintained patency throughout this period of altered flow, 25% of the unseeded grafts occluded. A similar report from Hunter et al. [Hunter et al. 1983] confirmed the benefits of endothelial seeding in low flow rates and suggested that seeding of prosthetic grafts may be particularly effective in conditions of high thrombogenicity.

Further increases in prosthetic graft patency may be achieved with the use of adjunctive antiplatelet therapy. Schmidt et al. [Schmidt et al. 1985a] reported that the patency of 4 mm dacron carotid interposition grafts was increased from 56% to 80% by endothelial seeding and that the patency of seeded grafts could be further improved to 100% by concurrent aspirin and dipyridamole administration. The beneficial effect of aspirin was confirmed by Campbell et al. [Campbell et al. 1988] who suggested that the maximum improvement in patency of ePTFE grafts would be obtained by endothelial seeding in combination with a 14 day course of antiplatelet agents.

The improved patency associated with endothelial seeding is presumably due to the
anti-thrombotic influence of the luminal endothelial cells, with a subsequent decrease in platelet deposition on the luminal graft surface. Dekker et al. [Dekker et al. 1989] measured the deposition of indium-111 labelled platelets on polyethylene capillary tubes covered with human endothelial cells in an *in vitro* perfusion model. Using this model, platelet deposition decreased with increasing endothelial cell coverage and was virtually abolished when the endothelial cell density exceeded $5 \times 10^4$ cells/cm$^2$.

Using *in vivo* models, many groups have demonstrated reduced platelet deposition on the surface of seeded grafts by direct measurement of platelet uptake and also by demonstrating increased platelet survival times [Wakefield et al. 1986; Sharefkin et al. 1982; Ramberg et al. 1985; Sicard et al. 1984]. Allen et al. [Allen et al. 1984] quantified platelet deposition on dacron grafts implanted into the carotid and femoral arteries of dogs, who received aspirin for the initial two weeks of the study. Platelet deposition on seeded grafts 24 hours after implantation was significantly higher than on unseeded controls, but this trend was reversed after two weeks when platelet deposition on the seeded grafts decreased to levels lower than controls. The lower thrombogenicity of the seeded grafts was maintained in serial studies up to 7 months following implantation. In a similar experiment Shepard et al. [Shepard et al. 1986] demonstrated that platelet deposition on seeded grafts was less than on paired controls 2 and 4 weeks after bypass grafting but that no difference between seeded and unseeded grafts was demonstrated at 24 hours.

It has been suggested that low density seeding confers little immediate benefit on graft thrombogenicity, as initial cell coverage is sparse and during replication to confluence the endothelium may express pro-thrombotic tendencies. This theory was examined by Budd et al. [Budd et al. 1991c] who showed that by using preformed confluent endothelial monolayers, the thrombogenicity of seeded grafts could be reduced to less than that of controls one week after implantation.

As has previously been discussed in chapter 2, the anti-thrombotic properties of the endothelium reside in the anionic charge of the cell membrane, the expression of thrombomodulin and the secretion of vasoactive mediators. The easiest of these mediators to quantify is prostacyclin (PGI$_2$) which may be assayed by the measurement of its stable metabolite 6-keto-prostaglandin F$_{1\alpha}$. Several groups have reported that seeded grafts produce higher levels of PGI$_2$ than unseeded controls [Sharp et al. 1986; Sicard et al. 1984; Ortenwall et al. 1988; Boyd et al. 1988; Jensen et al. 1992; Budd et al. 1992], which confirms that the decreased thrombogenicity of seeded grafts may be attributed to anti-thrombotic properties of the seeded endothelium.
Endothelial Seeding

Prosthetic Graft Infection.

Infection of prosthetic vascular grafts is a devastating complication in vascular surgery, resulting in high mortality and amputation rates [Liekweg, Greenfield, 1977]. Prosthetic grafts are sensitive to bacteraemia shortly after implantation [Moore et al. 1969], but this sensitivity diminishes with time due to the healing of the graft surface [Malone et al. 1975]. Endothelial seeding has been shown to decrease the adherence of haematogenously administered bacteria to the surface of the graft 4-8 weeks following implantation [Rosenman et al. 1985a; Birinyi et al. 1987].

This effect may be explained by the increased healing and thrombus free surface area exhibited by seeded grafts. Haematogenous bacteria adhere to grafts at sites of thrombus formation [Rosenman et al. 1985a], and so by decreasing the proportion of the graft surface covered by thrombotic debris, endothelial seeding reduces the likelihood of bacterial adherence. Endothelial seeding only has a significant effect on bacterial adherence shortly after implantation, as in well established grafts the luminal surface will have healed and will provide effective resistance to infection [Keller et al. 1988].

3.6 Clinical Trials of Endothelial Seeding.

Although the in vitro and in vivo studies discussed previously support the use of endothelial seeding, there are fundamental dangers in extrapolating these data to encompass human clinical trials. Regardless of interspecies differences [Ortenwall et al. 1988] all experimental animals endothelialise prosthetic graft surfaces whether seeded or not. This may reflect a difference in the ability of circulating precursor cells to stimulate endothelial growth in animal models, or the presence of atherosclerosis and continued atherogenic stimulation in humans. Despite these difficulties, the encouraging results from experimental seeding of prosthetic vascular grafts have stimulated preliminary clinical trials of endothelial seeding in the USA and in Europe.

One of the constant problems encountered throughout all clinical trials has been the inability to determine the degree of graft endothelialisation by direct biopsy. This difficulty has forced many investigators to use indirect methods of assessing graft healing such as platelet deposition or platelet morphology, whilst others have utilised graft patency as the single clinical endpoint.

The first clinical trial of endothelial seeding was performed by Herring et al. at the University of Indiana between 1978 and 1982 [Herring et al. 1984b; Herring et al. 1985b]. Dacron femoro-popliteal grafts were seeded with mechanically derived endothelium using a single staged technique, and their patency rates compared to both unseeded dacron controls and to autologous saphenous vein. Overall, seeded dacron grafts and the
unseeded controls had similar actuarial patency rates which were both inferior to those achieved by autologous vein. In a sub-group of patients who were non-smokers, endothelial seeding significantly improved patency, but in patients who smoked during the study, the reverse was true. Mechanical cell harvesting was also utilised by Walker et al. [Walker et al. 1988] who compared the patency of control and seeded ePTFE grafts implanted in 50 patients. Follow up 14 months after surgery revealed no significant differences between the two groups. It is possible that the relative failure of endothelial seeding to increase patency rates in these two studies was due to contamination of the mechanically derived endothelial cell isolate with smooth muscle cells, which acted as a focus for the development of myointimal hyperplasia in grafts subjected to continued atherogenic stimuli.

To eradicate smooth muscle contamination, enzymatic harvesting was employed in a second trial of endothelial seeding conducted at the University of Indiana [Herring et al. 1987; Herring et al. 1989]. In this study, preclotted ePTFE femoro-popliteal grafts were seeded with autogenous endothelial cells harvested from the patient's external jugular vein. The preliminary results from this study were encouraging, the one year actuarial patency being 82% in seeded grafts as compared to 31% in unseeded controls, which represents a significant improvement. In addition, throughout the trial 20 patients underwent revisional vascular surgery in the region of their graft, which allowed the graft to be biopsied [Herring, Legrand, 1989; Herring et al. 1985a]. Thirteen grafts were chronically occluded and histological examination for signs of healing was impossible. However, in six of the seven patent and newly occluded grafts, evidence of mid graft endothelialisation was observed, illustrating for the first time the clinical success of endothelial seeding in healing a prosthetic graft. Although this trial was not strictly randomised and the level of the distal anastomosis between seeded and control grafts varied, these findings must be considered a qualified success. Unfortunately, one disturbing element was that three graft infections occurred within the seeded group which raises concerns over the sterility of the seeding procedure [Herring et al. 1987].

The difficulty in recruiting sufficient numbers of patients to conduct a randomised, strictly controlled clinical trial of graft patency, has prompted several groups to use indirect measures of graft healing. Ortenwall et al. [Ortenwall et al. 1987; Ortenwall et al. 1990] studied the effects of endothelial seeding on platelet deposition in 22 dacron aorto-bifemoral grafts. One limb of the graft was seeded with enzymatically harvested autogenous endothelium at a low seeding density of 2.2x10^3 cells / cm^2; the contralateral limb remained unseeded and acted as a paired control. The deposition of indium-111 labelled platelets was quantified on both limbs of the graft 1,4 and 12 months after surgery. At all three time points the seeded graft limbs exhibited less platelet deposition than controls. In a succeeding trial the same group implanted seeded ePTFE femoro-
Endothelial Seeding

popliteal grafts into 23 patients [Ortenwall et al. 1989]. One half of the graft was seeded with $3.5 \times 10^3$ enzymatically harvested cells/cm$^2$; the other half was sham seeded with culture medium. Again, it was demonstrated that the seeded graft segment accumulated fewer platelets than the control segments 1 and 6 months following implantation. The findings from these two studies led the authors to suggest that endothelial seeding in humans has the potential to decrease graft thrombogenicity even when low seeding densities are employed.

Using similar techniques, Zilla et al. [Zilla et al. 1987] compared the platelet parameters in 9 patients receiving seeded ePTFE femoro-popliteal bypass, to 9 patients receiving unseeded grafts. After 14 weeks follow up, plasma levels of platelet factor 4 and β-thromboglobulin showed significantly improved results in the seeded group. Deposition of indium-111 labelled platelets and measurement of the post-operative ankle systolic pressure also favoured the seeded group, but the difference did not reach statistical significance. However, at 1 year follow up [Fasol et al. 1989], there were no differences between any of the platelet parameters and the investigators concluded that endothelial seeding failed to induce the development of a non-thrombogenic lining in ePTFE grafts.

The early results of endothelial seeding were equivocal at best and represented a relative failure when compared to animal studies. It is possible that in human trials a low inoculum of endothelial cells is insufficient to stimulate graft healing through the recruitment of circulating multipotential cells and stimulation of the host endothelium. If this is so, it becomes imperative that the graft is as completely covered with endothelial cells as possible, prior to implantation. Unfortunately, this has not been the case in the clinical trials described so far, which have been characterised by low seeding densities and short incubation times. Seeding densities of the order of $10^3$ cells/cm$^2$, which are the maximum possible using immediate seeding techniques, are approximately one hundred fold below those required to achieve confluent graft coverage. To increase both seeding density and incubation time, staged seeding procedures with amplification of the endothelial cell harvest in tissue culture must be used.

Recently, two groups have reported their initial results of such procedures. Magometschnigg et al. [Magometschnigg et al. 1992] undertook a non-randomised clinical study of 26 patients requiring revisional femorodistal procedures in whom no autologous vein was available. Endothelial cells were harvested in 13 patients from a segment of cephalic vein which was removed 4 - 7 weeks prior to operation. Cells were grown in tissue culture for two cell passages and were then seeded onto fibronectin coated ePTFE grafts at a density of $4 \times 10^4$ cells/cm$^2$, and incubated on the graft surface for 3 hours prior to implantation. The seeding efficiency was 74%. During follow up, the 13 seeded grafts were compared to 13 unseeded grafts which acted as a control group. Early graft patency was 92% for the seeded grafts and 83% for controls but, more significantly, the
18 month limb salvage rate was 85% in the seeded group and only 69% in those receiving unseeded grafts. The platelet accumulation index of the endothelialised prostheses was less than controls for all follow up investigations and additionally, in one case a graft biopsy was obtained which revealed a complete endothelial cell monolayer.

This trial appears to show convincing benefit of two stage endothelial seeding, but there are some criticisms that may dilute this apparent success. Initially 34 patients were selected to receive seeded grafts but successful endothelial cell culture was only achieved in 50% of these. The patients with unsuccessful culture were not included in the seeded group for analysis; they were discharged from the study or used as controls. This practice obviously skews the subsequent analysis as this is not based on an intention to treat policy. In addition three patients initially selected for seeding, clinically deteriorated during the time between cell harvest and graft removal and were discharged from the study.

Deutsch et al. [Deutsch et al., 1992] have recently presented their results of two stage seeding in 25 patients undergoing femoro-popliteal bypass. Endothelial cells were harvested from segments of external jugular vein removed under local anaesthesis, and grown until confluent. Cells were then incubated on the graft surface for 9 days prior to surgery. The 31 month actuarial patencies were 80% for endothelialised grafts and 70% for controls. No details regarding complications or efficiency of cell culture are available.

The preceding two studies have illustrated that the complete endothelialisation of prosthetic vascular grafts with cultured endothelial cells is clinically feasible and technically possible. Two stage seeding seems to offer the best opportunity to improve synthetic graft patency, but further advances in endothelial procurement techniques are required before widespread clinical application is possible.

3.7 Summary

This chapter has reviewed the available literature relating to endothelial seeding from its inception till the present time. Since 1978 the techniques of endothelial cell isolation and culture have improved immeasurably, and with an abundant source of readily available endothelium, understanding of endothelial attachment forces and seeding kinetics has advanced. Although results from animal studies were very favourable, the initial clinical trials of endothelial seeding proved disappointing. However, by utilising more advanced methodology and absorbing fundamental lessons from in vitro studies, promising results are now beginning to emerge. Eventually, a prospective, multi-centre randomised trial will be required to establish the role of endothelial seeding in modern vascular practice.

With greater understanding of the seeding process, several expanded applications of endothelial transplantation have been suggested. Endothelial cells may be genetically
modified to express recombinant DNA products and may thus be used to deliver gene therapy both systemically and at the sites of arterial surgery. The possibility of seeding the damaged vascular surfaces exposed following arterial reconstructions has also been raised and forms the basis of this thesis.
### CHAPTER 4
PERCUTANEOUS TRANSLUMINAL ANGIOPLASTY IN LOWER LIMB PERIPHERAL VASCULAR DISEASE

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Introduction.</td>
<td>45</td>
</tr>
<tr>
<td>4.2 Mechanism of Angioplasty.</td>
<td>46</td>
</tr>
<tr>
<td>4.3 Indications for Angioplasty.</td>
<td>47</td>
</tr>
<tr>
<td>4.4 Results of Balloon Angioplasty.</td>
<td>50</td>
</tr>
<tr>
<td>- Iliac artery angioplasty</td>
<td>50</td>
</tr>
<tr>
<td>- Femoropopliteal angioplasty</td>
<td>53</td>
</tr>
<tr>
<td>- Aortic angioplasty</td>
<td>56</td>
</tr>
<tr>
<td>- Tibial angioplasty</td>
<td>56</td>
</tr>
<tr>
<td>- Angioplasty for vein graft stenoses</td>
<td>58</td>
</tr>
<tr>
<td>4.5 Complications of Balloon Dilatation.</td>
<td>58</td>
</tr>
<tr>
<td>4.6 Summary.</td>
<td>60</td>
</tr>
</tbody>
</table>
Balloon Angioplasty

PERCUTANEOUS TRANSLUMINAL ANGIOPLASTY IN LOWER LIMB PERIPHERAL VASCULAR DISEASE

4.1 Introduction.

Percutaneous transluminal angioplasty (PTA) is nowadays accepted as a definitive treatment option in patients with arterial occlusive disease. The first percutaneous treatment of peripheral vascular disease was undertaken by Charles Dotter in 1964 [Dotter, Judkins, 1964]. Using a system of rigid co-axial catheters passed antegradely through the femoral artery, an adductor hiatus occlusion was dilated in an 84 year old woman, who refused amputation or reconstructive surgery. This technique, although subsequently modified and used successfully in the USA and Europe [van Andel, 1976], suffered from two fundamental disadvantages. Firstly, the passage of a solid catheter through an atheromatous lesion caused shearing forces to be applied in a longitudinal rather than a radial direction, which minimised arterial dilation and encouraged embolisation. Secondly, to dilate iliac artery stenoses, a large bore catheter must be passed through the femoral artery which created a sizeable defect and led to bleeding problems.

Both of these difficulties were overcome by the development of the balloon angioplasty catheter, which was described in its modern form by Gruentzig in 1974 [Gruentzig, Hopff, 1974], who reported the use of a double lumen balloon catheter with separate channels for balloon guidance and inflation. One of the most important features of this design was that the balloon could be inflated at high pressures to a predetermined cylindrical shape, and so apply a radial dilating force to an atheromatous lesion. Additionally, large diameter balloons could be introduced into the arterial tree on relatively small diameter catheters which minimised arterial trauma and bleeding complications. Since its inception the Gruentzig balloon catheter has undergone several modifications, but the essential design remains in use today. Catheters of this type are easily manoeuvrable and it is possible to position the angioplasty balloon in any peripheral or coronary artery via percutaneous groin puncture.

Although initially faced with scepticism [Roberts, Ring, 1982], PTA has become a universal technique in the treatment of peripheral and coronary artery occlusive disease. Angioplasty is often used as an alternative to bypass surgery, but has an equally effective role in facilitating surgical procedures by improving arterial inflow [Wilson et al. 1990] or maintaining existing graft patency [Berkowitz et al. 1992; Berkowitz, Greenstein, 1987]. As technology in catheter manufacture has improved, there has been a dramatic increase in the number of balloon dilatations performed for peripheral vascular disease. Tunis et al. [Tunis et al. 1991] investigated the rate of PTA amongst residents of Maryland between 1979 and 1989, and reported that the annual procedure rate had increased from 1 to 24 procedures per
1,000,000 residents during the time period studied. In a similar study, the population based rate of PTA within the Leicestershire Health Authority has been demonstrated to have increased significantly between 1974 and 1990 [Thompson et al. 1992] (Fig 4.1).

![Graph illustrating rate of percutaneous transluminal angioplasty for lower limb ischaemia. Points are fitted with the best-fit linear regression line, $R^2 = \text{regression co-efficient, } p = \text{probability.} $](image)

This chapter reviews the indications, use and results of percutaneous balloon dilatation in the treatment of lower limb arterial occlusive disease.

### 4.2 Mechanism of Angioplasty.

Dotter originally considered that balloon angioplasty acted through radial compression of atheromatous material, with release of fluid components from the plaque [Dotter, 1978]. At first sight, this seems an unlikely explanation as the vast majority of human atheromatous plaques are composed of incompressible, dense, calcified fibrocollagenous tissue. However, Kaltenbach et al. [Kaltenbach et al. 1984] demonstrated that the weight and thickness of atheromatous lesions could be partially reduced by prolonged application of pressure, but not to the extent that could totally explain the luminal widening achieved by PTA.

The dominant mechanism involved in PTA was simultaneously elucidated in the early 1980's by several groups who reported the results of animal and post-mortem studies. Block et al. [Block et al. 1980a; Block et al. 1980b] investigated the effect of balloon dilatation on experimentally induced atherosclerotic plaques in an animal model, and
Balloon Angioplasty

demonstrated that balloon angioplasty of aortic plaques caused endothelial desquamation, exposure of the sub-endothelial tissue and splitting of the atheromatous lesion. These observations in animal models were confirmed by Sanborn et al. [Sanborn et al. 1983] and by Faxon et al. [Faxon et al. 1982] who concluded that widening of the arterial lumen following balloon dilatation, was due to the fracture of the atheromatous plaque with dissection into the media and consequent stretching of the non-atheromatous portion of the vessel wall.

Similar findings were described by other groups who dilated cadaveric atheromatous arteries in vitro. Castaneda-Zuniga et al. [Castaneda-Zuniga et al. 1980] found no evidence supporting compression or redistribution of plaques, which verified the theory that atheromatous material is relatively incompressible. In a similar study, Zarins et al. [Zarins et al. 1982] demonstrated that balloon dilatation resulted in plaque disruption, with separation of the plaque from the tunica media and intimal flap protrusion into the arterial lumen. Plaque separation occurred mainly in the longitudinal direction, with the plaque remaining attached to the media at the limits of the angioplasty site. Dilation of the arterial lumen occurred by stretching and occasional rupture of the tunica media, with additional thinning of the tunica adventitia.

These experimental findings were substantiated when Block et al. [Block et al. 1981] described plaque disruption and dissection in post-mortem studies from three patients who had undergone angioplasty less than a week prior to death. The post-mortem appearances have since been confirmed by other investigators [Bodrog et al. 1986]. Recently, with the advent of more sophisticated imaging techniques, the plaque disruption mechanism of angioplasty has been proven in vivo. Losordo et al. [Losordo et al. 1992] used intravascular ultrasound to examine 40 patients undergoing iliac angioplasty. Analysis of pre- and post-angioplasty appearances confirmed that plaque fractures and compression of the plaque elements are the major factors responsible for increasing luminal diameter, with stretching of the arterial wall providing an additional but minor contribution. Tomaru et al. [Tomaru et al. 1988] visualised similar post-angioplasty morphology by fibre-optic angioscopy.

4.3 Indications for Angioplasty.

Utilising conventional techniques, balloon dilatation may be accomplished in virtually all peripheral arteries irrespective of the degree of atheromatous disease. Despite this potentially widespread application, the role of PTA in arterial occlusive disease has not yet been clarified, and the indications for this procedure remain controversial. Many of the difficulties in defining specific indications for PTA, stem from problems associated with early reports of angioplasty, which were often retrospective and employed diverse reporting standards [Walden et al. 1986]. There are widespread discrepancies in the reported results of
PTA which may be attributed to differences in patient selection, standards for defining success and methods of data analysis [Johnston et al. 1987]. One of the main problems in most series is the inability to report results on an intention to treat basis. It remains common practice to report the initial success rate of the procedure, and to then calculate long term patency on successfully dilated segments alone. By ignoring technical failures, the cumulative patency rates are erroneously high, and give a false impression of the procedure. In addition, the technical failure rate itself comprises several distinct entities. Technical failure implies an inability to reach the lesion, traverse the lesion with a guidewire or accomplish sufficient dilatation. However, many reports equate technical failure with early temporal failure and include acute arterial rethrombosis in the technical failure rate.

Further confusion arises due to the lack of uniform criteria for defining technical success and maintenance of patency. In general, investigators utilising clinical assessment alone report higher patency rates than those employing both clinical and haemodynamic measurements. Rutherford [Rutherford, 1991] demonstrated these differences by applying various reporting standards to 66 patients undergoing iliac angioplasty (Table 4.1). The cumulative 5 year patency rates varied from 52-84%.

<table>
<thead>
<tr>
<th>Criterion</th>
<th>5-year Patency(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thigh-brachial index increased &gt;0.1</td>
<td>84</td>
</tr>
<tr>
<td>As above: sustained without deterioration</td>
<td>74</td>
</tr>
<tr>
<td>Ankle-brachial index increased &gt;0.1</td>
<td>64</td>
</tr>
<tr>
<td>As above: sustained without deterioration</td>
<td>54</td>
</tr>
<tr>
<td>As above: plus symptomatic improvement</td>
<td>52</td>
</tr>
</tbody>
</table>

Table 4.1: Table illustrating a comparison of the results of iliac angioplasty judged by different criteria. (After Rutherford 1991).

The difficulties in comparing results between different reported series are compounded by the types of patients selected and the morphology of lesion treated. Many series do not detail the proportion of patients with critical limb ischaemia or the ratio of arterial stenoses to occlusions. Without this information, evaluation of results is essentially meaningless. The rapid expansion of endovascular techniques has made accurate comparison between different treatment modalities essential. To this end, an Ad Hoc Committee on Reporting Standards was established by the Society for Vascular Surgery and the International Society for Cardiovascular Surgery, who provided a set of guidelines to
Balloon Angioplasty

standardise reports of percutaneous procedures [Ad Hoc Committee on Reporting Standards SVS/ISCVS, 1986] (Table 4.2). These recommendations are now being widely applied with a consequent improvement in reported series.

<table>
<thead>
<tr>
<th>Recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients undergoing treatment should not be excluded from consideration due to technical failure, failure to complete the treatment or failure to produce haemodynamic or symptomatic improvement. All exclusions should be specified.</td>
</tr>
<tr>
<td>Chronic ischaemic limbs should be stratified into seven categories with the aid of objective non-invasive testing criteria.</td>
</tr>
<tr>
<td>Initial failures should be reported and analysed as to cause.</td>
</tr>
</tbody>
</table>
| To determine patency there must be:  
  - conventional arteriography or other established imaging techniques  
  - maintenance of achieved improvement in segmental blood pressure index (i.e. ABPI must have improved by 0.1 initially and not have deteriorated by more than 0.15 from initial post-operative level). |
| Life table methodology is recommended for analysing patency. |

Table 4.2: Table summarising the recommendations of the Ad Hoc Committee on Reporting Standards, 1986.

In an attempt to define the exact role of PTA, it is tempting to compare the results of PTA with those of surgery. In most cases [Spence et al.1981; Kwasnik et al.1987] these comparisons are inappropriate, as the characteristics of the two groups are dissimilar, with the patients undergoing surgery having a higher incidence of critical ischaemia, as well as a higher incidence of arterial occlusion and diffuse stenotic disease. However, in a prospective randomised comparison, Wilson et al. [Wilson et al.1989] compared PTA to surgery in a well matched group of patients with iliac, superficial femoral or popliteal arterial occlusive disease. Although the initial technical failure rate was higher in patients treated with PTA, this study concluded that in patients whose disease permitted a choice between PTA and surgery, both therapies gave similar long term results. In addition, it was emphasised that unsatisfactory PTA may be followed by vascular reconstruction without adversely affecting the outcome. Although encouraging, this study excluded patients with more severe arterial disease who were not considered suitable for PTA.

As well as comparing PTA to surgery, the results of balloon dilatation should also be considered in relation to conservative treatments. Most patients treated by PTA have moderate claudication, which in the past would have been managed conservatively. No trial
Balloon Angioplasty

has yet addressed the question as to whether the indications for percutaneous intervention should be extended to include all patients with mild claudication, and similarly to whether all patients with a superficial femoral artery (SFA) stenosis should be prophylactically treated to prevent occlusion [Moore, 1991].

Specific indications for PTA have essentially evolved through consensus opinion. At present, the only undisputed indication is a haemodynamically significant isolated iliac artery stenosis, which is causing severe symptoms of ischaemia. Apart from this particular circumstance all other treatment decisions must be made by an individual risk-benefit analysis. Ideally, a local audit of results should predict the success of angioplasty in different circumstances, i.e. differing anatomical locations, the state of the distal vasculature, arterial stenoses versus occlusions, and localised versus diffuse disease. Using these data, it should then be possible to decide on a treatment option by balancing the risks and benefits of each treatment modality with the individual requirements of the patient. With reliable and careful reporting, it may be possible to standardise the indications for angioplasty on a widespread basis [Scobie, 1986], but further comparative studies will be required before this aim is realised.

4.4 Results of Balloon Angioplasty

Iliac Artery Angioplasty

Percutaneous transluminal angioplasty of iliac artery stenoses (Fig 4.2) is perhaps the least controversial and most widely accepted of all percutaneous vascular interventions. This procedure is highly effective for the treatment of symptomatic atherosclerotic disease of the iliac arteries and is also utilised to improve arterial inflow prior to distal reconstructive surgery. The results of iliac artery angioplasty from some of the better studies are illustrated in Table 4.3. Most dilatations are performed for intermittent claudication and the vast majority of lesions are stenotic rather than occlusive. The immediate technical and clinical success rate is high, varying between 89% and 96%. The technical difficulty of iliac angioplasty is directly related to the severity of the occlusive lesion, with occlusions being associated with a much higher initial failure rate [Morin et al.1986].

The reported actuarial 5-year patency rates vary between 43% and 90%. However, these figures are misleading as some series only report the patency of technically successful dilatations, excluding all failed procedures. Consequently, if an intention to treat basis, with strict follow up criteria are used, the approximate 5-year patency falls to 60%. In an study designed to identify the variables associated with long term success, Johnston et al. [Johnston et al.1987; Johnston, 1992] reported a prospective series of 667 iliac dilatations with an overall 5 year actuarial patency of 33%. Using a Cox regression analysis, the
Balloon Angioplasty

Factors predicting prolonged patency were the indication for the procedure, claudication being associated with a higher success rate than rest pain or tissue necrosis; the site of the angioplasty, common iliac angioplasties having higher patencies than external iliac angioplasties; the severity of the lesion, stenoses were dilated more successfully than occlusions and; the state of the run-off, patients with good distal run-off having a better outcome than patients with poor outflow vessels.

<table>
<thead>
<tr>
<th>Author</th>
<th>No (</th>
<th>Claud (%)</th>
<th>Sten (%)</th>
<th>Tech Success (%)</th>
<th>Failure Included</th>
<th>FU</th>
<th>Patency Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tegtmeyer 1991</td>
<td>340</td>
<td>59</td>
<td>97</td>
<td>93</td>
<td>N</td>
<td>C</td>
<td>96</td>
</tr>
<tr>
<td>1988</td>
<td>55</td>
<td>100</td>
<td>98</td>
<td>89</td>
<td>Y</td>
<td>HD+C</td>
<td>85</td>
</tr>
<tr>
<td>Cole 1987</td>
<td>159</td>
<td>NA</td>
<td>97</td>
<td>91</td>
<td>Y</td>
<td>HD+C</td>
<td>75</td>
</tr>
<tr>
<td>Johnston * 1987</td>
<td>667</td>
<td>88</td>
<td>88</td>
<td>91</td>
<td>Y</td>
<td>HD+C</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>80</td>
</tr>
<tr>
<td>Tegtmeyer 1988</td>
<td>36</td>
<td>97</td>
<td>100</td>
<td>89</td>
<td>N</td>
<td>HD+C</td>
<td>88</td>
</tr>
<tr>
<td>Janeski 1985</td>
<td>68</td>
<td>NA</td>
<td>100</td>
<td>90</td>
<td>N</td>
<td>HD+C</td>
<td>98</td>
</tr>
<tr>
<td>Blankensteijn 1986</td>
<td>37</td>
<td>89</td>
<td>100</td>
<td>95</td>
<td>N</td>
<td>C</td>
<td>94</td>
</tr>
<tr>
<td>van Andel 1985</td>
<td>194</td>
<td>NA</td>
<td>100</td>
<td>96</td>
<td>N</td>
<td>C</td>
<td>98</td>
</tr>
<tr>
<td>Gallino 1984</td>
<td>153</td>
<td>92</td>
<td>100</td>
<td>95</td>
<td>Y</td>
<td>HD+C</td>
<td>86</td>
</tr>
<tr>
<td>Waltman 1982</td>
<td>100</td>
<td>69</td>
<td>99</td>
<td>92</td>
<td>N</td>
<td>HD+C</td>
<td>98</td>
</tr>
<tr>
<td>Spence 1981</td>
<td>160</td>
<td>86</td>
<td>100</td>
<td>93</td>
<td>N</td>
<td>C</td>
<td>93</td>
</tr>
</tbody>
</table>

Table 4.3: Table illustrating the results of iliac angioplasty from studies utilising life-table analysis [Tegtmeyer et al. 1991; Wollenweber et al. 1986; van Andel et al. 1985; Johnston et al. 1987; Spence et al. 1981; Henriksen et al. 1988; Waltman et al. 1982; Gallino et al. 1984; Janeski et al. 1986; Cole et al. 1987; Blankensteijn et al. 1986]. No = number of angioplasties performed; Claud = percentage claudicants; Sten = percentage stenoses; Tech Success = percentage of lesions that were successfully dilated; Failure included = technical failures included in subsequent analysis (Y = yes, N = no); FU = follow up criteria (C = clinical follow up only; HD+C = haemodynamic and clinical follow up). * = common iliac artery; ** = external iliac artery.

These predictive factors have been confirmed by several other studies [Morin et al. 1986; Gallino et al. 1984; Cambria et al. 1987] which have also suggested that the presence of diabetes or a previous angioplasty at the same site may be associated with high failure rates. In most studies, the majority of initially successful dilatations that subsequently
fail, do so within the first year after the procedure [Cambria et al. 1987].

In the past, occlusion of the iliac artery was considered a contra-indication to PTA, due to the risk of distal embolisation or retroperitoneal bleeding [Graziani, 1987; Standards of Practice Committee of the Society of Cardiovascular and Interventional Radiology, 1990]. However, with increased experience, results following balloon dilatation of iliac occlusions are improving [Korogi, Takahashi, 1986; Colapinto et al. 1986]. Colapinto et al. [Colapinto et al. 1986] reported a series of 64 iliac occlusions that were dilated with a 78% technical success rate and a one year patency of 88%. In occlusions less than 5 cm in length, the immediate success rate was 92%. Recent technical advances such as the use of thrombolytic therapy and pull through approaches [Loose, Ryall, 1988; Ginsburg et al. 1989; Tegtmeyer et al. 1991] have further facilitated this technique, and iliac occlusions should no longer be considered a contra-indication to balloon dilatation.

Fig 4.2: Radiograph illustrating an iliac artery stenosis before (left) and after (right) angioplasty.

The success of iliac angioplasty has expanded the treatment options available for the management of patients with symptomatic iliac atherosclerosis. In mild disease, the efficacy of PTA has shifted the criteria for intervention to an earlier clinical stage, and patients in whom the decision to operate would be premature, may be ideally treated with PTA [Gallino et al. 1984]. Conversely, many patients with critical limb ischaemia are medically unsuitable
Balloon Angioplasty

for surgical revascularisation, and in this group PTA offers a low risk acceptable alternative [Jorgensen et al.1988].

In patients suitable for both PTA and reconstructive surgery, the decision between the two treatment modalities is more difficult, especially in the absence of a prospective randomised trial. Kwasnik et al. [Kwasnik et al.1987] retrospectively compared the results of aortic bifurcation grafts to iliac PTA in 61 patients, and reported that the long term results of balloon dilatation were limited by restenosis; but that reconstructive surgery had a higher complication rate. No conclusions can be drawn from studies of this kind, as the two patient groups had completely differing clinical and angiographic features.

It seems reasonable to suggest that the choice between PTA and reconstructive surgery should be made after considering the results of local practice. The two treatment modalities are not mutually exclusive and the level of surgical bypass is not compromised by failed angioplasty [Waltman et al.1982].

Femoropopliteal Angioplasty.

The vast majority of angioplasties performed in the femoropopliteal region involve the superficial femoral (SFA) or proximal popliteal arteries (Fig 4.3). From a general consensus of the available literature (Table 4.4), the overall technical success for angioplasty in this region is approximately 80%. The initial success rate varies with the experience of the radiologist and the type of catheter system used (co-axial versus balloon) [Krepel et al.1985], but the prime determinant of successful angioplasty in this segment, is the morphological characteristic of the arterial lesion [Standards of Practice Committee of the Society of Cardiovascular and Interventional Radiology, 1990]. Arterial occlusions are associated with a much higher technical failure rate than stenoses [Capek et al.1991], although this effect appears to be independent of the length of the lesion [Hewes et al.1986]. In addition, Capek et al. [Capek et al.1991] reported that the complication rate following dilatation of arterial occlusions was significantly higher than dilation of stenoses in the same location.

Although PTA of occlusive lesions is associated with high rates of initial technical failure, several studies [Capek et al.1991; Krepel et al.1985; Hewes et al.1986] have demonstrated that successfully dilated occlusions have identical long term patencies to similarly treated stenotic lesions. Newer techniques (e.g. thrombolytic therapy and retrograde catheterisation via the popliteal artery [Tonnesen et al.1988; Verstraete et al.1988]) which have been designed to improve initial success, are thus also likely to improve long term arterial patency following PTA for femoropopliteal occlusion.
In the long term, the patency of femoropopliteal angioplasty is influenced by the same general factors that predict the outcome of iliac angioplasty. Variables associated with a favourable outcome include claudication [Cambria et al. 1987; Adar et al. 1989; Capek et al. 1991], good distal run-off [Cambria et al. 1987; Krepel et al. 1985; Johnston et al. 1987; Gallino et al. 1984], and absence of diabetes mellitus [Gallino et al. 1984; Cambria et al. 1987; Hewes et al. 1986; Capek et al. 1991]. In addition to these general factors, plaque morphology and the distribution of the atherosclerotic disease has a significant effect on actuarial patency rates. Highly eccentric lesions [Krepel et al. 1985; Capek et al. 1991], the

![Table 4.4: Table illustrating the results of femoropopliteal angioplasty from studies utilising life-table analysis [Capek et al. 1991; Johnston et al. 1987; Murray et al. 1987; Walden et al. 1986; Janevski et al. 1986; Wollenweber et al. 1986; Hewes et al. 1986; Krepel et al. 1985; Gallino et al. 1984; Waltman et al. 1982; Spence et al. 1981]. No = number of angioplasties performed; Claud = percentage claudicants; Sten = percentage stenoses; Tech Success = percentage of lesions that were successfully dilated; Failure included = technical failures included in subsequent analysis (Y = yes, N = no); FU = follow up criteria (C = clinical follow up only; HD+C = haemodynamic and clinical follow up). * = stenoses only; ** = occlusions only.

<table>
<thead>
<tr>
<th>Author</th>
<th>No Cloud (%)</th>
<th>Sten (%)</th>
<th>Tech Success (%)</th>
<th>Failure Included</th>
<th>FU</th>
<th>Patency Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1y  2y  3y  4y  5y</td>
</tr>
<tr>
<td>Capek 1991</td>
<td>217</td>
<td>74</td>
<td>68</td>
<td>80</td>
<td>N</td>
<td>HD+C</td>
</tr>
<tr>
<td>Johnston 1987</td>
<td>241</td>
<td>79</td>
<td>62</td>
<td>82</td>
<td>Y</td>
<td>HD+C</td>
</tr>
<tr>
<td>Murray 1987</td>
<td>193</td>
<td>60</td>
<td>65</td>
<td>83</td>
<td>N</td>
<td>HD+C</td>
</tr>
<tr>
<td>Walden 1986</td>
<td>23</td>
<td>52</td>
<td>29</td>
<td>81</td>
<td>N</td>
<td>HD+C</td>
</tr>
<tr>
<td>Janevski 1986</td>
<td>12</td>
<td>NA</td>
<td>100</td>
<td>83</td>
<td>N</td>
<td>HD+C</td>
</tr>
<tr>
<td>Wollenweber* 1986</td>
<td>71</td>
<td>82</td>
<td>45</td>
<td>88</td>
<td>N</td>
<td>HD+C</td>
</tr>
<tr>
<td>Hewes 1986</td>
<td>137</td>
<td>70</td>
<td>57</td>
<td>87</td>
<td>N</td>
<td>HD+C</td>
</tr>
<tr>
<td>Krepel 1985</td>
<td>164</td>
<td>90</td>
<td>77</td>
<td>84</td>
<td>N</td>
<td>HD+C</td>
</tr>
<tr>
<td>Gallino 1984</td>
<td>280</td>
<td>61</td>
<td>59</td>
<td>87</td>
<td>Y</td>
<td>HD+C</td>
</tr>
<tr>
<td>Waltman 1982</td>
<td>98</td>
<td>50</td>
<td>65</td>
<td>88</td>
<td>N</td>
<td>HD+C</td>
</tr>
<tr>
<td>Spence 1981</td>
<td>122</td>
<td>71</td>
<td>100</td>
<td>84</td>
<td>N</td>
<td>C</td>
</tr>
</tbody>
</table>

Table 4.4: Table illustrating the results of femoropopliteal angioplasty from studies utilising life-table analysis [Capek et al. 1991; Johnston et al. 1987; Murray et al. 1987; Walden et al. 1986; Janevski et al. 1986; Wollenweber et al. 1986; Hewes et al. 1986; Krepel et al. 1985; Gallino et al. 1984; Waltman et al. 1982; Spence et al. 1981]. No = number of angioplasties performed; Claud = percentage claudicants; Sten = percentage stenoses; Tech Success = percentage of lesions that were successfully dilated; Failure included = technical failures included in subsequent analysis (Y = yes, N = no); FU = follow up criteria (C = clinical follow up only; HD+C = haemodynamic and clinical follow up). * = stenoses only; ** = occlusions only.
presence of diffuse stenotic disease [Cambria et al. 1987; Hewes et al. 1986] and lesions exceeding 3 cm in length [Gallino et al. 1984; Capek et al. 1991; Krepel et al. 1985] have all been described to adversely influence the long term restenosis rate following SFA angioplasty.

