The Role of Inflammation in the Expression of Matrix Metalloproteases in Abdominal Aortic Aneurysms

A Thesis for the Degree of Doctor of Medicine

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Synopsis

Abdominal aortic aneurysms are a common and life-threatening condition. At the current time the only treatment available is major surgery. This thesis is concerned with attempts to find a pharmacotherapy in order to reduce both the need for surgery and the devastating consequences of aneurysm rupture. The first two chapters are literature reviews to establish an understanding of the pathogenesis of aneurysms and to review past attempts at medical therapy. From these, the twin processes of inflammation and matrix metalloprotease activity were identified as potential therapeutic targets. It was hypothesised that either a COX-2 inhibitor or a statin might have the required activity against both of these processes, and in vitro experiments to determine this were carried out in Chapters Four and Five. From these experiments it became clear that rofecoxib (a COX-2 inhibitor) would not be a suitable drug, whereas simvastatin did affect both inflammation and MMP activity. Chapter Six describes experiments undertaken in order to elucidate the pathways involved in mediating these beneficial effects, whilst Chapter Seven deals with the effects of simvastatin on human aneurysmal tissue in a double-blinded, controlled and randomised clinical trial.

Although recruitment into the clinical trial had to be curtailed for ethical reasons following the publication of the Oxford Heart Protection Study, the preliminary results from both this study and the laboratory work suggest that statins may well have a role to play in the prevention of aneurysm development.
Chapter 1

Pathogenesis of Abdominal Aortic Aneurysms

1.1 Introduction

This thesis was undertaken with a simple aim in mind. It was hoped that by gaining an understanding of the processes involved in the development of abdominal aortic aneurysms, a pharmacological means of preventing aneurysm growth might be found. The first part of the work, then, involved comprehensive internet searches to identify research into aneurysm pathogenesis.

In recent years, the pathophysiology of abdominal aortic aneurysms (AAAs) has become better understood. Whilst it is accepted that biomechanical effects of hypertension and increased physical forces do have a role to play, there is now increasing interest in the biochemical and cellular events occurring within the aortic wall. It has been recognised that a greater understanding of these events may well explain why aneurysms rupture at different sizes in different individuals, despite risk factors remaining the same. In addition, it is hoped that an understanding of these events may lead to new strategies for medical treatment of aneurysms.

The fundamentals of research into aneurysm pathogenesis can be divided into seven broad areas.

1. Epidemiology
2. Histology
3. Genetics
4. Biomechanical effects of blood flow within the aorta
5. Abnormal proteolysis
6. Inflammation and the immune response, and more recently
7. Oxidative stress

By necessity, there is much overlap between these areas, but for the purposes of this review, each will be considered separately.

1.2 Epidemiology

One of the first steps in studying a disease process is to examine the epidemiology. The identification of disease associations is never sufficient to prove causality, but will at least provide a scientifically sound basis for further study. Associations may be both positive and negative, and some remain unclear. Proven associations with abdominal aortic aneurysms may be divided into eight areas;

1. Family History
2. Smoking
3. Gender
4. Age
5. Lipids
6. Blood pressure
7. Race
8. Diabetes
1.2.a Family History

Familial studies may well help to identify whether there is a genetic component to the disease pathogenesis. Family studies into AAA began in earnest in 1977, when Clifton first reported a family of three brothers, all of whom underwent surgery for AAA (Clifton, 1977). By 1984, Tilson had a series of 50 families in whom the incidence of AAA was too high to be explained by random chance. In 1989, Bengtsson et al published the results of a trial of screening siblings of patients with known AAA (Bengtsson et al. 1989). Of 87 patients who accepted the invitation for screening, 10 brothers (29%) and 3 sisters (6%) were found to have dilatation of the aorta, a much higher prevalence than in the general population. Bengtsson has also looked at the prevalence of AAA in the offspring of patients dying from ruptured AAA, and in that study 8 out of 39 sons (20.5%) were found to be affected. Salo's more recent study (Salo et al. 1999) showed that having an affected sibling increased the relative risk by 4.33 times, such that the prevalence in male siblings over 60 was as high as 18%. This risk factor was independent of the sex or age of the proband.

1.2.b Smoking

Smoking has been clearly and irrefutably associated with both AAA and atherosclerosis in numerous studies. Wilmink et al reported a case-control study including 447 individuals, and concluded that smoking increased the relative risk by 7.6 times (Wilmink et al. 1999). Moreover, even ex-smokers had a three-fold increase in risk. Duration of smoking was important, with each year of smoking increasing the relative risk of AAA by 4%. The amount smoked was not shown to
influence the prevalence of AAA, and after stopping smoking the risk declined only very gradually. Interestingly, smoking gave a higher relative risk of small rather than large aneurysms. The amount smoked was estimated by the level of plasma cotinine, a metabolite of nicotine. This was higher in patients with small AAAs. Perhaps unexpectedly, the levels of cotinine did not differ between patients with stable or expanding aneurysms. These findings suggest that smoking is a risk factor for aneurysm initiation rather than expansion.

