The effect of novel immunosuppressants on extracellular matrix remodelling and the expression of fibrosis associated genes in an experimental model of allograft vasculopathy

a thesis submitted for the degree of Doctor of Medicine at the University of Leicester, May 2002

by

Gavin J Murphy

The work contained in this thesis is completely original and has not been submitted previously for any university degree. The body of experimental work described in this thesis has been performed in the most part by myself however I am grateful for the technical assistance provided by Dr Gareth Bicknell PhD, with respect to the development of the molecular biological techniques, and to Dr P Furness MRCPath for his technical assistance with the picrosirius red computerised histomorphometry. I am also grateful to my supervisor, Professor ML Nicholson and Mr RD Sayers for their advice and financial support.
Publications arising from this thesis

Murphy GJ, Bicknell GR, Nicholson ML

Murphy GJ and Nicholson ML.

Murphy GJ, Bicknell GR, Nicholson ML
The effect of combined rapamycin/cyclosporin on the changes in pro-fibrotic gene expression that occur during the development of allograft vasculopathy in rats compared to cyclosporin or rapamycin in isolation. Transplant International, 2003, in press.

GJ Murphy and ML Nicholson
Rapamycin has no effect on the progression of early or established allograft vasculopathy in rats. Journal of Thoracic and Cardiovascular Surgery, 2003 in press.
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<td>A disintegrin and metalloproteinases</td>
</tr>
<tr>
<td>Aza</td>
<td>Azathioprine</td>
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<tr>
<td>CAD</td>
<td>Chronic allograft dysfunction</td>
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<tr>
<td>CAV</td>
<td>Cardiac allograft vasculopathy</td>
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<tr>
<td>CDK</td>
<td>Cyclin dependent kinases</td>
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<tr>
<td>cDNA</td>
<td>Copy deoxyribonucleic acid</td>
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<td>Cyclosporin</td>
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<td>DS</td>
<td>Dermatan sulphate</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK Binding Protein</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
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<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
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<tr>
<td>I/R</td>
<td>Ischaemia reperfusion</td>
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<td>ICUS</td>
<td>Intracoronary ultrasound</td>
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<td>Ig</td>
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<td>IGF</td>
<td>Insulin-like growth factor</td>
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<td>Interleukin-</td>
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<td>MAC</td>
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<td>Matrix metalloproteinase</td>
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<td>Matrix metalloproteinase inhibitor</td>
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<tr>
<td>mRNA</td>
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<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>NB</td>
<td>Northern blot</td>
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<td>NF</td>
<td>Nuclear Factor</td>
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<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PA</td>
<td>Plasminogen activator</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>PTA</td>
<td>Percutaneous balloon angioplasty</td>
</tr>
<tr>
<td>Rapa</td>
<td>Rapamycin</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-Glycine-Aspartate</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TX</td>
<td>Thromboxane</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot</td>
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<tr>
<td>Z</td>
<td>Zymography</td>
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The effect of novel immunosuppressants on extracellular matrix remodeling and the expression of fibrosis associated genes in an experimental model of allograft vasculopathy

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Abstract

Background: Chronic allograft dysfunction (CAD), the leading cause of solid organ transplant failure, is characterised by histological evidence of parenchymal extracellular matrix (ECM) accumulation (fibrosis) and allograft vasculopathy. The aim of this study was to compare the effects of rapamycin and cyclosporin therapy, individually as well as in combination, on fibrosis associated gene expression and ECM remodeling in an experimental model of allograft vasculopathy.

Methods: Vascular remodeling and ECM accumulation (picrosirius red) were measured by computerised histomorphometry of F344 to Lewis rat aortic allograft sections harvested at serial time points. Expression of fibrosis associated genes was studied by means of semi-quantitative RT-PCR.

Results: Rapamycin (0.5mg/kg/day) or cyclosporin (5mg/kg/day) inhibited intimal hyperplasia, medial ECM accumulation and expansive vascular remodeling (increasing vessel circumference) in rat aortic allografts. This was associated with attenuation of the graft inflammatory infiltrate and a reduction in intra-graft gelatinase, collagen III and TIMP 1 mRNA levels. At a lower dose (0.25mg/kg/day) Rapamycin inhibited intimal hyperplasia and medial ECM accumulation however there was a lesser effect on vascular remodeling compared to cyclosporin or rapamycin 0.5mg/kg/day. Combined rapamycin and cyclosporin also inhibited intimal hyperplasia however there was a lesser effect on both vascular remodeling and medial extracellular matrix accumulation. Combined treatment or rapamycin 0.25mg/kg/day monotherapy aortic allografts were also seen to have a more severe inflammatory infiltrate and larger amounts of intra-graft MMP 9, TGFβ and TIMP 1 mRNA than cyclosporin or rapamycin 0.5mg/kg/day monotherapy.

Conclusion: These data suggest that in addition to the tissue response to injury, the alloimmune injury itself may directly contribute to vascular and ECM remodeling. In this experimental model rapamycin inhibited vascular remodeling and ECM accumulation only in the presence of effective immunosuppression.
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Introduction

Transplantation has evolved as the treatment of choice for many patients with end stage organ failure. Early graft survival rates have steadily increased over the years due to improvements in perioperative care, advanced operative and tissue typing techniques, deeper insight into the process of organ rejection and development of more effective immunosuppressant drugs and drug regimens. In contrast, the rate of organ loss has not changed in kidney (Cecka et al 2000), heart and lung (Hosenpud et al 2000) and liver (Starzl et al 1997) transplantation as improvements in short term survival of heart and kidney recipients have merely shifted the survival curves upwards (Gjertson & Cecka, Wallwork 1994). The leading cause of long term allograft failure in solid organ transplantation is a pathological fibroproliferative process that has more recently been called Chronic Allograft Dysfunction (CAD) (Gjertson & Cecka, Wallwork 1994). This is characterised by a progressive decline or failure of graft function in association with gradual fibrosis and obliteration of hollow structures within the graft, regardless of whether they are renal tubules, blood vessels, bile ducts or bronchioles (Billingham 1987, Sibley 1994, Wallwork 1994). While the functional and morphological findings are well recognised, pathophysiological mechanisms remain obscure. Clinical studies and experimental models of CAD suggest that immunological mechanisms operating in a milieu of non immunological risk factors constitute the principal stimuli that result in myointimal hyperplasia of the graft vasculature, graft fibrosis and ultimately failure (Weis & von Scheidt 1997, Tullius and Tilney 1998, Hayry 1998).

There is no satisfactory treatment for this condition and in particular the introduction of effective inhibitors of the cell mediated immune response (cyclosporin and Tacrolimus) have failed to inhibit or reduce the development and severity of CAD despite reducing graft loss due to acute rejection. It is hoped that newer immunosuppressant agents currently being used in clinical trials such as rapamycin or Mycophenolate mofetil (MMF) may
prevent or arrest its development perhaps due to a direct effect on the fibroproliferative donor vasculature as well as the host immune response. This chapter will summarise the functional and histological features, risk factors and pathogenesis of CAD in heart and renal transplants in particular as well as in other organs and experimental models of allograft rejection and vascular disease. The role of newer immunosuppressant drugs in the treatment of CAD will also be discussed in the second part of this chapter and the molecular basis of the fibroproliferative response to injury will be discussed in part 3 and 4. The role of different experimental models in the investigation of CAD will be considered in section 5.
Part 1. Chronic allograft dysfunction

1.1 Definition of chronic allograft dysfunction

Chronic allograft dysfunction is a phenomenon occurring in solid organ transplants that display a gradual deterioration of graft function months to years after transplantation and this process ultimately results in graft failure. This progressive decline in function is accompanied by characteristic histological features including 1, diffuse fibrous intimal thickening in arteries and arterioles termed allograft vasculopathy, (AV), although several other terms are also used (Table 1), 2, progressive parenchymal fibrosis and 3, obliterative fibrosis of hollow structures within the graft such as bronchioles, bile ducts and glomeruli (Tilney et al 1991).

This progressive obliterative vasculopathy and organ fibrosis represents a chronic fibroproliferative response to injury that shares many similarities with other chronic inflammatory diseases including non-transplant atherosclerosis, chronic renal disease and rheumatoid arthritis (Ross 1999). The histopathological and functional changes that characterise CAD in different solid organ allografts are detailed below.

1.2 Histopathological changes and clinical presentation

CAD of the heart, lungs, liver and kidneys is characterised by similar proliferative changes in vasculature and histological features specific to each organ that often precede clinical symptoms.

Heart

Macropathology

Cardiac allograft vasculopathy developed in the second human heart transplant recipient within a few months of engraftment in 1968 and caused the patient's death by heart failure 19 months later. At autopsy, the extent of CAV was profound despite the donor being only
24 years old (Thompson et al 1968). Billingham and co workers at Stanford (1987, 1990) published the first clinical series describing the nature of these pathological lesions. Pre-eminent among gross pathological findings at autopsy or explant are cord like, orange-coloured coronary vessels on the outside of the organ. Calcification is rarely present and platelet aggregation is virtually absent. In contrast to ordinary atherosclerosis, in which the larger vessels preferentially develop asymmetric deposits of occlusive plaque, graft vascular disease is often characterised by symmetrical concentric narrowing or occlusion along the entire length of large and small vessels (Russell et al 1993). Large vessel occlusion producing large infarcts is infrequent. CAV is more commonly characterised by a largely symmetrical occlusion of small vessels resulting in small triangular shaped infarcts (Billingham et al 1990). Eccentric plaques similar to ordinary atheroma do occur (Billingham 1987) with a heterogeneous distribution within the arterial tree. Histological analysis demonstrates eccentric plaques predominantly in smaller epicardial and mural arteries differing from the distribution of ordinary atheroma. Angiographic (Gao et al 1990) and Intra Coronary Ultrasound (ICUS) studies (Yeung et al 1995) however describe focal lesions predominantly in proximal vessels similar to ordinary atherosclerosis with diffuse circumferential lesions more commonly in mid and distal segments. The aorta (including its vasa vasorum) is also involved although the pulmonary artery is usually spared (Russell et al 1993). A multi centre ICUS study demonstrated that the most rapid rate of intimal thickening occurs within the first year post transplant (Yeung et al 1995). There is also a very strong correlation between the presence and severity of arterial thickening and the presence of venous thickening (Oni et al 1992). These thickened veins show similar myointimal proliferation to those seen in allograft arteries leading to the adoption of the more accurate term cardiac allograft vasculopathy (CAV) rather than arteriopathy or atherosclerosis.
Table 1. Alternative terms for allograft vasculopathy

Graft Vascular Disease
Transplant Arteriosclerosis
Allograft Arteriosclerosis
Transplant Arteriopathy
Accelerated transplant atherosclerosis
Graft arteriosclerosis, atherosclerosis or coronary disease
Chronic rejection
Vascular immuno-obliterative disease
Obliterative arteriopathy

Micropathology

Microscopic examination demonstrates that the vessel narrowing is attributed to a dramatic concentric proliferation of the intima. The internal elastic lamina shows small breaks but retains its shape and the media in some compromised vessels is actually thinner than normal. The endothelial layer is generally intact (Billingham 1987, 1994). Complicated atherosclerotic plaques that bear a close resemblance to non-transplant atherosclerosis are also seen (Pucci et al 1990).

A time dependent spectrum of histopathological changes has been described (Johnson et al 1991). Early post transplant there is a diffuse intimal reaction and it is common to see a distinct infiltration of lymphocytes along the margin of the lumen just deep to the endothelial layer. Staining of these cells indicates that they are primarily T Cells and Macrophages. This "endothelialitis" tends to be absent later in the disease (Johnson et al 1991). Perivascular infiltrates are sometimes seen in the larger coronary arteries but these are usually attributed to acute rejection episodes. Late after transplantation focal atherosclerotic plaques, diffuse intimal thickening or a mixture of both is found. With time...
excessive lipid deposition occurs in the form of lipid stuffed macrophages or foam cells with extracellular lipoprotein aggregates (McManus et al 1994). Johnson et al described a diverse array of lesions (1991). These ranged from cellular and acellular concentric lesions with abundant or virtually no foam cells to cellular and mixed fibrofatty plaque lesions with scattered foam cells resembling natural atherosclerosis. The lesion distribution was heterogeneous but all types occurred in a single allograft. The cellular infiltrate of intimal proliferative lesions consists of modified smooth muscle cells, macrophages, monocytes and T lymphocytes (Oguma et al 1988, Hruban et al 1990).

Fibrotic changes may appear throughout the substance of the myocardium and in the pericardium, usually late in the process although whether this is the result of progressive ischaemia or a direct injury from immunological and non-immunological factors is unclear.

**Clinical presentation**

Although there is some evidence of partial reinnervation of cardiac allografts most heart transplant patients cannot experience typical angina pain associated with myocardial ischaemia or infarction. The first clinical manifestations therefore are often ventricular arrhythmias and congestive heart failure from repeated small infarcts or sudden death from an acute infarct or progressive loss of myocardium (Wallwork 1994). Actuarial survival curves of patients undergoing heart transplantation from the Registry of the International Society of Heart and Lung Transplantation demonstrate a 79.1%, 67.8% and 55.8% survival at 1, 5 and 10 years respectively (Kaye 1993).
Pathophysiology of CAD in other organs

Kidney

Multi-centre multivariate analysis of renal transplants reported to the United Network for Organ Sharing Registry demonstrates that the 10-year survival of cadaver kidney grafts is 50% and this graft loss is largely due to CAD (Gjertson 1999).

Chronic renal allograft dysfunction is manifest clinically as progressive proteinuria, hypertension and declining function (Fellstrom et al 1989). It was first described in one of the original renal transplant recipients, an individual who was sustained by his kidney transplant for almost 6 months without immunosuppression. Diagnostic histological features specific to renal CAD as opposed to other renal disorders are firstly transplant glomerulopathy, characterised by widespread duplication of the glomerular basement membrane, a modest increase in the mesangial matrix and interposition of matrix and mesangial cells, and secondly myointimal hyperplasia of the allograft vasculature (Solez 1994). Other less specific glomerular changes occur with increased mesangial matrix, partial or complete collapse of the capillary tuft and eventual glomerulosclerosis. These latter lesions may represent ischaemic damage to the kidney from progressive vascular occlusion rather than a direct response of the rejection process per se (Paul 1994). Other lesions such as interstitial fibrosis and tubular atrophy are much less specific but much more common in renal transplant patients and have been used to grade "chronic transplant nephropathy" as described in the Banff classification (Solez et al 1996). As these lesions are also present in hypertensive vascular disease and chronic cyclosporin toxicity others have recommended that the diagnosis of chronic allograft dysfunction cannot be made unless myointimal hyperplasia is present in the core biopsy (Paul et al 1993). These observations have been incorporated in the revised Banff classification (1999).
**Lung**

The actuarial survival for adult lung transplants at 5 years is 43.9% (Kaye 1993). The most common lesion seen in long surviving lung allografts is a progressive fibrosis and obliteration of terminal bronchioles called bronchiolitis obliterans (Griffiths et al 1993). This is thought to occur in greater than 50% of surviving lung transplant patients at 5 years and is a major cause of morbidity and mortality in lung transplant patients (Hosenpud et al 1995). Patients with this condition lose small and medium airways and are particularly susceptible to infection. Most patients die of respiratory failure (Wallwork 1994). The condition is manifest by bronchiolar epithelial ulceration, peri-bronchial inflammatory infiltrate and intra-lumenal plugs of mucus and necrotic debris. As the chronic process evolves, numbers of infiltrating macrophages and lymphocytes decrease and the bronchiolar lumen becomes obliterated by fibrosis (Yousem et al 1996). Progressive intimal hyperplasia and obliteration of the pulmonary arteries and veins is also present (Yousem et al 1996).

**Liver**

CAD of liver allografts develops in as many as half of long term recipients and contributes to about 20% of graft failure (Snover et al 1993). CAD of the liver is characterised by progressive focal bile duct occlusion or "vanishing bile duct syndrome" that affects predominantly interlobular bile ducts (Freese et al 1991, Hubscher et al 1991). The obliterative process is characterised by a cellular infiltrate consisting of T cells and macrophages. It is closely correlated with occlusive intimal thickening (Demetris et al 1997). Progressive arterial insufficiency is also associated with non-specific changes such as hepatocellular change, centrilobular ballooning, degeneration, atrophy and canalicular cholestasis are also seen. This is followed by gradual development of asymptomatic
jaundice with increasing levels of serum bilirubin, alkaline phosphatase and glutamyl transpeptidase (Snover et al 1993, Demetris et al 1997).

1.3 Incidence of CAD

Heart

The actuarial survival rate in heart transplant recipients is 60% at 5 years and 40% at 10 years. (Hosenpud et al 1996) and has remained largely unchanged despite improvements in short-term survival. In large multi-centre studies CAV is the major cause of death in-patients surviving 1 year after transplantation (Kaye 1993, Billingham 1987) with a 25% mortality due to CAV at 5 years. It is estimated to significantly affect more than 40% of recipients who survive beyond 4 years after transplantation (Gao et al 1990), although the overall frequency of the disease is even greater. The onset of the pathological lesions of chronic cardiac rejection may occur quite early, often within the first three months, and in a substantial number of cases within 3 to 5 years (Billingham 1994). Previous estimates of CAV were falsely lowered by the use of coronary angiography as a diagnostic tool. At 1, 2 and 4 years the actuarial likelihood of any angiographically visible CAV is 11%, 22% and 44% respectively (Costanzo et al 1998) whilst intimal thickening is detectable with ICUS in up to 75% of patients at year 1 (Yeung et al 1995).

Kidney, lung and liver

The rate of decline of other organ allografts are relatively similar: Pooled data from both North America and Europe show that the half life of cadaver renal allografts remains constant at 7 to 8 years despite 80% behaving satisfactorily at 1 year (Cecka et al 2000, Gjertson 1999). Lung allograft survival rates are poorer with less than 40% of patients surviving at 4 years (Hosenpud et al 1996). The lower incidence in liver allografts (50-60%
functioning at 5 years) has been attributed to several factors including their putatively lower immunogenicity (Demetris et al 1997). CAD is responsible for most late graft loss regardless of the type of tissue transplanted (Tilney et al 1991, Fellstrom et al 1989, Sibley 1994)

1.4 Aetiology of CAD

The clinical symptoms and decline in graft function that characterise CAD arise due to an abnormal fibroproliferative tissue response to injury. Subsequently much clinical and experimental work has studied the nature of the injurious stimuli and pathophysiology of these stimuli on the tissue response. A review of our current understanding of the aetiological factors is summarised below.

The aetiology of CAD is multi-faceted. Risk factors have been divided into antigen dependent events such as the influence of HLA matching and early acute rejection and antigen independent factors which include donor and recipient related factors, effects of brain death, drug toxicity, viral infections and other stimuli (Tullius and Tilney 1995, Weis & von Scheidt 1997, Hayry 1998).

Allograft vasculopathy bears certain similarities to normal coronary artery and peripheral vascular disease and it is possible that similar pathogenetic mechanisms may be at work (Ross 1999). Artherosclerosis as found in non transplant patients can be seen simplistically as focal intimal thickening and vascular remodelling occurring in response to low grade inflammation (mediated by macrophages and T lymphocytes). The inflammation occurs in response to various metabolic (hyperglycaemia and hyperlipidaemia, toxins i.e. smoking) and non-metabolic factors (hypertension and shear stress, chlamydiae pneumoniae infection) (Ross 1993). Whilst similar metabolic and non-metabolic processes are important in CAD the inflammatory component (due to ischaemia reperfusion, cellular and vascular rejection or viral infection) is substantially greater. As such the disease process is
considerably more rapid in onset and progression and more generalised in nature.

Minimising the frequency and severity of these risk factors is currently at the centre of clinical strategies to slow the progression of CAD and improve allograft survival.

Table 2. Etiological factors for CAD

<table>
<thead>
<tr>
<th>Antigen Dependent</th>
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<tbody>
<tr>
<td>Histocompatibility</td>
<td>Ischaemia/Ischaemia</td>
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<tr>
<td>Acute Rejection</td>
<td>Hypertension</td>
</tr>
<tr>
<td>Sub-optimal immunosuppression</td>
<td>Hyperlipidaemia</td>
</tr>
<tr>
<td>Non-compliance</td>
<td>Hyperglycaemia</td>
</tr>
<tr>
<td>Vascular Rejection</td>
<td>Age</td>
</tr>
<tr>
<td>Anti-donor antibodies</td>
<td>Gender</td>
</tr>
<tr>
<td></td>
<td>Race</td>
</tr>
<tr>
<td></td>
<td>Body Mass Index</td>
</tr>
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<td>Infection (CMV)</td>
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1.4.1 Alloantigen dependent events

The importance of host alloresponsiveness in the development of CAD is supported by the observation that allografts develop typical changes of CAD much more rapidly than isografts (Tullius et al 1994, Knight et al 1997) and that although the entire vascular tree of cardiac allografts is affected by CAV the native vasculature is spared (Russell et al 1993). Clinically, antigenic differences between host and donor increases the possibility that CAD will occur. HLA mismatches between donor and recipient influence survival of kidney
grafts in humans (Opelz et al 1993, 1994, Cecka and Terasaki 1994). Kidney transplants between identical twins have half lives of up to 20 years compared to 10 years for living related transplants with one haplotype mismatched (Cecka and Terasaki 1994). In addition kidney transplants from living related donors are less likely to develop CAD than those from cadaver donors although these figures are undoubtedly also affected by alloantigen independent factors such a brain death or ischaemic time. Experimentally, graft arteriosclerosis evolves more rapidly in rat recipients of heart grafts after pre-immunisation with donor splenocytes (Cramer et al 1990). Mouse heart allografts transplanted to SCID mice do not develop CAV unless infused with a donor-directed cytotoxic antibody (Russell et al 1994). Experiments in knock out mice demonstrate that that depletion of T cells, B cells, CD4 cells or macrophages all reduce CAV (Shi et al 1996) as do a number of immunosuppressive regimens including cyclosporin (Little et al 1996), rapamycin (Schmid et al 1995) mycophenolic mofetil (Raisanen-Sokolowski et al 1994) or CTL-4Ig (Russell et al 1996) in rat cardiac and aortic allografts.

1.4.1.1 HLA mismatch

The ability of the immune system to distinguish self from non-self protects the integrity of all host cells and allows reactivity against microorganisms and allografts. The cell surface structures recognised by T lymphocytes as self include both major (MHC) and minor histocompatibility antigens. Three classes of MHC antigen, denoted I, II and III are present on human chromosome 6. MHC genes exhibit extreme polymorphism (Stepkowski et al 1997a). The human class I MHC contains at least 17 genes with 3 well characterised loci encoding class I molecules: HLA-A, -B and -C as well as the lesser well known -E to -J. The HLA-A, -B and -C are highly polymorphic with 32,25, and 8 serologically defined specificities respectively. The class II MHC region of the human HLA complex contains at least 13 loci (6 α and 7 β chains) arranged in four clusters HLA-DP, -DR, -DQ and -DZ.
An obvious strategy for reducing transplant allorecognition is through donor recipient matching for the MHC loci. In humans histocompatibility matching considers primarily the class I HLA-A and HLA-B loci and the class II HLA-DR (and sometimes the HLA-DQ loci) (Duquesnoy and Demetris 1995).

Studies in kidney transplantation have shown that the degree of histocompatibility between donor and recipient is a major risk factor for the development of chronic allograft nephropathy and reduced long term survival (Cecka and Terasaki 1994, Opelz and Wujciak 1994). In particular HLA-DR mismatch is thought to be the most important (Opelz and Wujciak 1994). Several multi-centre and large single centre studies have demonstrated that HLA matching, especially for HLA-DR, reduces rejection (Zerbe et al 1991, Kerman et al 1993, Jarcho et al 1994) and increases graft survival in heart transplant patients (Opelz and Wujciak 1994). The role of MHC donor recipient differences in the pathogenesis of CAV is not clear however. Early reports on the role of HLA mismatch on the development of CAV have been conflicting. Some report no relationship between HLA incompatibility and CAV (Costanzo 1995), others report an increase in CAV with HLA mismatch (Uretsky et al 1987, Oliviari et al 1989) whilst a few report a reduction in CAV with HLA mismatch (Narrod J et al 1989, Billingham et al 1990). This confusion has been attributed to the small numbers included in single centre studies, polymorphism of HLA antigens, different immunosuppressive regimens between centres and the lack of sensitivity in the diagnosis of CAV by angiography (Costanzo 1995). In addition HLA mismatch is associated with increased deaths from both infection and rejection thereby making the interpretation of survival data difficult (Frist et al 1987). In cardiac transplantation, because ischaemic time is restricted to 4 hours, a prospective cross match is logistically difficult to perform, and therefore the number of well-matched patients in any study is often small. Single centre studies with small numbers of patients are therefore prone to conflicting results. Opelz et al (1994) reported on the effect of HLA matching on
cardiac allograft survival in 8331 patients from the Collaborative Transplant Study, 128 of which were well matched. HLA mismatch was clearly seen to affect long term survival of cardiac allografts, however, the greatest effect was evident within the first three months post transplant following which the slope of the survival curves for matched and poorly matched patients were similar. This may therefore reflect primarily an effect on early rejection and not on CAV per se. A report on 1072 patients from the Cardiac Transplant Research Database (Jarcho et al 1994) demonstrated that HLA mismatch was associated with increased frequency of acute rejection at 1 year and decreased patient survival at 2 years due to acute rejection. Another feature complicating interpretation of clinical studies of HLA matching is that until recently tissue typing has been by serological techniques rather than with DNA genotyping. Furthermore the use of DNA based molecular typing has demonstrated that a high proportion of HLA-DR locus typings used to assess histocompatibility in human renal transplantation were incorrect (Opelz 1997).

Animal studies have also highlighted the complexity of the effect of HLA matching on the development of CAD. CAD can develop in rats transplanted with a MHC identical graft (Guttman et al 1997, Schmid et al 1997) and also in genetically engineered animals lacking MHC genes (Xiao et al 1995). MHC deficient mice reject allografted skin as rapidly as fully immunocompetent recipients do however (Fedoseyeva 1994). This suggests other allograft specific antigens distinct from the MHC may play a role in the development of CAD. Little is known about the nature of the non HLA antigens although vascular endothelial cell, heart specific antigens, heat shock proteins and autoantigens have been suggested as likely targets for alloresponsiveness (Duquesnoy & Demetris 1995, Crisp et al 1994).

Finally an added complication in the consideration of the role of HLA mismatch in the development of CAD is the limited understanding of the immunopathophysiological mechanisms involved. There is conflicting evidence as to whether donor specific anti HLA
antibodies play a role in CAD (Rose 1996), or whether they play a role in cellular
allorecognition. Prolonged activated circulating T cell alloreactivity to donor HLA-DR in
patients with CAV occurs suggesting that indirect allorecognition of donor HLA-DR
peptides may occur in CAV whilst lymphocytes cultured from coronary arteries with CAV
do not exhibit much HLA specificity (Duquesnoy et al 1992). Furthermore although
formation of donor reactive antibodies is associated with lower graft survival there is no
compelling evidence that anti HLA antibodies play a role in CAV (Rose 1996). On the
other hand increased frequency of acute rejection episodes and a persistence of donor
reactive T cells are associated with more CAV (see below).

Overall there is little hard evidence for the role of HLA mismatch in the development of
CAD. There is good evidence that HLA mismatch is associated with reduced graft survival
however it is possible that this is related to the increase in frequency and severity of
rejection episodes and infection. The discrepancy between findings in heart and renal
transplantation patients is difficult to explain. Cardiac transplant patients tolerate acute
rejection less well than do renal transplant patients and correspondingly take larger doses
of immunosuppressants. In addition the lack of sensitivity in diagnosing CAV as well as
the low numbers of well-matched cardiac transplant patients further complicates the issue.
HLA mismatch may play a role in CAV, possibly by increasing acute rejection, but its
importance is questionable and other non-MHC antigens appear to be equally as important.

1.4.1.2 Acute rejection

The relative importance of acute rejection on the development of CAD is dependent on the
timing, severity, diagnosis and effective treatment of this process. Failure to account for
these factors in many clinical studies has produced conflicting results however there is now
a large body of clinical and experimental evidence that acute cellular rejection plays an
important role in the development of CAD.
Early angiographic studies in clinical cardiac transplantation from Stanford suggested no association between the incidence, onset, frequency or severity of acute rejection episodes as diagnosed by biopsy and/or composite scoring systems and the development or rate of onset of CAV (Gao et al 1990, 1996). Alternatively Uretsky et al (1987) demonstrated that patients who experienced two or more episodes of heart rejection were at higher risk of developing CAV, an observation supported by others, (Radovancevic et al 1990) whilst Costanzo-Nordin et al showed a correlation between the number of mild and total rejection episodes and the incidence of CAV (Costanzo-Nordin et al 1993). This latter study suggested that mild untreated rejection episodes may have a significant role in the development of CAV. This has been further supported by an autopsy study (Winters et al 1990) where clinically treated acute rejection episodes were weakly correlated with the severity of CAV but when all rejection episodes were considered there was a strong correlation between acute rejection and CAV. A more recent study using quantitative angiography has demonstrated that the number of acute rejection episodes is an independent risk factor for the early development but not late development of CAV, a factor that may also confound some cross sectional studies (Hornick et al 1997). These early studies have many deficiencies of which the most significant is that they were based largely on angiographic diagnosis of CAV. Angiography is known to drastically underestimate the presence of CAV, and the development of Intra Coronary Ultrasound (ICUS) as a diagnostic tool has led to more accurate estimates of the incidence and severity of CAV. A multi-centre ICUS study has demonstrated an association between the development of CAV and acute rejection—specifically early mild untreated acute rejection (Kobashigawa et al 1995, Brunner-la Rocha 1998). The timing of rejection episodes is also important and episodes of acute rejection occurring after 1 year are associated with subsequent development of CAV (Narrod et al 1989).
Acute rejection is also strongly related to CAD in other organ grafts including lung (Kroshus et al 1997) liver (Starzl 1997) and kidney (Almond et al 1993, Matas et al 1994). In renal transplantation several large single centre studies report that the absence of an acute rejection episode within the first year is a powerful predictor of long-term graft survival (Cecka and Terasaki 1994, Matas et al 1994, Ferguson 1994). Basadonna et al (1993) reported that in a cohort of 205 cadaveric renal transplant recipients the incidence of biopsy proven CAD was 0% in the 109 patients without acute rejection, 36% in the 69 patients with an acute rejection episode within the first two months after transplantation (P<0.001), and 63% in the 27 patients with acute rejection 60 days after transplantation (P<0.001). Matas et al (1994) demonstrated that multiple episodes of rejection or late rejection significantly reduced long-term renal allograft survival, whilst Massey et al (1996) showed that early rejection episodes (within the first three months) had no effect on late graft survival (Massey et al 1996). Acute rejections with complete functional recovery do not have a deleterious effect on the long-term outcome whereas an increased baseline serum creatinine level after treatment of a rejection episode is associated with CAD (Cosio et al 1997). The way acute rejection is treated can also influence long-term graft loss from CAD. In a retrospective analysis of patients treated for acute renal allograft rejection with either the anti-CD3 monoclonal antibody OKT3, prednisone alone or prednisone and Minnesota ALG, the use of OKT3 greatly reduced the incidence of CAD (Ferguson 1994).

Experimental studies in animal models have also demonstrated that acute rejection is a significant risk factor for CAD. Acute rejection episodes are associated with increased CAV in cardiac, aortic and renal allografts in rats (Cramer et al 1990, Hullet et al 1996, Yilmaz et al 1993). Rat cardiac allografts between major or minor histocompatibility mismatched strains develop a short lived acute rejection response followed by the histological development of CAD like histological changes. Attenuation of these acute
rejection episodes reduced the onset and severity of the CAD lesion (Geerling et al 1994, Cramer et al 1990) and delaying the initial immunosuppression accelerated its development compared to controls (Hullet et al 1996). Similar results were seen in rabbit aortic allografts (Nakagawa et al). Increasing the degree of acute inflammatory response by transplanting rat cardiac allografts with a stronger MHC mismatch increased the development of CAV (Schmid et al 1997, Geerling et al 1994). The role of acute rejection in the development of CAV is further supported by re-transplantation studies. In weakly immunogenic models that require no initial immunosuppression (Forbes et al 1997) re-transplantation to the donor strain up to 40 days reversed the inflammatory infiltrate and prevented the development of CAV. In stronger models however re-transplantation was seen to reduce but not prevent the development of CAV. Moreover in these stronger models re-transplantation after a critical period reversed the associated cellular infiltrate whilst the intimal proliferation not only did not diminish but continued to worsen (Schmid et al 1996, Hullet et al 1996, Mennander et al 1996). Taken together the results of these studies suggest that the severity of acute rejection is associated with the rate of progression of CAV in the rat and that this early immune mediated insult to the allograft can trigger the later development of CAV even when the allogenic stimulus is removed. This may explain the apparent failure of immunosuppressant agents such as cyclosporin to prevent CAD as well as the role of non-immune factors in the development of CAV.

CAD may develop as a result of insufficient immunosuppression and continued subclinical rejection episodes. The type of maintenance immunosuppression is therefore also an important factor when considering the effect of cellular rejection on the development of CAD. Low doses of cyclosporin in some studies has been associated with CAD (Almond et al 1993, Soin et al 1995, Salomon et al 1991). At 5 years post-transplantation the percentage of renal transplant recipients who were free of CAD as determined by biopsy
was 86% for those using cyclosporin>5mg/kg/day versus 77% for those on <5mg/kg/day (Almond et al 1993). Isomeni et al (1991) found that CAD was less apparent in patients receiving triple versus double therapy immunosuppressive regimens however this is not supported by other studies. Non compliance also indicates that CAD may result from inadequate immunosuppression (Bittar et al 1992, Dunn et al 1990, Troppman et al 1995).

In a study by the Minneapolis group, 34% of patients were non-compliant and this was associated with late deterioration in graft function. Against this argument is the fact that although cyclosporin is an efficient inhibitor of the T cell mediated immune response it has not reduced the incidence of CAD in heart, liver or renal transplantation since its introduction in the 1980s. More recently it has been suggested that, particularly in renal allografts, two different patterns of CAD may be present that reflect the variability of individual patients as well the multifactorial aetiology of the process (Paul 1999). On the one hand one group of patients with histological CAD may be under immunosuppressed and have ongoing subclinical rejection whilst the other is over immunosuppressed with inappropriately high levels of cyclosporin. High levels of cyclosporin as well as other immunosuppressants such as FK506 can themselves promote CAD by increasing the incidence of non-alloimmune risk factors (e.g. hyperlipidaemia, hypertension and hyperglycaemia) or as in the case of renal allografts by a direct toxic effect perhaps mediated by elevated TGFβ. Such patterns are evident in studies where immunosuppressive treatment is reduced in patients with CAD followed by an improvement in the renal function in some patients but a decline in the renal function of others (Paul 1999).

1.4.1.3 Vascular rejection and humoral mechanisms

Vascular rejection is humorally mediated. It is associated with characteristic deposits of immunoglobulin and complement in the coronary vasculature in combination with
endothelial swelling or vasculitis. Characteristically mononuclear cell infiltrates are absent (Olsen et al 1993). In cardiac transplantation vascular rejection is associated with a more rapid development of CAV and reduced cardiac allograft survival (Hammond et al 1992). Furthermore patients with cellular rejection had better long-term graft and patient survival whilst patients with mixed cellular and vascular rejection had intermediate survival (Hammond et al 1992). Heart transplant patients with vascular rejection are more likely to die from CAV related causes or to lose their graft secondary to CAV than patients demonstrating only acute cellular rejection. Patients with vascular rejection also had a higher incidence of graft loss due to cellular rejection. Similarly in renal transplantation vascular rejection has also been found to be an important early risk factor for early and late graft loss and CAD (van Sasse et al 1995, Norman et al 1991). In addition anti-B cell antibodies and/or anti-endothelial antibodies are associated with the development of accelerated CAV in human transplant patients (Crisp et al 1994).

Clinical and experimental data such as this would therefore suggest that patients with predominantly humoral alloreactivity (as opposed to cellular reactivity) have worse long-term outcome. However the multiplicity of mechanisms of antibody damage, the fact that many antibodies produced after transplantation are harmless and some may actually be beneficial, has made it difficult to evaluate the role of humoral responses on the development of CAV. It is extremely likely that every time recipient T cells are stimulated by an allograft, B lymphocytes are stimulated to make antibody (Rose 1996). The antibody response will include many specificities, some against alloantigens but some against "autoantigens" that is, antigens not normally encountered by the immune system but released as a result of T cell mediated damage. In addition many cardiac transplant patients develop circulating IgG and IgM type antibodies reactive both specifically with the heart and non-specifically with other tissues (Rose 1996). Hosenpud et al, in a study of patients with CAV, demonstrated low levels of alloantibody by flow cytometry whilst indices of
cell mediated reactivity were high (Hosenpud et al 1995) suggesting the finding of alloreactive antibodies may merely represent an epiphenomenon resulting from prior T cell mediated processes rather than contributing to the pathogenesis of CAV per se.

Most attention has concentrated on donor reactive or anti-HLA antibodies but it is now clear that antibodies against endothelial cells and other non-HLA antigens are possibly of equal importance. In addition the development of anti-idiotypic antibodies may contribute to immunological tolerance in some patients. In general studies of the role of the humoral response on the development of CAV suffer from confusion of nomenclature, short follow up and uncertainty as to the mechanisms involved in humoral responses.

1.4.1.4 Panel reactive antibody

Transplant patients often become allosensitised to foreign HLA alloantigens and HLA reactive antibody can be detected in patient serum following blood transfusions, prior transplants or pregnancy. The degree of humoral sensitisation can be assessed as the percentage of panel reactive antibody (PRA) by screening recipient sera against a panel of HLA-typed lymphocytes, representing all the common HLA antigens, in a complement dependent lympocytotoxic assay. The advantage of this test is that it can be performed at any time before transplantation. Although PRAs are commonly referred to as anti-HLA antibodies, unless they have been shown to be anti-HLA by blocking studies using monoclonal antibodies against MHC determinants, they do not necessarily have HLA specificity. In addition determination of donor specificity of these antibodies requires cross reactivity with donor lymphocytes. Most heart transplant centres now screen their patients for PRA’s before transplant. A majority of patients undergoing cardiac transplantation produce detectable panel reactive lymphocytotoxic antibodies following operation (Smith et al 1992). Patients with higher panel reactive antibody (>10-15%) experience lower cardiac allograft survival in some studies (Lavee et al 1991, Loh et al

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1994, Rose et al 1992) but not others (McCloskey et al 1989, Smith et al 1992, Kerman et al 1993). In particular, high responders (>50%) demonstrated a marked trend towards reduced survival in a study of 699 cardiac transplant patients (McCloskey et al 1989). In a study of 240 patients high PRA levels (>10%) were associated with increased frequency of angiographically evident CAV as well as increased mortality at 4 years (Rose et al 1992).

1.4.1.5 Anti HLA antibodies and donor reactive antibody

There is no compelling evidence that an antibody response to donor HLA determinants predisposes to the development of CAV. Anti-HLA antibodies are associated with acute rejection (Fenoglio et al 1989, Smith et al 1992), reduced long term survival in heart and renal allografts (Suciu-Foca et al 1991a, Suciu-Foca et al 1991b) and in one study HLA specific PRA formation was associated with the development of CAV (Smith et al 1992). In addition however anti HLA antibodies are found in up to 20% of long term cardiac transplant survivors (Suciu-Foca et al 1991b). A confounding factor in these studies may be the presence of soluble HLA antigen released by the damaged allograft cells, or anti-idiotypic antibodies binding to circulating immunoglobulin rendering their specificity difficult to interpret (Suciu-Foca 1991a, Suciu-Foca et al 1991b). Following depletion of circulating HLA antigen with immunomagnetic beads, high levels of anti HLA antibodies became detectable in the sera of patients with CAV, whilst long term survivors had much lower levels (Suciu-Foca et al 1991b). In addition the presence of HLA antigen/anti HLA immunoglobulin complexes was associated with reduced graft survival. A complex relationship between anti-HLA antibodies, soluble HLA antigens shed from the graft and anti idiootypic antibodies may influence graft outcome (Suciu-Foca et al 1991a, Barr et al 1993). In a study of 18 cardiac transplant patients Sucia-Foca et al demonstrated the presence of anti-anti HLA (anti-idiotypic) antibodies in the sera of long term survivors but not in that of patients who lost their graft to rejection. Similar findings are reported in
patients with long term renal allograft survival (Suciu-Foca et al 1991b). The presence of anti-idiotypic antibodies may explain the presence of detectable anti-HLA antibodies in long-term survivors and it may play a role in the development of hyporesponsiveness. Hyporesponsiveness to donor antigens whilst retaining responsiveness to third party antigens is reported in approximately one third of all kidney, heart and lung transplants in several single centre series (Reinsmoen et al 1993, Kubo et al 1995). These patients show no difference in their early acute rejection rate from non-hyporesponsive patients but they had significantly fewer instances of CAD and graft loss due to CAD. The appearance of donor reactive antibody is associated with increased severity of rejection (Suciu-Foca et al 1991b, Smith et al 1992) and increased vascular rejection (Cherry et al 1992). Approximately half of patients produce anti-HLA antibodies of donor specificity and approximately 80% produce anti-leucocyte antibodies with no identifiable HLA specificity (Cerilli et al 1987, Smith et al 1992). Other studies have shown that in the sera of patients who lost their grafts the frequency of anti HLA antibody in donor reactive antibody was 55% at 1 year and 74% at 2 years. Conversely long term transplant survivors showed low frequencies of anti–HLA specificity (Sucia-Foca et al 1991a,b).

1.4.1.6 Lymphocytotoxic antibodies

A lymphocytotoxic cross match is performed by co-incubating recipient serum with donor lymphocytes. Lysis of 10% to 50% of donor lymphocytes is defined as a positive cross match. This is routinely performed in renal transplantation but not in cardiac transplantation because of time constraints. Approximately 10% of patients have a positive cross match, which appears to be associated with reduced graft survival in some studies (Smith et al 1993, Yacoub et al 1987, McCloskey et al 1989) but not in others (Kerman et al 1993, Lavee et al 1991). In addition a positive cross match has not been associated with acute rejection in several studies (Lavee et al 1991, Zerbe et al 1991).
1.4.1.7 Significance of non HLA antigens

Little is known about the nature of the non HLA antigens. Antibodies against antigens not apparently found on lymphocytes can lead to hyperacute rejection of cardiac allografts (Brasile et al 1985, Trento et al 1988, Lavee et al 1991). Several studies have reported patients experiencing hyperacute rejection (thought to involve preformed antibodies) with negative lymphocytotoxic cross matches were found to have lymphocytotoxic antibodies against donor endothelium (Trento et al 1988) and Ig against cardiac muscle proteins (Dunn MJ et al 1992). Several authors have implicated non-MHC encoded alloantigens including a tissue specific antigen system that is shared by circulating monocytes and endothelial cells but not lymphocytes (Cerilli et al 1985). In patients with early rejection Western blotting of serum from lymphocytotoxicity negative cross match sera show strong bands of reactivity, often for self antigens, and often for heat shock proteins (Latif et al 1995). Western blotting of sera in cardiac transplant patients by Crisp et al (1994) demonstrated IgM reactive to endothelial membrane bound proteins associated with accelerated CAV (Dunn MJ et al 1992). These proteins have been identified as vimentin, a fibrillar protein found in endothelial cells, fibroblasts and proliferating smooth muscle cells (Crisp et al 1994). In addition retrospective studies of the sera from these patients demonstrated that the antibodies were formed in the immediate post transplant period, persisted until the time of diagnosis and were not associated with increased acute cellular rejection (Costanzo 1995). These are non-cytotoxic antibodies and it is possible that they exert their effect by endothelial dysfunction or activation rather than cytolysis. What is not clear is whether these antibodies are responsible for endothelial activation or represent a response to autoantigen following T cell mediated damage.
1.4.2 Alloantigen independent events

The importance of non alloantigen dependent events on the development of CAD is supported by the fact that in both clinical and experimental settings, kidney isografts develop vascular lesions comparable to those seen in CAD although at a slower rate (Tullius et al 1994). Late changes comparable to those of chronic allograft rejection have been observed to develop in human kidney transplants between identical twins (Tilney et al 1991). Similarly long term kidney isografts in rats develop functional and histological changes associated with macrophage infiltration and cytokine expression that had been documented in rat renal allografts at an earlier stage (Tullius et al 1994). In other experiments rat renal allografts were re-transplanted back into the donor strain at serial intervals after the initial engraftment to determine at what stage the CAD process could be reversed by removing the alloantigen dependent stimulus. It was shown that re-transplantation to the donor strain could reverse the process up to a certain time interval (12 weeks) after which time structural changes, particularly fibrosis, not only did not diminish but continued to worsen (Tullius et al 1994). In the same rat strain combination re-transplantation of cardiac allografts to the donor strain was seen to reduce levels of mononuclear infiltration but not levels of myointimal proliferation (Schmid et al 1996). These findings suggest that progressive organ injury beyond a certain point may become autonomous as well as alloantigen independent. Of equal importance is the suggestion that the initial insults, whether alloantigen dependent or independent occurring at an early stage post transplant are crucial in the later development of CAD.

Several non immunological mechanisms could contribute to the progression of CAV. These include donor characteristics (donor ischaemic time, age, pre-existing disease, brain death and donor sex) and recipient characteristics (age, sex, obesity, hypertension, hyperlipidaemia, insulin resistance and CMV infection). Much clinical and experimental
work in this field involves the kidney however it is likely that these conditions can be equally applied to cardiac and other allografts.

1.4.2.1 Ischaemic time and ischaemia reperfusion injury

Pre-transplant ischaemia

Ischaemia and reperfusion are unavoidable consequences of organ transplantation. There is strong evidence that the duration of cold ischaemia is involved in the pathogenesis of CAD specifically in renal transplantation but there is no compelling evidence for its role in clinical cardiac transplantation. ICUS (Escobar et al 1992, Hauptman et al 1995), angiographic (Wallwork et al 1994) and histopathological studies (Billingham 1990) have all demonstrated no significant role of cold ischaemic time on CAV. Similarly International Society of Heart and Lung Transplantation (ISHLT) data shows no effect of cold ischaemic time on patient survival post cardiac transplantation (Hosenpud et al 1996). This is no doubt due to the relatively short cold ischaemic time tolerated by hearts prior to transplantation (less than 4 hours) however there is experimental evidence in rat heart allografts (Schmid et al 1997) that it may potentially contribute to CAD. In renal transplantation the long-term survival of living donor transplants (related and non-related) is significantly better than that of cadaveric donors (Terasaki et al 1995). Furthermore UNOS registry data has demonstrated that >24 hours cold ischaemia is associated with poor long-term graft survival. In experimental rat heart (Knight et al 1997), aortic (Wanders et al 1994) and renal (Tullius et al 1994) allografts the degree of intimal hyperplasia is much more extensive after a long period of ischaemia. Ischaemia produces vascular lesions similar to CAV in cardiac and renal isografts (Schmid et al 1997, Tullius et al 1994) whilst subjecting the single native kidney of uninephrectomised rats to an ischaemic insult similar to that occurring during engraftment also results in late functional
and structural changes comparable to those observed in rat kidney isografts harvested at similar time intervals (Tullius et al 1994).

In addition to the cold ischaemic time factors such as pre transplant status of the donor, the inotropic support the donor received or the quality of the organ preservation mean that it is likely that allografts are exposed to a small but significant warm ischaemic insult that is difficult to quantify. Endothelial cell activation and infiltration of cell populations occurs following warm ischaemia in human renal allografts prior to transplantation similar to those seen following cold ischaemia (Goes et al 1995)

In an animal model of ischaemia reperfusion injury, rat heart allografts subjected to 3 hours of cold ischaemia before transplantation experienced significantly more CAV by 90 days in non ischaemic grafts (Knight et al 1997). Syngeneic grafts injured in a similar fashion did not develop CAV suggesting that the process was dependent on alloantigen dependent mechanisms. This is not a universal finding however and longer periods of ischaemia (4hours) have been shown to produce changes similar to CAV in syngeneic rat heart allografts (Schmid et al 1995)

Ischaemic reperfusion injury

Both warm and cold ischaemia result in ischaemic reperfusion injury (I/R). Several mechanisms of injury from early ischaemia and reperfusion have been suggested. The generation of free radicals and inflammatory eicosanoids before and subsequent to reperfusion are thought to be important mediators of the inflammation that characterises I/R injury. Significantly the administration of human recombinant super oxide dismutase (a free radical scavenger) or thromboxane synthase inhibitors has been associated with prolonged graft survival in clinical and experimental trials (Land et al 1994). Reperfusion following prolonged ischaemia in renal allografts leads to acute tubular necrosis resulting,
in severe cases, in delayed function. Other clinical studies have suggested that ischaemia reperfusion injury may only contribute significantly to late graft loss only in the presence of acute rejection (Halloran et al 1997). Graft inflammation following I/R appears to trigger upregulation of MHC antigens promoting the fenestration of activated lymphocytes in the allograft. This potentially increased immunogenicity of the engrafted organ may lead to increased allodependent injury and renal grafts subjected to >24 hours cold ischaemia have significantly more CD4 cells following reperfusion than those with <24 hours cold time. Organ grafts with prolonged ischaemia or delayed graft function experience an early acute rejection episode more often than grafts that functioned immediately. This apparent relationship between ischaemia, delayed graft function and acute rejection produces worse results over both the short and long-term than either injury alone in human renal transplants (Cole et al 1995, Pirsch et al 1996).
1.4.2.2 Donor related factors

Pretransplant donor related factors may be as important in determining the genesis of cardiac allograft vasculopathy as post transplant immunological and non immunological factors.

Brain death

The influence of brain death on the outcome of kidney transplantation has been suggested by the clinical observation that the behaviour of grafts from living related and unrelated donors has been consistently superior to those of cadavers (Terasaki et al 1995). Following brain death donor organs are subjected to a series of adverse haemodynamic, hormonal and metabolic changes that affect performance. This derangement is most marked in patients with explosive brain death (intracranial bleed, trauma). In an ICUS study of heart transplant recipients, hearts from explosive brain death donors had significantly more severe CAV than those from gradual brain death donors (Mehra 2000).

The pathophysiological mechanisms underlying these changes have been investigated in experimental models. The hyperdynamic state that follows brain death is believed to be due to an immense release of catecholamines into the circulation or "autonomic storm". Shortly following explosive brain death in baboons a Cushing's reflex is observed with massive increases in serum catecholamine levels, systolic pressures in excess of 400 mmHg, and an increase in cardiac output by 50%. Within 6 hours systolic pressure falls to below normal values but this is associated with physiological indices of myocardial damage, notably reduced compliance and contractility and impaired right ventricular function, a leading cause of mortality after heart transplantation (Chen EP et al 1996, Bittner et al 1996, Bruinsma et al 1997). In both humans and animal models this damage is characterised histologically by subendocardial disruption of myocytes and areas of focal infarction thought to be due to microvascular spasm secondary to catecholamine toxicity.
An additional metabolic insult is the rapid decrease in thyroxine, ADH and cortisol levels following brain death. This promotes further haemodynamic derangement, anaerobic metabolism and donor organ damage (Novitsky et al 1987, 1988).

The events surrounding brain death may alter the organ so that it is more prone to recipient alloimmunity. Such changes may profoundly influence early and late behaviour of donor organs. As well as producing direct end organ injury brain death may result in profound haemodynamic instability that can lead to periods of systemic inflammatory response, hypoxia or warm ischaemia (Tilney et al 1998). This can produce massive releases of cytokines, adhesion molecules and inflammatory mediators that may alter the immunogenicity of donor organs by increasing MHC and costimulatory molecule expression (Takada et al 1998). The suggestion of increased immunogenicity is supported by clinical studies that show that kidney transplants from non heart beating donors have fewer rejection episodes than those from traditional cadaveric donors who have often been subjected to length periods of physiological support following brain death and prior to organ donation (Nicholson et al 199199).

Donor age

Large scale multi-centre multivariate analysis has shown donor age to be a significant risk factor for mortality at both 1 and 5 years post cardiac transplant (Hosenpud 2000). ISHLT data demonstrates that donor age has a linear impact on patient mortality, with survival most adversely affected with a donor age >30 years. Multivariate and univariate analysis of the Papworth and Harefield data demonstrated that grafts from patients greater than 40 years were at significantly greater risk of developing CAV but were only slightly more likely to lose their grafts CAV (Sharples et al 1991). This is also in agreement with the Stanford analysis (Gao et al 1996). More recent ICUS studies have also demonstrated a correlation between older donor age and severity of CAV in heart transplant recipients.
Donor/recipient sex mismatch

Female sex is a significant risk factor for patient death following cardiac transplantation. Studies of cardiac transplant patients demonstrated significantly greater intimal thickening in male recipients of female allografts. Male recipients develop CAV earlier and more frequently than in females. This may be due to insufficiency of a small transplant in larger recipients due to increased haemodynamic shear stresses, a putative mechanism for the progression of CAD in renal allografts with a smaller nephron mass. Alternatively, this gender effect may be due to oestrogen. In rat aortic isografts, female gender protects against myointimal hyperplasia. Oestrogen protects against cardiovascular disease, and it has been demonstrated that oestradiol effectively inhibits transplant arteriosclerosis in experimental models.
1.4.2.3 Recipient related

CMV infection

Several clinical studies have suggested that viral infections, especially CMV, play a role in the pathogenesis of allograft arteriosclerosis in heart transplant patients (Grattan et al 1989, McDonald et al 1989, Everett et al 1992, although in some series no such association could be found (Sharples et al 1991, Weimar et al 1991, Scott et al 1990). It has also well established as a cofactor in CAD of human liver transplants and in obliterative bronchiolitis in lung transplantation (Koskinen et al 1997). Early angiographic studies in heart transplant patients associated CMV infection with increased severity of CAV and increased graft loss (Grattan et al 1989), increased frequency of CAV (McDonald et al 1989, Koskinen et al 1993) and patients with CAV were more likely to have CMV infection than those without CAV (Everett et al 1992). Koskinen and colleagues (1993b) demonstrated that CMV infection was associated with accelerated CAV particularly in the first two post transplant years but tailed off thereafter. Conversely a large multivariate analysis with adjustment for immunosuppression showed no such association between infection and angiographic evidence of CAV (Sharples et al 1991) and a more recent ICUS study has shown no association between CMV infection and severity of CAV (Escobar et al 1994). Multivariate analysis on risk factors for CAD in renal allograft recipients has shown no difference in the incidence of CMV infection in patients who did or did not lose their graft due to CAD (Massy et al 1996). CMV infection may therefore not be an independent risk factor but inter-related with for example immunosuppression or acute rejection or alternatively may be associated with early graft loss and therefore not detected by later or cross sectional analysis.