Despite the popularity of profundaplasty in revascularisation of the critically ischaemic lower limb, reports of profunda femoris angioplasty are surprisingly sparse. This may be partly due to the technical difficulty associated with this procedure [Dacie, Tennant, 1990]. Passage of a guide wire into a diseased profunda femoris artery through an ipsilateral antegrade puncture may be difficult due to disease at the arterial origin [Dacie, Daniell, 1991], high bifurcation of the common femoral artery, or wide angle of origin of the profunda. These problems have recently been overcome by utilising cross-over techniques, axillary [Villavicencio, Meier, 1991], or retrograde profunda approaches [Dacie, Tennant, 1990]. Dacie and Daniell [Dacie, Daniell, 1991] reported a series of 29 profunda angioplasties with a 90% technical success rate, whilst Hoffmann et al. [Hoffmann et al. 1992] described 43 balloon dilatations with 95% initial success. Utilising modern techniques, profunda femoris angioplasty may be performed with high reliability, and provides an effective alternative to surgical profundaplasty.
Balloon Angioplasty

**Aortic Angioplasty.**

The first descriptions of successful balloon dilatation of the abdominal aorta and its bifurcation came from Velasquez et al. [Velasquez et al. 1980] and Grollman et al. [Grollman et al. 1980] in 1980. Despite these early reports, it was not until recently that aortic angioplasty became an established technique for treating abdominal aortic stenoses. Initially, the development of aortic PTA was retarded due to difficulties in manufacturing large diameter balloons, but of late, concerns over distal embolisation have hampered acceptance of this technique [Ravimandalam et al. 1991]. Both of these potential problems have been overcome by adopting the so called "kissing balloon" technique, with a single balloon catheter introduced via each iliac artery. In patients with plaques close to the aortic bifurcation, the double balloon technique avoids plaque debris being displaced into the contralateral iliac artery. As the initial success and long term patency rates achieved by balloon dilatation are superior in larger vessels with high flow rates [Johnston, 1992], the results of aortic angioplasty may potentially surpass those of iliac PTA. One major advantage of PTA over surgery in this area is the absence of impotence or other sexual dysfunction associated with iatrogenic damage to the hypogastric plexus. One concern with this technique is that the aorta may be prone to rupture, as less force is required to rupture a vessel of large diameter [Yakes et al. 1989]. To date there has been one report of a post-angioplasty aortic rupture [Berger et al. 1986], in a patient with a calcified aortic plaque. Many series [Morag et al. 1987; Odurny et al. 1989] describing aortic angioplasty have predominantly reported a sub-group of patients, usually female smokers [Costantino et al. 1979; De Laurentis et al. 1978] who are prone to develop localised aortic disease in vessels of small diameter. In these cases aortic PTA is almost universally successful. More recent reports [Ravimandalam et al. 1991; Yakes et al. 1989; Belli et al. 1989] include patients with diffuse disease and longer stenoses. In these cases, the primary success rates exceeded 90% and although follow up is generally short, cumulative patency rates of 70% have been described [Odurny et al. 1989]. Although confirmation of the long term efficacy of this technique is required, aortic PTA may now be recommended for treatment of stenoses in the non-aneurysmal aorta. The place of PTA in calcified, eccentric or long segment stenoses has not yet been determined.

**Tibial Angioplasty.**

Up until recently, the risks of percutaneous intervention in vessels below the knee have restricted the indications for tibial angioplasty (Fig 4.4) to patients with critical limb
ischaemia [Standards of Practice Committee of the Society of Cardiovascular and
Interventional Radiology, 1990]. However, with the advent of coronary angioplasty,
technology has been applied to develop specialised small diameter angioplasty catheters. The
availability of low profile balloon angioplasty catheters, steerable guidewires and
vasodilatory drugs [Flueckiger et al. 1992] has enabled PTA to be safely applied to the crural
arteries.

Fig 4.4: Radiograph illustrating multiple stenoses in the crural vessels, before (left) and after (right) angioplasty.

Utilising these techniques, recent reports have documented initial success rates for
tibial angioplasty of between 86% and 97%, with cumulative 2 year patencies of
approximately 70% [Schwarten, 1991; Bakal et al. 1990; Horvath et al. 1990; Flueckiger et
al. 1992; Bull et al. 1992; Buckenham et al. 1993]. In all series the technical success rate was
reduced in occluded vessels. The recent success of tibial angioplasty has expanded the
indications for this procedure. It has been suggested that infrapopliteal angioplasty may now
be utilised in patients with severe claudication and may also have a role in maintaining the
patency of proximal reconstructions by treating diseased run-off vessels.
Angioplasty for Vein Graft Stenoses.

In addition to treating diseased lower limb arteries, PTA may be utilised to improve infrainguinal vein graft patency. The recent introduction of non-invasive vein graft surveillance programmes [Harris, 1992; Buth et al. 1991; Green et al. 1990] has facilitated detection of vein graft stenoses, which, if untreated would probably result in graft occlusion [Moody et al. 1989; Grigg et al. 1988]. Berkowitz et al. [Berkowitz et al. 1992; Berkowitz, Greenstein, 1987] were the first group to describe PTA for vein graft stenoses, and have recommended balloon dilatation as the primary therapy for this condition. In their most recent report, this group treated 81% of reversed vein graft stenoses with PTA, and reported a 31% recurrence rate.

London et al. [London et al. 1993] reported the percutaneous treatment of vein graft stenoses in both reversed and in situ bypass. In this series, 29 out of 33 vein graft stenoses were successfully treated with PTA, although repeated dilations were required in 6 cases. The importance of PTA in maintaining graft patency was illustrated by this study, in which the 42 month primary graft patency of 40% was improved to 65% by vein graft surveillance and treatment of stenoses.

4.5 Complications of Balloon Dilatation.

The complications affecting PTA may be classified as major, minor or radiographic. Major and minor complications cause clinical manifestations to a greater or lesser degree, whereas radiographic complications have no clinical sequelae and are only observed during angiography, i.e. contrast extravasation and subintimal dissection. The two major causes of mortality and morbidity complicating PTA are initial technical failure and the development of chronic restenosis at the angioplasty site. These two occlusive complications form the major topic to be investigated in this thesis, and will be discussed in the following chapter.

Reports of complications vary considerably between series, but from a consensus of the available literature (Table 4.5), it appears that major complications occur in approximately 7% of dilatations, with minor complications in 5%. Angioplasty related problems that require surgical repair are usually well documented and vary between 2-3% [Adar et al. 1989]. Fraedrich et al. [Fraedrich et al. 1987] reported a series of over 4000 lower limb angioplasties with a surgical intervention rate of 2.8%. The major causes of surgery in this series were haematoma formation or haemorrhage at the puncture site.

In general, bleeding problems at the puncture site are the commonest complications following angioplasty and reach 6% in most reports. Distal embolisation of atheromatous material is a constant threat, particularly during dilation of large vessels. Although the reported incidence of embolisation is around 1.5%, the need for surgery is much lower than
Balloon Angioplasty

this [Casarella, 1986], which implies that not all emboli have significant clinical effects.

<table>
<thead>
<tr>
<th>Author</th>
<th>Haem Puncture Site</th>
<th>Emboli</th>
<th>Perforation</th>
<th>Overall Comp</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capel 1991</td>
<td>-</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Tegtmeyer 1991</td>
<td>1.2</td>
<td>0.3</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adar 1989</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>Ameli 1989</td>
<td>1.8</td>
<td>0.9</td>
<td>2.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wilson 1989</td>
<td>-</td>
<td>9.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Henrikson 1988</td>
<td>9.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Weibull 1987</td>
<td>2.9</td>
<td>-</td>
<td>1.5</td>
<td>12.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Johnston 1987</td>
<td>5.1</td>
<td>0.1</td>
<td>1.4</td>
<td>3.4</td>
<td>4.6</td>
</tr>
<tr>
<td>Graziani 1987</td>
<td>1.2</td>
<td>-</td>
<td>8.2</td>
<td>0</td>
<td>9.4</td>
</tr>
<tr>
<td>Cambria 1987</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.5</td>
<td>11.3</td>
</tr>
<tr>
<td>Korogi 1987</td>
<td>3.8</td>
<td>2.6</td>
<td>1.1</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>Walden 1986</td>
<td>10</td>
<td>-</td>
<td>1.3</td>
<td>5.8</td>
<td>16.7</td>
</tr>
<tr>
<td>Hewes 1986</td>
<td>-</td>
<td>-</td>
<td>2.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>van Andel 1985</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Krepel 1985</td>
<td>1.8</td>
<td>-</td>
<td>2.4</td>
<td>1.9</td>
<td>8</td>
</tr>
<tr>
<td>Gallino 1984</td>
<td>3.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Waltman 1982</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Spence 1981</td>
<td>-</td>
<td>0.3</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Greenfield 1980</td>
<td>-</td>
<td>5.7</td>
<td>5.6</td>
<td>2.8</td>
<td>11.4</td>
</tr>
</tbody>
</table>

| Median          | 2.9                | 2.6    | 0.9         | 1.5          | 2.8   |
| 95% CI          | 1.2                | 1.1-2.3| 4.2        | 1.1-3.7      | 6.4-  |

Table 4.5: Table illustrating reported complications (%) after aorto-iliac and femoropopliteal angioplasty [Wilson et al.1989; Johnston et al.1987; Walden et al.1986; Weibull et al.1987; Waltman et al.1982; Graziani, 1987; Hemiksen et al.1988; Korogi et al.1987; Gallino et al.1984; Spence et al.1981; Cambria et al.1987; Adar et al.1989; van Andel et al.1985; Ameli et al.1989; Krepel et al.1985; Greenfield, 1980; Hewes et al.1986; Capel et al.1991; Tegtmeyer et al.1991]. Haem = haematoma; Bleed = bleeding complication; FA = false aneurysm; Maj = major; Surg = surgical intervention required; Min = minor; Median = median value of all results; 95% CI = 95% confidence interval. Median figures in italics are derived from a low number of series and the 95% CI are thus not given.

Similarly, vessel perforation does not always necessitate surgical repair, but is more likely to do so in the aorto-iliac region due to the risk of retroperitoneal bleeding. In addition to the major complications discussed, a plethora of less common problems have also been described to adversely affect PTA, e.g. septicemia [Weibull et al.1987], arterial rupture [Chong et al.1950; Murphy et al.1987], femoral neuralgia [Hallett et al.1990], arteriovenous fistulae [Johnston et al.1987] and contrast induced renal failure [Spence et al.1981].
Balloon Angioplasty

4.6 Summary.

The introduction of PTA has enhanced the treatment options available for relieving lower limb ischaemia. Although firmly established as a therapeutic technique, the indications for lower limb PTA have yet to be defined, and extensive randomised clinical trials will be required for this purpose. The major disadvantage of PTA is the mechanism of action, which results in severe vascular injury at the site of balloon dilatation. Damage to arterial wall during angioplasty is the initiating event producing acute arterial reocclusion and chronic restenosis, which are the two main complications limiting the long term success of this procedure. The cellular events involved in the pathogenesis of these complications will be discussed in the following chapter.
CHAPTER 5
ACUTE ARTERIAL CLOSURE AND CHRONIC RESTENOSIS
FOLLOWING PERCUTANEOUS TRANSLUMINAL ANGIOPLASTY

5.1 Introduction. 62

5.2 Acute Arterial Closure. 62
- Cellular events in acute thrombosis 64
- Mechanism of arterial spasm 65

5.3 Restenosis Following Angioplasty. 66
- Restenosis after percutaneous transluminal coronary angioplasty 66
- Histology of restenotic lesions 67

5.4 Myointimal Hyperplasia. 68
- Cellular biology of myointimal hyperplasia 68
- Effects of vascular trauma 69
- Role of platelets 70
- Role of growth factors 70
- Role of haemodynamic shear stress 72
- Role of the endothelium 73

5.5 Strategies for Preventing Restenosis Following Angioplasty. 76
- Pharmacologic prevention of restenosis 76
- New angioplasty techniques 79
- New approaches 80

5.6 Scope of This Thesis. 81
5.1 Introduction.

Despite significant advances in the technology and application of peripheral percutaneous transluminal angioplasty, acute arterial closure and the development of chronic restenosis remain the two complications which limit the efficacy of this procedure. Both of these complications may be attributable to the mechanism of angioplasty itself, and the consequent interaction of the damaged vessel wall with the circulating constituents of the blood. In this chapter, the pathophysiological events mediating acute arterial occlusion and chronic restenosis will be discussed, and a strategy to prevent both events will then be presented.

Most research concerning the haemorrhheologic response to balloon angioplasty has been performed on patients undergoing percutaneous transluminal coronary angioplasty (PTCA). Although performed on smaller coronary vessels, the mechanism of PTCA is identical to peripheral PTA, and the incidence of both acute vessel closure and chronic restenosis is similar in the two procedures. Although the cellular events that result from lower limb PTA have not been extensively researched, it appears reasonable to extrapolate data from studies of PTCA as the cellular responses to injury are likely to be analogous.

5.2 Acute Arterial Closure.

Acute arterial closure in peripheral vessels may be defined as reclosure of the dilated vessel within 24 hours of an attempted angioplasty. The incidence of acute closure is difficult to ascertain from many published series, as results are presented as initial technical and clinical success rates, without detailed analysis of the causes for procedural failure. Technical failure of an attempted balloon dilatation may be due to inability to successfully cross the lesion with a guidewire or accomplish adequate dilatation; but may also include causes of acute closure such as arterial rethrombosis or spasm. A limited number of series specifically report the acute closure rate (Table 5.1), which averages approximately 4%. Recently, Jorgensen et al. [Jorgensen et al. 1990] investigated the rate of acute arterial closure following femoro-popliteal angioplasty, and reported a 24 hour rethrombosis rate of 27%, which rose to 40% in cases of femoro-popliteal occlusion.

Acute closure following PTCA has been carefully documented in many studies, as acute arterial occlusion in the coronary circulation may necessitate emergency coronary artery bypass [Hollman et al. 1983]. The acute closure rate after PTCA is approximately 4% [Hollman et al. 1983; Holmes et al. 1984; King, 1990], and is higher in patients with
long stenoses, stenoses at branching points or with multivessel disease [King, 1990].

<table>
<thead>
<tr>
<th>Author</th>
<th>Distribution</th>
<th>Stenosis (%)</th>
<th>Acute Occlusion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capek 1991</td>
<td>FP</td>
<td>68</td>
<td>3.2</td>
</tr>
<tr>
<td>Jorgensen 1990</td>
<td>FP</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>Jorgensen 1990</td>
<td>FP</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>Ameli 1989</td>
<td>II + FP</td>
<td>82</td>
<td>1.8</td>
</tr>
<tr>
<td>Kwasnik 1987</td>
<td>II + FP</td>
<td>84</td>
<td>3</td>
</tr>
<tr>
<td>Walden 1986</td>
<td>FP</td>
<td>29</td>
<td>2.2</td>
</tr>
<tr>
<td>Wollenweber 1986</td>
<td>II + FP</td>
<td>64</td>
<td>5</td>
</tr>
<tr>
<td>Hewes 1986</td>
<td>FP</td>
<td>57</td>
<td>1.3</td>
</tr>
<tr>
<td>Gallino 1984</td>
<td>II + FP</td>
<td>73</td>
<td>9</td>
</tr>
<tr>
<td>Waltman 1982</td>
<td>FP</td>
<td>65</td>
<td>4.1</td>
</tr>
<tr>
<td>Spence 1981</td>
<td>II + FP</td>
<td>100</td>
<td>2.4</td>
</tr>
</tbody>
</table>


The pathophysiology of acute vessel closure is intimately related to the arterial injury caused during balloon dilatation of an atheromatous plaque. Balloon angioplasty causes severe mechanical damage to the arterial wall which is manifest in endothelial desquamation, exposure of the sub-endothelial matrix [Block et al.1980b; Block et al.1980a; Sanborn et al.1983], intimal dissection [Zarins et al.1982], splitting of the plaque [Losordo et al.1992] and stretching of the tunica media [Faxon et al.1982]. Acute vessel closure may result from mechanical obstruction due to a large intimal flap produced during dilatation; or due to interactions between the damaged vessel wall and the bloodstream, which may cause mural thrombosis and vasospasm [Simpfendorfer et al.1987; Waller et al.1984; Ellis et al.1988]. It is now considered that pure mechanical obstruction is rare and that occlusion following formation of an intimal flap is due to secondary intravascular thrombosis [King, 1990] or vasospasm.
Acute Arterial Closure and Chronic Restenosis

Cellular Events in Acute Thrombosis.

The cellular mechanism of acute arterial rethrombosis, is an exaggerated version of the normal haemostatic mechanism. Any form of mechanical arterial injury causing endothelial denudation will activate the haemostatic system by exposing the sub-endothelial matrix to the circulating blood [Mustard et al. 1987]. This reaction may be propagated by loss of the local anticoagulatory influence of the intact endothelial monolayer, and the expression of a procoagulatory phenotype in neighbouring cells [Stern et al. 1988]. The initial stage in the thrombotic process is the adhesion of platelets to the damaged arterial tissue, a process which is mediated through the interaction of platelet membrane glycoprotein I₃ (GPI₃) with sub-endothelial collagen fibres and von Willebrand factor [Berndt, Phillips, 1981; George et al. 1984]. Platelet adhesion stimulates platelet aggregation and degranulation which results in the local release of adenosine diphosphate (ADP), platelet factor 4 (PF4), β-thromboglobulin, and platelet derived growth factor (PDGF), in addition to activation of platelet membrane phospholipase and synthesis of thromboxane A₂ (TXA₂) [Harker, 1987; Zucker, 1980]. These mediators act synergistically to recruit circulating platelets to the damaged arterial site. With continuing aggregation a platelet thrombus may form as a result of platelet-vessel wall and platelet-platelet interactions. During aggregation, platelets express the platelet membrane fibrinogen receptor IIb/IIIa (GP IIb/IIIa), which binds to fibrinogen and results in cross linking of platelets and stable thrombus formation [Phillips et al. 1988; Coller, 1990; Leung, Nachman, 1986].

In addition to platelet deposition, arterial injury activates the coagulation cascade by both the intrinsic and extrinsic pathways, through expression of tissue factor and exposure of sub-endothelial collagen [Stern et al. 1988]. Release of platelet mediators accelerates the generation of thrombin, which in turn cleaves fibrinogen to fibrin and completes the thrombotic process. In homeostasis, the thrombotic process is regulated by plasma protease inhibitors [Harker, 1987], thrombomodulin [Preissner, 1988], protein C [Marlar, 1982], and the fibrinolytic system [Van Hinsbergh, 1988] to ensure that thrombosis is limited and that arterial patency is maintained.

Percutaneous transluminal angioplasty results in widespread mechanical damage to the arterial wall. In addition to superficial endothelial desquamation, balloon dilatation causes deeper arterial injury, with exposure of the sub-endothelium, media and atheromatous debris to circulating blood, thus providing a strong stimulus to platelet activation and thrombus formation. Increased platelet deposition following lower limb PTA has been demonstrated by several investigators using indium-111 platelet scintigraphy. In these studies, the platelet uptake index was increased approximately twofold following PTA, and maximal platelet deposition occurred in vessels with
Acute Arterial Closure and Chronic Restenosis

extensive intimai dissection [Pope et al.1985; Minar et al.1987; Minar et al.1989; Poskitt et al.1991]. Jorgensen et al. [Jorgensen et al.1992] illustrated activation of the coagulation cascade following angioplasty by quantifying an increase in systemic cross linked fibrin degradation products (D-dimer). Interestingly, in this study, the level of D-dimer was predictive of acute vessel closure with higher levels reported in patients with early rethrombosis.

Despite the administration of heparin and anti-platelet agents, platelet deposition and the formation of mural thrombi have been extensively reported in post-mortem studies examining coronary arteries after early post-angioplasty deaths [Waller et al.1984]. These post-mortem appearances have recently been confirmed by Uchida et al. [Uchida et al.1989] who demonstrated the presence of angioscopically identifiable mural thrombus 15 - 30 min following angioplasty.

Given that the formation of a mural thrombus is an almost universal event following balloon dilatation, the reported incidence of acute vessel closure in 4% of patients seems surprisingly low. Mustard et al. [Mustard et al.1990] have suggested that this may be due to local flow patterns within the dilated arterial segment, as in animal models, large thrombi do not from in conditions of laminar flow. It is possible therefore that if balloon angioplasty restores laminar flow through a dilated arterial segment, the formation of platelet-fibrin thrombi will be limited and reocclusion would be unlikely. If however, there is extensive arterial dissection or the formation of a large intimai flap, the resultant flow may be turbulent which would encourage thrombus formation and consequent arterial closure.

Mechanism of Arterial Spasm.

Arterial spasm may be a contributing factor in abrupt vessel closure, and has been described in 3-5% of coronary angioplasties [Cowley et al.1984; Dorros et al.1983] and up to 26% of renal angioplasties [Beinart et al.1983]. Sanders et al. [Sanders, 1985] noticed that there was a 16-62% reduction in intraluminal diameter within 30 min of coronary angioplasty, an effect which has been attributed to the occurrence of vascular spasm. Although well described both clinically and in animal models [Lam et al.1987; Steele et al.1985], the pathophysiology of post-angioplasty spasm remains incompletely defined.

Although it has been proposed that endothelial injury may cause arterial spasm due to the loss of EDRF [Fischell, Ginsberg, 1987], several animal studies have suggested that angioplasty induced vasospasm is primarily caused by the action of local vasoactive mediators (e.g. thromboxane A2, serotonin), released from aggregating platelets [Lam et al.1987; LeVeen et al.1985]. Lam et al. [Lam et al.1987] quantified platelet deposition on
Acute Arterial Closure and Chronic Restenosis

dilated porcine carotid arteries, and revealed that there was a direct correlation between the extent of platelet deposition and localised vasoconstriction. However, in the same experimental series, it was demonstrated that treatment with anti-platelet agents did not entirely abolish vascular reactivity in response to injury, and it was concluded that although platelets are important in initiating arterial spasm, other mechanisms may also be operative.

Recent studies have illustrated that endothelial cells may respond to mechanical forces by synthesising and releasing vasoactive substances capable of activating calcium dependent smooth muscle contraction [Katusic et al. 1987]. Fischell et al. [Fischell et al. 1989] demonstrated that stretch induced endothelium dependent myogenic activation is partially responsible for angioplasty induced vasoconstriction. This stretch induced effect is abolished by cyclooxygenase inhibitors and the vasoactive mediator concerned may be a prostanoid derived product.

Animal experiments have documented that spasm is most pronounced distal to the dilated arterial segment. The angioplasty site itself is severely damaged during balloon dilation, with associated smooth muscle injury and arterial paralysis. The arterial segments immediately adjacent to the angioplasty site are less severely damaged and are thus very reactive to vasoactive mediators [LaVeau et al. 1990], lowering the threshold for vasospasm.

5.3 Restenosis Following Angioplasty.

Rapid progress in catheter based technology has allowed the successful application of PTA in increasingly complex lesions. Unfortunately, the primary success rate of this technique is tempered by long term failure in over 40% of patients, due to restenosis at the angioplasty site [Nicolini, Pepine, 1992]. Although restenosis affects at least 30% of lower limb angioplasties (Tables 4.3 and 4.4), the vast majority of research concerning the pathology and prevention of post-angioplasty restenosis has been performed on patients undergoing PTCA. However, as stated previously, the mechanism of restenosis in both peripheral and coronary angioplasty is likely to be identical, and data may be extrapolated between the two procedures.

Restenosis After Percutaneous Transluminal Coronary Angioplasty.

Coronary angioplasty is an established treatment for coronary artery disease, with over 300,000 procedures being performed in the U.S.A. during 1989 [Califf et al. 1991]. Recurrence of the original obstructing lesion (restenosis), may occur in 17% - 53% of cases, depending on the diagnostic criteria used [Califf et al. 1991; Holmes et al. 1984;
Acute Arterial Closure and Chronic Restenosis

Ernst et al. 1987; McBride et al. 1988; Oershlick, de Bono, 1990. Restenosis occurs rapidly following angioplasty, with the maximal reduction in diameter occurring between the second and third month [Serruyes et al. 1988]. This rapid time course suggests that restenosis is due to a pathological process at the angioplasty site, rather than a deterioration of pre-existing atherosclerotic disease in the run-off vessels.

Coronary restenosis may present as a recurrence of angina or decreased exercise tolerance, but in up to 30% of cases may be asymptomatic [Nicolini, Pepine, 1992]. Restenosis rates are consistently higher in patients with diabetes, severe angina or myocardial infarction [Califf et al. 1989; Margolis et al. 1989; Halon et al. 1989]; and in some studies, persistent smoking [Galan et al. 1988], hyperlipidemia [Bergelson et al. 1989] and abnormalities of the fibrinolytic system [Kirschstein et al. 1989] have been identified as independent risk factors for restenosis. Interestingly, a number of coronary morphologic factors have been related to an increased risk of restenosis. Higher restenosis rates have been reported in lesions that are calcified [Guiteras Val et al. 1987], long [Hall, Gruentzig, 1984], or eccentric [Mata et al. 1985]. Lesions treated with an oversized balloon [Rubin et al. 1988], using multiple dilations, or high inflation rates [Mylar et al. 1987; Marantz et al. 1984; Dangoisse et al. 1982; Meier et al. 1984] also exhibit higher rates of restenosis which suggests that the degree of restenosis may be related to the extent of iatrogenic arterial injury.

Histology of Restenotic Lesions.

Despite the successful application of PTCA from 1977 onwards [Gruentzig et al. 1977], it is only recently that the pathological processes responsible for coronary restenosis have been defined. Waller et al. [Waller et al. 1991b; Waller et al. 1991a; Waller et al. 1990] examined restenotic lesions from 14 patients who had died several months after clinically successful coronary angioplasty. Histological analysis of the angioplasty sites demonstrated that fibrocellular intimal proliferation was responsible for restenosis in 60% of cases, and in three quarters of these, there was evidence of severe arterial injury. The lesions not showing evidence of intimal proliferation were composed of atheroma that exhibited no evidence of previous arterial injury. It was considered that, in these lesions, inadequate initial dilation or chronic elastic recoil was responsible for restenosis. The observations of Waller et al. have confirmed the findings of other smaller studies [de Morais et al. 1986; Duber et al. 1986; Bruneval et al. 1986; Ueda et al. 1987; Kohchi et al. 1987; Walley et al. 1988; Potkin, Roberts, 1988], that intimal proliferation is the principal histological feature in restenotic lesions.

With the advent of atherectomy, it has recently become possible to obtain tissue from restenotic lesions in vivo. Histological examination of this material verified that
Acute Arterial Closure and Chronic Restenosis

intimal hyperplasia was the predominant mechanism accounting for human restenotic lesions [Forrester et al.1991].

5.4 Intimal Hyperplasia.

All forms of arterial reconstruction cause iatrogenic injury to the arterial wall, with the initiation of a wound healing response [Ip et al.1990]. Although essential in maintaining vascular continuity, this response may cause wall thickening and eventual luminal reduction due to excessive myointimal hyperplasia [Clowes, Reidy, 1991]. In animals and man, this process starts immediately after injury, continues for a brief time, and is usually complete within 6 months. The fully formed intimal lesions appear white and fibrous, and consist of smooth muscle cells with surrounding extracellular matrix [Clowes, 1992].

Unfortunately, the arterial response to injury is difficult to investigate in man, as arterial biopsies cannot be routinely obtained at varying time points following reconstruction. Information regarding the cellular process of myointimal hyperplasia has therefore been largely derived from animal studies.

Cellular Biology of Myointimal Hyperplasia.

Balloon deendothelialisation has been used by many investigators to study the arterial response to injury [Painter, 1991]. In the balloon injury model, removal of the endothelium is followed by immediate platelet adherence to the sub-endothelial matrix [Groves et al.1979], with the local release of mitogenic growth factors [Goldberg et al.1980; Ip et al.1991]. Although the arterial injury destroys approximately 20% of the medial SMC's [Clowes, Clowes, 1983], high levels of proto-oncogenes are expressed within 30 minutes of injury [Miano et al.1990; Bauters et al.1992] and 24 hours after the initial insult, 20-30% of the remaining SMC's start to synthesise DNA [Clowes, Schwartz, 1985] and synchronously enter the growth cycle [Majesky et al.1987]. Several days after this initial wave of proliferation, SMC's start migrating from the media to intima, a process aided by the expression of proteolytic enzymes, which are capable of degrading the extracellular matrix [Clowes et al.1990; Sperti et al.1992].

Not all of the migrating cells undergo initial medial proliferation, Clowes and Schwartz [Clowes, Schwartz, 1985] estimated that non-dividing cells accounted for half the SMC's migrating across the internal elastic lamina. Once in the media however, the migrating cells continue to proliferate for at least 8 weeks and form a thick intimal layer [Clowes, Schwartz, 1985]. During proliferation and migration, the vascular SMC's undergo a phenotypic change from contractile to synthetic states. Synthetic cells exhibit an
Acute Arterial Closure and Chronic Restenosis

increased capacity to divide, and in addition, synthesise four or five times the amount of extracellular matrix produced by contractile cells [Liu et al. 1989]. The neointimal layer is thus further enlarged by the accumulation of extracellular matrix synthesised by the phenotypically modified SMC's. A steady state is reached 3 months after injury, at which time the intimal layer is composed of 80% matrix and 20% SMC's [Clowes, Clowes, 1983].

From this brief description of the cellular events involved in the generation of myointimal hyperplasia, it is apparent that the vascular SMC plays a pivotal role in this process. The molecular mechanisms that induce smooth muscle cell growth have not yet been fully elucidated, but probably involve a complex series of interactions between several biologic processes, which will be discussed in isolation.

**Effects of Vascular Trauma.**

Vascular injury may initiate SMC replication by antagonising the growth inhibitory influence of the intact arterial wall [Ip et al. 1991]. In the quiescent state, less than 1% of the medial SMC's actively proliferate, compared with 40-50% following injury [Webster et al. 1990]. It may be possible that the extent of the arterial injury determines the degree of myointimal proliferation.

Balloon deendothelialisation causes severe medial damage in addition to endothelial denudation [Consigny et al. 1986]. Following this degree of injury, it is impossible to ascertain whether the degree of SMC replication is determined by absence of the endothelial monolayer, or by primary damage to the medial SMC's. Experimental studies have demonstrated that removal of the endothelial monolayer without damage to the underlying media, causes increased medial SMC replication, but no intimal proliferation or thickening, irrespective of the size of the endothelial defect [Reidy, Silver, 1985; Fingerle et al. 1990; Tada, Reidy, 1987]. In contrast, Walker et al. [Walker et al. 1983] demonstrated that a narrow arterial injury, reendothelialised within 6 days, was able to stimulate intimal proliferation providing the depth of injury extended into the media.

The pattern of arterial repair following injury implies that medial damage is required to initiate myointimal proliferation. The mechanism of this action has not yet been fully defined, but it has been suggested that proliferation may be stimulated by an intracellular factor, possibly basic-fibroblast growth factor (b-FGF), that is released from damaged SMC's [Clowes, Reidy, 1991].
Acute Arterial Closure and Chronic Restenosis

Role of Platelets.

As has previously been discussed, platelets are deposited on the denuded arterial surface following vascular injury, in numbers proportional to the extent and depth of injury [Ip et al. 1990]. Adherent platelets release 97% of their α granules within 40 minutes of injury, which results in local release of platelet derived growth factor (PDGF), epidermal growth factor (EGF), and transforming growth factor beta (TGF-β) [Baumgartner, Muggli, 1976]. In the past it was postulated that platelet derived mitogens acted synergistically [Assoian et al. 1984] to stimulate activation and proliferation of vascular SMCs. Support for the mitogenic role of platelets in vivo was derived from the studies of Moore and Friedman [Moore et al. 1976; Friedman et al. 1977], in which the authors demonstrated that intimal hyperplasia was significantly reduced by prolonged thrombocytopenia. These studies were interpreted to suggest that platelets play an important role in inducing SMC replication.

This interpretation has recently been challenged by Fingerle et al. [Fingerle et al. 1989], who quantified SMC proliferation and intimal thickening, in rats made transiently thrombocytopenic by administration of a polyclonal anti-platelet antibody. Although in this study, the degree of myointimal hyperplasia was again reduced in thrombocytopenic animals, the SMC replication rate was identical in thrombocytopenic and control groups. These data suggest that platelets do not initiate the wave of SMC proliferation after injury, but probably regulate cellular migration into the intima. This conclusion is further supported by an in vivo study which demonstrated that an intravenous infusion of PDGF BB, the predominant isomer in rat platelets, is only a weak stimulant of SMC proliferation [Jawien et al. 1990].

Role of Growth Factors.

Platelet deposition stimulates migration of SMCs into the media, but appears to have little effect on SMC replication. Autocrine and paracrine growth factors regulate endothelial replication, SMC proliferation and migration, and extracellular matrix secretion. It has been suggested that growth factors may influence the progression of myointimal hyperplasia through these actions. Growth factors may be divided into competence and progression factors. Competence factors allows cells to move from G0 to G1 phase of the cell cycle, whereas progression factors are required before the cell enters S phase and starts to synthesise DNA [Cerceke et al. 1991].
Platelet Derived Growth Factor (PDGF).

Platelet derived growth factor is released by platelets [Ross et al. 1974], activated endothelial cells [Di Corletto, Bowen-Pope, 1983], and vascular SMCs [Nilsson et al. 1985]. PDGF is composed of two polypeptide chains A and B, with three possible dimer combinations AA, BB and AB. PDGF AA has strong mitogenic activity but is not as effective as PDGF BB or AB in promoting SMC migration [Siegbahn et al. 1990; Fagin, Forrester, 1992]. The differing effect of the various PDGF isomers may be due to differential expression of PDGF receptors on the SMC surface [Majesky et al. 1990].

Immediately following vascular injury, platelets adhere to the damaged vascular surface and release PDGF BB and AB into the microenvironment of the arterial wall. These dimers of PDGF stimulate SMC migration, but in addition activate medial SMC's to produce PDGF AA. PDGF AA is a competence factor for SMC's allowing them to move from the quiescent (G0) to G1 phase of the cell cycle. The SMC's then require a progression factor to enable DNA synthesis to occur [Fagin, Forrester, 1992]. Thus, although PDGF probably drives the migration of SMC's from media to intima, it also has a significant effect in preparing medial cells for proliferation.

Insulin-type growth factor type 1 (IGF-1) is the most important progression factor for PDGF. IGF-1 is produced in high levels by medial SMC's after arterial trauma, and it is possible that the biochemical inducer of IGF-1 expression is PDGF itself [Cercek et al. 1991].

Basic Fibroblast Growth Factor (bFGF).

Basic fibroblast growth factor is one of the heparin binding growth factors [Lobb et al. 1986] that is synthesised by both endothelial and smooth muscle cells [Vlodavsky et al. 1987b; Gospodarowicz et al. 1988]. Under quiescent conditions, bFGF is concentrated in the nucleus of these cells, and stored in the sub-endothelial matrix [Vlodavsky et al. 1987a]. This sub-endothelial store forms a pool of bFGF in the arterial wall which is released during vascular injury to act on the surrounding cells [Gajdusek, Carbon, 1989; McNeill et al. 1989].

Basic FGF is a powerful stimulus to replication of both SMC's and endothelial cells [Lindner et al. 1990]. Lindner et al. [Lindner et al. 1991] demonstrated that systemically administered bFGF increased the SMC replication rate following balloon deendothelialisation from 11.5% to 54.8%. In the same experiment it was reported that a similar increase in SMC proliferation followed bFGF infusion after superficial injury affecting the endothelium alone. These results suggested that bFGF was a potent mitogen for SMC's in vivo, and that the release of endogenous bFGF may initiate SMC
Acute Arterial Closure and Chronic Restenosis

proliferation after vascular injury. Further support for this hypothesis was derived from a separate study in which Lindner and Reidy [Lindner, Reidy, 1991] demonstrated that an antibody directed against bFGF could decrease the extent SMC proliferation following injury by 80%. Interestingly, although SMC proliferation was reduced, the size of the intimal lesion was unaffected by this treatment, which suggested that bFGF had no effect on SMC migration.

In addition to its mitogenic role, bFGF is a potent stimulus to its own expression [Vlodavsky et al.1987a], which provides an important mechanism for amplifying SMC proliferation after injury. Further amplification of this response may be achieved by enhanced expression of high affinity bFGF receptors following arterial trauma [Casscells et al.1990].

_transforming growth factor beta (TGF-β)_.

In the clinical setting, restenosis occurs approximately 3 - 6 months following angioplasty [Serruys et al.1988], at which time the bulk of the neointimal plaque is composed of extracellular matrix proteins synthesised by vascular SMC's [Clowes, Clowes, 1983]. Transforming growth factor beta is a polypeptide released in large quantities by platelets [Sporn, Roberts, 1983], macrophages [Sporn et al.1987], endothelial and smooth muscle cells [Antonelli-Orlidge et al.1985] which stimulates the production of glycosaminoglycans, collagen, and fibronectin [Fagin, Forrester, 1992]. Liberation of TGF-β following arterial injury may be a major factor regulating extracellular matrix deposition during vascular repair.

_role of haemodynamic shear stress_.

Anatomic studies have revealed that intimal thickening tends to be located in areas of low shear stress e.g. the lateral wall of arterial branches [Ku et al.1985]. This association has been confirmed in studies of venous bypass grafts, where an inverse relationship has been demonstrated between the rate of blood flow and the degree of myointimal hyperplasia [Zarins et al.1981; Dobrin et al.1989]. Areas of low shear stress are usually associated with marked flow disturbances [Liu et al.1989], and it has been suggested that the fluctuation in flow is more important in initiating intimal thickening than the absolute value of wall shear stress [Bassiony et al.1992]. Inadequate balloon dilatation of an atherosclerotic plaque may cause a residual stenosis with consequent fluctuation in local blood flow. In these cases, abnormal haemorheology in the neighbouring arterial segment, distal to the residual lesion, may favour accelerated intimal thickening and luminal reduction [Ip et al.1991].
Role of the Endothelium.

The role of the endothelium in modulating the arterial response to injury is both complex and controversial. The majority of data investigating this subject have been obtained from experiments studying the cellular events following mechanical endothelial denudation. Recently, tissue and organ culture techniques have been utilised to further elucidate the inter-relationship between endothelial and vascular SMCs, which may form the basis of the cellular response to arterial injury.

Denudation of the endothelium during arterial reconstruction is accompanied by an immediate response which aims to reconstitute the endothelial monolayer. Small endothelial defects are rapidly repaired by migration and proliferation of neighbouring endothelial cells [Reidy, Schwartz, 1979]. After more extensive injury, endothelial cells at the edge of the wound begin to proliferate, and the growing edge advances at a rate of 0.2 mm/day [Clowes, Reidy, 1991]. However, after 4-6 weeks, the rate of endothelial repair slows, and stops spontaneously within 8-12 weeks [Reidy et al. 1983], leaving a large area of unhealed tissue which may remain deendothelialised for up to 3 years [Reidy, 1985]. The mechanisms limiting endothelial regrowth are not completely understood, but may relate to a loss of replicative capacity in cells forming the leading edge of the endothelial sheet [Reidy, 1985].

The first evidence that endothelial cells had an inhibitory effect on myointimal hyperplasia derived from the observation that rapid reendothelialisation after limited arterial injury, was associated with little or no intimal thickening [Reidy, Schwartz, 1979]. Similarly, Haudenschild and Schwartz [Haudenschild, Schwartz, 1979], demonstrated that after complete deendothelialisation of the rat thoracic aorta, SMCs only migrated into areas uncovered by regenerating endothelium. From these findings it was concluded that injured intimal areas were protected from the development of myointimal hyperplasia by reendothelialisation. This conclusion was supported by Bjorkerud and Bondjers [Bjorkerud, Bondjers, 1973] who demonstrated that arterial areas uncovered by regenerating endothelium were associated with the greatest degree of intimal thickening, whereas in reendothelialised segments, intimal lesions regressed.

Although the endothelium may modulate the degree of myointimal hyperplasia following arterial injury, there is substantial evidence to suggest that it is not the sole determinant of SMC proliferation and migration. In chronically denuded arterial segments, the neointimal thickness reaches a peak within 8 weeks, and remains the same up to 1 year after injury, despite the continued endothelial absence [Clowes, Clowes, 1983; Clowes et al. 1983; Clowes et al. 1986]. In addition, injuries designed to remove the endothelium without associated medial damage, result in a wave of SMC proliferation but no intimal
Acute Arterial Closure and Chronic Restenosis

thickening [Reidy, Silver, 1985; Fingerle et al. 1990; Tada, Reidy, 1987]. These findings suggest that absence of the endothelium alone is not sufficient to induce an intimal lesion, and that intimal proliferation may cease prior to reendothelialisation.