This contrasts markedly with MacSweeney’s report in The Lancet (MacSweeney et al. 1994). This study followed 43 patients with small aneurysms for 3 years, and assessed the size of their aneurysms with serial ultrasound scans. Growth rates in patients who did not smoke averaged 0.9 mm per year compared to 1.6 mm per year in smokers (p=0.038). These higher growth rates correlated significantly with serum cotinine levels. Vardulaki’s results (Vardulaki et al. 2000) also contrast with those of Wilmink – in this study the level of exposure to cigarette smoke was found to be more significant than the duration of smoking.

As yet, nobody has proven a causative link between smoking and AAA formation, but one paper looking at the effect of cigarette smoking on fluid extracted from iatrogenic skin blisters provides a possible theory (Knuutinen et al. 2002). In this study, blister fluid from the skin of smokers showed lower rates of collagen synthesis, lower levels of the tissue inhibitor of metalloprotease TIMP-1 and higher levels of the matrix metalloprotease, MMP-8.
1.2.c and 1.2.d Gender and Age

In common with atherosclerotic disease, males have consistently been shown to be affected more than females (Castleden and Mercer, 1985; Lawrence et al. 1999; Lederle et al. 2001). Similarly, the age is a well-recognised risk factor, the incidence increasing with age. Papers dealing with these factors are summarised in table 1.

1.2.e Plasma lipid levels

There is a more complicated relationship between plasma lipid levels and the risk of AAA. Blanchard et al failed to show any correlation between cholesterol levels, LDL or HDL and aneurysm risk (Blanchard et al. 2000). Tomwall, however, showed an increased risk in patients whose plasma cholesterol was high and a protective effect was seen in patients whose serum HDL was high (Tomwall et al. 2001). Conversely, Singh showed that low serum HDL gave an increased risk of AAA. Lederle’s work with the American veterans also showed high cholesterol to be an independent risk factor (Lederle et al. 2001; Lederle et al. 1997).

1.2.f Blood Pressure

There is some disagreement in the literature regarding the effect of hypertension on aneurysm risk. The American Veterans study represented the largest of its type, and showed hypertension to be an independent risk factor. Singh, however only showed that taking medication for high blood pressure was a risk factor, whereas hypertension itself was significant in women. Tornwall and Blanchard both showed both systolic
and diastolic hypertension to be risks. A study of all men born in Malmo in the year 1914 failed to demonstrate hypertension as a risk factor at all (Bengtsson et al. 1991). Experimentally, AAAs artificially induced into hypertensive rats were found to grow larger than those in normotensives, (Anidjar et al. 1992) and the dilatation correlated well with systolic pressure.

1.2g Race

AAA is predominantly a disease of Caucasians; indeed one recent study of familial AAAs identified 233 multiplex families in a multinational investigation and all of them were white (Kuivaniemi et al. 2003). Racial studies have predominantly come from America and have concentrated on the different prevalences in the Afro-American and Caucasian communities. Lederle has shown a reduced risk in black people and LaMorte has shown a black:white odds ratio of 0.29 (LaMorte et al. 1995). Whether or not this racial disparity is related to the distribution of diabetes is an interesting theory but remains unproven.

One English study has looked at the racial differences in patients presenting with aneurysms in Bradford, UK (Spark et al. 2001). Over a 7-year period, 233 AAAs were identified. Given the racial mix of the catchment area, 28 of these could have been expected to be in Asian patients. In reality, none were. Although assumptions made on this evidence alone would be fraught with difficulties given differing rates of racial uptake of healthcare facilities, it does provide some evidence that AAA is more common in people of Caucasian origin than Asians.
1.2. Diabetes

Diabetes is known to be a risk factor for atherosclerotic disease, but has been shown consistently to be protective against abdominal aneurysms (Blanchard et al. 2000; LaMorte et al. 1995; Lederle et al. 1997) (See table 1).

1.3 Histology

Microscopic examination of the structure of the wall of AAAs and comparison with the normal structure of the aortic wall gave some of the earliest insights into the development of AAAs. The normal structure of the aortic wall consists of well-organised elastin lamellae held together in a collagen matrix. By contrast, aneurysms are characterised by a loss of elastin lamellae, changes in the elastin:collagen ratio, and a marked inflammatory infiltrate. Zatina demonstrated that loss of the normal lamellar architecture can lead to aneurysm formation, and Terpin showed a decrease in the number of aortic elastin layers in a group of lathyritic turkeys that were highly susceptible to aortic rupture (Terpin and Roach, 1987; Zatina et al. 1984).

Inflammation is a feature of nearly all aneurysms, and in vitro experiments by Anidjar et al showed that the intensity of the inflammatory infiltrate correlated well with the size of the enlarging aneurysm. A standardised scale of inflammatory response has been suggested, the Histological Inflammation Scale of Aneurysms. This categorised aneurysms into five groups;

A. Mixed acute and chronic inflammation

O. No inflammation
1. Mild chronic inflammation
2. Moderate chronic inflammation
3. Severe chronic inflammation

The majority of aneurysms fell into group 1, whereas 5.4% fell into group 3, marking them out as “inflammatory” aneurysms (Rijbroek et al. 1994).