Experimentally CMV infection produces increased CAD in aortic, cardiac and lung allografts in rats (Lemstrom et al 1993, 1995, 1996). In rat cardiac allografts accelerated intimal hyperplasia associated with CMV infection took place earlier and was more intense
than that seen in non infected allografts and treatment with the antiviral agent gancyclovir reversed this process (Lemstrom et al 1994). The effect of CMV infection was self limiting however and tailed off such that no discernable difference existed between infected and non infected groups at three months (Lemstrom et al 1993) in keeping with the clinical findings mentioned above (Grattan et al 1989, Koskinen et al 1993b). In rat renal allografts CMV infection produced the characteristic changes of CAD in rat renal allograft glomeruli, tubules and interstitium. This was associated with increased episodes of acute rejection (Lemstrom et al 1995).

CMV is a DNA virus belonging to the herpes virus family. It is widely prevalent infecting 50-100% of adults depending on the population studied and is the most important infectious complication after organ transplantation. Various studies have reported the active infection rate in heart transplant patients at between 30-50% (Grattan et al 1989, McDonald et al 1989). CMV is able to turn into a latent state and reactivation can occur. It is thought that reactivation in the host is the most common cause of active CMV infection although both asymptomatic and symptomatic patients have been shown to share the same risk and neither primary nor reactivation/ re-infection alters that risk (Grattan et al 1989).

The site of residence of CMV is not known, but several cell types have been suggested such as vascular endothelium (Smiley et al 1988) and smooth muscle cells (Tumilowicz et al 1985) of the vascular wall. CMV nucleic acids have been identified in coronary arteries of patients with severe CAV (Hruban et al 1990). In heart and kidney transplantation CMV is associated with higher rates of acute rejection (Grattan et al 1989, Weimar et al 1991), (this is not seen in other studies see Koskinen et al 1993). This has been attributed to increased endothelial MHC expression, seen during CMV infection in renal transplants (Von Willebrand et al 1997), or cross reactivity between the CMV membrane protein and the HLA-DR B chain (Fujimani et al 1988) with subsequently increased immunogenicity. CMV infection has also been implicated directly in the pathogenesis of non-transplant
atherosclerosis (Melnick et al 1995). Theoretically reactivation of latent CMV infection in the vessel wall may lead to endothelial activation/injury with subsequent "response to injury" leading to generation of an atherosclerotic lesion (Ross 1993).
Metabolic recipient risk factors

Hyperlipidaemia

Hyperlipidaemia, reported in 60-80% of heart transplant patients, has been correlated with the development of CAV in angiographic (Gao et al 1996, Eich et al 1991), necroscopic (Winters et al 1990) and ICUS (Escobar et al 1994) studies. The combination of a lipid lowering agent, simvastatin, and a cholesterol lowering diet has been shown to reduce CAV and prolong cardiac allograft survival (Wenke et al 1995, 1997). Hyperlipidaemia is also associated with reduced graft survival in renal transplantation (Hamar et al 1997). In experimental studies hypercholesterolaemia has been shown to increase the severity of CAV in rat aortic allografts, rabbit cardiac allografts (Raisanen-Sokolowski et al 1994b, Alonso et al 1977) and mouse carotid artery allografts (Shi et al 1997). Administration of the lipid lowering agent simvastatin has been shown to prevent the development of allograft vasculopathy in rats (Meiser et al 1993). Several angiographic studies from Stanford showed no correlation between the incidence or severity of early CAV (<2 years) with hyperlipidaemia (Gao et al 1996) and Heroux et al (1994) reported total cholesterol as a risk factor in late but not early CAV (<2 years) in a small angiographic study. Hyperlipidaemia may therefore have a more insidious effect on the development of CAD relative to other factors. Early accelerated CAD may be associated with other factors such as, for example, rejection whilst hyperlipidaemia may be a more important factor in the development of CAD in longer surviving grafts where acute rejection has presumably been less severe with subsequently a less marked effect on the development of CAD.

Other clinical and experimental studies have shown no association between hyperlipidaemia and CAD (Scott et al 1992, Mennander et al 1993). This confusion arises due to a number of factors including the fact that serum lipid levels are affected by several other risk factors for CAD including immunosuppressive drugs, glucose intolerance and
obesity (Kemna et al 1994, Johnson et al 1992). Cyclosporin is thought to have its own independent effect on post transplant hyperlipidaemia (Kobashigawa et al 1997). It inhibits bile acid synthesis in animal models and reduces cholesterol gut loss thereby increasing total cholesterol (Kobashigawa et al 1997). Lipoprotein bound cyclosporin may also alter LDL clearance by interfering with the LDL receptor molecule (Kobashigawa et al 1997).

In addition cyclosporin increases the atherogenicity of hypercholesterolaemia by promoting oxidation of LDL at high doses (Apanay et al 1994). Oxidative modification of LDL has an important pathophysiological role, because it has been implicated in the generation of macrophage-derived foam cells, the hallmark of atherogenesis (Steinberg D et al 1989). Prednisolone also increases serum lipids however it tends to elevate HDL but not LDL cholesterol. Another confusing factor is the lipoprotein profile of the hyperlipidaemia considered in different studies. Some lipoproteins are considered injurious (triglycerides, LDL, VLDL) whilst others are considered to have a protective role in CAD (HDL, IDL). In clinical studies in heart transplantation CAD has been associated with elevated total cholesterol (Escobar et al 1994, Eich et al 1991), LDL cholesterol (Escobar et al 1994), and triglycerides (Gao et al 1996, Escobar et al 1994, Winters et al 1990) and reduced HDL cholesterol and apolipoprotein A1 (Valantine et al 1992) levels. Studies in the rat demonstrate that feeding recipients with cholesterol-cholic acid rich diet (increasing total and HDL cholesterol but not triglycerides) does not enhance intimal thickening in aortic (Mennander et al 1993) or cardiac (Adams et al 1993) allografts, but feeding them with a mixed cholic acid and glycerol diet (increasing total cholesterol and triglycerides) does. Elevated triglyceride levels have been shown to be an independent risk factor for the severity of intimal thickening ICUS in heart transplant patients.

Lipoproteins are also important components of the inflammatory response and experimental studies have suggested that lipoprotein profile may be important in mediating alloimmune injury. It has been suggested that other insults to the allograft such as acute
rejection or I/R injury may promote and enhance oxidised LDL injury of the endothelium, a mechanism suggested by the apparent enhancing effect of hypercholesterolaemia in strongly immunogenic models, but not in weak ones. In the DA to PVG allograft model, which generates a strong acute inflammatory response due to major and minor MHC mismatch, cholesterol rich diets did generate an accelerated CAV (Fellstrom et al 1990). In the less immunogenic model (DA to WF) cholesterol rich diets alone had no effect on allograft arteriosclerosis in rat aortic allografts (Raisanen-Sokolowski et al 1994b).

In summary elevated total cholesterol, (oxidised) LDL cholesterol and serum triglycerides appear to have an important role in the generation of CAV. This clearly represents a parallel with the role of hyperlipidaemia, notably oxidised LDL, in the generation of ordinary atherosclerosis (Ross 1999).

**Body mass index**

Grady et al (1991) demonstrated that cardiac transplant patients have a persistent elevation in their body weight post transplantation and others have correlated this with hyperlipidaemia (Keogh et al 1988). Winters et al (1990) demonstrated that Body Mass Index (BMI) was one of the most significant predictors of severity of CAV in failed human allografts. This has been supported by recent ICUS studies that have demonstrated a correlation between BMI and the incidence and severity of intimal thickening (Escobar et al 1994, Hauptman et al 1995). Similar findings are seen in renal transplants (Cecka and Terasaki 1992). Patients with a BMI>30 have significantly reduced graft survival at three months however the deleterious effect on graft survival is not seen at one year.

**Hyperglycaemia**

The role of diabetes in the development of CAD is unclear. A recent ICUS study has suggested that glucose intolerance and insulin resistance both frequently observed after
heart transplantation (Kemna et al 1994) were strong predictors of intravascularly visible intimal thickening in heart transplant patients. Similar findings have been reported in angiographic studies (Narrod et al 1989). In renal transplantation hyperglycaemia/ diabetes mellitus is associated with reduced graft survival. In heart transplantation other angiographic and ICUS studies have reported no association between diabetes and CAV however (Escobar et al 1994, Eich et al 1991, Gao et al 1996), whilst in renal transplantation death with a functioning graft, particularly for cardiovascular diseases, is the major reason for graft loss in diabetic patients.

**Hypertension**


**Recipient age**

Multivariate analysis of the data from the Registry of the International Society for Heart and Lung Transplantation suggests that older recipient age is a significant independent risk factor for graft loss at 1 and 5 years. The impact of recipient age is not linear but begins to have an adverse effect on survival in the middle of the sixth decade. This is in agreement
with other multivariate analysis (Sharples et al 1991, Gao et al 1996). A multi-centre ICUS study has shown no association between recipient age and intimal thickening however (Hauptman et al 1995) suggesting that recipient may not be an independent factor in the development of CAV. Recipient age is also a risk factor for graft loss in renal transplant recipients (Cecka and Terasaki 1994), however if graft loss caused by patient death is excluded the impact of recipient age is excluded. Young recipients (<5 years) also have decreased transplant survival in heart transplantation (Hosenpud et al 1996).

**Recipient race**

Race of allograft recipients is an important determinant of renal transplant survival in the United States. Five-year graft survival rates were 66% for Asians, 61% for Caucasians and Hispanics, and 47% for black recipients (Cecka 2000). Black recipients of heart transplants develop also CAD earlier than non blacks (Costanzo et al 1998). Multivariate analysis however has suggested that a substantial part of the effect of race is related to socioeconomic factors. This was demonstrated in the study where blacks transplanted in Canada showed the same survival rates as whites, thus indicating the influence of health care system and socioeconomic factors (Koyama H et al 1994). Multivariate analysis has also demonstrated that blacks in the US tended to have worse HLA matching and higher presensitisation than whites (Cecka 2000). This is because most blood and organ donors are white. The absence of the Duffy antigen receptor for chemokines on erythrocytes in African Americans seems an important risk factor for the development of CAD.
1.5 Summary

CAD, characterised in all organs by the development of transplant arteriosclerosis and organ fibrosis, has a multifactorial aetiology. This includes donor-related factors that relate to the size and functional status of the organ prior to brain death, donor age and sex, the adequacy of organ protection both before (brainstem death) and after harvest (cold ischaemia and preservation technique). Once transplanted into the donor the severity of the early inflammation within the graft is particularly important (acute rejection, ischaemia reperfusion injury) whilst other factors such as recipient metabolic derangements (hyperlipidaemia, hyperglycaemia), hypertension and metabolic requirements (body mass index, sex) may have a more insidious effect. A chronic inflammatory infiltrate develops within allografts possibly in response to these stimuli however a subsequent CAD-specific alloimmune response is also likely to be important. The combination of these injurious stimuli produces a characteristic response in allografted organs, i.e. extracellular matrix deposition in the neointima and parenchyma analogous to scar formation. The end result varies in the rapidity of its onset and in its morphology and this spectrum reflects the interaction of different risk factors to different extents on the disease process. The pathophysiological mechanisms by which they may each affect the development of CAV is considered below.
Part 2. Pathogenesis of CAD

Part 2A. The inflammatory response

CAD, characterised by vascular neointimal thickening and organ extracellular matrix accumulation, can be seen as a response to injury phenomenon. The most important injury, at least in the initial period post transplantation is the inflammatory response that occurs due to a number of factors including rejection, ischaemia reperfusion, and potentially CMV infection.

2A.1 Ischaemia/ischaemia reperfusion injury

Ischaemic damage

The changes produced by ischaemia are well known. Initially hypoxia causes a breakdown of oxidative metabolism and a diminution of high energy phosphates. The depletion of adenosine triphosphate stores inhibits active ion transport systems. This leads to an increase in intracellular sodium, calcium and subsequently water with cell swelling (Weight et al 1996, Day et al 1995). This loss of extracellular water is compensated for by translocation from the intravascular compartment to the interstitium producing haemoconcentration, hyperviscosity, capillary sludging and diminished perfusion (Weight et al 1996). This diminished perfusion is further exacerbated by endothelial cell swelling and vascular occlusion. In addition, the increase in intracellular calcium leads to activation of membrane phospholipase A2 with subsequent generation of the pro coagulant platelet activating factor (PAF) and the release of leucotrienes. High intracellular calcium levels also lead to the increased calmodulin dependent activation of constitutive nitric oxide synthase (cNOS) with increased nitric oxide production (Weight et al 1997). Hypoxia also leads to the production of inducible nitric oxide synthase (iNOS). Cold ischaemic time has been correlated with levels of endothelial iNOS expression in a rat aortic allograft model (Akyurek et al 1996). Once synthesised iNOS is active and generates NO. High levels of
NO themselves can activate or damage endothelial cells and leucocytes. Another feature of tissue hypoxia is the activation of the complement cascade (Day et al 1995). Transient ligation of coronary arteries in animal models results in the deposition of C1q and C5b-C9 (the membrane attack complex or MAC) on vascular endothelial cells, the accumulation of leucocytes (Weisman et al 1990, Litt et al 1989) and the generation of the anaphylotoxins C3a and C5a. C1q activates platelets, neutrophils and macrophages (Peerschke et al 1993, Bobak et al 1987) whilst the insertion of the MAC into cell membranes leads to endothelial activation with increased leucocyte adherence, growth factor and cytokine release. In addition the anaphylotoxins promote an acute inflammatory response with tissue oedema, recruitment of granulocytes and monocytes (Baldwin et al 1995). The end result of prolonged ischaemia is intravascular platelet accumulation and thrombosis, leucocyte aggregation, tissue acidosis and acute inflammation. Ultimately cellular autolysis (swelling of lysosomes, dilatation and vesiculation of endoplasmic reticulum, leakage of enzymes and proteins and loss of cellular compartmentalisation) occurs; membrane integrity cannot be maintained and tissue necrosis follows (Day et al 1995, Weight et al 1996)

Reperfusion damage

Re-establishing blood flow has two beneficial consequences for ischaemia: the energy supply is restored and toxic metabolites are removed. This reperfusion also induces further local tissue injury the degree of which correlates with the duration of the ischaemia. Reperfusion injury is mediated by the interaction of free radicals, nitric oxide, endothelial cells and leucocytes.
Free radicals

Several studies have implicated oxygen derived free radicals (unstable molecules containing one or more unpaired electrons) as the mediators of the reperfusion component of the ischaemia reperfusion injury (for review see Grace 1994). Several oxygen free radicals can be produced by reduction or excitation of molecular oxygen including superoxide, perhydroxyl and singlet oxygen. Superoxide is produced in small quantities by normal cellular metabolism but is produced in large quantities following I/R (Welbourne et al 1991). This reacts with molecular water leading to the production of hydroxyl radicals. The hydroxyl radical is the most reactive of the free radicals in biological systems and is probably responsible for most of the cellular damage that occurs from free radicals (Weiss 1986). It causes lipid peroxidation, oxidises sulphydryl groups, inactivates cytochrome enzymes and alters membrane transport systems. Superoxide may also react with endothelial cells to promote the formation of inflammatory mediators such as platelet activating factor (PAF) (Lewis et al 1988) or leucotriene B4 (LTB4) (Lehr et al 1991).

In ischaemia reperfusion the enzyme xanthine oxidase appears to be a major source of free radicals. During ischaemia its substrate hypoxanthine (an adenosine 5 monophosphate (AMP) derivative) accumulates in the tissues as do levels of the enzyme itself. On reperfusion of the tissue the second substrate of the enzyme, oxygen, is introduced and the reaction proceeds producing a "respiratory burst" of free radical production (Welbourne et al 1991, McCord et al 1985). Other sources of free radical production are the Fenton reaction and the plasma membrane during the production of eicosanoids (Weight et al 1996). In the Fenton reaction ferric iron, probably derived from heme, is reduced to the ferrous state by superoxide and is then able to donate an electron to hydrogen peroxide, which then dismutates to hydroxyl radicals. These free radicals cause peroxidation of membrane lipids with subsequent production of further reactive species and membrane
damage (Grace 1994). Other cellular components at risk of damage from free radicals are proteins and nucleic acids. Reactive oxygen species have been shown to influence the expression and activation of nuclear factor kB (NF/kB), a nuclear factor that plays a key role in the transcriptional role of several cytokines involved in endothelial and leucocyte activation, initiate changes in local eicosanoid synthesis and interact with NO.

**Nitric oxide**

Nitric Oxide is also thought to be an important mediator in I/R injury however its precise role is unclear. In renal I/R injury conflicting evidence from in vitro and in vivo work suggests that NO may be either cytotoxic or cytoprotective respectively (Weight et al 1998b). NOS activity is elevated early in ischaemia but evidence for its elevation during reperfusion is conflicting (for review see Weight et al 1998b). In studies of rat renal reperfusion injury it has been suggested that NO is essential in maintaining renal blood flow post I/R. High levels of NO produce cell damage but they also reduce the neutrophil respiratory burst and inhibit the activity of cyclo-oxygenase thereby reducing eicosanoid production (Weight et al 1998b). In combination oxygen radicals and NO can combine to produce peroxynitrite which breaks down to the toxic hydroxyl radical producing further membrane damage, endothelial activation, leucocyte and platelet aggregation and a reduction in protective species (Weight et al 1996, Day et al 1995, Grace 1994).

**Leucocytes**

The first cells to infiltrate allografts following I/R are neutrophils. Neutrophil activation and infiltration is mediated by free radicals, pro inflammatory eicosanoids and activated complement (Welbourn et al 1991b) Infiltrating neutrophils in turn further aggravate inflammation and endothelial activation within the graft by releasing free radicals and proteases (Welbourn et al 1991b). The importance of neutrophils in I/R has been
demonstrated in experimental studies in dogs where systemic depletion of neutrophils inhibited the acute inflammatory response to I/R (Romson JL et al 1983). Inhibition of P-selectin, the key adhesion molecule involved in the earliest events in the adherence of circulating leucocytes to endothelium, post I/R, prevented early neutrophil infiltration, increased expression of class II MHC and later T cell infiltration (Takada et al 1997). Neutrophil activation is therefore important for the activation of other leucocyte subgroups by producing an early increase in inflammatory cytokines.

Ischaemia and reperfusion is also associated with lymphocyte infiltration (predominantly CD4) and this initially is independent of allogenicity. Heeman et al demonstrated that the pattern of cellular infiltration and cytokine expression in both syngeneic and allogenic cardiac grafts was similar within the first 24-48 hours after engraftment (Heeman et al 1994). The importance of these lymphocytes in endothelial activation and the subsequent allogenicity of the graft have been demonstrated in experimental models. Inhibition of T lymphocytes by either blocking of the CD28 molecule or administration of cyclosporin (Takada et al 1997c, Kouwenhaven et al 1999) attenuates the severity of I/R whilst in non transplant models CD4 deficient mice have significantly less hepatic damage following in situ ischaemia compared to wild type mice.

2A.2 Response of vascular endothelium to ischaemia and reperfusion: endothelial activation and the cytokine adhesion molecule cascade

The infiltration of transplanted organs by lymphocytes and macrophages, the key features of CAD development, are dependent upon the specific adhesion of these cells to graft endothelium followed by migration (Libby 1989). "Resting endothelium" is immunologically unreactive, negative for MHC Class II and has low levels of expression of co-stimulatory molecules. In normal conditions inflammatory cells come into contact with endothelium in a random fashion. In organ transplantation the endothelial cells are
activated by ischaemia and reperfusion injury. Upon activation endothelial cells retract, express adhesion molecules such as P selectins and integrins and release increased amounts of cytokines establishing a cytokine gradient near the vessel wall. Cytokine release in turn promotes growth factor release, increased platelet aggregation, eicosanoid production and increased lipid accumulation (Waltenburger et al 1996, Takada 1997b, Libby 1989, Young-Ramsaran 1993. Binding of L selectins on lymphocytes to their ligands on endothelial cells initiates slowing and transient sticking of the leucocytes to the endothelium (Azuma et al 1994). As this early selectin binding is weak flowing blood forces the cells along the endothelium producing a rolling effect. Activation of different cell populations may be dependent on the type of selectin expression and cellular infiltration is possibly controlled by expression of these molecules. These rolling leucocytes are further activated in the cytokine gradient (Azuma et al 1994, Jutila 1994) and express other classes of adhesion molecules including the β 2 integrin LFA-1 and β 1 integrin VLA-4 that interact with ICAM-1 and VCAM-1 on the endothelium respectively (Azuma et al 1994, Jutila 1994, Orosz 1994). Interactions between β 1 and β 2 integrins and their ligands may facilitate leucocyte extravasation and migration through the vessel wall. β 1 integrins may facilitate a change in shape of leucocytes as well as influencing their interaction with extracellular matrix proteins and migration through the tissue (Azuma et al 1994, Orosz 1994, Jutila 1994).

These processes are accompanied by up regulation of MHC Class I II molecules and costimulatory ligands for T cell and macrophage activation such as CD28 and CD40. (Azuma et al 1995, Takada 1997). Even short periods of ischaemia have been shown to upregulate the expression of MHC molecules in humans (Goes et al 1995) and in animal models (Takada et al 1997a, Azuma et al 1995). In a rat kidney I/R model this MHC upregulation occurs at 24-48 hours post I/R and correlated with a prior increase in IFN γ which itself can be upregulated by the release of TNF α by neutrophils (Takada et al
Donor endothelial cells can now act like antigen presenting cells acting in the direct pathway of T cell alloantigen recognition (Pober et al 1996). Specific interactions between T cell receptors of lymphocytes and allogenic peptide MHC complexes expressed by the vascular endothelium permit the release of specific lymphokines, and in the presence of costimulation facilitates T cell proliferation and differentiation (Fellstrom et al 1998). Ischaemic injury to the heart causes the release of heat shock proteins (Day et al 1995), furthermore damaged endothelium may release donor endothelial antigens (Day et al 1995). In combination with the upregulation of MHC these factors may promote an increased alloimmune response by promoting peripheral T cell activation and immunoglobulin production.

Ischaemia reperfusion injury can therefore be considered as a link between the cytokine adhesion molecule cascade and the alloimmune response via activation of lymphocyte endothelial interaction and indirectly via immunoglobulin deposition. Evidence to support this hypothesis has been shown in both clinical and experimental studies. In a double blind, prospective, randomised, placebo controlled trial, it was shown that a single dose of recombinant superoxide dismutase, given intravenously at the time of transplantation significantly reduced both acute rejection episodes and CAD in kidney transplants (Land et al 1994). In addition prolonged ischaemia is associated with a higher incidence of both acute rejection and CAD in human liver transplantation (Gomez et al 1994). Ischaemic reperfusion injury may also interact with other aetiological factors in CAD. Ischaemic reperfusion injury may promote CMV infection either through increasing the grafts susceptibility to infection (CMV is known to have a tropism for damaged tissue) or by reactivating latent CMV infection (Day et al 1995). This is supported by the histological identification of CMV infected cells in areas of ischaemic injury seen in endomyocardial biopsies from heart transplant patents (Gaudin et al 1994). I/R also interacts with non
alloimmune factors. A feature of reperfusion injury is the appearance of oxidatively modified lipoproteins (Fellstrom et al 1998). It has been shown elevated levels of oxidised lipoproteins and diminished levels of anti-oxidants have been associated with CAD in human renal allografts (Ghanem et al 1996) and oxidatively modified LDL cause changes in endothelial cells, smooth muscle cells and macrophages in vitro (Frostegaard 1991, 1990a,b). Ischaemia reperfusion may interact with other metabolic abnormalities in the production of CAV. Ischaemia reperfusion injury in diabetic rat kidneys results in a progressive deterioration in renal function with histological changes similar to CAD after only 30 minutes of cold ischaemia. These changes were not present after a similar insult in non diabetic kidneys (Melin et al 1997). Ischaemia may initiate the process of chronic injury to the endothelium thereby amplifying the effect of other aetiological factors and leading to the development of the CAV lesion.

2A.3 Alloimmune injury

The immunological injury to the graft may be mediated by donor HLA antigens, endothelial cell antigens or by other tissue specific antigens (Duquesnoy & Demetris 1995). Alloantigens of the graft may stimulate the poliferation of T helper and T cytotoxic cells. The T helper cells can release cytokines that may promote B cell proliferation and the secretion of antibodies against donor antigens (Lemstrom et al 1995, Azuma et al 1994).

Allorecognition

Allorecognition involves genetically different molecules encoded by the major histocompatibility complex (MHC) that can activate T lymphocytes. Two pathways have been recognised (see Duquesnoy & Demetris 1995).
1. The direct pathway involves peptides in the groove of allogenic MHC molecules expressed on donor antigen presenting cells (APCs).

The direct pathway has been considered the primary mechanism of donor specific cytotoxic T cell mediated graft injury in acute rejection. Direct recognition also promotes adhesion of alloreactive T cells to the vascular endothelium, their activation, release of lymphokines and other inflammatory mediators.

2. The indirect pathway involves the TCR recognition of donor MHC peptides in the groove of self-APCs. This requires the release of donor peptides by donor cells and their uptake and processing by recipient APCs. Indirect T cell activation is associated with humoral alloimmune mediated injury.

2A.3.1 T cell mediated immunological injury

Direct T cell activation and acute cellular rejection

T cells recognise antigens through the T cell receptor (TCR). The TCR is a heterodimer of two covalently bonded polypeptides. MHC molecules carry many peptides in their grooves that can be presented to the T-cell receptor (TCR), and this interaction involves the binding of the TCR to the α helix of the MHC molecule. Allorecognition results from amino acid sequence differences between donor and recipient MHC molecules. Activation of T cells results from complex and interdependent signals delivered by the TCR, costimulatory molecules and cytokines (Duquesnoy & Demetris 1995). To initiate T cell proliferation, a second non-specific signal is delivered by binding of the CD28/B7 molecules. CD28 receptors are constitutively expressed on most resting CD4 cells and about one half of CD8 cells in human peripheral blood. Their expression is increased by T cell activation. B7 ligands are expressed rapidly by antigen presenting cells (APCs) following activation. Following T cell activation a second high affinity receptor for B7, CTL4 is expressed. In addition T cell activation may be facilitated by co-receptors. CD4 and CD8 ligands
recognise MHC Class II and Class I molecules respectively and act as accessory molecules to increase the avidity and stability of this interaction. Activated CD4+ cells (T helper 0; Th0) may become Th1 which produce IL-2, INF γ and TNF, or Th2 (see below). Cell mediated injury is mediated by direct activation of Th0 cells by donor MHC molecules which in turn promotes differentiation into the Th1 type. Donor macrophages and other antigen presenting cells (including activated endothelium) modify and present donor specific antigen in association with MHC class II molecules. In conjunction with IL-1 production this produces activation and clonal expansion of T helper cells. The subsequent release of IL-2, INF γ, TNF α and other cytokines by activated Th1 cells results in the recruitment, activation and clonal expansion of cytotoxic T cells (CD8), natural killer cells and B cells. IL-2 is particularly important in promoting clonal proliferation of alloreactive Th1 cells and stimulates CD8 cells to develop into mature cytotoxic effector cells. Perforin and Granzyme B, proteins present in the granules of activated CD8 cells and natural killer cells, are the effectors of cell mediated injury. Perforins are pore-forming molecules that can polymerise and perforate cell membranes (Persechini et al 1990). Granzyme B belongs to a family of serine proteases that is co-secreted with perforins from cytoplasmic granules in cytotoxic T lymphocytes (Pasternack et al 1985). Granzyme B entry into cells via perforin created pores catalyses target proteins thereby activating a pathway leading to apoptosis (Nakajima et al 1994). Fas Ligand, a transmembrane protein belonging to the tumour necrosis family of ligands is expressed on cytotoxic T lymphocytes and binds with the Fas receptor on target cells, inducing a distinct pathway leading to target cell apoptosis (Rouvier et al 1993). In the absence of immunosuppression this injury is sufficient to produce graft failure.
2A.3.1.1 The role of acute rejection on the pathogenesis of CAD

Acute rejection may promote the development of CAV by a number of mechanisms. The first and perhaps the most important of these is the direct response to injury of the graft to cell mediated immunological damage. Graft injury is associated with increased production of growth factors by activated endothelium and vascular smooth muscle cells whilst the release of INF γ and TNF α ligands by T cells may directly stimulate smooth muscle cell proliferation prior to migration and extracellular deposition. Failure to adequately reverse this process may therefore result in graft damage. Clinically this may be manifest as multiple rejection episodes, failure to revert to normal graft function post treatment, ongoing subclinical rejection, under immunosuppression and non-compliance.

Experimentally the role of direct cell mediated damage in the development of CAD has been demonstrated in animal models. The blockade of T cell costimulatory molecules CD28/B7 and CD40/CD40L decreases T cell infiltration and inhibits intimal hyperplasia in murine aortic and cardiac allografts (Basadonna et al 1993, Geerling et al 1994, Russell ME et al 1996, Sim et al 1997). Furthermore carotid artery allografts in CD4 deficient mice develop intimal thickening to only 40% of that in controls (Shi et al 1996).

Cell mediated rejection may also promote CAD by the recruitment of macrophages to the allograft. The continuous presence, the activated state and the upregulation of macrophage associated cytokines in allografts with CAD suggests a pivotal role for the macrophage in this process. In rodent models of CAD mononuclear cell infiltration in the later stages is dominated by macrophages whilst T cell numbers dwindle (Azuma et al 1994b, Russell et al 1995). CAD is prevented in rat renal allografts by the administration of the macrophage inhibitor gammalactone (Azuma et al 1995), whilst carotid allografts in mice deficient in macrophages develop only slight intimal hyperplasia (Shi et al 1996). T Helper cells promote macrophage recruitment and activation by the release of γ interferon, TNF α and
the macrophage chemoattractant RANTES (Regulated upon activation, normal T cell expressed and secreted) (Nadeau et al 1995, Russell et al 1993). T cell and monocyte derived TNF α and interferon γ also serve to further upregulate MHC expression as well as that of the adhesion molecules ICAM and VCAM (Orosz 1994). Other cytokines such as IL-8, Macrophage chemoattractant protein (MCP) -1, Macrophage inflammatory Protein 1β, (MIP-1) and osteopontin released by activated endothelial, interstitial and smooth muscle cells in response to injury are also chemotactic for macrophages (Russell et al 1993, 1995). The release of IL-8 including MCP-1 augments the expression of MAC-1 on macrophages and facilitates its interaction with ICAM-1. Upregulated adhesion molecules contribute to their localisation within the graft where they are activated by IFN-γ.

Activated macrophages produce a number of cytokines including TNF α, IL-1 β, PDGF, bFGF and TGF β (Lemstrom et al 1995, Azuma et al 1994). These growth factors in particular are crucial mediators in the development of neointimal hyperplasia and allograft fibrosis.

2A.3.1.2 Other mechanisms of cell mediated immunological damage

Activated Th cells (predominantly CD4) can be categorised as Th1 and Th2 based on their pattern of cytokine production and effector function. Whilst Th1 cells are associated with cell mediated injury as described above Th2 cells synthesise IL4, IL5, IL10 and IL13, and are involved in humoral responses (Duquesnoy & Demetris 1995). Whilst cell mediated injury is an important aetiological factor in CAD potent inhibition of direct CD4 Th1 cell activation by cyclosporin, FK 506 or anti IL-2R monoclonal antibodies these therapies have failed to prevent the development of CAD in clinical transplantation. It has therefore been suggested that other immunological mechanisms may also be important in the development of CAD. One suggestion (reviewed in Shirwan 1999) is that by inhibiting Th1 activation Th2 cell activation is promoted and that this may contribute to the
development of CAD by mechanisms other than direct cell mediated injury. This is supported by the observation that activated CD4 Th1 cells are not seen in substantial numbers in long surviving allografts with CAD, Th2 cytokine profiles predominate in grafts with CAD, Th2 activation is not inhibited by calcineurin inhibitors (Shirwan 1999). Th2 cells are preferentially activated by the indirect pathway, which is not IL2 dependent and in fact this pathway may be promoted by the effective inhibition of the IL2 dependent Th1 pathway by cyclosporin and Tacrolimus. Graft infiltrating macrophages have the ability to act as antigen presenting cells and these activate T cells via the indirect pathway by presenting processed antigen in association with self MHC molecules as well as other sites such as spleen or recipient lymph nodes. The Th2 response is characterised B cell antibody production (considered below) and it is possible that this may be an important factor in the development of CAD in longer surviving allografts. The release of Th2 cell growth factors such as TGF β, IGF and PDGF may directly result in activation of smooth muscle cells leading to neointimal hyperplasia and extracellular matrix accumulation (fibrosis). Th2 cytokines, particularly IL4 and IL10, as well as TGF β directly influence metalloproteinase expression as well as that of their inhibitors by smooth muscle.

An apparent paradox in the pathogenesis of CAD is the lack of donor alloresponsiveness in T cells cultured from long surviving allografts with CAD (Murase et al 1995). As well as being categorised by their TCR costimulatory molecules (CD4/CD8) T cells can also be categorised according to their TCR polypeptides. These are predominantly α and β chains. Both chains have variable regions and their α and β genes undergo rearrangements to generate T cell specificity towards antigenic polypeptides presented by MHC class molecules. Some categories of TCR are composed of Gamma/delta chains and these TCRs appear to interact predominantly with non-MHC antigens. Little is known about the function of gamma/delta cells although they appear to show reactivity towards heat shock
proteins (HSP) and E selectin (Jutila 1996). Heat shock proteins are highly conserved molecules expressed on endothelium and vascular smooth muscle following stress such as hypoxia, ischaemia reperfusion or following endothelial activation that are thought to have immunomodulatory and protective functions. Gamma/ delta T cells are found in large numbers in lymphocyte cultures propagated from coronary arteries with CAV (Vaessen LM et al 1992, Duquesnoy et al 1992) and graft infiltrating T cells from chronically rejecting rat hearts reacting to self APC proliferate markedly in the presence of HSP (Duquesnoy et al 1999). The significance of this pathway in the development of CAD is poorly understood but this may be associated with ongoing cellular alloreactivity towards non-MHC antigens. Failure of current immunosuppressive regimens to inhibit this pathway (Duquesnoy et al 1999) may also in part explain the failure of these drugs to prevent the development of CAD in clinical transplantation.

2A.3.2 B Cell mediated injury

Resting B cells bind antigen by membrane bound immunoglobulin (Ig) that serves as a B cell receptor (BCR) and initiates activation via a similar mechanism to the TCR. Diversity of the BCR is generated by the v regions of light and heavy chains. B cells process foreign antigen and present it in association with recipient MHC II to T helper cells (B cells express CD40 molecules constitutively that bind to CD40 ligands expressed by activated T cells). Th cells in turn promote activation and clonal expansion of immunoglobulin producing cells by the release of cytokines including IL-4, IL-5, IL-6, IL-10 and IL-13 (Th2 type) (Duquesnoy & Demetris 1995). The clinical evidence to support a role for the humoral response in the generation of CAD is often conflicting. A significant proportion of patients produce antibodies following transplantation, both against vascular and myocyte components of the graft as well as donor specific HLA antigens and HLA antigens not found on the donor (see above). Formation of these antibodies is associated with increased
number or severity of acute rejection episodes, and in some cases with poor survival (Orosz 2000). The precise specificity of antibody responses has been shown to be HLA antigens in a minority of cases, but in most cases the nature of the target antigen remains undefined (Shirwan 1999). Furthermore CAV can occur in the absence of alloreactive immunoglobulin as demonstrated by flow cytometry. In contrast there is a great deal of experimental evidence for a role of humoral immunity in CAD. Mice deficient in functioning B cells show the absence or diminished levels of CAD in allografts however CAD can develop following passive transfer of immunoglobulin in plasma from sensitised non B cell deficient mice (Russell et al 1994, Shi et al 1996). Alloantibodies found in chronic rejection in several experimental models are of IgG1 isotype, a type usually associated with Th2 cell activation, whilst in other studies IgG was seen to localise to vascular smooth muscle cells in rat allografts with CAD not endothelial cells (Plissonnier et al 1995). These immunoglobulins are thought to directly induce smooth muscle cell activation by the cross linking of MHC I molecules. Other targets of the humoral response, such as non-HLA antigens may also be involved as suggested by the association between anti-vimentin antibodies and CAD in clinical studies in heart transplantation (Jurcevic et al 2001). These IgM antibodies were not cytotoxic but may potentially promote endothelial and or smooth muscle cell activation. Again indirect T cell mediated alloimmune injury would appear to be important in the development of such antibodies and the humoral immune response is not inhibited by calcineurin inhibitors.

2A.4 CMV infection

There are two possible mechanisms whereby CMV infection may increase allograft arteriosclerosis: either 1. Indirectly via an amplification of the insults to the vascular endothelium or 2. Directly via a proliferative effect on vascular SMC (Lemstron et al 1994).
CMV infection may enhance CAV by intensifying several molecular, inflammatory and immunological cascades. Endothelial activation and/or enhanced alloimmune activation may follow leading to accelerated CAV. Both clinical studies in heart transplant patients (Koskinen et al 1994) and rat aortic and cardiac allograft models (Lemstron et al 1993, 1995) demonstrate an enhanced acute/early inflammatory response associated with CMV infection. Clinical studies have demonstrated an increase in acute rejection episodes in cardiac transplant patients infected with CMV (Grattan et al 1989, Weimar at al 1991). In rat aortic allografts CMV infection was associated with an increase in helper and cytotoxic T cells, an increase in endothelial adhesion molecules ICAM and VCAM, MHC Class II expression, growth factor and cytokine production (Lemstron et al 1994, 1995b). This included the growth factors PDGF-BB and TGF-β both potent SMC growth factors for rat aortic SMC in vitro. The enhanced early inflammatory infiltrate in infected allografts was only significant early in the CAD process whilst there was a significant difference in intimal hyperplasia but not inflammatory cells late in the process. This may suggest that early insults affect late development of CAD via as yet poorly understood mechanisms. Similar changes were seen in CMV infected cardiac allografts with strong CMV antigen staining seen in infiltrating monocytes (Lemstrom et al 1995c), however CMV antigen staining was also seen in the media of stenosed vessels (see below) and the staining of monocytes could be attributed to uptake of virus particles. No similar staining of the endothelium was observed despite the evident endothelial activation.

Whether this acute inflammatory response is primarily due to increased immunogenicity of the endothelium or increased leucocyte activation or another mechanism is unclear. The immediate early gene of CMV can code for a protein that as sequence homology and immunological cross reactivity with HLA-DR B chain. Expressed on the surface of infected endothelial cells this could enable direct T cell activation. IFNγ expression is increased during viral infection (von Willebrand et al 1997) and this can increase the
expression of MHC class II leading to increased immunogenicity of the endothelium. CMV infection of human monocytes leads to increased production of IL-1β and increased inflammatory responses (Iwamoto et al 1990) and has also been shown to upregulate TNFα, IL-2 and IL-2R genes thereby enhancing T cell activity (Geist et al 1991, 1996).

In support of the direct hypothesis it has been demonstrated that CMV is able to infect vascular SMC (Tumilowicz et al 1995) and induce changes in SMC cellular metabolism (Melnick et al 1993, Hajjar 1991). Immunocytochemical staining of CMV infected rat cardiac allografts showed strong focal CMV antigen staining of vascular wall SMC in stenosed intramyocardial arterioles (Lemstron et al 1995). Immediate early protein of human CMV can be a trans-acting factor and thus possibly could induce alteration in the transcription of host cell genes (Melnick et al 1993).

2A.5 Other pathophysiological mechanisms

2A.5.1 Hyperlipidaemia

It is likely that hyperlipidaemia has multiple effects on the development of CAV such as the effect on eicosanoid production, direct activation of macrophages and endothelium and possibly interaction with NO metabolism. The evidence for a role of hyperlipidaemia (specifically LDL) in the generation of atherogenesis (Ross 1993) and the potential role it plays in the generation of the CAV lesion suggest that similar mechanisms are at work. It is possible that the toxic effects of LDL and its modified form are amplified by other aetiological factors such as acute rejection and I/R by their generation of oxygen free radicals and endothelial/macrophage activation. This may then lead to an enhanced and accelerated proliferative response in the intima due to cytokine and growth factor production with subsequent lipid uptake by SMC and macrophages leading to lipid

Lipid laden foam cells in the intima and media of vessels are not a universal feature of transplant arteriosclerosis however (Billingham et al 1990) this may reflect different patterns of CAV produced by different combinations of risk factors.

LDL, modified by oxygenation (free radicals), glycation (in diabetes), aggregation or association with proteoglycans is a major factor in the activation of inflammatory cells as well as directly injuring endothelium and smooth muscle. Renal transplants patients with CAD had higher indices of lipid peroxidation (Ghanem et al 1996) whilst the administration of antioxidants in addition to cyclosporin prolonged cardiac allograft survival in rats (Slakey et al 1993). Modified LDL is internalised by macrophages by means of specific LDL scavenger receptors, presumably as part of an initial protective response in inflammatory states. Once internalised however LDL accumulation results in the formation of foam cells, activating these cells leading to cytokine and growth factor production as well as inhibition of NO dependent vasodilatation (Ross 1993). Mediators of inflammation such as TNF-α and IL-1 increase the expression of LDL receptors on endothelium and smooth muscle cells. After binding to these scavenger receptors in vitro endothelial and VSMC cells are activated and may further modify LDL by oxidation. Activation of these cells is characterised by the expression of MHC and adhesion molecules as well as further production of inflammatory cytokines and growth factors.

Endothelial cells also produce macrophage colony stimulating factor (MCSF) and MCP-1, chemotactic factors for macrophages, which may expand the inflammatory response (Fellstrom 1999, Ross 1993). Oxidised low-density lipoprotein also increases expression of MHC class II and IL-2R in human T cells in vitro (Fellstrom 1999, Ross 1993).

Proteoglycan molecules bind to specific apolipoproteins such as apolipoprotein B which facilitate their uptake by macrophages. Macrophage derived cytokines such as IL-1, TNF-
α, and INF-γ can stimulate the synthesis of proteoglycans in SMC and endothelial cells (Klein et al 1992). Proteoglycans are produced in excess quantities in CAV, and in particular two proteoglycans decorin and biglycan are associated with areas of lipid accumulation in the vessel wall (Lin H et al 1996). Hyperlipidaemia is associated with an elevation in levels of the proinflammatory eicosanoid thromboxane B2 (TXB2) in rat aortic allografts (Mennander et al 1993) and eicosanoids may play a role in the pathogenesis of CAD (see below).

2A.5.2 Eicosanoids

Eicosanoids are biologically active metabolites of arachidonic acid (AA). They are formed by the enzymatic transformation of AA after its release from membrane phospholipids and function as inter and intra-cellular signal molecules in the generation of an inflammatory response. The main classes of AA-derived lipid mediators are the leucotrienes and cyclooxygenase products that include prostaglandins, thromboxane and prostacyclins. Leucotrienes are formed by the products of the 5-lipoxygenase (LO) pathway and are formed in phagocytic and mast cells. The major bioactive leucotrienes are LTB$_4$ and LTD$_4$. In vitro studies have shown that LTB$_4$ is synthesised by neutrophils, macrophages and monocytes can stimulate neutrophil degranulation, endothelial activation and enhance immunoglobulin production from B cells. They are also able to stimulate DNA synthesis in SMC in vitro (Palmberg et al 1987). The major cyclooxygenase products are prostaglandins E$_2$ and D$_2$ (PGE$_2$ and D$_2$), thromboxane A$_2$ and prostacyclin. Prostaglandins are produced by a wide variety of cells, have cytoprotective effects including vasodilatation, and can inhibit proliferation of SMC in vitro (Nilsson 1993). Prostacyclin (PGI$_2$) is synthesised by the enzyme prostacyclin synthase in vascular endothelial cells and acts along with PGE$_2$ to promote vasodilatation and prevent platelet aggregation. Thromboxane A$_2$ is produced in platelets, and released on platelet activation promoting
vasoconstriction and platelet aggregation. These two opposing influences represent a
dynamic equilibrium that can be influenced by among other things inflammation and drugs
such as NSAIDs.

Arachidonic acid derivatives play a role in the development of CAD in animal models.
Increased TXA$_2$ and reduced prostacyclin levels (or TXA$_2$/PGI$_2$ ratio) are associated with
the development of CAV in (DA to WF) rat allografts (Mennander et al 1992). Aortic
allografts developing CAV in hypercholesterolaemic rats developed elevated TXB$_2$ (a
TXA$_2$ derivative) (Mennander et al 1993). The administration of a TXB$_2$ receptor blocker
to this model did not significantly reduce the development of intimal hyperplasia however
(Mennander et al 1992). Others have demonstrated that the administration of a prostacyclin
analogue and a thromboxane A$_2$ synthase inhibitor reduces the TXA$_2$/PGI$_2$ ratio and
attenuates CAV in a fully MHC mismatched aortic allograft model (Hirano et al 2001) in
rats. Other evidence for the role of eicosanoids in CAD comes from studies in the F344 to
Lewis rat cardiac allograft model. This model is mismatched at minor MHC loci (RT-3)
only. Adams and co-workers (Adams et al 1993) fed these rats a diet deficient in linolenic
and linoleic acid producing a deficit in their ability to produce arachidonic acid. The rats
subsequently displayed an inability to produce LTB$_4$, prostaglandins and thromboxane
with an associated impairment of the inflammatory but not cellular immune response. This
was associated with a reduction in both the incidence and severity of CAV suggesting that
diet induced alterations in leucocyte metabolism of arachidonic acid caused a reduction in
CAV.

In clinical renal transplantation CAD is associated with elevated plasma TXB2 and
reduced prostacyclin levels (Teraoka et al 1987). Furthermore the administration of
cyclooxygenase inhibitors such as indomethacin (Michielsen and Vanrentereghem 1993)
thromboxane inhibitors (Teraoka et al 1993) and anti-platelet agents (Teraoka S et al 1997)
have all been shown to improve graft survival in patients with established CAD in clinical trials. In clinical renal transplantation administration of aspirin, a potent cyclo-oxygenase inhibitor was associated with significantly improved renal function at one year compared to a cohort of historical controls (Murphy et al 2000).

The mechanism by which thromboxane may affect the development of CAD is unclear. TXA2 may act by producing vasospasm and promoting further platelet aggregation and activation at sites of endothelial activation. This aggregation and subsequent release of smooth muscle cell mitogens in response to damaged endothelium may be important in CAD, an observation supported by the prolongation of renal allograft survival by the anti-platelet agent Satrigel (Teraoka et al 1997). Eicosanoid production is also affected by several risk factors in the development of CAV. This includes alloantigen dependent factors such as oxidised LDL cholesterol levels and I/R injury. It is also altered in acute allograft rejection (Teraoka et al 1987). Persistent elevation of eicosanoids may serve to amplify the ongoing inflammatory response in which macrophages, LDL cholesterol and hypertension also play a role. In addition increased thromboxane production is also a feature of cyclosporin induced nephrotoxicity and may be significant in the development of glomerular hypertension. Eicosanoids, particularly thromboxane are important inflammatory mediators in the pathogenesis of ischaemia reperfusion injury, (Weight et al 1996) an inevitable consequence of solid organ transplantation and a major aetiological factor for CAD. In humans the administration of aspirin to machine perfused kidneys reduces intrarenal thromboxane production, attenuates the severity of subsequent I/R injury and improves graft survival at three years in comparison to controls (Abendroth et al 1997).
2A.5.3 Increased wall shear stress/abnormal vascular responses/ hyperfiltration

Risk factors for CAD such as hypertension, low nephron mass, donor age and sex discrepancies are thought to contribute to the development of CAD by the effect on wall tension and shear stress on organ vasculature. Positioned between the blood flow and the vessel wall, endothelial cells function as mechanosensors and can transduce the physical forces produced by the blood flow into biochemical signals. In this way blood flow regulates the internal diameter of arteries both acutely, by relaxation and contraction of smooth muscle cells, and chronically, by the reorganisation of vascular wall cellular and extracellular components.

In pathological states, such as systemic hypertension, increases in wall tension coupled with reduced shear stress and turbulence at vessel bifurcations directly activate vascular endothelial cells leading to upregulation of adhesion molecules and release of growth factors that can in turn stimulate proliferation and migration of vascular smooth muscle cells to form a neointima (Ross 1999). Such a mechanism has been attributed to the role of hypertension in the development of atheroma in non transplant patients (Ross 1999) however it is also likely to occur in transplant patients with uncontrolled hypertension. Hypertension also has direct proinflammatory actions, increasing plasma levels of free radicals that can lead to reduced NO levels, endothelial activation and increased leucocyte adhesion (Ross 1999). Concentrations of Angiotensin II, the principal product of the renin-angiotensin system, are often elevated in patients with hypertension, particularly in renal transplant patients. In addition to producing hypertension due to its vasoconstrictor actions Angiotensin II can also stimulate the proliferation of VSMC and endothelial cells and promote cytokine production via specific angiotensin II receptors (Mehra et al 1995). It also increases smooth muscle lipoxygenase activity, which can increase inflammation and the oxidation of LDL (Ross 1999). In animal models of CAD angiotensin converting enzyme
inhibitors reduce the severity of CAD in aortic, cardiac and renal allografts (Paul 1999). ACE inhibitors have also been shown to inhibit the progression of CAV in clinical heart transplantation (Mehra et al 1995). This effect is possibly mediated via a direct effect on the tissue response to injury.

Elevated shear stress is also thought to underlie the reduced graft survival in size mismatched allografts where disproportionate metabolic demands are placed on the allograft. This has been particularly well demonstrated in renal allografts in rats. Experimentally, kidneys with decreased functioning mass develop CAD like changes at an accelerated rate, irrespective of whether the graft is an isograft or an allograft (Azuma et al 1997). This is attributed to high blood flow rates in residual nephrons producing glomerular hypertension with subsequent endothelial and mesangial cell activation. (Azuma et al 1997) Experimental reductions in nephron mass of rat renal allografts were associated with accelerated T cell and macrophage infiltration within the grafts in association with high levels of MCP, RANTES, Endothelin 1 and ICAM followed by accelerated CAD development (Azuma et al 1997). Such a process may be increasingly important in longer surviving renal transplants once graft function has deteriorated to such a point that hyperfiltration and increased shear stress result.
Part 2B. Intervention in transplant arteriosclerosis: immunosuppressants

Prior to 1982, standard immunosuppression consisted of the combination of corticosteroids and azathioprine, complimented in some centres with the prophylactic use of antilymphocyte sera in the first few post transplant weeks. The addition of the calcineurin inhibitor cyclosporin in the early 1980s resulted in a decreased incidence of acute rejection episodes and an improved one year graft survival rate, but it did not seem to affect the rate of graft attrition after the first post transplant year in both heart and renal transplantation (Paul 1999). Possible explanations for the failure of cyclosporin to reduce the incidence of CAD, as well as the potential of other immunosuppressant drugs to reduce CAD is discussed below.

2B.1 Cyclosporin

Cyclosporin is a fat soluble cyclic peptide antibiotic derived from the fungus Tolypocladium inflatum. Initially discovered in 1970 its immunosuppressive properties were discovered two years later and it became widely used in transplantation in the early 1980's (Vella and Sayegh 1997). Cyclosporin blocks the calcium dependent T-cell receptor signal transduction pathway by binding to cyclophilin, a cytoplasmic protein member of the immunophilin family. This complex inhibits calcineurin a calcium and calmodulin dependent serine threonine phosphatase. Calcineurin binding is necessary for the dephosphorylation of cytosolic NF-AT (Nuclear Factor of Activated T cells) prior to its translocation to the nucleus where it inhibits the progression of the T cell cycle from the G0 to the G1 phase (Vella and Sayegh 1997) This includes preventing the transcription of cytokines such as IL-2 by binding to and inhibiting their promoter sequences. By preventing IL-2 mRNA production and thereby inhibiting IL-2 secretion further T cell activation and proliferation is prevented. This direct IL-2 effect means that cyclosporin has
greater specificity for cell mediated immune mechanisms than either steroid or azathioprine. Cyclosporin is absorbed from the upper small intestine in a variable manner resulting in an initial bioavailability of between 4 and 26% (Vella and Sayegh 1997). Furthermore the half life varies from 14 to 27 hours and in combination with other factors, such as drug interactions or liver enzyme induction, this has led to difficulties in maintaining stable therapeutic levels. This leads to periods of under-immunosuppression as well as intermittent drug toxicity. Recently a microemulsion formulation of cyclosporin (Neoral) has been produced with greater bioavailability and a more stable pharmacological profile. Whilst Neoral has been associated with lower rates of acute cellular rejection in heart, liver and renal transplantation there is no evidence that it reduces the incidence of CAD in the long-term compared to non microemulsion formulations (Paul 1999).