Recently Jamal et al. [Jamal et al. 1992] compared the arterial responses to differing forms of hyperdistension injury. It was demonstrated that severe medial damage, in the absence of endothelial injury did not result in intimal proliferation, and that both endothelial and medial injury were required to initiate intimal thickening. These findings suggested that although loss of the endothelium alone was unable to stimulate myointimal hyperplasia, an intact endothelial monolayer was able to inhibit the medial response to injury, and thus prevent the development of intimal lesions.

The inhibitory effect of the endothelium may be due to its action as a permeability barrier, or due to the secretion of growth inhibitory substances. Deendothelialisation removes the normal permeability barrier of the arterial wall and allows growth factors in the plasma to access medial SMCs. In this manner, plasma derived mitogens (e.g. PDGF, IGF, EGF and serotonin) may induce cellular proliferation and migration [Fagin, Forrester, 1992]. Plasma proteins may also cause a cellular response, fibronectin promotes modulation of SMCs from the contractile to synthetic phenotype [Thyberg et al. 1990].

In addition to providing a barrier to plasma derived products, the endothelium secretes SMC specific growth inhibitors. Spontaneous phenotypic modulation of isolated SMCs is prevented by growth in co-culture with a confluent endothelial cell monolayer [Chamley-Campbell, Campbell, 1981], whilst endothelium conditioned growth medium inhibits vascular SMC proliferation in vitro [Castellot et al. 1981]. The anti-proliferative effect of the endothelium is abolished by treatment with heparinase, which suggests that endothelial cells produce a heparinlike inhibitor of SMC function [Castellot et al. 1987]. Heparin is a glycosaminoglycan which is a highly effective inhibitor of SMC proliferation as well as migration [Clowes, Clowes, 1985; Majesky et al. 1987; Clowes, Clowes, 1986]. Heparin also alters the composition of the extracellular matrix to reduce the collagen content and increase production of heparin sulphate proteoglycans [Snow et al. 1990]. The anti-proliferative and anti-migratory actions of heparin are probably mediated through inhibition of growth factors [Saksela et al. 1988], interference with enzymes required to degrade the extracellular matrix [Clowes et al. 1992; Au et al. 1992b; Au et al. 1992a], and a direct receptor mediated inhibition of cell division [Fagin, Forrester, 1992]. It is probable that continuous production of heparinlike factors from endothelial cells is responsible for the low rate of SMC proliferation in the quiescent arterial wall.

Recently, Weidinger et al. [Weidinger et al. 1990] demonstrated a significant correlation between the degree of SMC proliferation and the loss of endothelium-
Acute Arterial Closure and Chronic Restenosis

dependant vasodilation following vascular injury. Nitrovasodilators inhibit SMC replication in vitro [Garg, Hassid, 1989], and it is possible that reduced release of EDRF from the damaged endothelial monolayer may contribute to the generation of injury induced myointimal hyperplasia.

Although the inhibitory, anti-mitogenic capacity of the endothelium has been well documented, organ culture studies have suggested that in some circumstances the endothelium can stimulate SMC proliferation. Koo and Gotlieb [Koo, Gotlieb, 1991] demonstrated in porcine aortic organ culture that the rate of SMC proliferation accelerates when the endothelium is actively dividing, and conversely that SMC proliferation slows when endothelial regeneration ceases. These observations prompted the authors to hypothesise that dividing endothelial cells may regulate SMC growth by the release of soluble growth factors, and that when endothelial replication stops, growth factor secretion is diminished with the consequent stabilisation of the intimal lesion. Similar findings have been reported by Angelini et al. [Angelini et al.1991] in an organ culture of human saphenous vein.

Further evidence supporting the pro-mitogenic role of endothelial cells has been described by Clowes et al. [Clowes et al.1985], who demonstrated that in healing prosthetic grafts, the rate of SMC proliferation was enhanced beneath areas of regenerating endothelium. In this study, the authors also reported that perfusate from the healing graft exhibited significantly more mitogenic activity than perfusate from a native artery. It seems likely therefore that endothelial cells have the ability to stimulate intimal proliferation through the secretion of soluble growth factors. This scenario is supported by evidence demonstrating that the endothelium is capable of synthesising and releasing a large number of growth factors including PDGF [Di Corletto, Bowen-Pope, 1983], bFGF [Schweigerer et al.1987], and interleukin-1 [Miossec et al.1986].

One of the recurring themes in the study of endothelial cell function, as outlined in chapter 2, is the ability of the endothelium to promote diametrically opposed reactions in different circumstances. The same characteristic is recognised in the control of SMC growth, with both pro- and anti-mitogenic capabilities being expressed after arterial injury. Following balloon dilatation of an atheromatous plaque, the endothelial monolayer is severely damaged, and endothelial replication is stimulated at the edge of the wound. Endothelial proliferation may be associated with the release of mitogenic growth factors, which enhance the SMC response to injury. Growth factor secretion continues until endothelial continuity is restored or replication ceases. At this time the quiescent endothelium stops producing mitogenic growth factors and commences synthesis and release of heparin and EDRF, which will inhibit SMC proliferation and migration. The degree of intimal thickening after balloon angioplasty may thus be determined by the balance between pro- and anti-mitogenic factors derived from the endothelium.
5.5 Strategies for Preventing Restenosis Following Angioplasty.

Restenosis following angioplasty is a serious clinical problem and accordingly, a vast amount of basic and clinical research has been directed towards reducing chronic restenosis rates. Much of this research has been performed in experimental models, but unfortunately, agents which prevent myointimal hyperplasia in animals do not have an equivalent effect in man. The strategies aiming to prevent chronic restenosis may be divided into pharmacological approaches, aiming to modify the cellular response to vascular injury, and mechanical techniques which attempt to alter the process of angioplasty itself. Despite considerable effort, as yet no technique has been definitively proven to reduce the incidence of restenosis following PTA [Califf et al. 1991].

Pharmacologic Prevention of Restenosis.

Numerous agents have been investigated in an attempt to define a single therapeutic agent which will reduce clinical restenosis. A brief review of the most widely used drugs is presented.

Anti-Platelet Agents.

Aspirin, an anti-aggregatory agent, has been extensively investigated in clinical restenosis trials. Several studies have shown that administration of acetyl-salicylic acid decreases the acute arterial reocclusion rate following PTCA [Barnathan et al. 1987; Schwartz et al. 1988], and most patients undergoing this procedure now receive some form of anti-platelet therapy. However, in clinical trials [Schwartz et al. 1988; Thornton et al. 1984; White et al. 1987; Finci et al. 1988], no significant difference in the rate of restenosis was observed between patients receiving aspirin therapy and non-treated controls, although there was a trend towards lower restenosis rates when low dose aspirin was used [Mufson et al. 1988]. Similarly, dipyridamole has no effect on restenosis after PTCA [Schwartz et al. 1988; White et al. 1987].

Anticoagulants.

Heparin has been considered a promising agent for preventing restenosis, as it has both anticoagulatory and anti-proliferative effects. Early discontinuation of heparin after PTCA is associated with high rates of arterial re-occlusion, and again heparin therapy is routinely given during coronary angioplasty [Hermans et al. 1991]. However, in a
Acute Arterial Closure and Chronic Restenosis

prospective randomised trial of 416 patients, Ellis et al. [Ellis et al. 1989] demonstrated no difference in angiographically defined restenosis between patients receiving either heparin or placebo.

In animal models heparin is effective in reducing intimal thickening if started just before or after arterial dilation, but is considerably less effective if started more than 48 hours following injury [Clowes, Clowes, 1986]. Low molecular weight heparin (LMWH), which has no systemic anti-coagulatory effect, has been shown to be effective in inhibiting intimal thickening [Dryjski et al. 1988], and appears to offer the greatest potential for preventing intimal hyperplasia. A multicentre double blind controlled clinical trial with LMWH is currently underway in the U.S.A. and results of this study are awaited with interest.

Anti-Proliferative Agents.

The mechanism of restenosis following angioplasty is known to involve SMC proliferation and migration in response to injury. In an attempt to modify this response, several investigators have utilised systemically administered anti-proliferative agents to reduce the degree of myointimal hyperplasia. Currier et al. [Currier et al. 1989] demonstrated that colchicine, a cytotoxic agent, could decrease restenosis in an animal model, although the dose used was such that clinical application is likely to be limited by a high incidence of side effects.

An alternative strategy would be to reduce SMC proliferation through inhibition of mitogenic growth factors released after arterial injury. Triazolopyrimidine inhibits PDGF stimulated cell replication in vitro, and has been demonstrated to decrease the degree of restenosis following angioplasty in a rabbit model [Liu et al. 1990]. Similarly, the extent of injury induced SMC proliferation may be reduced by treatment with an anti-bFGF antibody [Lindner, Reidy, 1991], and a clinical trial of growth factor antagonists seems warranted.

Angiotensin Converting Enzyme Inhibitors.

Angiotensin II is a potent SMC mitogen in vivo [Dalmen et al. 1991], which causes a rapid induction of proto-oncogenes c-fos, c-jun and c-myc [Naftilan et al. 1990; Naftilan et al. 1989]. In addition to these mitogenic effects, angiotensin II stimulates protein [Berk et al. 1989] and glycosaminoglycan synthesis [Fagin, Forrester, 1992] which may act to increase extracellular matrix deposition in the generation of an intimal plaque. Although angiotensin converting enzyme (ACE) inhibitors do not dramatically inhibit SMC proliferation in vitro, Powell et al. [Powell et al. 1991b; Powell et al. 1991a] demonstrated
that ACE inhibitors could inhibit the formation of intimal hyperplasia following injury. Two large multi-centre clinical trials were designed to investigate the effects of cilazapril on restenosis following PTCA, but the results of the recently published MERCATOR trial have concluded that long term ACE inhibition does not prevent restenosis [Serruys, 1992].

Calcium Channel Blockers.

Calcium channel blockers decrease platelet aggregation, prevent coronary spasm, and reduce the incidence of restenosis in animal models [Faxon et al. 1984]. However, in several placebo controlled clinical trials, neither nifedipine [Whitworth et al. 1986], or diltiazem [O'Keefe et al. 1991], had any significant effect preventing restenosis.

Fish Oil.

Epidemiologic studies have revealed that a diet rich in eicosapentanoic acid (EPA) may account for the low incidence of coronary artery disease in Eskimos. Eicosapentanoic acid replaces arachidonic acid in the prostaglandin synthetic pathway (Fig 2.1) with a consequent decrease in the activity of thromboxane derived products and an enhancement of PGJ2 derived compounds, which combine to inhibit platelet aggregation [Nicolini, Pepine, 1992]. Administration of omega-3 fatty acids has also been associated with improved endothelial dependent relaxation [Vekshtein et al. 1989] and an anti-proliferative effect on SMC's due to the inhibition of PDGF [Fox, D'Corleto, 1988].

In 1988, one small controlled clinical trial demonstrated that prolonged administration of fish oil was associated with a significant reduction in the restenosis rate [Dehmer et al. 1988], but since that time several other trials have failed to demonstrate any convincing benefit [Bowles et al. 1991; Grigg et al. 1989; Reis et al. 1989]. Overall, it seems unlikely that fish oil therapy is effective in preventing post-angioplasty restenosis.

Anti-Inflammatory Agents.

Immunologic mechanisms are now recognised to be important in atherogenesis (chapter 1), and glucocorticoids have been shown to reduce atherosclerosis in animal models [Gordon et al. 1988]. Hydrocortisone can inhibit SMC proliferation in culture [Gordon et al. 1987], and steroids have been suggested as potential inhibitors of restenosis. A multi-centre double blinded placebo controlled trial failed to show any benefit of methylprednisolone therapy following PTCA, although it was suggested that some effect may have been present in low risk lesions [Pepine et al. 1990]. It is possible
Acute Arterial Closure and Chronic Restenosis

that the severe damage created in high risk lesions was too great to be influenced by one
dose of steroids and further studies are required.

New Angioplasty Techniques.

Laser Angioplasty.

Great enthusiasm accompanied the introduction of percutaneous laser angioplasty,
which offered the potential to ablate atherosclerotic plaques with minimal damage to the
arterial wall. In the majority of systems in current use, continuous wave laser energy is
transmitted through a fiberoptic delivery system to a catheter tip, which converts the laser
energy to controlled thermal energy. Atherosclerotic plaques may then be ablated or
debulked by direct contact with the "hot tip" laser probe. Laser angioplasty may be used in
isolation or in combination with balloon angioplasty.

Histologically, laser angioplasty causes severe thermal injury to the arterial wall,
which remains smooth in appearance with an intact internal elastic lamina [Sanborn
et al.1987]. This form of injury may reduce the thrombogenicity of the angioplasty site by
welding and sealing the arterial wall. Abela et al. [Abela et al.1990] demonstrated that
platelet deposition was less after laser than after balloon angioplasty.

Clinically, femoropopliteal and iliac artery laser angioplasty has been performed
with good initial success rates averaging approximately 70% [Blebea et al.1991; Criado
Sanborn et al.1989; Seeger et al.1989; White et al.1990]. As with conventional
angioplasty techniques, technical success depends upon the diameter of the arterial
segment, the length of the lesion and the degree of stenosis or occlusion. However,
although excellent results have been reported by some centres [Lammer et al.1991,
Sanborn et al. 1989], the long term patency rates in series using life table analysis rarely
exceed 50% at 1 year [Blebea et al.1991; Perler et al.1989; White et al.1990], suggesting
that restenosis rates may be equal to or greater than those achieved with conventional
balloon angioplasty. In a recent prospective randomised trial, Lammer et al. [Lammer
et al.1992] demonstrated that 12 month patency rates were identical between continuous
wave and pulsed wave laser angioplasty, as compared with conventional angioplasty.
From these results the authors concluded that laser assisted angioplasty should only be
considered after failure of conventional PTA.

Atherectomy.

Atherectomy involves the controlled mechanical removal of atheroma from
diseased arteries. Atherectomy was introduced with the hypothesis that removal of the atherosclerotic mass would create a larger lumen and reduce the incidence of intimal dissection, thus improving the technical success rate. Removal of the atherosclerotic plaque without damage to the arterial wall should create a smooth surface with minimal flow disturbance.

At present at least 4 types of atherectomy devices are available [Simpson et al. 1988; Wholey, Jarmolowski, 1989; Ahn et al. 1990; Cull et al. 1991] and experience with each type is limited. However, despite the theoretical attraction of this technique, in a recent report, Simpson et al. [Simpson et al. 1988] reported a restenosis rate of 36%, and similarly high restenosis rates have been observed during cardiac atherectomy [Stack et al. 1990].

**Stent Implantation.**

Intravascular stents are presently utilised in an attempt to overcome elastic recoil and intimal dissection following balloon angioplasty [Becker et al. 1989]. Intravascular stents provide a radial dilating force to overcome elastic recoil and compress plaque dissections against the vascular wall, to create a rounded channel. To date, stents have been most extensively used in the iliac arteries, with several investigators reporting technical success rates greater than 90% in recanalising iliac occlusions [Gunther et al. 1991; Gunther et al. 1989; Strecker et al. 1990]. There is also some evidence to suggest that the immediate haemodynamic results of stenting are superior to those of balloon dilation [Richter et al. 1991].

Recently, Palmaz et al. [Palmaz et al. 1990] reported the results of a prospective randomised trial comparing conventional balloon dilatation with primary stenting. At 24 months follow up, patency was significantly higher in the stented group, suggesting a reduction in the degree of restenosis. Further research is required to substantiate this effect.

**New Approaches.**

Despite the vast quantity of data collected on the generation of myointimal hyperplasia following angioplasty, there is still no single therapeutic technique that reduces restenosis rates. Clowes et al. [Clowes et al. 1991; Clowes, 1992] have suggested that a combination of therapeutic agents may be required, and experimentally a limited course of heparin with a longer course of cilazapril produces a beneficial effect.

One of the problems with systemically delivered pharmacologic agents is that the maximum dosage achievable at the site of action may be limited by systemic side effects.
Acute Arterial Closure and Chronic Restenosis

This problem may be overcome by the local delivery of therapeutic agents. Edelman et al. [Edelman et al. 1990] demonstrated that the controlled adventitial release of heparin may be more effective in inhibiting SMC replication than systemic therapy. Similarly, Gimple et al. [Gimple et al. 1991] used a perforated balloon catheter to inject heparin directly into the arterial wall with a consequent reduction in the restenosis rate.

As the mechanism of restenosis has been elucidated, it has become possible to target certain crucial steps in the generation of myointimal hyperplasia. The promise of specific growth factor inhibitors has been alluded to previously. Recently, Simons et al. [Simons et al. 1992] used a synthetic antisense oligonucleotide probe to inhibit proto-oncogene expression in the vessel wall. Local application of this oligonucleotide significantly reduced the degree of intimal thickening following arterial injury, and similar molecular based techniques offer a new therapeutic approach to preventing restenosis.

In this thesis a cellular therapy to prevent restenosis is suggested, and the basis of this hypothesis is described below.

5.6 Scope of This Thesis.

Percutaneous transluminal angioplasty is increasingly used as a first line treatment for both peripheral and coronary arterial occlusive disease. This endovascular technique has advantages over conventional bypass surgery in that it may be performed under regional anaesthesia and usually necessitates a short hospital stay. However, the efficacy of balloon angioplasty is limited by the incidence of both acute arterial closure and chronic restenosis which may affect PTA in up to 50% of cases. Both of these complications are directly attributable to the mechanical arterial damage produced during angioplasty itself, which is manifest in total endothelial desquamation, intimal dissection and severe injury to the tunica media.

Loss of the endothelial monolayer is a pivotal event initiating both early arterial reocclusion and chronic restenosis. In the acute phase, loss of the endothelial cell monolayer exposes the highly thrombogenic sub-endothelial matrix to the circulating blood, with the consequent deposition of platelets and activation of the coagulation cascade. A mural thrombus is thus formed on the damaged vascular surface, which may progress to complete arterial occlusion through continuing intra-vascular thrombosis or vasospasm.

Chronic restenosis following angioplasty is due to the generation of myointimal hyperplasia at the angioplasty site. Loss of the overlying endothelium may affect the formation of a neointimal lesion by removing the permeability barrier between the blood and the media, and also by altering the balance between inhibitory and stimulatory endothelial derived mediators. Loss of the mechanical endothelial barrier allows plasma
Acute Arterial Closure and Chronic Restenosis

and platelet derived growth factors to come into direct contact with medial SMC's, thus stimulating the wave of SMC proliferation and migration that accompanies arterial injury. In the quiescent state, the endothelium secretes heparin sulphate glycosaminoglycans which inhibit SMC replication and migration. Damage to the endothelium during balloon dilatation not only removes the tonic inhibitory influence of the intact endothelial monolayer, but also promotes the secretion of endothelial-derived mitogens from replicating endothelial cells. Secretion of these mitogenic growth factors continues until endothelial continuity is restored or until endothelial replication ceases.

The central tenet of this thesis is the hypothesis that rapid restoration of the endothelial monolayer following angioplasty may have the potential to decrease both early arterial reocclusion and chronic restenosis. It is proposed to use endothelial seeding techniques to restore the endothelial monolayer immediately following angioplasty. Seeding of native vascular surfaces is theoretically attractive as the damaged angioplasty site should provide an excellent substrate for endothelial cell retention.

Restoration of the endothelial monolayer may reduce the immediate thrombogenicity associated with balloon dilatation by partially restoring a mechanical permeability barrier, and by providing a functioning source of anti-thrombotic mediators e.g. PGI₂, EDRF. In the longer term, seeding the post-angioplasty surface may shorten the time period of endothelial regeneration, and thus decrease the production of endothelial-derived SMC mitogens. Rapid restoration of a confluent monolayer should also stimulate secretion of heparin glycosaminoglycans which have the potential to decrease the degree of intimal thickening associated with arterial injury.

Due to the absence of substantial research in this field, this thesis combines both in vitro and in vivo studies. Chapters 7-9 investigate endothelial seeding in an in vitro model of vascular damage, in an attempt to study the degree of endothelial attachment, retention and function on native vascular surfaces. These in vitro studies also aimed to determine the optimum seeding parameters for use during in vivo experiments which are reported in chapters 10-12. Chapter 10 describes a method to transluminally seed endothelial cells onto experimental angioplasty sites. This methodology is then utilised to investigate the effect of immediate endothelial seeding on thrombogenicity (chapter 11) and myointimal hyperplasia (chapter 12) following balloon dilatation.
## CHAPTER 6  
**MATERIALS AND METHODS**

### 6.1 Introduction.

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>84</td>
</tr>
</tbody>
</table>

### 6.2 Human Umbilical Vein Endothelial Cells (HUVEC's).
- Isolation and culture of HUVEC's
- Quantification of cell number and viability
- Identification of HUVEC's
- Immunohistology for von Willebrand factor

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>84</td>
</tr>
<tr>
<td>85</td>
</tr>
<tr>
<td>86</td>
</tr>
</tbody>
</table>

### 6.3 Rabbit Aortic Endothelial Cells.
- Isolation and culture of rabbit aortic endothelial cells
- Identification of rabbit aortic endothelial cells

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>87</td>
</tr>
<tr>
<td>88</td>
</tr>
</tbody>
</table>

### 6.4 Autogenous Rabbit Endothelial Cells.
- Harvest of internal jugular venous endothelium
- Microvascular cell harvest

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>89</td>
</tr>
<tr>
<td>90</td>
</tr>
</tbody>
</table>

### 6.5 Radiolabelling Techniques.
- Radiolabelling of endothelial cells
- Isotope leakage from endothelial cells
- Isotope leakage from HUVEC's
- Isotope leakage from rabbit endothelial cells
- Radiolabelling of rabbit platelets

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>92</td>
</tr>
<tr>
<td>93</td>
</tr>
<tr>
<td>93</td>
</tr>
<tr>
<td>94</td>
</tr>
<tr>
<td>95</td>
</tr>
</tbody>
</table>

### 6.6 Preparation of Histological Samples.
- Light microscopy
- Scanning electron microscopy

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
</tr>
<tr>
<td>96</td>
</tr>
</tbody>
</table>

### 6.7 Equipment.
- 5 mm seeding chamber
- Silicone gel plate

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>97</td>
</tr>
</tbody>
</table>

### 6.8 Statistical Methods.

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>97</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>83</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS

6.1 Introduction.

This chapter details the general methods used throughout the following experimental chapters, and describes tissue culture techniques in depth. The vast majority of the methods delineated here were previously established in our laboratory and do not derive from this thesis. Some tissue culture techniques, however, specifically for the isolation of autogenic rabbit endothelial cells were developed due to the failure of conventional techniques and will be described in detail. The source and composition of tissue culture reagents is outlined in Appendix A.

6.2 Human Umbilical Vein Endothelial Cells (HUVEC's).

Isolation and Culture of HUVEC's.

Endothelial cells were isolated from human umbilical veins by a method derived from Jaffe et al. [Jaffe et al. 1973]. Sterile techniques were used in all manipulations of the umbilical cord. Umbilical cords were severed from the placenta soon after birth and placed in a sterile container filled with cord collecting solution (Appendix A). Containers were stored at 4°C and cords utilised within 24 hours of collection.

The cord was inspected under a laminar flow hood (Gelaire, UK), and all areas damaged by clamps or needle holes were excised. The umbilical vein was identified and cannulated at either end using cannulae specifically made for this purpose [Budd, 1991d]. The cannulae were secured with a tie. Three way taps were connected to each cannula (Monoject, St Louis, USA) and the cord flushed with 20 ml Minimal Essential Medium (MEM - Appendix A) to remove any blood clots. The vein was then distended with 5 ml prewarmed 0.1% collagenase solution (Appendix A), and incubated in a sterile plastic bag in a 95% air, 5% CO₂ atmosphere at 37°C for 15 minutes (Queue cell culture incubator, West Virginia, USA). After this time, the collagenase and separated endothelial cells were flushed from the cord by perfusion with 20 ml MEM. The effluent was collected in a 50 ml conical polypropylene tube (Fisons, Loughborough, UK), and the resulting cell suspension centrifuged at 300g, at 4°C for 7 minutes. Following centrifugation, the supernatant was removed and the cell pellet resuspended in 5 ml of complete culture medium (Appendix A). The final cell suspension was plated onto a 25 cm² tissue culture plate (Fisons, Loughborough, UK) and incubated in a 95% air, 5% CO₂ atmosphere at 37°C.

The culture medium was completely changed the day following harvesting and
after this, half changed on alternate days. Cells were grown to confluence and then
subcultured in a ratio of 1 to 3. For subculture, cells were harvested with 0.1% trypsin in
0.02% EDTA (Appendix A). Prior to trypsinisation, the culture medium was removed and
the tissue culture plate thoroughly washed with MEM to remove any traces of fetal calf
serum, which would inhibit the action of trypsin. After washing, 1 ml of 0.1%
prewarmed trypsin was added to the culture flask and the flask agitated. The endothelial
cells were subsequently observed using a phase contrast microscope (Nikon, Phase
contrast ELWD 0.3, Japan), until they assumed a rounded appearance and detached from
the plate. At this point, the trypsin was neutralised by the addition of 11 ml MEM with 5%
fetal calf serum. The resulting cell suspension was then divided into three aliquots and
plated onto three 25 cm² tissue culture flasks. These cells were then grown to confluence.

Quantification of Cell Number and Viability.

Specific concentrations of endothelial cells were required for a number of
experiments. The required number of endothelial cells were harvested from tissue culture
flasks by trypsinisation (one 25 cm² flask contains approximately 1x10⁶ endothelial cells
at confluence), and suspended in a known volume of complete culture medium. Twenty
microliters of this suspension were removed and mixed with 20µl of trypan blue
(Appendix A) in an ependorf (Sarstedt, Leicester, UK). The number of cells in this
suspension were counted in a haemocytometer (Weber, UK) and cell viability determined.
Viable endothelial cells do not take up dye and so viability was expressed as the
percentage of unstained endothelial cells in the total cell population [Patterson, 1979;
Phillips, 1973]. The total cell number present in the cell suspension was calculated and
following centrifugation, the required cell concentration was obtained by resuspending the
cell pellet in a predetermined volume of complete culture medium.

Identification of HUVEC's.

Prior to use all HUVEC's were examined to confirm their identity by both
morphologic and antigenic criteria. Cell morphology was determined by phase contrast
microscopy (Phase contrast ELWD 0.3, Nikon, Japan). Endothelial cells grow in
confluent monolayers without a definable whorling pattern. The cells are homogeneous,
closely opposed with an oval centrally located nucleus and indistinct cell borders [Jaffe et
al.1973] (Fig 6.1). After satisfactory morphologic examination, cell identity was
confirmed by staining for von Willebrand factor which is synthesised and stored by
vascular endothelial cells (chapter 2). The method for von Willebrand identification is
detailed below.
Materials and Methods

Figure 6.1: Photomicrograph illustrating the characteristic morphology of human umbilical venous endothelial cells. Magnification x 20.

**Immunohistology for von Willebrand Factor.**

Endothelial cells were harvested from a confluent 25 cm² tissue culture flask by trypsinisation as has previously been described. The cell suspension was centrifuged and the resulting cell pellet resuspended in 1 ml of MEM with 5% fetal calf serum. Two hundred microliters of the endothelial suspension was then cryospun onto a plain microscope slide (1 mm thick, BDH, Merck Ltd, Poole, UK) by centrifugation at 800g for 1 minute (Shandon Cytospin II, Southern Products, Runcorn, UK). The cryospun slides were allowed to dry and then fixed in acetone (Fisons, Loughborough, UK) for 10 minutes. The area containing cells was ringed with a diamond pen to enable fluid to be retained on the slide.

The cells were incubated at 4°C with 100μl of a 1 in 10 dilution of rabbit anti-human von Willebrand antibody (Sigma, Poole, UK) for 12 hours. Control slides were incubated for the same time period with MEM. The cells were washed carefully with Tris buffered saline and were then incubated with 100μl of a 1 in 100 dilution of a biotin conjugated mouse anti-rabbit immunoglobulin (IgG) (Sigma, Poole, UK). The slides were washed and dried, after which 100μl of extravidin (1 in 400 - Sigma, Poole, UK) was added. Following a further 20 minute incubation at room temperature the cells were washed and 100μl of chromogenic substrate (Appendix A) added to each slide. The slides were incubated for 15 minutes, and were then counterstained with 100 ml acid haematoxylin (Sigma, Poole, UK) for 5 minutes. The slides were washed and examined.
Materials and Methods

under a Olympus BH2 microscope (Fig 6.2) (supplied by Gallencamp, Fisons, Loughborough, UK).

Fig 6.2: Light micrograph illustrating positive staining for von Willebrand factor (right) and negative control (left). Magnification x 75.

6.3 Rabbit Aortic Endothelial Cells.

Isolation and Culture of Rabbit Aortic Endothelial Cells.

Rabbit aortic endothelial cells were harvested by the method of Rone et al. [Rone, Goodman, 1987] from segments of abdominal aorta obtained from freshly sacrificed animals. Immediately after sacrifice the abdominal aorta was dissected free of adventitial tissue and excised. The intact aortic segment was washed in MEM to remove all traces of blood, opened longitudinally and pinned onto a silicone gel plate with the intimal surface uppermost. The segment was then covered with 5 ml of prewarmed 0.1% collagenase solution (CLS 1, Worthington Biochemical, Freehold, New Jersey, USA - Appendix A), and incubated in a 5% CO₂, 95% air atmosphere for 10 min. Following incubation the intima was gently scraped in one direction with a scalpel blade and the cells obtained suspended in complete culture medium. The aortic surface was washed three times with complete culture medium and these washings were also collected. The cell suspension
thus obtained was centrifuged at 300 g for 7 min at 4°C. The resulting cell pellet was re-suspended in 3 ml of complete culture medium and the cells plated onto one well of a six well plate (Fisons, Loughborough, UK). Cells were grown to confluence and repeatedly passaged onto 25 cm² tissue culture plates (Fisons, Loughborough, UK) until required.

**Identification of Rabbit Aortic Endothelial Cells.**

Endothelial cell identity was confirmed by appearance at phase contrast microscopy. Rabbit endothelial cells show heterogeneity in culture, with two distinct cell types being recognisable on the basis of immunohistochemical staining for angiotensin converting enzyme and Factor VIII [Rone, Goodman, 1987]. Angiotensin converting enzyme positive cells are rounded and exhibit a characteristic cobblestone appearance at confluence; whereas Factor VIII positive cells are more elongated and grow in distinct patches. Both cell types were recognisable in primary aortic cell cultures (Fig 6.3)

![Image](image_url)

**Fig 6.3:** Light micrograph illustrating the appearance of rabbit aortic endothelial cells in primary culture. Two distinct cell types are recognisable. Magnification x 30.

Cell identity was confirmed by immunohistochemical staining with an anti-thrombomodulin antibody specific for rabbit endothelial cells (QB-END 40, Serotec,
Oxford, UK). Immunohistochemistry was performed using the method for von Willebrand staining described previously.

6.4 Autogenous Rabbit Endothelial Cells.

Throughout this thesis, it proved difficult to harvest autogenous rabbit endothelium. Two approaches to this problem were undertaken by attempting to isolate both large vessel endothelium from internal jugular veins, and microvascular endothelium from inguinal fat pads. Isolation of microvascular endothelium was unsuccessful due to recurrent contamination of the endothelial cell isolates with fibroblasts. Harvest of large vessel endothelium from internal jugular veins was successful, but had a low primary success rate. Despite this disadvantage large vessel endothelium was utilised in the experiments described in chapter 12, which required autogenous endothelial cell seeding.

Harvest of Internal Jugular Venous Endothelium.

The method described below for harvesting rabbit endothelial cells from internal jugular veins was kindly provided by Dr LK Birinyi, Brigham and Women’s Hospital, Boston, USA, in a personal communication.

Anaesthesia was induced in female New Zealand White rabbits with intravenous hypnoval (Roche Pharmaceuticals, Herts, UK), and maintained by an inhalational mixture of halothane (ICI Pharmaceuticals, Cheshire, UK) and oxygen. A midline neck incision was made and skin flaps reflected to expose the right internal and external jugular veins. The right internal jugular vein was dissected free of the surrounding connective tissue using loupe magnification and a no touch technique. The vein was exposed from the angle of the mandible to the thoracic inlet. All venous tributaries, including the external jugular vein, were ligated and divided, and the cranial end of the vein cannulated with a 24G cannula. The cannula was secured and the vein flushed gently with 5 ml of complete culture medium. The vein was then removed, placed in chilled complete culture medium and transported immediately to the tissue culture laboratory.

Under sterile conditions, the vein was flushed with a further 5 ml of complete culture medium to remove any residual blood. After flushing the non-cannulated end of the vein was clamped with a microvascular clamp and the vein distended with 0.5 ml prewarmed 0.1% collagenase solution (CLS 1, Worthington Biochemical, Freehold, New Jersey, USA - Appendix A). The vein was incubated at 37°C in a 95% air, 5% CO₂ atmosphere for 10 min. After this time, the distal clamp was removed and the vein flushed with 10 ml complete culture medium. The cell suspension was collected and then centrifuged at 300 g for 7 min at 4°C. The resulting cell pellet was re-suspended in 3 ml
Materials and Methods

Of complete culture medium and the cells plated onto one well of a six well plate (Fisons, Loughborough, UK). Cells were grown to confluence and repeatedly passaged onto 25 cm² tissue culture plates (Fisons, Loughborough, UK) until required.

Endothelial cell identity was confirmed by appearance at phase contrast microscopy, and immunohistochemical staining for an anti-thrombomodulin antibody, specific for rabbit endothelial cells (QB-END 40, Serotec, Oxford, UK). Immunohistochemistry was performed using the method for von Willebrand staining described previously.

Although this method was successful in isolating autogenous rabbit endothelium, the success rate was low. From 30 attempted harvests, adequate primary cell cultures were only obtained in 12 cases (40%). The 18 failures were due to an insufficient number of cells isolated from the primary harvest.

Microvascular Cell Harvest.

It was attempted to harvest microvascular endothelial cells from the inguinal fat pads of New Zealand White rabbits. Rabbits were anaesthetised with intravenous hypnoval (Roche Pharmaceuticals, Herts, UK) and anaesthesia maintained by an inhalational mixture of halothane (ICI Pharmaceuticals, Cheshire, UK) and oxygen. The inguinal fat pads were removed through a small parainguinal incision, immediately stored in chilled MEM, and transported to the tissue culture laboratory. Twelve experimental harvests were performed, the median weight of tissue used was 25g (range 20 - 40g). Rabbits were allowed to recover after this procedure.

On arrival in the tissue culture laboratory, the fat was placed in 20 ml prewarmed 0.1% microvascular collagenase solution (Appendix A), and agitated in this suspension for 20 min. The supernatant was removed and centrifuged at 4°C for 7 min at 300 g. The resulting cell pellet was resuspended in 5 ml of complete culture medium and plated onto a 25cm² tissue culture plate (Fisons, Loughborough, UK). These cells were grown in tissue culture and were identified as mesothelial in origin by their appearance at phase contrast microscopy.

Following this preliminary incubation, the fat was washed with MEM and then finely minced using two scalpel blades. The minced tissue was divided into 4 equal aliquots and each aliquot placed in a 20 ml universal container (Fisons, Loughborough, UK) which contained 7 ml 0.1% collagenase. The containers were incubated on a roller table for 30 min, and the resultant suspension was then passed through a 250μm pore nylon mesh (Lockertex, Warrington, UK) to separate the undigested fat. The filtered solution was cooled on ice for 30 min to allow the fat to separate. The fat was removed with a pipette and the resulting suspension placed in a 50 ml conical polypropylene tube.
Materials and Methods

(Fisons, Loughborough, UK) and centrifuged at 300g for 7 min.

Separation of the microvascular cells was attempted by density gradient centrifugation. Bovine serum albumin (BSA - Advanced Protein Products, Brierley Hill, UK) gradients from 35% to 20% were made up from 35% BSA and MEM as illustrated in Table 6.1. Three gradients were used in each experiment, the gradients were varied between experiments in an attempt to achieve optimal cell separation. The cell pellet obtained following centrifugation was resuspended in 10 ml of the highest density BSA to be used. Five milliliters was placed in the bottom of two sterile Dupont tubes (Dupont Pharma, Herts, UK). Four milliliters of the next highest density gradient was then carefully layered onto the cell pellet to produce an interface, and 4 ml of the lowest density BSA was in turn then layered onto this. The gradients were completed by 1 ml of MEM layered onto the lowest density BSA (Fig 6.4).

<table>
<thead>
<tr>
<th>BSA Gradient</th>
<th>35% BSA (ml)</th>
<th>MEM (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35%</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>29%</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>26%</td>
<td>10</td>
<td>3.5</td>
</tr>
<tr>
<td>23%</td>
<td>10</td>
<td>5.2</td>
</tr>
<tr>
<td>20%</td>
<td>10</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Table 6.1: Table illustrating composition of BSA gradients.

The Dupont tubes were then centrifuged at 4°C for 30 min at 18,000g. Following centrifugation, the interface between different BSA densities contained a cell layer. These layers were removed and placed in a sterile 10 ml tube (Fisons, Loughborough, UK), where they were washed three times with MEM by repeated centrifugation. The final cell pellets (4 from each density gradient centrifugation) were then resuspended in 5 ml complete culture medium and plated onto a 25 cm² tissue culture plate (Fisons, Loughborough, UK). Cells were grown to confluence and identified by appearance at phase contrast microscopy.

In all, 12 separate experiments were performed with differing combinations of BSA gradients between 35% and 20%. Although microvascular endothelial cells were obtained between 23% and 26% BSA gradients, the isolates were always heavily contaminated with fibroblasts and other cell types. It was concluded that high numbers of uncontaminated endothelial cells could not be obtained by this method, which was
therefore not used in autogenous seeding experiments.

Fig 6.4 : Diagrammatic illustration of density gradient centrifugation showing
distribution of gradients and cell layers following centrifugation.

6.5 Radiolabelling Techniques.

Radiolabelling of Endothelial Cells.

Endothelial cells were radiolabelled with indium-111 oxine using a method derived
from Sharefkin et al. [Sharefkin et al. 1983]. The required number of endothelial cells
were released from tissue culture by trypsinisation (0.1% trypsin / 0.02% EDTA), and
following determination of cell number, the resulting cell suspension was centrifuged at
300g and 4°C for 7 minutes. The cell pellet was then resuspended in 0.5 ml MEM.

The required amount of indium-111 oxine (Amersham International, Amersham,
Bucks, UK) was made up to 0.6 ml volume with MEM, and 0.5 ml of this solution was
added to the endothelial cell suspension. The remaining 0.1 ml was diluted with 9.9 ml of
MEM (control wash) and was used in the determination of labelling efficiency. The
indium / endothelial cell suspension was allowed to incubate at room temperature for 15
min, and was then washed twice by centrifugation with a combined volume of 49 ml
MEM. The supernatants from these washings were saved for determination of labelling
efficiency (cell wash). The final cell pellet was resuspended at the required volume and
20μl withdrawn for cell counting and viability studies.

To determine labelling efficiency, the gamma activity of 20 μl of the control and
cell wash solutions were determined in a LKB-Wallac 1280 UltroGamma II counter
(LKB-Produkter AB, Sweden). Percentage labelling efficiency was calculated by the formula:

\[
\text{Efficiency (\%) = } \frac{\text{Counts (control wash)} - \text{counts (cell wash)}}{\text{Counts (control wash)}} \times 100
\]

**Isotope Leakage from Endothelial Cells.**

One of the disadvantages of using indium-111 labelling of endothelial cells to monitor cell attachment and retention, is that spontaneous intracellular leakage of the isotope occurs [Miyata et al. 1991; Newman et al. 1991]. To correct for this effect, the rate of isotope leakage from rabbit endothelial cells was calculated as described below. The isotope leakage rate from human umbilical venous endothelial cells had previously been determined [Budd, 1991d].

Endothelial cells were released from tissue culture and 1x10^5 cells labelled with 60μCi of indium oxine as described previously. Following labelling, the cells were resuspended in MEM with 5% fetal calf serum at a concentration of 2x10^5 cells/ml. Five hundred microliters of the labelled endothelial cell suspension was pipetted into 8 separate ependorf tubes (Sarstedt, Leicester, UK) which were then incubated in a 95% air, 5% CO_2 atmosphere at 37°C. Eight incubation times were studied, 0, 10, 20, 30, 45, 60, 90, and 120 min.