A study of mRNA levels in normal and aneurysmal aortic walls showed that there was an increase in the expression of mRNA coding for collagen I, but that mRNA coding for elastin was not increased. This may, in part, explain the changes seen in the elastin:collagen ratio in AAAs (Mesh et al. 1992). Whether or not the amount of elastin cross-linkages is relevant to AAA formation is a matter of some debate, but Gandhi et al showed that an apparent decrease in elastin cross-linkage was only in proportion to the absolute decrease in elastin content (Gandhi et al. 1994). Conversely, Minion argued that elastin actually increased in AAAs, but not to the same degree as collagen (Minion et al. 1994).

1.4 Biomechanical Effects

It has long been known that aortic aneurysms occur mainly in the infrarenal aorta, and as far back as 1967 it was suggested that this might be due to a reduction in the elastic lamellae in this region when compared to the thoracic aorta (Wolinsky and Glagov, 1967). Zatina et al investigated the role of the medial lamellar architecture in the formation of aneurysms in porcine thoracic aorta (Zatina et al. 1984). Two hypotheses were postulated as to why the human infra-renal aorta was particularly susceptible to aneurysm formation: firstly that the blood supply to the vessel wall was
less at this point than is seen in other areas of the aorta, and secondly that the number of elastin lamellae was smaller than in other mammals of comparable size.

To test these hypotheses, the vasal blood flow of the porcine aortas was surgically ablated, and the aortas were physically crushed to reduce the number of intact lamellae. The findings were revealing; whilst ligation of the vasa vasorum led to cell death in the medial compartment it did not lead to significant alterations in the extracellular matrix, nor to the formation of aneurysms in the 2-month period of follow-up. However, destruction of the elastic lamellae led to aneurysm formation in both ischaemic and non-ischaemic aortas, providing the number of intact lamellae fell beneath a critical threshold. The authors pointed out, however, that prolonged mural ischaemia may eventually lead to alterations in the matrix composition and so could not be discounted as a contributing factor.

Elastin supplies most of the expansile properties of the aorta, as well as contributing to tensile strength. Decreased elastin therefore leads to higher pressures, greater shearing forces and a weaker aortic wall. Combined with the fact that elastin concentration declines with age and is not replaced, this explains why the peak age incidence for AAAs is in the eighth decade (Collin, 1992; Scott et al. 1991).

In 1991 Anidjar and Dobrin demonstrated that a reduction in elastin alone was sufficient to allow dilatation of the vessel in arteries cultured in vitro, and that loss of elastin alone caused the characteristic tortuosity seen in ectatic vessels (Dobrin and Anidjar, 1991). However, loss of collagen from the vessels also led to dilatation, and perhaps more significantly also caused the vessel to rupture in every case. This
experiment utilised both canine carotid arteries and human iliac arteries in vitro, but considerable work has been done using a similar in vivo model (Anidjar et al. 1990).

In this model, healthy adult rats underwent laparotomy, at which point ligatures were placed around a section of the infra-renal aorta. Once the section was isolated, it was infused with elastase, which initiated the protein degradation. The elastase was then washed out and the circulation restored. This technique always led to aneurysm formation when pancreatic elastase was infused, but other agents have also been investigated (Dobrin and Anidjar, 1991).

Quantifying the extent to which biomechanical variables contribute to aneurysm formation has not been easy. Many experimental studies have suggested that hypertension should be a risk factor, notably those of Tilson and Reilly (Anidjar et al. 1992; Reilly and Tilson, 1989). However, there remains no consistent epidemiological data to support this hypothesis. Nevertheless, experimental models of aneurysmal disease have shown that hypertension can increase both the size of the aneurysm and the rate of rupture (Ricci et al. 1996b). What is not clear is the mechanism by which hypertension can induce or support the formation of aneurysms. In one study, patients on beta-blocking medication had a reduced incidence of ruptured aneurysms, and there has been considerable work undertaken to investigate the reasons for this (Gadowski et al. 1994; Leach et al. 1988; Ricci et al. 1996a).

Clinical experiments into the use of beta blockade to retard aneurysm growth were thus thought to be of utmost importance, and a multi-centre, double-blinded, randomised and controlled trial was initiated. The results of this trial were reported in 2002 (Anonymous 2002b). Five hundred and forty-eight patients with small AAAs
were randomised to receive either propranolol or placebo and were followed-up for a mean of 2.5 years. The trial showed that growth rates in the placebo and study arms were similar (0.26 cm per year for the placebo group and 0.22 cm per year in the study group, p 0.11). There was a slight increase in the tendency to perform elective repair in the placebo group, but there was no difference in survival rates. During follow-up, 42% of patients taking propranolol stopped taking their medication compared to only 27% of those taking placebo. In addition, when the quality of life of patients in the two arms of the study was assessed using the SF-36 questionnaire, those taking beta-blockers performed significantly worse. These results show that propranolol does not significantly affect growth rates or survival, and is also poorly tolerated by patients.