In heart transplantation early studies suggested that the introduction of cyclosporin had no beneficial effect on the development of CAD (Billingham 1987, Gao et al 1996) however two recent ICUS studies have suggested that low cyclosporin dose may have an adverse effect on the development of CAD. In one cross sectional study a positive correlation was found between a coronary intimal thickness greater than 3mm and a lower daily cyclosporin dose (Rickenbacker et al 1996). In another multi-centre study patients receiving more than 5mg/kg/day of cyclosporin had significantly less development of CAD than patients receiving less than 3mg/kg/day at one year (Mehra et al 1997). This difference was not seen at two years however. In animals studies intimal thickening in rat aorta and cardiac allografts is inhibited by cyclosporin in some studies but not in others (Cramer et al 1990, Mennander et al 1991a, Koskinen et al 1995, Geerling et al 1994, Little et al 1996). Much of this confusion arises from different experimental models and dosages. In high responder (MHC mismatched) rat allograft models cyclosporin at high doses inhibits intimal hyperplasia (Little et al) whilst in low responder models (mismatched at
non MHC loci only) the opposite appears to be the case (Mennander et al 1991a). This clinical and experimental evidence therefore suggests that the success of cyclosporin in heart transplantation is limited by the unacceptable side effects at larger doses and that cyclosporin at the correct dose may attenuate CAD.

In renal transplantation the picture is complicated by the nephrotoxicity of cyclosporin. Cyclosporin at high doses can produce intimal hyperplasia and graft fibrosis in non transplanted kidneys that is virtually identical to clinical renal CAD. Cyclosporin is thought to be responsible for the development of end-stage renal failure in up to 7% of all heart transplant patients (Goldstein et al 1997, Greenberg et al 1990). Several large renal transplant registries (UCLA/UNOS, Eurotransplant Collaborative study and UKTSSA) all demonstrate that the introduction of cyclosporin has not reduced the rate of late renal allograft loss (for review see Paul 1999). There are several indirect mechanisms whereby cyclosporin can damage renal allografts such as cyclosporin induced hypertension, hyperlipidaemia and hyperglycaemia (for review see Mihatsch et al 1998). Cyclosporin also has a direct effect on renal glomeruli producing glomerular vasoconstriction, microvascular hypertension and hyperfiltration. This results in the release of pro-fibrotic growth factors (PDGF, TGF β, bFGF) and cytokines (IFN, RANTES) that can promote the mesangial cell differentiation, proliferation and extracellular matrix accumulation characteristic of cyclosporin toxicity. Cyclosporin at very high levels is also associated with a range of renal histological features suggestive of toxicity including isometric vacuolation of tubular cells, microangiopathy and in chronic cases tubular atrophy (Mihatsch et al 1998). As suggested by differing results from the high and low responder animal models above, patients with a marked cellular response post transplantation may benefit from higher cyclosporin levels whereas those with low cyclosporin levels would be under immunosuppressed. Conversely those with high cyclosporin levels and less cell
mediated rejection may not receive any benefit from over immunosuppression and be subjected to the adverse effects of cyclosporin that may themselves promote CAD. The nature of patient populations dictate that the relative contribution of differing aetiological factors to CAD will vary. As yet there is no laboratory or clinical test that can differentiate between those who might benefit from cyclosporin dose increase or reduction.

TGF-β is thought to be an important growth factor in the development of cyclosporin mediated injury to renal allografts (Jain et al 2000). Cyclosporin increases the expression of TGF-β in various cells including T lymphocytes, endothelial and renal cells (Khanna et al 1998). A sustained elevation of TGF-β may potentially promote smooth muscle proliferation, excessive matrix deposition and further contribute to fibrosis and neointimal hyperplasia. Other explanations for the failure of cyclosporin to prevent the development of CAD in organ allografts have been mentioned above. In particular cyclosporin by promoting the differentiation of Th2 cells with subsequent humoral alloreactivity may be responsible for the failure of cyclosporin to prevent the development of CAD.

2B.2 Tacrolimus (FK506)

Tacrolimus is a macrolide antibiotic which possesses similar, though more potent immunosuppressive properties than cyclosporin on a milligram to milligram basis. The mechanism of action is similar to that of cyclosporin. Tacrolimus binds to an immunophilin, FKBP (FK506-binding protein), which prevents the phosphorylation of NFAT by calcineurin thereby inhibiting T cell receptor signal transduction and inhibition of IL-2. In heart, liver and renal transplantation tacrolimus has been shown to reduce the incidence and severity of acute rejection episodes relative to cyclosporin however it has not been shown to increase survival in the long term (Henry 1999), except in a few single centre studies (Morris Stiff et al 1998). Although the efficacy of tacrolimus appears to be
greater tacrolimus itself has considerable toxicity and therefore the therapeutic window is small. Tacrolimus induced nephrotoxicity is similar to that found in cyclosporin toxicity (Henry 1999, Mihatsch et al 1998). It produces post transplant hypertension and whilst it has less of a tendency to promote hyperlipidaemia compared to cyclosporin it does promote more hyperglycaemia relative to cyclosporin by a directly toxic effect on Islet of Langherhans B cells.

This along with its failure to inhibit indirect T cell activation may serve to reduce any long-term benefit derived from lower rates of acute rejection.

2B.3 Azathioprine

Azathioprine is an imidazoyl derivative of mercaptopurine and act as an S phase specific purine synthesis inhibitor. It is not cell specific and therefore causes dose limiting bone marrow suppression, gastrointestinal toxicity, sterility, hepatotoxicity and acute pancreatitis. It has no effect on CAD in rat aortic allografts or rabbit cardiac allografts (Mennander et al 1991a, Laden 72). In clinical heart transplantation lower azathioprine levels were associated with more severe intimal thickening on ICUS (Rickenbacker et al 1996) however several studies in renal function have shown no difference in the incidence of CAD or graft survival between patients treated with cyclosporin/ prednisolone/ azathioprine triple therapy and those treated with cyclosporin prednisolone dual therapy (Paul 1999).

2B.4 Corticosteroids

Prednisone is believed to inhibit the action of nuclear transcription factors such as NF-κB (Nucleus Factor-kappa B) (Nair and Morris 1995). NF-κB is found in a number of inflammatory cells and acts on genes for cytokines, immune receptors and adhesion molecules. This gives prednisone a variety of non-specific anti-inflammatory and
immunosuppressive effects. Prednisone has no effect on CAD in rat aortic allografts (Nair and Morris 1995) and in humans has no effect on the incidence of CAD in heart transplantation (Keogh et al 1992, Price et al 1992) whilst in renal transplantation the evidence is conflicting. In the Collaborative Transplant Study Database the five year survival was superior in patients who had steroids withdrawn whilst in a Canadian multi-centre steroid withdrawal study patients on higher doses of steroids had superior long-term graft survival (Paul 1999). These differences may be attributable to differences in study design, or alternatively may reflect different patient groups. In the former study (non-randomised) steroid withdrawal may have been more frequent in more stable patients thereby influencing the result. Prednisone has deleterious effects on patient blood pressure, insulin resistance and lipid profiles and it is likely that any potential benefit associated with the anti-inflammatory effect of prednisone may be offset by the exacerbation of other risk factors for CAD.

2B.5 Mycophenolate mofetil

Mycophenolate is a morpholinoethyl ester of mycophenolic acid. It inhibits the synthesis of guanosine monophosphate via blockade of the enzyme inosine monophosphate dehydrogenase. The drug blocks proliferative responses of T and B lymphocytes, and inhibits antibody formation and the generation of cytotoxic T cells. It is not nephro- or hepato-toxic and may have less bone marrow toxicity than azathioprine. In animal studies MMF inhibits the development of CAD in rat renal, cardiac and renal allografts (Raisanen Sokolowski et al 1994, Nadeau et al 1996, Morris et al 1991). In clinical transplantation MMF in combination with cyclosporin and Tacrolimus is associated with dramatic reductions in clinical rejection episodes. More recently evidence is emerging of a beneficial effect of MMF treatment in patients with CAD (Jain et al 2001).
2B.6 Rapamycin

Rapamycin is a macrocyclic fermentation product of *Streptomyces hygroscopicus*, an actinomycete originally isolated from a soil sample on Easter Island (Rapa Nui) in 1975. Rapamycin was initially investigated as an anti-fungal and anti-tumour agent however its lymphopenic properties heralded its role as an immunosuppressant (Sehgal et al 1975, Baker et al 1978, Vezina et al 1975). Initially tested in animal models of cellular rejection however it was apparent that as well as prolonging allograft survival, histological changes associated with CAD were also inhibited (Meiser et al 1991). It has been this apparent inhibitory effect on the development of CAD, particularly in rodents that has led to an upsurge of interest in the use of rapamycin in clinical transplantation.

2B.6.1 Rapamycin as an immunosuppressant

Rapamycin inhibits cytokine driven cell proliferation and maturation by blocking post receptor events (reviewed in Kahan 1997, Saunders 2001). Rapa has a similar structure to FK506 and also binds to FKBP in cell cytoplasm but whereas the FK506-FKBP complex acts to inhibit calcineurin phosphatase, Rapa-FKBP has no effect on calcineurin phosphatase and instead binds to one or more proteins known as "targets of rapamycin" (mTOR). In untreated lymphocytes cytokines such as IL-2 and the CD28/B7 costimulatory pathway activate TOR resulting in downstream events critical for cell cycle regulation. In the presence of rapamycin the rapamycin-FKBP/ TOR complex inhibit several molecular effectors including the enzymatic regulation of the phosphorylation status of several sarcoma (src) like, receptor type and cell cycle dependent kinases (CDK), enzymes involved in the progression of the cell cycle. It is by this mechanism that rapamycin inhibits phosphorylation and inactivation of the gene encoding the retinoblastoma protein (pRb) a critical regulator of VSMC proliferation. The inhibition of pRb phosphorylation by rapamycin results in cell-cycle arrest in VSMC and inhibition of proliferation. pRb
phosphorylation is regulated by the interaction of Cyclin Dependent Kinases that are themselves inhibited by CDK inhibitors including p16, p21 and p27<sup>kip1</sup>. Over expression of CDKI results in inhibition of VSMC proliferation and rapamycin inhibits the downregulation of the CDKI p27<sup>kip1</sup> both in myogenic cell lines in vitro as well as in pigs following balloon angioplasty in vivo. Another important molecular effector target of this complex is the p70<sup>S6</sup> kinase which hyperphosphorylates the 40S ribosomal protein p70<sup>S6</sup> an essential step in protein synthesis. Rapamycin inhibits this hyperphosphorylation, and therefore inhibits the protein synthesis associated with G<sub>1</sub> progression, at the same concentration at which it blocks cell proliferation. In addition rapamycin increases the stability of p21<sup>kip</sup>, thereby inhibiting the action of down stream serine-threonine protein kinases (p34<sup>cdc2</sup>) and preventing the generation of the active p34<sup>cdc2</sup>-cyclin D complex. This complex forms the critical 'maturational promoting factor' as well as the generation of the cyclin dependent kinase cdk2, the catalytic partner of cyclin E, two factors which control the rate of cell progression into the S phase of the cell cycle. In summary the rapamycin-FKBP complex binds to TOR and subsequently inhibits DNA and protein synthesis, resulting in arrest of the cell cycle in late G1 as it progresses to the S phase of the cell cycle (Kahan 1997, Saunders 2001).

**Immunosuppressive properties in vitro**

Rapamycin inhibits T cell proliferation induced by cytokines (IL-1, -2, -3, -4, -6, -7, -12 and -15), alloantigens and mitogens in a dose dependent manner (Sehgal 1998, Sehgal and Bansbach 1993). At 10-100 fold higher concentrations rapamycin also inhibits natural killer cells and cytotoxic CD8 cells (Sehgal et al 1994). Rapamycin also inhibits the expression of proinflammatory cytokines involved in both the Th1 and Th2 response in mice in some studies by an as yet undetermined mechanism however this is not a universal finding. In concalvin A- stimulated spleen cells rapamycin inhibited the expression of IL-2,
IFN γ, IL-4 and IL-10 more effectively than cyclosporin (Zheng et al 1994) whereas in mitogen activated T cells rapamycin did not inhibit the expression of IL-2, -3, -4 or TNF α (Sehgal 1998). Rapamycin also acts on B cells, causing an inhibition of antigen and cytokine driven B cell proliferation and differentiation into antibody producing cells (Kim et al 1994, Dumont et al 1994). This B cell effect is independent of its effects on T helper cells.

Immunosuppressive properties in vivo

By inhibiting cytokine-activated signal transduction as well as cytokine transcription rapamycin is a potent immunosuppressive agent in vitro as well as in vivo with 20-100 times the equimolar potency of cyclosporin at inhibiting cell mediated rejection in animal models. Rapamycin prolongs allograft survival in cardiac, renal, pancreatic, skin and small bowel allografts in a number of species including rats, mice rabbits, pigs, dogs (Saunders 2001, Stepkowski et al 1991). Higher doses used in early rodent experiments (0.2-10mg/kg/day) are poorly tolerated by larger animal models however, particularly primates, where a high incidence of adverse gastrointestinal side effects has been noted. In human studies, where much lower doses are tolerated (2-5mg/day or less than approximately 0.1 mg/kg/day), rapamycin has been shown to be an effective immunosuppressant when compared to cyclosporin based regimens in randomised trials. In two European multi-centre trials comparing rapamycin triple therapy (Prednisolone and either Azathioprine or MMF) with cyclosporin based triple therapy in renal transplantation (Groth et al 1999, Kreis et al 2000) there was no difference in the rate of acute rejection, patient or graft survival at one year. There was, however a tendency to require treatment for acute rejection with steroid boluses more often in rapamycin treated patients compared to cyclosporin treated patients in both trials. This suggests that in clinical transplantation, at the lower doses of rapamycin tolerated by primates compared to rodents, rapamycin may
be slightly less effective at inhibiting acute rejection than cyclosporin. The effect of rapamycin on CAD remains to be seen and long-term follow up of these two studies are awaited.

2B.6.2 Rapamycin and CAD

As well as being an effective immunosuppressant in vivo and in vitro rapamycin has been shown to inhibit the development of CAD in heart, aortic, femoral artery and lung allografts in rodents. In clinical transplantation there is insufficient evidence as yet to indicate whether rapamycin will indeed inhibit the development of CAD however experimental evidence suggests that this could potentially occur via a variety of mechanisms.

Mechanisms by which rapamycin may inhibit the development of CAD

Inhibition of cell mediated rejection

As discussed above there is abundant clinical and experimental evidence to demonstrate effective inhibition of cell mediated rejection by rapamycin and that this is effected both by the inhibition of cytokine production as well as by inhibition of cytokine dependent T cell proliferation. Immunohistochemical studies in BN to Lew rats have demonstrated that rapamycin significantly inhibits the level of graft infiltration by CD4 and CD8 cells as well as macrophages (ED-1) by high doses (3mg/kg/day i.p.) but not by lower doses 1.5mg/kg/day (Schmid et al 1995). This was also associated with inhibition of MHC II, IL-1, IL-2 and INF γ immunostaining at higher doses. In high responder BN to sensitised Lewis rats mRNA levels of IL-2, IL-1, IL-6, IFN γ and TNF α were reduced by rapamycin at 0.25 mg/kg/day in another study (Wasoswska et al 1996) although Schmid et al reported that rapamycin at 0.5mg/kg/day did not reverse cellular rejection in this model (Schmid et al 1995). Rapamycin has also been shown to inhibit the expression of MIP as well as
cytotoxic CD8 and natural killer cell specific expression of granzyme B in rejecting cardiac allografts (Sehgal 1998). In 1991 Meiser et al reported that rapamycin at 1.5mg/kg/day, in a "high responder" Brown Norway to Lewis model, inhibited CAD in heterotopic rat cardiac allografts more effectively than either cyclosporin or FK506. As BN and Lew rats are mismatched at major HLA loci (RT-1n versus RT-11) the development of CAD in this model is felt to be directly related to cell mediated rejection and intimal thickening occurs predominantly between 30 and 40 days. In this model rapamycin at the same dose was reported by the same group to also inhibit the development of allograft vasculopathy in femoral artery allografts by 40 days (Morris et al 1995). The importance of inhibiting the early cellular response is underscored by the diminished efficacy of rapamycin if administration was delayed. In the BN to Lew model delaying onset of rapamycin treatment by 14, 21 or 30 days inhibited the progression of CAD only at large doses (3mg/kg/day at 14 and 21 days and 6mg/kg/day at 30 days) and was of marginal statistical significance only at this later time point (28% versus 49%, P=0.03) (Morris et al 1995).

**Inhibition of Th2 response**

Whereas rapamycin strongly inhibits Th1 type cytokines in the sensitised BN-Lew rat cardiac allograft model there is no apparent inhibition of Th2 type cytokine expression. Specifically IL-4 and IL-10 expression was preserved in rapamycin (0.8mg/kg/day i.p.) treated hosts although this contradicts the finding that rapamycin strongly inhibited IL-10 expression in concalvin A-stimulated splenocytes in vitro (Wasowska et al 1996). It is possible that these changes represent some form of rapamycin induced tolerance however in combination with the apparent persistence of IgG in rats expressing Th 2 cytokines it is possible that rapamycin may fail to inhibit all the pathophysiological mechanisms underlying the development of CAD.
**Inhibition of humoral rejection**

Rapamycin strongly inhibits in vitro immunoglobulin production by human lymphocytes. Furthermore in rats rapamycin (0.8mg/kg/day) alleviates accelerated rejection in presensitised animals and inhibits antigen specific cytotoxic antibody formation in the high responder Buffalo to WF models (Chen et al 1991). In low responder models (PVG (RT1\(^c\)) to ACI (RT1\(^a\)), where the cellular immune response in ACI rats is 'weak', humoral responses are thought to contribute more to the CAD lesion. This develops between 60 and 1990 days post-transplant in cardiac allografts in this model (Poston et al 1999). Anti-RT-1\(^c\) IgG antibodies are significantly elevated between 60 and 90 days, and this was inhibited by delayed rapamycin (3mg/kg/day i.p day 60 to 90) but not by cyclosporin. Rapamycin also reversed the development of CAD in this model when introduced at 60 days. In sensitised Lewis recipients of BN cardiac allografts (Wasowska et al 1996) rapamycin (0.25mg/kg/day) administered at the time of transplantation diminished the IgM alloantibody response as well as the switch from IgM to IgG production in the early post transplant period. There was however a persistent level of IgG expression in long-term survivors with rapamycin, primarily of the IgG2b subclass but also to a lesser extent IgG2a. IgG2b subclass are associated with complement activation whilst IgG2a is the most effective subclass in triggering antibody dependent cell cytotoxicity. Rapamycin therapy was not seen in mice however (Kahan 1997). In mice rapamycin did not inhibit the action of IL-4 to promote the switch from IgM to IgG1 but did inhibit the ability of IFN \(\gamma\) to switch to IgG2a, IgG3 and of TGF \(\beta\) to switch to IgG2b. Alloantigen specific IgM antibody production was halved and IgG2a, IgG2b and IgG3 production was nearly completely abrogated whilst levels of non complement binding IgG1 increased. Furthermore transfer of serum from rapamycin treated mice to irradiated secondary recipients induced allo-unresponsiveness in the recipients (Kahan 1997).
2B.6.3 Direct inhibition of the response to injury: lessons from models of ischaemic and mechanical injury models

Rapamycin significantly inhibits neointimal hyperplasia and fibrosis in animal models of balloon injury and ischemia reperfusion injury and this has led to the suggestion that by the chief effect of rapamycin on the development of CAD may be via direct inhibition of the graft response to injury. This is supported by the observation that rapamycin (0.5mg/kg/day i.p.) had no effect on the level of perivascular infiltrate in F344 to Lew cardiac allografts but completely abolished neointimal thickening (Schmid et al 1995) suggesting that rapamycin may inhibit the response to injury without necessarily inhibiting the level of inflammation. The evidence for a direct role of rapamycin on the tissue response to injury is summarised below.

1. Inhibition of the response to injury in non alloimmune models

Morris and co-workers first reported that rapamycin (1.5mg/kg/day i.p.) inhibited intimal hyperplasia by 65% following balloon injury to rat carotid arteries (Gregory et al 1993) whilst in pigs rapamycin (1.5mg/kg/day i.m.) reduced post angioplasty restenosis by 50% (Saunders 2001). In a rat model of obliterative bronchiolitis rapamycin (6mg/kg/day) (Morris et al 1995b) prevented the development of lumenal fibrosis whilst at a dose of 0.5mg/kg/day rapamycin has also been shown to inhibit the progressive decline in renal function and proteinuria that characterises progressive graft fibrosis following ischaemia reperfusion injury (Jain et al 2001).

2. Decreases expression of growth factors

In the high responder BN to Lew model, as well as in rat models of balloon injury immunostaining demonstrated that levels of TGF β, bFGF and PDGF were inhibited by
large doses (3mg/kg/day) of rapamycin, an effect not seen at 1.5mg/kg/day (Gregory et al 1993). The administration of rapamycin to rats subjected to varying periods of renal ischaemia (0.5mg/kg/day) inhibited level of TGF β mRNA expression as well as subsequent renal fibrosis (Jain et al 2001). In contrast rapamycin increased the expression of TGF β in human lymphocytes in vitro (Kahan 1997) and it is possible that these in vivo observations were simply the result of the many other effects of rapamycin, notably inhibition of the cellular and humoral immune responses, macrophage infiltration and the antiproliferative effect on smooth muscle and endothelial cells that can themselves produce these growth factors.

3. In vitro inhibition of growth factor driven vascular smooth muscle cell proliferation

Rapamycin inhibits the growth factor mediated proliferation of cells involved in the pathogenesis of graft fibrosis and allograft vasculopathy in vitro. This anti-proliferative effect is seen in non-stimulated as well as bFGF, IGF-1 and PDGF driven vascular smooth muscle cells. Rapamycin exerted similar effects on bFGF induced bovine aortic and human umbilical vein endothelial cells as well as PDGF/bFGF stimulated rat cardiac and human lung fibroblasts (Akselband et al 1991, Nair et al 1997) and also inhibits the migration of activated VSMC through a matrix gel. The mechanism by which rapamycin inhibits VSMC proliferation is unknown but it is possible that this occurs at several levels. As mentioned above rapamycin inhibits phosphorylation and inactivation of the gene encoding the retinoblastoma protein (pRb), a critical regulator of VSMC proliferation, resulting in cell-cycle arrest in VSMC and inhibition of proliferation. Another postulated mechanism whereby rapamycin may inhibit VSMC proliferation is by the upregulation of iNOS. iNOS is expressed at high levels in rat aortic allografts treated with rapamycin whereas such high levels were not seen in cyclosporin treated or untreated control allografts (Pham et al
Over expression of iNOS gene following gene transfer in rat allografts diminishes
the severity of allograft vasculopathy (Shears et al 1997) as does the administration of the
NO donor FK409 (Fukada et al 2001). High NO levels inhibit VSMC proliferation as well
as inducing apoptosis and it is possible that high local levels due to increased iNOS
expression with rapamycin treatment may have an inhibitory effect on the progression of
CAD.

4. Attenuation of calcineurin mediated nephrotoxicity in renal transplantation
Rapamycin is non-nephrotoxic and has the potential to allow early dose reduction or
elimination of cyclosporin, limiting the adverse effects of this drug on the development of
CAD. In trials of de novo transplantation rapamycin in combination with low doses of
cyclosporin had equivalent rejection rates compared to combinations with higher
cyclosporin doses. Significantly lower cyclosporin dose patients had superior renal
function, significantly so at 3 months, than those on higher doses (Kahan et al 1999). This
suggests that the addition of rapamycin may reduce cyclosporin related nephrotoxicity and
therefore reduce the progression of CAD. In another study the addition of rapamycin
permitted cyclosporin withdrawal in those patients who had not had an episode of cellular
rejection (Abstract 958;ATS/ASTS Meeting, May 2000). This was achieved in 66% of
patients, without increasing the rejection rate, and was associated with significantly
improved renal function at 6 months.

The potential benefit of rapamycin in combination with cyclosporin is also underscored by
the abundant experimental and clinical evidence suggesting synergy between the two
drugs. In vitro combinations of rapamycin and cyclosporin inhibit cell mediated
lympholysis and IL-2 dependent T cell proliferation at significantly reduced doses
compared to their independent effects (Kahan et al 1991). This combined effect is much
more than one would expect from a purely additive effect suggesting a synergic
interaction. Rapamycin and FK506 do not appear to have the same synergy in vitro, in fact
FK506 appears to antagonise the inhibition of VSMC cells seen with rapamycin seen in
vitro (Gregory et al 1993b). In animal studies in vivo cyclosporin and rapamycin again
demonstrate synergistic properties. Sub therapeutic doses of rapamycin (0.01 to 0.04
mg/kg/day) and cyclosporin (0.5 to 2 mg/kg/day) prolonged rat cardiac and kidney
allograft survival compared with either drug alone or the additive effect of a combination
of both (Stepkowski et al 1997). In rats in vivo this effect is mediated in part by a
rapamycin induced elevation in the serum level of cyclosporin however this effect in
isolation is insufficient too explain the observed reduction in cellular rejection seen with
sub-therapeutic doses of the two drugs seen in isolation (Stepkowski et al 1997). In rats
rapamycin/ cyclosporin synergy is evident in the prolongation of cardiac and renal allograft
survival and this is mirrored by evidence of synergy in the reduction of cytokine gene
expression (IL-4, IL-10, IL-2 and IFN γ) (Stepkowski et al 1997). The inhibitory effect of
rapamycin on cytokine signal transduction is cytokine concentration dependent and
cyclosporin acts via the inhibition of cytokine production. By reducing cytokine production
cyclosporin may therefore enhance the inhibitory activity and specificity of sub-therapeutic
amounts of rapamycin on signal transduction. Synergy is also seen in vivo in rat cardiac
and murine small bowel allografts between rapamycin and FK506 (Vu et al 1997, Chen et
al 1998). The apparent contradiction between in vivo and in vitro data for rapamycin may
be due to the small amount of FKBP occupied by rapamycin or FK506 need to achieve
maximal immunosuppression. Whereas FKBP is present in abundance in vivo and
competitive antagonism is unlikely, this is not the case in vitro and therefore the opposite
applies.
There is some evidence of synergy between rapamycin and cyclosporin in clinical transplantation although the underlying mechanisms are unclear. Low cyclosporin and rapamycin doses appear to produce as effective immunosuppression with low rates of acute rejection compared to higher doses of the two drugs (Kahan et al 1999). Rapamycin increase serum cyclosporin levels in humans, particularly if the two drugs are ingested simultaneously (Kaplan 1998). One potential drawback of this apparent synergy is that it may extend to the direct nephrotoxicity of cyclosporin. In a salt depleted rat model of cyclosporin nephrotoxicity the addition of rapamycin was associated with morphological and functional deterioration in renal function (Andoh et al 1996). Furthermore in two multi-centre clinical trials of rapamycin and cyclosporin versus either cyclosporin/Pred or cyclosporin/Pred/Aza (Abstracts 957, 960, 962; American Society of Transplantation and Transplant Surgeons, May 2000) patients on rapamycin and cyclosporin had higher serum creatinine values than controls. This is not a universal finding in renal transplantation however and other studies have suggested that the addition of rapamycin to patients on cyclosporin is not associated with any significant reduction in renal function (Murgia et al 1996). Although the nephrotoxic effect of cyclosporin may not overlap with reported adverse effects of rapamycin the effects of the two agents do overlap with regard to hyperlipidaemia, a recognised risk factor for CAD. Cyclosporin primarily increases LDL cholesterol levels however whereas rapamycin primarily increases triglycerides (Saunders 2001).

2B.7 Summary

Effective attenuation of cell mediated acute rejection by agents such as cyclosporin and FK506 has not been shown to prevent CAD. This is because of the toxicity of these drugs, in particular their nephrotoxicity which leads to a high incidence of renal failure in heart and liver transplant patients as well as contributing to CAD in renal transplants via a direct
toxic effect. They also induce hypertension, hyperlipidaemia and hyperglycaemia, all of which are risk factors for CAD as well as death from other causes. Newer agents such as rapamycin may offer effective immunosuppression without nephrotoxicity. In addition rapamycin may attenuate the progression of CAD by a direct effect on the fibroproliferative response to injury. Early results from clinical trials have not supported earlier findings in experimental studies however and this may be attributed to the co-administration of rapamycin and cyclosporin in many trials, which have a synergic nephrotoxic effect.
Part 3. Intimal hyperplasia and fibrosis

3.1 Common response to chronic inflammation: the response to injury hypothesis
The cellular interactions in the development of CAD are fundamentally no different from those in other chronic inflammatory- fibroproliferative diseases notably atherosclerosis, but also vein graft stenosis and even cirrhosis, rheumatoid arthritis, glomerulosclerosis, pulmonary fibrosis and chronic pancreatitis (Ross 1999). Whilst the response of each tissue or organ depends on its characteristic cells and the nature of the offending stimulus, central to each of these processes is an initial inflammatory infiltrate predominantly macrophages and also T cells. Whilst the initial inflammatory response is initially to remove the injurious response and instigate repair of damaged tissue if the stimulus persists the response changes from a protective to an injurious process. Constant or repetitive injury can stimulate each tissue to repair the damage by means of a fibroproliferative response. Replacement of normal tissue with fibrous tissue in combination with apoptosis diminishes the functional capacity of the tissue or organ and becomes part of the disease process. In allografts where a host of immunological and non immunological stimuli persist this is associated with cytokine and growth factor excess, disruption of normal extracellular matrix architecture, cellular apoptosis, proliferation of smooth muscle cells and extracellular matrix accumulation that is characterised histologically as neointimal hyperplasia and parenchymal fibrosis.

3.2 Growth factor and cytokine excess
Inflammation and the release of cytokines and growth factors are seen to lead to neointimal hyperplasia in all types of vascular injury including mechanical, ischaemic, alloimmune or chemical injury (Ross 1999, Azuma et al 1994, Lemstrom et al 1995). The aetiological factors for CAD referred to previously, notably ischaemia reperfusion, alloimmune injury,
hyperlipidaemia, infection and hypertension results in T cell and macrophage infiltration associated with the activation of endothelial cells, vascular smooth muscle cells and tissue fibroblasts. The result is an alteration in the cellular milieu whereby the excessive production of growth factors causes changes in cell phenotype that leads to smooth muscle cell proliferation and migration to the neointima and the deposition of excess extracellular matrix by smooth muscle cells and fibroblasts (Delafontaine 1999). Both experimental and clinical studies suggest that an excess of growth factors is associated with the development of CAD. Allogenic lymphocytes induce endothelial cell expression of bFGF, PDGF and TGF β whilst cytokines such as IL-1, IL-6 and TNF α have been shown to induce PDGF expression in endothelial cells and fibroblasts (Azuma et al 1994, Lemstrom et al 1995). Furthermore the development of vasculopathy in rat aortas is associated with increased expression of PDGF B, TGF β, bFGF and IGF-1 relative to isograft controls (Lemstrom 1995 review). In clinical studies patients with CAD have higher levels of PDGF and TGF β expression (Delafontaine 1999, Lemstrom et al 1999). Experimental studies suggest that these growth factors act synergistically and interact at multiple levels. Notably IGF-1 receptors on VSMC are upregulated by PDGF and bFGF whilst in turn upregulation of IGF-1 receptors enhances the growth responses to these agonists (Delafontaine 1999).

**bFGF**

Basic fibroblast growth factor is a glycoprotein of approximately 15kD. It is synthesised by monocytes/macrophages and endothelial cells and acts as a paracrine growth factor for endothelial cells, VSMC and fibroblasts as well as being chemotactic for macrophages. In balloon angioplasty studies bFGF has been shown to be important in initiating the initial VSMC proliferative response following mechanical injury by promoting entry of the cells into the G1 phase (Ross 1993). bFGF has a high affinity for heparan sulphate proteoglycans and is found in normal tissues in the extracellular matrix or bound to basement membranes. Direct mechanical trauma and disruption of tissue architecture
exposes bFGF to smooth muscle cells leading to their activation. In small bowel allografts in rats induced expression of bFGF during chronic rejection is localised to infiltrating macrophages (Kouwenhoven et al 1999b).

**PDGF**

Platelet derived growth factor is a cationic glycoprotein of approximately 30kD. It consists of 2 polypeptide chains, the A and B chains. PDGF BB homodimer is produced principally by activated macrophages (Lemstrom et al 1997) and is a potent growth factor for smooth muscle cells which can themselves produce PDGF when activated (Hahn et al 1991). Two types of PDGF receptors have been characterised, α and β. The α-receptor binds to all PDGF isoforms whilst the β type does not bind the AA type. PDGF BB is elevated in rat aortic renal and cardiac allografts and PDGF BB and PDGF β-receptor are elevated in chronically rejecting human renal allografts (Lemstrom et al 1995). In cardiac transplantation strong PDGF immunostaining is seen in advanced CAD lesions in post mortem studies on failed transplants as well as in endomyocardial biopsies in patients with allograft vasculopathy (Meliss et al 1999, 2001). Inhibition studies in balloon injury models indicate that PDGF BB is predominantly associated with smooth muscle cell migration (Ferns et al 1991, Bornfeldt et al 1994) however it also promotes VSMC proliferation in conjunction with bFGF (Bornfeldt et al 1995).

**TGF β**

Transforming growth factor β is a dimeric homodimer or heterodimer protein of 28kD produced by activated T cells, monocytes/macrophages and proliferating vascular smooth muscle cells (Jain et al 2000). The exact role of TGF β in the development of CAD is unclear due to the fact that it has both immunomodulatory activities during the inflammatory response as well as being central in the tissue response to injury. It has an inhibitory effect on T cell and monocyte proliferation and maturation (Border and Noble
1997) and can induce lymphocyte apoptosis. TGF β has been shown to have a central role in several fibroproliferative disease states by promoting extracellular matrix protein production and inhibiting the degradation of newly formed matrix protein (Border and Noble 1997). During progressive neointimal thickening after balloon angioplasty transfection of the TGF β gene is associated with increased extracellular matrix deposition although it is also chemotactic for VSMC and fibroblasts. It is strongly expressed during CAD in human and experimental models (Lemstrom et al 1997) and may be the key cytokine that initiates and terminates tissue repair and whose sustained production underlies the development of tissue fibrosis. In renal transplantation TGF β expression correlates with severity of CAD, decline in renal function in renal transplants and collagen III immunostaining (Jain et al 2000).

**IGF**

Insulin like growth factor-1 is produced by fibroblasts and smooth muscle cells and appears to act in an autocrine or paracrine manner as a weak mitogen, although its presence is thought to be important for the actions of other mitogens. Several recent studies have highlighted the potential importance of IGF-1 in CAD (Lemstrom et al 1995). Aortic allografts in rats and rabbits show increased levels of IGF-1 mRNA and protein levels respectively, whilst several agents that inhibit the development of intimal hyperplasia in these models also attenuate IGF-1 expression. IGF-1 expression is also seen in the neointima of atherosclerotic plaques and following balloon injury (Bornfeldt et al 1994). Administration of an IGF-1 analogue that inhibited the interaction of the IGF-1 molecule with its receptor reduced smooth muscle replication by 60-70%. These studies suggest that IGF-1 may be a crucial rate-limiting gene in VSMC proliferation.

**IL-1**

The interleukin-1 (IL-1) molecules are multi-potent inflammatory mediators, with biological activity residing in two major 17kD polypeptides (IL-1α and IL-1β). These both
bind to the same receptors and have essentially similar functions. Their major cellular source is the activated monocyte/macrophage in response to stimulation by TNF α, and IL-1 itself (Azuma et al 1994) and also to some extent by T and B cells. IL-1 acts chiefly on monocytes/macrophages and vascular endothelial cells to induce synthesis of IFN γ, TNF, IL-6, IL-8 and IL-1 itself and cytokines that cause upregulation of adhesion molecules on endothelial cells. It increases IL-2 production and synergises with IL-4 to increase B cell proliferation and Ig production. It also upregulates eicosanoid production thereby promoting inflammation and has a mitogenic effect on vascular smooth muscle primarily through increased production of PDGF (Azuma et al 1994, Lemstrom et al 1995).

**TNF α**

TNF α is trimer of 17kD fragments produced by monocytes/macrophages at sites of inflammation and to a lesser extent by T cells and activated NK cells in response to TNF α itself, IL-1 and IL-6 (Lemstrom et al 1995, Azuma et al 1994). It acts on monocytes/macrophages, lymphocytes, neutrophils and endothelial cells promoting upregulation of adhesion molecules, including MHC I and II, and cytokines such as IL-1, IL-6, IL-8 and the TNF α itself. It is a co-stimulator for T cell and B cell activation and promotes release of the growth factor PDGF release by vascular endothelium and promotes angiogenesis (Lemstrom et al 1995). Many of its effects are augmented by INF-γ (Azuma et al 1994).

### 3.3 Structure of uninjured arteries

The artery wall consists of three distinct layers: the intima, media and adventitia. In normal arteries the inner layer, the intima comprises a continuous endothelial monolayer seated on a specialised extracellular matrix, the basement membrane, whilst the outer adventitia contains fibroblasts in a loose connective tissue which also contains small blood vessels
and fat. The media is composed largely of VSMC embedded in an extracellular matrix (ECM). Individual medial VSMC are surrounded by their own basement membrane composed of type IV collagen, laminin and heparan sulphate proteoglycans such as perlecan and syndecan. These are in turn tightly packed into an interstitial matrix which contains types I and II collagen, fibronectin and chondroitin sulphate proteoglycans such as versican. The media of elastic and muscular arteries also contain lamellae rich in elastin, localised to the inner and outer margins of the media in most arteries with a honeycomb of lamellae between these margins that varies in density depending on the type of artery.

3.4 The extracellular matrix

The ECM is a dynamic superstructure of self aggregating macromolecules including collagen, proteoglycans and fibronectin as well as a infinite variety of smaller molecules that contribute to the composition of the extracellular milieu.

Collagens are a family of proteins that constitute the major structural components of the ECM. To date at least 18 different collagen types, in addition to several non structural proteins that contain non-collagenous domains have been identified (Tryggvason 1995). Collagens are characteristically formed from three polypeptide α chains that interact to form a triple helix with the polypeptides most commonly composed of Glycine-Proline-Hydroxyproline repeats. Collagen types I, II, III, IV and XI form fibrils and are present in a variety of connective tissues including tendons, ligaments and cartilage where they confer tensile strength. In blood vessels collagen I and III are located in the interstitial ECM. Collagen IV is specifically present in cellular basement membranes where it forms a network structure.
Proteoglycans are a heterogeneous group of macromolecules composed of various sulphated polysaccharides termed glycosaminoglycans (GAG) covalently linked to a core protein with both core protein and GAG chains conferring specific properties (reviewed in Wight 1989). Historically proteoglycans have been classified according to their glycosaminoglycan chains. Three different types of chain are recognised in which the disaccharides are composed of the basic units for: (1) chondroitin sulphate (CS)/dermatan sulphate (DS), (2) heparin/heparan sulphate (HS), and (3) keratan sulphate. The first two of these are located in parenchymal tissue such as kidney or the walls of blood vessels whilst the keratan sulphate is found principally in skin. CS/DS and HS GAG have different structure, function and location within tissues. A further level of functional complexity is conferred by heterogeneity of the core proteins, as well as certain core proteins having mixed HS and CS GAG chains (e.g. syndecan). In general cell associated proteoglycans are characterised by HS GAG, that is often found linked to or intercalated with the cell membrane. Syndecan proteoglycans are also distinguished by the fact that they have transmembrane domains. Large aggregating CS GAG (predominantly chondroitin sulphate but also versican, aggrecan) are characteristic of the extracellular matrix proteoglycans whilst the major proteoglycan of basement membranes is perlecan, a HS proteoglycan with a large core protein (Davies et al 1992, Wight 1989).

Fibronectin is a large multi-domain body fluid and matrix glycoprotein capable of inducing the adhesion of many cell types and in blood vessels is located in the interstitial matrix. It has a central domain, which contains a characteristic cell binding sequence of peptides Arginine-Glycine-Aspartate or RGD sequence that binds to many integrin subtypes promoting cellular proliferation and migration (Ruoslathi 1984).
3.5 Cellular- extracellular matrix interactions and integrins

Dynamic adhesive interactions between cells and components of the ECM play a central role in cell proliferation and migration by inhibiting or promoting these processes as well as providing cell substrate traction for the generation of intracellular mechanical stresses (Newby and Zaltsman 2000). The cell surface molecules believed to be central to the interaction of the cell with the ECM are the integrins (Ruoslathi 1994). These are a family of cell membrane glycoproteins composed of two subunits, an α and a β, that mediate cell adhesion principally to the ECM but also to other cells. Cytoplasmic domains in each subunit interact with components of the cytoskeleton and cell signalling apparatus enabling the integrins to serve as a link between the cytoskeleton and ECM. Each β subunit can associate with multiple αs and likewise a single α subunit can be paired with one or more βs. The specific ligand binding site of the integrin appears to be formed on the cell surface by sequences from both subunits (Ruoslathi 1994) providing a wide diversity in the functional capabilities of integrins on cell proliferation and migration. The recognition site for many of the integrins that bind to ECM is the tripeptide sequence RGD common to many ECM molecules including fibronectin, osteopontin, fibrin, tenascin, thrombospondin and vitronectin. Other ECM molecules such as collagens and laminin bind via alternative motifs (for review see Ruoslathi 1994).

3.6 The central role of smooth muscle cells in neointimal hyperplasia

The accumulation of VSMC in the intima of blood vessels is the most prominent cellular feature following vessel injury. Most of our understanding of this process has been derived from rodent models, particularly the rat carotid balloon injury model introduced by Clowes et al (Clowes A et al 1987). In this model vascular smooth muscle cells in the media of carotid arteries subjected to mechanical injury undergo an initial proliferative phase between 4 and 48 hours followed by a migratory phase from media to intima between 24
hours and seven days. Once in the intima VSMC undergo further proliferation followed by excessive deposition of extracellular matrix. In the rat carotid this leads to lumenal occlusion however this is offset by a compensatory increase in medial volume and vessel diameter referred to a vascular remodelling. This sequence of events has been seen in many animal models of neointimal hyperplasia, including pigs, rabbits and even primates (Newby and Zaltsman 2000) although direct comparisons between these models and more chronic fibroproliferative processes in humans such as atheroma and CAD remain guarded. In rat, murine, porcine and primate models of allograft vasculopathy an initial inflammatory phase is followed by medial SMC proliferation followed by the emergence of proliferating VSMC in the neointima. In rodents neointimal VSMC are derived from the adjacent media in allograft vasculopathy (Geraghty et al 1996, Aziz et al 1995) as well as in balloon injury models of neointimal hyperplasia whilst in human arteries the origin of the cells seen in neointimal lesions is not as clear (Newby and Zaltsman 2000). In humans the normal intima has a population of mesenchymal cells, probably modified VSMC that some have suggested may be the source of cells in neointimal thickening (whilst others suggest that adventitial fibroblasts may be responsible (Shi et al 1996, 1997). Human atherosclerotic plaques are almost always associated with underlying medial thinning with disruption of the internal elastic lamina however and it is likely that at least some of the cells seen in human neointimal thickening arise from the underlying media (Newby and Zaltsman 1999).

3.6.1 Regulation of VSMC proliferation, migration and ECM turnover

Studies in rodents have enabled the unravelling of many of the cellular mechanisms and molecular mechanisms associated with intimal hyperplasia. It is sensible to suggest that these mechanisms are common to all neointimal thickening regardless of aetiology and are summarised below. Central to this process is the dynamic interaction between the cell and
its external milieu, both in terms of its exposure to growth factors that may alter cell behaviour (see above), but also in the interaction of the cells with structural matrix proteins. The importance of both these influences on cell behaviour is demonstrated by the different actions of growth factors on cells in culture as opposed to organ culture or in vivo. Exposure of arterial or vein organ cultures to serum that is profoundly mitogenic to cells in culture either fails to stimulate VSMC proliferation or does so only if the vessel is also injured. The so-called 'brake hypothesis' (Newby and Zaltsman 2000) suggests that the interaction of VSMC with their immediate ECM, the basement membrane, acts as a brake on proliferation and migration. Changes in the constituency of the extracellular matrix as well as the nature of ECM-cell interaction is therefore an essential regulatory feature in this process.

3.6.2 Smooth Muscle cell activation and change in phenotype
Medial VSMC of uninjured adult arteries retain the ability to migrate and divide rapidly in response to injury. This change in behaviour requires a switch in the spectrum of active genes referred to as phenotypic modulation. This phenotypic modulation commonly referred to as a switch from the contractile to the secretory state can occur in parallel with, or after the onset of VSMC proliferation (Clowes et al 1983).

3.6.3 VSMC proliferation
VSMC proliferation can be divided temporally into Gap 1 (G1), DNA synthesis (S), Gap 2 (G2) and mitosis (M). The molecular basis of cellular proliferation is reviewed elsewhere (Newby and Zaltsman 2000) however in summary it is dependent on the release of the transcription factors such as E2F which causes induction of DNA polymerase and the commencement of the S phase. Commitment of a cell to pass completely through to mitosis is achieved around this point. Release of E2F is dependent on the inactivation of the Retinoblastoma protein (pRB) by cyclin dependent kinases (D and E) that are in turn regulated by the competing effects of multiple pathways, including the MAP kinase
cascade, protein kinase B and C, transcription factors (NF-kB, AP-1) and growth factor
dependent GNP binding proteins (Newby and Zaltsman 2000). pRB phosphorylation is
jointly regulated by growth factors and the extracellular matrix and cyclin activation does
not occur unless certain cell surface integrins are engaged with specific extracellular matrix
components (Assoian and Marcantonio 1996). For example interaction of integrins with
moieties containing the RGD sequence such as fibronectin, osteopontin and vitronectin
fragments promotes VSMC proliferation, whilst blocking the interaction of integrins with
RGD containing moieties with synthetic RGD sequences inhibits VSMC proliferation in
vivo (Matsuno et al 1994). Conversely interaction with basement membrane constituents
such as laminin and heparan sulphate (HS) proteoglycans inhibit VSMC proliferation
(Clowes et al 1991). Heparin and also presumably HS is known to inhibit transcription
factor AP-1 (Au et al 1994) important in the transcription of metalloproteinases and tissue
plasminogen activator via an inhibitory effect on protein kinase C whilst fibrillar collagen
upregulates the p27 cyclin dependent kinase inhibitor (Koyama et al 1996).

The type of integrin expressed at the cell surface and the production of different ECM
constituents by proliferating VSMC are of particular importance in determining the nature
of the cell ECM interaction. Integrin dependent activation of MAP kinase may play an
important role in cyclin expression and subsequent pRB inactivation. Wary et al (1996)
demonstrated that MAP kinase activation was found to be dependent on the α subunit of
the integrins expressed on the cell surface, occurring with integrins containing the α 1,5 or
v subunits but not with integrins containing the α 2 or 3 subunits. Injured VSMC express
α5b1 and α v containing integrins as well as synthesising increasing quantities of the
interstitial matrix components fibronectin and vitronectin (Topol et al 1994). Interaction of
fibronectin with a5b1 promotes proliferation in VSMC (Wary et al 1996). The type of β
subunit is also important. Synthetic VSMC increase their production of fibrillar collagen (1
and 3) as well as switching their collagen binding integrins α1β1 to the growth promoting α1β2 variety. Conversely damaged VSMC production of basement membrane proteins such as laminin is reduced as is the expression of VSMC laminin receptors α2β1 (Wary et al 1996).

3.6.4 Migration

Directed VSMC migration requires the combined action of a chemoattractant gradient (in this case from the intima to the media) and remodelling of the extracellular matrix. (The role of different growth factors and chemokines in chemotaxis is reviewed in Abedi and Zachary 1995). The signal transduction mechanisms underlying the movement of cells remain largely unknown however the generation of force by the cell cytoskeleton is believed to be mediated by actin stress fibres and focal adhesions or focal contacts, specialised juxtaembrane regions which form at the termini of stress fibres and at sites of attachment of cells to the ECM. The assembly of focal adhesions is altered during cell movement, in particular it is increased by PDGF (Ridley & Hall 1992). Integrins aggregate at focal contacts and many non-structural cytoskeletal components are localised to these structures. Dynamic interaction between cell surface integrins and components of the ECM provide cell substrate traction for the generation of intracellular mechanical stresses as well as transducing signals into cells which may be important in the regulation of cell movement. Focal Adhesion Kinase p125, a protein tyrosine kinase localised to focal adhesions with receptors for both integrins and focal adhesion constituent proteins is thought to have an important role in regulating cytoskeletal and focal adhesion assembly and cell movement (Abedi and Zachary 1995). Activation of B1 integrin by fibronectin induces phosphorylation of p125 FAK (Hanks et al 1992).
Cell migration is associated with changes in the type, concentration and location of integrins on the cell surface. VSMC express B1 and B3 integrins as well as a variety of a subunits including av and al (Ruoslathi 1994, Abedi and Zachary 1995). B1 is constitutively expressed in most cells in the body, is important in cellular adhesion to ECM substrate and in resting VSMC is localised to focal adhesions. B3 integrins on the other hand are expressed in VSMC stimulated with bFGF, TGF β and PDGF BB (Janat et al 1992). In migrating VSMC B1 integrins are located on the base of the cells and antibodies to B1 inhibits the adhesion of VSMC on fibronectin substrates (Yue et al 1994). B3 integrins are located on the leading edge of migrating VSMC and antibodies to B3 but not B1 inhibited migration in response to osteopontin, an RGD motif ECM molecule, produced by proliferating VSMC (Yue et al 1994).

As well as changes in focal adhesion formation and integrin expression VSMC migration is also associated with significant changes in ECM production. ECM molecules such as fibronectin and vitronectin promote migration, others, principally basement membrane substrates such as Collagen IV, laminin and HS inhibit it (Koyama et al 1991, Naito et al 1991, Ross 1999). Injury to rat arteries is associated with an increase in CS proteoglycan production with a reciprocal decrease in HS synthesis (Alavi and Moore 1987, Thyberg et al 1997). Expression of collagen I, and V, elastin and fibronectin is increased following injury (Rocnik et al 1998) whilst that of laminin is inhibited (Thyberg et al 1997). Vitronectin a serum glycoprotein accumulates in neointimal lesions (Brown et al 1994) and is thought to promote VSMC migration via RGD recognition by avb3 integrin. Fibrillar collagens are thought to inhibit migration however denatured or newly synthesised monomeric Collagen fibrils expose RGD sequences that can promote migration (Mogford et al 1996, Rocnik et al 1998). Procollagen or collagen breakdown products may therefore serve to promote migration, indeed the loss of the fibrillar architecture of the interstitial
ECM may also serve to facilitate VSMC production. This is supported by the observation that in the period immediately post injury in rat arteries the GAG hyaluron is produced in large quantities. This forms a loose hydrated ECM that may facilitate migration and proliferation (Riessen et al 1996). Collagen VIII, is a short chain collagen produced at the leading edge of migrating VSMC in balloon injured rat and pig arteries (Hou et al 2000) and interacts with α2β1 and α1β1 integrins to stimulate VSMC attachment and focal adhesion formation. It is strongly chemotactic for VSMC (Hou et al 2000) and stimulates the release of proteases by VSMC particularly MMPs. By acting as a provisional matrix substrate following injury, a potent chemotactic agent and as a modulatory molecule Collagen VIII is thought to be a primary regulator of VSMC migration (Hou et al 2000).

3.6.5 Extracellular matrix accumulation

Migrating VSMC eventually form only 10 to 20% of mature atherosclerotic or intimal lesions and production and deposition of ECM constitutes most of the volume of the neointima. Human atherosclerotic plaques are characterised by high levels of collagens 1 and 3, as well as elastin and CS and DS GAG (Wight et al 1997). These plaques appear heterogeneous with distinct zones rich in either GAG or collagen. Mature atherosclerotic plaques classically demonstrate a collagenous fibrous cap. The composition and relative structural integrity of this cap is thought to be a critical determinant of plaque stability and subsequent vascular events such as stroke, myocardial infarction and critical limb ischaemia. In intimal lesions associated with allograft vasculopathy early inflammatory lesions stained for laminin and collagen IV and not collagens 1 and 3 whilst mature intimal hyperplasia in allografts was associated with high levels of the interstitial collagens 1 and 3 (Kemeny et al 1991). Following balloon injury to rat and pig arteries in vivo intimal ECM expansion was associated with increased VSMC expression of the CS versican, the DS biglycan, type 1 procollagen and elastin (Nikkari et al 1994, Strauss et 1994).
3.6.6 Vascular remodelling

In addition to neointimal hyperplasia, vascular injury is also accompanied by changes in the geometric dimensions of the vessel by a process termed vascular remodelling. Vascular remodelling may be expansive or constrictive and it is the sum of this geometric change and the degree of neointimal hyperplasia that determines ultimate lumen diameter, i.e. expansive remodelling prevents, and constrictive remodelling enhances, lumenal narrowing by intimal hyperplasia. Indeed in animal models of intimal hyperplasia as well as in humans with angioplasty restenosis, constrictive remodelling is the most important determinant of lumenal narrowing (Post et al 1994, Lafont et al 1995). Understanding of the molecular basis of this process is therefore as important as our understanding of intimal hyperplasia.

Remodelling is seen in virtually the entire spectrum of vascular disease including atherosclerosis, vein graft stenosis, post angioplasty restenosis, hypertension and allograft vasculopathy (Pasterkamp 2000). In atherosclerosis, an inflammatory process, the introduction of ICUS has greatly increased our understanding of this process. In particular the absence of a relation between plaque rupture and angiographic lumenal stenosis, for reasons that were previously unclear, can now be explained by the correlation between expansive remodelling and plaque instability. Expansive remodelling prevents lumenal narrowing but masks plaque volume as detected by angiography (Pasterkamp et al 1998). Furthermore plaques associated with expansive remodelling are also more prone to rupture (Pasterkamp et al 1998, Fishbein and Seigel 1996). They demonstrate increased metalloproteinase activity, accumulation of interstitial ECM with less tensile strength and increased plaque neovascularisation all factors thought to promote plaque rupture (Pasterkamp 2000). Allograft vasculopathy in particular is associated with expansive remodelling. This is apparent in ICUS studies where intimal thickening is present in the
majority of patients by three months after heart transplantation, but is only detected in 30% of patients using quantitative angiography.