At the end of each incubation period, the ependorf was removed from the incubator and centrifuged at 13,000 rpm for 5 min (MSE centrifuge, Fisons, Loughborough, UK). Following centrifugation, 100μl of the supernatant (solution A) was removed and the cells resuspended in the remaining solution (solution B). A 100μl sample of the suspended cells was removed and the gamma activity of both samples was determined in a LKB-Wallac 1280 UltroGamma II counter (LKB-Produkter AB, Sweden). The percentage leakage at each time period was calculated by the formula:

\[
\text{Leakage (\%) = } \frac{\text{Counts (Solution A)}}{\text{Counts (Solution B)}} \times 125
\]

**Isotope Leakage from HUVEC's.**

The leakage rate of indium-111 oxine from HUVEC's has previously been determined in our laboratory to be 3.5% / hour [Budd, 1991d] for the first two hours after labelling. This value has been used to correct the gamma activity recorded in chapters 8
Isotope Leakage from Rabbit Endothelial Cells.

The isotope leakage from rabbit aortic endothelial cells was determined as described above. Cells from the second to fourth passage were used and 7 duplicate experiments were performed at each time period studied. The median labelling efficiency achieved during this experiment was 25% (95% confidence intervals 11.1% - 40.3%). The rate of leakage is illustrated in Fig 6.5:

![Graph illustrating spontaneous leakage of indium-111 from rabbit endothelial cells. Results are presented as means with 95% confidence intervals.](image)

Indium loss from the endothelial cells was greatest in the first 30 min after labelling and following this phase a plateau was reached as has been reported by other observers [Kesler et al. 1986]. The loss of indium-111 as determined in this experiment allowed gamma counts to be corrected, when monitoring the loss of gamma activity from seeded angioplasty sites (chapter 10). Individual leakage rates for ten minute periods from 0-120 min were determined and counts corrected at each individual time point. The correction factors for rabbit endothelial cells are tabulated in Table 6.2:
Materials and Methods

Table 6.2: Table illustrating the correction factors for spontaneous leakage of indium-111 from rabbit endothelial cells. EEC = rabbit endothelial cells.

Rabbits were anaesthetised with intravenous hypnoval (Roche Pharmaceuticals, UK) and an inhalational mixture of halothane (ICI, UK) and oxygen. The right femoral vein was exposed and cannulated with an 18G cannula. Seventeen milliliters of blood was withdrawn into a 20 ml syringe containing 3 ml anhydrous dextrose/sodium acid citrate (ACD); a further 9 ml of blood was drawn into a 10 ml syringe containing 1 ml sodium citrate (Sigma, Poole, UK). The two syringes were transported to the radio-pharmacy where, under aseptic conditions, the blood from the 20 ml syringe was transferred to a universal tube (Fisons, Loughborough, UK) and centrifuged for 10 min at 180g to produce platelet rich plasma (PRP). The blood from the 10 ml syringe was also placed in a sterile universal tube (tube A). Five milliliters of the PRP was removed to avoid contamination with red blood cells, and was then placed in a sterile tube containing 5 ml of calcium free Tyrode buffer supplemented with 300 ng/ml prostaglandin E1 (tube B). Tubes A and B were then spun for 10 min at 640g. The supernatant in tube A represented
Materials and Methods

platelet poor plasma (PPP).

The supernatant from tube B was removed and stored for later use. A further 2.5 ml of calcium free Tyrode buffer supplemented with 300 ng/ml prostaglandin E₁ was added to the pellet in tube B and centrifuged for 640g for 90 sec. The supernatant was then discarded and the resulting pellet resuspended in 2.5 ml calcium free Tyrode buffer with 300 ng/ml prostaglandin E₁. The required quantity of indium-111 oxine (Amersham International, Amersham, Bucks, UK) was then added to the platelet suspension and incubated for 2 min, after which the suspension was made up to 10 ml volume by the addition of the previously reserved supernatant. This platelet suspension was then centrifuged at 640g for 10 min, after which the supernatant was removed and reserved for calculation of labelling efficiency. The platelet pellet was resuspended in 3 ml of PPP and the gamma activity of this and the supernatant determined in a gamma counter.

Labelling efficiency was given by the following equation:

\[
\text{Labelling efficiency (\%) = } \frac{\text{cpm platelets}}{\text{cpm platelets + supernatant}} \times 100
\]

Following determination of labelling efficiency, platelets were ready for re-infusion.

6.6 Preparation of Histological Samples.

Light Microscopy.

Tissue samples for light microscopy were stored in 10% formyl saline (BDH, Merck Ltd, Poole, UK) for at least 18 hours prior to processing. The samples were then dehydrated in 95% IMS (Sigma, Poole, UK) for 2 hours followed by immersion in 99% IMS for a further 7 hours. The specimens were transferred into xylene (Sigma, Poole, UK) for 3 hours prior to embedding in paraffin wax for at least 4 hours. The wax embedded tissue was serially sectioned to 4 μm thickness and sections dried at 37°C prior to staining. Staining techniques are described in Appendix B.

Scanning Electron Microscopy.

Tissue for scanning electron microscopy was fixed under tension in 4% paraformaldehyde (BDH, Merck Ltd, Poole, UK) / 2% gluteraldehyde solution (Sigma, Poole, UK) for at least 48 hours prior to processing. The tissue was washed in 0.1M cacodylate buffer (Sigma, Poole, UK) at a pH of 7.2 for 60 min, and was then transferred
Materials and Methods

into a solution of 1% osmium tetroxide (Sigma, Poole, UK) in 0.1M cacodylate buffer for a further hour. At this point the specimen was dehydrated through graded acetone solutions (50% acetone for 30 min; 70% for 60 min; 90% for 15 min and 100% for 15 min), prior to storage in 100% acetone.

Specimens were then prepared for viewing by critical point drying (Polaron E3000 critical point dryer - Bio-Rad Microscience Ltd, Hemel Hempstead, UK) and sputter coating with gold (Polaron E5150 coater - Bio-Rad Microscience Ltd, Hemel Hempstead, UK). Sections were viewed on a dual stage scanning electron microscope (DS 130, International Scientific Instruments, Buxton, UK).

6.7 Equipment.

5 mm Seeding Chamber.

Seeding chambers were manufactured from 1 ml automatic pipette tips (Fisons, Loughborough, UK) that were filled with silicone gel (BDH, Merck Ltd, Poole, UK) to an internal diameter of 5 mm (Fig 7.3).

Silicone Gel Plate.

Silicone gel plates were manufactured by filling a 5 cm diameter petrie dish (Fisons, Loughborough, UK) with silicone gel (BDH, Merck Ltd, Poole, UK) to a depth of 1 cm. Silicone plates were used to pin out arterial samples during preparation for scanning electron microscopy.

6.8 Statistical Methods.

Individual statistical methods are described in each chapter. In general, the number of experiments performed in each phase of this thesis are small and therefore non-parametric statistics have been used. Statistical analysis was performed using the Minitab Release 8.1 statistical programme (Minitab Inc, Pennsylvania, USA) on a Macintosh LC personal computer (Apple Computer Inc, California, USA). Additional confidence interval analysis was performed with the CIA software package (SB Gardner, PD Winter, MJ Gardner - British Medical Journal, London, UK) on an Atari PC3 personal computer (Atari, California, USA).
CHAPTER 7
THE EFFECT OF SEEDING TIME AND SEEDING DENSITY ON ENDOTHELIAL ATTACHMENT TO DAMAGED VASCULAR SURFACES

7.1 Introduction.

7.2 Methods.
- Development of an in vitro model of vascular damage
- Isolation and culture of endothelial cells
- Radiolabelling of endothelial cells
- Attachment of endothelial cells to vein surface
- Attachment of endothelial cells with time
- Attachment of endothelial cells with seeding density
- Statistical analysis

7.3 Results.
- Histological examination of dilated saphenous vein segments
- Endothelial cell attachment with varying incubation times
- Endothelial cell attachment with varying seeding densities

7.4 Discussion.

7.5 Summary.
THE EFFECT OF SEEDING TIME AND SEEDING DENSITY ON ENDOTHELIAL ATTACHMENT TO DAMAGED VASCULAR SURFACES

7.1 Introduction.

The prerequisite conditions for successful endothelial cell seeding involve transplanting viable endothelial cells to the appropriate site, where they must attach and resist the shear stresses of the circulation, whilst preserving their anti-thrombotic functions. The initial step in this seeding process is the interaction between the seeded endothelial cells and the substrate onto which attachment takes place. Previous experiments investigating seeding of prosthetic vascular grafts have identified three variables which may influence initial cell attachment; the surface-substrate composition, the length of endothelial cell incubation and the number of cells available [Pratt et al. 1988].

In this thesis, the effect of seeding endothelial cells onto post-angioplasty vascular surfaces will be examined. The surface to be seeded will remain relatively constant throughout these experiments, and initial cell attachment will therefore be determined by the seeding time and the density of the seeded endothelial cells. To date, research into seeding damaged vascular surfaces has been minimal and optimum seeding conditions have not been defined. Experience from prosthetic graft seeding has suggested that it is important to characterise these parameters prior to in vivo studies. Many of the early clinical trials of endothelial cell seeding failed due to low seeding densities, which hampered the formation of an endothelial monolayer on the graft surface.

In this chapter, the static attachment of human umbilical venous endothelial cells to an in vitro model of vascular damage will be described. Cell attachment at different seeding times and seeding densities has been quantified and the optimum seeding parameters defined. Optimisation of these parameters should facilitate the rapid establishment of an endothelial cell monolayer in later in vivo experiments.

7.2 Methods.

Development of an In Vitro Model of Vascular Damage.

Segments of previously undistended long saphenous vein, taken at ankle level, were obtained from patients undergoing aorto-coronary bypass grafting and transported to the laboratory in chilled Minimal Essential Medium (MEM - ICN, High Wycombe, Bucks, UK). The use of these vein segments was approved by our local ethical committee. Once in the laboratory, the vein segments were immersed in MEM and
threaded over a 5 mm balloon angioplasty catheter (Medi-Tech, UK), which was in turn supported by a guide wire (Meadox [UK] Ltd, Caddington, Beds, UK) (Fig 7.1).

Fig 7.1: Photograph illustrating a segment of long saphenous vein threaded over a 5 mm balloon angioplasty catheter, which is in turn, passed over a guide wire. The petrie dish is filled with MEM.

The angioplasty balloon was inflated to 9 atm pressure for 30 sec (Fig 7.2) using a constant pressure syringe (Schneider Meditag AG, Zurich, Switzerland - Fig 10.3), after which time the vein was removed from the catheter. Vein segments were opened longitudinally, pinned onto a silicone gel plate and fixed under tension in 4% paraformaldehyde (BDH, Merck Ltd, Poole, UK) / 2% gluteraldehyde solution (Sigma, Poole, UK).

Segments of vein were prepared for light and scanning electron microscopy (SEM) as has previously been described (chapter 6). To validate this model of vascular damage 10 undistended control veins were compared to 10 veins that had been dilated with the balloon catheter. Ten representative scanning electron micrographs of each vein were assessed semi-quantitatively to determine the degree of endothelial coverage. Assessment was performed blinded of the specimen type, and the degree of endothelial coverage estimated as the percentage of the luminal surface covered with endothelium.
Isolation and Culture of Endothelial Cells.

Endothelial cells were harvested from human umbilical veins and grown in tissue culture as described earlier (chapter 6). In this experiment, cells from the second to fourth passage were used. Endothelial cell identity was confirmed by appearance at phase contrast microscopy and positive immunohistochemical staining for von Willebrand factor (Dakopatts, High Wycombe, Bucks, UK - described in chapter 6).

Radiolabelling of Endothelial Cells.

Human umbilical vein endothelial cells (HUVEC's) were labelled with indium-111 oxine by a method modified from Sharefkin et al. [Sharefkin et al. 1983], described in detail in chapter 6. Cells were released from tissue culture by trypsinisation (0.1% trypsin in 0.02% EDTA), and following cell counting in a haemocytometer (Weber, UK) were centrifuged at 300 g for 7 min. The cell pellet was re-suspended in 0.5 ml MEM and incubated with 60 μCi indium-111 oxine (Amersham, International, Amersham, Bucks, UK) for 15 min. The cells were washed twice with MEM and finally re-suspended in MEM with 5% fetal calf serum at a known cell concentration. Labelling efficiency and cell viability were determined at this time.
Attachment of Endothelial Cells to Vein Surface.

The same methodology was used in experiments investigating both seeding time and seeding density. A 5 mm diameter punch biopsy of dilated saphenous vein was placed on silicone gel in a 5 mm diameter seeding chamber. Seeding chambers were manufactured from 1 ml automatic pipette tips (Fisons, Loughborough, UK) that were filled with silicone gel (BDH, Merck Ltd, Poole, UK) to an internal diameter of 5 mm (Fig 7.3). A 100μl aliquot of an indium-111 labelled endothelial cell suspension, at a known concentration, was added to the seeding chamber so that the cells only came into contact with the luminal vein surface. After incubation in 95% air, 5% CO$_2$ for the allotted incubation time, the unattached cells were removed by washing the luminal vein surface three times with MEM. The vein segment was removed and the seeding chamber washed twice further with MEM. All washings were collected. The gamma radioactivity on the vein surface and in the washings (which contained the unattached cells) was measured in an LKB-Wallac 1280 UltroGamma II counter (LKB-Produkter AB, Sweden). The percentage cell attachment was calculated by the following formula:

$$\text{Cell attachment (%) = } \frac{\text{Counts on vein}}{\text{Counts on vein + Counts in washings}} \times 100$$

Vein segments were fixed under tension after cell attachment had been determined, and prepared for light and SEM.

Attachment of Endothelial Cells with Time.

In studying endothelial cell attachment with time, a seeding density of 1x10$^5$ cells/cm$^2$ (i.e. cell concentration = 1.96x10$^5$ cell/ml) was used in all experiments, as this represents confluence [Miyata et al. 1991], thus ensuring both maximal cell coverage and maximum percentage cell retention. Attachment of endothelial cells at incubation times of 5, 10, 20, 30, 40, and 60 min were studied. The number of experiments in each group is shown in Table 7.1.

Attachment of Endothelial Cells with Seeding Density.

A seeding time of 30 min was used in all experiments. Seeding densities from 1x10$^4$ to 1x10$^7$ cells/cm$^2$ were studied. The number of experiments in each group is shown in Table 7.1, as is the required cell concentration to achieve the specified seeding
Seeding Time and Density

densities.

Fig 7.3: Photograph illustrating a 5 mm seeding chamber manufactured by filling a 1 ml automatic pipette tip with silicone gel to an internal diameter of 5 mm.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Number of Experiments</th>
<th>Seeding Density (Cells/cm²)</th>
<th>Number of Experiments</th>
<th>Cell Concentration (Cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>6</td>
<td>$1 \times 10^4$</td>
<td>5</td>
<td>$1.96 \times 10^4$</td>
</tr>
<tr>
<td>10</td>
<td>11</td>
<td>$1 \times 10^5$</td>
<td>6</td>
<td>$1.95 \times 10^5$</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>$2 \times 10^5$</td>
<td>5</td>
<td>$3.92 \times 10^5$</td>
</tr>
<tr>
<td>30</td>
<td>8</td>
<td>$5 \times 10^5$</td>
<td>10</td>
<td>$9.8 \times 10^5$</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>$6.2 \times 10^5$</td>
<td>6</td>
<td>$12.1 \times 10^5$</td>
</tr>
<tr>
<td>60</td>
<td>8</td>
<td>$1 \times 10^6$</td>
<td>5</td>
<td>$1.96 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$2 \times 10^6$</td>
<td>5</td>
<td>$3.92 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1 \times 10^7$</td>
<td>6</td>
<td>$1.96 \times 10^7$</td>
</tr>
</tbody>
</table>

Table 7.1: Table illustrating the number of individual experiments performed to investigate cell retention at different seeding times and seeding densities. The required cell concentrations to achieve individual seeding densities are also shown.
**Statistical Analysis.**

Results are presented as medians with 95% confidence intervals (CI). The difference in endothelial cell coverage between control and dilated vein segments, and differences between cell retention at different seeding times and densities, are compared using the Mann Whitney test. Linear regression was used to analyse the relationship between seeding parameters and cell retention.

**7.3 Results.**

**Histological Examination of Dilated Saphenous Vein Segments**

The median endothelial cell coverage, as assessed by SEM, of the control saphenous vein segments (Fig 7.4) was 70% (95% CI 60% to 80%), as compared to 0% (95% CI 0% to 0%) for the dilated vein segments (W = 3915, 95% CI 60% to 70%, p < 0.0001, Mann Whitney). Scanning electron microscopy of the dilated vein segments illustrated that the sub-endothelial tissue was exposed over the luminal surface and that both longitudinal and circumferential tears into the media were present (Fig 7.5). These changes were confirmed by light microscopic examination which revealed endothelial desquamation, rupture of the internal elastic lamina, tangential and circumferential tears in the tunica media and stretching of the tunica media (Fig 7.6).

![Fig 7.4](image_url) Scanning electron micrograph illustrating a control segment of long saphenous vein with 60% endothelial cell coverage. Magnification x 252.
Fig 7.5: Scanning electron micrograph of a dilated saphenous vein segment exhibiting complete endothelial desquamation, exposure of the sub-endothelial matrix and a longitudinal tear extending into the media. Magnification x 209.

Fig 7.6: Light micrograph illustrating a dilated vein segment. There is complete endothelial desquamation with rupture and stretching of the tunica media. EVG stain. Magnification x 25.
Endothelial Cell Attachment with Varying Incubation Times.

The median labelling efficiency in this set of experiments was 34.5% (95% CI 30.7% to 40%). All endothelial cell suspensions had a viability of greater than 95% as assessed by trypan blue exclusion. The results for endothelial cell attachment have been expressed as the percentage cell retention at given incubation times (Fig 7.7, Table 7.2). There was a linear relationship between cell attachment and seeding time between 5 and 30 min ($r^2 = 0.98$, $p = 0.007$ - linear regression analysis), after which no further significant increase in cell retention occurred (difference between median cell attachment at 30 and 60 min $W = 63$, 95% CI -13.7 to 6.6, $p = 0.64$ - Mann Whitney).

Endothelial cell seeding was confirmed by both light (Fig 7.8) and SEM (Fig 7.9).

![Graph illustrating the percentage endothelial cell retention with varying incubation times. Results are presented as medians with 95% confidence intervals.](image-url)
Fig 7.8: Light micrograph illustrating seeded endothelial cells on the surface of a dilated saphenous vein. H&E stain. Magnification x 75.

Fig 7.9: Scanning electron micrograph illustrating seeded endothelial cells on the surface of a dilated saphenous vein. The sub-endothelial matrix is exposed in areas not covered with endothelial cells. Magnification x 249.
Endothelial Cell Attachment with Varying Seeding Densities.

The median labelling efficiency in this set of experiments was 27.5% (95% CI 16% to 36.3%). All endothelial cell suspensions had a viability of greater than 95% as assessed by trypan blue exclusion. In this experiment results have been expressed as the number of seeded endothelial cells attached per cm$^2$ of dilated vein (Fig 7.10, Table 7.3). There was a linear relationship between cell coverage and seeding density between densities of $1 \times 10^4$ and $2 \times 10^6$ cells/cm$^2$ ($r^2 = 0.94$, $p < 0.0001$, linear regression analysis), after which little further useful increase in cell attachment occurred (difference between median cell attachment at $2 \times 10^6$ and $1 \times 10^7$ cells/cm$^2$, $W = 30$, 99% CI $-1.9 \times 10^5$ to $1.8 \times 10^5$, $p = 1.0$ - Mann Whitney). Confluent endothelial cell coverage of a vascular surface is usually assumed at $1 \times 10^5$ cells/cm$^2$ [Miyata et al.1991]; in this experiment confluence is reached at a seeding density of $3.2 \times 10^5$ cells/cm$^2$.

![Graph illustrating endothelial cell attachment with differing seeding densities. Results are presented as medians with 95% confidence intervals of the logarithmic transformation of the data. The dotted line represents the point at which confluence ($1 \times 10^5$ cells/cm$^2$) is reached. This equates to a seeding density of $3.2 \times 10^5$ cells/cm$^2$.](image)
Seeding Time and Density

<table>
<thead>
<tr>
<th>Seeding Time (Min)</th>
<th>Cell Attachment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>6.2 (5.4-7.4)</td>
</tr>
<tr>
<td>10</td>
<td>10.8 (5.4-16.7)</td>
</tr>
<tr>
<td>20</td>
<td>20.5 (16.0-33.7)</td>
</tr>
<tr>
<td>30</td>
<td>26.1 (19.7-35.1)</td>
</tr>
<tr>
<td>40</td>
<td>22.8 (21.0-37.7)</td>
</tr>
<tr>
<td>60</td>
<td>31.1 (18.1-38.8)</td>
</tr>
</tbody>
</table>

Table 7.2: Table illustrating cell attachment with increasing seeding times. Results are presented as medians with 95% confidence intervals in parentheses.

<table>
<thead>
<tr>
<th>Seeding Density (Cells/cm²)</th>
<th>Cell Coverage (Cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x10⁴</td>
<td>3.5x10³ (2.0 - 4.3x10³)</td>
</tr>
<tr>
<td>1x10⁵</td>
<td>3.1x10⁴ (2.7 - 3.9x10⁴)</td>
</tr>
<tr>
<td>2x10⁵</td>
<td>6.4x10⁴ (3.6 - 9.1x10⁴)</td>
</tr>
<tr>
<td>5x10⁵</td>
<td>1.8x10⁵ (1.6 - 1.9x10⁵)</td>
</tr>
<tr>
<td>6.2x10⁵</td>
<td>1.7x10⁵ (1.1 - 2.4x10⁵)</td>
</tr>
<tr>
<td>1x10⁶</td>
<td>3.5x10⁵ (2.6 - 4.0x10⁵)</td>
</tr>
<tr>
<td>2x10⁶</td>
<td>4.9x10⁵ (3.2 - 6.6x10⁵)</td>
</tr>
<tr>
<td>1x10⁷</td>
<td>4.7x10⁵ (4.3 - 5.3x10⁵)</td>
</tr>
</tbody>
</table>

Table 7.3: Table illustrating cell retention onto damaged vascular surfaces with varying seeding densities. Figures in parentheses are 95% confidence intervals.

7.3 Discussion

Percutaneous transluminal angioplasty of an atheromatous plaque causes severe mechanical damage to the arterial wall, which is manifest in complete endothelial desquamation, splitting and compression of the atheromatous lesion with stretching of the medial and adventitial tissues [Block et al. 1980a,b; Faxon et al. 1982; Sanborn et al. 1983; Zarins et al. 1982; Losordo et al. 1992]. To achieve successful endothelial transplantation onto these sites, seeded endothelial cells must attach to the damaged vascular surface...
exposed following PTA. This surface is partly composed of compressed, fractured atheromatous debris, but also exhibits dissected areas where the sub-endothelial and medial tissues are exposed. In this experiment an in vitro model of vascular injury, produced by hyperbaric distention of long saphenous vein segments, has been used to study endothelial attachment to damaged vascular surfaces.

Although this model is limited due to the absence of atheromatous disease, it provides a reasonable model of damaged vascular surfaces. Dilated vein segments exhibited complete endothelial desquamation, exposure of the sub-endothelial matrix, with splitting of the tunica media and stretching of the adventitial tissues. The exposed luminal surface provides an acceptable approximation of a post-angioplasty vessel in which optimum seeding parameters may be determined.

Cell attachment has been defined as the initial phase of interaction between a cell and a substrate [Knox, 1981]. This interaction involves the formation of an adhesive bond between the cell and the substrate and results in a spherical cell attaching to the substrate-surface and becoming flattened ("spread") as anchorage matures. Fully developed attachment sites are characterised by large numbers of intracellular myofilaments and secretion of extracellular connective tissue [Toselli et al.1984]. Utilising this knowledge many investigators have demonstrated that coating prosthetic graft surfaces with extracellular matrix proteins dramatically increases endothelial cell adherence [Dalsing et al.1989; Thomson et al.1991; Hasson et al.1986; Koveker et al.1991]. Theoretically therefore, native vascular surfaces should form an ideal substrate for endothelial cell adherence as they present large quantities of basement membrane and extracellular matrix proteins.

In this experiment the ability of human umbilical vein endothelial cells (HUVEC's) to adhere to damaged vascular surfaces was quantified. Human umbilical vein endothelial cells were used for this purpose as they are relatively easy to harvest and grow rapidly in tissue culture. Although HUVEC's have been used extensively in previous endothelial seeding experiments [Kesler et al.1986; Anderson et al.1987; Budd et al.1989], the cells are derived from an organ at the end of its physiological life and exhibit different metabolic characteristics to adult vascular endothelial cells [Fry et al.1984]. Some investigators have suggested that adult endothelial cells should be preferentially used in seeding experiments [Jarrell et al.1984] as this would be more relevant to clinical application. However, both Lundgren et al. [Lundgren et al.1986] and Zilla et al. [Zilla et al.1989] have demonstrated that adult vascular and umbilical venous endothelial cells exhibit identical attachment and retention characteristics to prosthetic vascular grafts. It therefore seems reasonable to utilise HUVEC's in experiments investigating static cell attachment.

The method of using indium-111 labelled endothelial cells to determine cell attachment on segments of vascular grafts has been well described in the literature [Budd
In this study, the methodology was adapted for use with native vascular surfaces, and using a constant seeding density of $1 \times 10^5 \text{cells/cm}^2$, the maximal cell attachment achieved was approximately 27%. This level of endothelial adherence compares favourably with attachment to uncoated prosthetic grafts, which has been reported to be less than 10% \cite{Budd1989, Vohra1991, Kent1988}. From these initial observations, it would appear that damaged vascular tissue provides an excellent substrate for endothelial cell attachment, but it should be noted that endothelial adherence to prosthetic vascular grafts may be increased to 80% by pretreatment with extracellular matrix proteins \cite{Budd1989, Vohra1991}.

The good attachment characteristics of native vascular surfaces has been noted before by other observers. Hoch \etal\cite{Hoch1989} used supra-confluent seeding densities to study endothelial coverage of denuded arterial tissue and reported that 80% of the luminal surface was covered by an endothelial cell monolayer 1 hour after seeding. From these observations, it was concluded that very rapid and complete endothelial attachment occurred on denuded arterial tissue. Similarly, Bowersox and Anderson \cite{Bowersox1988} and Lalka \etal\cite{Lalka1989} have demonstrated excellent endothelial adherence to acellular vascular matrices designed to mimic arterial basement membrane.

Having established that endothelial cells would adhere to native vascular surfaces, the optimum seeding parameters for this interaction were defined. By varying incubation times, it was demonstrated that maximal cell attachment was complete within 30 minutes, after which time little useful increase in cell adherence occurred. These rapid attachment kinetics are similar to those reported for endothelial adhesion to prosthetic vascular grafts \cite{Budd1990, Vohra1991}. Anderson \etal\cite{Anderson1987} demonstrated that maximal cell adherence to fibronectin coated grafts was complete within 15 minutes, but also showed that prolonged incubation periods (> 1 hour) may lead to cell detachment. From results of this experiment, it was concluded that the optimum incubation period for seeding native vascular surfaces was 30 minutes.

Utilising the optimum seeding time of 30 min, the effect of endothelial seeding density on endothelial cell retention was quantified. In this experiment it was demonstrated that cellular coverage of damaged vascular surfaces was directly related to the number of cells available, up to a seeding density of $2 \times 10^6 \text{cells/cm}^2$, at which point numerical cell attachment remained constant. The maximum number of cells attaching to the luminal surface was $4.86 \times 10^5 \text{cells/cm}^2$ (95% CI 3.16 - 6.63 $\times 10^5 \text{cells/cm}^2$), which is approximately 5 times greater than the endothelial density of undamaged native vessels. This apparent overpopulation of the vascular surface is explained by the morphology of immediately seeded cells, which are initially spherical and only become spread onto the
Seeding Time and Density

surface as the attachment site matures. Similar relationships between seeding density and endothelial cell coverage have been demonstrated in seeding prosthetic surfaces [Newman et al. 1991; Anderson et al. 1987].

One of the aims of this thesis is to utilise endothelial seeding to produce a confluent monolayer on angioplasty sites. Assuming that confluent endothelial cell coverage occurs at a cell density of $1 \times 10^5$ cells/cm$^2$, a seeding density of greater than $3.2 \times 10^5$ cells/cm$^2$ will be required to achieve this aim. The advantages of using high seeding densities to achieve confluence have been previously investigated in experiments of two stage endothelial cell seeding [Tannenbaum et al. 1987; Hussain et al. 1991]. Miyata et al. [Miyata et al. 1991] demonstrated that maximal resistance to shear stress was achieved at confluence, which may be explained by the fact that the amount of fibronectin deposited on seeded surfaces increases with the density of seeded cells [van Wachem et al. 1988]. Schneider et al. [Schneider et al. 1990] confirmed the effects of high seeding densities on native vascular surfaces in an experiment looking at the attachment of baboon aortic endothelial cells to segments of endarterectomised aorta. Using a seeding density of $6 \times 10^5$ cells/cm$^2$, $3.8 \times 10^5$ cells/cm$^2$ became attached within 20 minutes of seeding.

7.5 Summary.

In this experiment, an *in vitro* model to facilitate the study of endothelial seeding of damaged vascular surfaces has been developed. Using this model, it has been shown that the optimum endothelial seeding time for attachment to damaged vascular surfaces is 30 min, and that to achieve confluent cell attachment, a seeding density of in excess of $3.2 \times 10^5$ cells/cm$^2$ should be used.
# CHAPTER 8
THE EFFECT OF SHEAR STRESS ON ENDOThelial ATTACHMENT TO NATIVE VASCULAR SURFACES

## 2.1 Introduction.

## 2.2 Methods.
- Isolation and culture of endothelial cells  
- Radiolabelling of endothelial cells  
- *In vitro* model of vascular damage  
- Seeding procedure  
- Perfusion chamber  
- Vein segment perfusion  
- Haemodynamic profile  
- Determination of cell retention  
- Histological examination  
- Statistical analysis

## 2.3 Results.
- Cell viability and labelling efficiency  
- Vein dimensions  
- Effect of shear stress on endothelial cell retention  
- Histological examination

## 2.4 Discussion.

## 2.5 Summary.
THE EFFECT OF SHEAR STRESS ON ENDOTHELIAL RETENTION TO NATIVE VASCULAR SURFACES

8.1 Introduction.

In the previous chapter, the optimum seeding parameters for static endothelial cell attachment to damaged vascular surfaces were quantified. The results of these experiments suggested that it was possible to form a confluent monolayer on damaged vascular surfaces by using high endothelial seeding densities and an incubation time of 30 min. Having established initial cell attachment, the next step in the seeding process involves the retention of cells in arterial flow conditions. It has been suggested that the main determinant of a successful seeding procedure, is the ability of endothelial cells to resist the shear stresses exerted by the bloodstream [Rosenman et al. 1985b; Pratt et al. 1988].

Rosenman et al. [Rosenman et al. 1985b] illustrated the importance of cell retention in an experiment investigating cellular attachment to prosthetic grafts in a canine model. After 30 min of arterial flow, 30% of the seeded endothelial cells became detached from the ePTFE prosthesis, and final cell retention on the graft after 24 hours was only 17%. This magnitude of cell loss is not exceptional, and has been confirmed by several other investigators [Patterson et al. 1989; Ramalanjaona et al. 1986].

It is apparent that the measurement of adherent cells under static conditions fails to predict cell attachment when exposed to flow. The aim of this experiment was to determine the ability of immediately seeded endothelial cells to resist detachment from damaged vascular surfaces exposed to physiological shear stresses.

8.2 Methods.

Isolation and Culture of Endothelial Cells.

Endothelial cells were harvested from human umbilical veins as has previously been described (chapter 6). Cell number was amplified in tissue culture and cells from the second to fourth passage were used in this experiment. Cell identity was again confirmed by appearance at phase contrast microscopy and by immunohistochemical staining for von Willebrand factor (chapter 6).

Radiolabelling of Endothelial Cells.

For each separate experiment, $3 \times 10^5$ cells were released from tissue culture and labelled with 60μCi indium-111 oxine as described in chapter 6. Following labelling and
Cell Retention in Shear Stress

determination of labelling efficiency, the cells were resuspended in complete culture medium at a concentration of 3x10^6 cells/ml. Cell viability was determined at this time by trypan blue exclusion.

In Vitro Model of Vascular Damage.

Segments of long saphenous vein at least 2 cm in length were obtained from patients undergoing aorto-coronary bypass grafting, and transported to the laboratory in chilled MEM (ICN, High Wycombe, Bucks, UK). Each segment was dilated with a 5 mm balloon angioplasty catheter (Medi-Tech, UK) for 30 seconds at 9 atm pressure, as described in chapter 7. Following dilatation each end of the vein was cannulated with a small metal cannula made specifically for this purpose. The cannulae were secured in place, and 3-way taps (Monoject, St Louis, USA) were then connected at each end.

Seeding Procedure.

The vein segment was gently perfused with 10 ml MEM to remove any debris resulting from balloon dilatation. A 1 ml syringe was then connected to one of the 3-way taps and 0.4 ml of the indium-111 labelled endothelial cell suspension instilled into the vein segment and retained by closure of both 3-way taps. The vein segment was placed into a petri dish (Fisons, Loughborough, UK) containing prewarmed MEM and incubated in a 5% CO₂, 95% air atmosphere at 37°C for 30 min. During incubation, the vein segment was rotated through 25° every 7.5 min to ensure even distribution of the seeded cells. After 30 min incubation, the vein segment was gently flushed with a further 10 ml MEM to remove any unattached cells. The 3-way taps were removed and the seeded vein segment, still cannulated at either end, was placed in a perfusion chamber prior to analysis in pulsatile flow.

Perfusion Chamber.

The perfusion chamber consisted of a modification of the seeding chamber described by Budd et al. [Budd et al. 1991b]. The chamber was manufactured in the Department of Surgery, University of Leicester, and was originally developed to facilitate confluent endothelial seeding of prosthetic vascular grafts. The chamber was composed of a polymethylpentene tube (Just Plastics Ltd, London, UK) onto which two stainless steel caps could be threaded and sealed with silicone rings. The steel caps contained two ports; a central port through which a stainless steel perfusion cannula was placed, and a peripheral port composed of a luer fitting and cap. The perfusion cannulae could be moved...
Cell Retention in Shear Stress

within the perfusion chamber, and could also be locked in place. The external end of both perfusion cannulae held a luer fitting to enable easy connection to the pulsatile flow circuit (Fig 8.1).

Fig 8.1: Diagrammatic illustration of the perfusion chamber with vein segment in place.

The chamber was modified from its original specification by the addition of a plastic connector (manufactured in the Department of Surgery) that joined the perfusion cannulae to the cannulated vein segment.

To analyse cell loss in pulsatile flow, the seeded vein segment was connected to the two perfusion cannulae and the perfusion chamber filled with prewarmed MEM via the peripheral ports. The external ends of the two perfusion cannulae were then connected into a pulsatile flow circuit which enabled the seeded native vascular surface to be subjected to varying shear stresses.

**Vein Segment Perfusion.**

A perfusion circuit consisting of a roller pump unit, a reservoir, and a perfusion chamber was designed and constructed to deliver pulsatile flow. A 250 ml reservoir (Duran glass bottle, FSA, Loughborough, UK) was filled with a gelatin based colloid solution (Haemaccel - Hoechst, Hounslow, UK) and placed in a water bath at 37°C. The reservoir cap contained an inlet port and an outlet port which was connected to a length of tubing sited in the Haemaccel reservoir. The outlet port was connected to the roller pump with blood administration tubing (Travenol Laboratories, Thetford, UK), and this was in turn connected to the proximal perfusion cannula of the perfusion chamber. The distal
Cell Retention in Shear Stress

perfusion cannula was similarly connected to the inlet port of the reservoir to complete the circuit (Fig 8.2, 8.3).

Fig 8.2: Photograph illustrating the complete perfusion circuit. During use the reservoir would be placed in a water bath.

Fig 8.3: Diagrammatic illustration of the pulsatile flow circuit.
The roller pump consisted of a pump motor (7554-30, CP instruments Ltd, Bishops Stortford, UK), a pump head (7017-29, CP instruments Ltd, Bishops Stortford, UK), silicone tubing and a speed control (CP instruments Ltd, Bishops Stortford, UK), and was capable of delivering pulsatile flow at rates between 2.8 and 280 ml/min.

Each vein segment was placed in the perfusion chamber immediately after seeding and exposed to 120 min of pulsatile flow using Haemaccel perfusate. The diameter and length of each vein segment was measured during perfusion. Flow rates of 50 and 100 ml/min were studied and 7 duplicate experiments were performed at each flow rate.

**Haemodynamic Profile.**

Prior to perfusion of the seeded vein segments the pump was hand calibrated by timed volume measurements to flow rates of 50 and 100 ml/min. To assess the haemodynamic profile of the system a 4 mm ePTFE (WL Gore Ltd, Livingstone, UK) graft was perfused at these two flow rates with Haemaccel containing preswollen glass beads of a fixed diameter (Sephadex G-25 fine, Pharmacia, Upsala, Sweden). The introduction of these beads allowed the graft to be scanned under pulsatile flow conditions with a Diasonics DRF 400 duplex scanner (Diasonics, Bedford, UK). A 4 mm ePTFE graft was used for this analysis as the vein segments were of insufficient length to be imaged and the approximate diameter of the dilated vein segments was 4 mm.

Duplex scanning provided a profile of flow velocity waveforms (Fig 8.4) and also measured the maximum flow velocity. Using these measurements in conjunction with the mean vein diameters, and the kinematic viscosity of the Haemaccel perfusate, the haemodynamic characteristics of the pulsatile circuit could be calculated (Table 8.1, calculations are detailed in Appendix C). The Reynolds number ($N_r$) gives an indication of the likelihood of turbulent flow. In artificial flow circuits turbulence does not usually occur until the $N_r$ reaches 1600 [Milnor, 1989]. As the maximum $N_r$ generated at 100 ml/min is less than this figure, turbulent flow is unlikely and calculations of shear stress may be based on laminar flow. The maximum shear stress generated at a flow of 100 ml/min was calculated to be 16.5 dynes/cm², a value within the reported range of stresses in the femoropopliteal arterial system [Kadletz et al.1987; Eskin et al.1985; Allen et al.1984].

**Determination of Cell Retention.**

Seeded endothelial cell loss from the dilated vein segments was calculated by monitoring the gamma activity of the vein segment during flow. After the vein segment was placed in the perfusion circuit, an Isotope Localisation Monitor 235, fitted with a type
235 probe (DA Pitman Ltd, Weybridge, UK) (Fig 8.5) was fixed in position on the outside of the perfusion chamber opposite the centre of the vein segment. The probe collimater was set at 1 cm.

Fig 8.4: Duplex scan illustrating flow velocity waveform generated in a 4 mm ePTFE graft perfused at 100 ml/min.

|                        | Flow Rate
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 ml/min</td>
</tr>
<tr>
<td>Mean flow velocity (cm/s)</td>
<td>5.9</td>
</tr>
<tr>
<td>Maximum flow velocity (cm/s)</td>
<td>22.5</td>
</tr>
<tr>
<td>Reynolds Number (mean)</td>
<td>165</td>
</tr>
<tr>
<td>Reynolds Number (max)</td>
<td>630</td>
</tr>
<tr>
<td>Mean shear stress (dyn/cm^2)</td>
<td>1.76</td>
</tr>
<tr>
<td>Maximum shear stress (dyn/cm^2)</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Table 8.1: Table illustrating haemodynamic parameters of the pulsatile flow circuit at 50 and 100 ml/min flow.
The flow circuit was primed with Haemaccel by slowly activating the pump. When the circuit was full a baseline reading was taken to establish the gamma activity of the seeded vein segment prior to commencement of flow.

Flow was then instituted at 50 or 100 ml/min and serial recordings of gamma activity over the vein segment were made. The position of the radioisotope monitor did not vary between readings. Readings were taken every 10 min up to 120 min when the experiment was terminated. To correct for spontaneous leakage of indium-111 from the seeded endothelial cells, the gamma counts were corrected at the rate of 3.5% per hour (chapter 6).

Having corrected the gamma counts at all time periods, cell retention was calculated and expressed as the percentage of initially attached cells that were retained at the seeded site. Percentage cell retention was determined by the formula:

\[
\text{Cell retention (\%) = } \frac{\text{cpm } t^x}{\text{cpm } t^0} \times 100
\]

where cpm \( t^x \) represents corrected counts per minute over the seeded vein site at time \( x \) and cpm \( t^0 \) represents corrected counts per minute over the seeded vein site immediately before institution of flow.
Cell Retention in Shear Stress

**Histological Examination.**

After 120 min of perfusion, the vein segment was removed from the perfusion chamber and prepared for scanning electron microscopic examination as has been previously described (chapter 6).