The Stanford group has developed a model to look at atherosclerosis in hypertension (Tropea et al. 1996). New Zealand white rabbits were fed an atherogenic diet for three weeks to initiate plaque formation. Dacron bands were then placed around the thoracic aorta to create a hypertensive segment proximal to the stenosis. That this area did indeed become hypertensive was confirmed by comparison to both operated controls with no dacron bands and to non-operated controls. In the banded group, the mean systolic pressure was 89 mmHg +/- 3 compared to 64 mmHg +/- 4 in the operated controls and 74 mmHg +/- 3 in the non-operated group. The main findings in this experiment were that when the hypertensive section of aorta was analysed immunohistochemically and using epifluorescent microscopy, there was a dramatic increase in monocyte binding, and in the expression of endothelial Vascular Cell Adhesion Molecule-1 (VCAM-1). These were accompanied by increased intimal thickness and an accumulation of macrophages. A follow-up experiment looked at the same model of hypertension but this time added either a loose or firm wrap around the
hypertensive segment (Tropea et al. 2000). This had the effect of preventing excessive wall motion when compared to the coarcted group with no wrap, leading to a level of wall motion similar to that seen in the non-coarcted aorta. Interestingly, despite the hypertension, the level of intimal thickening in the aortas in which excessive wall motion was inhibited was much less than that seen in the coarcted group without a wrap. These results suggest that it is the motion of the vessel wall that is a key factor and not purely the rise in blood pressure.

Another series of aortic banding experiments investigated the effects of coarctation on the nitric oxide system, and will be dealt with in the section concerning oxidative stress (Barton et al. 2001a; Barton et al. 2001b).

1.6 Aspects of Aneurysm Genetics

Aortic aneurysms share many of the same risk factors as atherosclerosis such as age, sex and smoking, and for this reason were considered to be a product of the same disease process. However, in the mid-1980s evidence began to accumulate to suggest that abdominal aortic aneurysms were more common in caucasians than in Afro-Caribbeans, whilst the reverse is true of atherosclerosis (LaMorte et al. 1995). In addition, the fact that aneurysms tend to run in families was first documented in 1977, and since then several other observers have identified a familial component (Clifton, 1977) (Collin and Walton, 1989; Tilson and Seashore, 1984). Proving a family link is not easy, and Collin has outlined some of the problems involved (Collin, 1996).

Questionnaires suffer three major drawbacks. Firstly, asking patients whether any of their relatives have aneurysms will only identify the incident cases, whereas the
lifetime prevalence is at least three times the incidence. Secondly, due to the age of most AAA patients, it is possible that they may be separated from other members of their family and may be unaware of or have forgotten their families' medical histories. Thirdly, as AAA is a disease of old age, the relatives of the patient may simply be too young to have developed an aneurysm at the time of the questionnaire. Similarly, many patients with a propensity to develop aneurysms will die of other causes before the disease manifests itself. Nonetheless, telephone questionnaires have been undertaken. Verloes et al performed a segregation analysis on data obtained from 324 probands, concluding that a single gene effect with dominant inheritance was the most likely explanation for the pattern of inheritance seen in their subjects (Verloes et al. 1995).

Family screening studies have been conducted successfully using ultrasound, giving an average prevalence of 24% in first order male relatives compared with between 5-10% in males with no affected relatives (Bengtsson et al. 1989; Webster et al. 1991) (Adams et al. 1993; Baird et al. 1995; Fitzgerald et al. 1995). With this strong evidence that aneurysms were familial and not simply an advanced form of atherosclerosis, the search for genetic factors began in earnest.

The pattern of inheritance of possible candidate genes for aneurysmal disease has been investigated in an important recent paper. Kuivaniemi led an international team that was able to identify 233 families with multiple affected members, every single family being Caucasian (Kuivaniemi et al. 2003). There was an average of 2.8 members of each family affected, although some had 6 or 7, and one family even had eight members affected. It was found that no single inheritance mode could explain
the pattern of inheritance seen; 72% of cases could be explained by an autosomal recessive gene, whilst 25% could have been autosomal dominant. The remainder would have had to be an autosomal dominant with incomplete penetrance.

A variety of genes have been suggested as likely candidates. These include genes coding for the matrix proteins, for proteolytic enzymes and their inhibitors and two genes on chromosome 16 coding for the haptoglobin alpha chain and its neighbouring cholesterol-ester transfer protein gene (Verloes et al. 1996).

Collagen is the major constituent of the extracellular matrix, particularly types I and III. Type III has been shown to be deficient in a group of patients with familial AAA (Menashi et al. 1987) and a variation of the gene COL3A1 has been associated with the development of AAAs. Interestingly, the aneurysm wall in patients with this variant was found to be less elastic (Powell et al. 1991). Kontusaari investigated two families with mutations in this gene that developed aneurysms in a dominantly inherited manner (Kontusaari et al. 1990). In one of these families, collagen type III synthesis was severely impaired, and they went on to develop a range of symptoms from AAA to full-blown Ehlers-Danlos syndrome. Conversely, by sequencing DNA from 50 unrelated patients with AAA, Tromp et al showed that mutations in the gene coding for collagen type III were in fact a rare cause of AAAs (Tromp et al. 1993).