The pathogenesis of vascular remodelling remains poorly defined. High shear stress on the intact endothelium is thought to mediate expansive remodelling via a nitric oxide mediated mechanism in response to high flow under normal physiological conditions (Tronc et al 1996, Guzman et al 1997). Similarly low flow induces vessel constriction and this serves to maintain shear stress at a relatively constant level. Pathological remodelling occurs in atherosclerotic lesions perhaps as a result of alterations in shear stress induced by lumenal encroachment. This fails to explain the coexistence of expansive and constrictive remodelling side by side in a single vessel as seen in human studies however (Pasterkamp et al 1996) although the presence of dysfunctional endothelium may play a role.

Remodelling also occurs following balloon angioplasty and appears to be regulated by different factors than those responsible for intimal thickening (Post et al 1997). In pigs in vivo lumen loss following PTA was chiefly due to constrictive remodelling whilst lumen loss following stenting was chiefly due to neointimal hyperplasia (Post et al 1997). Shear stress and wall tension again are thought to be important in this process. The reduction in shear stress associated with the acute lumen gain, following PTA, is closely correlated with the degree of constrictive remodelling (Krams et al 1998) despite the loss of endothelium associated with this injury. Immediately following PTA wall stress increases (due to increased vessel diameter (law of Laplace) whilst shear stress falls as flow velocity across the stenosis drops. In the presence of a MMPi constrictive remodelling is attenuated such that shear stress normalises however wall stress does not (i.e. due to an increase in the thickness of the wall or wall area (Wentzel et al 2001)
Other postulated pathophysiological mechanisms important in remodelling include formation of the neoadventititia and alterations in ECM turnover. Adventitial thickening is related to the severity of constrictive remodelling, more so than changes in intimal or medial dimensions, (Lafont et al 1995) however its precise role in this process is unclear. In rat allografts neoadventitial thickening was associated with intense Collagen I synthesis and it has been suggested that this may serve as a constrictive band around the vessel. Given that collagen constriction can occur, mediated by MMP dependent mechanisms, it is possible that this could contribute to changes in vessel diameter. Formation of this 'neoadventitia' in balloon injured porcine arteries in vivo is associated with proliferation of adventitial fibroblasts and modulation of their phenotype to myofibroblasts and an accumulation of ECM (Shi et al 1996). During remodelling of porcine autologous vein grafts in vivo adventitial myofibroblasts migrate to the neointima (Shi et al 1997). The overall significance of this process to remodelling and neointimal formation is unknown however as similar changes are not seen in cultured human saphenous vein and the time course of neoadventitial thickening (occurring at three days post injury) differs from that of constrictive remodelling (commencing at one week) (de Smet et al 1998).

Differences in the nature and level of ECM deposition between constrictive and expansive remodelling have prompted suggestions that factors regulating ECM turnover may therefore be important. In rabbit arteries in vivo collagen content is significantly lower in restenotic versus non restenotic vessels after balloon angioplasty (Coats et al 1997). In almost direct contradiction to this inhibition of constrictive remodelling in rats in vivo by the inhibition of TGF β was associated with a reduction in collagen I and collagen III mRNA expression (Smith et al 1999). In rats collagen mRNA expression increases starting at two days post angioplasty whilst collagen accumulation only becomes significant at 14 days (Strauss et al 1994). It is therefore possible that observed differences in collagen
content may be mediated not only by collagen synthesis, but also by collagen breakdown. This is supported by serial angiographic studies in humans with documented atherosclerosis. Those homozygous for the 6A promoter (6A6A) of MMP 3, which has lower promoter activity (with less MMP activity and hence less collagen breakdown) had greater progressive lumenal narrowing on serial angiography than those not homozygous for 6A6A (Ye et al 1998). This would suggest that decreased ECM breakdown and hence turnover reduces the vessel's ability to expansively remodel. Atheromatous plaques associated with expansive remodelling paradoxically have not only greater plaque volume (and hence ECM content), but also increased MMP activity (Pasterkamp 2000). Moreover in the rat carotid balloon injury model transfection of MMP 9 was associated with expansive remodelling (Mason et al 1999).

In inflammatory models of intimal hyperplasia, such as allograft vasculopathy, expansive remodelling is more prominent. It is possible that NO and protease production by inflammatory cells may contribute to increased ECM breakdown and subsequent expansive remodelling. This is supported by the observation in rats that expansive remodelling is seen in arterial allografts (Wehr et al 1997) but not isografts. In addition attenuation of the inflammatory response by cyclosporin also attenuated the remodelling (Wehr et al 1997). By inhibiting expansive remodelling cyclosporin may therefore potentially accelerate lumenal narrowing. This could in part contribute to graft dysfunction whilst more importantly immunosuppressant that fail to suppress remodelling could have the opposite effect.

3.7 **Fibrosis**

Chronic allograft dysfunction is characterised not only by allograft vasculopathy but also by the development of prominent perivascular and interstitial fibrosis within the allograft.
Fibrosis may be defined as a progressive and inappropriate accumulation of connective tissue, dominated by collagen, within the tissue. This fibrosis is ultimately associated with loss of allograft function whether it is due to renal tubular atrophy and glomerulosclerosis, cardiac failure or obliterative bronchiolitis. The injurious stimuli responsible for this fibrosis are likely to include a combination of end organ ischaemia secondary to allograft vasculopathy as well as the specific response to injury of the tissue concerned by native fibroblasts or other mesenchyme derived cells such as mesangial cells or renal tubular cells. The rate of interstitial fibrosis does not necessarily parallel the progression of allograft vasculopathy, as demonstrated in murine models of cardiac allograft dysfunction (Armstrong et al 1997) although the fundamental pathophysiological process of growth factor and cytokine excess operating in a milieu extracellular matrix turnover are similar. They result in the recruitment, proliferation of mesenchyme derived cells followed by a marked increase in their production of extracellular matrix and a reduction in the rate of ECM breakdown (Strutz 1995). Major ECM constituents produced in excess in fibrotic disease include the major fibrillar collagens 1 and 3, fibronectin and CS and DS proteoglycans, whilst ECM breakdown is inhibited in part by the increased production of protease inhibitors (Strutz 1995, Franklin 1995). Few studies have been directed at elucidating the mechanisms involved in allograft fibrosis with the exception perhaps of renal transplants. In injured or transplanted human or rat kidneys the degree of tubulointerstitial fibrosis correlates closely with the rate of decline of renal function (Strutz 1995), and in renal transplants specifically collagen 3 protein levels and TIMP 1 mRNA transcript levels correlate with the rate of decline in graft function (Nicholson et al 1997, 1999). The Puromycin aminonucleoside nephrosis rat model of renal parenchymal fibrosis has demonstrated two phases of ECM synthesis (Jones et al 1992). The first is associated with increased interstitial collagen and fibronectin production as well as an increase in the activity of enzymes that digest ECM such as MMPs. This phase appears at least in some
animal models to be reversible. The second phase was associated with increased ECM synthesis but diminished MMP activity attributed not to a decline in protease expression but an increase in their inhibitors (Jones et al 1992).

**TGF β**

Several lines of evidence point to transforming growth factor β as a key cytokine that initiates and terminates tissue repair and whose sustained production underlies the development of tissue fibrosis (reviewed in Border and Noble 1994). There are three isoforms TGF β1, 2 and 3 with similar biological properties although TGF β1 is the isoform associated with fibrosis. TGF β1 is synthesised as a 391 amino acid precursor molecule that is proteolytically cleaved to yield an active 112 amino acid subunit. Active TGF β1 is composed of two subunits linked by a disulphide bond. The inactive form is excreted by cells bound to a dimeric peptide, the latency associated peptide. Latent TGF β is then stored at the cell surface or in the ECM in this way and activated by cleavage by an as yet unknown mechanism. There are three TGF β receptor types- 1,2 and 3. Receptor type 1 is associated with the remodelling of the ECM whilst the effects of TGF β on cell growth and proliferation are mediated by the type 2 receptor. The biological activity of TGF β can therefore be regulated at the level of synthesis, post translational modification, excretion, activation or receptor type. This diversity is reflected in the wide range of activity attributed to TGF β not only in fibrosis and later stages of tissue repair but also in modulating tissue injury (Border and Noble 1994).

TGF β has an anti-inflammatory and immunomodulatory role in tissue inflammation, and is for example elevated in acutely rejecting allografts, in response to hypercholesterolaemia and hypertension and (Paul et al 1996) and following arterial balloon angioplasty injury. TGF β in damaged tissue is chemotactic for macrophages and induces activation of these cells at sites of injury promoting them to produce more TGF β. TGF β can also however
inhibit proliferation and activation of lymphocytes, macrophages and neutrophils at higher concentrations thereby acting as both an activator and inhibitor of the inflammatory process (Border and Noble 1994). One suggested physiological role for TGF β is that it serves to reduce inflammation in preparation for healing.

One major effect of TGF β is the regulation of ECM turnover. This effect is characterised by changes in ECM production (increased production of interstitial collagens and proteoglycans), altered integrin expression in cells and a reduction in the rate of ECM breakdown by fibroblasts, VSMC and other types of mesenchyme cell stimulated by TGF β (Border and Noble 1994). Paradoxically TGF β has also been ascribed an inhibitory effect on ECM expansion by promoting greater adhesiveness between ECM and cells via altered integrin expression (Kagami et al 1993). TGF β therefore appears to be a central regulatory molecule in the tissue response to injury and repair. It is a disruption of normal repair processes often associated with TGF β excess that is thought to characterise fibrotic disease. Excessive TGF β production has been associated with the development of fibrosis in chronic allograft dysfunction (Jain et al 2000) as well as fibrotic diseases in lung, liver and native kidney (Border and Noble 1994). In the antithymocyte serum induced glomerulosclerosis rat model of renal fibrosis a single dose of antithymocyte globulin is associated with a transient elevation in mesangial cell TGF β production associated with a commensurate rise in ECM synthesis. After a single dose this ECM accumulation is completely reversible, however if a second dose is given prior to resolution TGF β levels underwent a sustained increase and this was associated with progressive irreversible fibrosis (Yamamoto et al 1994). Repetitive injury may therefore induce dysregulation in the TGF β mediated regulation of tissue inflammation and repair leading to TGF β excess and fibrosis. This 'two shot ' hypothesis could explain the elevated TGF β levels and progressive ECM accumulation that characterise chronic inflammatory lesions such as seen
in chronic allograft dysfunction where multiple acute and chronic injurious stimuli could react to produce dysregulation of TGF β mediated tissue repair mechanisms.

3.8 Summary

Neointimal hyperplasia and parenchymal fibrosis represent a common response to a chronic inflammatory injury whether this is infective, traumatic, allo- or autoimmune in nature. Central to this process is a switch in the pattern of active genes in cells, whether they be VSMC or fibroblast like cells, in response to disruption of tissue architecture and cytokine excess. The change in the pattern of active genes leads to changes in the nature of the cell's interaction with the ECM. This occurs via changes in focal adhesion and cytoskeletal assembly, integrin type expression on cell membranes and production of ECM molecules that promotes cellular proliferation and migration and ECM accumulation. Central to this hypothesis of ECM remodelling and changes in cell behaviour is a dynamic component attributed to changes in the nature and type of interaction between cells and ECM molecules. These changes are thought to be effected in large part by the activity of enzymes that can collectively degrade the components of the ECM permitting changes in ECM integrin interaction. The expression, activation and control of these proteases are interlinked with those processes mentioned above. One large family of proteases, the Matrix Metalloproteinases appear particularly important. Their role in neointimal hyperplasia, vascular remodelling and ECM accumulation will be discussed in the next section.
Part 4: Matrix metalloproteinases and their inhibitors in vascular disease

4.1 Introduction

Vascular injury leads to excessive fibroproliferative and inflammatory lesions that produce vessel lumenal encroachment with resultant end organ ischaemia or infarction. This process is seen in neointimal hyperplasia in arterial bypass conduits, arteriovenous fistulas for haemodialysis, atherosclerotic lesions that are responsible for myocardial, cerebral and peripheral ischaemia (Ross 1999) and in particular in allograft vasculopathy in solid organ allografts. Smooth muscle cell proliferation migration and extracellular matrix deposition are central to this process, the molecular basis of which has been referred to in the previous section.

A family of zinc dependent extracellular proteinases, the metalloproteinases, and their inhibitors appear to be particularly important in all stages of the development of these lesions and represent an attractive target for therapeutic intervention (George 1998). A great deal of current research in the field of vascular disease is therefore directed at the development of novel treatments based on the modulation of metalloproteinase activity. MMPs have also been implicated in a variety of other disease processes including arthritis, and most significantly in the invasion and metastasis of malignant cells in the pathogenesis of cancer. In spite of the theoretical benefits of MMP inhibitors in these diseases they have not proved beneficial in clinical trials. This may reflect poor efficacy of the drugs in question but may also reflect our lack of a clear understanding of the processes involved. The purpose of this section therefore is to review our current understanding of the role of MMPs and TIMPs in the development of intimal hyperplasia and atherosclerosis with a view to understanding their role in the neointimal hyperplasia, remodelling and extracellular matrix accumulation that characterises allograft vasculopathy.
4.2 The matrix metalloproteinase family

4.2.1 Structure and function of MMPs

The MMPs are a family of highly conserved zinc dependent endopeptidases, which,
collectively, are capable of degradation of most, if not all, of the basement membrane and
extracellular matrix. Although ECM components can also be degraded non-specifically by
aspartyl, cysteinal or seryl proteases it thought that MMPs are primarily responsible for the
proteolysis of specific structural components of the matrix. There are now over 25
enzymes that are classified as MMPs (Nelson et al 2000) and these are divided into
different groups based on substrate specificity and structure. This includes 1) collagenases
that degrade structural fibrillar collagens found in the interstitial matrix, 2) gelatinases
which act predominantly on basement membrane components and non fibrillar collagens,
3) stromeolysins which have a broad substrate specificity including proteoglycans and
fibronectin, and 4) membrane type MMPs (MT MMP). A summary of this classification is
listed Table 1. Each of the collagenases and stromeolysins MMPs have a similar structure
consisting of 5 domains including a signal peptide to target for secretion, a ‘pro’ region
that binds to the active site to maintain latency and is cleaved to achieve activation and an
active catalytic region that contains the zinc-binding active site. (Woessner 1994). This in
turn is connected via a hinge region to a –COOH terminal domain such as a haemopexin
like domain or fibronectin like domain thought to be important in substrate recognition or
inhibitor binding. Gelatinases also have this 5 domain structure, but within their catalytic
domain are three fibronectin domains that are thought to facilitate substrate binding. The
MT MMPs possess not only the five domains common to other MMPs but also a
transmembrane domain that anchors MT-MMPs to the cell surface (Sato et al 1994).
4.2.2 Regulation of MMP activity

MMP activity is regulated at multiple levels including transcription, activation and inhibition (Nelson et al 2000, Woessner 1994). These represent potential targets of therapeutic strategies aimed at treating vascular disease.

Transcriptional regulation


The promoter regions of many of the inducible MMP genes share a common activator protein 1 (AP-1) whilst constitutively expressed MMP 2 has a different promoter region (Huhtala et al 1990). The interaction of the AP-1 site with other elements such as the polyomavirus enhancer A binding protein 3 (PEA 3) site are required for basal transcription and this site is involved in the repression of MMP transcription by TGF β and corticosteroids (Benbow et al 1997). TGF β decreases mRNA transcription by binding to an inhibitory element of the common MMP promoter known as the TGF β inhibitory element (TIE). The MMP 2 promoter lacks this component and indeed TGF β may in fact increase MMP 2 expression in vitro (Brown et al 1990, Overall et al 1991). The promoter of the MMP 2 gene also lacks the AP 1 and PEA 3 sites but contains two sites for the binding of transcription factor SP 1 (Corcoran et al 1996). Unlike other MMPs, MMP 2 is expressed constitutively in uninjured rat, rabbit and pig arteries and pig and human
saphenous vein as well as in organ culture models and in VSMCs in culture that are not
stimulated to proliferate although MMP 2 expression is also increased following injury.

Transcription is also regulated at the level of the individual MMP gene (Brown et al 1990,
Vicenti et al 1998) by individual promoter sequences and polymorphisms of these
promoter regions have been shown to affect the rate of transcription of the MMP genes.
Specifically polymorphisms of the MMP 1 promoter sequence have been associated with
increased transcription in certain tumour cell lines (Rutter et al 1998) and causes increased
matrix degradation by certain tumour cells. In humans the 6A polymorphism of the MMP
3 promoter sequence is associated with lower promoter activity compared to the 5A
polymorphism. Homozygosity of this 6A allele was associated with progression of
atherosclerosis in patients with ischaemic heart disease, potentially via a reduction in
matrix degradation with subsequent matrix accumulation within atherosclerotic plaques
(Ye et al 1998). Polymorphisms with greater promoter activity within the MMP 2 and
MMP 12 promoter regions were associated with worse angiographic disease (the
expression of which is increased by insulin). Two polymorphisms, the A and G alleles
have been identified, with greater AP 1 binding promoter activity in the A allele (Jormsfo
et al 2000). In clinical studies the A allele was associated with smaller lumenal diameter in
diabetic patients with manifest coronary artery disease. A similar study of the promoter
regions for human MMP 9 promoter regions demonstrated that polymorphisms that had
greater promoter activity (T polymorphisms) had more severe angiographic ischaemic
heart disease (Zhang et al 1999).

**Activation of proenzyme**

MMPs such as collagenases, gelatinases and stromelysins are secreted as inactive
membrane boundzymogens and require proteolytic cleavage of the N terminal pro peptide
for activation (Woessner et al 1994). Initiation of catalytic activity is believed to occur via
a cysteine switch mechanism (Corcoran et al 1996). An unpaired cysteine residue in the prodomain maintains the latency of the zymogen via a thiol interaction with the active site zinc atom. Activation of the proenzyme occurs in several stages. Partial activation occurs when the prodomain is cleaved by other proteases e.g. plasmin, trypase, chymase, kallikrein and other MMPs. Once partially activated full activation can occur by autcatalytic or exogenous cleavage generating a proteolytic cascade that includes several positive feedback mechanisms. For example activation of one MMP may be initiated by interacting with a second activated MMP. MMP 3 can activate MMP 2 and 9 (see below), whilst MMP 7 and MMP 2 can activate MMP 3 and MMP 9 (Fridman et al 1995, Imai et al 1995). The pathways that initiate these proteolytic cascades are unclear however several parallel mechanisms that potentially localise proteolytic activity to the cell membrane or immediate pericellular environment may be responsible. These include extracellular activation by plasmin, MT-MMPs, ADAMs, integrins, exogenous MMPs and extracellular matrix components, release of lysosomal cathepsins (serine proteases) and intracellular activation by golgi associated furin.

Plasmin

One of the first mechanisms identified as initiating the proteolytic cascade was the serine protease plasmin the activity of which is increased in blood vessels following injury. In vitro, plasmin directly activates pro MMPs -1, -3, -9, -10 and 13 (Lijnen and Collen 1999), and also activates MMP-2 in conjunction with MT-MMP 1 (Baramoova et al 1997). The plasminogen/plasmin system contains a pro-enzyme, plasminogen, which is converted to the active enzyme plasmin by tissue-type (t-PA) or urokinase-type (u-PA) plasminogen activator. Gene knockout studies in mice have demonstrated that only uPA had a significant role in MMP activation following vessel injury (Lijnen and Collen 1999). The activity of uPA is localised to the surface of macrophages and VSMC by interacting with its membrane bound receptor (uPA-r). The subsequent production of plasmin is thought to
localise proteolysis and MMP activation at the leading edge of the cell. MMP 2 activation is plasmin independent (Corcoran et al 1996). In vitro cell culture studies have shown that MMP-2 activation occurs independently of plasmin in cultured mice smooth muscle cells and fibroblasts whilst MMP-9 is activated independently of plasmin by fibroblasts but not smooth muscle cells or macrophages (Lijnen et al 1998a). In knockout mice in vivo MMP-2 activation following vascular injury occurs independently of plasmin however MMP 9 activation in this model was plasmin dependent (Lijnen et al 1998a). MMP-3 is also activated by plasmin which can in turn activate MMP 9 but not MMP 2 although activation of MMP 9 also occurs via non MMP 3/ plasmin dependent pathways (Lijnen and Collen 1998b). MMP 3 can also activate interstitial collagenase (MMP 1). MMP 3 specifically interacts with uPA increasing its serine protease activity as well as increasing its affinity with the uPA receptor on smooth muscle and macrophage cell membranes in vitro (Ugwu et al 1999)

**MT-MMPs**

MMP 2 activation as well as collagenase 3 (MMP-13) is thought to be localised at the cell membrane by the action of MT MMPs which also degrade fibrillar collagens and other ECM constituents. MT1 MMP mediated activation of pro MMP 2 occurs in conjunction with TIMP 2 (Corcoran et al 1996). TIMP 2, by binding to pro MMP 2 as well as MT MMP, stabilises their interaction and enables the formation of active MMP 2 at the leading edge of the cell (Butler et al 1998). Conversely molar excess of TIMP 2 inhibits the activation of MMP 2 (Strongin et al 1995). The rate of activation of MMP 13 by MT1 MMP is enhanced by the presence of pro-MMP 2 (Knauper et al 1996).

MT MMP may be activated by plasmin whilst its activity is also influenced by interaction with the ECM. VSMC stimulation by lectins increases MT MMP activation whilst inhibiting the interaction of a2b1 integrin with collagen inhibits MT MMP (Jenkins et al 1998). This may represent a direct connection between ECM cell interaction and MMP
activation. MT1 MMP activation occurs only in the earliest phase of VSMC migration (Jenkins et al 1998) following balloon injury in rat arteries whilst MMP 2 activity continues until the migration phase ceases. MMP 2 is also activated at the cell membrane directly by interaction with avb3 integrin, the expression of which is increased in migrating VSMC (Brooks et al 1996) and it is possible that activation by avb3 integrin levels may maintain MMP 2 activation during migration after MT1 MMP activation has ceased.

**ADAMs**

Metalloproteinase activity is also possessed by another group of cell surface proteases called A Disintegrin And Metalloproteinase proteins (ADAMs). These are a group of 30 cell membrane proteins that contain protease like regions but also a disintegrin region that can mediate cell adhesion and fusion events (Wolfsberg et al 1995). Sixteen of these are known to contain a classical MMP active site and members are capable of degrading extracellular matrix components, cell surface receptors and extracellular proteins. It is possible that such molecules could be important in mediating cell matrix interaction in part by initiating or promulgating the MMP proteolytic cascade however there is little evidence to date regarding the role of these molecules in vascular disease. One important reason for considering these molecules is that they may be inhibited by synthetic MMP inhibitors and such may confuse the interpretation of experiments aiming to study the effects of MMPs by adding synthetic inhibitors.

**Lysosomal proteases**

Extracellular MMPs can also be activated by the release of lysosomal proteases. Chymase and tryptase released from activated mast cells that accumulate in the shoulder regions of atherosclerotic plaques activate MMPs and may contribute to plaque instability (Johnson et al 1998). The release of free radicals by inflammatory cells may also be involved in extracellular MMP activation possibly by disrupting the binding of the prodomain to the catalytic site as well as by inactivating MMPs (Frears et al 1996, Rajagopalan et al 1996).
**Furin activation**

Intracellular activation of pro-MMP by the Golgi associated serine protease furin is another mechanism whereby the catalytic cascade may be initiated with activated rather than proenzyme released into the extracellular space. This has been only demonstrated for MMP 11 however MMP 3 and MT MMP 1,2 and 3 all contain furin recognition sequences (Pei et al 1995) and it is possible that these too may be activated intra-cellularly by this process. Furin induced activation of MT1 MMP is not a prerequisite for MMP 2 activation however (Cao et al 1996) and whether this mechanism is responsible for the activation of cell bound MT1 MMP is unclear.

The presence of multiple activation cascades involved in regulating cell surface proteolysis suggests that there is likely to be a high degree of redundancy in the systems controlling cell surface proteolysis. Pathological (and possibly pharmacological) events that block one aspect of matrix breakdown may cause recruitment of an alternative rescue mechanism. Another alternative is that they have different roles in that they mediate responses to different stimuli, possibly in parallel but also in concert, with overall cell behaviour being dependent on the sum effect of different stimuli.

**Inhibition of MMP activity**

The most important physiological inhibitors of the MMPs are the tissue inhibitors of metalloproteinases (TIMPs), a family of low molecular weight proteins capable of specific inhibition of the active forms of the MMPs. Several different TIMPs (TIMPs 1-4) (Gomez et al 1997) have been identified in vascular tissue, each with differing specificities. Binding of the TIMPs to the catalytic site of activated MMPs in a stochiometric 1:1 ratio produces a complex that is unable to bind to substrate. In addition binding of specific TIMPs to the latent form regulates activation of the proforms. The transcriptional/post
transcriptional regulation of the TIMPs is independent of the MMPs (Fabunmi et al 1996). Important characteristics of the TIMPs are summarised in Table 4. As well as inhibiting MMPs, TIMPs also have multiple other functions. They have growth factor like properties mediated by a part of the TIMP molecule different to that which binds to MMPs. This is a direct cellular effect mediated by cell surface receptors (Bertaux et al 1991) and is independent of their effect as MMP inhibitors. Furthermore TIMP 2 either unbound or bound to MMP 2 can inhibit the proliferative response of vascular endothelial cells in response to bFGF (Hayakawa et al 1994) by a mechanism independent of its MMP action.
Table 1. Nomenclature and substrate specificity of individual MMPs.

<table>
<thead>
<tr>
<th>MMP Family</th>
<th>Descriptive Name</th>
<th>Number</th>
<th>Principal Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenases</td>
<td>Interstitial Collagenase</td>
<td>MMP 1</td>
<td>Fibrillar Collagens III &gt;&gt; I</td>
</tr>
<tr>
<td></td>
<td>Neutrophil Collagenase</td>
<td>MMP 8</td>
<td>Fibrillar Collagen I &gt;&gt; III</td>
</tr>
<tr>
<td></td>
<td>Collagenase 3</td>
<td>MMP 13</td>
<td>Fibrillar Collagen</td>
</tr>
<tr>
<td></td>
<td>Xenopus Collagenase</td>
<td>MMP 18</td>
<td></td>
</tr>
<tr>
<td>Gelatinases</td>
<td>Gelatinase A</td>
<td>MMP 2</td>
<td>Gelatin, Collagen I, IV, V,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fibronecctin, Elastin</td>
</tr>
<tr>
<td></td>
<td>Gelatinase B</td>
<td>MMP 9</td>
<td>Gelatin, Collagen IV, V</td>
</tr>
<tr>
<td>Stromeolysin</td>
<td>Stromeolysin 1</td>
<td>MMP 3</td>
<td>Proteoglycans, laminin, fibronectin,</td>
</tr>
<tr>
<td></td>
<td>Stromeolysin 2</td>
<td>MMP 10</td>
<td>non-fibrillar collagens</td>
</tr>
<tr>
<td></td>
<td>Matrileysin</td>
<td>MMP 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stromeolysin 3</td>
<td>MMP 11</td>
<td>Serine Proteinase Inhibitors</td>
</tr>
<tr>
<td>Membrane Type</td>
<td>MT MMP 1</td>
<td>MMP 14</td>
<td>Progelatinase A, Gelatin, Collagen I</td>
</tr>
<tr>
<td>Type</td>
<td>MT MMP 2</td>
<td>MMP 15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MT MMP 3</td>
<td>MMP 16</td>
<td>Progelatinase A, Gelatin, Collagen I</td>
</tr>
<tr>
<td></td>
<td>MT MMP 4</td>
<td>MMP 17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MT MMP 5</td>
<td>MMP 21</td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>Macrophage Elastase</td>
<td>MMP 12</td>
<td>Elastin</td>
</tr>
<tr>
<td></td>
<td>Enamelysin</td>
<td>MMP 20</td>
<td>Amelogenin</td>
</tr>
<tr>
<td></td>
<td>Rheumatoid Arthritis Associated</td>
<td>MMP 19</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMP 23</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMP 24</td>
<td></td>
</tr>
</tbody>
</table>

MMPs have both a descriptive name (e.g. interstitial collagenase I, an enzyme found in the interstitial space that degrades interstitial collagens) and a number (in this case MMP 1) based on the numerical order that they were sequenced. Although the numbering system recognises over 25 MMPs, the nomenclature does not accurately reflect the actual number of enzymes, because MMP 4, MMP 5, and MMP 6 have been eliminated as result of duplication.
<table>
<thead>
<tr>
<th>TIMP</th>
<th>Structure</th>
<th>Origin</th>
<th>Transcription/Expression</th>
<th>Action</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28.5kD</td>
<td>Soluble</td>
<td>Constitutive: Uninjured Blood Vessels and VSMC in culture (Kranzhofer 1999)</td>
<td>Inhibits all activated collagenases, stromelysins, gelatinases and ADAMs but not MT MMPs</td>
<td>Increased following angioplasty to rat carotid (9 hours) (Webb et al 1997) and rabbit aorta (neointima) (Wang et al 1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inducible: antioxidants, TNF α, IL-1, bFGF, TGF β, PDGF (Galis et al 1994, Hannemaaijer et al 1993)</td>
<td>Particular affinity for MMP 9</td>
<td>Increased in cultured human LSV (localised to neointima) (Kranzhofer et al 1999)</td>
</tr>
<tr>
<td>2</td>
<td>21kD</td>
<td>Soluble</td>
<td>Constitutive: Uninjured Blood Vessels and VSMC in culture (Corcoran et al 1996)</td>
<td>Inhibits all activated collagenases, stromelysins, gelatinases and MT MMPs</td>
<td>Increased following angioplasty to rat carotid (during migration phase, 2-7 days, in parallel with MMP 2) (Hasentab et al 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inducible: PDGF Inhibited: TGF β</td>
<td>Particular affinity for MMP 2. Stabilises pro MMP 2 but required for MMP 2 activation by MT1 MMP (Corcoran et al 1996)</td>
<td>Increased in cultured human LSV (media, adventitia but predominantly neointima,) (Kranzhofer et al 1999)</td>
</tr>
<tr>
<td>3</td>
<td>kD</td>
<td>Insoluble</td>
<td>Constitutive: Uninjured Blood Vessels (Kranzhofer et al 1999)</td>
<td>Inhibits all activated collagenases, stromelysins, gelatinases and ADAMs</td>
<td>Binds to ECM. Not increased during neointima formation in rat allografts (Murphy et al unpublished observations), porcine vein grafts (George et al 2000) or human LSV culture (Kranzhofer et al 1999)</td>
</tr>
<tr>
<td>4</td>
<td>23kD</td>
<td>Soluble</td>
<td>Constitutive (medial VSMC in uninjured blood vessels) Dollery et al 1999b</td>
<td>Inhibits MMP 1,2,3,7,9 (and MT1 MMP in cultured fibroblasts, Bigg et al 2001)</td>
<td>Localises preferentially to vascular tissue (Dollery et al 1999b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Affinity for MMP 2 (Dollery et al 1999b)</td>
<td>Increased during neointimal ECM accumulation in the rat (Dollery et al 1999)</td>
</tr>
</tbody>
</table>

**Table 4. Structure and role of TIMPs in injured and uninjured blood vessels**

ECM, Extracellular matrix, VSMC, Vascular Smooth Muscle Cell, LSV, Long saphenous vein, ADAMs, A Disintegrin and Metalloproteinases
4.3 MMPs and their inhibitors in occlusive vascular disease

In the uninjured blood vessel constitutive expression of MMP 2 (in rat, rabbit and pig in vivo as well as in cultured VSMC cells in vitro) and TIMP 1 and 3 (in human LSV in vivo) may serve to regulate cell growth and ECM turnover via modulation of cytoskeletal interaction basement membrane components with their subsequent effects on VSMC gene expression. Following injury however each step in the development of lesions such as atherosclerosis, allograft vasculopathy or vein graft stenosis (i.e. infiltration of inflammatory cells, medial VSMC proliferation and migration, extracellular matrix deposition and vascular remodelling) is characterised by a dramatic change in the rate and nature of ECM synthesis and degradation. This involves severance of the attachments of the cells to the matrix permitting phenotypic change and cell movement, breakdown of the ECM to remove physical barriers to migration and alteration of the constituency of the ECM to change the effect they have on cells i.e. basement membrane constituents or non-fibrillar or degraded collagens (Newby 2000). The action of selective proteases, capable of fulfilling each of these steps is therefore central to the vessel response to injury. Similarly the release of protease inhibitors (TIMPs) may serve to tightly control this process including restricting proteolysis to the immediate pericellular environment as well as fulfilling other functions such as inducing apoptosis or inhibiting cell growth.

The importance of MMPs and TIMPs in the development of vascular disease is underscored by the observation that they are increased during neointimal thickening and vessel remodelling in humans and in animal models as well as in organ cultures of saphenous vein and rabbit aorta in vitro. In an apparent paradox however synthetic MMPi's that inhibit medial smooth muscle migration and proliferation following vessel injury in rats fail to inhibit final neointimal thickness (Zempo et al 1996, Bendeck et al 1996). Furthermore the administration of synthetic MMP inhibitors often inhibits processes associated with MMP activity in some regions but not in others. For example batimastat, a
non-selective MMP inhibitor, inhibits VSMC proliferation in the media but not in the intima and subsequently fails to reduce the final neointimal thickness following balloon angioplasty in rats (Bendeck et al 1996). Over expression of TIMPs following gene transfer inhibits neointimal development in rat carotids (TIMP 1 and TIMP 2, Dollery et al 1999a, Cheng L et al 1998), cultured human saphenous vein segments (TIMPs 1,2 and 3) (George et al 1998a, George et al 1998b, George et al 2000) and in porcine autologous vein grafts (TIMP 3). It is unclear however whether their effects are entirely due to MMP inhibition or other TIMP mediated actions. The question remains therefore whether MMPs are realistic targets for therapeutic intervention. The roles of MMPs and their inhibitors in occlusive vascular disease can be considered as 1. The transduction of the injury stimulus, 2. Vascular smooth muscle cell migration and proliferation, 3. Extracellular matrix deposition and 4. Vascular remodelling.

4.3.1 MMPs and the transduction of the injury stimulus

The three principal stimuli in disease states that result in intimal hyperplasia and vascular remodelling in arteries and veins are 1. Inflammation, 2. Mechanical injury, and 3. Shear stress. Each of these stimuli is characterised by the increased metalloproteinase activity associated with the stimulus itself as well as the subsequent changes in MMP expression associated with VSMC proliferation and migration. These may be influenced in experiments seeking to elucidate the role of MMPs in the response to injury by the administration of MMP inhibitors or TIMP over expression.

4.3.1.1 Inflammation

The infiltration of T lymphocytes and macrophages that characterises the early stages in development of intimal hyperplasia in inflammatory states (e.g. human atherosclerosis and allograft vasculopathy (Ross 1999) is characterised by MMP activation. The principal MMP expressed by T cells is MMP9 which is released in its unbound form and is
subsequently activated by other proteases such as MMP 3 (Goetzl et al 1996). Adhesion of T lymphocytes to activated endothelial cells through VCAM 1 induces MMP 2 and 9 expression in endothelial cells in vivo (Romanic et al 1994, Madri et al 1996). MMP 2 and 9 expression may also serve to facilitate T cell migration through the basement membrane and interstitial matrix. Whilst MMP production by monocytes and macrophages may also facilitate cell movement macrophages produce a broader range of MMPs which may contribute substantially to overall ECM turnover and connective tissue remodelling.

Monocytes constitutively express MMP 9 (Galis et al 1994b) bound as a complex to TIMP 1 thereby protecting the complex from activation by MMP 3. Macrophages can also secrete MMP 1,2,3, 7 and 12 (macrophage metalloelastase) as well as expressing MT1 MMP (Goetzl et al 1996). MMP expression is increased by contact with ECM molecules such as collagen I (MMP 1 but not MMP 9) and laminin (MMP 1 and MMP 9) (Wesley et al 1998, Khan et al 1997) as well as by exposure to oxidised LDL (Xu et al 1999). The interaction of monocytes with collagen I may also induce MMP activation by stimulating superoxide production and secretion of IL-1 which can activate latent MMP 2 and 9 (Gudewicz et al 1994, Rajagopalan et al 1996). Pro MMP 2 and 9 in cultured VSMC are also directly activated by exposure to reactive oxygen species released by activated macrophages (superoxide, hydrogen peroxide and nitrous oxide) (Rajagopalan et al 1996) possibly by disrupting the thiol interaction between the prodomain and the Zn ion of the catalytic site.

In cell culture oxidised LDL, a major aetiological factor in the development of inflammatory vascular lesions increases MMP 9 expression in monocytes and MT1 MMP and MMP 2 expression activation in VSMC and macrophages in culture (Rajavashisth et al 1999b). In cultured macrophages oxidised LDL also reduces TIMP 1 expression, an effect that is abrogated by co-incubation with HDL (Xu et al 1999). In rabbits in vivo lipid lowering reduced macrophage accumulation, MMP activity and increased interstitial collagen staining in hypercholesterolaemic rabbits (Aikawa et al 1998). MMP 9/ TIMP 1
complexes also bind and are internalised by LDL protein receptors in embryonic fibroblasts suggesting that competitive activation of this receptor may directly regulate ECM protease activity (Hahn-Dantona et al 2001).

MMPs may also modulate the inflammatory response by their involvement in the processing of inflammatory mediators such as TNF α, the p80 TNF, p60 TNF and IL-6 receptors, L selectin and Fas ligand (George 1998). MMP 12 can process TNF α (Chandler et al 1996) releasing it from its membrane bound precursor and allowing it to stimulate VSMC proliferation. Similarly MMPs 2, 3 and 9 can process recombinant precursor IL-1 β (pIL-1β) to its active form in vitro, as well as promote breakdown of the active form (Schonbeck et al 1998). MMP 3 promoted slow activation that remained stable for 8 to 24 hours whilst MMP 9 promoted rapid activation that remained stable for 72 hours. In vitro expression of IL-1β and MMPs frequently occurs simultaneously at sites of inflammation and the balance of different MMPs and pIL-1β may therefore regulate the level and duration of IL-1 activity in these areas.

4.3.1.2 Trauma/ endothelial denudation

Restenosis following balloon angioplasty occurs in approximately 70% of patients and represents an enormously potent stimulus for intimal hyperplasia. The relationship between endothelial denudation and mechanical disruption of the media and neointima hyperplasia and remodelling is complex and multiple pathways are involved including MMP activation in the vessel wall as a direct consequence of injury. Platelet adhesion to the injured wall activates the adhesion cascade leading to high concentration of thrombin adjacent to the damaged media. Thrombin as well as being important in coagulation also has numerous other important effects on vascular cells including the promotion of VSMC proliferation and migration, partly by interacting with thrombin receptors on these cells (Galis et al
High local concentrations of thrombin may activate pro MMP 2 even when bound to TIMP 2 (Galis et al, 1997). This occurs in vitro in the absence of endothelial or VSMC cells. Similarly in cultured human endothelial cells (Zucker et al, 1995) thrombin activates pro MMP 2 independent of its interaction with the thrombin receptor. Thrombin also increases expression of MMP 1 and 3 in human endothelial cells by a G protein coupled thrombin receptor.

4.3.1.3 Shear stress and wall tension

Shear stress and wall tension are important factors in both physiological and pathological vascular remodelling responses and this has a direct effect on VSMC MMP expression. Following mechanical injury to rabbit carotid arteries MMP 2 expression increased (Baissiouny et al, 1998). Activation was further enhanced by low flow and shear stress and relatively inhibited by high flow. Furthermore MMP 2 activation was enhanced in uninjured arteries subjected to low flow (Baissiouny et al, 1998). This last observation is important as it suggests that increased MMP 2 activity may be induced by low flow independent of endothelial and VSMC injury. Such mechanisms may be due to direct release of MMP 2 from shear activated VSMC or indirectly via cytokine mediated pathways. Evidence of a direct effect of shear on MMP expression is supported by the observation that shear stress on cultured VSMC was associated with inhibition of MMP 2 and MT-MMP expression (Palumbo et al, 2000), whilst human VSMC in vitro subjected to biaxial strain showed inhibition of PDGF and TNF α induced expression of MMP 1 but not MMP 2 or TIMP 2 (Yang et al, 1998). This direct effect of mechanical stretch on MMP synthesis was associated with downregulation of the ets-1 MMP 1 transcriptional promoter. Increased wall tension (pressure) may also directly affect MMP expression. Cyclical mechanical stretch on cultured human VSMC increases fibronectin and collagen
synthesis as well as increased MMP 2 activity, which occurs at least in part through mechanical strain induced TGF β production in VSMC (O'Callaghan et al 2000).
SPECIAL NOTE

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<table>
<thead>
<tr>
<th>Species</th>
<th>Medial VSMC Proliferation</th>
<th>VSMC Migration</th>
<th>Neointimal VSMC Proliferation</th>
<th>Extracellular Matrix Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic allografts</td>
<td>MMP 2 and 3 increased 6 hours to 5 days (Webb et al 1997)</td>
<td>Increased MT1 MMP at 2 days precedes MMP 2 activation (Jenkins et al 1998 Z, NB, WB, IM). TIMP 2 (Z) maximal at 3 days (Hasentab et al 1997 Z, NB, WB, IM) but continuously elevated (Jenkins et al 1998)</td>
<td>No increase in TIMP 1 expression detected (Webb 1997, Hasentab et al 1997 Z, NB, WB, IM)</td>
<td>Increased MMP 9 (Murphy et al, unpublished)</td>
</tr>
<tr>
<td>Rabbit Balloon Angioplasty</td>
<td>TIMP 1 transiently increased (at 9 hours). TIMP 2 increased at 24 hours with MMP 2 (Hasentab et al 1997, Z, NB, WB, IM)</td>
<td>12-16 weeks post transplant Increased MMP 2, MMP 9 (Murphy et al, unpublished, RT-PCR)</td>
<td>TIMP 1 increased during neointimal proliferation (Wang et al 1996, NB, WB, ISH, IM)</td>
<td>Higher collagen content negatively correlates with gelatinase activity Coats et al 1997</td>
</tr>
<tr>
<td>Pigs Balloon Angioplasty</td>
<td>Increased MMP 2 and MMP 9 (Southgate et al 1996 Z, ISH)</td>
<td>Increased MMP 2 and 9 (Southgate et al 1996 Z, ISH) (also from cultured arterial segments (Southgate et al 1992))</td>
<td>Increased MMP 9 localises to neointimal VSMC. Some increase in MMP 2 (Southgate et al 1996 Z, ISH)</td>
<td>Increased TIMP 1 expression (Kranzhofer et al 1999 Z, WB, IM)</td>
</tr>
<tr>
<td>Interposition Vein Graft</td>
<td>Increased MMP 9 (Southgate et al 1999 Z, IM)</td>
<td>Increased MMP 2 and 9 (Southgate et al 1999 Z, IM)</td>
<td>Increased MMP 9 localises to neointimal VSMC (Southgate et al 1999 Z, IM)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Changes in the expression of MMPs and their inhibitors during neointima formation *in vivo* and *in vitro* experimental studies. Z, Zymography, NB, Northern Blot, WB, Western blot, ISH, In-situ Hybridisation, IM, Immunostaining, RT-PCR, Reverse Transcription-Polymerase Chain Reaction
4.3.2 The role of MMPs in the response to injury

As summarised above the response of blood vessels to a variety of injurious stimuli includes an initial wave of medial VSMC proliferation followed by migration to the neointima where a further wave of proliferation occurs. In addition expansive as well as constrictive remodelling also occurs. MMPs and their inhibitors are involved in the regulation of ECM turnover in each of these processes.

4.3.2.1 The role of MMPs in medial VSMC proliferation

There is abundant evidence to suggest that MMP activity is increased during medial VSMC proliferation (Table 5). BB64 (Batimastat) a non-specific MMPi did inhibit early medial VSMC proliferation following balloon injury in rats as well as the proliferation of VSMC in culture. Conversely the synthetic MMP inhibitor Ro-31-9370 had no effect on VSMC proliferation in culture. Similarly GM6001 (hydroxamic acid) did not inhibit medial VSMC proliferation in rats and rabbits but did inhibit migration (Bendeck et al 1996, Strauss et al 1996). The difference in these results may be due to the relative specificities or lack thereof of synthetic MMPi. Some MMPi also inhibit other classes of protease such as those involved in the cross linking of collagens whilst others also inhibit ADAMs, a family of proteases with a metalloproteinase like region important that may serve as important regulators of cell-ECM interaction. Alternatively MMP activity may only be important in the initiation of proliferation by the initial breakdown of the basement membrane. VSMC proliferation can be effected by multiple pathways (Newby and Zaltsman 2000) of which MMP activation is only one. MMP inhibition may simply be bypassed by other parallel pathways in the presence of such inhibitors and this could for example explain the effect of Batimastat on early but not late medial proliferation.

The over expression of TIMPs in cultured VSMC as well as to blood vessels in vivo following vascular injury have also produced conflicting results. TIMP 1 adenovirus
transfection had no effect on medial VSMC proliferation in rats following balloon injury (Dollery et al 1999a) or in cultures of human saphenous vein (George et al 1997). In cultured rat VSMC TIMP 1 gene transfer using retrovirus technology demonstrated a reduction in proliferation (Forough et al 1996) however the opposite was observed when TIMP 1 was over expressed using adenovirus mediated transfer (Baker et al 1998) a discrepancy attributed to clonal variation as a result of the retroviral gene transfer technique. Adenovirus mediated TIMP 2 over expression inhibited proliferation in cultured rat VSMC (Baker et al 1998). This effect was not mimicked by exogenous TIMP 2 however and the anti-proliferative effect may not have been due to extracellular MMP 2 inhibition but to another TIMP action. Adenoviral transfer of TIMP 2 also failed to inhibit medial proliferation in cultured human saphenous vein (George et al 1998b). Over expression of TIMP 3 induced apoptosis in cultured rat VSMC as well as in pig interposition vein grafts with no effect on medial VSMC proliferation (George et al 2000). Adenoviral transfection of TIMP 4 also had no effect in the rat carotid balloon injury model (Dollery et al 1999b).

4.3.2.2 Role of MMPs in smooth muscle cell migration
VSMC migration occurring between 2 and 7 days in rat carotids in vivo following angioplasty was inhibited by the synthetic MMPi’s Batimastat and gm6001 (Zempo et al 1996, Bendeck et al 1996). Furthermore PDGF, a growth factor with potent migratory and chemotactic properties, induces MMP 2 and MMP 9 expression in VSMC in vivo and in vitro (Kenagy et al 1994). Migration of VSMC in vitro through a matrigel filter (predominantly Collagen IV and other basement membrane proteins) was associated with increased MMP 2 expression and activity and was inhibited by a synthetic MMPi (Pauly et al 1994, Cheng et al 1998). Migration of VSMC from pig (Southgate et al 1992) and baboon (Kenagy et al 1994) arterial segments in culture are also associated with increased
expression of MMP 2 and MMP 9. VSMC migration from the latter was neutralised by antibodies to MMP 2 and MMP 9 (Kenagy et al 1994) and the synthetic MMPi Batimastat (Kenagy et al 1996) and from the former by administration of a synthetic MMPi (Southgate et al 1992). In contrast more recent studies comparing the effect of MMP inhibition in vitro have demonstrated that although invasion of VSMC is inhibited by synthetic MMPi and TIMP over expression, VSMC motility in the absence of a collagen barrier was not affected (Kanda et al 2000).

Potential roles for MMPs in cell migration include removal of the barrier to cell movement, removal of inhibitory signals to migration and proliferation by either altering ECM matrix proteins, altering their interaction with the VSMC i.e. breaking down fibrillar collagen or preventing the interaction of ECM proteins with integrins. MMP 9 over expression increases the migration of VSMC through not only matrigel coated filters but also vitrogen coated filters (95-98% Collagen I, 2-5% Collagen III) (Mason et al 1999) despite the fact that fibrillar collagen I is not a known substrate for MMP 9. Suggested mechanisms to explain this effect include cleavage of Collagen III by MMP 9 to reveal a promigratory sequence (RGD) (Collagen fragments are promigratory in vitro) or MMP 9 may degrade other ECM constituents produced by VSMC themselves to produce promigratory sequences. Alternatively MMP 9 may act in a proteolysis-independent manner by altering cell matrix interaction. For example MMP 9 binds to collagen III without degrading it (Allan et al 1995) as well as the VSMC membrane receptor CD44 (Yu et al 1999). MMP 9 and MMP 2 can also bind to integrins (B1 and avB3 respectively (Brooks et al 1996, Partridge et al 1997). Several lines of evidence suggest that MT1 MMP/ MMP 2 activation is particularly important for VSMC migration, a mechanism first suggested for tumour cell invasion in malignant tumours (Sato et al 1994). In rats following balloon injury (Jenkins et al 1999) MT1 MMP expression occurred just before VSMC migration and just preceded MMP 2 activation. Furthermore MT1 MMP in
invasive tumour cells also cleaves CD44, a major receptor for hyaluron, promoting motility, a step that is critical for migration in these cells (Kajita et al 2001). Although this has not been seen in VSMC it reflects a potential level of complexity in MMP function greater than removal of a barrier to cell movement and proliferation. Inhibition of the CD44/hyaluron interaction also prevents collagen contraction by VSMC and this potentially may be important in constrictive remodelling. The integrin avb3 is a binding site for MMP 2 that can also cause its activation. MT1 MMP levels fall rapidly after 2 days in rats post PTA in vivo however avb3 expression is increased in migrating VSMC and may serve to maintain MMP 2 activation during migration as seen in some tumour cell lines (Deryugina et al 2001).

4.3.2.3 Role of MMPs in neointimal VSMC proliferation

Once medial VSMC reach the intima they undergo a second wave of proliferation such that 50% of cells in the rat neointima at 14 days following angioplasty are thought to arise from migration and 50% from proliferation (Clowes et al 1985). Several studies indicate that this second wave of proliferation is regulated by different processes than those occurring in the media. (Koyama H et al 1997). Batimastat, has no effect on intimal VSMC proliferation in the rat (Zempo et al 1996) whilst rat intimal VSMC proliferation was actually increased three fold in the presence of the synthetic MMPi GM6001 (Bendeck et al 1996). In addition over expression of MMP 9 in adenoviral gene transfection studies was not associated with increased intimal proliferation in rat arteries in vivo. Viral transfer of TIMPs also failed to inhibit intimal VSMC proliferation in rats in vivo (TIMP 1, Forough et al 1996, TIMP 2, Cheng et al 1998) as well as in pig vein grafts (TIMP 3) and in human saphenous vein culture (George et al 1998a, George et al 1998b, George et al 2000). This is surprising given the strong association between MMP expression and intimal VSMC proliferation (Table 5).

4.3.2.4 The role of MMPs in neointimal VSMC extracellular matrix deposition

ECM accumulation ultimately contributes to approximately 90% of the neointimal volume and is thought to occur as a result of an imbalance between overall synthesis and degradation of ECM proteins, with TIMP activity in particular being associated with reduced degradation in many models of fibrosis (Franklin 1995). This is supported by studies of human saphenous vein cultures where TIMP 1 is strongly expressed in the neointima at day 14 with net TIMP 1 secretion seen to exceed total MMP secretion, when extracellular matrix accumulation is occurring (Table 5) (Kranzhoffer et al 1999). Overall TIMP secretion exceeds that of MMP production at all periods during formation of the neointima in cultured human saphenous vein however, a balance probably central to
maintaining tissue integrity and closely restricting proteolysis. This suggests a level of complexity in the process of ECM accumulation that is as yet poorly understood.

In the rat carotid angioplasty model ECM accumulation is characterised by increased expression of predominantly type I collagen as well as elastin HS, CS and DS glycosaminoglycans by intimal VSMC (Nikkari et al 1994). Retroviral transfer of TIMP 1 to VSMC subsequently seeded onto balloon injured rat arteries caused elastin accumulation, attributed to a reduction in metalloelastase (MMP 12) activity (Forough et al 1998). This was not however associated with any difference in the relative proportion of intimal VSMC volume to matrix volume ratio indicating a change in the composition but not volume of ECM protein. Adenoviral transfer of TIMP 1 to rat carotids (Dollery et al 1999a) was not associated with increased collagen density although total collagen content was reduced in association with inhibition of neointimal development. Adenoviral mediated TIMP 1 over expression in hypercholesterolaemic mice also led to an overall reduction in the severity of intimal thickening however this was associated with an increase in elastin and collagen in the intima.