**Statistical Analysis.**

Results of cell retention have been presented as median values with 95% confidence intervals (CI). The rate of cell loss at differing time intervals has been analysed with linear regression, the slope of the regression line giving the rate of loss. Continuous variables have been analysed using the Mann Whitney test. Cell retention at the two flow rates was analysed by calculating the areas under each cell retention curve, and then comparing these with a Mann Whitney test.

**Results.**

**Cell Viability and Labelling Efficiency.**

Cell viability as assessed by trypan blue exclusion was > 95% in all cases. The median labelling efficiency in experiments performed at a flow rate of 50 ml/min was 30% (95% CI 22%-34%), and 32% (95% CI 23%-34%) in experiments at at 100 ml/min. There was no significant difference between the labelling efficiencies of the two experiments (W=56.5, 95% CI = -2 to 11, p = 0.66, Mann Whitney).

The median seeding density at the lower flow rate was 6.7x10^5 cells/cm^2 (95% CI 5.9-9.2x10^5 cells/cm^2), as compared to 5.3x10^5 cells/cm^2 (95% CI 4.3 - 9.6x10^5 cells/cm^2) at the higher flow rate. Again there was no statistical difference between these two figures (W = 44, 95% CI -3.1 to 3.1x10^5 cells/cm^2, p = 0.31, Mann Whitney).

**Vein Dimensions.**

Median vein lengths and diameters are illustrated in Table 8.2. There was no significant difference in vein length or diameter between flow rates of 50 and 100 ml/min (W = 62.5, 95% CI -0.5 to 1, p = 0.23, and W = 45.5, 95% CI -0.04 to 0.01, p = 0.4, respectively, Mann Whitney.)
Cell Retention in Shear Stress

<table>
<thead>
<tr>
<th>Flow Rate</th>
<th>Vein Diameter (cm)</th>
<th>Vein Length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 ml/min</td>
<td>0.4 (0.38-0.43)</td>
<td>1.5 (1-1.5)</td>
</tr>
<tr>
<td>100 ml/min</td>
<td>0.39 (0.38-0.41)</td>
<td>2 (1-2.16)</td>
</tr>
</tbody>
</table>

Table 8.2: Table illustrating median vein lengths and diameters. Figures in parentheses are 95% confidence intervals.

**Effect of Shear Stress on Endothelial Cell Retention.**

The retention of the seeded endothelial cells during exposure to pulsatile flow at 50 and 100 ml/min is illustrated in Fig 8.6. Cell retention on the vascular surface was significantly higher at the lower flow rate (median area under curve at 50 ml/min = 929 arbitrary units [95% CI 836-990], area under curve at 100 ml/min = 816 [735-859], W = 70, 95% CI = 19.9 to 228, p = 0.03, Mann Whitney). After 120 min of pulsatile flow at 50 ml/min, 68% (95% CI 54%-74%) of the initially adherent endothelial cells remained attached to the damaged luminal surface of the vein segment. At flows of 100 ml/min, the final cell retention was 51% (95% CI 44%-60%).

![Graph illustrating median cell retention in flows of 50 and 100 ml/min.](image)

Fig 8.6: Graph illustrating median cell retention in flows of 50 and 100 ml/min. Error bars are 95% confidence intervals.
To assess the rate of cell loss from the seeded vascular surface, linear regression lines were plotted through the data [Rosenman et al. 1985b]. The rate of cell loss (% loss / min) was given from the slope of the regression line which was characterised by the equation:

\[ y = a + bx, \]

where \( a \) is the \( y \) intercept, and \( b \) is the slope of the graph [Bland, 1990].

**FLOW 50 ML/Min.**

During this degree of pulsatile flow, which generated a maximum shear stress of 6.7 dyn/cm², cell loss was rapid (1.2% / min) during the first 20 min of perfusion (\( r^2 = 0.68, p < 0.001 \), linear regression). After this time the rate of cell loss declined considerably to 0.12% / min (\( r^2 = 0.17, p = 0.58 \)). This relationship is illustrated in Fig 8.7 which analyses the rate of cell detachment from the seeded venous surface in two phases; during the initial 20 min of flow and then from 20-120 min.

![Fig 8.7 : Graph demonstrating the relationship between the percentage of initially adherent endothelial cells and the time of exposure to flow (50 ml/min).](image-url)
Cell Retention in Shear Stress

FLOW 100 ML/MIN.

In the higher shear stresses generated by flows of 100 ml/min, cell loss was again rapid during the initial 20 min of perfusion, the rate being 1.48% / min ($r^2 = 0.63$, $p < 0.001$). After this phase there was a period of intermediate cell loss (0.25% / min) from 30 min till 90 min ($r^2 = 0.29$, $p < 0.001$) and after this time the rate of cell detachment was minimal (cell loss = 0.089% / min, $r^2 = 0.012$, $p = 0.58$) (Fig 8.8).

![Graph demonstrating the relationship between the percentage of initially adherent endothelial cells and the time of exposure to flow (100 ml/min).](image)

Fig 8.8: Graph demonstrating the relationship between the percentage of initially adherent endothelial cells and the time of exposure to flow (100 ml/min).
**Histological Examination.**

Scanning electron microscopy confirmed the presence of seeded endothelial cells on the post-perfusion vein segments (Fig 8.9).

![Scanning electron micrograph illustrating the presence of seeded endothelial cells on a vein segment following perfusion at 100 ml/min for 120 min. Magnification x 950.](image)

**Discussion.**

The ability of seeded endothelial cells to resist the shear stress of the circulation is a prime determinant of successful seeding. Prior to commencing any *in vivo* seeding of angioplasty sites, it was necessary to investigate the adhesion of endothelial cells to native vascular surfaces, in conditions simulating arterial flow.

The pulsatile flow circuit used in this experiment was designed to produce physiological shear stresses. The maximum shear stress generated by this circuit at flow rates of 100 ml/min was 16.5 dynes/cm², which is comparable to levels recorded in the femoropopliteal arteries [Kadletz *et al.* 1987; Eskin *et al.* 1985; Ivarsson *et al.* 1989]. However, the artificial flow circuit did not exactly mimic physiological conditions as it was unable to generate shear stresses in the range 30 - 100 dynes/cm², which occur at arterial branch sites [Dewey, 1979]. The difference between *in vivo* and *in vitro*...
Cell Retention in Shear Stress

conditions was exacerbated by the use of a colloid perfusate in place of human blood. Blood was not utilised as pump induced damage to the cellular elements and activation of leucocytes and platelets by the artificial circuit, would have introduced considerable intra-experiment variability [Miyata et al. 1991].

Cell detachment was quantified by measuring the decrease in gamma activity that accompanied loss of radionlabelled endothelial cells. The use of indium-111 labelling in perfusion experiments has been criticised in the past, as quantification of cell loss relies upon secondary measurements. In addition, it has been suggested that there are inaccuracies in radionlabelling techniques due to spontaneous intracellular leakage of the isotope, the affinity of indium-111 for other biologic materials and potential toxicity during the labelling procedure [Miyata et al. 1991; Newman et al. 1991]. However, despite these drawbacks, indium labelling is relatively simple to perform and has the advantage of being quantitative and reproducible [Patterson et al. 1989; Kesler et al. 1986]. Spontaneous isotope leakage from endothelial cells does occur, but the rate may be easily predicted and appropriate corrections made [Kesler et al. 1986]. Toxicity may be a problem at high doses [Vohra et al. 1990a, b], but in this experiment, post-labelling cell viability exceeded 95% in all cases.

Analysis of cell detachment in artificial flow circuits has been used extensively in studies of endothelial cell retention on prosthetic graft surfaces. Kent et al. [Kent et al. 1988] measured endothelial cell loss from uncoated graft surfaces after a 90 min incubation period. Following perfusion at 220 ml/min for 60 min, only 40% of the initially attached endothelial cells remained on the graft. Similar rates of cell loss, after short incubation periods have been documented in other studies. Vohra et al. [Vohra et al. 1990b] reported a 45% cell detachment rate from fibronectin coated surfaces during 120 min perfusion at 1.7 dynes/cm², and similarly, Budd et al. [Budd et al. 1991a] reported that 50% of initially adherent cells were lost from fibronectin coated ePTFE grafts within 120 min flow.

In an attempt to improve cell resistance to shear stress, several groups have utilised increased incubation times prior to initiation of flow. Zilla et al. [Zilla et al. 1989] cultured endothelial cells on ePTFE graft surfaces for 9 days before perfusion, and showed that only 23% of the seeded cells were detached in the first 24 hours of flow. These findings were confirmed by both Miyata et al. [Miyata et al. 1991] and Budd et al. [Budd et al. 1991a], who demonstrated that enhanced cell retention could be achieved by prolonged (>1 day) incubation periods. The increased resistance to flow associated with longer incubation was presumably due to the maturation of endothelial attachment sites, with the development of junctional complexes and deposition of extracellular matrix [Miyata et al. 1991].

In this study, endothelial cells were seeded onto damaged vascular surfaces using
the optimum seeding parameters defined in the previous chapter. At flows of 50 ml/min, 32% of the initially attached endothelial cells were lost from the damaged vascular surface after 120 min of pulsatile flow. The corresponding loss from vein segments perfused at 100 ml/min was 49%, which confirms the findings of others, that cell detachment is greater at increasing levels of shear stress [Pratt et al. 1988].

At both perfusion rates, cell loss occurred in two distinct phases. Initially, cell detachment was rapid in the first 20 min of perfusion with 24% and 27% of cells being lost at flow rates of 50 and 100 ml/min respectively. The second phase involved a slower rate of cell detachment, which commenced 20 min following perfusion and continued for the remainder of the experiment, when a plateau was reached. At flow rates of 100 ml/min, the second phase could itself be divided in two periods with the rate of cell loss slowing as the experiment progressed.

This biphasic pattern of cell detachment has been observed during perfusion of seeded vascular grafts both in vitro and in vivo [Vohra et al. 1990a, b; Kesler et al. 1986; Rosenman et al. 1985b]. In this experiment, at the higher flow rate, seeded cell detachment from native vascular surfaces reached 88% / hour in the rapid phase of cell loss, but decreased to 5% / hour after 90 min of flow. Similar rates of cell loss were reported by Rosenman et al. [Rosenman et al. 1985b] who demonstrated that cellular loss from endothelial seeded canine carotid interposition grafts was 60% / hour during the first 30 min of flow and 3.7% / hour from 30 min to 24 hours, after which time cell loss ceased. The initial phase of rapid cell loss is a constant observation in all perfusion experiments of seeded surfaces, and is attributed to the loss of poorly attached, rounded endothelial cells [Vohra et al. 1990b]. Following this initial loss, the subsequent slow detachment of seeded cells is caused by spontaneous loss of initial attachment due to cell death or cellular damage produced during tissue culture [Rosenman et al. 1985b; Glassberg et al. 1982]. Eventually a plateau phase is reached where the cells are stably attached to the underlying substrate and no further loss occurs. Stable attachment is enhanced by spreading of the endothelial cells on the underlying substrate, a process which which is actually stimulated by shear stress [Anderson et al. 1987; Schneider et al. 1988].

The results from this experiment demonstrate that endothelial cells seeded onto damaged vascular surfaces may have the ability to resist the shear stresses of the circulation in the short term. Unfortunately, in this study long term retention of seeded endothelial cells could not be studied due to the short half life of indium-111 (67 hours) and an equivalent loss from intracellular leakage [Kesler et al. 1986]. However, findings from other studies have suggested that quantification of successful short term cellular resistance to flow may predict long term attachment [Rosenman et al. 1985b].

The retention of seeded endothelial cells to damaged vascular surfaces has also been studied by Schneider et al. [Schneider et al. 1990] who seeded endarterectomised
segments of baboon aorta with endothelial cells at a density of $6 \times 10^5$ cells/cm$^2$. Initially $3.8 \times 10^5$ cells/cm$^2$ attached to the arterial segment which was then placed in a perfusion circuit. After 2 hours perfusion, $2.8 \times 10^5$ (74%) cells/cm$^2$ were retained on the luminal surface which showed the presence of a confluent, flattened monolayer.

**Summary.**

In this experiment, an *in vitro* model of vascular damage has been utilised to investigate the ability of seeded endothelial cells to resist shear stresses generated in a perfusion circuit. At perfusion rates of 100 ml/min, maximum shear stress reached 16.5 dyn/cm$^2$. At this level, the rate of cell detachment from the damaged vascular surface was 88% / hour for the first 20 min of flow but gradually decreased to 5% / hour after 90 min. These findings suggest that endothelial cells may be adequately retained on damaged vascular surfaces in conditions approximating arterial flow.
# CHAPTER 9
PROSTACYCLIN RELEASE FROM SEEDED NATIVE VASCULAR SURFACES

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.1 Introduction</td>
<td>130</td>
</tr>
<tr>
<td>9.2 Methods.</td>
<td>131</td>
</tr>
<tr>
<td>- Isolation and culture of endothelial cells</td>
<td>131</td>
</tr>
<tr>
<td>- <em>In vitro</em> model of vascular damage</td>
<td>131</td>
</tr>
<tr>
<td>- Seeding procedure</td>
<td>132</td>
</tr>
<tr>
<td>- Prostacyclin measurement</td>
<td>132</td>
</tr>
<tr>
<td>- Statistics</td>
<td>133</td>
</tr>
<tr>
<td>9.3 Results.</td>
<td>133</td>
</tr>
<tr>
<td>- Basal prostacyclin release</td>
<td>133</td>
</tr>
<tr>
<td>- Stimulated prostacyclin release</td>
<td>135</td>
</tr>
<tr>
<td>9.4 Discussion.</td>
<td>135</td>
</tr>
<tr>
<td>9.5 Summary.</td>
<td>138</td>
</tr>
</tbody>
</table>
9.1 Introduction.

In the previous two chapters, it has been demonstrated that cultured endothelial cells are able to adhere to damaged native vascular surfaces, and subsequently remain attached when exposed to physiological shear stress. From these results, it can be concluded that endothelial cells may be satisfactorily transplanted and retained on sub-endothelial tissues, thus fulfilling two of the three requirements for effective endothelial cell seeding. The final prerequisite for successful seeding is that the transplanted cells retain their anti-thrombotic and anti-coagulatory properties once attached to the vascular surface.

In the quiescent state the endothelial cell monolayer presents a non-thrombogenic surface to the flowing blood. This property is largely attributable to an anionic surface charge on the endothelial cells [Nievelstein, de Groot, 1988], together with the constant release of endothelium derived relaxing factor (EDRF) [Furchgott, 1983], a powerful anti-platelet agent [Radomski et al. 1987]. Damage or disruption to the endothelial cell monolayer diminishes the anti-thrombogenic influence of the endothelial cells, and also exposes the highly thrombogenic sub-endothelial matrix to the circulating blood. Platelets subsequently adhere and aggregate on the sub-endothelium thus initiating the thrombogenic process [Faxon et al. 1987].

Prostacyclin (PGI₂) is an arachidonic acid derivative synthesised by the vascular endothelium, that is the most potent naturally occurring inhibitor of platelet aggregation [Moncada et al. 1976; Moncada et al. 1987]. As well as its anti-platelet action, PGI₂ has a powerful vasorelaxant activity [Bunting et al. 1983], and also modulates the generation of superoxide anions from activated neutrophils [Fantone, Kinnes, 1983; Faber et al. 1988]. Prostacyclin further inhibits thrombus formation by limiting the extent of fibrinogen-platelet interactions [Graber, Hawiger, 1982], in addition to mediating a direct thrombolytic action [Usonomiya et al. 1980]. Despite these properties, prostacyclin does not contribute to the anti-thrombogenic nature of non-damaged endothelium [Czervionke et al. 1979], but is released in large quantities during vascular damage, and acts synergistically with EDRF [Moncada et al. 1987] to limit platelet aggregation and vasospasm [Dusting, Macdonald, 1990].

One rationale for endothelial seeding of native vascular surfaces is to utilise the transplanted endothelial cells as a source of vasoactive mediators. It has been hypothesised that high levels of PGI₂ and EDRF may contribute to the formation of a thromboresistant flow surface following iatrogenic arterial damage. In order for this hypothesis to be valid,
Prostacyclin Release

it is initially necessary to demonstrate that the transplanted endothelial cells retain their ability to function physiologically. In this chapter, the ability of endothelial seeded native vascular surfaces to synthesise and release PGH$_2$ will be investigated.

9.2 Methods.

Isolation and Culture of Endothelial Cells.

Endothelial cells were harvested from human umbilical veins, and subsequently grown in tissue culture as has previously been described (chapter 6). Cells from the second and third passage were used in this experiment. Endothelial identity was confirmed by the characteristic morphology under phase contrast microscopy and by immunohistochemical staining for von Willebrand factor. Endothelial cell viability was >95% in all cases.

In Vitro Model of Vascular Damage.

Segments of distal long saphenous vein at least 2 cm in length were obtained from patients undergoing aorto-coronary bypass grafting and transported to the laboratory in chilled Minimal Essential Medium (MEM - ICN, High Wycombe, Bucks, UK). On arrival, the vein was divided into two equal parts. One segment was dilated at 9atm pressure with a 5 mm angioplasty balloon as has been described in chapter 7; the other segment remained undamaged and was stored in chilled MEM. Both undilated and dilated vein segments were then opened longitudinally and placed in a MEM filled petrie dish (Fisons, Loughborough, UK), with the luminal surface uppermost. A 5 mm diameter punch biopsy (biopsy punch manufactured in the Department of Surgery, Leicester University) was taken from the undilated vein and 2 similar biopsies were obtained from the dilated vein segment. One of the dilated vein biopsies was then seeded with endothelial cells, whilst the remaining dilated vein biopsy and the undistended vein biopsy were sham seeded with complete culture medium.

Therefore from one initial vein segment three vein biopsies were obtained (Fig 9.1):

- undistended vein biopsy = control vein
- dilated vein biopsy = dilated vein
- distended and seeded vein biopsy = seeded vein.
Prostacyclin Release

Fig 9.1: Diagrammatic representation of the experimental design resulting in three vein biopsies for prostacyclin assay.

Seeding Procedure.

Human umbilical venous endothelial cells were released from tissue culture by trypsinisation (0.1% trypsin in 0.02% EDTA) and resuspended in complete culture medium at a concentration of 9.8x10^5 cells/ml. The 5 mm diameter punch biopsy of distended saphenous vein was placed on silicone gel in a 5 mm diameter seeding chamber. Seeding chambers were manufactured from 1 ml automatic pipette tips as described in chapter 7 (Fig 7.3). A 100µl aliquot of the endothelial cell suspension was added to the seeding chamber so that the cells came into contact with the luminal vein surface. After incubation in 95% air, 5% CO₂ at 37°C for 30 min, the unattached cells were removed by washing the luminal vein surface three times with MEM. The seeding density throughout this experiment was 5x10^5 cells/cm².

The control and dilated segments were similarly placed in seeding chambers but were incubated with acellular complete culture medium for 30 min (sham seeded).

Prostacyclin Measurement.

The control, dilated and seeded vein segments were removed from the seeding chambers and washed with MEM. Each vein biopsy was then placed in one well of a 24 well plate (Fisons, Loughborough, UK) which contained 1 ml of MEM (pH 7.4) prewarmed to 37°C. The samples were incubated in a 95% air, 5%CO₂ atmosphere at 37°C for 30 min, after which time 200µl of MEM was removed from each well. This
Prostacyclin Release

A sample was later assayed for PGI$_2$ release and represented the basal release of PGI$_2$ from the three paired vein biopsies.

Following the removal of basal PGI$_2$ samples, 300µl of a thrombin solution (2units/ml, Sigma, Poole, UK) was added to each well. The samples were then incubated for a 10 min period, after which further 200µl samples of MEM were removed from each well. These samples represented the stimulated release of PGI$_2$ from the vein samples. Samples of MEM for PGI$_2$ assay were frozen at -70°C until required. Prostacyclin concentration in each of the samples was assayed by measurement of 6-keto-prostaglandin F$_1$α, the stable metabolite of PGI$_2$ breakdown at pH 7.4. The assay was performed using a commercially available radioimmunoassay kit (Amersham International, Amersham, Bucks, UK), details of which are given in Appendix D. In this study, 8 replicate experiments were performed; in each experiment PGI$_2$ was assayed from control, dilated and seeded vein biopsies obtained from the same original vein segment.

Statistics.

Results of PGI$_2$ release are presented as median values with 95% confidence intervals (CI). The data from all three vein groups was initially analysed by a Kruskal-Wallis one way analysis of variance to determine specific differences within the data. Having established these differences, specific inter- and intra-group differences were analysed using the Wilcoxon paired rank test with a level of significance set at 1 in 20.

9.3 Results.

Prostacyclin release was expressed as the quantity of 6-keto-prostaglandin F$_1$α released from standard vein biopsies per minute (pg/cm$^2$/min). The results for basal and stimulated PGI$_2$ release are illustrated in Figs 9.2, 9.3, and Table 9.1. These results were analysed using a Kruskal-Wallis one way analysis of variance to determine specific differences between the data (H = 18.15, p = 0.003).

Basal Prostacyclin Release.

Basal PGI$_2$ release from control veins was higher than from dilated veins, but the difference did not reach statistical significance (W = 30, 95% CI = -5 to 98, p = 0.107, Wilcoxon paired rank test). Seeded veins produced significantly more PGI$_2$ than either control or dilated veins (W = 33, 95% CI = 7 to 497, p = 0.042; W = 35, 95% CI = 50 to 556, p = 0.021 respectively, Wilcoxon paired rank test).
Fig 9.2: Graph illustrating basal prostacyclin release (measured as 6-keto-prostaglandin \( \text{F}_1\alpha \)) from control, dilated and seeded vein biopsies. Results are presented as medians with 95% confidence intervals.

Fig 9.3: Graph illustrating stimulated prostacyclin release (measured as 6-keto-prostaglandin \( \text{F}_1\alpha \)) from control, dilated and seeded vein biopsies. Results are presented as medians with 95% confidence intervals.
Stimulated PG\textsubscript{I\textsubscript{2}} Release.

Thrombin stimulation of the vein segments significantly increased PG\textsubscript{I\textsubscript{2}} release from control veins ($W = 36$, 95\% CI = 60 to 200, $p = 0.014$, Wilcoxon), but did not increase production from either seeded or dilated veins ($W = 11$, 95\% CI = -427 to 136, $p = 0.36$; $W = 32$, 95\% CI -2 to 107, $p = 0.059$ respectively, Wilcoxon).

Stimulated PG\textsubscript{I\textsubscript{2}} release was significantly higher from both control and seeded veins than from dilated veins ($W = 36$, 95\% CI = 14 to 209, $p = 0.014$; $W = 33$, 95\% CI 19 to 257, $p = 0.042$ respectively, Wilcoxon). Seeded and control veins however, showed statistically indistinguishable PG\textsubscript{I\textsubscript{2}} release ($W = 15$, 95\% CI = -133 to 152, $p = 0.73$, Wilcoxon).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Basal PG\textsubscript{I\textsubscript{2}} Release (pg/cm\textsuperscript{2}/min)</th>
<th>Stimulated PG\textsubscript{I\textsubscript{2}} Release (pg/cm\textsuperscript{2}/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Dilated</td>
</tr>
<tr>
<td>1</td>
<td>129</td>
<td>133</td>
</tr>
<tr>
<td>2</td>
<td>126</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>208</td>
<td>105</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>70</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>105</td>
<td>54</td>
</tr>
<tr>
<td>7</td>
<td>251</td>
<td>163</td>
</tr>
<tr>
<td>8</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>Median</td>
<td>116</td>
<td>62</td>
</tr>
</tbody>
</table>

Table 9.1: Table illustrating basal and stimulated prostacyclin release from the 8 paired vein samples. The median values and 95\% confidence intervals are shown at the foot of the table.

9.4 Discussion.

Prostacyclin is generated by blood vessels from arachidonic acid or from unstable PG endoperoxides [Dusting, MacDonald, 1990]. The ability of the vasculature to synthesise PG\textsubscript{I\textsubscript{2}} is greatest at the luminal surface and decreases progressively towards the adventitia [Moncada et al. 1977]. Although vascular endothelial cells are responsible for the majority of PG\textsubscript{I\textsubscript{2}} synthesis, SMCs also have the ability to generate PG\textsubscript{I\textsubscript{2}} from arachidonic acid, as the PG\textsubscript{I\textsubscript{2}} synthase concentrations in SMCs and endothelial cells are equal. The difference in synthetic capacity between the two cell types is determined by the
concentration of cyclo-oxygenase, which is 20 fold higher in the endothelium [De Witt et al.1983; Smith et al.1983].

The role of the endothelium in synthesising and secreting PGI$_2$ has been investigated in several studies which have examined the effects of acute arterial injury on the metabolic activity of the arterial wall. Eldor et al. [Eldor et al.1981] reported that PGI$_2$ production in the rabbit aorta was dramatically decreased after balloon deendothelialisation, and from these findings concluded that the endothelium was responsible for virtually all PGI$_2$ secretion from normal blood vessels. However, following deendothelialisation, PGI$_2$ production from the denuded arterial segment returned to normal within 70 days. The recovery of PGI$_2$ generating capacity in this experiment was associated with a corresponding recovery in thromboresistance of the arterial wall.

Using the same experimental model, Mattson et al. [Mattsson et al.1990; Mattsson et al.1992] investigated the effects of balloon dilatation on the production of PGI$_2$ from intact aortic segments. Immediately after balloon dilatation PGI$_2$ release from the dilated segments was significantly lower than from undilated controls. After one month, the luminal surface of the dilated segments was covered with multiple layers of SMC's, with PGI$_2$ production returning to normal. In contrast to these findings, Boeynaems et al. [Boeynaems et al.1985] reported that arterial injury caused a sustained mobilisation of arachidonic acid and a transient increase in PGI$_2$ production immediately after injury. This increase lasted for 30 min after which time secretion declined to sub-normal levels.

In summary, the findings from the above studies suggest that loss of the endothelial monolayer causes a transient increase in PGI$_2$ production which then evolves into a prolonged depression of synthetic capability. This depression resolves with the formation of a SMC dominated neointima, which is associated with a recovery in the thromboresistant properties of the vessel wall.

Throughout this thesis, endothelial seeding has been proposed as a mechanism to rapidly increase endothelial coverage of native vascular surfaces, thereby increasing their PGI$_2$ synthetic capacity. This concept has previously been investigated in seeding of prosthetic vascular grafts. Several studies have demonstrated a long term increase in PGI$_2$ release associated with endothelial seeding [Budd et al.1992; Boyd et al.1988; Ortenwall et al.1988]. Jensen et al. [Jensen et al.1992] quantified PGI$_2$ release from carotid interposition ePTFE grafts in sheep. Three weeks after implantation, native arteries had a significantly higher PGI$_2$ release than endothelial seeded grafts which in turn had a higher release than non seeded grafts. Endothelial cell seeding was thus able to increase PGI$_2$ production from seeded vascular grafts.

In this experiment, prostacyclin release from punch biopsies of long saphenous vein was measured by a radioimmunoassay of 6-keto-prostaglandin F$_{1alpha}$ performed
directly in the incubation medium. 6-keto-prostaglandin F1a is the stable metabolite of PGI2 at pH 7.4 [Cho, Allen, 1978] and has been used by many investigators as an indirect measurement of PGI2 release [Mattson et al.1990; Mattsson et al.1992; Kent et al.1983; Leschke et al.1989], although some reactivity with PGF2a and PGE has been reported [Boeynaems et al.1985]. In all experiments basal and stimulated PGI2 was measured. Prostacyclin release was stimulated by thrombin [Baenziger et al.1981], which acts via a receptor mediated activation of phospholipase A2, which in turn releases arachidonic acid from membrane phospholipids [Weksler et al.1978].

In this study, basal PGI2 release from control vein biopsies was higher than from dilated biopsies, but the difference did not reach statistical significance. This finding may be explained by damage to the original vein segments during preparation for aortic-coronary bypass grafting, and by the assay technique used. In chapter 7 it was demonstrated that 30% of the endothelial monolayer was erased during surgical handling. This is similar to the findings of Angelini et al. [Angelini et al.1967] who demonstrated that this degree of endothelial cell loss could result in severe depression of endothelial function.

In addition to the pre-existing vein damage, assaying PGI2 release from punch biopsies may be unable to reveal small differences between samples. Punch biopsy techniques have been criticised as the SMC's exposed at the cut edge of the specimen may produce PGI2, and the mechanical damage produced during the biopsy procedure may stimulate PGI2 synthesis. Eldor et al. [Eldor et al.1981] reported that full thickness punch biopsies were unable to differentiate normal from dilated vessels, and to do so more sophisticated techniques should be used. However, despite these disadvantages the biopsy technique has been extensively used and has the advantage of being simple and reproducible [Oku et al.1990; Chaikhouni et al.1986; Boeynaems et al.1985]. The results of basal and stimulated PGI2 release from control vein biopsies in this experiment are comparable to those reported elsewhere [Constantini et al.1990; Chaikhouni et al.1986; Dion et al.1990].

Although not statistically significant, dilated vein biopsies exhibited a decrease in basal PGI2 release when compared to controls. The initial increase in PGI2 production after vascular injury, reported by Boeynaems et al. [Boeynaems et al.1985] was not observed in this study. This was probably due to the 1 hour time delay (30 min sham seeding and 30 min incubation) between vein dilation and PGI2 assay. The most notable finding from basal PGI2 measurements was that the seeded vein biopsies had significantly higher PGI2 release than dilated vein segments. This is an important observation as it confirms that the seeded endothelial cells are able to function normally following transplantation. The seeded biopsies also released more PGI2 under basal conditions than control biopsies, but specific comparisons between these two samples are invalid as the
endothelial identities on each sample are different.

Thrombin stimulation of the vein biopsies increased the PGI\(_2\) production from control veins, but not from either dilated or seeded veins. In the stimulated state, control veins had significantly higher PGI\(_2\) release than dilated veins which reflects the difference in endothelial coverage between these two samples. Although PGI\(_2\) release from seeded veins was not enhanced by thrombin stimulation, the release was still significantly higher than from dilated veins. The failure of thrombin to increase PGI\(_2\) release from seeded vein biopsies was attributed to the preparation of the seeded endothelial cells. Cells were centrifuged prior to seeded and this process probably results in maximal cell stimulation [Baenziger et al. 1981].

As has been discussed previously, acute arterial occlusion complicating percutaneous transluminal angioplasty may be due to the mechanism of angioplasty itself. Physical loss of the endothelial cell monolayer during balloon dilatation exposes the highly thrombogenic sub-endothelial matrix to the circulating blood, with the subsequent deposition of platelets and activation of the coagulation cascade [Steele et al. 1985; Faxon et al. 1985; Badimon et al. 1988; Miller et al. 1991; Harker, 1987]. This series of events produces a mural thrombus on the damaged arterial surface, which, if severe may progress to complete arterial re-occlusion. High levels of PGI\(_2\) at the angioplasty site could reduce both platelet aggregation and vasospasm and therefore reduce acute arterial recoagulation. The potential benefits of PGI\(_2\) administration during coronary angioplasty were demonstrated by Knudtson et al. [Knudtson et al. 1990] who observed a reduction in acute vessel closure in patients treated with a peri-angioplasty PGI\(_2\) infusion.

This series of experiments has demonstrated that endothelial cells transplanted onto native vascular surfaces can produce high levels of anti-thrombotic mediators immediately after seeding. Endothelial seeding of angioplasty sites may thus have the capability to increase local PGI\(_2\) levels at sites of balloon dilatation, and therefore reduce acute arterial occlusion.

9.5 Summary.

In this, and the preceding two chapters, it has been demonstrated that cultured endothelial cells may be transplanted onto native vascular surfaces, and retained in arterial flow conditions whilst preserving their anti-thrombotic functions. These findings fulfill the three basic requirements for successful endothelial cell seeding of native vascular surfaces. Having established that seeding of native surfaces is feasible in vitro, the next step in this investigation is to apply these in vitro conditions to an in vivo experimental model of angioplasty, the details of which are described in the following chapters.
## CHAPTER 10

**TRANSLUMINAL ENDOTHELIAL SEEDING OF ANGIOPLASTY SITES: ATTACHMENT KINETICS AND RETENTION IN ARTERIAL FLOW**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.1 Introduction.</td>
<td>140</td>
</tr>
<tr>
<td>- Experimental models in vascular research</td>
<td>140</td>
</tr>
<tr>
<td>10.2 Methods.</td>
<td>141</td>
</tr>
<tr>
<td>- Experimental design</td>
<td>141</td>
</tr>
<tr>
<td>- Isolation and culture of endothelial cells</td>
<td>142</td>
</tr>
<tr>
<td>- Radiolabelling of endothelial cells</td>
<td>142</td>
</tr>
<tr>
<td>- Cell delivery system</td>
<td>142</td>
</tr>
<tr>
<td>- Angioplasty and seeding procedure</td>
<td>144</td>
</tr>
<tr>
<td>- Sacrifice</td>
<td>145</td>
</tr>
<tr>
<td>- Endothelial cell retention</td>
<td>145</td>
</tr>
<tr>
<td>- Histological studies</td>
<td>147</td>
</tr>
<tr>
<td>- Flow rates during angioplasty</td>
<td>147</td>
</tr>
<tr>
<td>- Statistical analysis</td>
<td>148</td>
</tr>
<tr>
<td>10.3 Results - Pilot Experiment.</td>
<td>148</td>
</tr>
<tr>
<td>10.4 Results - Definitive Experiment.</td>
<td>149</td>
</tr>
<tr>
<td>- Labelling efficiency, cell viability and seeding density</td>
<td>149</td>
</tr>
<tr>
<td>- Arterial measurements</td>
<td>149</td>
</tr>
<tr>
<td>- Endothelial cell retention</td>
<td>150</td>
</tr>
<tr>
<td>- Histological examination</td>
<td>151</td>
</tr>
<tr>
<td>10.5 Discussion.</td>
<td>153</td>
</tr>
<tr>
<td>10.6 Summary.</td>
<td>157</td>
</tr>
</tbody>
</table>
10.1 Introduction.

In the previous three chapters, it has been demonstrated that endothelial seeding of native vascular surfaces is technically feasible in vitro, with the seeded cells having the ability to resist physiological shear stresses and secrete anti-thrombogenic mediators following attachment to the sub-endothelial matrix. In this chapter, a method to transluminally seed endothelial cells onto experimental angioplasty sites will be described and the seeding kinetics determined. In subsequent chapters, the effect of transluminal endothelial seeding on platelet deposition and myointimal hyperplasia following angioplasty will be investigated.

Experimental Models in Vascular Research.

Experimental models of vascular injury have advanced the understanding of the pathophysiological processes encountered during vascular reconstructive procedures [Fuster et al. 1991]. There are a large number of well established animal models of vascular injury, and in this thesis a rabbit model will be used. In general terms, rabbit models may be divided into atherosclerotic or non-atherosclerotic categories. Many investigators [Sanborn et al. 1983; Wilentz et al. 1987; Block et al. 1980a,b; Sarembock et al. 1989] have induced atherosclerotic changes in rabbit arteries by high cholesterol diets in association with mild vascular injury, produced by balloon deendothelialisation or air drying. Although these hypercholesterolemic models are widely used, their main disadvantage is that the atherosclerotic lesions have a preponderance of foam cells, which are absent in human disease. These lesions also lack areas of calcification, necrosis or fibrosis which are the hallmarks of human atheroma [Weidinger et al. 1991].

In this investigation, the effects of endothelial cell transplantation on occlusive complications following angioplasty will be studied in a non-hypercholesterolemic, non-atherosclerotic rabbit model. Dilation of rabbit iliac arteries causes an increase in arterial diameter with histologic findings confirming complete endothelial desquamation and thinning of the tunica media [Consigny et al. 1986]. Immediately following angioplasty the dilated surface becomes covered with platelets and white cells [Jorgensen et al. 1988b; Jorgensen et al. 1988a; Plate et al. 1989; Cole et al. 1987], which may result in mural thrombus formation [Sarembock et al. 1989], vasospasm [Fischell et al. 1989] or acute arterial reocclusion. Several days after injury vascular smooth muscle cells begin to
Transluminal Endothelial Seeding

proliferate and migrate into the intimal layers where replication continues. A neointimal lesion thus forms which is maximal 4 weeks after injury and shows no sign of regression within 3 months [Hanke et al. 1990; Richardson et al. 1990]. Reendothelialisation of the angioplasty site does occur [Weidinger et al. 1990], but is slow, and remains incomplete 4 weeks after dilatation [Mattsson et al. 1992].

Balloon dilatation of non-atherosclerotic rabbit arteries is a useful model for studying angioplasty, as the immediate and long term morphological responses mimic the acute and chronic complications that affect PTA in man. The accuracy of this model may be enhanced by employing repeated arterial dilatations using an oversized balloon (3 mm diameter), and high inflation pressures (8 atm), which produce severe arterial injury with characteristic features of intimal dissection, medial necrosis and mural thrombus formation [Sarembock et al. 1989]. These conditions also maximise the degree of intimal hyperplasia as the extent of vascular smooth muscle cell proliferation following angioplasty is related to the severity of the arterial injury [Weidinger et al. 1990].

The advantage of the non-hyperlipidemic rabbit model in this situation is that the neointimal lesions are composed predominantly of vascular smooth muscle cells and extracellular matrix, which is typical of human restenotic lesions, rather than the foam cell rich lesions observed in the hypercholesterolemic model [Liu et al. 1990]. The obvious disadvantage of the non-atherosclerotic model is the lack of arterial disease which necessarily affects the response to dilatation [Demer, 1991].

10.2 Methods.

Experimental Design.

This experiment was designed to investigate the feasibility of transluminally seeding angioplasty sites with cultured endothelial cells. Two separate series of experiments were performed; an initial pilot series that utilised human umbilical venous endothelial cells in the seeding procedure, followed by a definitive series of experiments using allogeneic rabbit endothelial cells. The pilot experiment was performed to assess the adequacy of the experimental model and the design of the cell delivery system. In the pilot experiment the only parameter measured was the dynamic retention of seeded cells at the angioplasty site, whereas in the definitive experiment, dynamic and static measures of cell retention were recorded.

The experimental methods were the same between pilot and definitive experiments and will be described together. Nine replicate experiments were performed in the pilot series, with 7 replicate experiments in the definitive series.
Isolation and Culture of Endothelial Cells.

Two types of endothelial cells were used in this experiment. Human umbilical venous endothelial cells (HUVECs) and rabbit aortic endothelial cells were separately harvested and grown in tissue culture as has been described previously (chapter 6). In this study HUVECs were used in the pilot series of experiments, whereas the allogeneic rabbit aortic endothelial cells were utilised in the definitive study. In both cases, cells from the second to fourth passage were used. Endothelial cell identity was confirmed by appearance at phase contrast microscopy and positive immunohistochemical staining; for von Willebrand Factor (Dakopatts, High Wycombe, Bucks, UK) in human cells, and for an anti-rabbit endothelial, anti-thrombomodulin antibody (QB-END 40, Serotec, Oxford, UK) in the rabbit aortic endothelial cells.

Radiolabelling of Endothelial Cells.

In each separate experiment $1 \times 10^6$ HUVECs or rabbit aortic endothelial cells were released from tissue culture and labelled with $100\mu\text{Ci}$ indium-111 oxine as described in chapter 6. Following labelling and determination of labelling efficiency, the cells were resuspended in complete culture medium at a concentration of $2 \times 10^5$ cells/ml. Cell viability was determined at this time by trypan blue exclusion.

Cell Delivery System.

Throughout the in vivo work to be described in the present and subsequent two chapters, the same method has been used to deliver and retain seeded endothelial cells at angioplasty sites. This technique relied on the use of a double balloon seeding catheter (Fig 10.1) which was transluminally positioned at the angioplasty site immediately after balloon dilatation. Once in position, two low pressure isolation balloons were inflated to isolate the angioplasty site from the remainder of the circulation. This had the effect of creating an enclosed arterial segment into which endothelial cells could be instilled through a separate channel and instillation port. The endothelial cells could then be retained at the angioplasty site by continued inflation of the isolation balloons. After a 30 min incubation period, flow was restored through the arterial segment by deflation of the isolation balloons and removal of the seeding catheter.