An examination of the influence of differing collagen gene expression revealed some interesting results. Whereas mRNA for type I and type III collagen was increased in patients with aneurysms and in patients with aorto-occlusive disease, the actual concentration of collagen in aneurysmal aortas was reduced. This implies that far
from varying rates of collagen synthesis causing aneurysm development, the real key
is more likely to lie in different rates of collagen breakdown (McGee et al. 1991).

By contrast, Mesh at al (Mesh et al. 1992) sought to explain the different ratios of
elastin:collagen in AAAs in terms of increased collagen synthesis rather than an
increase in elastolysis. They showed that the expression of mRNA for type I collagen
was indeed upregulated in aneurysms and that the synthesis of elastin failed to
increase accordingly. This effect is independent of age, and the decrease in elastin
may explain why AAAs develop in these patients.

The Tissue Inhibitors of Metalloproteases (TIMPs) are natural antagonists of the
MMPs, and one hypothesis suggests that aneurysm development may be a result of
either a mutation in the genes coding for these molecules or a failure in expression.
However, when Tilson looked at the expression of TIMPs by fibroblasts from AAAs,
he found these were all at normal levels. In addition, whilst a single-point mutation of
the gene coding for TIMP-1 was found in two out of six patients, this did not affect
the amino acid transcription (Tilson et al. 1993).

The cathepsins are cysteine proteases with powerful elastolytic properties. They have
been demonstrated in atherosclerotic plaque, and cultured smooth muscle cells
secreted cathepsin-S when stimulated with interferon-γ or interleukin 1β (Sukhova et
al. 1998). In arterial smooth muscle cells they are naturally inhibited by cystatin C,
for which a polymorphism has been identified in the signal peptide. In a study of 424
patients with AAAs, the growth rate of the aneurysm was significantly less in patients
with the homozygous allele, AA. This suggests that the cathepsins have a role in aneurysm development (Eriksson et al. 2004).

Autoimmunity has been postulated as a possible cause of AAAs, and a study from Japan looked at the link between HLA haplotype and aneurysm prevalence (Hirose et al. 1998). They observed that the HLA-DR2 (15) antigen was found in twice as many patients with AAA as without. Another Japanese case-control study aimed to investigate the relationship between mutation of the gene coding for platelet-activating factor acetylhydrolase (PAF-AH) and aneurysms. They found a significant increase in the mutant allele in patients with aneurysms than in the control group (Unno et al. 2002). Another polymorphism that has been investigated consists of a single base pair deletion in the plasminogen activator inhibitor, PAI-1. The proportions of patients with the three genotypes (4G/4G, 4G/5G and 5G/5G) in the group with AAAs matched that of the general population. However, those with the a heterozygous pattern displayed faster aneurysm growth than the homozygous 4G/4G, and the 5G/5G showed faster growth still (p=0.07) (Jones et al. 2002).

Genetic variation in the haptoglobin gene and neighbouring cholesterol ester transfer protein gene on the long arm of chromosome 16 were found to be increased in patients with AAAs when compared to healthy control subjects (Powell et al. 1990). These genotypes were associated with an increased rate of aneurysm expansion, and homogenates from the aortic walls of such patients also displayed increased proteolysis in vitro.
An exciting prospect for the future is the use of human gene array technology. One recent study was able to evaluate 265 different genes in a group of patients with either AAA, aorto-occlusive disease (AOD) or neither (Armstrong et al. 2002). Of the 265 genes studied, 11 showed significant differences when corrected and standardised. The results for these 11 genes are summarised in table 2.

1.6 Abnormal Proteolysis

The elastin: collagen ratio has consistently been shown to be reduced in AAAs when compared to normal aortas, (Dobrin et al. 1996; Grange et al. 1997; Satta et al. 1998; Treharne et al. 1999) leading to loss of elasticity and weakening of the aneurysmal wall. This may not be simply due to increased elastin degradation, as Minion has shown that the total elastin content of the aneurysmal wall may actually increase, but that the corresponding increase in collagen is much greater (Minion et al. 1994). Despite this evidence, there is little doubt that proteolysis plays an important role in aneurysm development (Anidjar and Kieffer, 1992). Aneurysmal disease differs from stenotic disease by the intensity of proteolytic activity within the extracellular matrix. The established association with chronic lung disease supports the argument that elastolysis is a major contributory factor, and indeed this is an area in which there has been much research (Shah, 1997; Thompson et al. 1995; Vine and Powell, 1991).