In the atherosclerotic rabbit angioplasty induces a 4-10 fold increase in collagen, elastin and GAG synthesis (Coats et al). Collagen makes up >50% of total vascular protein content by 30 days post angioplasty with type I collagen accounting for 85% to 90% and Collagen III accounting for 5%-10%. In the rabbit model peak collagen synthesis occurs at 1-week post angioplasty returning to normal by 12 weeks with increased collagen lysis until 4 weeks when the balance tips in favour of collagen accumulation. MMP 2 expression (but not MMP 1) is also elevated for the first 4 weeks after which it decreases to normal levels, corresponding to the period of increased collagenolysis (Strauss et al 1996). In vitro studies have suggested that MMP 2 may be able to cleave fibrillar collagens in the presence of membrane bound MT1 MMP and therefore increased collagenolysis could be attributed to increased MMP 2 activity (Atkinson et al 2001) however other studies suggest
that MMP 2 binds to, is stabilised in the pro form by, but does not cleave Collagen I (Ellerbroek et al 2001). In the same model however administration of a synthetic MMPi, GM6001, led to an apparently paradoxical overall reduction in total vessel collagen accumulation. GM6001 inhibited collagen synthesis as well as overall neointimal area and it is possible that the inhibition of MMP mediated processing of inflammatory cytokines such as TNF α or IL-1 may have a significant effect on overall neointimal development. Inhibition of MMP mediated collagenolysis and subsequent formation of collagen degradation products, which can themselves promote VSMC proliferation and migration, could also explain this result. Following angioplasty in the rabbit total collagen content was increased in non-restenotic vessels relative to restenotic vessels (Coats et al 1997). Higher collagen content correlated with lumen area and also had a significant negative correlation with gelatinase activity although gelatinase activity was not found to be higher in restenotic compared to non-restenotic vessels. In contrast a study of human atherectomy specimens demonstrated that rates of collagen synthesis were higher and gelatinase activity lower in restenotic lesions (Tyagi et al 1995). Furthermore patients who expressed higher atherectomy TIMP levels and lower levels of MMP expression had increased duration form first angioplasty to restenosis (Tyagi et al 1996).

4.3.2.5 Vessel remodelling

In addition to neointimal hyperplasia, vascular injury is also accompanied by changes in the geometric dimensions of the vessel by a process termed vascular remodelling. Vascular remodelling may be expansive or constrictive and it is the sum of this geometric change and the degree of neointimal hyperplasia that determines ultimate lumen diameter, i.e. expansive remodelling prevents and constrictive remodelling enhances lumenal narrowing by intimal hyperplasia. Indeed in animal models of intimal hyperplasia as well as in humans with angioplasty restenosis constrictive remodelling is the most important
determinant of lumenal narrowing (Post et al 1994, Lafont et al 1995). Shear stress regulates arterial remodelling that occurs in normal physiological conditions as a response to changes in blood flow in association with changes in MMP activity. In uninjured vessels lumenal diameter varies with changes in flow to maintain shear stress within a narrow physiological range thereby achieving homeostasis of shear stress and wall tension. Diameter changes are mediated acutely via endothelium dependent (nitrous oxide) changes in smooth muscle tone but in persistent states of altered flow this is achieved by structural remodelling a process that requires changes in ECM turnover. Similarly low flow induces reductions in vessel diameter. In both expansive and constrictive flow induced modelling activation of MMP 2 and 9 is increased (Bassiouny et al 1998). This enhanced MMP activity proves to be an essential feature in the remodelling response since expansive remodelling in high flow states is inhibited by synthetic MMPi (Abbruzzese et al 1998). Pathological remodelling is also dependent on MMP activity. Constrictive remodelling following balloon angioplasty in pigs is inhibited by the synthetic MMPi's Marimastat and Batimastat. Furthermore expansive remodelling occurred in injured rat carotids is altered by adenoviral mediated over expression of MMP 9 (Jenkins et al 1998).

4.3.2.6 Pathogenesis of the atherosclerotic plaque
Plaque instability manifesting as ulceration of the fibrous cap, plaque rupture or intraplaque haemorrhage is largely responsible for the complications of atherosclerosis. The histopathological features that characterise a plaque that is prone to rupture are an increased lipid content, an increased macrophage, foam cell and T lymphocyte content and a reduced collagen and smooth muscle cell content. Fibrous plaques with a lower lipid content are essentially stable lesions and therefore the balance between ECM degradation and synthesis within the cap may be important in determining whether plaques rupture or remain stable.
Sites of rupture occur particularly in the shoulder regions of the fibrous cap and these areas contain numerous inflammatory cells of which macrophages are predominant with fewer T lymphocytes and mast cells. These areas are often rich in MMP 1, MMP 3, MMP 9, MMP 7 and MMP 12 which localise mainly in macrophages although expression is also found in VSMC as well as lymphocytes and endothelial cells (George 1998). It is thought that increased proteolysis in these shoulder regions is responsible for plaque rupture and this is supported by in situ zymography studies showing overall caesinolysis and gelatinolysis in these regions (Galis et al 1994a, Johnson et al 1998). Shoulder regions are subjected to the maximal mechanical stress. In human coronary atherosclerotic lesions MMP 1 activity was found to be increased several fold in regions of increased mechanical stress (Lee et al 1996) perhaps as part of ongoing remodelling as a direct response to wall stress. It is therefore possible that that the combination of increased protease activity due to remodelling as well as the inflammatory infiltrate at sites of excess mechanical stress leads to plaque instability and rupture.

Several studies have linked increased protease activity to clinical syndromes associated with plaque rupture. Rupture prone lipid laden plaques demonstrate increased MMP 1 and MMP 13 with less collagen I on immunohistochemical and Western Blot analysis compared to fibrous plaques (Sukhova et al 1999), and monocytes co-cultured with fibrous caps from human atheroma induced increased MMP 1 and MMP 2 activity along with collagenolysis (Shah et al 1995). Increased MMP 9 and MMP 12 expression was evident in the shoulder regions of symptomatic carotid endarterectomy specimens compared to those that produced no symptoms (Loftus et al 1999). Similarly in human coronary atherectomy specimens increased MMP 9 activity was identified in lesions from patients with unstable syndromes compared to those with stable symptoms (Brown et al 1995). Nikkari et al (1995) demonstrated a strong correlation between the percentage of the lipid core of human carotid plaques occupied by haemorrhage and the percentage of the lipid core
perimeter positive for MMP 1. TIMPs are expressed in all regions of atherosclerotic
plaques, although TIMP activity in rabbit atherosclerosis (Zaltsman et al 1999) appeared
predominantly in the layer between the fibrous cap and the lipid core.

Potential mechanisms for MMP activation include exposure to mast cell derived proteases,
inflammatory cytokines, mechanical injury, free radicals or CD40 ligation in macrophages
by T lymphocytes. ECM molecules degraded by mast cell proteases include Type IV
collagen (chymase) and fibronectin (tryptase) however they can also activate MMP 1
(chymase) and MMP 3 (tryptase and chymase). Mast cells account for up to 5% of
nucleated cells in the shoulder region of atherosclerotic plaques and degranulation of these
cells can occur in vivo in the presence of activated T cells and macrophages. In vitro
degranulation of mast cells in human carotid plaques is associated with increased MMP 1
and MMP 3 activity (Johnson et al 1998). CD40 ligation induces MMP 1, MMP 3 and
MMP 9 expression in monocytes in culture (Mach et al 1997) and co-localisation of CD 40
ligand and CD 40 occurs in T cells, VSMC and macrophages in the shoulder regions of
atherosclerotic plaques. VSMC that express CD40 also co-localise with MMP 1 and MMP 3 in human atheroma (Schonbeck et al 1997). Macrophages that have accumulated lipid
(foam cells) constitutively express MMP 3 and MMP 1 when cultured (Galis et al 1995).
VSMC MMP production may also contribute to plaque instability. Cultured VSMC also
markedly increase synthesis of MMP 1 and MMP (Lee et al 1995) in the presence of
macrophage cytokines such as TNF α or IL-1β.

Neovascularisation is also associated with plaque instability, being closely associated with
the degree of plaque inflammatory infiltrate and potentially causing intra-plaque
haemorrhage. MMPs are essential in the process of angiogenesis characterised as it is by
cell migration and ECM remodelling. Endothelial cell tube formation is associated with
expression of MMP 1 and MMP 3 as well as TIMP 1 and TIMP 2 (Herron et al 86).
Furthermore MMP 2 and MMP 9 antibodies inhibit endothelial cell tube formation in vitro
(Schnaper et al 1993) and an synthetic MMPi inhibitor reduces corneal angiogenesis in vivo (Galardy et al 1994).

Plaques that have ruptured have not only twice as many macrophages as unruptured fibrous caps but also half as many smooth muscle cells (Bennett 1999). One possible explanation for this reduction in cell numbers is apoptosis (programmed cell death) of VSMC (Bennett 1999). The presence of the extracellular matrix prevents apoptosis in many cell types via specific integrin-mediated signalling (Re et al 1994, Meredith et al 1993). MMPs may disrupt this cell matrix interaction and therefore promote apoptosis. MMPs may also directly promote apoptosis by releasing both soluble Fas ligand (Kayagaki et al 1995) and membrane bound TNF α from cells (Black et al 1997).

4.4 Summary

The activation and regulation of MMP activity involves many parallel pathways. Furthermore the involvement of MMPs and TIMPs in the development of occlusive disease is multi-staged. Inhibition of one activation pathway or stage of lesion development may therefore be made redundant by the recruitment of another. Synthetic MMPi's, despite attenuating the injury stimulus and stages of the fibroproliferative response, have variable effectiveness on neointimal development. This can be attributed to a lack of MMP specificity as well as the fact that the action of MMPs is complex and diverse, involving interaction with cellular receptors, integrin and ECM binding as well as binding to cell membrane and ECM proteins with both proteolytic and non proteolytic activity. Gene transfer of TIMPs remains an attractive option and effectively inhibits neointimal development in animal models predominantly by inhibiting VSMC proliferation but not migration. Increases in TIMP expression have effects in addition to their MMPi activity and may also interact with as yet unidentified MMPs or pathways however. As better synthetic MMPi's are designed, our understanding of the
diversity of MMPs and TIMPs increases and with the wider introduction of gene knockout models hopefully these problems will be resolved. Finally, there is convincing evidence that MMPi's may have a role in the stabilisation of atherosclerotic plaques, thereby reducing the incidence of acute ischaemic events. MMPi's also influence vessel remodelling and ECM accumulation in diseased vessels and the effect of MMP inhibition on lumenal encroachment remains to be examined.
Part 5. Rat Models of allograft dysfunction

5.1 Introduction

Numerous investigators have demonstrated the development of lesions in chronically rejecting rat allografts which were virtually indistinguishable from similar processes occurring in human transplants (Karnovsky et al 1994, Mennander et al 1991, Diamond et al 1992). Although cardiac and renal allografts were performed early in transplant research it is only in the last decade with the wider application of molecular biological techniques that they have provided insight into the pathogenesis of CAV. Most studies have concentrated on inbred rat strains with recognised histocompatibility differences.

Cardiac allografts between MHC identical strains (i.e. the WF.1L- Lew model, Guttman et al 1997) can survive indefinitely without any immunosuppression. High responder combinations with a complete major and minor (Brown Norway to Lewis, Schmid et al 1997) or minor MHC mismatches only (F 344 to Lewis matched at major RT-1\(^1\) versus RT-1\(^1\) but not at minor (RT-3) MHC loci) acutely reject and ultimately fail unless appropriate long-term immunosuppression is given. Allografts between strains with only non-MHC mismatch (DA-WF Lemstrom et al 1996, Cramer et al 1990) fall between these two extremes. When interpreting the reports of experimental studies on rat allograft models it is therefore imperative that the organ, strain combination and the type of immunosuppression used are considered. The F344/Lew combination that differs at minor MHC loci only requires an initial short (2 weeks) period of sub optimal immunosuppression without further treatment thereafter (Schmid et al 1997, Adams et al 1992). Instead of an ensuing acute rejection reaction as would be the case in man a state of immunological tolerance is achieved. The rat is the only species in which this occurs. A chronic inflammatory process subsequently develops leading to the development of histological changes characteristic of CAD in man (see below). Due to extensive investigation of the molecular and cellular changes that occur, in particular by Tilney and...
co-workers, the F344 to Lew combination is the best characterised rat model of CAD. Investigation of this process in rat and cardiac allografts characterised sequential changes in the behaviour of different cell types, cytokine and growth factor expression during the development of CAD.

5.2 Rat cardiac allografts

Several groups have used cardiac allografts in rats as models of CAV (Adams et al, Karnovsky et al 1994, Guttman et al 1997). In the well studied Lewis to F344 model 25% of cardiac allografts survive 120 days in the absence of immunosuppression, increasing to 85% if the early acute inflammatory response is abrogated by a short course of cyclosporine (Adams et al 1992). By 120 days 95% of the arteries in transplanted hearts develop significant CAV quite similar to that in humans.

The timing of the development of graft arteriosclerosis in this model is reproducible occurring at sequential intervals of time (Adams et al 1993). Soon after transplantation (d7-15) there is an accumulation of monocytes and T cells in the graft seen on immunohistochemical staining to lie close to or adhere to the vascular endothelium. Between 30 and 70 days post transplant there is an infiltration of macrophages and T cells into the intima. At 70 days smooth muscle cells begin to accumulate in the intima whilst the number of leucocytes declines and by 120 days post transplant the predominant cell types are smooth muscle cells with occasional macrophages and very few T cells. By 120 days the cellularity of the intima was reduced with diffuse fibrosis accounting for much of the intimal thickening and was also associated with a generalised fibrosis of the myocardium (Adams et al 1993).

Semi-quantitative PCR and immunohistochemical techniques have identified the role of adhesion molecules, growth factors, cytokines and different cell groups in the development of CAV in cardiac allografts. A common pattern emerges in both high and low responder

There is an early humoral response with deposition of IgG and IgM in the first week (Wasowska et al 1996). At 4-6 weeks post transplant there is an increase in CD4, CD8 T lymphocytes and macrophages with increased expression of the cytokines INF-γ, IL-2, IL-4, IL-6, IL-10, IL-12, MCP-1, cellular adhesion molecules such as ICAM, MHC class II levels and growth factors such as PDGF and TGF β in the allograft (Russell et al 1995, Wasowska et al 1996). By day 60 in the BN-Lew combination the infiltrating cells are predominantly macrophages with increased levels of predominantly macrophage products INF γ, IL-12, MCP-1 and TGF β (Korom et al 1998). In F344-Lew cardiac allografts 100 days post transplant CD4 and ED-1 (macrophages) (Schmid et al 1995,1996) are the predominant infiltrating cell types. CD8 cells are present but B cells are absent. Low levels of adhesion molecule (VLA-4), growth factor (bFGF) and cytokine (TNF-α, IL-1α) expression persist at this stage (Schmid et al 1995).

The role of different lymphocyte types has been demonstrated in several studies using monoclonal antibodies directed against specific leucocyte antigens. Antibodies directed against CD4 lymphocytes and macrophages attenuate the development of CAV in this model (Karnovsky et al 1994). Administration of the mouse Ig2A anti CD4 antibody reduced both CD4 and CD8 infiltration as well as reducing the observed increases in growth factors, cytokines and adhesion molecules. In contrast anti CD8 antibody administered to the weakly immunogenic W1F-Lew combination produced little change in the progression of CAV where presumably the alloimmune response is less important in the generation of the CAD lesion (Guttman et al 1997). This highlights the importance of understanding the significance of different high and low responder strain combinations when interpreting the results of rat allograft models of CAD.
5.3 Rat kidney allografts

Although there are many differences in the morphological aspects of chronic rejection in renal and cardiac allografts there are also important similarities. When renal allografts are considered a familiar pattern emerges of the progressive development of CAD in rats identical to that in occurring in man with similar leucocyte, growth factor and cytokine expression that is observed in the rat aorta and cardiac allografts (Diamond et al 1992, Hancock et al 1992, Nadeau et al 1995, Azuma et al 1994b). In F344 kidneys transplanted into Lewis rats a progressive deterioration in renal function and eventual graft loss occurs. This is associated with an initial early cellular infiltrate in the first month followed by early fibrosis at 12 weeks proceeding to generalised glomerulosclerosis, tubulointerstitial fibrosis and allograft vasculopathy by 24 weeks (Diamond et al 1992).

Within 2 weeks of transplant glomeruli and large vessels show heavy deposition of IgM, complement, fibrin and neutrophils with increased ICAM expression. By 4 weeks there is a cellular infiltrate of activated (IL-2R positive) CD4 cells and macrophages in the interstitium with an associated massive increase in IL-1, IL-2, IFN γ, IL-4, IL-6, IL-7, IL-8, TNF α, TGF β, PDGF and EGF. Arterial smooth muscle cells also stained strongly positive for most cytokines and TGFβ (Hancock et al 1992, Tullius et al 1994). By 12 to 16 weeks there is a dramatic increase in macrophage numbers and infiltrating T cells and macrophages are localised to the glomeruli and perivascular areas. There is an associated increase in the T cell derived monocyte chemoattractant RANTES and MCP-1 just prior to this upsurge in macrophage numbers (Azuma et al 1994b). Immunoglobulin and complement deposition peaks at 12 weeks and starts to decline. Macrophages infiltrating glomeruli express predominantly PDGF, TGF β, EGF, IL-6, IL-7 and TNF α. Surface molecule expression (ICAM-1 on endothelial cells and VLA-4 on infiltrating cells), cytokine and growth factor expression (TNF α and TGF β on glomerular cells and PDGF on arterial smooth muscle cells) peak at this time (Azuma et al 1994b, 1995b). By
24 weeks their number of infiltrating leucocytes has markedly reduced with a reduction in the level of activation (IL-2R expression) and a reduction in their products, growth factors and surface molecules (Azuma et al 1994b), although IL-6 is seen to persist (Hancock et al 1992).

The changes seen in this model appear to follow a logical sequence. Early in this allograft model, T cells and their products (IL-2, TNF α, and INF γ) predominate during an early acute rejection period. Several weeks later an increase in levels of RANTES, a T cell produced chemoattractant for macrophages, and the macrophage activator MCP-1 is detected (Azuma 1994b). The subsequent increase in macrophages is then associated with observed an increase in macrophage products (IL-1, IL-6, TGF β, TNF α, and IFN γ Azuma 1994b). These products are thought to be important in the development of fibrosis.

5.4 Rat aortic allografts

A simplified model of CAV was developed using the rat aorta (Mennander et al 1991). A feature of aortic allografts in rats is that even high responder strains permit long-term graft survival in the absence of any immunosuppression. This enables the study of CAV in high responder strain combinations with quantitation of both acute and chronic immunological responses to a foreign graft (Hullet et al 1996, Mennander et al 1991). An early self-limiting acute rejection episode is followed by the development of intimal hyperplasia characteristic of the lesions seen in chronically rejecting organ allografts. Although not entirely analogous with the vessel wall of human allografts one advantage of this model is that it does allow investigation of the changes in vascular wall cellularity, cytokine and growth factor expression per se without the need for extensive dissection (Mennander et al 1991).

Several groups have used this model to study allograft vasculopathy (Mennander et al 1991, Geraghty et al 1996, Morris et al 1995). Various authors describe the development of
a pathological lesion in allografted aortas virtually indistinguishable from CAV in man. In
addition these lesions undergo the same chronological development seen in rat cardiac
allografts. For example in the low responder DA-WF combination described by
Mennander et al (1991) aortic transplants undergo a short acute inflammatory episode of
rejection, particularly in the adventitia, which spontaneously subsides after one month. The
typical changes of chronic rejection occur during the following 5 months: there is a
decrease in the inflammatory activity in the adventitia, myocytes migrate into the intima
and intimal thickening occurs. Occasional breaks are noted in the internal elastic lamina.
Interestingly smooth muscle cells also migrate into the adventitia in this model and the role
of adventitial inflammation in the development of CAV has not been explained. By 12
months post transplantation, there is a decrease in the intimal thickness and cellularity of
the aortic allografts reflecting deposition of extracellular matrix and remodelling of the
graft.
Analysis of the cell types, cytokines and growth factors involved in the development of the
intimal lesion are similar to that seen in cardiac and renal allografts. Immunocytochemistry
has demonstrated that the early inflammatory infiltrate is composed of CD4 and CD8
lymphocytes and macrophages whilst the later cellularity is attributed to smooth muscle
cells (Mennander et al 1991, Hullet et al 1996). In aortic allografts lymphocyte and
monocyte infiltration is confined largely to the adventitia and neointima, occasionally these
cells are seen in the media. MHC class II expressing cells and IL-2 Receptor positive cells
are seen in the adventitia although many IL-2R expressing cells are also seen in the media
Concomitantly the adventitial cells stain strongly for the cytokine IFN γ and the adhesion
molecules ICAM-1 and LFA-1 α chain (Lemstrom et al 1994). IL-2R, IFN γ and adhesion
molecule expression reaches a peak at one month and then declines. (Lemstrom et al
1994). Whilst the early infiltrate of lymphocytes has a significant proportion of CD8
lymphocytes in the later stages CD8 cells are virtually non-existent. In the WA-DF model
CD4 cells, IL-2R (at low levels) and ED-1 cells (macrophages) persist in the neointima and
the adventitia even in advanced stages of the disease (Hullet et al 1996, Mennander et al
1991). Semi-quantitative mRNA analysis demonstrated prominent upregulation of PDGF-
B, IGF-1, TGF β and aFGF during the first three months post transplant. Thereafter the
expression of PDGF-B, TGF β and aFGF declined to the non transplanted level but IGF-1
remained high. EGF and bFGF levels remain low during the development of the CAV
lesion (Raisanen-Sokolowski et al 1995). In similar experiments where femoral arteries
were allografted between the strongly immunogenic BN-Lew strains similar changes in
cytokine expression (IL-1, IL-2, TNF α) and growth factors (PDGF-A, bFGF, TGF β) is
seen at six weeks post transplant.
The rat F344 to Lew aortic allograft model has not been investigated as thoroughly as in
cardiac and renal allografts, however, it is apparent from both these models that the cellular
changes associated with the tissue response to injury i.e. intimal hyperplasia and allograft
parenchymal fibrosis commence between 8 and 12 weeks and it is therefore reasonable to
assume that the development of CAV in aortas will follow a similar time course. Time
points for analysis in this study will therefore commence at 4 weeks (T cell and
macrophage infiltration), 8 weeks (increase in macrophage derived growth factors and
cytokines), 12 weeks (early intimal hyperplasia), 16 weeks (intimal hyperplasia and
fibrosis) and 24 weeks (fibrosis).

There are several disadvantages to this model. The first is that in rat aortic allografts
intimal hyperplasia occurs in a non-linear fashion such that intimal thickening appears to
increase to a certain level following which it ceases. This is unlike that which occurs in
primate aortic allografts where intimal thickening occurs in a more linear fashion (Ikonen
et al 2000). Furthermore as vessel occlusion does not occur this is not homologous with the
progressive obliterative vasculopathy demonstrated in dysfunctional allografts in man. This is related to the third disadvantage in this model in that as allograft vasculopathy proceeds it is not associated with any level of allograft dysfunction and therefore the effect of therapeutic intervention on CAV in this model has no direct applicability to its potential effect on CAD in humans. A fourth disadvantage is that remodelling in untreated aortic allografts is primarily expansive rather than constrictive. Constrictive remodelling is thought to be an important determinant of vessel occlusion in CAD. This is perhaps related to the different dynamics that affect the elastic rat thoracic aorta as opposed to the muscular arteries supported by surrounding parenchyma found in transplanted organs or even the rat abdominal aorta which is more cellular with fewer elastin lamellae. Different clonal subtypes of VSMC in the thoracic aorta could also explain this difference in behaviour. Thoracic aortas in humans also exhibit different remodelling responses in response to injury. Occlusive intimal thickening and constrictive remodelling are not seen in the thoracic aorta however this is possible in the distal abdominal aorta in humans. One further disadvantage is that as a high responder model it may be difficult to differentiate a difference between the immnosuppressant effect of rapamycin and its direct effect on the response to injury. It could be argued that the latter effect should be best explored in non alloimmune models of injury such as balloon angioplasty models or ischaemic reperfusion models, however it is also important to study the effect of rapamycin on ECM remodelling in parallel with such models for more direct relevance to CAD. High responder models are also criticised, as they tend to become very fibrous and acellular in the absence of immunosuppression. As this thesis primarily considers dysregulation of ECM remodelling however this is not necessarily a disadvantage. In contrast to this evidence is emerging that the process by which vasculopathy develops in vessel allografts may be different to that which occurs in organ allografts. This occurs in particular in models where allografts receive little or no immunosuppression and subsequently undergo an unrestrained acute
rejection episode. It is now becoming apparent that recipient derived non-lymphoid cells may contribute most if not all of the cells involved in intimal thickening and vascular remodelling (Plissonier et al 1995). One final disadvantage of this model is the lack of homology between the pharmacokinetics of rapamycin in rats compared to that in other species. In particular the bioavailability of rapamycin following enteral administration in the rat is 15% and therefore dosages are approximately 10-50 times those used in humans.

In summary rat allograft models of CAV and CAD demonstrate a consistent pattern. This consists of an initial inflammatory response associated with the deposition of humoral reactants, an early elevation in MHC class II molecules, IL-2R expressing cells, macrophages and lymphocytes with associated expression of inflammatory cytokines such as INF \( \gamma \) and IL-1 and growth factors. This is followed by a reduction in the intensity of the inflammatory response a persistence of macrophages and CD4 helper cells within the graft and the expression of in particular macrophage products TNF \( \alpha \), IL-6, TGF \( \beta \) and PDGF.
Part 6. Thesis synopsis

Chronic allograft dysfunction is the leading cause of solid organ allograft failure in clinical transplantation. Improvements in our understanding of the aetiological factors that influence the onset and rate of progression of CAD, as well as in the understanding of the pathogenesis of these aetiological factors has not to date led to significant improvements in outcome. This is often due in part to an inability to control donor related factors in clinical practice but also due to the limitations of immunosuppressive drugs, notably their toxicity profiles that can also promote CAD. The decline in graft function occurs as a consequence of the tissue response to these donor and recipient related aetiological factors and the failure of calcineurin inhibitors to attenuate this aspect of CAD is an important factor in their failure to improve the rate of graft loss. By inhibiting the fibroproliferative response to injury it may therefore be possible to slow the rate of decline in graft function.

Rapamycin is a novel immunosuppressive agent which has been shown to attenuate the fibroproliferative response by poorly understood mechanisms.

The purpose of this study was to compare the effect of rapamycin with the calcineurin inhibitor cyclosporin on the expression of genes important in the fibroproliferative response. This was achieved by performing thoracic to abdominal aortic allografts in a well characterised experimental model of allograft vasculopathy (F344 to Lew), measuring histological indices of vessel remodelling, neointima formation and ECM accumulation by light microscopy and correlating these changes with MMP, TIMP, Collagen and TGF β gene expression measured using semi-quantitative RT-PCR.

6.1 The objectives of this thesis were as follows

1. A critical review of our current understanding of allograft vasculopathy and in particular of the molecular basis of occlusive vascular disease.

2. To measure the changes in pro-fibrotic gene expression that accompanies the development of allograft vasculopathy in a rat aortic allograft model.
3. To measure the effects of the novel immunosuppressant agents rapamycin and microemulsion cyclosporin (Neoral) on these changes in pro-fibrotic gene expression.

4. To study the effect of rapamycin and Neoral in combination on allograft vasculopathy and pro-fibrotic gene expression.

5. To measure the effects of the novel immunosuppressant agent rapamycin on these changes once allograft vasculopathy is established.
Chapter 2. Materials and Methods
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1.1 Introduction to methods in the investigation of genes responsible for extracellular matrix remodelling in allograft vasculopathy

The thoracic aorta to abdominal aorta allograft model as described by Mennander et al (1991) was used to study the effect of different immunosuppressants on the expression of genes associated with ECM remodelling that occurs during the development of allograft vasculopathy. A segment of the descending thoracic aorta was used as a transplant. F344 rats acted as donors and recipients and Lewis rats as recipients. Harvested grafts were analysed using computerised histomorphometry and RT-PCR.

Sections of the aortic graft were stained with picrosirius red as well as haematoxylin and eosin. The median ECM content as well as the area fraction of the intima and media were quantified using a computer image analysis system. Analysis of the expression of genes expressed during ECM remodelling was performed using a modification of the method developed in our laboratory for the extraction of mRNA from single human glomeruli (Bicknell et al 1996). Briefly, total mRNA was extracted from aortic tissue and complementary DNA molecules were synthesised by reverse transcription (RT). These cDNA species were then amplified by polymerase chain reaction and quantified in an ELISA system. Relative quantitation was achieved by comparison of the signal intensity to that of the housekeeping gene β Actin.

In order to take into account all the factors which influence smooth muscle cell proliferation, migration and deposition of ECM during neotima formation, the following species were studied with RT-PCR: MMP2, MMP9, TIMP1, 2 and 3, TGFβ, and collagen III.
The levels of gene expression were compared in the different study groups using non-parametric statistical analysis. Allograft gene transcripts were correlated with intimal thickening expressed as the intimal media ratio and vessel ECM content.
1.2 The Animal Model

Since CAV was first described, extensive efforts have been made to develop animal models to enhance our understanding of the cellular characteristics, rate of development and treatment of CAV. Most studies have concentrated on rat cardiac (Karnovsky et al 1994), renal (Diamond et al 1992) and aortic (Mennander et al 1991) allografts although rabbits (Libby et al 1994) and mice (Armstrong et al 1997) also offer excellent models of CAV. Murine models in particular not only share significant homology to human genes, they also provide easier manipulation of animal genetics, greatly improving our understanding of the roles different genes play in CAV. More recently large animal models of allograft dysfunction and allograft vasculopathy have been developed, with primate models such as aortic allografts in baboons offering greater homology to the condition as it occurs in humans (Ikonen et al 2001). Balloon injury models also provide insights into the proliferative response of the vessel wall to endothelial injury in the absence of an immunological response and also enable us to study the effect of newer immunosuppressants such as Rapamycin and mycophenolate mofetil on the vessel response to injury in isolation.

Whilst animal models yield useful information on discrete mechanisms potentially involved in CAV it is important to realise that they also have considerable limitations. Care must be taken extrapolating intimal hyperplasia in animal, and particularly rodent allografts, under very controlled conditions to the complex multifactorial process involved in chronic graft loss in man. Furthermore the rat aortic model undergoes important pathological changes seen in CAD in man notably remodelling and intimal thickening. Humans too are high responders and therefore the F344 to Lew model can provide insight into the effects of novel immunosuppressant drugs on ECM remodelling in allograft vasculopathy. In addition, as well as being well characterised an advantage of this model in the current experiments is that tissue
processing to selectively investigate the cellular and molecular processes in the vessel wall during the development of intimal hyperplasia is simplified. The F 344 to Lewis rat aortic allograft model was selected as it is a recognised allograft model that displays histological changes of chronic allograft failure in both renal and cardiac allografts virtually identical with corresponding chronic allograft failure in man (Schmid et al 1995, Diamond et al 1992). These animals differ at minor MHC genes and without immunosuppression cardiac and renal allografts are rejected within 2 weeks. Following a short course of immunosuppression both cardiac, renal and aortic allografts undergo a series of chronological changes with approximately the same time course. There is an initial diffuse T cell and macrophage infiltration at 4 weeks that becomes localised to perivascular tissues within 8 weeks. At this point there is a low incidence of intimal thickening (hyperplasia). At 16 weeks significant intimal hyperplasia has developed with reduced mononuclear cell infiltration and by 24 weeks generalised CAV is established.

1.2.1 Experimental animals

The experimental protocol for this work was performed under the terms of Home Office Project Licence PPL 80/1434. Adult male F344 and Lewis rats weighing 250-300g were purchased from Harlan (Bicester, Oxon., UK). Animals were kept in a controlled environment with unlimited access to feed and water. Any animals showing signs of distress, significant weight loss or infection were culled by an appropriate method as described in Schedule 1 of The Animals (Scientific Procedures) Act 1986.
A modification of the thoracic aorta to abdominal aorta allograft model as described by Mennander et al (1991) was used.

In all cases aseptic surgical technique was implemented. Donor rats were anaesthetised by inhalation of halothane and oxygen, placed supine and the thoracic aorta exposed (Figure 1.) The thoracic aorta was carefully dissected away from its surrounding fascia using a no touch technique from the distal arch to the diaphragm, excised and perfused with 2ml of phosphate buffered heparinised saline. Small lumbar arteries originating from the aorta were divided as far away from the aorta as possible using cautery. The donor aorta was measured and trimmed to a length of 2.5cm and used as a transplant. Ischaemic injury to the graft was minimised by immersion in an ice bath at 4°C between procedures. The mean ischaemia time (± Standard Deviation) was 50 (± 9) minutes of which a mean of 24 (± 6) minutes was spent in the ice bath.

F344 rats acted as donors and Lewis or F344 rats as recipients. The recipient was anaesthetised in a similar manner to the donor, placed supine and through a midline laparotomy the abdominal aorta was exposed by lateral displacement of the viscera. The aorta was dissected free from the adjacent vena cava from the level of the renal arteries to the bifurcation, clamped and divided. The 2.5cm of thoracic aorta was then transplanted into a heterotopic position forming a loop in the recipient abdomen by end to end anastomosis to the recipient abdominal aorta using a continuous 9/0 prolene suture (Figure 2). Post laparotomy closure all rats received 2.5ml of 0.9% normal saline solution subcutaneously and were observed closely until ambulatory.
1.2.3 Drug administration and dosage

All drug dosages to be administered were in accordance with previously published work describing long term administration of these drugs alone (Little et al 1996, DiJoseph et al 1996), or in combination (Andoh et al 1996). As such all drug dosages were designed to produce no adverse side effects. Little et al (1996) described oral administration of Neoral 5mg/kg/day to Lewis rats for three months without adverse side effects. DiJoseph et al (1996) reported toxic side effects such as decreased weight gain and lymphoid nodal atrophy at 0.8mg/kg/day of Rapamycin after 28 days oral administration. In addition Lewis rats have been administered intra-peritoneal Rapamycin at 0.5mg/kg/day for 100 days with no adverse side effects (Schmid et al 1996). Given in combination the two drugs are nephrotoxic at high doses (Kahan et al 1994) and reduced dosages of CyA by 3.2-4 fold and Rapamycin 8.9-10 fold are reported to minimise side effects with equivalence of immunosuppression demonstrated in vivo (Stepkowski et al 1997, Kahan et al 1994, Andoh et al 1996).

Between 0-24 weeks post transplant groups of rats (n=6) were given immunosuppressant drugs by daily gavage as follows: Neoral 0.15 or 5mg/kg/day, Rapamycin 0.01-0.5mg/kg/day or any combination of these 2 drugs. Drug administration was commenced at variable time points post-transplant for up to 24 weeks. Group sizes were similar or less than those reported in other studies of the effect of immunosuppressants on CAD. Accurate power calculation was made difficult, as there is no precedent to this work with regard to the genes being measured. There is also potential for animal loss through postoperative complications and inadvertent lung dosing during gavage and therefore it was specified in the project license that at least 6 surviving animals in each group would be included in the analysis.
1.2.4 Animal Husbandry

One hundred and ninety three aortic transplant in total were performed with a total of 31 animals not surviving until their proposed cull date. This figure can be broken down as follows:

**Cull for animal distress n=5**

All of these animals bar one were receiving Rapamycin 0.5mg/kg/day at the time of cull and at post mortem appeared to have severe inflammation of the small intestine.

Three were in group 5, one was in group 7, and one was in group 6.

**Intraoperative death: n=11**

This was chiefly due to complications relating to blood loss and anaesthesia in early procedures (all restricted to Experiment 4).

**Cull for hind limb paralysis: n=15**

This occurred as a consequence of a combination of serial anastomotic narrowing and low post operative blood pressure where there had been intraoperative blood loss. Almost all cases occurred in early transplants (restricted to Experiment 4 animals) and responded to movement of the anastomosis more proximally on the aorta until immediately adjacent to the renal vessels.

**Weight loss**

No group suffered statistically significant weight loss although Group 5 and Group 6 had lower mean weight gain in the two weeks post operatively than other groups.
Figure 1. Harvest of Donor Aortic Graft

Figure 1A. Donor aorta in situ

Anterior

Posterior
Figure 1B. Excised aorta, prior to shortening to constant length (2.5 cm)
Figure 2. Transplant of aortic graft

Figure 2A. View of rat retroperitoneum during dissection of abdominal aorta
Figure 2B. Thoracic aorta transplant in situ

Cotton bud indicates proximal anastomosis
1.2.6 Graft harvest and tissue preparation

Recipient rats were anaesthetised by inhalation of halothane and oxygen, placed supine and the transplanted aorta exposed through the old midline incision. The aorta was dissected free of any peritoneal adhesions, vascular clamps were applied to the recipient's native aorta distally and proximally and the allograft excised along with 2-3mm of recipient aorta at either end. Blood was then flushed from the graft by gentle perfusion with 2mls of PBS at 4 °C and approximately 4mm of the donor aorta adjacent to the anastomotic suture line was excised and discarded. The graft was then quickly cut into 8 approximately 2mm sections with a fresh scalpel blade and processed as follows.

2 x 2mm lengths were fixed in 20ml 10% formalin for 12 hours and then paraffin embedded until processed.

4 x 2mm lengths were immediately snap frozen in liquid nitrogen. Frozen weight of each segment was approximately 8mcg

2 x 2mm lengths were embedded in OCT resin on cork board and immediately snap frozen in liquid nitrogen.

These samples were stored in liquid nitrogen until processing.
1.3 Experimental design

Experiment 1: To study the effect of alloimmune injury on the development of CAV

Group 0: F344 to F344 Isografts without any immunosuppression

Group 1: F344 to Lewis Allografts. These rats were administered Neoral 5mg/kg/day for the first two weeks only, to reverse any early rejection and then no immunosuppression

Group 2: F344 to Lewis Allografts receiving Neoral 5mg/kg/day until sacrifice

6 surviving rats in each group were culled at 4, 8, 12 and 16 weeks

Group 0 and Group 1 will serve as positive and negative controls for all subsequent experiments.

Experiment 2: To study the effect of rapamycin on the development of CAV

All groups will be F344 to Lewis allografts.

The rats were administered immunosuppressive drugs orally by gavage at the following doses daily from the day of transplant until sacrifice.

Group 3: Rapamycin 0.25mg/kg/day

Group 4: Rapamycin 0.5mg/kg/day

6 surviving rats in each group were culled at 4, 8, 12 and 16 weeks

Experiment 3: To compare the effects of rapamycin only therapy, Neoral only therapy and combined rapamycin/Neoral therapy on CAV

All groups were F344 to Lewis allografts.

The rats will be administered immunosuppressive drugs orally by gavage at the following doses daily from the day of transplant until sacrifice.
**Group 2:** Neoral 5mg/kg/day

**Group 5:** Rapamycin 0.25mg/kg/day

**Group 6:** Rapamycin 0.05mg/kg/day and Neoral 1.5mg/kg/day

6 surviving rats in each group were culled at 4, 8, 12 and 16 weeks

**Experiment 4:** To study the effects of immunosuppressants on established chronic rejection

Initially all rats were administered Neoral 5mg/kg/day orally by gavage for 14 days. Drug dosages will then be as follows

**Group 7:** Rapamycin 0.5mg/kg/day week 8 to week 24

**Group 8:** Rapamycin 0.5mg/kg/day week 12 to week 24

**Group 9:** Rapamycin 0.5mg/kg/day week 16 to week 24

**Group 10:** No immunosuppression

6 surviving rats in each group were culled at 4, 8, 12 and 16 weeks

Post graft harvest gene expression in the vessel wall was measured by RT-PCR and correlated with histological indices of disease severity. The levels of gene expression were compared in the different study groups using non-parametric statistical analysis.
1.4 Morphometric quantification of ECM content and lumen area

The principal constituent of rat arterial ECM is collagen comprising 50% of all protein. The main collagen type is Collagen I (80-90%) with the next most common Collagen III (5-10%) and lesser quantities of Collagen IV and V. These proportions do not change following the development of allograft vasculopathy (Kemeny et al 1991). The relative quantification of Collagen levels in the vessel wall should therefore serve as an accurate marker of ECM deposition. There are several techniques available to study ECM content in the vessel wall. Among these the most commonly used include immunohistochemistry for specific Collagen proteins, measurement of total collagen content by hydroxyproline assay and picrosirius red staining, each having their own advantages and disadvantages.

Immunohistochemistry (Nicholson et al 1999), although reproducible is time consuming and allows only for relative quantitation for specific epitopes. Its reliability is limited by the specificity and purity of the immunoglobulin used. Such criticisms have led to the suggestion that it is best utilised as a qualitative rather than a quantitative technique although Collagen III immunostaining in human renal transplant biopsies has been shown to be reproducible and to correlate with the rate of decline of function (Nicholson et al 1999). Hydroxyproline assay is the gold standard as a quantitative technique (Strauss et al 1994) permitting absolute quantitation of collagen levels. However it does not permit discrimination of different collagen types or analysis of relative collagen content in different tissue compartments and is time consuming to perform. Picrosirius red staining has the advantage that it is a simple laboratory technique that permits comparison of relative ECM content in different areas of tissue and has been clearly demonstrated in animal and human studies to accurately assess the relative ECM content in tissues in a highly reproducible manner.
Picrosirius red staining was initially described in 1964 (Sweat et al 1964) and was subsequently shown to be a specific method for ECM detection in tissue sections (Grimm et al 1999, Moreso et al 1994). It is birefringent when observed under polarised light. In animal models sirius red staining has been used to quantitate radiation induced fibrosis in lung tissue (Krus et al 1991), murine cardiac fibrosis (Herrman et al 1993), renal fibrosis (Flores et al 1998) and the ECM content of infarcted myocardium following treatment with captopril (van Krimpen et al 1991). In animal models of vascular disease sirius red is now widely used as a relatively simple reproducible method for the relative quantitation of ECM content (Dollery et al 1999b, Forough et al 1996).

In human studies sirius red staining has been used to quantitate fibrotic changes in paediatric liver transplants (Moragas et al 1992) as well as measuring the effect of interferon therapy on hepatitis induced liver fibrosis (Menabe et al 1993). In addition this latter study showed that sirius red staining was highly significantly correlated with total liver collagen as determined by hydroxyproline assay. It has also been used in human cardiac transplantation where it correlated with ischaemic time (Pickering et al 1990). In human renal biopsies sirius red staining has been quantitated using automated image analysis and has been shown to correlate with the interstitial volume fraction of the cortex as measured by point counting (Moreso et al 1995). In addition sirius red staining of ECM analysed by computerised histomorphometry has been shown to be highly correlated with the glomerular filtration rate at the time of biopsy (Moreso et al 1994).

1.4.1 Tissue analysis

Starting at 500 μm depth 5 μm sections were removed from the paraffin block at three 50μm intervals and stained with picrosirius red and haematoxylin and eosin stains.
Measurements were only made on sections that were complete. Histological quantification of ECM content and lumen area was performed in each of the three sections from the rat aortas and the overall mean value used in all further data analysis.

1.4.2 Morphometric quantitation of ECM content

Picrosirius red-stained sections were examined on a Nikon E800 microscope (Figure 3) with the use of circularly polarised light. The analyser (upper polarising filter ) was rotated so that its transmission axis was aligned at 45° to the fast axis of a quarter plate above it. The polariser (lower polarising filter) was replaced by a circular Nikon polarising filter so that the field of view was dark. Images were observed on a monochrome Photonic Science Coolpix Monochrome cooled CCD integrating mounted video camera on the vertical tube of the microscope along with a x1 relay lens. The images were digitised by a video frame grabber in a Power Macintosh G3 computer (Scion CG7) in monochrome mode and displayed on a high resolution monitor and analysed by the use of Java video analysis software NIH Image (courtesy of Wayne Rasband, NIH) with macros written by Professor P Furness (Leicester General Hospital, Leicester, UK).

Picrosirius red-stained ECM fibres appear bright when viewed with polarised light; however other vascular elements such as smooth muscle cells appear dark (Figure 4). This optical property of the tissue was exploited to assess ECM content. ECM content in the non adventitial tissue was calculated as follows: To eliminate everything except ECM from the vessel image, a blue filtered, monochrome, bright field image of the picrosirius red-stained sections was subtracted from a circularly polarised image. This produced an image composed of bright ECM fibres on a black background. A histogram of the brightness of each pixel was
in the image was plotted. All of the non-ECM stained area was removed by the subtraction and had a grey scale area of zero, using a scale from 0 (black) to 255 (white). Thus any pixel with a grey scale level >0 represented ECM. The ECM content in each section was expressed as the area fraction (%) of pixels with a grey scale >0 and the total relative ECM content expressed as the product of medial area (mm$^2$) times the area fraction (%).

Digital planimetry of haematoxylin and eosin stained sections was performed with the same computer assisted morphometric program without the use of polarised light. The following measurements were made using the internal and external elastic laminae as the margins of the media and the endothelium and internal elastic lamina as the margins of the intima.

Lumen Area (mm$^2$)
Lumen plus Intimal Area (mm$^2$)
Lumen plus Intimal plus Media Area (mm$^2$)
Circumference of the internal elastic lamina (mm)

Using these measurements the following calculations were performed

Intimal Area (mm$^2$)
Intimal Thickness (mm)
Medial Area (mm$^2$)
Medial Thickness (mm)
Intima: Media Ratio
1.4.3 Assessment of the severity of the inflammatory infiltrate

The degree of severity of the inflammatory infiltrate on haematoxylin and eosin stained sections. Severity was determined by an observer blinded to the treatment group or time of cull and was categorised as follows

None- No inflammatory cells visualised

Mild- Endothelialitis, inflammatory cell localised to the endothelium

Moderate- Dense inflammatory cell infiltrate in the innermost layer of the vessel wall

Severe- Dense Inflammatory cell infiltrate throughout all layers of the vessel wall.
Figure 3. Computerised histomorphometric measurement

Haematoxylin and eosin stained section of rat aorta viewed at ×40 magnification. Lines represent endothelial layer (lumenal area) and internal elastic lamina (lumenal plus neointima area) measured by a suite of macros. Intimal area is then calculated by subtraction.
Figure 4. Picrosirius red stained sections of rat aorta viewed under polarised and non-polarised light

Figure 4A. Sirius red stained section aortic allograft viewed under non-polarised light at \( \times 40 \) magnification. This is the same section as seen in figure 3.
Figure 4B. Same section viewed under polarised light

Staining intensity is highest in the neointima and least in the cellular neointima
1.5 RT-PCR and detection

1.5.1 Introduction to methods in the investigation of RNA

The measurement of gene expression within tissues can provide vital information on the molecular mechanisms taking place within the normal or diseased tissue. Measurement of the levels of mRNA is widely accepted as a means of evaluating the rate of synthesis of the respective protein. There are several techniques available to study gene expression at the mRNA level. These include *in situ* hybridisation, Northern blotting and reverse transcription - polymerase chain reaction (RT-PCR), each having its advantages and disadvantages.

*In situ* hybridisation (John et al 1969) utilises the specificity of a probe sequence (or cocktail of probe sequences) to detect nucleic acids, including RNA. The probe can be labelled by a variety of methods permitting direct or indirect detection. *In situ* hybridisation permits localisation of RNA, as it is performed on tissue sections and does not require the extraction of the RNA from the cells; however it lacks the ability to detect low copy number mRNA transcripts. There is also a potential problem of consistency of quantification as the technique can be capricious.

Northern blotting is widely used in the detection of RNA species, including tRNA, mRNA etc. The technique requires the extraction of all RNA species from a sample with subsequent fractionation on a gel, based on size. The direct detection of specific sequences is achieved utilising RNA, single stranded DNA or oligonucleotide probes. The probes are then detected directly or indirectly. Northern blotting provides no information on localisation, but does permit demonstration of multiple isoforms of genes simultaneously based on size separation. Northern blotting is also quantifiable. Northern blotting requires relatively large amounts of
starting material (i.e. approximately 1µg of poly-A tailed mRNA and approximately 10 - 20 µg of total mRNA, per track), therefore although quantification can be achieved by this method it is not suited to tiny samples such as those available from rat aortas.

Reverse transcription- polymerase chain reaction is an amplification method suited to the detection of tiny amounts of RNA. Using gene specific primers, RT-PCR is used to generate and amplify first strand cDNA reverse transcribed from a target RNA gene sequence (Figure 5.) The main advantage of RT-PCR is its high degree of sensitivity. This makes it applicable to the investigation of gene expression in tiny quantities of tissue (each 2mm segment of rat aorta in this case weighed approximately 8µg, with 100X10 µg sections therefore weighing 4µg).
Figure 5. Representation of cDNA amplification by polymerase chain reaction

Diagrammatic representation of reverse transcription and polymerase chain reaction amplification of mRNA using the Dynabead method.
The use of an efficient RNA extraction method can further improve upon the sensitivity of this method, permitting the optimal extraction of good quality RNA. Phenol has been used widely in the process of extraction of total mRNA. In 1968 a modification of this method involving the use of guanidinium isothiocyanate was introduced. In 1987 Chomczynski and Saachi (1987) reported a one step method of extracting total mRNA using a modification of the guanidinium and hot phenol method first reported by Feramisco et al. (1982). Modifications of the Chomczynski method, including commercial kits are still widely used, however new methods of producing high yield RNA with minimal contamination are still being sought. Paramagnetic Dynabeads® permit the extraction of high yield good purity mRNA from tiny samples. This approach was used in this thesis and is described more fully below.

The method of detecting and measuring the products of RT-PCR is important. Many studies use agarose gel electrophoresis followed by densitometry (Figure 6). Gel electrophoresis allows the differentiation of products of amplification by separation based primarily on size, higher gel concentrations allowing separation of smaller products and lower concentrations allowing separation of larger products. The rate at which the cDNA fragments move through the gel is also determined by the sequence and conformation of the DNA, as well as the current applied and the gel concentration. The samples are run alongside an appropriate DNA size marker, permitting the estimation of the size of the representative PCR product on the gel. This method of detection may permit the recognition of amplification product contamination by genomic DNA, made possible by designing primers that amplify across exon boundaries. The PCR products are detected by the incorporation of radioisotopes into the PCR products during the process of amplification followed by autoradiography, or by the incorporation of dyes, such as ethidium bromide, which allows the PCR products to be visualised using ultraviolet light. The disadvantages of densitometry include the use of radioisotopes, as well as
increased variability due to differences in gel thickness and staining from gel to gel. The use of a phosphorimager can remove variability due to gel differences. However this is specialised equipment which is not present in many laboratories.
Figure 6. Photograph showing detection of PCR product using agarose gel electrophoresis

β-Actin

TGF β
Enzyme-linked immunosorbent assay (ELISA) based detection of products of amplification uses the specificity of a nucleotide sequence for a complimentary region of a single strand of the amplification product. The method is discussed in detail below however in brief the single strand of amplification product is bound via an avidin biotin complex to the surface of CovaLink plate (see Figure 8). The appropriately labelled nucleotide sequence is hybridised to its complementary sequence on the bound single strand product of amplification, permitting subsequent detection and measurement. ELISA requires relatively inexpensive equipment and is highly sensitive.
Figure 8. Representation of ELISA based detection of cDNA products of PCR.

ELISA assay detection of PCR product showing the binding of the biotinylated forward primer to the plate, and the probe detection following the release of the reverse primed strand.
1.5.2 Selection of the housekeeping gene

In order to allow the determination of relative levels of cDNA in samples comparison of the product of amplification of a housekeeping gene was used. Within a tissue type or cell culture a housekeeping gene is required to have a cellular base-line level of expression which does not vary. Therefore following identical amplification reactions differences in the levels of the product of amplification of a housekeeping gene were assumed to be due to variations in the levels of cDNA seeded into the amplification reaction and consequently represent an index of the number of cells from which the mRNA was extracted. To test the reliability of this assumption 100 x 10μm cryotome cut sections (-20°C) of snap frozen aorta stored in liquid nitrogen (approximately 4mg) from all 30 animals in groups 1 to 5 at each time point were analysed in one PCR ELISA analysis. This was to control for differences in efficiency of PCR and ELISA steps between reactions and to use volumes of tissue with approximately equivalent number of cells. By comparing mean ELISA readings for each group it was therefore possible to determine whether any of the drugs administered affected the level of housekeeping gene transcript levels in the rat aortas. The housekeeping genes considered were GAPDH, a gene that encodes for a protein used in glucose metabolism and βActin, a gene that codes for a structural protein involved in microtubular assembly. Analysis of variance of the relative βActin transcript level between groups demonstrated that there was no statistically significant difference between the groups (Table 1). Conversely when GAPDH was compared this showed that both Rapamycin and Neoral was associated with lower transcript levels (P<0.01, Mann Whitney) suggesting that they suppress GAPDH mRNA transcription in rats. Furthermore this also suggested that none of the drugs influenced the expression of βActin and therefore it was selected as housekeeping gene in these experiments.
Table 1. Relative mRNA levels for two proposed housekeeping genes GAPDH and βActin at 16 weeks

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rapamycin 0.25 mg/kg/day</th>
<th>Rapamycin 0.5 mg/kg/day</th>
<th>Neoral</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH*</td>
<td>0.61 ±0.32</td>
<td>0.53 ±0.33</td>
<td>0.30 ±0.21</td>
<td>0.32 ±0.21</td>
</tr>
<tr>
<td>GAPDH/β actin</td>
<td>0.53 ±0.26</td>
<td>0.47 ±0.28</td>
<td>0.25 ±0.19</td>
<td>0.30 ±0.17</td>
</tr>
<tr>
<td>β actin*</td>
<td>1.15 ±0.36</td>
<td>1.14 ±0.29</td>
<td>1.07 ±0.23</td>
<td>1.04 ±0.27</td>
</tr>
</tbody>
</table>

*Values expressed are ELISA readings of cDNA PCR product ±S.D. at 16 weeks. P Value = Kruskall Wallis. Similar values were obtained for all other time points.
1.5.3 Materials

Enzymes

Avian myoblastosis virus reverse transcriptase (AMV RT), RNasin® ribonuclease inhibitor, and TdT were obtained from Promega (Southampton, UK); *Thermus aquaticus* DNA polymerase (*Taq*), were from Advanced Biotechnologies Ltd. (Surrey, UK); proteinase K, was from Boehringer Mannheim UK Ltd. (East Sussex, UK).

Oligonucleotide primers and probes

All oligonucleotide primer and probe sequences were designed in house using the 'gcg' primer design software, PRIMER. All primers were designed to provide optimum annealing at 59-60°C, and were checked against the European Molecular Biology Library (EMBL) and GENBANK databases using the FINDPATTERNS software on the 'gcg' system, to ensure specificity of the primers and probes to the target gene.