In this experiment a 4F double balloon seeding catheter (Meadox, Dunstable, UK; Ideas for Medicine Inc, Tampa, USA) was manufactured to allow endothelial cell delivery (Fig 10.2). The distance between the isolation balloons was 1.75 cm.
Fig 10.1: Diagrammatic representation of a double balloon catheter illustrating two low pressure isolation balloons isolating an enclosed arterial segment, into which endothelial cells may be instilled.

Fig 10.2: Photograph of the seeding catheter used in this experiment. Both isolation balloons are inflated, and the instillation port may be seen midway between these.
Angioplasty and Seeding Procedure.

Female New Zealand white rabbits weighing between 2.9 and 4.4 kg (median weight 3.9 kg) were used throughout the experiments. Anaesthesia was induced with intravenous hypnoval (Roche Pharmaceuticals, Herts, UK) and maintained by an inhalational mixture of halothane (ICI Pharmaceuticals, Cheshire, UK) and oxygen. The right and left femoral arteries were exposed in entirety and ligated at the level of the knee joint. The inguinal ligament was divided and both iliac arteries visualised to the aortic bifurcation by a muscle cutting retroperitoneal approach. Prior to angioplasty 1000 units of unfractionated heparin (CP Pharmaceuticals, Wrexham, Clwyd, UK) was administered via an ear vein.

Following heparinisation a 3 mm balloon angioplasty catheter (2.0 cm length, Meadox, UK), was passed into the external iliac artery over a guide wire (Meadox, UK) introduced through a superficial femoral artery (SFA) arteriotomy. Bilateral external iliac artery angioplasties were performed under direct vision by inflating the angioplasty balloon to 8 atm pressure for 30 seconds on three separate occasions, with 1 minute between inflations. To ensure standard inflation parameters, a constant pressure syringe (Schneider Meditag AG, Zurich, Switzerland) was used (Fig 10.3). The lower end of the angioplasty site was marked with a 7/0 prolene (Ethicon Ltd, UK) adventitial suture to facilitate identification later in the experiment. The angioplasty catheter was then withdrawn and the double balloon seeding catheter introduced via the SFA arteriotomy. The seeding catheter was positioned at the level of the angioplasty site by visual reference to the marking suture.

Fig 10.3: Photograph illustrating constant pressure syringe.
Transluminal Endothelial Seeding

The isolation balloons on the seeding catheter were inflated to 2 atm pressure, which enabled the central portion of the angioplasty site to be isolated from the circulation. One angioplasty site was then seeded with indium-111 labelled endothelial cells (2x10^5 cells/ml), by instilling the endothelial cell suspension through the central instillation port of the seeding catheter. The cell suspension was infused until resistance to flow was felt (approximately 0.05 - 0.1ml), and at this point the instillation was stopped, the instillation channel closed by a three way tap, and the seeded volume recorded. A sample of the indium-111 labelled endothelial cell suspension was retained for determination of gamma activity later in the experiment.

The contralateral angioplasty site served as a control and was isolated from the circulation with a seeding catheter as described above. However, instead of being seeded with endothelial cells, the control site was sham seeded with MEM alone. Gamma activity was continuously monitored over the seeded and control angioplasty sites during the entire experimental procedure using an Isotope Localisation Monitor 235, fitted with a type 235 probe (DA Pitman Ltd, Weybridge, UK - Fig 8.5) which was fixed 2 cm above each angioplasty site.

The endothelial cell suspension was incubated at the angioplasty site for 30 min, after which the isolation balloons were deflated, the seeding catheter removed and the SFA ligated at least 5 cm from the angioplasty site, to ensure adequate run-off [Wilentz et al. 1987]. Deflation of the isolation balloons restored arterial blood flow through the angioplasty sites. The pre- and post-angioplasty arterial diameters were measured.

Sacrifice.

One hundred minutes following removal of the seeding catheter, an 18G cannula was sited in the infra-renal aorta and the animal sacrificed by barbiturate overdose (phenobarbitone, Rhone-Poulenc Rorer Ltd, Sussex, UK). The aorta and iliac vessels were rinsed with isotonic saline for 10 min, followed by perfusion fixation with 4% paraformaldehyde (BDH, Merck Ltd, Poole, UK) / 2% gluteraldehyde solution (Sigma, Poole, UK) at 70 mmHg for 20 min. The two angioplasty sites were identified by their dilated appearance and their relationship to the marking suture. Both angioplasty sites were then excised and carefully cleared of adventitia.

Endothelial Cell Retention.

Determination of endothelial cell retention was calculated from both the continuous gamma monitoring (dynamic measurement) during the course of the experiment, and the final radioactivity of the seeded and control angioplasty sites, which were referenced to the
activity of the endothelial cell suspension (static measurement).

**DYNAMIC MEASUREMENTS.**

Dynamic measurements gave an estimation of endothelial cell retention as a function of time, over the course of the experiment. The gamma activity of the seeded and control angioplasty sites was monitored for the duration of the experiment, and counts were corrected for spontaneous indium-111 leakage as has been described earlier (chapter 6). Endothelial cell retention, as a percentage of the initial cell inoculum, at a given time ($t^X$) was calculated from the following equation:

$$\% \text{cell retention } t^X = \frac{\text{cpm seeded } t^X - \text{cpm control } t^X}{\text{cpm seeded } t^0 - \text{cpm control } t^0} \times 100$$

where cpm $t^X$ represents corrected counts per minute over the seeded or control angioplasty sites at time $x$ and and cpm $t^0$ represents corrected counts per minute over the seeded or control angioplasty sites immediately after the endothelial cell suspension was instilled.

**STATIC MEASUREMENTS.**

At the termination of the experiment the gamma activity of the excised angioplasty sites was determined in a LKB-Wallac 1280 UltraGamma II counter (LKB-Produkter AB, Sweden). The gamma activity of an aliquot of the endothelial cell suspension was also determined at this time. Using these measurements the percentage of the initial inoculum retained at the termination of the experiment was given by the formula:

$$\% \text{cell retention} = \frac{\text{cpm seeded } - \text{cpm control}}{\text{cpm EC x VEC}} \times 100$$

where cpm seeded / control represents counts per minute of the seeded or control angioplasty sites; cpm$^{EC}$ represents counts per minute of 1 ml of the endothelial cell suspension and V$^{EC}$ represents the volume (ml) of the original endothelial cell instillate.
Transluminal Endothelial Seeding

**Histological Studies.**

After determination of radioactivity, the two angioplasty segments were opened longitudinally, pinned onto silicone gel and immersed in 4% paraformaldehyde (BDH, Merck Ltd, Poole, UK) / 2% gluteraldehyde (Sigma, Poole, UK) solution for 48 hours. The specimens were prepared for scanning electron microscopy (SEM) by dehydration through graded acetone solutions, prior to carbon dioxide critical point drying and sputter coating with gold. Sections were viewed on a dual stage scanning electron microscope (DS 130, International Scientific Instruments, Buxton, UK - chapter 6).

**Flow Rates During Angioplasty.**

In a separate experiment, the flow rates in dilated and control external iliac arteries were measured during the angioplasty procedure. One rabbit was anaesthetised as described earlier and both femoral and iliac arteries exposed by retroperitoneal dissection. Blood flow through each iliac artery was quantified using a Op Dop 130 (Sci Med, Bristol, UK) with an appropriately sized probe. One femoral artery was then ligated distally and a superficial femoral artery arteriotomy fashioned to allow passage of a guide wire. This artery was then dilated with a 3 mm balloon angioplasty catheter using the inflation pressures detailed before. Following angioplasty, the balloon catheter was removed and the superficial femoral artery ligated.

Throughout these procedures blood flow was measured in the angioplastied and contralateral arteries. The contralateral artery was not angioplastied or ligated during the experiment and acted as a control. The results are illustrated in Table 10.1.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Blood Flow-Angioplasty (ml/min)</th>
<th>Blood Flow-Control (ml/min)</th>
<th>Ratio Dilated/Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial exposure</td>
<td>30</td>
<td>32</td>
<td>0.94</td>
</tr>
<tr>
<td>Immediately post ligation</td>
<td>8</td>
<td>30</td>
<td>0.27</td>
</tr>
<tr>
<td>30 min post ligation</td>
<td>15</td>
<td>18</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Table 10.1: Table illustrating blood flow in dilated and control external iliac arteries.
**Statistical Analysis.**

Results of cell retention were expressed as a percentage of the initial cell inoculum retained at the angioplasty site and were presented as median values with 95% confidence intervals (95% CI). Non-parametric analysis was used for the comparison of static and dynamic cell retention, and also to compare pre- and post-angioplasty arterial measurements (Wilcoxon paired rank test).

**10.3 Results - Pilot Experiment.**

In the pilot experiment, the viability of HUVEC's was greater than 95% as assessed by trypan blue exclusion. The median labelling efficiency was 31% (95% CI 24%-45%).

The results of cell retention and attachment at the angioplasty site are illustrated in Fig 10.4. This graph is most conveniently analysed in three separate phases, during endothelial cell incubation, immediately after restoration of flow (shown as the dotted line), and during the subsequent exposure to arterial flow.

![Graph illustrating the percentage of initially instilled endothelial cells retained at the angioplasty site over the duration of the pilot experiment. Results are presented as median values with 95% confidence intervals of 9 replicate experiments. Cell loss during deflation of the isolation balloons on the seeding catheter is shown as the dotted line.](image-url)
During endothelial cell incubation the vast majority of the seeded cells were retained at the angioplasty site, with 74% of the cells retained after 30 min incubation. Immediately following deflation of the isolation balloons and restoration of flow, there was rapid cell loss as all the unattached cells were removed from the angioplasty site. The initial cell attachment after removal of these cells was 58%.

During exposure to arterial flow, cell loss was rapid over the initial 30 min, but after this time the rate of cell loss declined. This pilot experiment illustrated that endothelial cells could be delivered and retained at angioplasty sites using a double balloon catheter.

### 10.4 Results - Definitive Experiment.

**Labelling Efficiency, Cell Viability and Seeding Density.**

The median labelling efficiency was 27.0% (95% CI 14.9%-49.3%). Cell viability as assessed by trypan blue exclusion was greater than 95% in all experiments. The median seeding density was $1.58 \times 10^4$ cells/cm$^2$ (95% CI $1.29 \times 10^4$ to $2.02 \times 10^4$ cells/cm$^2$).

**Arterial Measurements.**

The pre and post-angioplasty diameters of the control and seeded angioplasty sites are illustrated in Fig 10.5. There was no significant difference in arterial diameter between seeded and control sites either before ($W=2.5$, 95% CI 0.05 to -0.15, $p = 0.225$), or after ($W=7.0$, 95% CI 0.2 to -0.2, $p = 1.0$ - Wilcoxon paired rank test) angioplasty.

![Graph (Fig 10.5)](image)

*Fig 10.5 : Graph illustrating pre- and post-angioplasty arterial diameters (mm). Results are median values with 95% confidence intervals.*
**Endothelial Cell Retention.**

**DYNAMIC MEASUREMENTS.**

The percentage of the initial cell inoculum retained at the angioplasty site over the duration of the experiment is illustrated in Fig 10.6. Again, the graph may be divided into three distinct phases; during endothelial cell incubation, immediately following restoration of flow, and during subsequent arterial perfusion. During the incubation period the isolation balloons on the seeding catheter remained inflated and cell leakage was minimal, with over 80% of the cells retained after 30 min. A large proportion of the cells were lost when the isolation balloons were deflated and flow was restored through the arterial segment (shown in the Fig 10.6 as a dotted line). Cell attachment immediately after restoration of flow was 49% (95% CI 35.0-60.0%). At the termination of the experiment, the median cell retention was 17% (95% CI 15.5-30.3%) of the original inoculum (which represented 35% of the initially attached cells).

![Graph](image)

**Fig 10.6 :** Graph illustrating the percentage of initially instilled endothelial cells retained at the angioplasty site during the definitive experiment. Results are presented as median values with 95% confidence intervals. Cell loss during deflation of the isolation balloons on the seeding catheter is shown as the dotted line.
To assess the rate of cell loss from the angioplasty site following restoration of arterial flow, linear regression analysis was used as has previously been described in chapter 8. Using this technique, two phases of cell loss were identified (Fig 10.7). Cell detachment during the first 30 min of perfusion was rapid (0.71% / min, p < 0.001), but declined after this time to much lower level (0.15 % / min, p = 0.22 - linear regression).

Fig 10.7 : Graph illustrating two phases of cellular loss from seeded angioplasty sites. Both graphs are fitted with best fit linear regression lines, whose equations are shown. The slope of the regression lines gives the rate of cell loss.

**STATIC MEASUREMENTS.**

Static measurements gave an estimation of the endothelial cell retention at the termination of the experiment i.e. after 100 min of arterial flow. The median cell retention calculated by this method was 18.8% (95% CI 10.5%-20.8%). There was no significant difference between the final cell retentions as calculated by dynamic and static methods (W = 6.0, 95% CI -12.4 to 3, p = 0.21 - Wilcoxon - Fig 10.8).

**Histological Examination.**

Seeded and control angioplasty sites were examined by scanning electron microscopy. Control sites showed complete endothelial desquamation with exposure of the sub-endothelial connective tissue and underlying smooth muscle cells (Fig 10.9).
Seeded angioplasty sites showed similar changes in the vessel wall, but in addition, seeded endothelial cells could be demonstrated to have spread and attached onto the damaged vascular surface (Fig 10.10).

![Graph illustrating comparison of static and dynamic methods for determining cell retention after 100 arterial flow. Median values are highlighted.](image)

**Fig 10.8:** Graph illustrating comparison of static and dynamic methods for determining cell retention after 100 arterial flow. Median values are highlighted.

![Scanning electron micrograph illustrating the appearance of a sham seeded (control) angioplasty site. There is total endothelial desquamation with exposure of the underlying sub-endothelial matrix, and scattered deposition of red blood cells. Magnification x 1020.](image)

**Fig 10.9:** Scanning electron micrograph illustrating the appearance of a sham seeded (control) angioplasty site. There is total endothelial desquamation with exposure of the underlying sub-endothelial matrix, and scattered deposition of red blood cells. Magnification x 1020.
Fig 10.10: Scanning electron micrograph illustrating the appearance of a seeded angioplasty site. The sub-endothelial matrix is exposed but three seeded endothelial cells are visualised to be spread on the arterial surface. One cell is rounded in appearance suggesting that attachment is incomplete. Magnification x 1230.

10.5 Discussion.

In the series of experiments detailed in this chapter, a method to transluminally seed experimental angioplasty sites with endothelial cells has been described. The pilot experiment confirmed that the method employed could deliver and retain endothelial cells at angioplasty sites. However, as the pilot series employed xenogeneic HUVEC's, the relevance of the seeding kinetics determined in this setting must be questioned and will not be discussed further.

Balloon dilatation was performed in non-hypercholesterolemic, non-atherosclerotic rabbit external iliac arteries using an oversized balloon with high inflation pressures, to maximise the degree of arterial trauma [Weidinger et al. 1990]. The presence of severe arterial damage was confirmed by post-angioplasty scanning electron micrographs which demonstrated complete loss of the endothelial monolayer, exposure of the sub-endothelial matrix and tears extending into the tunica media. Although this model does not accurately reproduce the conditions in human atherosclerotic arteries, the mechanism of angioplasty
is identical, and the post-angioplasty appearances provide a reasonable approximation of the severe arterial damage caused during PTA of an atheromatous plaque [Block et al. 1981; Bodrog et al. 1986; Losordo et al. 1992].

The major disadvantage of the angioplasty technique used in this study, was that the superficial femoral artery (SFA) had to be ligated following removal of the angioplasty and seeding catheters. This necessarily caused a decrease in blood flow through the iliac arteries, an effect which was minimised by performing the arterial ligation as distal as possible [Wilentz et al. 1987], to ensure adequate run-off through the profunda femoris and other thigh vessels. The effect of ligation on blood flow was assessed in one experiment and although blood flow decreased dramatically following ligation of the SFA, 30 min after ligation, flow had returned to 83% of the level recorded in the contralateral unligated iliac artery.

To analyse the initial retention and loss to flow of seeded endothelial cells, allogeneic rabbit endothelial cells were utilised. Allogeneic cells were used as they could be reliably harvested from cadaveric aortic segments and grown in tissue culture to provide a large number of cells (chapter 6). A method to isolate autogenous rabbit endothelium from internal jugular veins was developed for later use in this thesis, but suffered from a low primary success rate, with satisfactory cell harvest being obtained in only 40% of cases (chapter 6). Therefore, in experiments involving the study of short term endothelial cell retention, allogeneic cells were used to minimise the number of experimental procedures performed.

The possible risk of developing an immune mediated rejection response presents a theoretical objection to the use of allogeneic cell seeding. However, in separate experiments both Zamora et al. [Zamora et al. 1986], and Campbell et al. [Campbell et al. 1985] demonstrated that allogeneic cell seeding was not associated with a rejection response. Zamora et al. [Zamora et al. 1986] compared the long term retention of allogeneic endothelial cells to the retention of autogeneic cells onto ePTFE arteriovenous grafts in a canine model. It was demonstrated that there was no significant difference in retention between the two cell types, and in addition, light and electron microscopy failed to reveal any immunologic or inflammatory cellular reaction to allogeneic cell seeding. Campbell et al. [Campbell et al. 1985] compared autogeneic, allogeneic and xenogeneic endothelial cell retention onto canine carotid ePTFE grafts. All three cell types attached to the graft surface with the same efficiency and showed similar rates of cell detachment when exposed to flow. These findings suggested that the early loss of seeded endothelial cells from prosthetic graft surfaces was not due to an immunologic reaction.

The lack of a rejection response associated with allogeneic endothelial cell seeding may be related to the fact that less than 2% of cultured endothelial cells express HLA-D antigens [Nunez et al. 1983], and that growth in tissue culture appears to downregulate the
Transluminal Endothelial Seeding

immunogenicity of these cells [Dhesi, 1992]. This apparent tolerance to allogeneic cell seeding justifies the use of allogeneic cells in experiments evaluating the short term efficiency of transluminal endothelial cell seeding.

The seeding parameters used in this study were determined from the in vitro experiments described in chapter 7. An incubation time of 30 min was used as maximal cell attachment to damaged vascular surfaces was complete within this time, and it was considered that the period of arterial occlusion should be as short as possible. In order to measure the maximal percentage cellular attachment to the angioplasty site, a sub-confluent seeding density of $1.58 \times 10^4$ cells/cm$^2$ was utilised. In later experiments, where maximal cellular coverage will be required, a much higher supra-confluent seeding density will be employed.

In this experiment, cell retention at the angioplasty site was calculated by two methods. The dynamic measurements which relied upon a portable gamma monitor were considered to be potentially inaccurate by virtue of the in vivo experimental setting; whereas the static measurements were performed on ex vivo tissue and were considered to be the more accurate. However, there was no difference between the final cell retention calculated by the two methods and this validated the use of dynamic measurements throughout the experiment.

Cultured endothelial cells were delivered to the post-angioplasty luminal surface by means of a specially designed seeding catheter which consisted of two low pressure isolation balloons and a central instillation port. Inflation of the isolation balloons created a protected space within the arterial lumen, into which endothelial cells could be instilled. The use of a double balloon catheter for endovascular cellular transplantation was initially described in 1989 by Nabel et al. [Nabel et al. 1989], who introduced porcine endothelial cells, expressing recombinant β-galactosidase into deendothelialised arterial segments of Yucan minipigs. Double balloon catheters may potentially be used for the local delivery of any therapeutic agent, and recently Jorgensen et al. [Jorgensen et al. 1989; Jorgensen et al. 1990] infused tPA into a segmentally enclosed arterial site, with a consequent reduction in the acute rethrombosis rate following angioplasty for femoro-popliteal occlusion. Further research into local administration of therapeutic agents following endovascular procedures has led to the development of a perforated angioplasty catheter, which may be used to inject heparin directly into the arterial wall [Wolinsky, Thung, 1990].

The 4F double balloon catheter used in this experiment was very efficient in delivering seeded endothelial cells to the angioplasty site, and retaining the cells in the enclosed arterial segment throughout the incubation period. Over 80% of the initially instilled endothelial cells were retained after 30 min incubation.

Immediately after deflation of the isolation balloons, all unattached cells will be removed by the restored blood flow, and the percentage cell retention at this time
Transluminal Endothelial Seeding

represents the initial cellular attachment to the angioplasty site after the 30 min incubation period. In this experiment initial attachment was 49%. This level of attachment is substantially higher than the maximal cell attachment of HUVEC's to native vascular surfaces described in chapter 7, and is also higher than the attachment of vascular endothelial cells to uncoated prosthetic vascular grafts [Budd et al.1989; Vohra et al.1991; Kent et al.1988]. In their pioneering work using a double balloon catheter to transplant genetically modified endothelial cells onto denuded arterial segments, Nabel et al. [Nabel et al.1989] reported that only 2-11% of initially instilled endothelial cells successfully attached to the arterial wall after a 30 min incubation period. The difference between this level of cell retention and the 49% reported in this chapter may be explained by the use of a high seeding density (2x10^6 cells/cm^2) by Nabel et al., which increased the degree of cellular coverage but which also reduced the percentage cell attachment. The degree of attachment of allogeneic rabbit endothelial cells to in vivo angioplasty sites described in this chapter was encouraging, and suggested a high affinity of attachment of the seeded endothelial cells for the post-angioplasty vascular surface.

The rate of cell loss from the angioplasty sites during exposure to arterial perfusion was initially rapid for the first 30 min of flow, but after this time decreased to a lesser level. This biphasic pattern of cell loss was also observed during the loss of seeded endothelial cells from damaged vascular surfaces exposed to physiologic levels of shear stress in vitro (chapter 8). The rates of cell detachment in both in vivo and in vitro experiments are almost identical, and also compare to the rate of cell loss from prosthetic vascular grafts [Rosenman et al.1985b]. The biphasic pattern of cell detachment is attributed to the initial loss of poorly attached, rounded endothelial cells [Vohra et al.1990b], followed by the slower detachment of cells damaged during the seeding procedure [Rosenman et al.1985b]. Towards the termination of the experiment, a plateau phase was reached where the cells appeared to be stably attached to the underlying surface and cell loss was minimal. This finding was confirmed by the appearance of the seeded angioplasty sites on scanning electron microscopy, which revealed the seeded endothelial cells to be spread on the damaged arterial tissue. Spreading of endothelial cells is a sign of stable attachment, and in this condition the cells are able to resist high shear stresses.

The successful retention of seeded endothelial cells on native vascular surfaces has been described by several other investigators. Nabel et al. [Nabel et al.1989] reported that 20-100% of initially attached endothelial cells remained adherent to a denuded arterial segment 2-4 weeks following transluminal seeding. Sterpetti et al. [Sterpetti et al.1992] studied the effect of endothelial seeding on carotid endarterectomy sites in a canine model and demonstrated that 10% of the seeded endothelial cells remained attached to the endarterectomised surface after 24h of flow.

In this experiment the long term retention of seeded endothelial cells has not been
measured due to the inaccuracies of indium-111 labelling over longer time periods. However, the results from this series of experiments suggest that a proportion of the seeded endothelial cells are stably retained at the angioplasty site, and due to their morphological appearance could be expected to remain attached. The long term effects of endothelial seeding on reendothelialisation following angioplasty will be investigated further in chapter 12.

10.6 Summary

In this series of experiments, the use of a double balloon isolation catheter to transluminally seed angioplasty sites with allogeneic endothelial cells has been described. The seeding catheter was efficient at delivering and retaining endothelial cells at the angioplasty site during cell incubation. Following exposure to arterial flow the initial cell attachment was 49% which represented a high affinity of attachment between the seeded endothelial cells and the damaged vascular tissue. Cell loss to arterial flow was biphasic, but 100 min after restoration of arterial flow, a stable population of endothelial cells was firmly attached and spread onto the angioplasty site. In the subsequent two chapters, this method of endothelial cell seeding will be used to investigate the effects of rapid reendothelialisation on acute and chronic occlusive complications following angioplasty.
CHAPTER 11
THE EFFECT OF TRANSLUMINAL ENDOTHELIAL SEEDING
ON PLATELET DEPOSITION FOLLOWING ANGIOPLASTY

11.1 Introduction. 159

11.2 Methods. 160
- Isolation and culture of endothelial cells 160
- Cell delivery system 160
- Angioplasty and seeding procedure 160
- Sacrifice 162
- Platelet labelling and determination of platelet deposition 162
- Histological studies 163
- Statistical analysis 163

11.3 Results. 164
- Cell viability and seeding density 164
- Arterial diameters 164
- Platelet labelling 164
- Platelet deposition 165
- Scanning electron micrographs 167

11.4 Discussion. 169

11.5 Summary. 172
Platelet Deposition Following Angioplasty

THE EFFECT OF TRANSLUMINAL ENDOTHELIAL SEEDING ON PLATELET DEPOSITION FOLLOWING ANGIOPLASTY

11.1 Introduction.

The rate of acute arterial reocclusion following lower limb angioplasty is approximately 4% (Table 5.1), but may occasionally reach 40% in high risk cases [Jorgensen et al. 1990]. Acute vessel closure is a significant cause of morbidity and mortality complicating both peripheral and coronary angioplasty, and occurs in some instances despite the presence of anticoagulant and anti-platelet agents [Dorros et al. 1983]. Early vessel closure has been attributed to acute arterial thrombosis at the site of balloon dilatation, in association with angioplasty induced vasospasm [Simpfendorfer et al. 1987; Waller et al. 1984; Ellis et al. 1988]. The occurrence of both causative mechanisms may be directly related to the vascular injury resulting from balloon dilatation of an atheromatous plaque, with the consequent formation of a thrombogenic flow surface.

Damage to the superficial and deeper layers of the arterial wall during PTA exposes the sub-endothelial matrix to the circulating blood, with the subsequent activation of platelets [Mustard et al. 1987] and the coagulation cascade [Stern et al. 1988], which act synergistically to form a mural thrombus on the damaged arterial surface [Uchida et al. 1989]. The associated reduction in arterial diameter may be exacerbated by vasospasm, which is produced by a combination of platelet derived vasoactive mediators [Lam et al. 1987] and the direct effect of arterial dilation on the vessel wall [Fischell et al. 1989]. If these changes lead to turbulent blood flow, intravascular thrombosis may ensue, with eventual arterial occlusion [Mustard et al. 1990].

The adhesion and aggregation of platelets on the damaged post-angioplasty surface plays a pivotal role in initiating acute arterial occlusion. Aspirin induced inhibition of platelet function has been shown to decrease the acute post-dilation coronary occlusion rate, whilst the converse also holds true, namely that elevated platelet counts are associated with increased rates of coronary artery rethrombosis [Barnathan et al. 1987]. Any technique that decreases platelet deposition on damaged vascular surfaces, should therefore reduce the incidence of abrupt vessel closure following angioplasty.

In the previous chapter, it was established that endothelial cells could be transluminally delivered and retained at angioplasty sites using a double balloon catheter. Endothelial seeding of angioplasty sites should theoretically re-establish the endothelial monolayer denuded during balloon dilatation, and consequently reduce platelet deposition, by forming a physical barrier between the sub-endothelium and the bloodstream, as well as providing a source of powerful anti-thrombogenic mediators. The experiment described in this chapter was designed to test the hypothesis that rapid restoration of the endothelial
Platelet Deposition Following Angioplasty

cell monolayer, achieved by transluminal endothelial seeding, could decrease acute platelet
deposition following angioplasty.

11.2 Methods.

Isolation and Culture of Endothelial Cells.

Allogeneic rabbit endothelial cells were harvested from segments of cadaveric
rabbit aortas and grown in tissue culture as described in chapter 6. Endothelial cell identity
was again confirmed by appearance at phase contrast microscopy and positive
immunohistochemical staining for an anti-rabbit endothelial, anti-thrombomodulin
antibody (QB-END 40, Serotec, Oxford, UK). In this study, large numbers of cells were
required to attain a high seeding density and so cells from the fourth to sixth passage were
used.

Prior to seeding, cells were released from tissue culture and resuspended in
complete culture medium at a concentration of $3 \times 10^5$ cells/ml. Cell viability was
determined at this time by trypan blue exclusion.

Cell Delivery System.

A double balloon catheter was used to deliver and retain allogeneic endothelial cells
at the angioplasty site. The catheter design was modified slightly in this experiment, with
the distance between the isolation balloons being increased to 2.4 cm. This modification
ensured that the whole of the angioplasty site could be isolated from the circulation and
incubated with the seeded cells.

Angioplasty and Seeding Procedure.

Eight female New Zealand White rabbits (median weight 3.62 kg, range 3.1-4.2
kg) were anaesthetised with intravenous hypnoval (Roche Pharmaceuticals, Herts, UK)
and inhalational halothane (ICI Pharmaceuticals, Cheshire, UK). The left and right
femoral arteries were exposed and their diameters measured. Prior to angioplasty 1000
units of unfractionated heparin (CP Pharmaceuticals, Wrexham, Clwyd, UK) and a
suspension of autologous platelets labelled with indium-111 oxine were infused via a
central line inserted through the left internal jugular vein. A 3 mm diameter, 2.0 cm length
low profile angioplasty catheter (Olbert catheter, Meadox, UK) was introduced into the
external iliac artery over a guide wire (Meadox, UK) passed though a superficial femoral
artery (SFA) arteriotomy. Bilateral external iliac artery angioplasties were performed by
inflating the angioplasty balloon to 8 atmospheres (atm) pressure for 30 sec, on 3 separate occasions. The lower limit of the angioplasty site was marked with a 7/0 prolene adventitial suture (Ethicon Ltd, UK), to facilitate identification at the termination of the experiment.

The angioplasty catheter was then withdrawn and the seeding catheter (Meadox, UK; Ideas for Medicine Inc, Tampa, USA) introduced via the SFA arteriotomy. The seeding catheter was positioned to enclose the angioplasty site and the isolation balloons inflated to 2 atm pressure. One angioplasty site, chosen at random, was incubated with endothelial cells whilst the contralateral site was treated in exactly the same manner, except that acellular culture medium was infused in place of the endothelial cell suspension.

The seeding procedure in this experiment was modified to take into account the increased distance between the isolation balloons and the need to achieve a high seeding density. The seeding process comprised two sequential phases; an initial endothelial cell inoculum of $4.5 \times 10^5$ cells in 0.15 ml complete culture medium, which was followed by a continuous intra-segment infusion (1.7 ml/h - Syringe Driver, Graseby Medical, UK - Fig 11.1) of endothelial cells at a concentration of $3 \times 10^6$ cells/ml. It was anticipated that the continuous infusion of cells would compensate for any cell loss due to leakage around the isolation balloons or into small arterial branches, and thus ensure a supra-confluent seeding density.

![Fig 11.1](image.jpg) : Photograph illustrating the syringe driver used to infuse endothelial cells into the isolated arterial segment.
Platelet Deposition Following Angioplasty

The incubation period lasted 30 min, after which time flow was restored by deflating the isolation balloons and removing the seeding catheter. The superficial femoral artery was ligated and the post-angioplasty arterial diameters measured.

Sacrifice.

Thirty minutes after removal of the seeding catheter, the infra-renal aorta was exposed and cannulated with a 18G intra-arterial cannula. Following sacrifice by barbiturate overdose (phenobarbitone, Rhone-Poulenc Rorer Ltd, Sussex, UK) the infra-renal aorta and distal vascular tree were rinsed with isotonic saline for 10 min, followed by perfusion fixation with 4% paraformaldehyde (BDH, Merck Ltd, Poole, UK) / 2% gluteraldehyde solution (Sigma Chemicals, Poole, UK) at a pressure of 70 mmHg, for 20 min duration. The aorta and iliac arteries were exposed and their lengths measured prior to en bloc excision. The entire arterial specimen was then excised and pinned onto a silicone gel plate, with care taken to replicate the in situ arterial lengths. The arteries were covered with 4% paraformaldehyde / 2% gluteraldehyde solution and carefully cleared of adventitia.

Platelet Labelling and Determination of Platelet Deposition.

Two hours prior to angioplasty, rabbits were anaesthetised, and the right femoral vein cannulated with a 20G cannula. Twenty-six milliliters of blood were withdrawn, the platelets separated, and labelled with indium-111 oxine as described in chapter 6. A median of 7.0 (95% CI : 5.7 - 13.2 MBq) MBq of indium-111 oxine was used in each labelling procedure. Autogenous labelled platelets were then re-injected into each appropriate rabbit immediately before angioplasty.

Just prior to sacrifice a 5 ml blood sample was obtained from a direct aortic puncture. The platelet concentration in this sample was determined in a haemocytometer and the indium-111 activity of a 1 ml aliquot measured in a gamma cell counter (LKB-Wallac 1280 UltraGamma II counter - LKB-Produkter AB, Sweden). From these measurements the gamma activity of a known quantity of platelets was determined. Estimation of platelet deposition on the seeded and sham-seeded angioplasty sites was performed by two methods, radionucleotide scanning and direct platelet quantification.

Radionucleotide Scanning.

The intact arterial specimen which consisted of the infra-renal aorta and both iliac arteries pinned onto a silicone gel plate, was imaged for 15 min using a Siemens ZLC 37 tube.
Platelet Deposition Following Angioplasty

gamma camera with a 6 mm pin hole collimator. After capture and analysis of the image, identical areas of interest were drawn around each angioplasty site, and the counts per pixel determined. These were then expressed as a ratio of the counts determined over the sham seeded angioplasty site divided by the counts determined over the seeded site.

DIRECT PLATELET QUANTIFICATION.

Following gamma imaging, the two angioplasty sites and a segment of undamaged aorta were excised from the en bloc arterial specimen and opened longitudinally. The angioplasty sites were identified by their characteristic dilated appearance and by their relationship to the marking suture. Each specimen was measured to allow calculation of the surface area, and the indium-111 activity of all samples was then determined in a gamma well counter (LKB-Wallac 1280 UltraGamma II counter - LKB-Produkter AB, Sweden). By referencing the gamma activity of the arterial samples to the gamma activity of the platelets in the pre-sacrifice blood sample, the number of platelets deposited per unit area of each arterial sample was calculated from the following equation:

\[
\text{Platelet deposition (platelets/mm}^2) = \frac{\text{cpm}_{\text{ART}}}{\text{SA}_{\text{ART}} \times \text{cpm}_{\text{PLT}}}
\]

where cpm\textsubscript{ART} represents the gamma activity of the arterial samples, SA\textsubscript{ART} represents the surface area of the arterial samples and cpm\textsubscript{PLT} represents the gamma activity of a single platelet as determined from the pre-sacrifice blood sample.

HISTOLOGICAL STUDIES.

After determination of platelet deposition, the seeded and sham seeded angioplasty sites, and the segment of undamaged aorta were pinned onto silicone gel and fixed in 4% paraformaldehyde / 2% gluteraldehyde solution for 48 h. These specimens were then prepared for scanning electron microscopy as described before.

STATISTICAL ANALYSIS.

Results for continuous variables are presented as median values with 95% confidence intervals (95% CI). Statistical analysis utilises non-parametric tests throughout; the Kruskal-Wallis one way analysis of variance was used to test for differences in the extent of platelet deposition in the seeded, sham seeded and undamaged control arteries.
Specific differences between these groups in both platelet deposition and arterial diameter were analysed using the Wilcoxon paired rank test.

11.3 Results.

Cell Viability and Seeding Density.

Endothelial cell viability as assessed by trypan blue exclusion was greater than 95% in all experiments. The initial seeding density was $1.95 \times 10^6$ cells/cm$^2$ ($1.9 - 2.1 \times 10^6$ cell/cm$^2$).

Arterial Diameters.

The pre- and post-angioplasty arterial diameters for the seeded and non-seeded angioplasty sites are illustrated in Table 11.1. There was no difference between endothelial seeded and sham seeded sites in either pre- ($w=2.0$, 95% CI = 0.1 to -0.15, p=0.79 - Wilcoxon) or post-angioplasty diameter ($w=18.5$, 95% CI = 0.2 to -0.1, p=0.5 - Wilcoxon). Balloon angioplasty significantly increased the arterial diameters at both endothelial seeded and sham seeded sites ($w=36$, 95% CI = 1.0-1.4, p=0.014 - endothelial seeded, $w=36$, 95% CI = 0.95-1.3, p=0.014 - sham seeded - Wilcoxon paired rank test)

<table>
<thead>
<tr>
<th></th>
<th>Pre Angioplasty Diameter (mm)</th>
<th>Post Angioplasty Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial seeded</td>
<td>$1.2$ ($1.0-1.4$)</td>
<td>$2.5$ ($2.3-2.5$)</td>
</tr>
<tr>
<td>Sham seeded</td>
<td>$1.3$ ($1.1-1.4$)</td>
<td>$2.5$ ($2.3-2.6$)</td>
</tr>
</tbody>
</table>

Table 11.1 Table illustrating the arterial diameters of endothelial seeded and sham seeded angioplasty sites. Values are median arterial diameters in mm with 95% confidence intervals in parentheses.

Platelet Labelling.

Platelets obtained from 26 ml of blood were labelled with a median of 7.0 MBq of indium-111 oxine (95% CI: 5.7 - 11.1 MBq). The median labelling efficiency was 89% (95% CI: 77% - 92%).

The median platelet count determined from the pre-sacrifice blood sample was
Platelet Deposition Following Angioplasty

$1.55 \times 10^8$ platelets/ml ($1.08 \times 10^8 - 1.99 \times 10^8$ platelets/ml)

**Platelet Deposition.**

RADIONUCLEOTIDE SCANNING.

Satisfactory gamma images were only acquired in 4 of 8 cases (50%) (Fig 11.2 and 11.3). In all 4 unsatisfactory scans, images were not acquired due to insufficient radioactivity of the arterial samples. The median sham seeded to endothelial seeded ratio in the 4 satisfactory scans was 1.41 (range: 0.92 - 13.2), indicating that endothelial cell seeding reduced platelet deposition following angioplasty. The numbers of satisfactory scans however, were too small for reliable evaluation of this result.

Fig 11.2: Radionucleotide scan of an intact arterial segment. A = aorta, S = seeded angioplasty site, P = sham seeded angioplasty site. This scan gave a sham seeded / endothelial seeded ratio of 13.2.

Fig 11.3: Radionucleotide scan of an intact arterial segment. A = aorta, S = seeded angioplasty site, P = sham seeded angioplasty site. This scan gave a sham seeded / endothelial seeded ratio of 1.35.
Platelet Deposition Following Angioplasty

DIRECT PLATELET QUANTIFICATION.

The number of platelets deposited per unit area on the endothelial seeded, sham seeded and undamaged arterial segments are tabulated in Table 11.2 for each individual experiment, and illustrated in Fig 11.4. For each group a platelet index was calculated by dividing the platelet deposition on the angioplasty site, by the platelet count on the corresponding undamaged control artery. This index gave an estimation of the relative thrombogenicity of the angioplasty sites with reference to normal artery. Analysis of platelet deposition within all three groups revealed a significant difference between the groups ($H=10.04$, $p=0.007$ - Kruskal-Wallis). The number of platelets deposited on the sham seeded angioplasty site was significantly higher than those deposited on normal artery ($w=36$, $95\%$ CI = 0.30 to 1.39, $p=0.014$ - Wilcoxon). Endothelial seeding significantly reduced platelet deposition on the angioplasty site (endothelial seeded vs sham seeded angioplasty sites, $w=36$, $95\%$ CI = 0.36 to 0.75, $p=0.014$ - Wilcoxon) to levels not significantly different from normal artery (endothelial seeded angioplasty site vs normal artery, $w=19$, $95\%$ CI = 0.66 to -0.27, $p=0.94$ - Wilcoxon).

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Platelet Deposition (Platelets/mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Seeded Site</td>
</tr>
<tr>
<td>1</td>
<td>$16.1 \times 10^4$</td>
</tr>
<tr>
<td>2</td>
<td>$8.3 \times 10^4$</td>
</tr>
<tr>
<td>3</td>
<td>$2.4 \times 10^4$</td>
</tr>
<tr>
<td>4</td>
<td>$3.2 \times 10^4$</td>
</tr>
<tr>
<td>5</td>
<td>$4.0 \times 10^4$</td>
</tr>
<tr>
<td>6</td>
<td>$1.9 \times 10^4$</td>
</tr>
<tr>
<td>7</td>
<td>$7.5 \times 10^4$</td>
</tr>
<tr>
<td>8</td>
<td>$16.0 \times 10^4$</td>
</tr>
</tbody>
</table>

Median: $3.6 \times 10^4$ (1.85 - 16.1$\times 10^4$) $13.1 \times 10^4$ (5.5 - 42.7$\times 10^4$) $3.4 \times 10^4$ (1.5 - 5.1$\times 10^4$)

Platelet Index: 0.91 (0.47 - 7.0) 3.74 (1.44 - 18.7) 1

Table 11.2: Table illustrating platelet deposition on the arterial samples in each of the 8 experiments. The median values with 95% confidence intervals in parentheses are also tabulated, as are the platelet indices.
This reduction in platelet deposition is clearly illustrated in the platelet indices. Platelet deposition on the sham seeded angioplasty site was 3.74 higher than on undamaged aorta, whilst deposition on the seeded angioplasty sites was actually lower than on control segments. The difference between sham seeded and endothelial seeded angioplasty sites was statistically significant (w=36, 95% CI = 1.3 to 14.2, p = 0.014).