For some time, the cause of elastin degradation remained unknown, but even as early as 1980 Busuttil described increased collagenase activity (Busuttil et al. 1980).
Powell and co-workers in 1991 found a spectrum of collagenase activity in the aortic wall of both atherosclerotic and aneurysmal vessels ranging from 55-92 kDa (Vine and Powell, 1991). Importantly, although the collagenase activity was limited, it increased dramatically when tissue inhibitors of metalloproteases (TIMPs) were destroyed. Thompson et al also described the increased expression of a 92 kDa gelatinase in AAAs when compared to both normal aortas and aorto-occlusive disease, and localised this to the area around infiltrating macrophage (Thompson et al. 1995). This gelatinase is part of a family of zinc-dependent proteolytic enzymes, the matrix metalloproteases (MMPs), now known as MMP-9. In the same year, Powell’s group further elucidated the relative amounts of both MMP-9 and MMP-2 by a combination of gelatin zymography and immunoblotting (Freestone et al. 1995). This study demonstrated that the principal gelatinase in smaller aneurysms was MMP-2, but that in larger aneurysms MMP-9 predominated. McMillan investigated mRNA levels for MMPs in AAAs and found that MMP-9 was maximally expressed in moderate diameter (5-6.9cm) rather than large (>7cm) or small (<4cm) aneurysms. These findings suggested that whilst MMP-9 was responsible for the rapid growth that was seen in this size of aneurysm, other enzymes were responsible for initiation and rupture.

Pyo’s paper elegantly proves a link between MMP-9 and aneurysm pathogenesis by looking at the effect of inhibiting it both pharmacologically and by targeted gene disruption (Pyo et al. 2000). Mice that were deficient in the MMP-9 gene failed to develop aneurysms as their wild-type counterparts did when subjected to elastase perfusion of the aorta. Bone marrow transplant from each group to the other reversed
the response to elastase infusion, demonstrating that the expression of MMP-9 by inflammatory cells is crucial to aneurysm development.

Other MMPs have also been implicated in the development of AAAs, (Irizarry et al. 1993; Newman et al. 1994b; Newman et al. 1994c) particularly MMP-1 and MMP-3. Vine and Powell also found immunoreactive MMP-1 in extracts from AAAs (Vine and Powell, 1991), and more recently the expression of MMP-3 as measured by reverse transcriptase polymerase chain reaction (rt-PCR) was found to be elevated in AAAs when compared to aorto-occlusive disease (Carrell et al. 2002).

MMP-13 is a recently described enzyme also known as collagenase-3, and its expression is tightly regulated. Mao measured its concentrations in AAA and atherosclerotic disease using rt-PCR and compared them to normal aortas taken from organ donors (Mao et al. 1999a). Whilst MMP-13 was not expressed at all in normal tissue, it was found in atherosclerotic disease and also in significantly higher concentrations in AAAs. Expression was localised to medial smooth muscle cells (SMCs) in the aortic tissue, and could also be detected in SMCs.

Membrane type MMP-1 (MT- MMP-1) is an activator of MMP-2 and was found to be increased in aneurysmal aorta when compared to normal or atherosclerotic aorta (Nishimura et al. 2003). MT- MMP-1 was localised to aortic smooth muscle cells and macrophages in aneurysmal tissue by immunohistochemical analysis. The ability to activate MMP-2 was confirmed by the addition of radiolabelled pro MMP-2, and determination of the subsequent amount of radiolabelled active MMP-2. However,
other experiments by Yamashita failed to show any difference in the levels of MT-MMP-1 between aneurysmal aorta and aorto-occlusive disease.

*In vivo*, the activity of matrix metalloproteases is tightly controlled by their natural inhibitors, the tissue inhibitors of MMPs, or TIMPs. In 2000, Olson demonstrated that TIMP-1 bound to both the monomeric and dimeric forms of MMP-9, whereas TIMP-2 bound only to the active form (Olson et al. 2000). Whilst it has been shown that the TIMPs are present in large quantities in AAAs, it has been suggested that it is an imbalance between MMPs and TIMPs that leads to the net increase in proteolysis seen (Knox et al. 1997; Mao et al. 1999b). Knox in particular showed that whilst the expression of TIMPs was increased in both AAA and AOD when compared to normal aorta, the increase was not in proportion to that of MMPs. Tamarina also showed that the TIMP:MMP ratio was actually decreased in AAA, despite an absolute increase in TIMP levels (Tamarina et al. 1997).

Whilst there has been considerable work published in the area of collagenases and other metalloproteases in AAAs, less is known about the role of serine proteases. Elastases of approximately 20-30 kDa have been demonstrated in the inner aspect of the media in AAAs (Rao et al. 1996). This elastase works best in the alkaline range, and is inhibited by α-1 anti-trypsin. The fact that it is also inhibited by phenylmethylsulfonyl fluoride (PMSF) confirms that it is indeed a serine protease. Five distinct serine proteases have been separated by gel electrophoresis from aortic aneurysm tissue (Herron et al. 1991), suggesting there is a spectrum of enzymes at work.
The third type of elastase is the cysteine protease group. These differ from serine proteases by the substitution of an Asn residue for an Asp in the catalytic triad. Cathepsins S and K are examples of this type of elastase and have been shown to be produced in abundance by smooth muscle cells in atheroma (Sukhova et al. 1998). They are inhibited by cystatin C, the expression of which is governed by a polymorphism of its signal peptide. As discussed previously, patients in whom the cathepsins are not inhibited displayed faster growing aneurysms (Eriksson et al. 2004).