Where possible, primers were designed to amplify products across EXON boundaries. This permits the differential recognition of genomic DNA amplified product from cDNA amplified product by size. Oligonucleotide primers and probes were synthesised by GIBCO BRL, Oswell, and Genosys. Forward primers for all sequences were synthesised biotinylated.

Sequences are detailed in Table 2 Primer design parameters are detailed below.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer size</td>
<td>18-22</td>
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<tr>
<td>Primer 3’ clamp</td>
<td>yes</td>
</tr>
<tr>
<td>Primer GC content</td>
<td>40-50%</td>
</tr>
<tr>
<td>Primer melting temp.</td>
<td>50-60°C</td>
</tr>
<tr>
<td>Product Length</td>
<td>200-300 bp</td>
</tr>
<tr>
<td>Product annealing temp.</td>
<td>60°C</td>
</tr>
</tbody>
</table>
### Table 2. PRIMERS & PROBES

* = 5’-end biotinylated.

Oligonucleotide sets (e.g. two primers and a probe) are grouped by complementarity.

All oligonucleotides are 20-mers unless otherwise stated.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ</td>
<td>RTGBF</td>
<td>RTGBR</td>
<td>RTGBP (formerly RTGBPRT)</td>
<td>59°C/40</td>
</tr>
<tr>
<td></td>
<td>*TAC GTC AGA CAT TCG GGA AG</td>
<td>GAA GCG AAA GCC CTG TAT TC</td>
<td>TCA AAA GAC AGC CAC TCA G</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>GPDF (19-mer)</td>
<td>GPDR</td>
<td>GPDP (19-mer, formerly GPDPRT)</td>
<td>59°C/35</td>
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<tr>
<td></td>
<td>*AGA ACA TCA TCC GTG CCT C</td>
<td>GCC AAA TTC GTG TAT ATA CC</td>
<td>GTT GAA TGC AGA GGA GAC C</td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>ACTF (22-mer)</td>
<td>ACTR (22-mer)</td>
<td>ACTP (21-mer, formerly ACTPR)</td>
<td>59°C/35</td>
</tr>
<tr>
<td></td>
<td>*TCA TCA CCA TTG GCA ATG AGC G</td>
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<td>GGA GTA CTT GGC CTC AGG AGG</td>
<td></td>
</tr>
<tr>
<td>Collagen III</td>
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<td>RC31R</td>
<td>RC31P (formerly RC31PR)</td>
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<tr>
<td></td>
<td>*GAA ATT CTG CCA CCC TGA AC</td>
<td>GGC TGG AAA GAA GTC TGA GG</td>
<td>CTT CTC AGC ACC AGC ATC TG</td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>*ATT GAT GCG GTA TAC GAG GC</td>
<td>GCG ACC CTG GAA GTA GC</td>
<td>CTC CAG AAT TTG TCT CCA GC</td>
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<td>MMP-9</td>
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<td>RMP9R</td>
<td>RMP9P (formerly RMP9PR)</td>
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<tr>
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<td>*GCA TTT CTT CAA GGA CGG TC</td>
<td>GGC CAG AGA ACT CTG TAT CC</td>
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<td>RTMP1R</td>
<td>RTMP1P (formerly RTMP1PR)</td>
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</tr>
<tr>
<td></td>
<td>*GTT CCC CAG AAA TCA TCG AG</td>
<td>TGA ACA GGG AAA CAC TGT GC</td>
<td>GCA GTG ATG TGC AAA TTT CC</td>
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<tr>
<td>TIMP-2</td>
<td>RTMP2F</td>
<td>RTMP2R</td>
<td>RTMP2P (formerly RTMP2PR)</td>
<td>59°C/40</td>
</tr>
<tr>
<td></td>
<td>*CGA ATT TAT CTA CAC GGC CC</td>
<td>TCT TGA TGC AGG CAA AGA AC</td>
<td>TCC CAG GGC ACA ATA AAG TC</td>
<td></td>
</tr>
<tr>
<td>TIMP-3</td>
<td>RTMP3F</td>
<td>RTMP3R</td>
<td>RTMP3P (formerly RTMP3PR)</td>
<td>59°C/32</td>
</tr>
<tr>
<td></td>
<td>*CGG AAG CCT CTG AAA GTC TC</td>
<td>CAG GCG TAG TGT TTG GAC TG</td>
<td>GGT GGT AGC GGT AAT TGA GG</td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>RINOSF</td>
<td>RINOSR</td>
<td>RINOSP3</td>
<td>59°C/40</td>
</tr>
<tr>
<td></td>
<td>*TGT CAC CGA GAT CAA TGC AG</td>
<td>GCA CAG AAG CAA AGA ACA CC</td>
<td>CTG CAT GTG CTT CAT GAA G</td>
<td></td>
</tr>
</tbody>
</table>
1.5.4 mRNA extraction from rat aorta

mRNA was extracted from vessel wall using oligo dT linked paramagnetic beads. OCT embedded section of rat aorta, stored since harvest at -270°C were warmed to -20°C for 1 hour and cut into 10μm sections using a cryotome. Approximately 100 sections were placed in a 1.5ml Eppendorf containing 600μl of lysis/binding buffer and 100μl of 50μg/ml of proteinase K (to assist with the dissociation of the tissue and cellular structure), and the entire mixture was incubated for 1 hour at 37°C.

Next the extract was centrifuged for 60 seconds at high speed in a centrifuge, to permit separation of the cellular debris from the lysate. 90μl of the aortic lysate was then added to the prepared beads, resuspended and allowed to anneal for 15 minutes at room temperature. The beads were pelleted with a magnetic particle concentrator, utilising the paramagnetic nature of the beads, and the supernatant removed. To purify the bead bound mRNA, the beads were resuspended in 100μl of mRNA extraction wash buffer 1 (Appendix 2), followed by pelleting and removal of the supernatant. The wash procedure was repeated once, followed by three washes with 100μl of mRNA extraction wash buffer 2 (Appendix 2). The beads were resuspended in 10μl of DEPC water as preparation for reverse transcription.
1.5.6 Preparation of dynabeads

To prepare the oligo-dT dynabeads®, 10μl of beads, in storage buffer, were placed in a sterile Rnase-free Eppendorf and utilising the paramagnetic nature of the beads pelleted with a Dynal MPC® (magnetic particle concentrator) until the supernatant was clear, at which point the supernatant was removed and the beads resuspended with 10μl lysis/binding buffer. The decision to use 10μl of beads was made on the basis of a report in the DYNAL® HANDBOOK Dynabeads® mRNA DIRECT kit which recommends the following aliquots of beads.
Table 3. DYNABEAD BINDING QUANTITIES (DYNAL® HANDBOOK Dynabeads® mRNA DIRECT kit)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of Cells</th>
<th>mRNA</th>
<th>Beads</th>
<th>Lysis/Binding Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2 mg</td>
<td>1-1.5 x 10^5</td>
<td>60-120 ng</td>
<td>10 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>3-8 mg</td>
<td>5 x 10^5</td>
<td>180-360 ng</td>
<td>30 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>6-15 mg</td>
<td>1 x 10^6</td>
<td>450-900 ng</td>
<td>75 μl</td>
<td>250 μl</td>
</tr>
<tr>
<td>8-20 mg</td>
<td>1-1.5 x 10^6</td>
<td>600-1200 ng</td>
<td>100 μl</td>
<td>333 μl</td>
</tr>
<tr>
<td>12-30 mg</td>
<td>1-2 x 10^6</td>
<td>900-1800 ng</td>
<td>150 μl</td>
<td>500 μl</td>
</tr>
<tr>
<td>16-40 mg</td>
<td>1-3 x 10^6</td>
<td>1.2-2.4 μg</td>
<td>200 μl</td>
<td>750 μl</td>
</tr>
<tr>
<td>20-50 mg</td>
<td>1-4 x 10^6</td>
<td>1.5-3 μg</td>
<td>250 μl</td>
<td>1000 μl</td>
</tr>
<tr>
<td>40-100 mg</td>
<td>2-8 x 10^6</td>
<td>3-6 μg</td>
<td>500 μl</td>
<td>2000 μl</td>
</tr>
<tr>
<td>80-200 mg</td>
<td>4-16 x 10^6</td>
<td>6-12 μg</td>
<td>1000 μl</td>
<td>3000 μl</td>
</tr>
</tbody>
</table>

As approximately 4 mg of aortic tissue was used (equating to 2 x 10^5 to 1 x 10^6 cells) 30 μl of beads were used per segment for each extraction to achieve a surplus of poly A sequences on the dynabeads. This ensured that all of the available mRNA was captured.
1.5.7 Reverse transcription-polymerase chain reaction

Reverse transcription of mRNA

The 10μl bead bound mRNA extraction was split into two 0.5ml Eppendorfs in 8μl and 2μl volumes, for positive RT and negative control RT reactions respectively. These volumes were chosen to maximise the production of first strand cDNA, while allowing a negative RT reaction to test for contamination of the mRNA extract. Each Eppendorf contained: 1 X AMV RT Buffer; 10mM DEPC treated dNTPs; and 28U Rnasin (Appendix 2). Positive RT reactions each contained 5U of AMV RT (Appendix 2) enzyme. The reaction was carried out at a total volume of 25μl, incubating at 42°C for 1 hour. If the Dynabead bound cDNA was to be stored, it was resuspended in TE buffer and stored at 4°C.

Polymerase chain reaction

Each PCR reaction contained the following reagents. 1 X in-house PCR buffer; 10pmol forward primer; 10pmol reverse primer; Jump Start Taq polymerase 1U per PCR reaction (Appendix 2). These reagents were mixed in the appropriate quantities in an initial mastermix sufficient for all the intended PCR reactions and added in aliquots to the RT reaction product (1μl of cDNA from the positive RT reactions (4μl of cDNA from the negative RT reactions). Reactions were run in a total volume of 50μl. In this way variations in the efficiency of the reactions between separate eppendorff tubes in the thermal cycler are minimised. Sterile mineral oil was placed over the top of the PCR mixture, to prevent evaporation and the tubes were placed in a thermal cycler. All PCR reactions were cycled at the optimum annealing temperature (approximately 59°C) and number of cycles for each individual primer as determined by previous work in this laboratory (Sunjay Jain, University of Leicester, personal communication).
1.5.8 Detection and measurement of products of amplification

1.5.8.1 Agarose gel electrophoresis

All RT-PCR product samples were run on a 3% Agarose MP gel to maximise resolution and to check for the presence or absence of the specific product of amplification, as well as any contamination.

To prepare the electrophoresis gel an appropriate weight to volume of Agarose was melted in 1 X TAE Buffer. After the addition of 0.5μg/ml ethidium bromide the agarose was poured into a gel former containing the appropriate sized comb for the formation of the wells. The gel was left at room temperature to solidify, before placing the gel into an electrophoresis tank containing 1 X TAE Buffer with 0.5μg/ml ethidium bromide.

PCR product was added to 10 X PCR loading buffer (final concentration of loading buffer 1 X), and an appropriate volume of this solution was loaded into the wells on the gel. An appropriate DNA size marker was included along with the amplification samples. Electrophoresis gels were run at 240V/4A for 30 minutes and upon completion of the cDNA migration was visualised by ultraviolet light.

1.5.8.2 ELISA detection and measurement of PCR products

ELISA probe labelling

Digoxigenin labelled probes were used to detect specific PCR gene products. Probes were labelled as follows: 1X Boehringer TdT; 100pmol; 85μM Digoxigenin- 11- dUTP; 425μM ATP; and 45U of rTDT enzyme (Appendix 2). The reaction was run in a total volume of 20μl incubated at 37°C for 15minutes. Reactions were stopped by the addition of 1 μl of 0.5M
EDTA. Probes were topped up with sterile water to a total volume of 100μl and stored at -20°C.

**ELISA based assay for the detection and measurement of PCR products.**

To prepare the plate for ELISA, Biotynlated CovaLink plates were washed once in ELISA wash buffer and coated with an avidin layer by incubating them with avidin (50 μg/ml) in ELISA wash buffer 2) for 30 minutes at room temperature. Following avidin coating the plates were washed and blocked against non-specific binding of DNA to the plate by incubation in 3% PBS/BSA for 15 minutes at room temperature to minimise background. The PBS/BSA was aspirated and the PCR products were dissolved in 1:10 in sterile water and added to the appropriate wells, followed by an addition of 90μl of 3% PBS/BSA. This was incubated for 1 hour at room temperature allowing the DNA to bind via the biotinylated forward strand to the avidin molecule. Unbiotiylated (reverse strand) PCR products were denatured from the biotinylated forward strand by the addition of an equal volume of 0.25M NaOH to the DNA/PBS/BSA for 10 minutes at room temperature. Following washing the plates were incubated for 1.5 hours at 42°C with 0.2pmol of probe diluted in rapid hybridisation buffer. The plates were then washed and incubated with alkaline phosphatase conjugated anti-digoxigenin diluted 1:500 in PBS/BSA, for 30 minutes at room temperature. Finally the plates were washed and incubated in para-nitrophenylphospate 1mg/ml in 1M diethanolamine pH 9.8, for 0.25 to 2 hours at 37°C. Samples were read at 405nm with a differential of 630nm on a Multiskan EX microtitre plate reader (Billingshurst, UK).

All ELISA measurements were performed in duplicate with the following controls:
1. Plate check: βActin forward primer was labelled with digoxigenin-11-dUTP. This allowed for monitoring of the day to day variation in plate performance.

2. Substrate only blank: These wells contained only substrate, and gave a measure of the background plate reading.

3. Non specific binding control: These wells contained mismatch PCR product to probe, and therefore tested the specificity of the probe.

4. Positive control: These wells contained probe specific PCR product for each gene. The same PCR sample was used on every ELISA run testing for the day to day variation in probe efficiency.

5. A PBS/BSA blank (BSA) i.e. no DNA added
Interpretation of ELISA readings

Recordings from the plate reader were taken at 15-minute intervals for 2.5 hours and plotted against time as follows.

Figure 9. Plots of MMP 9, Collagen III and TGF ELISA readings with time

Figure 9A. Collagen 3 ELISA signal versus time
Figure 9B. MMP 9 ELISA signal versus time
These results indicate that the ELISA plate reader is sensitive to changes in optical density (ELISA absorbency) associated with conversion of para-nitrophenylphosphate in a linear fashion until the value on the plate reader is greater than 3 at which time the calculation is non-linear. All ELISA readings are therefore taken before any value in the ELISA has reached 3.
1.5.9 Reproducibility of RT-PCR

The ability of RT-PCR to target gene sequences, thereby permitting detection of extremely low copy number mRNA, makes it applicable to the investigation on mRNA in small volumes of tissue. This study used super paramagnetic beads containing a poly-Thymine tail, as described previously. The poly-Thymine tail was used to prime the reverse transcription of first strand cDNA off the mRNA sequence, permitting the reverse transcription of all mRNA sequences in one RT reaction (assuming all mRNA sequences of interest have poly-A tails). This also presumably eliminated variability as all cDNA was synthesised in one reaction and efficiency between RT reactions for multiple genes within a single sample was standardised. Although variation in RT efficiency within a single sample is assumed to be removed by this method, variation between RT efficiency between samples will remain. For this reason the expression of mRNA for each sample was expressed as a ratio of the ELISA reading for that particular gene relative to the ELISA reading of the housekeeping gene βActin for that particular sample. Furthermore day to day variability in the efficiency of the PCR step in these experiments was controlled for by performing the PCR step for each individual gene in all specimens simultaneously at any given time point i.e. 4, 8, 12 or 16 weeks. Similarly PCR products for a particular gene in all specimens for each individual time point were quantified in the same ELISA to prevent variability in the efficiency of the ELISA reactions affecting the results.

The reproducibility of RT-PCR ELISA analysis in the rat for these genes has been reported previously (see Ph.D. Thesis Leon Hall University of Leicester) as have the individual PCR and ELISA steps (Ph.D. Thesis Leon Hall, Bicknell et al 1996). For the purpose of this thesis
however it was important to demonstrate the reproducibility of PCR/ELISA in the rat aorta with these methods.

**Results of PCR reproducibility studies**

For each gene 6 individual aliquots of dynabeads from a single RT were amplified on their own, unique PCR mastermix in order to simulate PCR performed on 6 separate occasions. These amplifications were then quantified by ELISA and compared to 4 ELISA readings of the PCR from which results had been obtained (Table 4). These 6 PCR results were then normalised against a control cDNA samples that had undergone PCR at the same time (Table 5).
**Figure 4. Results of PCR reproducibility studies**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>MMP-9</th>
<th>TGF</th>
<th>TIMP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 separate PCR's of single RT Reaction</td>
<td>Mean</td>
<td>2.61</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation</td>
<td>.11385</td>
<td>.18571</td>
</tr>
<tr>
<td></td>
<td>Variance</td>
<td>0.02</td>
<td>0.03</td>
</tr>
</tbody>
</table>

| 4 separate ELISA readings of original PCR from same RT reaction used in results | Mean | 2.58 | 2.1 | 1.9 |
| | N | 4 | 4 | 4 |
| | Std. Deviation | 0.1 | 0.13 | 0.03 |
| | Variance | 0.01 | 0.02 | 0.01 |

| ANOVA | F | 0.18 | 0.05 | 0.02 |
| | Sig. | 0.69 | 0.82 | 0.83 |

Six separate PCR's quantified at the same time showed no significant variation in PCR efficiency and indicate that aliquots of RT product produce a reproducible result. The narrow variation within both the original PCR results and the reproducibility study PCR would also suggest that the ELISA is reproducible although the reproducibility of this technique has been demonstrated in previous work in this department (Sunjay Jain, University of Leicester, personal communication).

When values were corrected however for the control cDNA for each gene however (as used to compare relative levels of gene expression between different time points) the results were not as reproducible.
Figure 5. Results of PCR reproducibility studies controlled for PCR reactions at
different time points

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Normalised MMP9</th>
<th>Normalised TGF</th>
<th>Normalised TIMP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 separate PCR's</td>
<td>Mean</td>
<td>4.77</td>
<td>2.8</td>
</tr>
<tr>
<td>N</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>0.21</td>
<td>0.26</td>
<td>0.06</td>
</tr>
<tr>
<td>Variance</td>
<td>0.04</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>4 separate ELISA readings of original 9a7 PCR used in results</td>
<td>Mean</td>
<td>3.63600</td>
<td>3.35775</td>
</tr>
<tr>
<td>N</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>0.21</td>
<td>0.21</td>
<td>0.02</td>
</tr>
<tr>
<td>Variance</td>
<td>0.04</td>
<td>0.04</td>
<td>0.0004</td>
</tr>
<tr>
<td>ANOVA</td>
<td>F</td>
<td>70.964</td>
<td>13.119</td>
</tr>
<tr>
<td>Sig</td>
<td>.000</td>
<td>.007</td>
<td>.218</td>
</tr>
</tbody>
</table>

The results of these reproducibility studies would therefore suggest that PCR ELISA is a reproducible technique however the use of a control cDNA sample in each PCR to enable direct comparison of the level of specific Control cDNA in each RT sample between large PCRs introduces an unacceptable degree of error.

Given the design of the current experiments were all samples from all experiments culled at a given time point were massed together in a single large PCR comparison between groups at any given time point is possible. The variability between different PCRs introduced following 'Normalisation' with control cDNA prevents comparison of relative cDNA levels between different time periods.
Chapter 3. The effect of Neoral cyclosporin on the development of allograft vasculopathy and the expression of genes responsible for extracellular matrix remodelling in rats
Abstract

**Background** Chronic allograft dysfunction (CAD), the leading cause of solid organ transplant failure is characterised by histological evidence of extracellular matrix (ECM) accumulation (fibrosis). The aim of this study was to characterise the changes in ECM accumulation and fibrosis associated gene expression in an experimental model of CAD and to measure the effect of the immunosuppressant cyclosporin on these changes.

**Methods** Lewis recipients of F344 rat thoracic to abdominal transplants were administered cyclosporin (Neoral) or no treatment. Vascular remodelling and ECM accumulation (sirius red) were measured using computerised histomorphometry. Fibrosis associated gene expression was studied by semi-quantitative RT-PCR.

**Results** Cyclosporin inhibited medial ECM accumulation and vascular remodelling in allografts. This was associated with an attenuation of the graft inflammatory infiltrate and a reduction in intragraft MMP 2 and MMP 9 mRNA levels. There was a significant negative correlation between neoadventitial ECM density and MMP 9 expression as well as with vessel circumference. Neoadventitial ECM density was significantly higher in the cyclosporin treated group compared to untreated allografts as were Collagen 3 and TIMP 1 mRNA levels.

**Conclusion** The alloimmune injury itself may directly contribute to vascular remodelling and fibrosis in allograft vasculopathy. Cyclosporin effectively attenuated this component of the pathophysiology of CAD.
Introduction

Chronic Allograft Dysfunction is a leading cause of late allograft loss and current immunosuppressants have little or no effect on the progression of this condition in clinical transplantation (Cecka 1994, Hosenpud et al 1995). It is defined histologically by a chronic fibroproliferative tissue response to injury that includes allograft vasculopathy and parenchymal extracellular matrix (ECM) accumulation (fibrosis) (Wallwork 1994, Sibley 1994, Paul 1994). Allograft vasculopathy is characterised by neointimal hyperplasia and vascular remodelling, pathological processes that are associated with changes in the regulation of ECM turnover. Central to these processes in many models of vascular injury are the matrix metalloproteinases, a family of zinc dependant proteases that can collectively breakdown all the constituents of the ECM (George 1998). The activity of these proteases in turn is tightly regulated by a highly specific family of inhibitory proteins, the tissue inhibitors of metalloproteinases (TIMPs). The role of MMPs and TIMPs in the development of allograft vasculopathy however remains to be defined. The aim of this study was to characterise the changes in expression of genes important in regulating ECM turnover in an animal model of CAD and to assess the effect of the immunosuppressant cyclosporin on these changes.
Methods

Animal model

The thoracic aorta to abdominal aorta allograft model as described by Mennander et al (1991) was used. A segment of the descending thoracic aorta approximately 3cm in length was excised, thoroughly perfused with phosphate buffered saline (PBS) and used as a transplant. F344 rats acted as donors and recipients and Lewis rats as recipients. Ischaemic injury to the graft was minimised by immersion in an ice bath at 4°C between procedures. Through a midline laparotomy incision the segment of thoracic aorta was anastomosed end to end to the recipient abdominal aorta using 9/0 prolene suture. The graft was transplanted into heterotopic position below renal arteries and above the bifurcation forming a loop in the recipient abdomen.

Animals were kept in a controlled environment with unlimited access to feed and water. Graft harvest was performed in heavily anaesthetised rats following which the animal was allowed to die by exsanguination.

Drugs

Microemulsion cyclosporin (Neoral, Novartis, Basle, Switzerland) was prepared as a solution of 5mg/ml in olive oil, stored at 21°C, and administered orally by gavage.

Experimental groups

Group 1. F344 to F344 isografts. (Isografts, negative control)

Group 2. F344 to Lewis allografts receiving cyclosporin 5mg/kg/day orally by gavage for 14 days only then no immunosuppression until sacrifice. (Allografts, positive control)

Group 3. F344 to Lewis allografts receiving microemulsion cyclosporin (Cyclosporin) 5mg/kg/day orally by gavage from the day of transplant until sacrifice.
In this experiment six rats in each group is sacrificed at each of weeks 4, 8, 12 and 16 with an additional 6 rats in Group 1 sacrificed at 24 weeks.

**Histological analysis**

Segments of the aortic graft were embedded in paraffin wax and multiple sections were stained with haematoxylin and eosin. The level of inflammatory infiltrate was assessed semi-quantitatively by a blinded single observer and was categorised as follows: **Nil**- no inflammatory cells visualised, **Mild**- endothelialitis, inflammatory cell localised to the endothelium, **Moderate**- dense inflammatory cell infiltrate in the innermost layer of the vessel wall, **Severe**- dense inflammatory cell infiltrate throughout all layers of the vessel wall.

The vessel circumference and area fraction of the intima and media were quantified using a computer image analysis system. The intima was defined as the area between the endothelium and the internal elastic lamina and the media was defined as the area between the internal and external elastic laminae. Paraffin embedded sections were also stained with sirius red stain and the area fraction of ECM staining quantified using computerised histomorphometry.

**Allograft gene expression using RT-PCR**

Total mRNA was extracted from aortic tissue and complementary DNA molecules were synthesised by reverse transcription (RT). These cDNA species were amplified by polymerase chain reaction (PCR) and quantified in an ELISA system. Relative quantitation was performed by comparison of the signal intensity to that of the housekeeping gene β Actin.
Substances to be assessed

In order to take into account all the factors which influence smooth muscle cell proliferation, migration and deposition of ECM during neotima formation, the following species were studied with RT-PCR: MMP2, MMP9, TIMP1, 2 and 3, TGF beta and collagen III.

Analysis of results

The levels of gene expression were compared in the different study groups using non-parametric statistical analysis. Allograft gene transcripts for matrix proteins and growth factors were correlated with intimal thickening expressed as the intimal media ratio, vascular remodelling expressed as vessel circumference (of the internal elastic lamina) and collagen accumulation expressed as a product of the percentage mean media sirius red staining and the medial area. Statistical analysis was performed using the Statistical Package for the Social Sciences Version 8.0 (Chertsey, UK.)
Results

Results of histological analysis

By four weeks following transplantation there was a dense inflammatory infiltrate in allografts, in many cases transmural, that was not seen in isografts, and was also present at 8, 12 and 16 weeks (Figure 1). By 12 weeks early intimal hyperplasia was evident in untreated allografts and by 16 weeks intimal thickening appeared to have slowed such that there was no significant difference between intimal medial ratio at 16 and 24 weeks in untreated allografts (Figure 2A). Expansive vascular remodelling occurred in a linear fashion between 4 and 24 weeks (Figure 2B). Cyclosporin significantly inhibited both intimal hyperplasia (intima media ratio 0.08±0.09 versus 0.2±0.05, P=0.01 Mann Whitney) and expansive vascular remodelling (Internal Elastic lamina Circumference 3.8 ±0.2 versus 4.2 ±18mm, P=0.02) compared to untreated allografts as well as significantly attenuating the severity of the inflammatory response within the graft at 4 weeks (Figure 2C) and at all subsequent time points (data not shown).

Results of picrosirius red histomorphometry

There was increased medial ECM accumulation within the allografts relative to isografts at 16 weeks (area fraction stained 0.45± 0.01 versus 0.35± 0.03 mm², P=0.03 Mann Whitney, Figure 3A). This was not associated with a change in ECM density but was due to an increase in medial area. Allograft medial ECM accumulation was significantly reduced by cyclosporin (0.38± 0.04 mm², P=0.01, Mann Whitney).

There was very little ECM staining detected in the neointima at 16 weeks and therefore this was not included in our analysis. As this would have increased predominantly the total ECM content in the untreated allograft group however it would not have greatly altered our results. In the current model it was impossible to accurately distinguish true allografts adventitia from peritoneal adhesions and therefore neoadventitial thickness or ECM.
content was not assessed. Adventitial ECM density however was reduced in allografts (0.72±0.07) relative to isografts (0.85±0.07, P=0.048 Mann Whitney) and this effect was attenuated by the administration of cyclosporin (0.8±0.05, P=0.08 Mann Whitney)(Figure 3B).
Figure 1. Representative photomicrographs of haematoxylin and eosin stained section of rat aorta (×100 magnification) from A. Isografts, B Untreated Allografts and C. Cyclosporin treated allografts. Untreated allografts have a dense transmural inflammatory infiltrate associated with intimal and medial thickening. These changes are attenuated by cyclosporin.

Figure 1A.
Figure 2. Results of histological analysis

Figure 2A. The effect of cyclosporin on neointima formation

The neointima forms between 8 and 16 weeks in untreated allografts relative to isografts. This intimal thickening was significantly inhibited by cyclosporin, \( P=0.02 \), Mann Whitney). Values represent mean values + S.D.

The effect of Cyclosporin on allograft vasculopathy
(Intima media ratios)

![Graph showing the effect of Cyclosporin on allograft vasculopathy](image)
Figure 2B. The effect of cyclosporine on vessel remodelling
Allografts undergo a significant progressive expansive remodelling process between 0 and 24 weeks. This process was inhibited by cyclosporin, (16 weeks P=0.01, Mann Whitney). Values represent Mean + S.D.
Figure 2C. Inflammatory infiltrate at 4 weeks

There was a significant difference between the severity of the inflammatory infiltrate between isografts, untreated allografts and cyclosporin treated allografts at 4 weeks (Pearson's Chi Square = 28.0, P<0.001). Cyclosporin reduced the severity of the inflammatory infiltrate.

Qualitative assessment of Inflammatory infiltrate

<table>
<thead>
<tr>
<th>Group</th>
<th>Nil</th>
<th>Mild</th>
<th>Mod</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isograft</td>
<td>6.0</td>
<td>5.0</td>
<td>4.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Allograft</td>
<td>2.0</td>
<td>4.0</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Cyclosporin</td>
<td>1.0</td>
<td>3.0</td>
<td>1.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Figure 3. Results of sirius red histomorphometry

There was significantly greater extracellular matrix accumulation in the media of allografts relative to that of isografts. This was characterised by an increase in media volume rather than ECM density. Conversely adventitial ECM density was reduced in untreated allografts and this decrease was attenuated by cyclosporin.

Box and whisker plots represent Mean, S.D. and 95% Confidence intervals

Figure 3A.

Medial ECM Content at 16 weeks

\[
\text{area fraction stained (mm}^2\text{)}
\]

isografts \hspace{1cm} allografts \hspace{1cm} cyclosporin

\[\div, P=0.03, \div, P=0.01 \text{ Mann Whitney}\]
Adventitial ECM Density at 16 weeks

Group

Isograft  Allograft  Cyclosporin

\( \ddagger \), P = 0.04, \( \ddagger, \dagger \), P = 0.08 Mann Whitney
Results of RT-PCR ELISA

MMP 9 mRNA transcript levels were increased in untreated allografts relative to isografts at 4 weeks and this increase was attenuated by cyclosporin (data not shown). There was no significant difference between the groups for the other genes studied at 4 weeks. There was no apparent difference between the groups for TIMP 2 or TIMP 3 at any time point. TIMP 3 however correlated closely with MMP 2 at all time points (for example r=0.8, P<0.0001, Spearman rank at 8 weeks).

At 8 weeks there was a reduction in TIMP 1 and Collagen 3 gene expression in untreated allografts relative to isografts (Figure 4B and 4D) with a persistent significant increase in MMP 9 transcript levels (Figure 4A) in untreated allografts relative to isografts. When gene/actin ratios were correlated there was a significant negative correlation between MMP 9 and Collagen 3 (R=-0.6, P=0.01 Spearman's rank). Cyclosporin significantly attenuated the MMP 9 rise and the Collagen 3 fall as well as significantly reducing TGF Beta mRNA transcript levels (Figure 4C). MMP 2 expression was also reduced by cyclosporin at 8 weeks but this was not statistically significant (data not shown).

In 12 week aortas there were significant increases in MMP 2, TGF beta and MMP 9 expression in untreated allografts relative to isografts (Figure 5A,B, D). Cyclosporin inhibited MMP 2 transcription in allografts. There were no significant differences with respect to pro-fibrotic genes such as Collagen 3 and TIMP 1 at this time point (Figure 5C,E).

At 16 weeks there were significant reductions in the expression of TIMP 1 and Collagen 3 as well as significant increases in MMP 9 in untreated allografts relative to isografts however these later changes in collagen 3 and TIMP 1 expression were not attenuated by cyclosporin (Figure 6).
Figures 4-6. Graphs demonstrate the influence of cyclosporin on the expression of genes important in the regulation of ECM turnover compared to negative (isograft) and positive (allograft) controls. Gene expression is expressed as the ratio of mRNA transcript levels for the gene considered relative to the mRNA transcript levels of the housekeeping gene β Actin to control for differences in cellularity between samples. The effect of cyclosporin on individual gene expression is described in the text. Boxes represent median, S.D. Whiskers represent 95% C.I.

Figure 4A-D. The effect of cyclosporin on fibrosis associated gene expression in aortic transplants at 8 weeks

4A

MMP 9 mRNA/ actin mRNA ratios at 8 weeks

\[ \frac{\text{MMP 9 mRNA}}{\text{actin mRNA}} \] ratios at 8 weeks

\( \frac{\text{MM9 mRNA}}{\text{actin mRNA}} \) ratio

Group

\( \frac{\text{Isograft}}{\text{allograft}} \) Cyclosporin

‡, \( P = 0.006 \), †, \( P = 0.04 \) Mann Whitney
Collagen 3 mRNA/actin mRNA ratio at 8 weeks

Collagen 3 mRNA/actin mRNA ratio

Group

‡, P = 0.011, †, P = 0.1, Mann Whitney
TGF β mRNA/actin mRNA ratio at 8 weeks

Group

‡, P = 0.004, Mann Whitney
TIMP 1 mRNA/actin mRNA ratio at 8 weeks

‡, P= 0.04, Mann Whitney
Figure 5A-D. The effect of cyclosporin on fibrosis associated gene expression in aortic transplants at 12 weeks
See text. Boxes represent median, S.D. Whiskers represent 95% C.I.

5A

MMP 2 mRNA/ actin mRNA ratios at 12 weeks

‡ P=0.03, † P=0.05, Mann Whitney
MMP 9 mRNA/ actin mRNA ratios at 12 weeks

MMP 9 mRNA/ actin mRNA ratio

N = 5 5 5
Isograft Allograft Cyclosporin

† P=0.03, Mann Whitney
Collagen 3 mRNA/actin mRNA ratios at 12 weeks
TGF β mRNA/ actin mRNA ratios at 12 weeks

TGF β mRNA/ actin mRNA ratio

Group

† P=0.05, Mann Whitney
TIMP 1 mRNA/actin mRNA ratios at 12 weeks

Group

N = 5 5 5 5
isograft allograft Cyclosporin

*
Figure 6A-D. The effect of cyclosporin on fibrosis associated gene expression in aortic transplants at 16 weeks
See text. Boxes represent median, S.D. Whiskers represent 95% C.I.

6A

MMP 9 mRNA/actin mRNA ratios at 16 weeks

MMP 9 mRNA/actin mRNA ratio

Group

†, P=0.01, ‡, P=0.02, Mann Whitney
Collagen 3 mRNA/actin mRNA ratios at 16 weeks

†, P=0.014, Mann Whitney
TGF β mRNA/ actin mRNA ratio at 16 weeks

†, P=0.01 Mann Whitney
TIMP1 mRNA/ actin mRNA ratios at 16 weeks

†, P=0.01, ‡, P=0.02, Mann Whitney
Histological correlates of observed changes in MMP 9 gene expression

MMP 9 transcript levels in the vessel wall increase with increasing histological severity of the inflammatory infiltrate (Figure 7A). Gene actin ratios for MMP 9 as determined by ELISA assay were also shown to correlates significantly with medial collagen content, (Figure 7B), vessel circumference (Figure 7C) and intimal area (r=0.6, P=0.02, Spearman's rank, data not shown). There was a weak but statistically significant negative correlation between MMP 9 transcript levels and neoadventitial collagen density (r=0.5, P=0.05, Spearman's rank).
Figure 7. Correlates of histological extracellular matrix remodelling, vessel remodelling, severity of inflammation and MMP 9 gene expression. (see text)

MMP 9 expression versus Inflammatory Infiltrate

Bars represent mean value, Kruskall Wallis P=0.001
MMP 9 expression versus Medial ECM Content

R=0.7, P=0.02

area fraction staining (mm²)

MMP 9 mRNA/actin mRNA ratio
Vessel Circumference versus MMP 9

Expression at 16 weeks

R=0.8, P<0.001
Vessel Circumference versus Adventitial Sirius Red staining fraction density at 16 weeks

Optical density ratio

R = 0.6, P = 0.005
These data show that the development of vasculopathy in rat aortic allografts was associated with progressive expansive vascular remodelling, inflammation, increased medial extracellular matrix accumulation and a reduction in adventitial collagen density. These changes were associated with increased intragraft MMP 9 and decreased TIMP 1 and collagen 3 mRNA levels compared to negative controls (Isografts). Inhibition of graft inflammation by cyclosporin attenuated both the increases in gelatinase expression in allografts as well as the changes in pro-fibrotic gene activity and significantly inhibited neointima formation, medial collagen accumulation and expansive vascular remodelling.

Expansive remodelling in rat allografts correlated with MMP 9 gene expression. An association between MMP 9 and expansive vascular remodelling is also seen in balloon injury and high shear stress models of vascular injury (Bassiouny et al 1998, Wentzel et al 2001, Mason et al 1999) however the molecular basis of this process remains obscure. In rat allografts MMP 9 transcript levels were associated with severity of the inflammatory infiltrate in the grafts and also correlated inversely with adventitial collagen density. MMP 9 is expressed by infiltrating T cells and activated macrophages in vivo and in vitro (Goetzl et al 1996) and in mice aortic allografts MMP 9 is localised to inflammatory cells throughout the allograft but particularly the adventitia (Lijnen et al 1998). The negative correlation between MMP 9 transcript levels and adventitial collagen density may therefore represent a direct effect of inflammatory cells on neoadventitia formation and subsequent vascular remodelling. The severity of constrictive remodelling in pigs is related to adventitial thickening more so than changes in intimal or medial dimensions, (Lafont et al 1995) and it has been suggested that a collagen dense adventitia may represent a mechanical barrier to vessel enlargement. A collagenolytic inflammatory infiltrate may therefore produce the opposite effect resulting in expansion. Adventitial fibroblast collagen production is a critical determinant of constrictive remodelling in balloon injured rabbit
arteries (Shi et al 1997) and it is noteworthy that expansive remodelling was associated with decreased Collagen 3 and TIMP 1 expression in this model.

Remodelling could also arise as a response to degradation of elastin, a substrate for MMP 9 and a major structural component in these vessels (elastin lamellae). These findings imply that vascular remodelling in allografts is not solely a component of the tissue response to injury (Ross 1999) but may in part be mediated by the alloimmune injury itself.

Cyclosporin, by attenuating the T cell response and subsequent macrophage infiltrate inhibited expansion.

Rat aortic allografts undergo neointimal thickening between 12 and 16 weeks following an initial inflammatory infiltrate within the graft. These changes coincide with significant increases in MMP 2, TGF beta and MMP 9 expression in 12 week allografts. Similar changes in gene expression are associated with ECM turnover during formation of the neointima in other experimental models (Southgate et al 1996, 1999, Webb et al 1997, Kranzhofer et al 1999) and therefore it is likely that these changes underly smooth muscle cell migration and proliferation in the aortic allografts.

The inhibition of neointimal hyperplasia by cyclosporin (5mg/kg/day) is a similar finding to some rat studies but not in others (Cramer et al 1990, Mennander et al 1991a, Koskinen et al 1995, Geerling et al 1994, Little et al 1996). Much of this confusion arises from different experimental models and dosages. In a high responder model as used in the current study effective inhibition of T cell mediated damage by cyclosporin inhibited allograft vasculopathy in rat aortic allografts and was associated with a reduction in MMP 2 transcription. MMP 9 was not inhibited by cyclosporin at 12 weeks compared to untreated allografts. This is unexplained, however, the severity of inflammatory infiltrate within the untreated allografts at 12 weeks appears less than at other time points, with subsequently lower MMP 9 transcription in the untreated allografts producing this result.
A beneficial effect of cyclosporin on allograft vasculopathy is also apparent in clinical heart transplantation. Two recent ICUS studies have suggested that high cyclosporin dose may have a beneficial effect on the development of CAD. In one cross sectional study a positive correlation was found between a coronary intimal thickness greater than 3mm and a lower daily cyclosporin dose (Rickenbacker et al 1996). In another multi-centre study patients receiving more than 5mg/kg/day of cyclosporin had significantly less development of CAD than patients receiving less than 3mg/kg/day at one year (Mehra et al 1997). Both clinical and experimental evidence therefore suggests that the success of cyclosporin in heart transplantation is limited by unacceptable side effects at larger doses and that cyclosporin in a proper dose may attenuate CAD.

Allograft medial fibrosis was reduced by the administration of cyclosporin, in association with reductions in graft inflammation. From the available data it would appear that medial ECM accumulation and inflammation are related processes. In apparent contradiction there was no increase in pro-fibrotic genes in allografts relative to isografts at any time point. This can be explained by several factors. The observation that TIMP 1 and Collagen 3 do not appear to be elevated during ECM accumulation and intimal VSMC proliferation in rat allografts may be the failing of the study to examine the distribution of mRNA or protein throughout the vessel wall. It is possible that a large reduction in TIMP or Collagen mRNA levels in for example the adventitia may mask different and more subtle changes in other parts of the vessel wall. Furthermore although TIMP 1 is constitutively expressed in uninjured rat carotids (Webb et al 1997), and levels were detected in isografts in this model, TIMP 1 expression is not increased during periods of ECM accumulation following balloon injury in the rat carotid (Webb et al 1997). TIMP 1, therefore, may not be as important in determining the rate of collagen accumulation in the rat as seen in other
species (Southgate et al 1999, Kranzhofer et al 1999, Kenagy et al 1994). In support of this Adenovirus mediated TIMP 1 over expression in injured rat arteries was associated with increases in elastin but not collagen accumulation (Dollery et al 1999a, Forough et al 1998). Sirius red stains not only collagen but also multiple other ECM components (Grimm et al 1999) and it is possible that the observed medial fibrosis involves the accumulation of predominantly non-collagen molecules.
Chapter 4. The effect of rapamycin on allograft vasculopathy and the expression of genes responsible for extracellular matrix turnover
Abstract

Background Chronic allograft dysfunction, the leading cause of solid organ transplant failure is characterised by histological evidence of Extracellular Matrix (ECM) accumulation (fibrosis). The aim of this study was to assess the effect of rapamycin on the expression of fibrosis associated genes in the Lewis to F344 rat aortic allograft model.

Methods Vascular remodelling and ECM accumulation (sirius red) were measured by computerised histomorphometry of aortic sections harvested at serial time points. Expression of fibrosis associated genes was studied by means of semi-quantitative RT-PCR.

Results Rapamycin (0.5mg/kg/day) inhibited intimal hyperplasia, ECM accumulation and vascular remodelling (increasing vessel circumference). This was associated with attenuation of the inflammatory infiltrate and a reduction in intragraft gelatinase, Collagen 3 and TIMP 1 mRNA levels. Lower dose (0.25mg/kg/day) rapamycin inhibited intimal hyperplasia and ECM accumulation however there was a lesser effect on vascular remodelling. Lower dose allografts also had a more severe inflammatory infiltrate and larger amounts of intragraft MMP 9 mRNA than those treated with the higher dose.

Conclusions As well as the tissue response to injury, the alloimmune injury itself may directly contribute to the vascular remodelling that occurs in allograft vasculopathy. Rapamycin at higher but not at lower doses inhibited both of these pathological processes.
Introduction

Chronic Allograft Dysfunction, the leading cause of late allograft loss in solid organ transplantation, is characterized histologically by a chronic fibroproliferative tissue response whereby progressive allograft fibrosis and allograft vasculopathy results clinically in organ dysfunction and failure (Kouwenhaven et al 1999). Rat aortic allografts develop a fibroproliferative vasculopathy that is characterized by vascular remodelling, intimal hyperplasia and medial extracellular matrix accumulation with many similarities to that occurring in human solid organ transplants (Mennander et al 1991 and Chapter 3). The changes in cell behaviour and extracellular matrix turnover that occur during the progression of allograft vasculopathy are associated with specific changes in the expression of genes that regulate the synthesis and degradation of the extracellular matrix (Chapter 3). In particular the matrix metalloproteinases, a family of zinc dependant proteases that can collectively breakdown all the constituents of the ECM and their inhibitors the Tissue Inhibitors of Metalloproteinases (TIMPs) (Nelson 2000) are central to this process.

In clinical practice calcineurin inhibitor based immunosuppression has no effect on the progress of this condition (Cecka et al 2000, Hosenpud et al 2000). More recently however the macrolide antibiotic rapamycin has entered clinical practice following the observation that in animal models at least it attenuated the tissue response to injury as well as being an effective immunosuppressant (Morris et al 1995, Stepkowski et al 1991). In rats rapamycin inhibits intimal hyperplasia following balloon angioplasty as well as renal fibrosis following ischaemic reperfusion (Morris et al 1995, Jain et al 2001) and this is associated with attenuation of changes in MMP and TIMP mRNA expression (Jain et al 2001, Waller JR, Nicholson et al, unpublished observations). The aim of this study was to measure the
effect of rapamycin at different doses on the changes in expression of genes important in regulating ECM turnover in an animal model of CAD.
Methods

Experimental model

The thoracic aorta to abdominal aorta allograft model as described by Mennander (1991) was used. A segment of the descending thoracic aorta approximately 3 cm in length was excised, thoroughly perfused with phosphate buffered saline (PBS) and used as a transplant. F344 rats acted as donors and recipients and Lewis rats as recipients. Ischaemic injury to the graft was minimised by immersion in an ice bath at 4°C between procedures. Through a midline laparotomy incision the segment of thoracic aorta was anastomosed end to end to the recipient abdominal aorta using 9/0 prolene suture. The graft was transplanted into a heterotopic position below renal arteries and above the bifurcation forming a loop in the recipient abdomen.

Animals were kept in a controlled environment with unlimited access to feed and water. Graft harvest was performed in heavily anaesthetised rats following which the animal was allowed to die by exsanguination.

Drugs

Rapamycin (Sirolimus; Wyeth-Ayerst, Princeton, NJ) was prepared twice weekly as a working solution of 5 mg/ml in distilled water. Solutions were stored at 4°C and protected from UV light exposure. Drugs were administered orally by gavage from the day of transplant until sacrifice.

Experimental groups

Group 1. F344 to F344 isografts. (Isograft)

Group 2. F344 to Lewis allografts receiving Neoral 5 mg/kg/day for 14 days and then no immunosuppression until the day of sacrifice. (Allograft)
Group 3. F344 to Lewis allografts receiving rapamycin 0.25mg/kg/day orally by gavage from the day of transplant until sacrifice. (Low Rapa)

Group 3. F344 to Lewis allografts receiving rapamycin 0.5mg/kg/day orally by gavage from the day of transplant until sacrifice. (High Rapa)

In this experiment six rats in each group is sacrificed at each of weeks 4,8,12 and 16.

**Allograft gene expression using RT-PCR**

Total mRNA was extracted from aortic tissue and complementary DNA molecules were synthesised by reverse transcription (RT). These cDNA species were amplified by polymerase chain reaction (PCR) and quantified in an ELISA system. Relative quantitation was performed by comparison of the signal intensity to that of the housekeeping gene β-Actin.

**Histological analysis**

Segments of the aortic graft were embedded in paraffin wax and multiple sections were stained with haematoxylin and eosin. The level of inflammatory infiltrate was assessed semi-quantitatively and was categorised as follows: None- no inflammatory cells visualised, Mild- endothelialitis, inflammatory cell localised to the endothelium, Moderate- dense inflammatory cell infiltrate in the innermost layer of the vessel wall, Severe- dense Inflammatory cell infiltrate throughout all layers of the vessel wall.

The vessel circumference and area fraction of the intima and media were quantified using a computer image analysis system. The intima was defined as the area between the endothelium and the internal elastic lamina and the media was defined as the area between the internal and external elastic laminae. Paraffin embedded sections were also stained with sirius red stain and the level of ECM staining quantified using computerised histomorphometry.
**Substances to be assessed**

In order to take into account all the factors which influence smooth muscle cell proliferation, migration and deposition of ECM during neotima formation, the following species were studied with RT-PCR: MMP2, MMP9, TIMP1 and collagen III.

**Analysis of results**

The levels of gene expression were compared in the different study groups using non-parametric statistical analysis. The Mann Whitney test was used to compare any two groups where Kruskall Wallis testing had indicated significant differences between all the means considered (P<0.05). Comparisons of the level of inflammatory infiltrate were performed using Pearson's Chi square where more than two groups were compared and Fisher's Exact test where only two groups were compared. Statistical analysis was performed using the Statistical Package for the Social Sciences Version 8.0 (Chertsey, UK.)
Results

Results of histological analysis

Untreated allografts underwent a progressive expansive remodelling process between 0 and 16 weeks whilst the neointima formation occurred between 12 and 16 weeks (Figure 1 and Figure 2A,B). Low dose rapamycin significantly inhibited neointimal hyperplasia at 16 weeks (intima media ratio 0.02±0.02 versus 0.2±0.05, P=0.004 Mann Whitney, Figure 2A) but not expansive remodelling (Internal Elastic Lamina Circumference 4.1 ±0.14 versus 4.2 ±0.14mm, P=0.4, Figure 2B) compared to untreated allografts. High dose rapamycin inhibits both intimal hyperplasia (Intima Media ratio 0.01±0.1, P=0.008 Mann Whitney) and expansive remodelling (Internal Elastic Lamina Circumference 3.9 ±0.15mm, P=0.016 Mann Whitney) compared to untreated allografts. At all time points following transplantation there was a dense inflammatory infiltrate in untreated allografts, in most cases transmural, that was not seen in isografts (Figure 1 A-D). There was a reduction in the severity of inflammatory infiltrate within the high dose rapamycin group, significantly more so than that seen in the low dose rapamycin group at all time points (for example at 4 weeks Fisher's Exact test P=0.04, Figure 2C).
Results of picrosirius red histomorphometry

There was a significant increase in extracellular matrix accumulation in the media of untreated allografts relative to that of isografts at 16 weeks (area fraction staining 0.45±0.01 versus 0.35±0.03 mm², P=0.03 Mann Whitney, Figure 3A). This was characterised by an increase in media volume rather than collagen density (data not shown). Total ECM accumulation was reduced by both low (0.41±0.04 mm², P=0.05, Mann Whitney) and high dose rapamycin (0.4±0.01 mm², P=0.03, Mann Whitney) although ECM density was not changed.

There was a reduction in adventitial ECM density in untreated allografts relative to isograft controls (0.71±0.07 versus 0.85±0.08 optical density ratio, P=0.01 Mann Whitney, Figure 3B). This reduction in ECM density was significantly attenuated by both low (0.84±0.04, P=0.01, Mann Whitney) and high dose rapamycin (0.86±0.04, P=0.02, Mann Whitney). It was impossible to accurately distinguish true allografts adventitia from peritoneal adhesions and therefore relative adventitial ECM content could not be assessed.
Figure 1. Representative photomicrographs (×100 magnification) of haematoxylin and eosin stained section of rat aorta from A. Isografts, B Untreated allografts C. Rapamycin 0.25mg/kg/day treated allografts and D. Rapamycin 0.5mg/kg/day treated allografts

Untreated allografts had a dense transmural inflammatory infiltrate associated with intimal and medial thickening. These changes were attenuated by high dose rapamycin. Lower dose rapamycin inhibited neointimal hyperplasia but not the inflammatory infiltrate or medial thickening.

Figure 1A.
Figure 1C.
Figure 1D.
Figure 2. Results of histological analysis.

2A. Changes in Intimal Medial ratio with time. Both high and low dose rapamycin inhibit intimal thickening that occurs between 12 and 16 weeks. Values represent mean ± S.D.
Figure 2B. Changes in vessel circumference with time
Values represent mean plus SD.
Higher dose rapamycin but not lower dose rapamycin significantly inhibited the expansive remodeling that occurred in allografts relative to isografts.
Figure 2C.
There was a significant difference between the severity of the inflammatory infiltrate between isografts, untreated allografts and rapamycin treated allografts at 4 weeks (Pearson’s Chi Square =26.6, P=0.002). When compared separately high dose rapamycin was shown to significantly reduce the level of inflammatory infiltrate compared to low dose rapamycin (Pearson’s Chi Square =5.8, P=0.02).

Semi-Quantitative assessment of Inflammatory infiltrate

Number of Allografts

Severity of Inflammation
Figure 3. Results of sirius red computerised histomorphometry
There was significantly greater extracellular matrix accumulation in the media of allografts relative to that of isografts. This was characterised by an increase in media volume rather than ECM density (data not shown). Conversely adventitial ECM density in allografts was reduced. Medial ECM accumulation was inhibited by both low dose and high dose rapamycin. Box and whisker plots represent Median, SD, and 95% Confidence intervals

\[3A\]

Relative Medial ECM Content at 16 weeks

\[\frac{\text{area fraction staining (mm}^2\text{)}}{\text{Group}}\]

\[\frac{\text{Isografts Allografts Low Rapa High Rapa}}{\text{P=0.05, } \ddagger, \text{ P=0.03, Mann Whitney}}\]
Adventitial Sirius Red ECM staining density

\[\text{Sirius Red Staining Density}\]

\[\frac{\text{Isografts}}{\text{Allografts}}: \text{Low Rapa}, P=0.01, \text{High Rapa}, P=0.02, \text{Mann Whitney}\]

\[\ddagger, P=0.01, \dagger, P=0.02, \text{Mann Whitney}\]
Results of RT-PCR

At 8 weeks there were significantly greater MMP 9 transcript levels and lower Collagen 3 and TIMP 1 transcript levels in allografts relative to isografts (Figure 4). High dose rapamycin but not low dose significantly attenuated the increase in MMP 9 expression in allografts. There was no discernible effect of rapamycin on pro-fibrotic genes (Collagen 3 and TIMP 1) at this time point. In 12 week aortic transplants there were increased MMP 2, MMP 9 mRNA levels in untreated allografts relative to isograft controls (Figure 5). This corresponded with neointima formation. Rapamycin at low and high doses attenuated the increase in MMP 2. Neither high nor low rapamycin significantly inhibited MMP 9 at this time point although the level of inflammation (and subsequently MMP 9) was lower in untreated allografts at this time point than at other times which could explain this finding (see below). There was no difference in pro-fibrotic gene expression between isografts and untreated allografts at 12 weeks, unlike at other time points, however TIMP 1 and collagen 3 mRNA levels were further reduced by rapamycin at high and low doses (Figure 5). In 16 week aortic transplants there was a significant increase in MMP 9 expression and down regulation of profibrotic genes (TIMP 1, collagen 3) in untreated allografts relative to isograft controls (Figure 6). High dose rapamycin inhibited the observed increase in MMP 9 expression whereas low dose rapamycin did not. Both high and low dose rapamycin further reduced expression of TIMP 1 and Collagen 3 however.
**Figures 4-6.** Graphs demonstrate the influence of rapamycin at high and low doses on the expression of genes important in the regulation of ECM turnover. Gene expression is expressed as the ratio of mRNA transcript levels for the gene considered relative to the mRNA transcript levels of the housekeeping gene β-Actin to control for differences in cellularity between samples. The effect of rapamycin on individual gene expression is described in the text. Box and whisker plots represent median, SD and 95% C.I.