![Graph illustrating platelet deposition on control, sham seeded and endothelial seeded angioplasty sites.](image)

**Fig 11.4** : Graph illustrating platelet deposition on control, sham seeded and endothelial seeded angioplasty sites. Results are presented as medians with 95% confidence intervals of the logarithmic transformation of the data.

**Scanning Electron Micrographs.**

Scanning electron microscopy confirmed that the aortic segments used as negative controls (i.e. undamaged artery) had a confluent endothelial cell monolayer (Fig 11.5). The sham seeded angioplasty site showed complete endothelial desquamation with exposure of the sub-endothelial connective tissue and consequent platelet deposition (Fig 11.6). The endothelial seeded angioplasty site showed similar morphological appearances, but in addition, seeded endothelial cells could be visualised (Fig 11.7), and platelet deposition was minimal.
Fig 11.5: Scanning electron micrograph illustrating the appearance of a control segment of aorta. The endothelial monolayer is intact and there is no platelet or fibrin deposition. Magnification x 563.

Fig 11.6: Scanning electron micrograph illustrating the appearance of a sham seeded angioplasty site. The endothelium has been denuded and the sub-endothelial matrix is exposed. There is widespread deposition of platelets and fibrin on the damaged arterial surface. Magnification x 1050.
Fig 11.7: Scanning electron micrograph illustrating the appearance of an endothelial seeded angioplasty site. Although the sub-endothelial matrix is exposed over much of the arterial surface, spread endothelial cells are seen on the damaged surface. There is no platelet or fibrin deposition on the arterial surface. Magnification x 1320.

11.4 Discussion.

In this study, the method of transluminal endothelial seeding, as described in chapter 10, was modified in an attempt to maximise endothelial coverage of the angioplasty site. The distance between the two isolation balloons on the seeding catheter was increased so that the entire angioplasty site could be isolated from the circulation and incubated with seeded cells. The increased length of the isolated segment however, also enhanced the probability of cellular loss from leakage into small arterial branches. This theoretical effect was minimised by adopting a two stage seeding procedure in which the initial endothelial cell instillate was followed by a continuous intra-segment infusion, to replenish any lost cells. In order to obtain the maximal cellular attachment on the damaged vascular surface, a supra-confluent seeding density of $1.95 \times 10^5$ cells/cm$^2$ was utilised. This seeding density exceeds the minimum number of cells required to attain confluence ($3.2 \times 10^5$ cells/cm$^2$), as determined in chapter 7. Allogeneic rabbit endothelial cells were again used in this study, as the risk of rejection within the 1 hour time course of the experiment was very low [Zamora et al. 1986; Campbell et al. 1985].
Platelet Deposition Following Angioplasty

To determine the effect of transluminal endothelial seeding on platelet deposition following balloon dilatation, an experimental model of angioplasty was used. Previous studies in similar models have demonstrated that activated platelets are deposited at sites of balloon dilatation [Badimon et al. 1988; Miller et al. 1991]. The extent of platelet adhesion to the angioplasty site is determined by local haemodynamic conditions [Heras et al. 1988], and by the extent of arterial injury [Lam et al. 1987; Wilentz et al. 1987]; severe injury resulting in greater platelet deposition. The model used in this experiment has previously been demonstrated to produce the pathophysiological changes of severe arterial injury [Weidinger et al. 1990], a finding that is confirmed in this study as the sham seeded angioplasty sites exhibited complete endothelial denudation with exposure of the sub-endothelial and medial tissues.

In this study, platelet deposition on endothelial and sham seeded angioplasty sites was compared to platelet deposition on undamaged aorta. Platelet deposition was quantified 30 min following restoration of arterial flow through the dilated arterial segment. Previous studies have demonstrated that platelet deposition following angioplasty is virtually complete within 30 minutes, at which time the dilated surface is completely covered with a platelet monolayer [Groves et al. 1979; Wilentz et al. 1987; Jorgensen et al. 1988a; Jorgensen et al. 1988b]. Platelet uptake was measured by two methods, radionucleotide scanning and direct calculation of platelet deposition. Unfortunately, satisfactory gamma scintillation scans were only acquired in 50% of cases, making estimation of platelet deposition by this method unreliable. The failure of radionucleotide scans to satisfactorily image platelet deposition at sites of acute arterial injury has previously been demonstrated in a study by Finklestein et al. [Finklestein et al. 1982], in which only 56% of the lesions caused by severe arterial injury were detected.

Direct calculation of the number of platelets deposited per unit area on the three arterial samples was performed by a method described by Plate et al. [Plate et al. 1989]. Using this technique, it was demonstrated that platelet deposition on sham seeded angioplasty sites was significantly higher than deposition on the control aortic segments, confirming increased platelet deposition after balloon dilatation. The absolute quantification of platelet deposition in this study is similar to that reported by Plate et al. [Plate et al. 1989] who measured platelet uptake on angioplasty (2.2x10^5 platelets / mm^2) and undamaged control sites (3.6x10^4 platelets / mm^2), 2 hours after balloon dilatation of atherosclerotic rabbit iliac arteries.

The most significant finding from this study was that transluminal endothelial seeding significantly reduced post-angioplasty platelet deposition, to a level that was indistinguishable from that of undamaged arterial segments. These results represent a considerable success of seeding native vascular surfaces, and suggest that
thrombogenicity following angioplasty may be abolished by endothelial cell transplantation.

The results of direct platelet quantification were qualitatively confirmed by the appearances of the arterial samples during scanning electron microscopy. An intact endothelial cell monolayer was demonstrated in all the undamaged aortic segments, which validated the use of these samples as a negative control. By contrast, the sham seeded angioplasty sites exhibited the characteristic changes of severe arterial injury and were almost completely covered with platelet and fibrin thrombus. Seeded endothelial cells were spread onto the damaged vascular surface of the endothelial seeded sites, which were almost completely devoid of platelets, thus confirming the reduction in platelet deposition associated with endothelial seeding.

The decrease in platelet deposition produced by endothelial seeding is not completely attributed to restoration of the mechanical endothelial cell barrier between the blood and the sub-endothelial matrix, as 100% endothelial coverage was not observed. It is likely that high levels of endothelial derived anti-platelet agents will contribute to the anti-thrombotic effect, as it has been previously shown in chapter 9 that seeded endothelial cells secrete high levels of PGI$_2$ following attachment to native vascular surfaces.

Endothelial seeding of native vascular surfaces has previously been utilised as a technique to reduce platelet deposition on the highly thrombogenic flow surface produced during endarterectomy. Schneider et al. [Schneider et al.1990] seeded endarterectomised baboon aortic segments with $6 \times 10^5$ baboon endothelial cells/cm$^2$, prior to determining platelet deposition in an in vivo femoro-femoral arteriovenous shunt. Platelet deposition on the unseeded endarterectomised segments, was $2.8 \times 10^9$ platelets/cm$^2$ after 1 hour of flow. This level of deposition was significantly reduced to $0.32 \times 10^9$ platelets/cm$^2$ by endothelial cell seeding, which again compared favourably with the $0.24 \times 10^9$ platelets/cm$^2$ deposited on control non-endarterectomised vessels. In a similar study, Krupski et al. [Krupski et al.1990] demonstrated that human adult endothelial cells seeded onto endarterectomised baboon aorta, could significantly reduce platelet deposition on the endarterectomised segments following implantation into exteriorised arteriovenous shunts. Using an entirely different model, Sterpetti et al. [Sterpetti et al.1992] reported a significant reduction in the thrombogenicity of canine carotid intimecomy sites after supra-confluent endothelial cell seeding. All of these experiments demonstrate that endothelial seeding of damaged native vascular surfaces may reduce thrombogenicity. The findings of Schneider et al. [Schneider et al.1990] are similar to those reported in this chapter, in that endothelial seeding actually abolished thrombogenicity following iatrogenic arterial injury.
11.5 Summary.

In this study, rapid partial restoration of the endothelial cell monolayer, achieved by endothelial seeding, has been shown to abolish the increased platelet deposition associated with balloon angioplasty. Platelets play a crucial role in initiating acute vessel closure, and a reduction in platelet deposition could be expected to decrease the incidence of acute arterial reclosure complicating balloon dilatation. In addition to the effect on acute occlusion, a reduction in platelet deposition may decrease chronic restenosis due to myointimal hyperplasia, as platelets and platelet derived growth factors are important in stimulating migration of smooth muscle cells into the arterial intima [Fingerle et al. 1989]. An investigation into the effect of transluminal endothelial seeding on myointimal hyperplasia will be described in the following chapter.
CHAPTER 12
THE EFFECT OF TRANSLUMINAL ENDOTHELIAL SEEDING ON MYOINTIMAL HYPERPLASIA FOLLOWING ANGIOPLASTY

12.1 Introduction.
174
12.2 Methods.
175
- Isolation and culture of endothelial cells
- Angioplasty and seeding procedure
- Sacrifice
- Preparation of samples for histological studies
- Histology of arterial lesions
- Quantification of myointimal hyperplasia
- Quantification of smooth muscle proliferation
- Degree of reendothelialisation
- Statistical analysis
176
177
178
178
179
179
179
180
181
185
186
186
188
179
180
181
185
186
186
188
12.3 Results.
- Cell viability and seeding density
- Arterial diameters
- Ranges of agreement
- Degree of intimal hyperplasia
- Degree of medial thickening
- Arterial patency rates
- Degree of reendothelialisation
- Smooth muscle cell proliferation
179
179
180
181
185
186
186
188
12.4 Discussion.
190
12.5 Summary.
194
THE EFFECT OF TRANSLUMINAL ENDOTHELIAL SEEDING ON MYOINTIMAL HYPERPLASIA FOLLOWING ANGIOPLASTY

12.1 Introduction.

Restenosis following balloon dilatation occurs within 6 months of the procedure [Serruys et al. 1988], and may complicate PTA in up to 40% of cases [Califf et al. 1991; Holmes et al. 1984; Ernst et al. 1987; McBride et al. 1988; Gershlick, de Bono, 1990]. The development of chronic restenosis is a major problem after both peripheral and coronary angioplasty, and is the limiting factor that affects long term patency. At present, no therapeutic or mechanical technique has been clinically proven to reduce the incidence of restenosis.

Chronic restenosis is due to the formation of myointimal hyperplasia at the angioplasty site [Waller et al. 1991b; Waller et al. 1991a; Waller et al. 1990]. Myointimal hyperplasia is a pathologic process characterised by SMC proliferation and migration, which leads to the formation of a thickened fibrocellular layer between the vessel lumen and the internal elastic lamina. This process is observed after all forms of vascular reconstruction, and represents a manifestation of the generalised wound healing response [Clowes, Reidy, 1991].

Although myointimal hyperplasia is probably initiated by mechanical damage to medial SMCs, the endothelium plays a crucial regulatory role in this process (chapter 5). In the quiescent state, the endothelium secretes heparin glycosaminoglycans which inhibit SMC proliferation and migration [Clowes, Clowes, 1985; Majesky et al. 1987; Clowes, Clowes, 1986]. Loss of the endothelial monolayer during balloon dilatation removes the tonic inhibitory effect of the intimal layer, and may also stimulate the production of mitogenic, growth-stimulatory mediators from replicating endothelial cells at the edge of the angioplasty site [Koo, Gotlieb, 1991]. In addition to these direct effects, endothelial damage may stimulate the generation of myointimal hyperplasia by facilitating platelet deposition on the exposed sub-endothelium, with the subsequent release of mitogenic growth factors.

The original hypothesis of this thesis was that rapid restoration of the endothelial cell monolayer following angioplasty would decrease the incidence of restenosis by providing a functioning source of anti-mitogenic mediators and restoring the permeability barrier between the arterial wall and the bloodstream. In chapter 11, it was demonstrated that transluminal endothelial seeding reduced the extent of acute platelet deposition following balloon dilatation. In this chapter, the effect of endothelial seeding on post-angioplasty myointimal hyperplasia will be investigated.
12.2 Methods.

Isolation and Culture of Endothelial Cells.

Four to six weeks prior to angioplasty, rabbits were anaesthetised and the right internal jugular vein removed. This vein was used as the tissue source for autogenous endothelial cell harvest, which was performed as described in chapter 6. From 30 attempted harvests, adequate primary cell cultures were only obtained in 12 cases (40%), although all satisfactory primary cultures were subsequently successfully grown through at least 4 cell passages. As in the previous two experiments, endothelial cell identity was confirmed by appearance at phase contrast microscopy and positive immunohistochemical staining for an anti-rabbit endothelial, anti-thrombomodulin antibody (QB-END 40, Serotec, Oxford, UK). Failure of primary culture in all 18 cases was due to an insufficient number of cells obtained during primary harvest.

Successfully harvested cells were grown in tissue culture until $6 \times 10^6$ cells were realised, which required 4 - 6 cell passages. When the required number of cells was achieved, cells were released from culture and resuspended in complete culture medium at a concentration of $3 \times 10^6$ cells / ml. Cell viability was determined by trypan blue exclusion prior to seeding.

Angioplasty and Seeding Procedure.

Bilateral external iliac angioplasties were performed in 12 New Zealand White rabbits using the technique described in chapter 11. Each animal received 1000 units of unfractionated heparin (CP Pharmaceuticals, Wrexham, Clwyd, UK) prior to the experimental procedure, and the lower limit of the angioplasty site was marked with a 7/0 prolene adventitial suture (Ethicon Ltd, UK). Following bilateral angioplasty, two seeding catheters were introduced via the right and left SFA arteriotomies and positioned at each angioplasty site. The isolation balloons on the seeding catheters were inflated to isolate both sites from the circulation. One angioplasty site, chosen at random was seeded with autogenous endothelial cells, whilst the contralateral site was sham seeded with acellular complete culture medium.

In an attempt to ensure maximal endothelial coverage of the angioplasty site, a two stage seeding procedure was used. An initial endothelial cell inoculum of $4.5 \times 10^5$ cells in 0.15 ml complete culture medium was instilled into the enclosed arterial segment, which was then followed by a continuous intra-segment infusion (1.7 ml / h - Syringe Driver, Graseby Medical, UK - Fig 11.1) of endothelial cells at a concentration of $3 \times 10^6$ cells / ml. The endothelial cells were incubated with the dilated arterial segment for 30 min, after
which time the isolation balloons were deflated and the seeding catheter removed.
Following removal of the seeding catheter, the superficial femoral artery was ligated
proximal to the SFA arteriotomy site, and the skin closed. The animal was allowed to
recover.

Sacrifice.

Following angioplasty and seeding, rabbits were maintained on normal rabbit
chow and unlimited water for either 1 or 3 weeks. One rabbit suffered a femoral nerve
injury following angioplasty and was excluded from the study. Five rabbits were
sacrificed one week post-angioplasty and six at three weeks. Immediately before sacrifice
rabbits were anaesthetised and the infra-renal aorta cannulated with an 18G cannula. The
iliac arteries were inspected and their patency determined by palpation. Rabbits were then
sacrificed with an overdose of phenobarbitone (Rhone-Poulenc Rorer Ltd, Sussex, UK),
and the infra-renal vessels fixed by perfusion with isotonic saline for 10 min, followed by
4% paraformaldehyde (BDH, Merck Ltd, Poole, UK) at a pressure of 70 mmHg, for 20
min duration. Gluteraldehyde was not used for perfusion fixation in this experiment as it
may interfere with in situ hybridisation techniques.

The angioplasty sites were identified by their characteristic dilated appearance and
reference to the marking suture. Both angioplasty sites were excised, cleared of adventitial
tissue, and immediately placed in 4% paraformaldehyde (BDH, Merck Ltd, Poole, UK)
prior to processing.

Preparation of Samples for Histological Studies.

Each angioplasty site measured 2 cm in length. The arterial segment was sectioned
transversely to produce 2 pieces; the proximal segment measured 1.5 cm in length and
was stored in 4% paraformaldehyde for light microscopic studies; the distal segment
measured 0.5 cm in length and was stored in 4% paraformaldehyde / 2% gluteraldehyde
solution (Sigma Chemicals, Poole, UK) for electron microscopy. Specimens were
prepared for light and scanning electron microscopy as detailed in chapter 6.

Histology of Arterial Lesions.

Characterisation of the morphology of the arterial lesions was achieved by light
microscopic examination of serial sections stained with haematoxylin and eosin, elastic
van Gieson's stain (EVG), alcian blue, periodic-acid Schiff (PAS), and
immunohistochemistry for smooth muscle actin.
Quantification of Myointimal Hyperplasia.

For each angioplasty site, two 4 μm sections, at least 1 cm apart were stained with EVG and viewed at x10 magnification on a Kontron Videoplan image analysis system (Kontron Electronics, Watford, Herts, UK), for planimetric determination of the degree of myointimal hyperplasia and medial thickening. Three parameters were measured on each section; the luminal area, the area enclosed by the internal elastic lamina (IEL), and the area enclosed by the external elastic lamina (EEL) (Fig 12.1). The area of myointimal hyperplasia was derived by subtracting the luminal area from the area enclosed by the IEL. The degree of myointimal hyperplasia for each section was expressed as a ratio of the hyperplastic area (IH), to the normalised area enclosed by the internal elastic lamina (IH / IEL) [Colburn et al.1992]. The IH / IEL ratio for each angioplasty site was calculated as the mean of the ratios from the two sections.

Fig 12.1 : Diagrammatic representation of an artery exhibiting myointimal hyperplasia, viewed in cross section. The measurements used to determine the degree of myointimal hyperplasia are illustrated.

Planimetry was performed by two independent observers (MM Thompson and MJ Underwood), blinded of the sample type. The inter-observer ranges of agreement were determined, and the final IH / IEL ratio for each angioplasty site was calculated as the mean of the ratios measured by the two observers. Additionally, one observer (MM Thompson), repeated all arterial measurements to allow calculation of the intra-observer ranges of agreement. The degree of medial thickening was also determined by calculating
Myointimal Hyperplasia Following Angioplasty

a media to external elastic lamina ratio.

Quantification of Smooth Muscle Proliferation.

The extent of smooth muscle cell proliferation in the intima and media of the arterial sections was determined by estimating the proportion of cells in S phase of the cell cycle. This was accomplished by two methods, immunohistochemistry for proliferating cell nuclear antigen, and \textit{in situ} hybridisation for histone genes H2b, H3 and H4 (Appendix B). Both methods identified smooth muscle cells undergoing DNA synthesis.

PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA).

Four serial sections were taken from each angioplasty site. Two sections were treated with a monoclonal antibody directed against PCNA (PC 10 - Dako Limited, Glostrup, Denmark) as described in Appendix B, and one section was used as a negative control. The fourth section was stained with haematoxylin and eosin (H and E). All sections were viewed at x75 magnification, and the number of positively staining cells in one high power field determined for both the intima and media, in four distinct sites around the arterial circumference (superior, inferior, medial and lateral). The total number of intimal and medial smooth muscle cells in equivalent high powered fields were then determined using the H and E stained serial sections.

The extent of smooth muscle proliferation was expressed as a percentage of the positively staining cells to the total number of cells. The percentage proliferation for both media and intima was calculated from an average of the proliferation indices at the four separate locations.

EXPRESSION OF HISTONE GENES.

Four serial sections were cut from each angioplasty site. Three sections were used for \textit{in situ} hybridisation of histone mRNA, as described in Appendix B; and the final section stained with H and E. The extent of SMC proliferation was again quantified as a percentage of positively staining cells to the total number of cells in four separate regions of the arterial section. Proliferation indices for the intima and media were separately calculated as an average of the four regional values.

Degree of Reendothelialisation.

A segment from each of the seeded and sham seeded angioplasty sites was
prepared for scanning electron microscopy, and examined using a dual stage scanning electron microscope (DS 130, International Scientific Instruments, Buxton, UK). Eight sites were chosen at random from each of the specimens, and these sites photographed at x1000 magnification. The resulting micrographs were qualitatively examined to determine the degree of endothelial cell coverage. The extent of reendothelialisation was calculated as an average of the eight representative micrographs.

**Statistical Analysis.**

Results for continuous variables are presented as median values with 95% confidence intervals (95% CI). Statistical analysis utilises non-parametric tests throughout. Paired samples were analysed by the Wilcoxon paired rank test, and non-paired samples by the Mann Whitney test. In cases where multiple comparisons were made, the data were initially analysed by the Kruskal-Wallis one way analysis of variance.

12.3 Results.

**Cell Viability and Seeding Density.**

Endothelial viability was greater than 95% in all experiments as assessed by trypan blue exclusion. The seeding density employed was 1.9x10^6 cells/cm^2 (95% CI 1.76 - 2.0x10^6 cells/cm^2).

**Arterial Diameters.**

The pre- and post-angioplasty arterial diameters for the seeded and sham seeded angioplasty sites are illustrated in Table 12.1. There was no difference between endothelial seeded and sham seeded sites in either pre- (w=22, 95% CI = 0.35 to -0.10, p=0.62 - Wilcoxon) or post-angioplasty diameter (w=32.55, 95% CI = 0.2 to -0.05, p=0.26 - Wilcoxon). Balloon angioplasty significantly increased the arterial diameters at both endothelial seeded and sham seeded sites (w=66, 95% CI = 1.0-1.2, p=0.004 - endothelial seeded, w=66, 95% CI = 1.0-1.25, p=0.004 - sham seeded- Wilcoxon paired rank test).
Table 12.1 Table illustrating the arterial diameters of endothelial seeded and sham seeded angioplasty sites. Values are median arterial diameters in mm, with 95% confidence intervals in parentheses.

**Ranges of Agreement.**

To assess the degree of consistency in quantifying the degree of myointimal hyperplasia (IH / IEL) both between observers and within an individual observer, intra- and inter-observer ranges of agreement were calculated. Inter-observer ranges of agreement were measured by plotting the difference between each of the observers' IH / IEL ratios (%) against the corresponding mean for each sample [Altman, Bland, 1983; Brennan, Silman, 1992] (Fig 12.2)

![Graph plotting the difference in IH / IEL (%) between two observers (MMT and MJU), against the mean IH / IEL ratio.](image)

The differences between the two observers were between +8% and -4%, and the
level of precision was not related to the degree of myointimal hyperplasia, i.e. high IH / IEL ratios did not result in larger inter-observer differences. The 95% range of agreement between the observers was -4.4% to +7%. A measure of inter-observer bias was calculated by determining the mean difference between the observers, based on the sample studied. The 95% confidence interval for this value was -0.05 to 2.65. As zero lies outside this interval it may be assumed that there was no bias between the two observers.

The Bland - Altman plot for intra-observer ranges of agreement is illustrated in Fig 12.3.

![Bland-Altman Plot](image_url)

**Fig 12.3 :** Graph plotting the difference in IH / IEL (%) between two individual measurements made by the same observer (MMT), at different times.

The 95% range of agreement was -2.8% to +3.1%. The 95% confidence interval for the mean intra-observer difference was -0.54 to 0.85, which implies no intra-observer bias.

**Degree of Myointimal hyperplasia.**

Intimal lesions produced after angioplasty consisted of smooth muscle cells in a surrounding extracellular matrix, as identified by staining with alcian blue, periodic-acid Schiff (PAS), and immunohistochemistry for smooth muscle actin (Figs 12.4 and 12.5). The degree of myointimal hyperplasia for each section was expressed as a ratio of the hyperplastic area (IH), to the area enclosed by the internal elastic lamina (IH/IEL).
Myointimal Hyperplasia Following Angioplasty

Fig 12.4: Photomicrograph illustrating the appearance of myointimal hyperplasia in a sham seeded angioplasty site. The internal elastic lamina can be seen separating the intima and media. Haematoxylin and eosin stain. Magnification x 25.

Fig 12.5: Photomicrograph illustrating an intimal lesion stained for smooth muscle actin. Numerous smooth muscle cells are identified in the intima. Immunohistochemistry for smooth muscle actin. Magnification x 25.
The degree of myointimal hyperplasia was compared between endothelial seeded and sham seeded angioplasty sites, 1 and 3 weeks following balloon dilatation (Fig 12.6, Table 12.2). Analysis of the \( \text{IH} / \text{IEL} \) ratio between both groups over the two time periods revealed significant differences within the data (\( H = 14.8, p = 0.002 \)- Kruskal-Wallis). At both sites, the degree of myointimal hyperplasia increased significantly from 1 to 3 weeks after injury (\( W = 30.0, 95\% \text{ Cl} \ 0.15 \) to 0.40, \( p = 0.037 \)- sham seeded; \( W = 51, 95\% \text{ CI} \ 0.076 \) to 0.23, \( p = 0.0075 \)- endothelial seeded- Mann Whitney test).

Fig 12.6: Graph illustrating the degree of myointimal hyperplasia in endothelial seeded and sham seeded angioplasty sites. Values are medians with 95% confidence intervals.

At both time periods, endothelial seeding was associated with a lesser degree of myointimal hyperplasia than observed at the sham seeded angioplasty sites. The differences in the \( \text{IH} / \text{IEL} \) ratio were not significant at independent time periods due to low numbers in each group (\( W = 0.0, 95\% \text{ CI} \text{ insufficient numbers, } p = 0.37 \)- 1 week; \( W = 0.0, 95\% \text{ CI} \ -0.022 \) to 0.15, \( p = 0.059 \)- 3 weeks - Wilcoxon paired rank test). However, over the entire duration of the experiment, endothelial seeding significantly
Myointimal Hyperplasia Following Angioplasty

reduced the degree of myointimal hyperplasia following angioplasty (W = 28, 95% CI 0.015 to 0.115, p = 0.022 - Wilcoxon paired rank test). This reduction is illustrated by the appearance of the neointimal lesions in the endothelial seeded and sham seeded angioplasty sites (Figs 12.7 and 12.8). Sham seeded angioplasty sites were characterised by concentric intimal lesions, whereas endothelial seeded sites generally exhibited minimal intimal proliferation around the majority of their circumference, with one area of localised thickening.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Duration (Weeks)</th>
<th>IML/IEL (%)</th>
<th>Media/IEL (%)</th>
<th>Endothelial Regeneration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Seed</td>
<td>Sham</td>
<td>Seed</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>4.0</td>
<td>5.5</td>
<td>11.3</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0</td>
<td>Th</td>
<td>10.1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>10.5</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>4.4</td>
<td>8.9</td>
<td>13.0</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>0</td>
<td>Th</td>
<td>19.2</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>12.5</td>
<td>27.6</td>
<td>25.6</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>8.0</td>
<td>Th</td>
<td>21.0</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>17.2</td>
<td>19.4</td>
<td>12.7</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>11.6</td>
<td>19.5</td>
<td>13.0</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>27.1</td>
<td>40.2</td>
<td>18.8</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>16.9</td>
<td>22.9</td>
<td>12.4</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td>8.1</td>
<td>19.5</td>
<td>13.0</td>
</tr>
</tbody>
</table>

Table 12.2: Table illustrating IML/IEL ratio, Media/IEL ratio, and the degree of endothelial regeneration in the 11 individual experiments. Median values with 95% confidence intervals in parentheses are also tabulated. Th = thrombosed artery.
Myointimal Hyperplasia Following Angioplasty

Fig 12.7: Photomicrograph illustrating a sham seeded angioplasty site 3 weeks following balloon dilatation. Elastic van Gieson stain. Magnification x 5.

Fig 12.8: Photograph illustrating an endothelial seeded angioplasty site 3 weeks following balloon dilatation. Elastic van Gieson stain. Magnification x 5.

Degree of Medial Thickening.

The degree of medial thickening was quantified and expressed as a media/EEL ratio (Fig 12.9, Table 12.2). There was no difference in the degree of medial thickening.
between the endothelial seeded and sham seeded groups over the duration of the experiment ($W = 16$, 95% CI -0.03 to 0.027, $p = 0.83$ - Wilcoxon).

**Fig 12.9**: Graph illustrating the degree of medial thickening in endothelial seeded and sham seeded angioplasty sites. Values are medians with 95% confidence intervals.

**Arterial Patency Rates.**

Three out of eleven sham seeded angioplasty sites were occluded at the time of harvest (1 at 3 weeks, and 2 at 1 week). All endothelial seeded sites were patent. The difference in patency between endothelial seeded (100%) and sham seeded angioplasty sites (73%) is statistically significant (95% CI between proportions 1.0 to 53.6 - confidence interval analysis).

**Degree of Reendothelialisation.**

The degree of reendothelialisation was compared between the endothelial seeded and sham seeded sites, 1 and 3 weeks following angioplasty (Fig 12.10, 12.11 and 12.12, Table 12.2). Analysis of variance between the control and treated groups illustrated significant differences within the data ($H = 7.79$, $p = 0.005$ - Kruskal-Wallis). At both time periods, endothelial seeding increased the degree of endothelial coverage of the angioplasty sites, and although this difference was significant over the duration of the experiment ($W = 52.5$, 95% CI 10.5 to 62.5, $p = 0.013$ - Wilcoxon), at 1 week there was no statistical difference between endothelial seeded and sham seeded angioplasty sites ($W$
Myointimal Hyperplasia Following Angioplasty

= 7.5, 95% CI -12 to 70, p = 0.47 - 1 week, W = 21, 95% CI 30.5 to 78.5, p = 0.036 - 3 weeks - Wilcoxon).

Fig 12.10: Graph illustrating the degree of reendothelialisation in endothelial seeded and sham seeded angioplasty sites. Values are medians with 95% confidence intervals.

Fig 12.11 Scanning electron micrograph of a sham seeded angioplasty site, 3 weeks after balloon dilatation. The endothelial coverage was estimated at 40%. Magnification x1000.
Fig 12.12 Scanning electron micrograph of an endothelial seeded angioplasty site, 3 weeks after balloon dilatation. The endothelial coverage was estimated at 90%.

**Magnification x1000.**

**Smooth Muscle Cell Proliferation.**

Smooth muscle cell proliferation was not determined in the thrombosed samples, as these had been subjected to a pathological process which may have affected the rate of SMC replication. Two samples from the 1 week group and one sample from the 3 week group were thus excluded from analysis. This left only 8 paired samples and due to the low number, results of cell replication from both experimental time periods have been presented together, with analysis being performed by the Wilcoxon paired rank test.

**PCNA Immunohistochemistry.**

The degree of intimal and medial cell proliferation as determined by proliferating cell nuclear antigen immunohistochemistry (Fig 12.13) is illustrated in Table12.3. There was no significant difference in the degree of SMC proliferation between endothelial seeded and sham seeded groups in either media or intima (W = 8, 95% CI -7.4 to 16.1, p = 0.35 - intima, W = 23, 95% CI -1.4 to 10.1, p = 0.15 - media).
Myointimal Hyperplasia Following Angioplasty

<table>
<thead>
<tr>
<th></th>
<th>Intima</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial Seeded</td>
<td>17.2 (6.7-30.7)</td>
<td>10.5 (3.0-14.0)</td>
</tr>
<tr>
<td>Sham Seeded</td>
<td>10.2 (4.5-35.2)</td>
<td>15.0 (1.7-17.8)</td>
</tr>
</tbody>
</table>

Table 12.3: Table illustrating the percentage of replicating intimal and medial SMC, determined by PCNA immunohistochemistry. Results are presented as median values with 95% confidence intervals in parentheses.

Fig 12.13: Light micrograph illustrating PCNA immunohistochemistry. Replicating cells are stained brown. Magnification x 75.

Expression of Histone Genes.

Similarly, the degree of SMC replication determined by in situ hybridisation for histone mRNA (Fig 12.14) is illustrated in Table 12.4. The proportion of SMC's synthesising DNA was higher in the sham seeded group, but this difference only reached statistical significance for medial cells (23.0 = 8, 95% CI -0.2 to 3.9, p = 0.15 - intima, W = 27, 95% CI 0.1 to 4.3, p = 0.035 - media).
Myointimal Hyperplasia Following Angioplasty

<table>
<thead>
<tr>
<th>Endothelial Seeded</th>
<th>Proliferating SMC (%)</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intima</td>
<td></td>
</tr>
<tr>
<td>Endothelial Seeded</td>
<td>2.75 (0-5.3)</td>
<td>2.5 (1.3-5.4)</td>
</tr>
<tr>
<td>Sham Seeded</td>
<td>5.5 (2.0-26.8)</td>
<td>4.1 (1.0-8.8)</td>
</tr>
</tbody>
</table>

Table 12.4: Table illustrating the percentage of replicating intimal and medial SMC, determined by *in situ* hybridisation for histone genes. Results are presented as median values with 95% confidence intervals in parentheses.

Fig 12.14: Light micrograph illustrating *in situ* hybridisation for histone mRNA. Replicating cells are stained blue. Magnification x 75.

12.4 Discussion.

Throughout the previous experiments described in this thesis, indium-111 labelling has been used to study *in vivo* and *in vitro* endothelial cell retention to seeded native vascular surfaces. Although this methodology gives a good estimation of endothelial retention in the short term, long term adherence cannot be calculated due to the short half life of the isotope and spontaneous intracellular leakage. In the experiments described in this chapter, the degree of reendothelialisation 1 and 3 weeks following
angioplasty has been determined for both endothelial seeded and sham seeded angioplasty sites. Determination of the degree of endothelial regeneration does not allow direct calculation of long term cell retention as endothelial cell replication will occur on the arterial surface. However, this study did permit the effect of endothelial seeding on restoration of the endothelial monolayer to be determined.

The experimental model of angioplasty utilised in this investigation causes severe mechanical damage to the arterial wall [Weidinger et al. 1990], with complete endothelial desquamation (chapters 10 and 11, Figs 10.9 and 11.6). In this study endothelial regeneration on the control (sham seeded) angioplasty sites was limited, with a median endothelial coverage of 0% and 8% at 1 and 3 weeks respectively. The inability of rabbit endothelial cells to repopulate denuded vascular surfaces has previously been described by Reidy et al. [Reidy et al. 1983], who investigated endothelial regeneration following balloon deendothelialisation of rabbit carotid arteries. Following balloon catheterisation endothelial regrowth progressed from the wound edges, but stopped spontaneously after 2 weeks, leaving a large denuded area. Similar findings were reported by Mattson et al. [Mattsson et al. 1992], who demonstrated that rabbit iliac angioplasty sites remained devoid of endothelium 4 weeks after balloon dilatation.

The degree of endothelial regeneration following angioplasty was significantly increased by supra-confluent endothelial cell seeding, to 12% after 1 week and 78% at 3 weeks. This degree of regeneration suggests that even with the use of supra-confluent seeding densities (1.9x10^6 cells/cm^2), it was not possible to achieve confluent coverage of the angioplasty site within a 30 min incubation period. This may be explained by the seeding kinetics determined in chapter 10, as it was demonstrated that although 35% of the initially adherent cells were retained at the angioplasty site 100 min after restoration of flow, there was a still a slow loss of cells from the seeded surface at this time point. However, even though confluent coverage was not immediately achieved, 3 weeks after angioplasty the seeded surfaces were 78% healed compared to the 8% healing achieved in control arteries. This confirms the ability of transluminal endothelial seeding to enhance restoration of the endothelial monolayer following angioplasty.

The mechanism by which endothelial cell seeding restores endothelial continuity cannot be determined from this experiment. It is likely that the seeded endothelial cells will replicate on the damaged vascular surface more readily than on prosthetic vascular grafts, as the post-angioplasty surface should provide an optimal substrate for endothelial regeneration. It is also possible that the seeded cells stimulate growth of the host endothelium through the secretion of mitogenic growth factors [Hussain et al. 1989; Wakefield et al. 1988].

The principle aim of this study was to investigate the effect of immediate endothelial seeding on the degree of myointimal hyperplasia following experimental angioplasty.
Myointimal Hyperplasia Following Angioplasty

balloon angioplasty. The extent of intimal thickening was quantified by determining the IH/IEL ratio. This ratio represents the proportion of the area enclosed by the internal elastic lamina which is occupied by neointima, and has previously been shown to be a satisfactory method of quantifying restenosis following balloon dilatation [Colburn et al., 1992]. The intra- and inter-observer limits of agreement were calculated for this measurement, and there was no bias either between observers or within individual observers.

Analysis of myointimal hyperplasia revealed that the degree of intimal thickening increased with time, and that transluminal endothelial seeding reduced the extent of luminal narrowing at both 1 and 3 week time periods. The morphological pattern of myointimal hyperplasia was also markedly different between treated and control groups. In the sham seeded angioplasty sites (Fig 12.7), the neointima formed a concentric ring on the luminal side of the internal elastic lamina. In the endothelial seeded arteries (Fig 12.8) however, the intimal thickening was usually reduced over two thirds of the arterial circumference, with an intimal plaque over the remaining third. It is possible that gravitational effects may explain this pattern, as seeded endothelial cells may preferentially attach to the bottom and sides of the arterial wall, leaving the superior aspect relatively devoid of cells.

As SMC proliferation is a principle component of the arterial response to injury, the effect of endothelial seeding on the vascular SMC replication rate was investigated. Proliferation was quantified by two methods, in situ hybridisation for histone gene mRNA, and immunohistochemistry for proliferating cell nuclear antigen (PCNA - appendix B). Smooth muscle cell proliferation was not quantified in the thrombosed samples, as these had been subjected to a pathological process which might have affected cell replication. As all these samples were in the sham seeded group, any variation in the cell proliferation rate would have biased the results. Due to the low number of patent samples at the two time periods, results have been presented from both time periods together, with a paired statistical analysis.

The rate of SMC proliferation determined by PCNA immunohistochemistry was consistently higher than that measured by histone gene expression. This may be attributable to the expression of PCNA, which is detected in G1 and G2 [Bravo, MacDonald-Bravo, 1987], as well as S phase of the cell cycle. In addition, it has been suggested that as PCNA is involved in DNA repair [Lee, Hurwitz, 1990], damaged cells may also stain positively for this antigen and the rate of cell proliferation may be erroneously high. In this study, there was no significant difference between treated and control sites in medial or intimal SMC replication rate, when assessed by PCNA staining.

Quantification of histone gene expression provides a highly accurate estimation of cells actively synthesising DNA. Using this methodology, the rate of SMC proliferation in
Myointimal Hyperplasia Following Angioplasty

both media and intima was lower in endothelial seeded than in sham seeded angioplasty sites, although the difference only reached statistical significance for medial cells. Taken together, these results suggest that transluminal endothelial seeding may decrease SMC replication after arterial injury, but clearly more experiments will be needed to confirm this effect.

The reduction in intimal thickening associated with endothelial seeding is possibly the most significant finding reported in this thesis, as it indicates that endothelial transplantation has the potential to decrease restenosis following angioplasty. In addition to this clinical application, these data provide a further insight into the role of the vascular endothelium following arterial injury. As was discussed in detail in chapter 5, the endothelium has the ability to promote or inhibit myointimal hyperplasia through the production of soluble mediators. Following arterial injury, activated, replicating endothelial cells produce mitogenic growth factors that stimulate SMC proliferation and migration [Herring, 1991a; Koo, Gotlieb, 1991; Williams, 1991]. Mitogenic factors are produced until spontaneous endothelial replication stops or when endothelial continuity is restored. At this time, the quiescent endothelial cells produce EDRF and heparin proteoglycans [Weidinger, 1991; Castellot et al. 1981], which inhibit SMC growth. It is postulated that endothelial seeding reduces the time taken to restore endothelial continuity, either through direct replication of the seeded cells or by stimulating growth of the host endothelium. Acceleration of endothelial repair will decrease the duration of growth factor production, and consequently shorten the time span between injury and restoration of the the endothelial monolayer with its associated tonic inhibitory influence on SMC replication. Alteration of the balance between growth factor / growth inhibitor secretion may provide one mechanism by which endothelial seeding decreases the hyperplastic response to injury. As well as affecting growth factor production, endothelial seeding abolishes platelet deposition following angioplasty (chapter 11). This action will also act to decrease myointimal hyperplasia through reduction of the SMC migration that accompanies platelet deposition [Fingerle et al. 1989; Friedman et al. 1977; Moore et al. 1976].