1.7 Inflammation and the Immune Response

Inflammation is a characteristic feature of all AAAs, not just those previously thought of as “inflammatory aneurysms” (Rijbroek et al. 1994; Satta et al. 1998) (Shah, 1997; Wassef et al. 2001). In 1992, Anidjar and Dobrin demonstrated that during the development of aneurysms using the rat model, the timing of the inflammatory infiltrate correlated well with aneurysm expansion (Anidjar et al. 1992). Elastolysis continued well after the infusion of elastase was discontinued, suggesting the inflammatory infiltrate was capable of activating endogenous elastolysis (Anidjar et al. 1994). Similarly, Freestone showed that in enlarging AAAs there was a denser inflammatory infiltrate when compared to small aneurysms (Freestone et al. 1995).

Another method of inducing aortic damage in an animal model is the application of calcium chloride to the outside of the aorta in rabbits (Freestone et al. 1997). In a series of experiments by Freestone et al, this caused both medial injury with
calcification as well as endothelial damage, echoing the pattern seen in most aneurysms. Interestingly, in this model, inflammation induced by the addition of thioglycollate to healthy aortas had no effect, but significantly increased the rate of dilatation in aortas that had previously been damaged by calcium chloride. Inflammation by itself would therefore seem to be insufficient to induce aneurysms in healthy aortas, but hastens their development in those that are already damaged. In a related result, it was found that the instillation of pure elastase into the isolated infrarenal aorta was unable, by itself, to induce aneurysm formation, but required the addition of an agent such as thioglycollate to initiate an inflammatory response (Carsten et al. 2001).

Koch has developed a scale for the measurement of inflammation in AAAs that allows some degree of standardisation between investigators (Koch et al. 1990). By immunostaining individually for T-cells, B-cells, macrophages, T-helper and T-suppressor cells, a gradient of inflammatory infiltration was demonstrated. Unsurprisingly, the highest level was described in “inflammatory” aneurysms, followed by normal AAAs, then aorto-occlusive disease and finally the normal aorta. These results also suggested an immune-mediated aetiology behind the development of AAAs.

Pro-inflammatory cytokines have been demonstrated in the aneurysmal wall. In 1994 Szekanecz analysed conditioned medium in which human aortic aneurysmal explants had been cultured (Szekanecz et al. 1994). The levels of interleukin-6 and interferon-\(\gamma\) were found to be significantly elevated when compared to both atherosclerotic and normal control tissue, although there was no difference seen in interleukin-2 and
interleukin-4. Using homogenates of aortic tissue, interleukin 1β (IL-1β) and tumour necrosis factor α (TNF-α) were also both shown to be raised (Newman et al. 1994a). Taking this one step further, the addition of TNF-α and IL-1β increased the expression of intercellular adhesion molecule 1 in human aortic endothelial cells, partially explaining the recruitment of inflammatory cells into the aneurysm. Conversely, by blocking the leukocyte adhesion molecule CD-18 using specific antibodies, the growth rate of experimental aneurysms was significantly slowed (Ricci et al. 1996c; Strindberg et al. 1996). Keen also found that the addition of IL-1β to vascular smooth muscle cell cultures resulted in a dose-dependent increase in the expression of collagenase (Keen et al. 1994).

Perhaps some of the best evidence that inflammation is crucial to aneurysm development can be seen in studies using indomethacin, a powerful non-steroidal anti-inflammatory. In 1996 it was shown that indomethacin prevented the development of aneurysms in the rat/elastase infusion model, (Holmes et al. 1996) and that this was due at least in part to a reduction in MMP-9. Three years later the same group went further to elucidate that the effect was manifested through cyclo-oxygenase 2 (COX-2) and not COX-1 (Miralles et al. 1999). Franklin et al combined in vitro studies of the effects of indomethacin on aneurysm development with a clinical case-control study to look at the effects in vivo (Franklin et al. 1999b; Franklin et al. 1999c; Walton et al. 1999). In the laboratory experiments, the secretion of inflammatory cytokines was inhibited, whilst the case-control study demonstrated slower aneurysm growth in patients taking non-steroidals.
Tilson has also hypothesised that there may be an auto-immune basis for aneurysm development (Gregory et al. 1996). The identification of immunoglobulin – G (IgG) within the aneurysmal aortic wall led to the search for a putative autoantigen. They identified a peptide belonging to the microfibril-associated glycoprotein family, MAGP-3. Further support for the autoimmune theory can be found in the relationship between AAA formation and the major histocompatibility locus, HLA-DR. Mutations at this locus are implicated in a variety of autoimmune disorders, notably rheumatoid arthritis. Tilson showed that the frequency of mutations in the HLA-DR locus in African Americans were far more common than could be explained by chance. These findings were especially interesting due to the fact that AAAs are extremely rare in Afro-Carribeans (Tilson et al. 1996).