**Figure 4A-D. The effect of rapamycin on fibrosis associated gene expression in aortic transplants at 8 weeks**

4A

![Graph showing MMP 9 mRNA/actin mRNA ratios at 8 weeks](image)

$\ddagger$, $P=0.04$, Mann Whitney
Collagen 3 mRNA/actin mRNA ratios at 8 weeks

Isografts  Allografts  Low Rapa  High Rapa

Collagen 3 mRNA/actin mRNA ratio

Group
TIMP 1 mRNA/actin mRNA ratios at 8 weeks

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<tr>
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Figure 5A-C. The effect of rapamycin on fibrosis associated gene expression in aortic transplants at 12 weeks
See text. Boxes represent median ±S.D. Whiskers represent 95% C.I.

**5A**

MMP 2 mRNA/ actin mRNA ratios at 12 weeks

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‡, P=0.01, †, P=0.05, Mann Whitney
Collagen 3 mRNA/ actin mRNA ratios at 12 weeks

‡, P=0.01, †, P=0.03, Mann Whitney
TIMP 1 mRNA/ actin mRNA ratios at 12 weeks

Group

\[ \frac{\text{TIMP1 mRNA}}{\text{actin mRNA}} \]

\[ \frac{\text{Isografts}}{\text{Alografts}} \frac{\text{Low Rapa}}{\text{High Rapa}} \]

\( \ddagger, P=0.01 \), Mann Whitney
Figure 6A-C. The effect of rapamycin on fibrosis associated gene expression in aortic transplants at 16 weeks

See text. Boxes represent median ± S.D. Whiskers represent 95% C.I.

6A

MMP 9 mRNA/actin mRNA ratios at 16 weeks

\[ \frac{\text{MMP 9 mRNA}}{\text{actin mRNA}} \]

\[ \text{Group} \]

\( \ddagger, P=0.01, \text{Mann Whitney} \)
Collagen 3 mRNA/actin mRNA ratios at 16 weeks

Collagen 3 mRNA/actin mRNA ratio

N = 4 5 5

Isograft Allograft Low Rapa High Rapa

P = 0.03, Mann Whitney

\( \ddagger, P=0.03, \) Mann Whitney
TIMP 1 mRNA/actin mRNA ratios at 16 weeks

‡, P=0.05, †, P=0.03, Mann Whitney
Discussion

This study demonstrated that rapamycin, administered orally at 0.5mg/kg/day inhibited intimal hyperplasia, medial ECM accumulation and expansive vascular remodelling (increasing vessel circumference) in rat aortic allografts. This was associated with attenuation of the graft inflammatory infiltrate and a reduction in intragraft gelatinase (MMP 2 and MMP 9), Collagen 3 and TIMP 1 mRNA levels. At a lower dosage (0.25mg/kg/day) rapamycin inhibited intimal hyperplasia and medial collagen accumulation however there was a lesser effect on vascular remodelling. Aortic allografts treated at the lower dose were also seen to have a more severe inflammatory infiltrate and larger amounts of intragraft MMP 9 mRNA than those treated with the higher dose.

The study was limited in that allograft vasculopathy in rat aortas has no clear correlation with allograft dysfunction. It is also important to avoid direct comparisons between the effect of rapamycin on allograft vasculopathy in rats and its role in human organ transplantation. The drug dosages used here bear little relation to those tolerated in humans (0.05-0.1mg/kg/day) due in part to the poor oral bioavailability of rapamycin in rats (15%). Serum drug concentrations of rapamycin following oral dosing (0.25mg/kg/day) in Lewis rats are approximately 0.4ng/ml (DiJoseph et al 1996). In humans 5-15ng/ml has been suggested as an appropriate therapeutic window for effective immunosuppression (Saunders et al 2001) and the serum levels in the current study would therefore be sub-therapeutic in humans. Higher doses of oral rapamycin are associated with unacceptable gastrointestinal side effects and weight loss in rats however (DiJoseph et al 1996). Nonetheless the doses of rapamycin in the current study were significantly less than those used in initial experiments with rapamycin in rodents (Stepkowski et al 1991, Gregory et al 1995). These results therefore also highlight the efficacy of rapamycin in the prevention of allograft vasculopathy in rats at what are relatively small doses.
The use of RT-PCR as a quantitative tool is also open to criticism. The ability of RT-PCR to target gene sequences, thereby permitting detection of extremely low copy number mRNA, makes it applicable to the investigation of mRNA in small volumes of tissue. The variability in the efficiency of the PCR step in these experiments was controlled for by performing the PCR step for each individual gene in all specimens simultaneously for any given time point i.e. 4, 8, 12 or 16 weeks. Similarly PCR products for a particular gene in all specimens for each individual time point were quantified in the same ELISA to prevent variability in the efficiency of the ELISA reactions affecting the results. In our laboratory this method is reproducible to within 5% on repetition for any given sample (Bicknell et al 1996) and therefore we consider these results to accurately represent relative gene expression in each group at the time specified. More information can be derived from other forms of analysis such as in situ zymography or hybridisation however the results presented here clearly demonstrate an effect of rapamycin on some of the genes responsible for fibroproliferative remodelling.

This is the first study to show an effect of rapamycin on vascular remodelling in rat allografts. Previous studies in the F344 to Lew aortic allograft model have demonstrated that the degree of expansive vascular remodelling correlates with intragraft MMP 9 transcript levels which in turn are associated with severity of the inflammatory infiltrate in the grafts and correlate inversely with adventitial collagen density (Chapter 3). In these experiments suppression of the inflammatory infiltrate was associated with attenuation of MMP 9 expression at almost all time points studied and it is our belief that the observed increases in gelatinase expression are derived from these cells. This is supported by observations that MMP 9 is expressed by infiltrating T cells and activated macrophages in vivo and in vitro (Goetzl et al 1996) and also by studies in mice aortic allografts where MMP 9 is localised to inflammatory cells throughout the allograft but particularly the
adventitia (Lijnen et al 1998). Macrophages constitutively express MMP 9 (Galis et al 1995) and can also secrete MMPs 1, 2, 3, 7, 12 and 14 (MT1-MMP) (Goetzl et al 1996). Macrophages therefore can themselves contribute substantially to overall ECM turnover and connective tissue remodelling as well generating the fibroproliferative response of other vascular wall cells by growth factor production (Ross 1999). Increased MMP 9 activity is also associated with expansive remodelling in response to high flow conditions in rats as well as following balloon injury in pigs (Bassiouny et al 1998, Wentzl et al 1998). In addition, over expression of MMP 9 following adenoviral mediated transfection balloon injury in the rat carotid was associated with expansive remodelling (Mason et al 1999). Remodelling in pig and rabbit balloon injury models is related to neoadventitial thickening (Lafont et al 1995, Shi et al 1996) and in rat allografts MMP 9, released by inflammatory cells, may actively degrade adventitial collagen. This is supported by the inverse correlation between intragraft MMP 9 mRNA levels and adventitial collagen density in this model (Chapter 3). Alternatively the inflammatory infiltrate may alter adventitial fibroblast activity (a critical determinant of remodelling in pigs (Shi et al 1998)) as evidenced by the reduction in collagen 3 and TIMP 1 levels in expansively remodelling grafts.

Rapamycin at 0.5mg/kg/day inhibited the level of inflammatory infiltrate more so than low dose rapamycin. This was reflected by a similar reduction in intragraft MMP 9 expression and attenuation in vessel expansion. This suggests that the vascular remodelling seen in rat allografts occurs not solely as a result of the response to injury by vessel wall cells but also as a direct result of the alloimmune injury itself. Inhibition of both of these pathological processes may therefore be important in the prevention of allograft vasculopathy in organ transplant patients.
Intimal hyperplasia in untreated allografts occurred predominantly between 12 and 16 weeks and was associated with increases in the expression of MMP 2 and MMP 9 in untreated 12 week allografts compared to isografts without the observed decrease in TIMP and collagen 3 expression seen at other times. This presumably reflects the extracellular matrix turnover associated with smooth muscle cell proliferation and migration during neointima formation and similar changes in MMP and Collagen gene expression have been reported during neointima formation in pigs, rats and rabbits following arterial injury in vivo (Southgate et al 1999, Galis et al 1994, Webb et al 1997, Coats et al 1997) as well as in tissue culture models of intimal hyperplasia in vitro (Southgate et al 1999). In the current study both high and low dose rapamycin significantly inhibited neointimal hyperplasia. This occurred apparently independently of the effect on the inflammatory infiltrate, a finding also reported by Schmid et al (1997) in F344 to Lew cardiac allografts where rapamycin (0.5mg/kg/day i.p.) had no effect on the level of perivascular infiltrate but completely abolished neointimal thickening. These findings therefore suggest that rapamycin inhibits the fibroproliferative response to injury by a direct effect on the expression of genes that regulate ECM turnover in vessel wall cells as distinct from its immunosuppressant action.

Rapamycin at high doses was also associated with a reduction in total medial ECM content. Inhibition of ECM accumulation (fibrosis) by rapamycin has also been reported in other rat models. For example in a rat model of obliterative bronchiolitis rapamycin (6mg/kg/day) (Morris et al 1995) prevented the development of lumenal fibrosis whilst an oral dose of 0.5mg/kg/day rapamycin has also been shown to inhibit the progressive decline in renal function and proteinuria that accompanies progressive fibrosis following ischaemia reperfusion injury (Jain et al 2001). In this latter study the action of rapamycin was associated with an inhibition of TIMP 1 expression (Jain et al 2001), and studies in
clinical renal transplantation have demonstrated a correlation between renal biopsy TIMP
1mRNA levels, allograft fibrosis and the rate of decline of graft function (ML Nicholson et
al unpublished observations). In the present study rapamycin profoundly suppressed TIMP
1 and collagen 3 expression relative to untreated allografts at 16 weeks however the
significance in relation to medial ECM accumulation is unclear. TIMP 1 expression is not
increased during ECM accumulation following balloon injury in the rat, nor indeed in rat
allografts compared to isografts as demonstrated here. Furthermore TIMP 1 over
expression following adenoviral transfer in balloon injured rat arteries was associated with
increased elastin but not collagen accumulation (Dollery et al 1999).

In conclusion this data suggests that rapamycin may have a significant inhibitory effect on
the vessel wall response to injury that is distinct from its immunosuppressant action.
Inhibition of all aspects of vessel remodelling and ECM turnover required effective
suppression of the inflammatory response however and this was only achieved at higher
rapamycin doses. Suppression of the vessel wall response to injury may not therefore be
sufficient to completely inhibit Chronic Allograft Dysfunction as inflammatory cells
themselves may directly remodel tissues by the direct production of metalloproteinases and
ECM constituents.
Chapter 5. The effect of combined rapamycin/Neoral cyclosporin on the changes in pro-fibrotic gene expression that occur during the development of allograft vasculopathy in rats compared to Neoral or rapamycin in isolation
Abstract

Background Chronic allograft dysfunction, the leading cause of solid organ transplant failure is characterised by histological evidence of Extracellular Matrix (ECM) accumulation (fibrosis). The aim of this study was to compare the effect of combined rapamycin and cyclosporin therapy on fibrosis associated gene expression and ECM turnover during the development of allograft vasculopathy, compared to either agent alone.

Methods Lewis recipients of F344 rat thoracic to abdominal aorta transplants were administered rapamycin, cyclosporin, combined rapamycin and cyclosporin or no treatment. F344 to F344 isografts served as controls. Six grafts in each group were harvested at 16 weeks. Vascular remodelling and collagen accumulation (sirius red) were measured by computerised histomorphometry of aortic sections. mRNA was extracted from frozen tissue and expression of fibrosis associated genes was studied by means of semi-quantitative RT-PCR.

Results Rapamycin (0.5mg/kg/day) and cyclosporin (5mg/kg/day) inhibited intimal hyperplasia, medial ECM accumulation and expansive vascular remodelling (increasing vessel circumference) in rat aortic allografts. This was associated with attenuation of the graft inflammatory infiltrate and a reduction in intra-graft gelatinase, Collagen 3 and TIMP 1 mRNA levels. Combined rapamycin and cyclosporin inhibited intimal hyperplasia however there was a lesser effect on vascular remodelling and medial extracellular matrix accumulation. Combined treatment aortic allografts were also seen to have a more severe inflammatory infiltrate and larger amounts of intra-graft MMP 9, TGF Beta and TIMP 1 mRNA than those treated with monotherapy.

Conclusion Rapamycin and cyclosporin act synergistically to inhibit intimal hyperplasia but not the inflammatory infiltrate, allograft fibrosis or vessel remodelling. In the high responder F344 to Lew model effective immunosuppression is required to reduce graft fibrosis.
Introduction

Despite the improvements in short-term survival of renal allografts since the introduction of cyclosporin in the 1980's several renal transplant registries (Cecka et al 2000, Opelz et al 1994) demonstrate no reduction in the rate of late renal allograft loss. This is largely due to its failure to prevent Chronic Allograft Nephropathy (CAN), a fibroproliferative response to injury characterised by allograft vasculopathy, glomerulosclerosis and interstitial fibrosis that results ultimately in transplant dysfunction and failure (Paul 1999).

Cyclosporin itself promotes CAN by direct and indirect (hypertension, hyperlipidaemia and hyperglycaemia) nephrotoxicity and at high doses can produce intimal hyperplasia and graft fibrosis in non-transplanted kidneys virtually identical to clinical CAN (Mihatsch et al 1998). Attempts to enhance the therapeutic window of cyclosporin by dose reduction and the addition of additional immunosuppressants have to date failed to improve long-term survival in clinical trials however (Paul 1999). Rapamycin, a macrolide antibiotic with immunosuppressant properties that has been shown to inhibit the fibroproliferative tissue response in rodents (Gregory et al 1993) has recently entered clinical practice. It has been shown to prevent allograft dysfunction in a wide range of animal models (Saunders et al 2000) and in rats in vivo cyclosporin and rapamycin act in synergy to attenuate cell mediated rejection in high responder combinations (Stepkowski et al 1997). In clinical trials low dose cyclosporin in combination with rapamycin has been shown to result in equivalent rejection rates but superior early allograft function compared to high dose cyclosporin in de novo renal transplants (Kahan et al 1999). The effect of these two agents on organ fibrosis and late allograft function remains to be determined however. The aim of this study was to compare the effect of combined rapamycin and cyclosporin therapy on the expression of genes central to the extracellular matrix accumulation that underlies the fibroproliferative response in rat allografts compared to either agent in isolation.
Experimental design and methodology

Animal model

The thoracic aorta to abdominal aorta allograft model as described by Mennander et al (1991) was used. A segment of the descending thoracic aorta approximately 3cm in length was excised, thoroughly perfused with phosphate buffered saline (PBS) and used as a transplant. F344 rats acted as donors and recipients and Lewis rats as recipients. Ischaemic injury to the graft was minimised by immersion in an ice bath at 4°C between procedures. Through a midline laparotomy incision the segment of thoracic aorta was anastomosed end to end to the recipient abdominal aorta using 9/0 prolene suture. The graft was transplanted into heterotopic position below renal arteries and above the bifurcation forming a loop in the recipient abdomen.

Animals were kept in a controlled environment with unlimited access to feed and water. Graft harvest was performed in heavily anaesthetised rats following which the animal was allowed to die by exsanguination. Experiments were performed under UK Home Office licence PPL 80/1434, The Animals (Scientific Procedures) Act 1986.

Drugs

Rapamycin (Sirolimus; Wyeth-Ayerst, Princeton, NJ) was prepared twice weekly as two solutions of 5mg/ml and 0.5mg/ml in distilled water for experimental groups 2 and 5 respectively. Solutions were stored at 4°C and protected from UV light exposure. Neoral cyclosporin (Novartis UK) was prepared as two solutions of 5mg/ml and 1.5mg/ml in olive oil and stored at 21°C. Drugs were administered orally by gavage from the day of transplant until sacrifice. These drug doses were chosen because of the demonstrated synergy between cyclosporin and rapamycin in prolonging graft survival in rats. Using median effect analysis Stepkowski et al (1997) demonstrated that equivalence in reducing
cell mediated rejection is achieved by a times 3 reduction in the cyclosporin dose and a
times 10 reduction in the rapamycin dose compared to the drugs in isolation.

**Experimental groups**

Group 1. F344 to F344 isografts. *(Isografts)*

Group 2. F344 to Lewis allografts receiving cyclosporin 5mg/kg/day orally by gavage for
14 days only then no immunosuppression until sacrifice. *(untreated Allografts)*

Group 3. F344 to Lewis allografts receiving cyclosporin 5mg/kg/day *(Cyclosporin)*

Group 4. F344 to Lewis allografts receiving rapamycin 0.5mg/kg/day *(Rapamycin)*

Group 5. F344 to Lewis allografts receiving combined cyclosporin 1.5mg/kg/day and
rapamycin 0.05mg/kg/day *(Rapa+Cyclosporin)*

Six rats in each group were sacrificed at 16 weeks.

**Allograft gene expression using RT-PCR**

Total mRNA was extracted from aortic tissue and complementary DNA molecules were
synthesised by reverse transcription (RT). These cDNA species were amplified by
polymerase chain reaction (PCR) and quantified in an ELISA system. Relative quantitation
was performed by comparison of the signal intensity to that of the housekeeping gene β
Actin. These techniques have been described in more detail elsewhere (Bicknell et al
1996). Probe and Primer sequences used in these experiments have also been published
previously (Jain et al 2000).

**Histological analysis**

Segments of the aortic graft were embedded in paraffin wax and multiple sections were
stained with haematoxylin and eosin. The level of inflammatory infiltrate was assessed
semi-quantitatively and was categorised as follows: **None**- no inflammatory cells
visualised, Mild- endothelialitis, inflammatory cell localised to the endothelium,
Moderate- dense inflammatory cell infiltrate in the innermost layer of the vessel wall,
Severe- dense inflammatory cell infiltrate throughout all layers of the vessel wall.

The vessel circumference and area fraction of the intima and media were quantified using a computer image analysis system. The intima was defined as the area between the endothelium and the internal elastic lamina and the media was defined as the area between the internal and external elastic laminae. Paraffin embedded sections were also stained with sirius red stain and the level of ECM staining quantified using computerised histomorphometry as described previously (Moreso et al 1995).

Substances to be assessed

In order to take into account all the factors which influence smooth muscle cell proliferation, migration and deposition of ECM during neotima formation, the following species were studied with RT-PCR: MMP9, TIMP1, 2 and 3, TGF beta and collagen III.

Analysis of results

The levels of gene expression were compared in the different study groups using non-parametric statistical analysis. Mann Whitney comparisons were made between individual groups only where Kruskall Wallis analysis suggested significance. Categorical values were compared with Pearson's Chi-Square. Statistical analysis was performed using the Statistical Package for the Social Sciences Version 8.0 (Chertsey, UK.)
Results

Results of histology

Untreated allografts developed significant neointimal thickening at 16 weeks compared to isografts and also undergo progressive expansive remodelling (Figure 1 and 2). Cyclosporin significantly inhibited both intimal hyperplasia (intima media ratio 0.08±0.09 versus 0.2±0.05, P=0.01 Mann Whitney, Figure 2A) and expansive vascular remodelling (Internal Elastic Lamina Circumference 3.8 ±0.2 versus 4.2 ±0.2mm, P=0.02, Figure 2B) compared to untreated allografts as well as significantly attenuating the severity of the inflammatory infiltrate ((Pearson's Chi Square P<0.001, Yates Correction, Figure 2D). Rapamycin also inhibited both intimal hyperplasia (Intima Media ratio 0.01±0.1, P=0.01 Mann Whitney), expansive remodelling (Internal Elastic Lamina Circumference 3.9±0.2mm, P=0.016 Mann Whitney) and inflammation compared to untreated allografts. Combination therapy inhibited intimal hyperplasia (Intima Media ratio 0.01±0.1, P=0.08 Mann Whitney) but failed to inhibit expansive remodelling (Internal Elastic lamina Circumference 4.0±0.2mm, P=0.016 Mann Whitney) or the allograft inflammatory infiltrate (Figure 2D).

Results of picrosirius red histomorphometry

There was a significant increase in extracellular matrix accumulation in the media of untreated allografts relative to that of isografts at 16 weeks (area fraction staining 0.45±0.01 versus 0.35±0.03 mm², P=0.03 Mann Whitney, Figure 2D). This was characterised by an increase in media volume rather than collagen density (data not shown). Total ECM accumulation was reduced by both cyclosporin (0.38±0.04 mm², P=0.01, Mann Whitney) and rapamycin (0.4±0.01 mm², P=0.03, Mann Whitney) although ECM was not changed. Combination therapy failed to significantly attenuate medial fibrosis (0.42±0.19 mm², P=0.08, Mann Whitney).
1. Figure 1. Representative photomicrographs (×100 magnification) of haematoxylin and Eosin stained section of rat aorta from A. cyclosporin, B. rapamycin 0.5mg/kg/day and C. Combined rapamycin 0.05mg/kg/day and cyclosporin 1.5mg/kg/day treated allografts and D. untreated allografts

Untreated allografts have a dense transmural inflammatory infiltrate associated with intimal and medial thickening. These changes are attenuated by rapamycin and cyclosporin in isolation however combined rapamycin cyclosporin fails to inhibit the inflammatory infiltrate in allografts.

Figure 1A.
Figure 1B.
Figure 1D.
Figure 2. Results of histological analysis and sirius red histomorphometry
Boxes represent median ±S.D. Whiskers represent 95% C.I.

Figure 2A. Effect of rapamycin, cyclosporin and the two in combination on neointima formation in rat transplants.
All three treatment groups inhibited intimal hyperplasia

Intimal Medial Ratios at 16 weeks

\[
\begin{align*}
\text{Intimal/Media Ratio} \\
\text{Isograft} & \quad \text{Allograft} & \quad \text{Cyclosporin} & \quad \text{High Rapa} & \quad \text{Rapa+CyA} \\
\end{align*}
\]

\*\*, \(P=0.0001\), \(\dagger\), \(P=0.01\) Mann Whitney
Figure 2B. The effect of rapamycin, cyclosporin and the two in combination on vascular remodelling in rat aortic transplants.
Expansive remodelling in allografts was inhibited by rapamycin and cyclosporin alone but not by the two in combination.

\[
\begin{array}{cccc}
\text{Vessel Circumference at 16 weeks} \\
\hline
\text{Vessel Circumference (mm)} & \text{Isograft} & \text{Allograft} & \text{Cyclosporin} & \text{Rapamycin} & \text{Rapa+CyA} \\
\hline
3.4 & \text{\#} & \text{\#} & \text{\#} & \text{\#} \\
3.6 & \text{} & \text{} & \text{} & \text{} \\
3.8 & \text{} & \text{} & \text{} & \text{} \\
4.0 & \text{} & \text{} & \text{} & \text{} \\
4.2 & \text{} & \text{} & \text{} & \text{} \\
4.4 & \text{} & \text{} & \text{} & \text{} \\
4.6 & \text{} & \text{} & \text{} & \text{} \\
\hline
\end{array}
\]

\[\ddagger, \ P=0.001, \text{ Mann Whitney}\]
Figure 2C. Inflammatory infiltrate in rat aortic transplants
There was a significant difference between the groups in the level of inflammatory infiltrate (Pearson's Chi square =18.6, P<0.005) with a more severe inflammatory infiltrate in the combined group.

Relative Severity of Inflammatory Infiltrate

Number of Allografts

Severity of inflammation

None  Mild  Moderate  Severe

Group
- Isograft
- Allograft
- Cyclosporin
- Rapamycin
- Rapa+CyA
Figure 2D. The effect of rapamycin, cyclosporin and the two in combination on medial fibrosis in rat aortic transplants.
There was significantly greater extracellular matrix accumulation in the media of allografts relative to that of isografts. Rapamycin and cyclosporin as single agents significantly inhibited fibrosis but not in combination at reduced doses.

Medial ECM Content at 16 weeks

Area Fraction Staining (mm$^2$)

<table>
<thead>
<tr>
<th>Group</th>
<th>Isograft</th>
<th>Allograft</th>
<th>Cyclosporin</th>
<th>Rapamycin</th>
<th>Rapa+CyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining</td>
<td></td>
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<tr>
<td>Isograft</td>
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<td>Allograft</td>
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<tr>
<td>Cyclosporin</td>
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<tr>
<td>Rapamycin</td>
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<tr>
<td>Rapa+CyA</td>
<td></td>
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</tbody>
</table>

‡, $P=0.001$, †, $P=0.08$ Mann Whitney
Results of RT-PCR
There were significant reductions in the expression of TIMP 1 and Collagen 3 as well as significant increases in MMP 9 and TGF Beta in untreated allografts relative to isografts (Figure 3). MMP 9 and TGF Beta transcript level were inhibited by both rapamycin and cyclosporin in isolation however this effect was not apparent in the combined treatment group. The observed changes in allograft Collagen 3 transcript levels were not significantly attenuated by immunosuppression (Figure 3C). TIMP 1 mRNA levels were significantly attenuated by cyclosporin and rapamycin monotherapy but not by combined treatment. There were no differences between any of the groups for TIMP 2 and TIMP 3, although TIMP 3 expression negatively correlated with TIMP 1 (r= -0.6, P<0.0001) and Collagen 3 expression (r= -0.8, P<0.0001, Spearmans rank, data not shown) implying that this may not be a pro-fibrotic gene.
Figure 3A-D The effect of rapamycin, cyclosporin and the two drugs in combination on fibrosis associated gene expression in aortic transplants relative to negative (isografts) and positive (allografts) at 16 weeks

Graphs demonstrate the influence of rapamycin/cyclosporin on the expression of genes important in the regulation of ECM turnover. Gene expression is expressed as the ratio of mRNA transcript levels for the gene considered relative to the mRNA transcript levels of the housekeeping gene β Actin to control for differences in cellularity between samples. The effect of rapamycin on individual gene expression is described in the text. Boxes represent median ±S.D. Whiskers represent 95% C.I.

3A

MMP 9 mRNA/ actin mRNA ratios at 16 weeks

‡, †, P=0.01, ‡, P=0.04 Mann Whitney
TGFβ mRNA/actin mRNA ratios at 16 weeks

\[ \begin{align*}
&\text{TGFβ mRNA/actin mRNA ratio} \\
&\begin{array}{c}
\text{Isograft} \\
\text{Allograft} \\
\text{Cyclosporin} \\
\text{Rapamycin} \\
\text{Rapa+CyA}
\end{array}
\end{align*} \]

\[ \begin{align*}
\text{Group} \\
\frac{\hat{\sigma}, \hat{\gamma}}{\hat{\sigma}, \hat{\gamma}, \hat{\gamma}} \quad \hat{\gamma}, \hat{\gamma} \\
\hat{\gamma}, \hat{\gamma} \\
\hat{\gamma}, \hat{\gamma}
\end{align*} \]

\[ \frac{\hat{\gamma}, P=0.01, \hat{\gamma}, P=0.02 \text{ Mann Whitney}}{\hat{\sigma}} \]
Collagen 3 mRNA/actin mRNA ratios at 16 weeks

Collagen 3 mRNA/actin mRNA ratio

Group

Isograft  Allograft  Cyclosporin  Rapamycin  Rapa+CyA
Discussion

The expression of TIMP-1 mRNA increased with cyclosporin but not with rapamycin. The effect of cyclosporin was more pronounced in isografts compared to allografts, suggesting that the expression of TIMP-1 mRNA may be influenced by the source of the graft. In contrast, the effect of rapamycin was similar in both isografts and allografts.

The data further support the hypothesis that the expression of TIMP-1 mRNA is regulated by the immune response and the allograft rejection process. The increased expression of TIMP-1 mRNA in isografts may be due to the absence of an immune response, while the similar expression in allografts may indicate a common regulatory mechanism.

Overall, the results suggest that the modulation of TIMP-1 expression by immunosuppressive therapy could be a useful tool for monitoring graft function and identifying potential complications.
Discussion

This data demonstrates that rapamycin and cyclosporin in combination at doses 10 fold and three fold lower than either rapamycin or cyclosporin respectively is as effective at preventing the neointimal thickening that characterises vasculopathy in rat aortic allografts compared to either agent alone. This effect was not solely dependent on the inhibition of alloimmune injury. Medial fibrosis, expansive remodelling, vessel inflammation and the expression of fibrosis associated genes were not inhibited by combination therapy at the doses administered however.

As this is a high responder model and the strength of the cellular immune response can obscure the relative contribution of other factors to the development of CAD. It is therefore possible that the measured histological and molecular changes may relate to the immunosuppressant effect of these drugs as opposed to a direct anti-fibroproliferative effect. That is, the attenuation of intimal hyperplasia, remodelling and ECM accumulation may represent a continuum of the immunosuppressant effect, with intimal hyperplasia in this model being suppressed by very low levels of immunosuppression and remodelling/medial fibrosis by high levels. Such as pattern is not consistent with the data however. There was less intimal thickening in the rapamycin/cyclosporin group compared to the cyclosporin only group (which had greater inhibition of the inflammatory infiltrate) and the greatest degree of intimal thickening among the treatment groups. This suggests that the effect of combination therapy on intimal hyperplasia is independent of the immunosuppressant effect. Similar inhibition of neointimal hyperplasia in the absence of immunosuppression in rats with low dose rapamycin monotherapy have been reported previously by ourselves and others (Murphy et al 2002, Schmid et al 1995). It could also be argued that this study would best be performed in a renal allograft model and indeed such a study is required however studying gene expression in arterial allografts prevents
confusion arising from the direct pro-fibrotic effect of cyclosporin on kidney mesangial
and interstitial cells.

In vitro combinations of rapamycin and cyclosporin inhibit cell-mediated lympholysis and
IL-2 dependant T cell proliferation at significantly reduced doses compared to their
independent effects (Kahan et al 1991). This combined effect is much more than one
would expect from a purely additive effect suggesting a synergic interaction. In animal
studies in vivo cyclosporin and rapamycin again demonstrate synergistic properties. Sub
therapeutic doses of rapamycin (0.01 to 0.04 mg/kg/day) and cyclosporin (0.5 to 2
mg/kg/day) prolonged rat cardiac and kidney allograft survival compared with either drug
alone or the additive effect of a combination of both (Stepkowski et al 1997). In the current
study there was no statistically significant difference in neointimal development between
the effect of these agents alone and that of the two in combination at dose reductions 10
fold and 3 fold for rapamycin and cyclosporin respectively and along with
histomorphometric data suggests that rapamycin and cyclosporin may act synergistically to
inhibit intimal hyperplasia in this model.

In rats in vivo this synergy effect is mediated in part by a rapamycin induced elevation in
the serum level of cyclosporin however this effect in isolation is insufficient too explain
the observed reduction in cellular rejection seen with sub-therapeutic doses of the two
drugs seen in isolation. The inhibitory effect of rapamycin on cytokine signal transduction
is cytokine concentration dependent and cyclosporin acts via the inhibition of cytokine
production. By reducing cytokine production cyclosporin may enhance the inhibitory
activity and specificity of sub therapeutic amounts of rapamycin on signal transduction
producing the observed synergy.
Synergy was not evident when other histological features of allograft vasculopathy were considered. Both rapamycin (0.5mg/kg/day) and cyclosporin (5mg/kg/day) significantly inhibit remodelling, medial ECM accumulation and the inflammatory infiltrate in rat aortic allografts. There was a more severe level of inflammation in the rapamycin/ cyclosporin group compared to the rapamycin and cyclosporin monotherapy groups and this was associated with higher MMP 9, TGF Beta and TIMP 1 transcript levels with combination therapy compared to monotherapy. Previous studies on the F344 to Lew aortic allograft model have demonstrated that expansive vascular remodelling and medial ECM accumulation correlates with intra-graft MMP 9 transcript levels that in turn are associated with severity of the inflammatory infiltrate (Chapter 3). Furthermore suppression of the inflammatory infiltrate was associated with attenuation of MMP 9 expression and it is our belief that the observed increases in gelatinase expression are derived from these cells. This is supported by observations that MMP 9 is expressed by infiltrating T cells and activated macrophages in vivo and in vitro (Goetzl et al 1996) and also by studies in mice aortic allografts where MMP 9 is localised to inflammatory cells (Lijnen et al 1998). Failure to suppress this inflammatory infiltrate may have contributed to expansive remodelling directly, by the release of MMP 9 from macrophages, or indirectly via the tissue response to the inflammatory injury. Elevated TGF Beta levels (a potent fibrotic cytokine released by macrophages as well as smooth muscle cells, Jain 2001) may have produced the higher TIMP 1 levels in the combined group (TIMP 1 expression is increased by TGF beta in vascular smooth muscle cells and fibroblasts in vitro (Galis et al 1994, Hanemaaijer et al 1993) with resulting ECM accumulation. ECM accumulation (fibrosis) results not only from the synthesis of extracellular matrix components but also by the inhibition of their breakdown by proteases such as the MMPs. TIMP 1 a 28.5kD glycoprotein, is synthesised by all connective tissue cells as well as macrophages and is capable of inhibiting all activated collagenases, stromelysins and gelatinases with a
particular affinity for MMP 9. In clinical studies in renal transplantation TIMP 1 levels correlate with histomorphometric calculations of allograft fibrosis in protocol biopsies and TIMP 1 is also strongly associated with interstitial and glomerular fibrosis in human renal disease (Strutz 1995) and animal models of renal fibrosis (Jain et al 2000). A role for TIMP 1 in medial collagen accumulation is far from proven however. TIMP 1 expression is not increased during intimal ECM accumulation following balloon injury in the rat (Webb et al 1997) or indeed in rat allografts compared to isografts as demonstrated here. TIMP 1 expression increases following balloon injury in rabbits (Wang et al 1996) in cultured saphenous vein (Kranzhofer et al 1999) and in atherosclerotic plaques (Nikkari et al 1996) however its expression is localised to the neointima in each case.

The failure of combination therapy to attenuate MMP 9 or the inflammatory infiltrate suggests that these two agents at the dose reductions described (ten fold reduction in rapamycin and three fold cyclosporin) were not as efficacious at inhibiting alloimmune injury as reported by Stepkowski et al (1997). This discrepancy may be explained by the use of continuous i.v. infusions for drug administration producing higher steady state concentrations of each drug in the former study (Stepkowski et al 1997) than those achieved with oral dosing. The doses of rapamycin in particular were very low and given the poor bioavailability of oral rapamycin (15%) these results do not exclude a synergistic effect of reduced dose cyclosporin and rapamycin on the fibroproliferative response in the presence of effective immunosuppression. No lack of efficacy with combined rapamycin cyclosporin therapy is reported in clinical trials. In de novo renal transplantation rapamycin in combination with low doses of cyclosporin had equivalent rejection rates compared to combinations with higher cyclosporin doses. Furthermore combined rapamycin cyclosporin treated patients had superior renal function, significantly so at 3 months, than those on higher doses (Kahan et al 1999). This suggests that the addition of rapamycin may
reduce cyclosporin related nephrotoxicity and therefore reduce the progression of CAD. Other studies do suggest increased nephrotoxicity with rapamycin and cyclosporin in combination however. In a salt depleted rat model of cyclosporin nephrotoxicity the addition of rapamycin was associated with morphological and functional deterioration in renal function (Andoh et al 1996). Furthermore in a randomised clinical trial patients with biopsy proven Chronic Allograft Nephropathy randomised to cyclosporin dose reduction and the addition of rapamycin had worse graft function at 6 months compared to those who underwent cyclosporin dose reduction alone (Saunders et al 2001). These latter reports would be supported by the finding of increased TGF Beta and TIMP 1 transcript levels with combined treatment in the current study.
Chapter 6. Rapamycin has no effect on fibrosis associated gene expression or extracellular matrix accumulation when administered to established or early allograft vasculopathy
Abstract

Background The majority of patients with functioning transplants have established and progressive Chronic Allograft Dysfunction (CAD) a fibroproliferative process for which there is no effective treatment. CAD is characterised by histological evidence of Extracellular Matrix (ECM) accumulation (fibrosis). The aim of this study was to compare the effect of rapamycin administered to rats with established allograft vasculopathy on histological indices of disease progression, extracellular matrix accumulation (fibrosis) and the expression of genes known to regulate ECM turnover in this model.

Methods Lewis recipients of F344 rat thoracic to abdominal aorta transplants were administered rapamycin starting at 8, 12 and 16 weeks post transplant or no treatment. Six grafts in each group were harvested at 24 weeks. Vascular remodelling and collagen accumulation (sirius red) were measured by computerised histomorphometry of aortic sections. mRNA was extracted from frozen tissue and expression of fibrosis associated genes was studied by means of semi-quantitative RT-PCR.

Results Rapamycin had no effect on the progression of early or established allograft vasculopathy with regard to intimal thickening, remodelling, extracellular matrix accumulation or pro-fibrotic gene expression, regardless of the time commenced.

Conclusion The attenuation of the fibroproliferative response in rodents by rapamycin is not seen if the onset of rapamycin therapy is delayed.
Introduction

Chronic Allograft Dysfunction (CAD) occurs as a consequence of a fibroproliferative response to tissue injury that is defined histologically by allograft vasculopathy and organ fibrosis (Kouwenhaven et al 1999) that results ultimately in organ dysfunction and failure. The majority of patients with functioning transplants have established and progressive CAD (Hosenpud et al 1995, Cecka and Terasaki 1994) with allograft vasculopathy detectable using Intracoronary Ultrasound (ICUS) in up to 75% of heart transplant patients at 1 year (Yeung et al 1993) and progressive fibrosis and allograft dysfunction detectable in 40% of renal transplants after the initial few post-transplant months (Paul 1999). No therapy to date has been shown to halt the progression of this process. Rapamycin, a macrolide antibiotic derived from the actinomycete Streptomyces hygroscopicus has been shown to attenuate the fibroproliferative response to injury in rats following mechanical injury and ischaemia reperfusion injury as well as in animal models of CAD in vivo (Morris et al 1995, Jain et al 2001). The aim of this study was to assess whether rapamycin administered to rats after the development of allograft vasculopathy had an effect on histological indices of disease progression, extracellular matrix accumulation (fibrosis) or the expression of genes known to regulate ECM turnover in this model.
Experimental design and methodology

Experimental model

The thoracic aorta to abdominal aorta allograft model as described by Mennander (1991) was used. A segment of the descending thoracic aorta approximately 3cm in length was excised, thoroughly perfused with phosphate buffered saline (PBS) and used as a transplant. F344 rats acted as donors and Lewis rats as recipients. Ischaemic injury to the graft was minimised by immersion in an ice bath at 4°C between procedures. Through a midline laparotomy incision the segment of thoracic aorta was anastomosed end to end to the recipient abdominal aorta using 9/0 prolene suture. The graft was transplanted into heterotopic position below renal arteries and above the bifurcation forming a loop in the recipient abdomen.

Animals were kept in a controlled environment with unlimited access to feed and water. Graft harvest was performed in heavily anaesthetised rats following which the animal was allowed to die by exsanguination.

Drugs

Rapamycin (Sirolimus; Wyeth-Ayerst, Princeton, NJ) was prepared twice weekly as a working solution of 5mg/ml in distilled water. Solutions were stored at 4°C and protected from UV light exposure. Drugs were administered orally by gavage.

Experimental groups

All experiments were on F344 to Lewis Allografts which received cyclosporin (Neoral) 5mg/kg/day for the first 14 days only, to attenuate early acute rejection and induce tolerance, followed by a period without immunosuppression, and were then started on rapamycin 0.5 mg/kg/day at the following times post-transplantation

Group 1. 8 weeks
Group 2. 12 weeks
Group 3. 16 weeks
Group 4. No rapamycin.
These time points correspond to macrophage and lymphocyte infiltration and expansive remodelling (8 weeks), early neointimal development and expansive remodelling (12 weeks), neointima thickening almost complete and expansive remodelling (16 weeks) (Figure 1D.)
In this experiment six rats in each group were sacrificed at 24 weeks.

**Allograft gene expression using RT-PCR**

Total mRNA was extracted from aortic tissue and complementary DNA molecules were synthesised by reverse transcription (RT). These cDNA species were amplified by polymerase chain reaction (PCR) and quantified in an ELISA system. Relative quantitation was performed by comparison of the signal intensity to that of the housekeeping gene β Actin.

**Histological analysis**

Segments of the aortic graft were embedded in paraffin wax and multiple sections were stained with haematoxylin and eosin. The level of inflammatory infiltrate was assessed semi-quantitatively and was categorised as follows: None- no inflammatory cells visualised, Mild-endothelialitis, inflammatory cell localised to the endothelium, Moderate- dense inflammatory cell infiltrate in the innermost layer of the vessel wall, Severe- dense inflammatory cell infiltrate throughout all layers of the vessel wall.

The vessel circumference and area fraction of the intima and media were quantified using a computer image analysis system. The intima was defined as the area between the endothelium and the internal elastic lamina and the media was defined as the area between the internal and external elastic laminae. Paraffin embedded sections were also stained with sirius red stain and the level of ECM staining quantified using computerised histomorphometry.
Substances to be assessed

In order to take into account all the factors which influence smooth muscle cell proliferation, migration and deposition of ECM during neotima formation, the following species were studied with RT-PCR: MMP2, MMP9, TGF Beta, TIMP1 and collagen III.

Analysis of results

The levels of gene expression and histomorphometric measurements of intimal thickening expressed as the intimal media ratio, vascular remodelling expressed as vessel circumference (of the internal elastic lamina) and collagen accumulation expressed as a product of the percentage mean media sirius red staining and the medial area were compared using non-parametric statistical analysis. Comparisons of the level of inflammatory infiltrate were performed using Pearson's Chi square. Statistical analysis was performed using the Statistical Package for the Social Sciences Version 8.0 (Chertsey, UK.)
Results

Results of histological analysis

Allografts undergo progressive expansive remodelling process that is associated with neointima as well as marked neoadventitia formation (Figure 1 and Figure 2A,B). There was no difference between any of the treatment groups in terms of intimal area, vessel circumference (Figure 2A,B), or the severity of the inflammatory infiltrate (Figure 2D). There was also no difference between the allograft groups in terms of relative medial ECM content (Kruskall Wallis P>0.05, Figure 3).

Results of RT-PCR

There were no significant differences between the groups for any of the genes studied (Figure 4) although it is noteworthy that all allograft groups had elevated TGF Beta, MMP 2 and MMP 9 levels relative to donor aorta.
Figure 1. Development of allograft vasculopathy in F344 to Lewis aortic allografts with time
Representative photomicrographs of haematoxylin and eosin stained section of 1A donor rat aorta and 1B aortic allografts at 24 weeks
Untreated allografts have a dense transmural inflammatory infiltrate associated with intimal and medial thickening as well as striking neoadventitia formation. These changes were not attenuated by rapamycin started at 8, 12 or 16 weeks (Figure 1C).

Figure 1A.
Figure 1B.
Figure 1C. Graph demonstrating stages of neointimal development at which rapamycin was commenced.
Arrows indicate times rapamycin treatment started in groups 1 to 3 (i.e. 8, 12 and 16 weeks respectively).
Values represent mean ±S.D.

Time course of the development of intimal hyperplasia in untreated F344-Lewis allografts versus F344-F344 isografts
(unpublished data)
Figure 2. Results of histological analysis
Values represent median, S.D. (Boxes) and 95% C.I. (Whiskers)

Figure 2A. Effect of rapamycin on neointimal development when introduced at 8, 12 and 16 weeks following transplantation.
Rapamycin has no effect on the development of the neointima, Kruskall Wallis, P>0.05
Figure 2B. Effect of rapamycin on vascular remodelling in rat aortic allografts when introduced at 8, 12 and 16 weeks post transplantation
Rapamycin had no effect on expansive remodelling, Kruskall Wallis P>0.05
Figure 2C. Effect of delayed rapamycin treatment on allograft inflammation at 24 weeks
There was no significant reduction in the severity of the inflammatory infiltrate between the rapamycin treated groups (Pearson's Chi-Square = 9.9, P > 0.05)

Qualitative assessment of Inflammatory infiltrate

Number of Allografts

Severity of inflammation

Group
- Rapa 8 weeks
- Rapa 12 weeks
- Rapa 16 weeks
- No Rapa
- Donor Aorta

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<td>6.0</td>
</tr>
<tr>
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<td>2.0</td>
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<tr>
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</table>
Figure 3. Relative medial ECM content at 24 weeks

There was no difference between the groups in medial collagen content and adventitial collagen density (Kruskall Wallis P>0.05). Boxes represent mean plus standard deviation. Whiskers represent 95% C.I.
Figure 4. Gene expression in allografts at 24 weeks

Graphs demonstrate the influence of rapamycin introduced at 8, 12 and 16 weeks on the expression of genes important in the regulation of ECM turnover in 24 week allografts. Gene expression is expressed as the ratio of mRNA transcript levels for the gene considered relative to the mRNA transcript levels of the housekeeping gene β Actin to control for differences in cellularity between samples. Box and whisker plots represent median, SD and 95% C.I.

4A

**MMP 2 mRNA/ actin mRNA ratios at 24 weeks**
MMP 9 mRNA/ actin mRNA ratios at 24 weeks

Group

N = 6 5 6 6 5

Rapa 8 weeks Rapa 16 Weeks Dono

Rapa 12 weeks No Rapa

MMP 9 mRNA/ actin mRNA ratio

14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0

0
2
4
6
8
10
12
14

Rapa 8 weeks Rapa 16 Weeks
Rapa 12 weeks No Rapa Dono
TGFβ mRNA/actin mRNA ratios at 24 weeks

![Graph showing TGFβ mRNA/actin mRNA ratios at 24 weeks with different groups and their respective ratios.]

- Rapa 8 weeks
- Rapa 16 Weeks
- Donor
- Rapa 12 weeks
- No Rapa

* Denotes statistically significant differences.
Collagen 3 mRNA/actin mRNA ratios at 24 weeks

Group

Rapa 8 weeks  Rapa 16 Weeks  Rapa 12 weeks  No Rapa  Donor

N = 6  5  6  6  5

Collagen 3 mRNA/actin mRNA ratio
TIMP 1 mRNA/actin mRNA ratios at 24 weeks

N = 6  5  6  6  5
Rapa 8 weeks  Rapa 16 Weeks  Donor
Rapa 12 weeks  No Rapa

Group
Discussion

Rapamycin had no effect on the progression of vasculopathy in rat aortic allografts when administered to grafts with early cellular changes but no neointima or to those with developing or established intimal hyperplasia. The beneficial effect of rapamycin on CAD development in this rodent model was therefore lost after 8 weeks. There was also no difference between any of the rapamycin treated allograft groups in the expression of genes that regulate the fibroproliferative response.

The rapamycin dose used was smaller than in other studies where rapamycin was shown to inhibit established (3mg/kg/day i.p) or de novo CAD (0.5-6mg/kg/dai i.p.) in rat allografts (Poston et al 1999, Morris et al 1995, Meiser et al 1991, Schmid et al 1995, Gregory et al 1993). Sirolimus at the 0.5mg/kg/day p.o. dose equates to a serum trough level of approximately 1ng/ml in Lewis rats (DiJoseph et al 1996). This dose has been shown in previous studies to significantly prolong rat renal and cardiac allograft survival in the high responder BN to Lew model (DiJoseph et al 1996) as well as significantly inhibiting the allograft inflammatory infiltrate, intimal hyperplasia and vessel remodelling in the F344 to Lewis model (Murphy et al 2001). These serum levels do not correlate with suggested therapeutic range levels in humans (5-15ng/ml) (Saunders et al 2001) however higher oral doses of rapamycin are poorly tolerated by rats, with significant weight loss reported at doses greater than 0.8mg/kg/day (DiJoseph et al 1996). It is possible that the rats may have increased their clearance of the drug over time and this study may have benefited from serum rapamycin levels on the day of cull. There was no significant inhibition of MMP 9 (a correlate of the severity of the inflammatory infiltrate in this model (Chapter 3)) in rapamycin treated versus untreated allograft however semi-quantitative assessment of the inflammatory infiltrate
suggested attenuation of the inflammatory response in the treated allografts. This would suggest that increased clearance of rapamycin has not occurred. Other criticisms relate to the use of a high responder model in the analysis of factors affecting the development of CAD (Poston et al 1999) as the intense cellular immune response tends to obscure the contribution of other important aetiological mechanisms.

In 1991 Meiser et al reported that rapamycin at 1.5mg/kg/day, in a high responder Brown Norway to Lewis model, inhibited CAD in de novo heterotopic rat cardiac allografts (Meiser et al 1991). BN and Lew rats are mismatched at major HLA loci (RT-1a versus RT-1b) and the development of CAD in this model occurs in association with an intense cell mediated response with intimal thickening occurring predominantly between 30 and 40 days. Morris et al (1995) reported similar findings with femoral artery allografts in the same high responder combination. In this latter study the importance of inhibiting the early cellular response was underscored by the diminished efficacy of rapamycin if administration was delayed. Delaying onset of rapamycin treatment by 14, 21 or 30 days inhibited the progression of CAD only at large doses (3mg/kg/day i.p. at 14 and 21 days and 6mg/kg/day i.p. at 30 days) and was of statistical significance only at this later time point (28% versus 49%, P=0.03) (Morris et al 1995). This study did not however consider the effect of rapamycin once intimal hyperplasia had become established as in the current study. Morris and colleagues subsequently demonstrated reversal of established allograft vasculopathy in the low responder PVG to ACI rat cardiac allograft model that develops significant intimal thickening between 30 and 90 days post transplantation (Poston et al 1999). Rapamycin (3mg/kg/day i.p.) significantly reversed intimal thickening when administered between 60 and 90 days (Poston et al 1999). Significant weight loss was noted in these animals however and therefore it is questionable.
whether the observed effect was due to a direct effect of rapamycin on the fibroproliferative response or simply as a result of drug toxicity.

There are few studies that have demonstrated any regression of CAD once established. In the weakly immunogenic WF to Lew model re-transplantation to the donor strain up to 40 days reversed the inflammatory infiltrate and prevented the development of CAD (Forbes et al 1997). In high responder models however re-transplantation was seen to reduce but not prevent the development of CAD. Moreover in these stronger models re-transplantation after a critical period reversed the associated cellular infiltrate whilst the intimal proliferation continued to worsen (Schmid et al 1996, Hullet et al 1996, Mennander et al 1991).

F344 to Lewis rat allografts re-transplanted to the F344 strain showed reversal of the process up to a certain time interval (12 weeks) after which time structural changes, particularly fibrosis continued to worsen (Tullius et al 1994b). In the same rat strain combination re-transplantation of cardiac allografts to the donor strain was seen to reduce levels of mononuclear infiltration but not levels of myointimal proliferation (Schmid et al 1995). These findings suggest that progressive organ injury (i.e. the fibroproliferative response) beyond a certain point may become autonomous as well as alloantigen independent.

Of equal importance is the suggestion that the initial insults, whether alloantigen dependant or independent occurring at an early stage post transplant are crucial in the later development of CAD. This study demonstrates that in F344 to Lewis allografts rapamycin does not appear to affect the fibroproliferative response after 8 weeks. This may be due to inadequate immunosuppression in a high responder model (as suggested by persistently elevated MMP 9 mRNA levels in allografts) however it may also be due to a failure of rapamycin to inhibit the later stages of the fibroproliferative response. This latter suggestion would be supported by the elevated MMP 2 and TGF levels in allografts at 24 weeks. In a randomised clinical trail in
renal transplant patients with biopsy proven CAD (Saunders et al 2001) cyclosporin dose reduction along with the administration of rapamycin was not shown to prevent the progressive decline in renal function. Together these present both clinical and experimental evidence that rapamycin may not attenuate the fibroproliferative response in allografts once established.
Chapter 7. Summary
Summary of main findings and conclusions

Changes in the expression of fibrosis associated genes during the development of allograft vasculopathy in F344 to Lewis aortic allografts

Aortic allografts undergo progressive expansive remodelling up to 24 weeks post transplantation and develop a neointima between 8 and 16 weeks. This is characterised by an inflammatory infiltrate within untreated allografts that is present at all time points.

MMP 9 expression correlates with the severity of the inflammatory infiltrate and previous studies have demonstrated that MMP 9 localises to adventitial macrophages and lymphocytes in untreated allografts.

8 weeks post transplantation there appears to be a period of increased extracellular matrix breakdown with increased MMP 9 and a reduction of pro-fibrotic genes (TIMP 1 and collagen 3) in untreated allografts relative to isografts. Similar changes are observed at 16 weeks. There is a positive correlation between MMP 9 expression and vessel circumference and medial collagen accumulation. There is also a strong negative correlation between vessel circumference and adventitial collagen density. It is hypothesised that the balance of extracellular matrix turnover in the adventitia is tending to promote extracellular matrix breakdown and this contributes to the observed expansive vascular remodelling.