The beneficial effect of endothelial seeding after iatrogenic arterial injury has been demonstrated in one previous study. Bush et al. [Bush et al. 1987] quantified myointimal hyperplasia after endothelial seeding of carotid endarterectomy sites in a canine model. In this model, the degree of intimal thickening was significantly reduced in the endothelial seeded site, which also demonstrated accelerated endothelial healing.

The two major factors that limit the long term success of PTA are acute arterial reocclusion and chronic restenosis. The initial aim of this thesis was to investigate a method to reduce the incidence of occlusive complications following angioplasty. In chapter 11, it was demonstrated that endothelial seeding abolished platelet deposition after
Myointimal Hyperplasia Following Angioplasty

balloon dilatation, and in this chapter it has been observed that endothelial transplantation reduced the degree of myointimal hyperplasia associated with arterial injury. The influence of endothelial seeding on both these parameters would be expected to have an effect on long term arterial patency, and in this study, the patency of endothelial seeded angioplasty sites was significantly higher than non seeded sites.

12.5 Summary.

This final experiment has demonstrated that immediate transluminal seeding of angioplasty sites results in accelerated endothelial healing of the denuded vascular surface. Rapid reendothelialisation is associated with a reduction in the degree of myointimal hyperplasia and an increased patency rate.
CHAPTER 13
CONCLUSIONS AND PROSPECTS FOR FUTURE RESEARCH

Prospects For Future Research

Conclusions and Prospects for Future Research 196
CONCLUSIONS AND PROSPECTS FOR FUTURE RESEARCH

During the past decade there has been a trend towards endovascular, catheter based interventions in the treatment of chronic arterial insufficiency. The most widely accepted of these "minimally invasive" techniques is percutaneous transluminal balloon angioplasty, which is now an established treatment option in both peripheral and coronary arterial occlusive disease. Unfortunately, balloon dilation of an atheromatous plaque causes severe mechanical damage to the arterial wall, which is manifest in endothelial denudation, intimal dissection and damage to the medial smooth muscle cells. In the acute phase, loss of the endothelial monolayer results in the formation of a thrombogenic flow surface which may initiate acute arterial recocclusion, a process which complicates PTA in approximately 5% of cases. In the longer term, the generalised wound healing response to arterial injury, causes chronic restenosis, through the formation of myointimal hyperplasia. Chronic restenosis may complicate balloon dilatation in up to 40% of patients, and is the major factor that limits the long term efficacy of this technique.

The administration of anti-platelet and anticoagulatory agents has been successful in decreasing the rate of abrupt arterial closure following angioplasty, but to date, no pharmacological or mechanical technique has had any proven benefit in reducing the incidence of chronic restenosis. The central proposition throughout this thesis, was that mechanical damage to the endothelial monolayer plays a significant role in the development of acute arterial occlusion and chronic restenosis following angioplasty. It was hypothesised that rapid restoration of the endothelial cell monolayer, achieved by transluminal endothelial seeding, could have the potential to decrease both early and late occlusive complications following balloon dilatation of an atheromatous plaque. This hypothesis was partially derived from previous work on the seeding of prosthetic vascular grafts, which demonstrated that endothelial seeding increased healing, decreased thrombogenicity, and increased the patency of prosthetic arterial conduits.

Up until the present time, there has been little research into the effect of seeding native vascular surfaces. Lessons learnt from experience with prosthetic grafts, indicated that the success of any seeding procedure may be determined by the seeding parameters used. The initial investigation into the possibility of seeding angioplasty sites, was thus to determine the optimum conditions for endothelial attachment to the native vascular substrate. By developing an in vitro model of vascular damage, it was possible to define optimum seeding times and densities for use in later in vivo investigations.

Having established these parameters, the feasibility of seeding native vascular surfaces relied upon two attributes; the ability of the seeded cells to resist the shear stresses of the circulation, and their ability to function normally following the seeding process. In chapter 8, it was demonstrated that seeded endothelial cells could resist in vitro
Prospects For Future Research

exposure to physiologic shear stress, with adequate cell retention on the damaged vascular surface. Endothelial function after seeding was quantified by measuring PGI₂ production from seeded damaged vascular surfaces. The PGI₂ synthetic capacity of these surfaces was significantly greater than that of unseeded controls, which suggested that the transplanted cells retained their anti-thrombotic functions during the seeding procedure.

Favourable results from the in vitro studies, suggested that endothelial seeding of angioplasty sites should be investigated in vivo. A non-atherosclerotic rabbit model was used throughout the in vivo studies. Initially, a pilot experiment was performed to assess the ability of a specially designed double balloon isolation catheter, to transluminally deliver endothelial cells to experimental angioplasty sites. This catheter was extremely efficient at retaining endothelial cells in contact with the damaged vascular tissue during balloon inflation. Subsequent experiments demonstrated that transluminally seeded endothelial cells could adhere to angioplasty sites within a 30 min incubation period, and resist the shear stress imposed during restoration of arterial flow. Cell loss from the angioplasty site was rapid during the initial 30 min of arterial flow, but after this period, the rate of cell loss declined, and a proportion of the cells became stably attached to the angioplasty site.

After establishing a successful method to transluminally seed angioplasty sites with endothelial cells, this technique was used to determine the effect of endothelial seeding on acute thrombogenicity and chronic restenosis following balloon dilatation. Using a supra-confluent density of seeded endothelial cells to maximise endothelial coverage, it was revealed in chapter 11 that transluminal seeding abolished the increased platelet deposition associated with balloon angioplasty. Platelet deposition is the pivotal event in the development of acute arterial occlusion, and it may be predicted that techniques that decrease platelet deposition after angioplasty, will also decrease the incidence of abrupt vessel closure.

The long term effects of endothelial seeding were investigated in chapter 12. As expected, seeding of the angioplasty sites dramatically increased the rate of endothelial regeneration over the damaged vascular surface. This increased degree of endothelial continuity was associated with a marked reduction in the extent of post-angioplasty myointimal hyperplasia, and perhaps more importantly with a significant increase in the arterial patency rate.

In conclusion, it can be stated that transluminal endothelial seeding of angioplasty sites can abolish thrombogenicity and improve long term patency in an animal model. On the basis of these results, it is tempting to suggest that this technique may be used therapeutically to reduce occlusive complications following PTA. However, previous experience with animal models has shown that data cannot be directly extrapolated from animal to clinical trials. Numerous pharmacologic agents which abolish experimentally
Prospects For Future Research

induced myointimal hyperplasia, have had no effect on human restenosis rates. In many cases, the failure of clinical trials was due to uncertainty regarding the dosage and duration of drug administration, but there still exists a fundamental difference between the clinical and experimental situation.

One of the advantages of using a cell based therapy, rather than a pharmacological approach to restenosis, is that the cellular response to arterial injury in man is similar to that observed in animals. The aim of endothelial seeding is to accelerate the process of reendothelialisation, and it may be that the effects of cellular healing in man are similar to animal models. However, even this point is uncertain, as the early promise of seeded prosthetic grafts in animals was not fulfilled in clinical trials, until the different characteristics of human endothelial behaviour were recognised.

Before transluminal endothelial seeding can be used clinically, further research is needed to answer several crucial questions. The most important of these, is the ability of seeded cells to adhere to the surface produced by dilation of an atheromatous plaque. Clearly, cells can attach to the sub-endothelial matrix following injury of normal vessels, but adherence to disrupted atheroma has not been defined. This must be investigated both in vitro and in vivo to determine the optimum seeding parameters for this surface. It is possible that adherence to atheroma may not be necessary, as most stenotic plaques are eccentric, with up to two thirds of the vessel wall being unaffected by atheroma. Cell adherence to the non-atherosclerotic wall may be effective in preventing occlusive complications.

One of the crucial differences between experimental models and the clinical setting is the presence of continued atherogenic stimulation in man. This has important sequelae for endothelial seeding as cells may behave differently if exposed to noxious stimuli. The possibility remains that in patients who continue to smoke, seeded cells may be pro-thrombogenic and pro-mitogenic and exacerbate arterial occlusion. This possibility requires careful in vivo evaluation.

In addition to suggesting a new therapeutic approach to improve arterial patency after balloon dilatation, the work from this thesis has further elucidated the role of the endothelium in the generation of myointimal hyperplasia. The data from chapter 12 demonstrate that seeded endothelium inhibits intimal thickening. This has important implications in all aspects of vascular surgery, as it suggests that measures designed to reduce or eliminate endothelial damage during arterial reconstruction may decrease the incidence of subsequent restenosis. Further experiments are required to investigate the mechanism through which the endothelium expresses its inhibitory action. Preliminary results have suggested that SMC replication may be inhibited, but these data are inconclusive and further investigation is planned. Early events in the formation of restenotic lesions may be studied by investigating proto-oncogene expression after arterial
Prospects For Future Research

injury, and the contribution of seeded cell mediated platelet inhibition to the generation of myointimal hyperplasia, may be determined by seeding angioplasty sites in thrombocytopenic animals.

Finally, the promise of cellular transplantation as a therapeutic technique has been enhanced by gene transfection, in which recombinant DNA products may be expressed by genetically modified cells. Transplantation of genetically modified cells may allow specific metabolic pathways to be targeted and inhibited by transfected, seeded endothelial cells.
Appendix A

APPENDIX A
REAGENTS

Cord Collecting Solution.

Five hundred milliliter bottles of sterile M199 (ICN, High Wycombe, Bucks, UK) were supplemented with penicillin and streptomycin (Northumbria Biologicals [NBL], Cramlington, Northumbria, UK) to a final concentration of 100U/ml.

Minimal Essential Medium.

Minimal Essential Medium (MEM) was prepared by diluting a solution of concentrated (x10) MEM (ICN, High Wycombe, Bucks, UK) with sterile water and supplementing the diluted solution to a final concentration of:

- penicillin - 100U/ml (NBL, Northumbria, UK)
- streptomycin - 100U/ml (NBL, Northumbria, UK)
- Hepes buffer - 20mmol/l (NBL, Northumbria, UK)
- NaOH - to a final pH of 7.4 (Sigma, Poole, UK)

0.1% Collagenase.

Collagenase was obtained in powder form, from Worthington Biochemical, New Jersey, USA (CLS I). Collagenase was dissolved in MEM to give a 0.1% solution supplemented with calcium chloride (Sigma, Poole, UK) to a final concentration of 15mmol/l. Prior to use the solution was sterilised by filtering through a 0.45µm filter (Falcon 7104, Becton Dickinson & Co, New Jersey, USA).

Microvascular 0.1% Collagenase.

0.1% collagenase was used in microvascular cell harvest. Collagenase was again obtained in powder form, from Worthington Biochemical, New Jersey, USA (CLS I). Collagenase was dissolved in MEM to give a 0.1% solution, which was supplemented with 4% bovine serum albumin (BSA - Advanced Protein Products, Brierley Hill, UK) and 5mmol/l glucose. The solution was sterilised by filtration prior to use.
Appendix A

Trypsin.

A 1% solution of ethylenediaminetetra-acetic acid (EDTA) (Sigma, Poole, UK) was made up with distilled water and subsequently autoclaved. A 0.1% trypsin (ICN, High Wycombe, Bucks, UK) solution was made up in Dulbeccos phosphate buffered saline (PBS - ICN, High Wycombe, Bucks, UK), and EDTA was then added to a final concentration of 0.02%.

Complete Culture Medium.

This medium was composed of MEM with supplements added to the final concentrations given below:

- fetal calf serum - 20% (Sera Lab, Lot 001010, UK)
- penicillin - 100 U/ml (NBL, Northumbria, UK)
- streptomycin - 100 μg/ml (NBL, Northumbria, UK)
- Hepes buffer - 20 mmol/l (NBL, Northumbria, UK)
- glutamine - 2 mmol/l ((NBL, Northumbria, UK)
- pyruvate - 1mmol/l (Sigma, Poole, UK)
- endothelial cell growth factor - 15 μg/ml (Sigma, Poole, UK)
- heparin - 90 μg/ml (Sigma, Poole, UK)

Trypan Blue.

Trypan blue was obtained in powdered form (Sigma, Poole, UK), and made up to a 0.2% solution using 9 parts distilled water to 1 part normal saline.

Chromogenic Substrate.

This reagent was freshly made prior to use in immunohistochemical staining, and used within 15 minutes of composition. Ten milliliters of Tris buffer (Sigma, Poole, UK) was added to 10 mg naphthol phosphate (Sigma, Poole, UK), and this solution then added to 10 mg fast red (Sigma, Poole, UK). After vortexing, 100μl 0.1M levamisole (Sigma, Poole, UK) was added and the resultant solution filtered through a Whartman No 1 filter paper.
APPENDIX B

HISTOLOGICAL METHODS

This section details brief histological methods for staining paraffin embedded tissue prior to light microscopy. The staining procedures themselves were carried out by the Department of Pathology, University of Leicester. The majority of these methods are referred to in chapter 12.

Haematoxylin and Eosin (H and E).

Haematoxylin and eosin denotes staining of nuclei by oxidised haematoxylin through chelate bonds of metals such as aluminium, followed by counterstaining by the xanthine dye eosin, which colours in varying shades the different tissue fibres and cytoplasm [Bancroft, Cook, 1984].

Reagents.

Mayer's Haematoxylin Solution.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematoxylin</td>
<td>1 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Potassium alum</td>
<td>50 g</td>
</tr>
<tr>
<td>Sodium iodate</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Citric acid</td>
<td>1 g</td>
</tr>
<tr>
<td>Chlormal hydrate</td>
<td>50 g</td>
</tr>
</tbody>
</table>

Acid Alcohol.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>99% IMS</td>
<td>700 ml</td>
</tr>
<tr>
<td>Pure water</td>
<td>300 ml</td>
</tr>
<tr>
<td>Hydrochloric acid (conc.)</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

Bicarbonate Solution.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium bicarbonate</td>
<td>20 g</td>
</tr>
<tr>
<td>Pure water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Eosin Solution.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous eosin</td>
<td>10 g</td>
</tr>
<tr>
<td>Pure water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
Method.

Sections were dewaxed in xylene and then rehydrated in 99% IMS followed by 95% IMS for 2 min each. Sections were rinsed in water and then covered with haematoxylin solution for 5 min. Sections were washed again in water and immersed briefly in bicarbonate solution prior to wet mounting and viewing. The sections were then differentiated by repeated application of acid alcohol, alternated with bicarbonate solution, until only the nuclei were stained blue. The tissue was covered with eosin for 2 min and washed in water. Following staining the section was dehydrated in 96% and 99% IMS prior to immersion in xylene and mounting.

**Miller’s Elastin and Van-Gieson Stain (EYG).**

This technique combines Miller's elastin stain with Van-Gieson's technique for demonstrating collagen fibres. The resultant stain allows easy identification between elastin fibres (blue / black), collagen (red), and muscle (yellow). This staining technique is particularly useful in demonstrating the internal and external elastic laminae during assessment of myointimal hyperplasia.

**Reagents.**

**Miller’s Elastin Stain.**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Victoria blue</td>
<td>1 g</td>
</tr>
<tr>
<td>New fuschin</td>
<td>1 g</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>1 g</td>
</tr>
</tbody>
</table>

These reagents were dissolved in 200ml of deionised water, to which the following were added:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resorcino</td>
<td>4 g</td>
</tr>
<tr>
<td>Dextrin</td>
<td>1 g</td>
</tr>
<tr>
<td>30% ferric chloride</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

The solution was boiled, the precipitate removed and dissolved in 200ml 95% alcohol. The resulting solution was then boiled for 20 min, prior to filtration and the addition of 2 ml concentrated hydrochloric acid.

**Van Gieson Stain.**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated aqueous picric acid</td>
<td>100 ml</td>
</tr>
<tr>
<td>1% aqueous acid fuchsin</td>
<td>10 ml</td>
</tr>
</tbody>
</table>
Method.

Sections were dewaxed and washed. The loose sections were then oxidised in 0.25% potassium permanganate solution for 5 min prior to bleaching in 1% oxalic acid for a further 1 min. Following this process, sections were washed and rinsed in 95% alcohol, before being immersed in Miller's staining solution for 2 hours. After staining the sections were washed and counterstained with Van Gieson's stain for 2 min. Completed sections were dehydrated in alcohol and mounted.

Alcian Blue Stain.

This stain identifies acid mucins by blue staining.

Reagents.

Alcian Blue Solution (pH 2.5).
Deionised water 97 ml
Acetic acid (3%) 3 ml
Alcian blue 1 g

1% Neutral Red.
Deionised water 100 ml
Neutral Red 1 g

Method.

Sections were dewaxed, washed, and incubated with alcian blue solution for 5 min. Following staining sections were washed and then counterstained with neutral red for 30s. Sections were finally washed, dehydrated and mounted.

Periodic-Acid Schiff Technique.

This technique identifies neutral mucins.
Appendix B

Reagents.

1% Periodic Acid.
50% periodic acid 1 ml
Deionised water 50 ml

Schiff’s Reagent.
BDH (BDH, Merck Ltd, Poole, UK) commercial preparation.

Method.

Sections were dewaxed, washed and treated with periodic acid for 10 min. After this time, sections were washed and immersed in Schiff’s reagent for 15 min. Following incubation, sections were washed again and counterstained with haematoxylin for 30 sec prior to dehydration and mounting.

Proliferating Cell Nuclear Antigen (PCNA).

Proliferating cell nuclear antigen (PCNA) is the polymerase delta accessory protein [Waseen, Lane, 1990; Lee, Hurwitz, 1990], which is essential for cellular DNA synthesis. The protein is most likely involved in DNA leading strand synthesis, and in DNA repair [Lee, Hurwitz, 1990], and as such, is maximally expressed during S phase of the cell cycle [Cenis et al. 1984; Matthews et al. 1984], although expression may continue during G1 and G2 [Bravo, MacDonald-Bravo, 1987]. Proliferating cell nuclear antigen may be detected by the binding of monoclonal antibodies, a technique which thus allows the detection of proliferating cells. PCNA immunohistochemistry is a reliable and sensitive method for the detection of proliferating vascular smooth muscle cells in intimal hyperplasia, and has been shown to compare favourably with other methods of detecting replicating cells, namely [3H] thymidine autoradiography and bromodeoxyuridine immunohistochemistry [Zeymer et al. 1992].

In chapter 12, replicating SMC’s were detected by using mouse monoclonal anti-PCNA antibody PC 10 (Dalco Limited, Glostrup, Denmark) with an avidin / biotin / diaminobenzidine detection system, which imparts a brown colour to replicating cell nuclei.
Djaitmimolbeinis solution.

- Diaminobenzidine 5 mg
- PBS 10 ml
- 3% hydrogen peroxide 70 µl

Sections were dewaxed and washed, and placed in 6% hydrogen peroxide for 10 min. After this period the sections were washed in PBS and pretreated with normal rabbit serum (dilution 1:20 - Dako Limited, Glostrup, Denmark) for 10 min, prior to application of the primary antibody (mouse monoclonal anti-PCNA antibody PC 10 [Dako Limited, Glostrup, Denmark]) at a 1:50 dilution. The primary antibody was incubated on the slide for 20 min at 4°C after which the slide was washed and a biotinylated rabbit anti-mouse immunoglobulin (Dako Limited, Glostrup, Denmark - 1:400 dilution) added for 30 min. After repeated washes with PBS, slides were incubated with avidin and biotinylated horseradish peroxidase (1:200) for 30 min.

Following this incubation period and further washing with PBS, diaminobenzidine tetrachloride was added for 5 min to act as a chromagen.

Smooth Muscle Actin.

This immunohistochemical technique allows identification of smooth muscle cells by staining for smooth muscle actin.

Method.

Sections were rehydrated, dewaxed and washed prior to incubation in 6% hydrogen peroxide for 10 min. After further washes with PBS, sections were incubated with normal rabbit serum (1:20 dilution) for 10 min after which the smooth muscle actin antibody (1:400, Dako Limited, Glostrup, Denmark) was added and incubated with the section for 20 min. Following this incubation period, a rabbit anti-mouse immunoglobulin (1:25, Dako Limited, Glostrup, Denmark) was added, and following washing, the section was treated with diaminobenzidine for 5 min. Sections were finally dehydrated and mounted.
**Appendix B**

**In Situ Hybridisation of Histone mRNA.**

As an alternative to PCNA immunohistochemistry, *in situ* hybridisation for histone mRNA was used to detect replicating smooth muscle cells [Colloby *et al.* 1993b; Colloby *et al.* 1993a]. This methodology was devised in the Department of Pathology, Leicester University, and was kindly made available by Dr James H Pringle.

Histones are small proteins with a high proportion of positively charged amino acids (lysine and arginine), that are present in eucaryotic cells and bind to DNA. Histones may be classified into two main groups, the nucleosomal histones and the H1 histones. The nucleosomal histones are small proteins responsible for coiling DNA into nucleosomes, thereby allowing orderly DNA packaging inside the nucleus. The four nucleosomal histones are designated H2A, H2B, H3 and H4, and form the inner core of the nucleosome. Unlike the majority of proteins which are synthesised throughout interphase, histones are synthesised in S phase of the cell cycle, when histone mRNA increases fifty fold. The histone mRNA's have a special sequence at the 3' end which is responsible for their instability when DNA synthesis ceases. Due to this instability, histone mRNA's are degraded within minutes at the end of S phase [Molecular biology of the cell. New York, Garland]. This property makes histone mRNA a specific marker of S phase and consequently an ideal tool for identifying cells actively synthesising DNA. *In situ* hybridisation for histone mRNA has a theoretical advantage over PCNA immunohistochemistry, as it will only detect cells in S phase whereas PCNA is expressed both in G1 and G2 as well as S phase [Bravo, MacDonald-Bravo, 1987]. In this thesis *in situ* hybridisation will be performed [Pringle *et al.* 1990], with an anti-digoxigenin alkaline phosphatase conjugate direct detection method [Kendall *et al.* 1991].

**Reagents.**

**Pre Hybridisation Solution.**

- 0.6 M NaCl
- 1 x PE/10% dextran sulphate
- 1.50μg/ml ss DNA (salmon sperm derived)
- 30% formamide
- DEPC water
Appendix B

Hybridisation Solution.

0.6 M NaCl
1 x PE 10% dextran sulphate
Labelled oligonucleotide probe 200 ng DNA/ml
150μg/ml ss DNA (salmon sperm derived)
30% formamide
DEPC water

Detection Solution.

4μl NBT/ml (75mg/ml in 70% dimethyl formamide)
4μl BCIP/ml (50mg/ml in 70% dimethyl formamide)
1μg 1M levamisole/ml
Substrate buffer (3% BSA, 0.1% Triton-X in TBS)

Method.

Pretreatments.

Pretreatments are required to unmask mRNA target sequences and to permeabilise the tissue sections for the detection reagents.

Sections were dewaxed and rehydrated through a series of clean xylene ethanol and diethylpyrocarbonate-treated ultra pure water (DEPC-water). Sections were immersed in 2x standard saline citrate buffer (SSC) at 70°C for 10 min, and then digested in proteinase K (Boehringer Mannheim, Germany) at 37°C for 60 min. Following digestion, sections were washed in DEPC water at 4°C for 10 min, prior to fixation in 0.4% paraformaldehyde, 0.1M PBS /DEPC water for 20 min at 4°C.

Oligonucleotide Synthesis.

Oligonucleotides were synthesised by Dr JH Pringle using a DNA synthesiser model 380B (applied Biosystems, U.S.A.). Oligonucleotides were labelled at the 3’ end using the TdT reaction previously described by Pringle et al. [Pringle et al.1989] to add a homopolymer tail of dUTP-11-digoxigenin.
Hybridisation.

Following fixation, slides were drained and covered with 25µl of pre-hybridisation solution for 1 hour at 37°C. After this time the sections were covered with 50µl of hybridisation solution and covered with silicone coverslips to ensure even coverage of the hybridisation solution. Slides were incubated overnight at 37°C.

Following hybridisation, slides were washed in the following order:

- 30% formamide / 2xSSC 37°C 10min
- 2xSSC 37°C 4min
- TBS 37°C 15min.

Detection.

Sections were incubated in a blocking solution for 5 min and then an antidigoxigenin-alkaline phosphatase conjugate (1:600, Boehringer Mannheim, Germany) added for 30 min. After this time slides were washed twice in TBS and alkaline phosphatase activity demonstrated using the 5-bromo-4-chloro-3-indolyl phosphate (BCIP enzyme substrate) and nitroblue tetrazolium (NBT chromagen) technique. Slides were incubated in detection medium for 60 min in a darkened room and after further washing under tap water sections were mounted in Apathy's medium for viewing.
APPENDIX C

HAEMODYNAMIC PARAMETERS

This appendix details the calculation of haemodynamic parameters for the pulsatile perfusion circuit described in Chapter 8.

Flow Rates.

Flow rates of 50ml/ min and 100 ml/min were used.

50 ml/min = 0.83 ml/s
100 ml/min = 1.67 ml/s.

Flow Velocities.

Mean flow velocities were calculated from the mean flows and the mean vein diameters recorded through the experiment.

Flow 50 ml/min : Mean vein radius = 0.21cm
∴ Mean vein area = 0.14 cm², and as velocity = flow/ area,
Mean flow velocity = 0.83 / 0.14 = 5.9 cm/s

Flow 100 ml/min : Mean vein radius = 0.20cm
∴ Mean vein area = 0.13 cm², and as velocity = flow/ area,
Mean flow velocity = 1.67 / 0.14 = 12.9 cm/s

Peak Flow Velocity (PTFE).

Peak flow velocity was measured in a 4 mm PTFE graft (WL Gore, Livingstone, UK), using a Diasonics DRF 400 duplex scanner (Diasonics, Bedford, UK). The maximum velocities (V_max) achieved were :

Flow 50 ml/min : V_max = 25 cm/s
∴ Assuming a diameter of 4 mm,
Peak flow generated at mean flow of 50 ml/min = 25 x 0.126 = 3.15 ml/s.

Flow 100 ml/min : V_max = 53 cm/s
∴ Assuming a diameter of 4 mm,
Peak flow generated at mean flow of 100 ml/min = 6.7 ml/s
Having calculated the peak flow rates with the duplex measurements, these were then applied to the experimental situation to calculate maximum velocities for the two flow rates.

Flow 50 ml/min: Mean vein radius = 0.21 cm
∴ Mean vein area = 0.14 cm$^2$,
Max flow velocity = $3.15 / 0.14 = 22.5$ cm/s

Flow 160 ml/min: Mean vein radius = 0.20 cm
∴ Mean vein area = 0.13 cm$^2$,
Max flow velocity = $6.7 / 0.14 = 51.5$ cm/s

**Reynolds Numbers.**

The Reynolds number is given by:

$$N_R = 2. \cdot \frac{r \cdot v}{\nu}, \quad \text{[Milnor, 1989]}$$

where; $N_R =$ Reynolds number; $r =$ radius (cm); $v =$ mean velocity (cm/s); and $\nu =$ kinematic viscosity.

Kinematic viscosity $= \frac{\eta}{\rho}$, where $\eta =$ viscosity (poise) and $\rho =$ density (g/cm$^2$).

Viscosity of Haemaccel (Hoechst, Hounslow, UK) was measured by a Coulter Viscometer II (Coulter, UK) at 1.54x10$^{-2}$ poise at 35°C.

Density of Haemaccel was 1.03 g/cm$^2$.

∴ Kinematic viscosity $= 0.015$

At 50 ml/min; $N_R$ (mean) $= 2 \times 0.21 \times 5.9 / 1.5 \times 10^{-2} = 168$.
$N_R$ (max) $= 630$. 

---

211
Appendix C

At 100 ml/min; $N_R$ (mean) = $2 \times 0.2 \times 12.9 \times 1.5 \times 10^{-2} = 364$.
$N_R$ (max) = 1373.

Shear Stress.

Shear stress in laminar flow is given by:

$$S_w = 4 \eta Q / \pi r^3.$$

where $S_w$ = shear stress (dyn/cm²); $\eta$ = viscosity (poise); $Q$ = flow rate (ml/s) and $r$ = internal radius. For this calculation we have assumed that the external and internal radius of the vein segments were equal.

At 50 ml/min; $S_w$ (mean) = $4 \times 1.54 \times 10^{-2} \times 0.83 / \pi \times 0.21^3 = 1.76$,
$S_w$ (max) = 6.70.

At 100 ml/min; $S_w$ (mean) = $4 \times 1.54 \times 10^{-2} \times 1.67 / \pi \times 0.20^3 = 4.1$,
$S_w$ (max) = 16.5.
Prostacyclin (PGI\textsubscript{2}) is unstable and undergoes spontaneous hydrolysis to 6-keto-prostaglandin F\textsubscript{1\alpha} with a half life of approximately 3 min [Cho, Allen, 1978]. The quantitation of 6-keto-prostaglandin F\textsubscript{1\alpha} is thus accepted as a measure of PGI\textsubscript{2} formation. Prostacyclin was assayed by a radioimmunoassay, supplied in kit form by Amersham (Amersham International, Amersham, UK). The assay is based on competition between unlabelled 6-keto-prostaglandin F\textsubscript{1\alpha} (in the sample to be assayed) and tritium labelled 6-keto-prostaglandin F\textsubscript{1\alpha} for binding to an high specificity antibody. With fixed quantities of antibody and radioactive ligand, the amount of radioactivity bound will be inversely proportional to the quantity of non labelled 6-keto-prostaglandin F\textsubscript{1\alpha} present in the sample.

**Reagents.**

Reagents supplied in the kit were:
- Dextran coated charcoal.
- Vial 1: assay buffer (phosphate buffered saline with gelatin and thimerosal).
- Vial 2: 37kBq [\textsuperscript{3}H] 6-keto-prostaglandin F\textsubscript{1\alpha} in assay buffer.
- Vial 3: 6-keto-prostaglandin F\textsubscript{1\alpha} antiserum in assay buffer.
- Standard concentrations of 6-keto-prostaglandin F\textsubscript{1\alpha} (0.14, 0.3, 0.75, 2.0, 5.0 ng/ml) for construction of a standard curve

**Method.**

**Preliminary Stage (Day 1).**

Samples were removed from the -70°C freezer and thawed to room temperature. A set sequence of procedures was then followed:
1. 200 µl of buffer was pipetted into 4 polypropene tubes (12 x 75mm, Falcon,Becton-dickinson, New Jersey, USA); 2 labelled for total count (TC), and 2 tubes for non-specific binding (NSB).
2. 100µl of the tritium labelled 6-keto-prostaglandin F\textsubscript{1\alpha} was then added to all 64 tubes (48 for samples, 6 for NSB, TC and Bo, and 10 for standard curve).
3. 100µl of the standard solutions was added to the appropriate tubes in duplicate.
4. 100 µl of the samples to be assayed was then placed into appropriately labelled tubes.
5.- 100µl of 6-keto-prostaglandin F1α antiserum was pipetted into B0, standard and sample tubes.
6.- All tubes were vortexed and then centrifuged at 1000g for 15 seconds. The tubes were vortexed again and then incubated, initially at room temperature for 1 hour and then at 4°C for 16-24 hours.

**SEPARATION OF UNBOUND 6-KETO-PROSTAGLANDIN F1α (DAY 2).**

7.- The assay tubes were placed on crushed ice.
8.- 1 ml of assay buffer was pipetted into the TC tube, the solution mixed and then decanted into 15 ml of scintillation fluid (OptiScint, FSA, Loughborough, UK).
9.- The dextran coated charcoal (at 4°C) was thoroughly mixed and 1 ml added to each tube whilst the tubes were being vortexed. The tubes were then mixed again and incubated on crushed ice for 9 min.
10.- The tubes were centrifuged at 1000g for 10 min at 4°C.
11.- The supernatant was removed from each tube and decanted into the scintillation fluid. The beta activity of each sample was determined in a beta scintillation counter (1217 RackBeta, LKB Instruments Ltd, Croydon, UK).
12.- A standard curve was plotted on the log-logit graph paper provided and the 6-keto-prostaglandin F1α content of each of the samples was then read from the graph.


Antonelli-Orlidge A, Saunders KB, Smith SR, D'Amore PA: An activated form of transforming growth factor β is produced by co-cultures of endothelial cells and pericytes. Proc Natl Acad Sci U S A 1985;3:4544-4548


Barrett ML, Willis AL, Vane JR: Inhibition of platelet derived mitogen release by nitric acid (EDRF). Agents Actions 1989;27:488-91


Bergelson BA, Jacobs AK, Small DM: Lipoproteins predict restenosis after PTCA. Circulation 1989;80 (Suppl II):II-65(Abstract)


Bodrog I, Mohasy J, Urli L: Light and electron microscopic study of pathomorphological changes on the arterial wall after transluminal angioplasty. *Int Angiol* 1986;5:13-19


Bibliography


Budd JS, Bell PRF, James RFL: Attachment of indium-111 labelled endothelial cells to pretreated polytetrafluoroethylene vascular grafts. *Br J Surg* 1989;76:1259-1261


Budd JS: *An investigation into the effect of endothelial cell seeding on the function of small calibre prosthetic vascular grafts*. MD Thesis. University of Leicester, 1991d

Bibliography


Bibliography


Celsi JE, Bravo R, Larsen PM, Fey SJ: Cyclin: a nuclear protein whose level correlates directly with the proliferative state of normal as well as transformed cells. *Leuk Res* 1984;8:143-157


Colapinto RF, Strobl RD, Johnston WK: Transluminal angioplasty of complete iliac obstructions. AJR Am J Roentgenol 1986;146:859-862


Collopy BS, Pringle JH, Balcer J, et al: Non isotopic in situ hybridisation (NISH) for histone mRNA as an indicator of proliferation in lymphoma - an alternative to PCNA for fixed and archival tissue. J Pathol 1993a;169 (Suppl):140 A


Consigny PM, Tuleleno TN, Nicossia RF: Immediate and long-term effects of angioplasty-balloon dilation on normal rabbit iliac artery. Arteriosclerosis 1986;6:265-276


Bibliography

Downing MR, Bloom Jw, Mann KG: Comparison of the inhibition of thrombin by three plasma protease inhibitors. *Biochemistry* 1978;17:2649-2653


Fair DS, Marler RA, Levin EG: Human endothelial cells synthesise Protein S. *Blood* 1986;67:1168-71
Bibliography


Faxon DP, Sanborn TA, Haudenschild CC: Mechanism of angioplasty and its relation to restenosis. Am J Cardiol 1987; 60:5B-9B


Forsberg EI, Feuerstein G, Shohami E, Pollard HB: Adenosine triphosphate stimulates inositol phospholipid metabolism and prostacyclin formation in adrenal medullary endothelial cells by means of P2-purinergic receptors. Proc Natl Acad Sci USA 1987; 84:5630-4


Fry DL: Certain histologic and chemical responses of the vascular interface to acutely induced mechanical stresses in the aorta of the dog. *Circ Res* 1969;24:93-108


Bibliography


Gordon T, Kanel WB: Predisposition to atherosclerosis in the head, heart and legs. *JAMA* 1972;221:661-6


Bibliography


Harker LA: Role of platelets and thrombosis in mechanisms of acute occlusion and restenosis after angioplasty. Am J Cardiol 1987;60:20B-28B

Harlan JM: Leucocyte-endothelial interactions Blood 1985;65 513-25


Hasson JE, Wiebe DH, Sharefkin JB, D’Amore PA, Abbott WM: Use of tritiated thymidine as a marker to compare the effects of matrix proteins on adult human vascular endothelial cell attachment: implications for seeding of vascular prostheses. Surgery 1986;100:884-892

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


Bibliography


232
Bibliography


Koo EW, Gotlieb AI: Neointimal formation in the porcine aortic organ culture I. Cellular dynamics over 1 month. Lab Invest 1991;64:743-753


Lau HK, Rosenberg RD: The isolation and characterisation of a specific antibody population directed against the thrombin-antithrombin complex. *J Biol Chem* 1980;255:5685-5693


Leary T: The genesis of atherosclerosis. *Arch Pathol* 1941;32:507-555

Lee SA, Hurwitz J: Mechanism of elongation of primed DNA by DNA polymerase delta, proliferating cell nuclear antigen and activator 1. *Proc Natl Acad Sci USA* 1990;87:5672-5676


Bibliography


Lindner V, Reidy MA: Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. Proc Natl Acad Sci U S A 1991;88:3739-3743


Lloyd RG, Priam GW, Gomez H, Laub J, Mortz GH: Failure of pentoxifylline for end stage peripheral arterial disease. Angiology 1987;38:304-308


Bibliography


Luscinskas FW, Cybulsld MI, Kiely JM, Perkins CS, Davis VM, Gimbrone MAJr: Cytokine activated human endothelial monolayers support neutrophil transmigration via a mechanism involving both endothelial - leucocyte adhesion molecules and intracellular adhesion molecules. J Immunol 1991;146:1617-1625


Maruyama I, Bell CE, Majerus PW: Thrombomodulin is found on endothelium of arteries, veins, capillaries and lymphatics, and on syncytiotrophoblast of human placenta. J Cell Biol 1985;101:363-71


236


Moncada S: Biological importance of prostacyclin. *Br J Pharmac* 1982;76:3-31


Moore S: Thromboatherosclerosis in normolipaemic rabbits: a result of continued endothelial damage. *Lab Invest* 1973;29:478-487


Bibliography


Bibliography


Bibliography


Rees DD, Palmer RM, Moncada S: Role of endothelium derived nitric oxide in the regulation of blood pressure. *Proc Natl Acad Sci USA* 1989;86:3375-8


Bibliography


Bibliography


Sanders M: Angiographic changes thirty minutes following percutaneous transluminal coronary angioplasty. *Angiology* 1985;36:419-424


243
Bibliography


Schwarten DE: Clinical and anatomical considerations for nonoperative therapy in tibial disease and the results of angioplasty. Circulation 1991;83:186-190


Schweigerer L, Nuefeld G, Friedman J, Abraham JA, Fiddes JC, Gospodarowicz D: Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. Nature 1987;325:257-259


Sentissi JM, Ramberg K, O'Donnell T, Connolly RJ, Callow AD: The effects of flow on vascular endothelial cells grown in tissue culture on polytetrafluoroethylene grafts. Surgery 1986;99:337-342


**Bibliography**


Sipehia R: The enhanced attachment and growth of endothelial cells on anhydrous ammonia gaseous plasma modified surfaces of polystyrene and poly(tetrafluoroethylene). *Biomater Artif Cells Artif Organs* 1990;18:437-446


Steinberg D: Research related to the underlying mechanisms in atherosclerosis. Circulation 1979;60:1559-1565


Sussman II, Rand JR: Subendothelial deposition of von Willebrand factor requires the presence of endothelial cells. J Lab Clin Med 1982;100:526-32


Bibliography


Thilo DGJ, Muller-Kusel S, Heinrich D, Kaufer I, Weiss E: Isolation of human venous endothelial cells by different proteases. *Artery* 1980;8:259-266


Van Hinsbergh VWM: Regulation of the synthesis and secretion of plasminogen activators by endothelial cells. Haemostasis 1988;18:307-27

van Andel GJ: Percutaneous transluminal angioplasty - the Dotter procedure. Amsterdam, Excerpta Medica, 1976


Bibliography
Bibliography


Waller BF, Gorfinkel HJ, Rogers FJ, Kent KM, Roberts WC: Early and late morphologic changes in major epicardial coronary arteries after percutaneous transluminal coronary angioplasty. *Am J Cardiol* 1984;53:42c-47c


Waller BF, Pinkerton CA, Orr CM, Slack JD, VanTassel JW, Peters T: Morphological observations late (greater than 30 days) after clinically successful coronary balloon angioplasty. *Circulation* 1991a;83:128-141

Waller BF, Pinkerton CA, Orr CM, Slack JD, VanTassel JW, Peters T: Restenosis 1 to 24 months after clinically successful coronary balloon angioplasty: a necropsy study of 20 patients. *J Am Coll Cardiol* 1991b;17:968-70B


Bibliography


Widmer LK, Biland L, DaSilva A; Risk profile and occlusive peripheral artery disease (OAPD), in *Proceedings of the 13th International Congress of Angiology, Athens 9-14 June 1985*


Williams SK: Regulation of intimal hyperplasia: do endothelial cells participate? [editorial]. Lab Invest 1991;64:721-723


Yeager A, Callow AD: New graft materials and current approaches to an acceptable small diameter vascular graft. ASAIO Trans 1988;34:88-94