At 12 weeks there appears to be a period of increased extracellular matrix turnover with increases in MMP 2 and MMP 9 as well as TIMP 1 and 2 and Collagen 3. This coincides with the early development of the neointima and presumably reflects the extracellular...
matrix turnover associated with smooth muscle cell proliferation, migration and extracellular matrix deposition during neointima formation.

By 24 weeks the mean neointimal thickness is similar to that at 16 weeks but expansive vascular remodelling continues in an almost linear fashion to 24 weeks. There are very similar changes in gene expression at 16 weeks and at 8 weeks although TGF Beta now appears to correlate with pro-fibrotic genes rather than MMP 9. TGF Beta appears to mediate both early inflammation and later fibrosis in this model.

Effect of cyclosporin

Cyclosporin inhibits all aspects of the inflammatory and fibroproliferative response. A reduction in the severity of the inflammatory infiltrate in this model with cyclosporin therapy was associated with a significant reduction in the level of ECM deposition, expansive remodelling and intimal thickening. This is associated with attenuation of the observed changes in gene expression. This suggests that the effectiveness of cyclosporin in clinical transplantation is limited by its toxicity, particularly nephrotoxicity, which contributes to CAD in renal transplants.

The severity of medial collagen accumulation correlates with the severity of the inflammatory infiltrate (using MMP 9 as a surrogate marker of inflammation).

Effect of rapamycin

Rapamycin at higher doses inhibits intimal hyperplasia, vascular remodelling and ECM accumulation in association with attenuation of the graft inflammatory infiltrate and a reduction in gelatinase and TIMP 1 mRNA expression. At a lower dosage rapamycin failed to inhibit vascular remodelling or inflammation however it did significantly inhibit intimal hyperplasia. This was associated with an attenuation in MMP 2 and TIMP 1 expression.
compared to controls during the period of intimal thickening only (12 to 16 weeks). This data suggests that rapamycin may have a significant inhibitory effect on the vessel response to injury that is distinct from its immunosuppressant action. Inhibition of all the aspects of the fibroproliferative response required effective suppression of the inflammatory response however and this was only achieved at higher doses. Suppression of the tissue response to injury may not therefore be sufficient to completely inhibit CAD as inflammatory cells themselves are capable of remodelling tissues by the direct production of metalloproteinases and ECM constituents.

**Effect of rapamycin and cyclosporin in combination: evidence of synergy in the prevention of CAD**

This data demonstrates that rapamycin and cyclosporin in combination at doses 10 fold and three fold lower than either rapamycin or cyclosporin respectively is as effective at preventing the fibroproliferative response that characterises vasculopathy in rat aortic allografts compared to either agent alone. This effect was not solely dependent on the inhibition of alloimmune injury.

**Effect of rapamycin on established allograft vasculopathy**

Rapamycin had no effect on the progression of vasculopathy in rat aortic allografts when administered to grafts with early cellular changes but no neointima, or to those with developing or established intimal hyperplasia. The beneficial effect of rapamycin on CAD development in rodents is therefore lost after 8 weeks. There was also no difference between any of the allograft groups in the expression of genes that regulate the fibroproliferative response in this model.
Conclusion

There are two components to extracellular matrix remodelling in rat aortic allografts

1. The fibroproliferative response to injury
2. Macrophage and lymphocyte infiltrates

This is manifest histologically by intimal hyperplasia, medial fibrosis, expansive remodelling and neoadventitial remodelling. In this model adventitial remodelling appeared to be an important determinant of vessel remodelling and vessel diameter.

Both clinical and experimental evidence suggest that rapamycin is effective at inhibiting both of these processes. In the current study rapamycin at lower doses effectively inhibited the first but failed to sufficiently inhibit the second of these two processes. Inflammatory cells themselves particularly macrophages can significantly contribute to extracellular matrix remodelling. This is clearly demonstrated by the central role attributed to the macrophage in the development of the atherosclerotic plaque and other fibroproliferative diseases (Ross 1999). The clinical significance of this observation is unclear, due largely to the limitations of the rat aortic allograft model. It would appear to suggest however that attenuation of neointima formation, as demonstrated in early experiments with rapamycin in rodent models may not transfer to attenuation of the allograft vasculopathy and organ fibrosis that characterises CAD in clinical practice. In the combined rapamycin/cyclosporin experiment it was also evident that failure to inhibit the inflammatory response also failed to inhibit aspects of vessel remodelling. In clinical practice this means that effective immunosuppression remains the cornerstone of attempts to reduce the progression and severity of CAD. This is further reinforced by the demonstration and observation here that there are no convincing clinical or experimental studies showing that the process of CAD can be reversed or even halted although there is some evidence that its progression can be slowed (Jain et al 2001).
**Future research**

Attempts to address the problem of CAD should be two fold: 1, minimise the injury to the allograft and 2, inhibit the fibroproliferative response to injury.

Addressing the first of these issues includes minimising exposure to aetiological risk factors (Chapter 1, Part 1) and more effective pharmacological inhibition of ischaemic reperfusion and alloimmune injury. The second of these issues has been less well addressed than the first with the introduction of rapamycin the only widely practised attempt to inhibit the fibroproliferative response. Novel agents that inhibit the tissue response to injury are in development however targeting extracellular mechanisms with for example MMP inhibitors or inhibition of cytokine receptors are unlikely to be successful. This is because their effects are likely to be bypassed by the multiple regulatory processes that influence this process. Increased understanding of vascular smooth muscle cell and fibroblast proliferation and migration at the intracellular level for example the processes regulating Cyclin Dependant Kinase inactivation of Retinoblastoma protein or phosphorylation of p125 FAK promoting cell migration are important. Introduction of pharmacological agents that affect these processes will have a better chance at effective inhibition of the fibroproliferative response.
Appendix 1

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<th>Extraction of mRNA from Frozen Sections Using Dynabeads</th>
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<td>dynafroz 06/09/01</td>
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**Method**

1. Once fully trained in the safe use of the refrigerated microtome (cryostat), cut and place 10 μm sections into a pre-chilled, sterile 1 ml Eppendorf. Make sure that the Eppendorf and anything used to touch the sections is maintained at -20°C (to prevent the section melting).

2. Add 500 μl of Lysis/Binding buffer to the Eppendorf. The contents are now safe to leave the cryostat.

3. Mix the contents. Add 25 μl of 1 mg/ml proteinase K to the lysate, mix again, and incubate for 1 h at 37-50°C.

4. Centrifuge the lysate for 30-60 sec at 10,000 g to pellet any debris.

**Preparation of Dynabeads**

Note: it is best to prepare Dynabeads in one go and then dispense 30 μl aliquots for mRNA extraction into an appropriate number of Eppendorfs. Preparation for a single lysate is as follows:

5. Dispense 30 μl Dynabeads into a sterile Eppendorf.

6. Pellet the Dynabeads with a Dynal MPC for 10 sec/until the supernatant is clear.

7. Remove the supernatant and resuspend the Dynabeads in at least 30 μl of Lysis/Binding Buffer.

8. Pellet the Dynabeads with the Dynal MPC for 10 sec/until clear.

9. Remove the supernatant and resuspend the Dynabeads in 30 μl of Lysis/Binding Buffer.

**Extraction of mRNA**

11. Using a P200 Gilson with a P10 (white) tip attached to the end of a filtered P200 (white) tip, suck/scoop out any pellet and discard it, taking care to minimise loss of liquid lysate. The pellet may be very hard to see.

12. Add the lysate to the Dynabeads. When adding the lysate, use a P200 Gilson with a P10 (white) tip attached to the end of a filtered P200 (white) tip, and always pipette from just below the meniscus. Pipette slowly, taking care not to disturb any remaining pellet (which may be very hard to see). If any pellet is accidentally disturbed, discard it in the lid of the Eppendorf and continue pipetting.

13. Mix the lysate with the Dynabeads and allow it to anneal for 10 min at room temperature.

14. Pellet the Dynabeads with the Dynal MPC for 10 sec. Increase the time if the solution is noticeably...
Viscosity is a sign of genomic DNA entangling with the beads. Reduce it by pressing at least 3 times through a 21-gauge needle with a sterile, RNase-free 1 ml syringe. (Give the syringe ~ 0.3 ml dead airspace before using it, so that as much lysate as possible can be ejected after pressing.)

15. Discard the supernatant.

16. Mix the Dynabeads thoroughly with 50 µl Washing Buffer + LiDS. Pellet the Dynabeads with the Dynal MPC until the solution is clear. Discard the supernatant. Repeat once.

17. Mix the Dynabeads thoroughly with 50 µl Washing Buffer. Pellet the Dynabeads with the Dynal MPC until the solution is clear. Ensure that all of the supernatant is discarded. Repeat twice.

18. If absolutely necessary, store beads in 50 µl TE buffer at 4°C. However, it is better to proceed immediately to reverse transcription, as detailed in dynarp.

**Reagents**

**Proteinase K**

See RPM 1

**Lysis/Binding Buffer**

See RPM 35

**Washing Buffer + LiDS**

See RPM 33

**Washing Buffer**

See RPM 32

**TE buffer**

1 ml 100x TE stock

99 ml sterile distilled water

**100x TE Stock**

See RPM 5

**Special Equipment**

Dynabeads kit and MPC; 0.5 ml / 1 ml Eppendorf tube for each biopsy; Gilsons; cryostat.
**Method**

1. Place the mRNA-loaded Dynabeads into a 0.5 ml Eppendorf tube.
2. Pellet Dynabeads using a Dynal MPC (magnet) for 10 sec. Discard supernatant.

**RT step**

3. If mRNA is rare, perform an unequal -RT/+RT split; otherwise perform an equal -RT/+RT split

   **Unequal -RT/+RT split**

   Resuspend Dynabeads in 10 µl DEPC-treated water. This is the +RT sample. Pipette 6.5 µl DEPC-treated water into a second 0.5 ml Eppendorf. This will be the -RT sample. Pipette 2 µl of Dynabeads from the +RT sample into the -RT sample.

   **Equal -RT/+RT split**

   Resuspend Dynabeads in 16 µl DEPC-treated water. This is the +RT sample. Pipette 0.5 µl DEPC-treated water into a second 0.5 ml Eppendorf. This will be the -RT sample. Pipette 8 µl of Dynabeads from the +RT sample into the -RT sample

4. Make an RT mastermix. Add 16.5 µl of mastermix to each -RT sample. Mix well. Always make up slightly more mastermix than is needed.

5. Into the remainder of the mastermix, mix 0.5 µl of 10 U/µl AMV RT (5 U) for every +RT sample. Add 17 µl of this mastermix to each +RT sample. Mix well.

6. Incubate at 42°C for 1 hour using the thermal cycler.

7. If cDNA-loaded Dynabeads are to be stored, store the +RT sample(s) in TE buffer at 4°C.

**PCR step**

Note 1: if you are performing PCR with only one primer set, make a PCR mastermix containing the primers. However, if you are performing PCR with a panel of primers (e.g. GPD, C42, C31), make a PCR mastermix of 1X AJ buffer only, and then aliquot it out to make ‘mini’ mastermixes of each primer set.

Note 2: it is advisable to run a “water blank”, i.e. a complete reaction mixture, but without the Dynabeads, to check for contamination of AJ buffer, primers, water and Taq.

Note 3: if performing PCR with multiple aliquots of a +RT sample, do not dispense multiple volumes of 1 µl, for example. Instead, it is more accurate to dispense the total volume needed, add four times the amount of sterile distilled water, and then dispense as 5 µl aliquots, e.g. take 9 µl (for 9 reactions), add 36 µl water (9 x 4 µl), and dispense as 9 x 5 µl aliquots.
Note 4: from Step 8 on, the ‘PCR ONLY*’ Gilsons must be used and no others.

8. Thaw a tube of AJ 10X buffer. Vortex it thoroughly. Spin at 3000-4000 g for 1 min to pellet precipitated BSA (which inhibits PCR). Make PCR mastermix(es) with or without primers as appropriate (see Note 1 above).

9. If using Dynabeads after 4°C storage of 1 week, pellet them using the Dynal MPC (magnet), discard the supernatant, and then resuspend the Dynabeads in TE to the correct volume.

10. If performing an experimental PCR (for example with a panel of primers), use only the +RT cDNA (e.g. at 1 µl per reaction). If checking the quality of an mRNA extraction by performing PCR on -RT and +RT samples, PCR should be performed according to which RT protocol was used:

**Unequal -RT/+RT split**

The -RT product is ¼ the concentration of the +RT product. Dispense volumes accordingly, e.g. 4 µl of -RT beads is equivalent to 1 µl of +RT beads.

**Equal -RT/+RT split**

Dispense equal volumes of -RT and +RT products for PCR, e.g. 1 µl of -RT beads and 1 µl of +RT beads.

11. If applicable, adjust the volume of +RT and -RT beads by adding sterile distilled water or removing supernatant, so that for each sample the addition of mastermix/Taq will bring the final PCR reaction volume to 50 µl.

12. Add PCR mastermix including primers to each sample (both -RT and +RT) as follows:-

**Normal Taq**

Add PCR mastermix so that the volume in each tube is 45 µl. Place 1 drop of mineral oil over each sample, using the dropper bottle.

Place samples in the thermal cycler, and hot-start the PCR process using an appropriate program (e.g. PCR59-40).

Dilute Taq polymerase so that each sample will receive 1 U of Taq in 5 µl of (1X) AJ buffer. Do not use PCR mastermix to dilute the Taq, unless the same gene is being investigated in every sample.

At the annealing temperature pause, add 5 µl of Taq dilution to each sample, taking care to mix the Taq with the reaction mixture and not with the oil overlay. Resume the PCR program.

**JumpStart Taq**

Add JumpStart Taq polymerase to the mastermix so that each sample will receive 1 U of JumpStart Taq.

Add PCR mastermix so that the volume in each tube is 50 µl. Place 1 drop of mineral oil over each sample, using the dropper bottle.

Place samples in the thermal cycler, and start the PCR process using an appropriate program (e.g. JMP59-40).

13. If products are to be stored, store at 4°C. If Dynabeads are to be re-used, thorough washing with 100 µl of TE, followed by 50 µl of AJ buffer, must be performed to get rid of amplicon contamination.
Reagents

RT Mastermix (per reaction)

5 µl AMV RT 5X buffer
2.5 µl of 10 mM DEPC-treated dNTPs (1 mM)
0.6 µl of 40 U/µl Rnasin (25 U)
8.4 µl DEPC-treated water

DEPC-treated dNTPs

See RPM 20

DEPC water

See RPM 31

PCR Mastermix (per reaction) – add primers separately if different genes are to be investigated simultaneously

5 µl AJ 10X buffer
2 µl of 5 pmol/µl forward + reverse primer mix (10 pmol each) or alternatively 1 µl each of 10 pmol/µl primer F and 10 pmol/µl primer R

Sterile distilled water to a final PCR reaction volume of 50 µl (don't forget to allow for the Dynabeads)

Forward + Reverse Primer Mix

See RPM 34

AJ 10X buffer

See RPM 36

Special Equipment

Dynal MPCs, 0.5 ml Eppendorfs, Gilsons and ‘PCR ONLY’ Gilsons, centrifuge, thermal cycler
Method

1. Set up the required casting tray, using yellow gel tape to seal the ends, if necessary. Make sure that the combs touch neither the bottom nor the sides of the tray.

2. Place 50 ml (mini gel), 100 ml (midi gel) or 250 ml (maxi gel) of TAE into a conical flask.

3. Add 3% by weight agarose (1.5 g for a mini, 3 g for a midi, 7.5 g for a maxi).

4. Microwave at full power until the gel starts bubbling and/or frothing upwards.

5. Using a heat-gauntlet, swirl the contents of the flask around a few times.

6. Microwave at full power until the gel starts bubbling and/or frothing upwards.

7. Using a heat-gauntlet, swirl the contents of the flask around a few times.

8. Microwave at full power until the gel starts bubbling and/or frothing upwards.

9. Using a heat-gauntlet, swirl the contents of the flask around a few times.

10. Swirl the contents of the flask under cold running water until you are able to hold the flask without the aid of the heat gauntlet.

11. Add 15 μl of ethidium bromide for every 100 ml of gel. Swirl until the ethidium bromide is evenly distributed.

12. Pour the gel into the casting tray and allow to set.

Running

13. Set out one 0.5 ml Eppendorf tube for every sample of DNA. Pipette 2 μl of 5 x loading buffer into each.

14. Fill an appropriately sized "submarine" tank with TAE.

15. When the gel is set, remove the yellow sealing tape. Remove the comb carefully to avoid tearing the gel. Check the wells for signs of damage. A gel that has wells "linked" to each other by a crack, or a gel with wells that are bottomless, will have to be re-set.

16. Submerge the gel in the tank, making sure that no air bubbles are trapped in the wells. Mix 15-20 μl of ethidium bromide into the "lower" (nearest) end of the tank.

17. Pipette 15-20 μl (depending on comb tooth size; 15 μl for 22-tooth combs) of 100 bp ladder into the first well. If possible leave a blank well between the 100 bp ladder and your first sample.

18. Mix 15-20 μl (depending on comb tooth size) of DNA with the 5 x loading buffer, and pipette the mixture into one of the wells. Take care not to allow the mixture to cross over into the next well. To
save on pipette tips, the same tip may be re-used for each sample, provided that the tip is flushed a few times between each sample with buffer from the lower end of the electrophoresis tank.

19. Attach the lid/power cords to the tank, and run the gel as detailed in powerpack. A midi will require approximately 30 mins at 150 V (45 mins, if analysing competitive PCR). Adjust time and voltage according to requirement.

20. When the run is finished, view/photograph gel using uv illumination as detailed in gds.

Caution: ethidium bromide is a known carcinogen (Red 2). Great care must be taken, particularly when weighing out any powder. Regard anything inside the taped area as being contaminated.

Reagents

TAE
200 ml 50x TAE
10 litres purified water

50x TAE
See RPM 8

Ethidium bromide
See RPM 12

5 x Loading buffer
See RPM 10

100 bp ladder
See RPM 23

Special Equipment
Gilsons, gel casting trays, combs, gel tanks, power packs, yellow gel tape, microwave, conical flask, weighing boats, heat gauntlets, Eppendorfs, uv documentation system

<table>
<thead>
<tr>
<th>Version 1.1</th>
<th>Use of the Gel Documentation System</th>
</tr>
</thead>
<tbody>
<tr>
<td>gds</td>
<td>01/06/00</td>
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</tbody>
</table>
Method

Note: try to pick up your gel from the electrophoresis tank with only one hand. Use your uncontaminated hand to open the gel documentation system’s door. If this is not possible, use a fresh glove to open and close the door. However, you should regard anything within the taped boundary as being contaminated. Under no circumstances is the computer, or any part of it, to be touched with anything but fresh gloves or bare hands. Failure to comply with these instructions will not be tolerated, as ethidium bromide is a known carcinogen.

1. Check that the computer is not on standby by jiggling the mouse. If the computer does not respond, switch it on using the power button on the front.

2. Log on under your name by clicking on it and then clicking on the password box. Type in your password and click OK. If someone else is already logged on (i.e. a working desk-top is visible), check that they have finished with the computer and log them out by clicking on Start, then Log Off…

3. Switch on the gel documentation system using the power switch at the back of the tower, about two-thirds of the way up on the right hand side.

4. Double click on the GeneSnap icon.

5. Select your name from the list and click OK.

6. If you are presented with a window asking you to open a file, close the window, click on New Configuration in the File menu, then select GeneGenius from the menu and click OK.

7. Open the lower door of the tower. With the door wide open, position the gel on the uv transilluminator using the live image on the computer as a reference. Close the door of the tower.

8. The gel documentation system can be controlled with the buttons on the front of the tower, or by opening the Control Darkroom window (click on the menu button that looks like the tower).

9. Switch on the UV by pressing on the real UV button or clicking the simulated UV button on the computer screen.

10. Adjust the zoom level, iris aperture and focus, if necessary, using the real buttons on the front of the tower or the simulated buttons on the computer screen.

11. When ready, click on the Stop Capturing menu button (the red camera). Having done so, if you want to try again, click on the Start Live Video menu button (the left green camera).

12. Adjust the picture by using the brightness and contrast scroll bars to the right of the image.

13. When ready, save the image by clicking on the Save Document menu button (the red floppy disk). DO NOT save in My Documents – use the pull-down to select the C: drive. Double click on GeneGenius and then double click on your folder. Type a recognisable name (e.g. the experiment number) in the File Name box and click Save.

14. If you want a print-out of your image, make sure that the Sony printer is switched on. Click on the Print Image menu button (the blue printer). Make sure that the printer name is SONY UP-D890. If it is not, select this name from the pull-down menu. Click OK. The printer will take approximately 10 sec to print.

340
15. Close the image window. If you wish to take another picture, click on the New Document menu button (the white piece of paper). Go to Step 7.

16. Close the main window. If prompted to save the configuration, click on No.

17. Log off the computer by clicking Start and then Log Off… DO NOT switch off or shut down the computer.

18. Switch off the gel documentation system using the power switch at the back.

19. Open the lower tower door and remove the gel. Clean the uv transilluminator with water and wipe it dry. Close the tower door. Dispose of the gel and your gloves in the yellow bags.

Special Equipment

Gel documentation system

<table>
<thead>
<tr>
<th>Version 2.6</th>
<th>End Labelling of Oligonucleotides with Digoxygenin</th>
</tr>
</thead>
<tbody>
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Method

1. Dispense 10 µl of 10 pmol/µl oligo into a 0.5 ml Eppendorf
2. Add 12 µl of labelling mastermix
3. Incubate at 37°C for 15 mins.
4. Stop reaction with 1 µl of 0.2 M EDTA.
5. Add 77 µl of ultrapure water (to give a final oligo concentration of 1 pmol/µl oligo)
6. Store as aliquots at -20°C

Reagents

Labelling Mastermix (per reaction)

- 4 µl Boehringer TdT 5X buffer
- 2 µl of Boehringer 25 mM CoCl₂
- 2 µl of 1 mM DIG-11-dUTP
- 1 µl of 10 mM dATP
3 μl of 15U/μl Gibco or Boehringer rTdT

0.2 M EDTA

See RPM 25

**Special Equipment**

0.5 ml Eppendorf for each reaction, Gilsons, thermal cycler or incubator, pH meter (for balancing pH of EDTA).

<table>
<thead>
<tr>
<th>Version 2.8</th>
<th>PCR Assay Using ELISA</th>
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<tbody>
<tr>
<td>elisa</td>
<td>09/11/00</td>
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</tbody>
</table>

**Method**

Note: ensure that there are the following controls:-

a. a substrate only blank (SOB), i.e. Steps 1-4

b. a PBS/BSA blank (BSA), i.e. no DNA added

c. a non-specific binding control (NSB) for each probe used, i.e. an equivalent dilution of unrelated test DNA instead of the target DNA (e.g. actin instead of GAPDH in a GAPDH test)

d. a positive DNA control for each target DNA tested (POS), i.e. an equivalent dilution of the same DNA species, previously amplified and then frozen in aliquots

e. a positive plate/enzyme control (PLATE), i.e. a 1:10 dilution of digoxigenin-labelled biotinylated PCR primer (e.g. digoxigenin-labelled AFHB)

Note: if there are ready-streptavidinylated plates available, this method maybe started at Step 5 ("DNA Binding"). In this case, the SOB is an untreated well.

**Preparation**

1. Dilute a stock solution of Biotin-NHS 1:500 in PBS (allow 1 ml of dilution per strip, or 10 ml of dilution per plate of 12 strips). Add 100 μl to each well of a Cova multiwell plate, cover with labfilm and leave overnight at room temperature. (It is also possible to leave them at room temperature over the weekend, if sealed properly in labfilm.)

2. Wash plate 3 times in Amersham buffer, using a primed Wellwash 4 Mk 2, as detailed in *wellwash*. The wells should be left dry. Plates can be stored like this for about 1 week at 4°C if sealed in labfilm, but are best made up the afternoon before use.

**Avidinylation**
3. Dilute a stock solution of avidin 1:200 in Cova buffer. Add 100 μl of the avidin dilution to each well of the treated Cova plate. Leave for 30 min at room temperature (shaking is optional).

4. Wash plate 3 times with Amersham wash buffer, using the Wellwash, as detailed in *wellwash*. The wells should be left dry.

### DNA Binding

5. Add 100 μl PBS/BSA to each well except SOB, and leave for 15 min at room temperature (shaking is optional).

6. Aspirate plate using the Wellwash, as detailed in *wellwash*.

7. Dispense 25 μl PBS/BSA into each well of the plate, except SOB.

8. Make a 1:10 dilution of the digoxigenin-labelled biotinylated primer (PLATE).

9. Add the desired quantity (e.g. 2 μl) of PCR product or DNA positive (POS) or unrelated DNA (NSB) or diluted digoxigenin-labelled biotinylated primer (PLATE) to the appropriate wells.

10. Add a further 75 μl PBS/BSA to each well except SOB, and leave for 30 min at room temperature (shaking is optional).

11. Add 100 μl of 0.25 M NaOH to each well except SOB. Leave for 10 min at room temperature (shaking is optional).

12. Wash plate 3 times with Amersham wash buffer, using the Wellwash, as detailed in *wellwash*. The wells should be left dry.

13. Dilute appropriate digoxigenin-labelled probe to 0.2 pmol per 100 μl of Rapid Hyb buffer (allow 1 ml of probe dilution per strip, 10 ml per plate of 12 strips). Add 100 μl probe dilution to each well except SOB, BSA and PLATE. Add 100 μl neat Rapid Hyb buffer to PLATE. Cover plate with labfilm and incubate for 1½-2 h at 42°C.

### Immunosorption

14. Wash plate 3 times with Amersham wash buffer, using the Wellwash, as detailed in *wellwash*. The wells should be left dry.

15. Dilute anti-digoxigenin-AP 1:500 in PBS/BSA (allow 1 ml of dilution per strip, or 10 ml of dilution per plate of 12 strips). Add 100 μl to each well except SOB and BSA, and leave for 30 min at room temperature (shaking is optional). While incubation continues, pre-warm an aliquot of DEA solution to 37°C. Multiples of 5 ml are used (see Step 17).

16. Wash plate 3 times with Amersham wash buffer, using the Wellwash, as detailed in *wellwash*. The wells should be left dry.

17. Dissolve 1 tablet PNPP (5 mg, from Sigma) per 5 ml DEA (allow 5 ml of resulting solution per 5 strips or part thereof). Add 100 μl PNPP solution to each well. Incubate for 2 h at 37°C.

18. Read plate at 405 nm (630 nm differential), as detailed in *wellscan*.

### Reagents
Stock Biotin-NHS

See RPM 28

If not solid when removed from storage, do not use.

Cova Buffer

See RPM 15

Stock Avidin

See RPM 29

Amersham Wash Buffer

See RPM 16

PBS/BSA (per 96-well plate)

30 ml of PBS

0.3 g BSA (1%)

This should be made up freshly, though it can reportedly be frozen in aliquots. If you need less than a plate's worth, allow 3 ml per strip of 8 wells.

PBS

See RPM 2

0.25 M NaOH

See RPM 17

DEA

See RPM 27

Special Equipment

CovaLink/streptavidinylated plates, Gilsons and multichannel pipette, 15 ml and/or 50 ml centrifuge tubes, labfilm, plate shaker, plate washer, bijous, weighing boats (for multichannel work), plate reader
Method

1. Ensure that the waste bottle (the largest bottle) is attached and at least half-empty. Ensure that the rinse bottle (the smallest bottle) is attached and at least half-full. Ensure that the correct wash-head is fitted and for the program(s) to be used (see instrument manual).

2. If the washer is off, switch it on by the switch at the back. Wait for the program summary to appear.

3. Fill the Buffer A (rear medium-sized bottle) or Buffer B bottle (front medium-sized bottle) of the Wellwash Ascent with the appropriate buffer as dictated by the program summary.

4. Select the desired program using the SELECT key (see Appendix). If the buffer is the first of the day, or if it has been changed, press the PRIME key once. When the washer is ready (up to 5 secs), it will prime once by filling and emptying the wash-head reservoir. The washer is now ready and can be left until needed.

5. Insert the microtitre plate in a ‘vertical’ orientation. If less than 12 strips are to be processed, ensure that the strips are at the end furthest from you.

6. Press twice the key that corresponds to the number of strips that you want to wash, then press the START key. Abort the operation by pressing the STOP RESET key twice. When the washer is ready (up to 5 secs), it will begin.

7. If at any time, the LIQUID LEVEL light illuminates, check that enough buffer is in the appropriate bottle, or that the rinse bottle contains enough water, or that the waste bottle is not full. Remedy as appropriate.

8. To change a program (e.g. to aspirate instead of wash), select a new program with the SELECT key (see Appendix). If this causes a change of buffer, you must prime with the new buffer.

9. Repeat steps 5-8 as often as needed.

10. At the end of the day, clean the washer as follows: use the < or > keys to bracket SETUP at the bottom of the display. Press the \_ key. Use the < or > keys to bracket MAINTAIN. Press the \_ key. SLEEP should already be bracketed. Press the \_ key again.

11. When prompted, switch off the washer. Empty the waste bottle if necessary, rinsing it with water.

Appendix

Currently, there are four programs:

Program 1.1 is a wash protocol using Buffer A for three passes.

Program 2.1 is an aspirate protocol.
Program 3.1 is a 30 min shake protocol.

Program 4.1 is a wash protocol using Buffer B for four passes with a 15 sec soak each time.

The programs are currently set up for twin-headed protocols. This means you must have a dummy strip if you do not require an even number of strips to be processed.

<table>
<thead>
<tr>
<th>Version 1.2</th>
<th>Use of the Microtitre Plate Reader</th>
</tr>
</thead>
<tbody>
<tr>
<td>wellscan</td>
<td>09/11/00</td>
</tr>
</tbody>
</table>

**Method**

1. Check that the computer is not on standby by jiggling the mouse. If the computer does not respond, switch it on using the power button on the front.

2. Log on under your name by clicking on it and then clicking on the password box. Type in your password and click OK. If someone else is already logged on (i.e. a working desk-top is visible), check that they have finished with the computer and log them out by clicking on Start, then Log Off...

3. Switch on the microtitre plate reader using the power switch at the back. Wait until the display says Ready! before placing your plate in the reader.

4. Double click on the Genesis icon.

5. Click on the Run Assay menu button (the running man).

6. If the Protocol frame does not include the name of the desired protocol (e.g. c:\progra~1\genesis\protocol\450v630.prt) click on the Select... button in the Protocol frame. Using the file and directory lists, select the desired protocol file (e.g. c:\progra~1\genesis\protocol\450v630.prt) and click OK.

7. If the Plate Layout frame does not include the name of the desired plate layout file (e.g. c:\progra~1\genesis\protocol\4strips.plt) click on the Select... button in the Plate Layout frame. Using the file and directory lists, select the desired plate layout file (e.g. c:\progra~1\genesis\protocol\4strips.plt) and click OK.

8. Your data file will automatically be named after the current date + a ‘W’ (to indicate a raw data file) + a filename extension equivalent to the number of plates read so far (e.g. 001, 002, 003 etc.). You cannot change this file name.

9. Click RUN to begin reading the plate.

10. When the plate has been read, a preliminary results screen will be shown. Using the mouse and the right-hand mouse button, ‘kill’ any numbers that you do not want shown in the final report. ‘Unkill’ them by clicking them again. When finished, click OK.

11. A final report screen will be shown. Click Print Report to print it. Data will be printed first as duplicates.
corrected for meniscus effects (at 630 nm), then as means of those corrected values. Any further corrections must be done by you in Excel.

12. If desired, read another plate by clicking on the Run Assay menu button and proceeding as before.

13. When you have finished, if you wish to export your file (e.g. to Excel), click Export under the Results menu. Your file will be found in the c:genesis directory under the name automatically given to it. Select this file and click OK. Your file will be exported as a list of averaged readings to the a: drive (if you wish to change the file name or drive letter, do so in the appropriate box).

14. Close the Genesis program.

15. Log off the computer by clicking Start and then Log Off… DO NOT switch off or shut down the computer.

16. Switch off the microtitre plate reader using the power switch at the back.

17. If you wish to import exported data into an Excel spreadsheet, use Excel's Plate template.

**Special Equipment**

Multiskan EX microtitre plate reader
Using paraffin sections

Time period must be precise to be reproducible

1. Deparaffinise and hydrate slides
   - 2X xylene 5 minutes
   - 2X 100% ethanol 2 minutes
   - 1X 95% ethanol 2 minutes
   - 1X 80% ethanol 2 minutes

2. Wash in running tap water for 10 minutes (minimum)

3. Rinse in purified water

4. Stain in 0.1% picrosirius red overnight

Exactly 12 hours later

5. Place slides in 0.01N HCl for 2 minutes

6. 70% ethanol for 45 seconds

7. 80% ethanol 2 minutes

8. 95% ethanol 2 minutes

All above timings must be precise, as SR stain is removed by water
9. 2X 100% ethanol 2 minutes

10. 2X xylene 2 minutes

11. Mount slides using DPX (flammables cupboard) and glass coverslip. Use only as much as required, in a plastic 1ml syringe
## Appendix 2

<table>
<thead>
<tr>
<th></th>
<th>Proteinase K 1 mg/ml</th>
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<tbody>
<tr>
<td>1</td>
<td>ProK</td>
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<tr>
<td></td>
<td>09/06/00</td>
</tr>
</tbody>
</table>

Storage: -20°C ("Moynihan")

Shelf Life: 1 year

Ingredients: protinase K, 0.05 M Tris pH 7.65

To make 5 ml, dissolve 5 mg proteinase K in 5 ml 0.05 M Tris pH 7.65 in a sterile bijoux

Dispense into 50μl aliquots in sterile 0.5 ml Eppendorfs

For RNA work, pretreat the bijoux and spatula in 3% $\text{H}_2\text{O}_2$ for at least 20 min. Rinse them with DEPC water. Use DEPC Tris.

<table>
<thead>
<tr>
<th></th>
<th>Phosphate Buffered Saline (PBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>PBS</td>
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<tr>
<td></td>
<td>31/05/00</td>
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</tbody>
</table>

Storage: RT

Shelf Life: 6 months

Ingredients: PBS tablets, purified water

Dissolve 1 tablet per 200 ml purified water (25 tablets in 5 litres, 50 tablets in 10 litres)

For RNA work, use DEPC-treated water

<table>
<thead>
<tr>
<th></th>
<th>1 M Tris</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Tris1M</td>
</tr>
<tr>
<td></td>
<td>31/05/00</td>
</tr>
</tbody>
</table>
Ingredients: Tris, sterile distilled water, concentrated HCl

To make 100 ml, dissolve 12.114 g Tris in 70 ml sterile distilled water
Transfer to a stirrer in the fume hood and adjust the pH to the desired value using concentrated HCl and a pH meter
Make up to 100 ml with sterile distilled water, using a volumetric flask
For RNA work, pretreat the beaker, bottle(s), volumetric flask, spatula, flea and the end of the pH meter probe in 3% H$_2$O$_2$ for at least 20 min. Rinse them with DEPC water. Use DEPC water to dissolve the Tris.

<table>
<thead>
<tr>
<th>5</th>
<th>100 x TE</th>
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</thead>
<tbody>
<tr>
<td>100TE</td>
<td>31/05/00</td>
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</table>

Ingredients: Tris, EDTA, sterile distilled water, concentrated HCl

To make 100 ml, dissolve 12.11 g Tris and 3.72 g EDTA in 50 ml sterile distilled water
Transfer to a stirrer in the fume hood and adjust the pH 8.0 using concentrated HCl and a pH meter
Make up to 100 ml with sterile distilled water
For RNA work, pretreat the beaker, bottle(s), volumetric flask, spatula(s), flea and the end of the pH meter probe in 3% H$_2$O$_2$ for at least 20 min. Rinse them with DEPC water. Use DEPC water to dissolve the Tris and EDTA.
1 x TE is made from this 100 x stock

<table>
<thead>
<tr>
<th>6</th>
<th>0.5 M EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA05M</td>
<td>31/05/00</td>
</tr>
</tbody>
</table>
Storage: RT
Shelf Life: 1 year
Ingredients: EDTA, sterile distilled water, 5 M NaOH

To make 100 ml, mix 18.612 g EDTA with 60 ml sterile distilled water
Transfer to a stirrer in the fume hood and adjust the pH to 8.0 using 5 M NaOH and a pH meter. Heating the solution may help with dissolving the EDTA, but the pH will rise as the solution cools again.
Make up to 100 ml with sterile distilled water, using a volumetric flask

<table>
<thead>
<tr>
<th>7</th>
<th>1 M MgCl₂</th>
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<tbody>
<tr>
<td>MgCl₂</td>
<td>MgCl₂1M 31/05/00</td>
</tr>
</tbody>
</table>

Storage: RT
Shelf Life: 1 year
Ingredients: MgCl₂, sterile distilled water

To make 100 ml, dissolve 20.33 g MgCl₂ in 70 ml sterile distilled water
Make up to 100 ml with sterile distilled water, using a volumetric flask

<table>
<thead>
<tr>
<th>8</th>
<th>50 x TAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAE</td>
<td>50TAE 31/05/00</td>
</tr>
</tbody>
</table>

Storage: RT
Shelf Life: 6 months
Ingredients: Tris, EDTA, glacial acetic acid, sterile distilled water

To make 1 litre, mix 242 g Tris in 600 ml sterile distilled water
Add 100 ml of 0.5 M EDTA
In the fume hood, add 57.1 ml glacial acetic acid

Once all the ingredients are fully dissolved, make up to 1 litre with sterile distilled water

<table>
<thead>
<tr>
<th>9</th>
<th>10 x Gel Loading Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>10GLB</td>
<td>31/05/00</td>
</tr>
</tbody>
</table>

Storage: RT
Shelf Life: 1 year
Ingredients: 5 x LB -xc, 50 x TAE, glycerol, sterile distilled water

To make 20 ml, mix
- 2 ml 5 x LB -xc
- 2 ml 50 x TAE
- 12 ml glycerol
- 4 ml sterile distilled water

<table>
<thead>
<tr>
<th>11</th>
<th>10% SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>31/05/00</td>
</tr>
</tbody>
</table>

Storage: RT
Shelf Life: 1 year
Ingredients: SDS

USE APPROPRIATE PRECAUTIONS

To make 100 ml, weigh 10 g SDS into a beaker and add 50 ml sterile distilled water in the fume hood
Cover with laboratory sealing film until dissolved
Make up to 100 ml in a measuring cylinder
For RNA work, pretreat beaker, measuring cylinder, spatula and flea in 3% H₂O₂ for at least 20 min. Rinse them with DEPC water. Use DEPC water to dissolve the SDS.

<table>
<thead>
<tr>
<th>12</th>
<th>10 mg/ml Ethidium Bromide</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtBr</td>
<td>09/06/00</td>
</tr>
</tbody>
</table>

**Storage:** 4°C, foil-wrapped ("Patey")

**Shelf Life:** 1 year

**Ingredients:** ethidium bromide, sterile distilled water

**USE APPROPRIATE PRECAUTIONS**

**YOU MUST NOT USE THIS METHOD WITHOUT CONSULTING THE LABORATORY MANAGER**

To make 20 ml, dissolve 0.2 g ethidium bromide in 20 ml sterile distilled water

<table>
<thead>
<tr>
<th>15</th>
<th>Cova Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cova</td>
<td>09/06/00</td>
</tr>
</tbody>
</table>

**Storage:** 4°C ("Patey")

**Shelf Life:** 6 months

**Ingredients:** PBS, NaCl, MgSO₄·7H₂O, Tween 20

To make 1 litre, weigh out 116.9 g NaCl

Add 10 g MgSO₄·7H₂O

Dissolve to 1 litre with PBS

Add 500 µl Tween 20

<table>
<thead>
<tr>
<th>16</th>
<th>Amersham Buffer</th>
</tr>
</thead>
</table>
Storage: RT  
Shelf Life: 6 months  
Ingredients: PBS, Tween 20

To make 1 litre, add 200 µl Tween 20 to 1 litre of PBS

Alternatively, to make 10 litres, dissolve 50 PBS tablets in 10 litres of purified water
Add 2 ml Tween 20
Mix by magnetic stirrer until completely dissolved

<table>
<thead>
<tr>
<th>18</th>
<th>0.05 M Tris</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris005M</td>
<td>31/05/00</td>
</tr>
</tbody>
</table>

Storage: RT  
Shelf Life: 1 year  
Ingredients: 1M Tris, sterile distilled water

To make 20 ml, add 1 ml of 1 M Tris to 16 ml sterile distilled water
Transfer to the fume hood and adjust the pH to the desired value using concentrated HCl and a pH meter
Make up to 20 ml with sterile distilled water

<table>
<thead>
<tr>
<th>19</th>
<th>1 M Ammonium Sulphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmSO1M</td>
<td>31/05/00</td>
</tr>
</tbody>
</table>

Storage: RT
Shelf Life: 1 year

Ingredients: (NH₄)₂SO₄, sterile distilled water

To make 100 ml, dissolve 13.213 g of (NH₄)₂SO₄ in 70 ml sterile distilled water

Make up to 100 ml with sterile distilled water, using a volumetric flask

<table>
<thead>
<tr>
<th>20</th>
<th>10 mM dNTPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dNTPs</td>
</tr>
<tr>
<td></td>
<td>09/06/00</td>
</tr>
</tbody>
</table>

Storage: -20°C ("Moynihan")

Shelf Life: 1 year

Ingredients: dATP, dTTP, dCTP, dGTP, DEPC water

Using a new set of 100 mM (250 µl) dNTPs, mix together

- 250 µl dATP
- 250 µl dTTP
- 250 µl dCTP
- 250 µl dGTP
- 1500 µl DEPC water

Dispense as 100 µl aliquots

<table>
<thead>
<tr>
<th>21</th>
<th>1 M MnCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MnCl₁M</td>
</tr>
<tr>
<td></td>
<td>31/05/00</td>
</tr>
</tbody>
</table>

Storage: RT

Shelf Life: 1 year

Ingredients: MnCl₂, sterile distilled water
To make 100 ml, dissolve 19.79 g of MnCl₂ in 70 ml sterile distilled water
Make up to 100 ml with sterile distilled water, using a volumetric flask

<table>
<thead>
<tr>
<th>22</th>
<th>1.2 M Sodium Cacodylate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaCaco</td>
</tr>
<tr>
<td></td>
<td>09/06/00</td>
</tr>
</tbody>
</table>

Storage: 4°C ("Patey")
Shelf Life: 6 months
Ingredients: Cacodylic acid, sterile distilled water, concentrated HCl

USE APPROPRIATE PRECAUTIONS
YOU MUST NOT USE THIS METHOD WITHOUT CONSULTING THE LABORATORY MANAGER
To make 10 ml, weigh out 1.92 g of cacodylic acid into a sterile universal
Make up to just under 10 ml with sterile distilled water
Adjust the pH to 7.2 using concentrated HCl and a cleaned pH meter
Make up to 10 ml with sterile distilled water
Clean pH meter thoroughly
DO NOT AUTOCLAVE SOLUTION

<table>
<thead>
<tr>
<th>23</th>
<th>100 bp ladder</th>
</tr>
</thead>
<tbody>
<tr>
<td>100bp</td>
<td>09/06/00</td>
</tr>
</tbody>
</table>

Storage: -20°C ("Moynihan")
Shelf Life: 1 year
Ingredients: 100 bp ladder, 5 x LB -xc, sterile distilled water
To make 5 ml, mix 250 μl of 100 bp ladder into 1 ml of 5 x LB -xc

Add 3.75 ml sterile distilled water

Dispense as 4 x 1 ml and 50 x 20 μl aliquots

<table>
<thead>
<tr>
<th>24</th>
<th>25 mM Cobalt Chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoCl25mM</td>
<td>09/06/00</td>
</tr>
</tbody>
</table>

Storage: -20°C ("Moynihan")
Shelf Life: 1 year
Ingredients: CoCl₂, sterile distilled water

To make 100 ml, dissolve 0.5948 g of CoCl₂ in 100 ml sterile distilled water, using a volumetric flask
Dispense into 20 ml and 1 ml aliquots

<table>
<thead>
<tr>
<th>25</th>
<th>0.2 M EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA02M</td>
<td>31/05/00</td>
</tr>
</tbody>
</table>

Storage: RT
Shelf Life: 1 year
Ingredients: 0.5 M EDTA, sterile distilled water

To make 100 ml, add 40 ml of 0.5 M EDTA to 60 ml of sterile distilled water

<table>
<thead>
<tr>
<th>26</th>
<th>15 mM Sodium Azide in PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaAz</td>
<td>31/05/00</td>
</tr>
</tbody>
</table>

358
Storage: RT  
Shelf Life: 1 year  
Ingredients: Sodium azide, PBS

To make 100 ml, dissolve 0.0975 g of NaN₃ in 100 ml PBS, using a volumetric flask

<table>
<thead>
<tr>
<th>27</th>
<th>DEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEA</td>
<td>31/05/00</td>
</tr>
</tbody>
</table>

Storage: RT  
Shelf Life: 1 year  
Ingredients: DEA, concentrated HCl, purified water

To make 1 litre, add 105 ml concentrated (99%) diethanolamine to 800 ml distilled water  
Transfer to a stirrer in the fume hood and adjust the pH to 9.8 using concentrated HCl and a pH meter  
Make up to 1 litre with purified water

<table>
<thead>
<tr>
<th>28</th>
<th>NHS-Biotin</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHS-Biotin</td>
<td>09/06/00</td>
</tr>
</tbody>
</table>

Storage: 4°C ("Patey")  
Shelf Life: 6 months  
Ingredients: NHS-biotin, DMSO

To make 1 ml, dissolve 10 mg of NHS-biotin in 1 ml DMSO  
Dispense as 100 μl aliquots
29  

<table>
<thead>
<tr>
<th>Avidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avidin</td>
</tr>
</tbody>
</table>

Storage: 4°C ("Patey")

Shelf Life: 3 months

Ingredients: avidin, 15 mM sodium azide in PBS

To make up 1 ml, dissolve 10 mg of avidin in 1 ml of sodium azide

30  

<table>
<thead>
<tr>
<th>Stock Dilution of Primer/Probe Sets</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPSet</td>
</tr>
</tbody>
</table>

Storage: -20°C ("Starzl")

Shelf Life: -

Ingredients: Oligonucleotides, TE pH 8.0

Using the 'PCR ONLY' P200 Gilson, resuspend the oligonucleotide in enough TE to make a 200 pmol/µl stock solution. (Tip: if the oligonucleotide is provided lyophilised in nmol quantities, the volume of TE required equals the number of nmol synthesised multiplied by 5.)

Mix by vortexing for at least 15 seconds

Dispense this stock solution into 0.5 ml Eppendorfs as ~ 100 µl aliquots, leaving the last aliquot in the original tube provided

Label each tube on the lid with the primer name and its concentration, e.g. for 200 pmol/µl of ActF

Store the aliquots of stock solution at -20 °C in the clear-topped primer boxes, leaving a space between each group of oligonucleotide aliquots. Store the original tube in the blue box

31  

| Preparation of DEPC Water |

360
DEPCwat  31/05/00

Storage: RT
Shelf Life: 6 months
Ingredients: DEPC, sterile distilled water

USE APPROPRIATE PRECAUTIONS
YOU MUST NOT USE THIS METHOD WITHOUT CONSULTING THE LABORATORY MANAGER
In a fumehood, add 1 ml DEPC to each litre of sterile distilled water
Shake until well mixed
Leave overnight in the (running) fumehood
Autoclave

<table>
<thead>
<tr>
<th>32</th>
<th>Dynal Washing Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DynaWB</td>
<td>20/07/00</td>
</tr>
</tbody>
</table>

Storage: 4°C/-20°C ("Patey"/"Starzl")
Shelf Life: 3 months from placing at 4°C
Ingredients: 1 M Tris pH 8.0, 0.5 M EDTA pH 8.0, LiCl, DEPC water

To make 450 ml, pretreat bottle(s), spatula, weighing boat and measuring cylinder in 3% H₂O₂ for at least 20 minutes. Rinse them with DEPC water
Add 4.5 ml of 1 M Tris pH 8.0 to 200 ml of DEPC water
Add 0.9 ml of 0.5 M EDTA pH 8.0
Add 2.862 g of LiCl
Make up to 450 ml with DEPC water. Dispense as 50 ml aliquots. Store at -20°C until needed, whereupon store at 4°C.
33 Dynal Washing Buffer + LiDS (SDS)

| DynaWBL | 20/07/00 |

Storage: 4°C ("Patey")

Shelf Life: 6 months from placing at 4°C

Ingredients: 1 M Tris pH 8.0, 0.5 M EDTA pH 8.0, LiCl, DEPC water, 10% SDS

To make 50 ml, add 0.5 ml of 10% SDS to 49.5 ml Dynal Washing Buffer

Alternatively, to make 50 ml from fresh, pretreat bottle(s), spatula, weighing boat and measuring cylinder in 3% H₂O₂ for at least 20 minutes. Rinse them with DEPC water

Add 0.5 ml of 1 M Tris pH 8.0 to 25 ml of DEPC water

Add 0.1 ml of 0.5 M EDTA pH 8.0

Add 0.318 g of LiCl

Add 0.5 ml of 10% SDS

Make up to 50 ml with DEPC water

34 Working Dilutions of Primer/Probe Sets

| PPSet2 | 09/06/00 |

Storage: -20°C ("Morris")

Shelf Life: -

Ingredients: Oligonucleotide stock solutions, sterile distilled water

PCR primers:

Using one of the stock solution aliquots in the clear-topped boxes, make 500 μl of 10 pmol/μl F primer by diluting the stock solution in sterile distilled water (e.g. add 475 μl of sterile distilled water to 25 μl of stock
solution)

Mix well by vortexing for at least 15 seconds. Return unused stock solution to storage

Repeat for the R primer

Mix together equal quantities of 10 pmol/µl F primer and 10 pmol/µl R primer (e.g. 500 µl of 10 pmol/µl F primer and 500 µl of 10 pmol/µl R primer makes 1 ml of 5 pmol/µl F+R primers).

Vortex for at least 15 seconds

Dispense the working dilution of F+R primers as aliquots of 50 or 100 µl. Store at -20°C in your personal box(es)

Probes:

Using one of the stock solution aliquots in the clear-topped boxes, make 100 µl of 10 pmol/µl probe by diluting the stock solution in sterile distilled water (e.g. add 95 µl of sterile distilled water to 5 µl of stock solution)

Mix well by vortexing for at least 15 seconds. Return unused stock solution to storage

Store the dilution at -20°C in your personal box(es)

<table>
<thead>
<tr>
<th>35</th>
<th>Dynal Lysis/Binding Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DynalLBB</td>
<td>20/07/00</td>
</tr>
</tbody>
</table>

Storage: 4°C/-20°C ("Patey"/"Starzl")

Shelf Life: 6 months from placing at 4°C

Ingredients: 1 M Tris pH 8.0, 0.5 M EDTA pH 8.0, LiCl, 10% SDS, 1 M DTT, DEPC water

To make 450 ml, pretreat bottle(s), spatula, weighing boat and measuring cylinder in 3% H₂O₂ for at least 20 minutes. Rinse them with DEPC water.

Add 45 ml of 1 M Tris pH 8.0 to 200 ml of DEPC water

Add 9 ml of 0.5 M EDTA pH 8.0

Add 9.537 g LiCl

Add 45 ml of 10% LiDS (SDS)

Add 2250 µl of 1 M DTT

Make up to 450 ml with DEPC-treated water. Dispense as 50 ml aliquots. Store at -20°C until needed, whereupon store at 4°C.
10 x AJ Buffer

AJBuff  09/06/00

Storage: -20°C ("Morris")

Shelf Life: 6 months

Ingredients: 1 M Tris pH 8.8, 1 M (NH₄)₂SO₄, 1 M MgCl₂, 100 mM ultrapure dNTPs, 20 mg/ml ultrapure BSA, b-mercaptoethanol, 1 mM EDTA pH 8, sterile distilled water

YOU MUST NOT USE THIS METHOD WITHOUT PRIOR TRAINING FROM THE LABORATORY MANAGER. USE THE 'PCR ONLY' GILSONS FOR ALL DISPENSING

To make 2 ml, dispense 900 μl of 1 M Tris pH 8.8 into a bijou

Add 220 μl of 1 M (NH₄)₂SO₄

Add 90 μl of 1 M MgCl₂

Add 40 μl each of 100 mM ultrapure dATP/dGTP/dCTP/dTTP

Add 110 μl of 20 mg/ml ultrapure Bovine Serum Albumin

Add 8.8 μl of 1 mM EDTA pH 8

Add 501.8 μl of sterile distilled water

In a fume hood, add 9.4 μl of neat b-mercaptoethanol

Wrap inside one of your gloves the pipette tip used to dispense the b-mercaptoethanol. Wrap this glove inside the other glove, and dispose

Mix the 10 x AJ buffer gently but thoroughly using a vortex mixer

Dispense as 100 μl aliquots

0.1% Picrosirius Red

RPM045_Picro  01/11/00

Storage: RT
Protocol for making 0.1% picrosirius red (500ml).

Must be done in fume hood

Picric acid crystals must be kept hydrated at all times (store under water). Explosive when dry.

1. Make 1% sirius red solution (1g in 100ml purified water, dissolve by stirring)
2. Prepare 3% picric acid saturated solution
   - 15g picric acid in 500ml purified water
   - heat and stir with caution
   - not all crystals will dissolve, dissolve as much as possible
3. Combine 50ml of 1% sirius red with 450ml saturated picric acid, final concentration of sirius red 0.1%
4. Add picric acid crystals to saturate the above mixture, until crystals visible at bottom of container. Use a pH meter to ensure pH 2.0.
5. Let dye stand in fume hood for 24 hours and check that crystals remain at bottom of container.
6. Picrosirius dye is stable over a long period, at least 3 months.
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