1.8 Oxidative Stress

The action of reactive oxygen species has been implicated in the aetiology of many disease processes (Berkenboom, 1998; Heinecke, 1999; Uemura et al. 2001). In particular, the effect of oxidative stress on many aspects of vascular biology has come under intense scrutiny over the past few years (Chilton, 2001; Escargueil-Blanc et al. 2001; Higashi et al. 2002; Miller et al. 2002). In order to demonstrate an improvement in endothelial function when oxidative stress was reduced, Higashi et al investigated patients with and without renal artery stenosis. Renal artery stenosis is known to activate the renin-angiotensin axis and thereby cause a global increase in oxidative stress. In this experiment, the degree of oxidative stress was measured in two ways. Firstly, by urinary excretion of 8-hydroxy 2-deoxyguanosine (8-OHdg), an oxidised base which is an indicator of the degree of oxidation of DNA, and secondly
by the levels of serum malondialdehyde modified low density lipoprotein (mm-LDL),
both of which are recognised indices of total body oxidative stress. Endothelial
function was assessed by the administration of acetylcholine (ACh), which causes
vasodilatation in healthy endothelium, and also by the administration of isosorbide
dinitrate, which causes vasodilatation independently of endothelial function. The
degree of vasodilatation after each stimulus was measured using forearm
plethysmography. In the patients with renovascular hypertension, the response to ACh
was reduced and there were higher levels of the markers of oxidative stress.
Following renal arterial angioplasty, the serum mm-LDL and the urinary 8-OHdg
dropped, and the degree of vasodilatation improved, thereby neatly demonstrating a
link between systemic oxidative stress and vascular endothelial function.

Other products of oxidation, namely mildly oxidised LDL (MoxLDL) have been
implicated in the process of atherosclerosis by activating platelet-derived growth
factor beta-receptor (PDGFβR) in cultured vascular endothelial cells (Escargueil-
Blanc et al. 2001). Reactive oxygen species were increased by up to 70% in bovine
aortic endothelial cells cultured in high concentrations of glucose for 2 weeks, and
this also caused an increase in both the expression and activity of MMP-9. The
addition of anti-oxidants significantly reduced the activity of MMP-9, whereas the
addition of inhibitors of protein kinase C had no effect. These results suggest that the
increased proteolytic activity seen in the extracellular matrix in patients with diabetes
mellitus is due, at least in part, to the effects of oxidation, and may help to explain a
link between aneurysm formation and oxidative stress.
A further series of aortic banding experiments have demonstrated that in areas of high pressure there is an upregulation of endothelial nitric oxide synthase (eNOS) when compared to tissues downstream of the artificial coarctation (Barton et al. 2001b). Measuring nitrotyrosine in the same tissues gave some indication of the degree of nitric oxide breakdown and sequestration by reactive oxygen species. In the areas above the banding (heart, brain and thoracic aorta) the levels of nitrotyrosine were much higher than in areas not exposed to high pressures (distal aorta). The inactivation of nitric oxide due to oxidative damage in areas of high pressure is another indication of vascular endothelial dysfunction, which may contribute to the pathogenesis of aneurysms.

Combining the in-vitro elastase perfusion rat model of Anidjar and Dobrin with modern cDNA microassay analysis, Yajima et al looked at the expression of 8799 in rats with induced aortic aneurysms, and compared them with genes expressed in rats that had undergone sham operations (Yajima et al. 2002). Using this technique they were able to identify over 200 genes whose expression had more than doubled in the aneurysm group. Significantly, this included many genes reflecting an increase in oxidative stress, notably heme oxygenase, inducible nitric oxide synthase (iNOS), 12 lipoxygenase and heart cytochrome C oxidase, subunit VIa. Conversely, antioxidant genes such as superoxide dismutase, reduced NAD-cytochrome b-5 reductase and glutathione S reductase were found to be down-regulated. These two complementary findings both point to oxidative stress playing a major role in AAA development.
1.9 The Next Stage

After researching the pathogenesis of aneurysms, the next logical step was to identify drugs that had already been investigated for their action on AAAs, and to find whether there had been any progress in preventing aneurysm development.
<table>
<thead>
<tr>
<th>Study</th>
<th>M</th>
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<th>Smoking</th>
<th>Age</th>
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Table 1. Risk factors associated with aneurysm development in published studies

M = Male sex
F = Female sex
FH = Positive family history
C = Caucasian race
Chol = Raised plasma cholesterol
LDL = Low density lipoprotein
HDL = High density lipoprotein
DM = Diabetes Mellitus
↑BP = Hypertension. * indicates studies in which both an increase in systolic and diastolic hypertension were found to be independent risk factors. # = studies in which taking medication for hypertension was more significant than hypertension itself.
Table 2. Differing gene expressions in patients with abdominal aortic aneurysms (AAA) or aorto-occlusive disease (AOD) when compared to control subjects.

<table>
<thead>
<tr>
<th>Gene</th>
<th>AAA</th>
<th>AOD</th>
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<td>Collagen type VI α 1</td>
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<td>Glycoprotein III A</td>
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<td>α-2 macroglobulin</td>
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<td>MMP-9</td>
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<td>Intercellular adhesion molecule-1</td>
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<td>Interferon-β receptor</td>
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<td>Laminin α-4</td>
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<td>Insulin-like growth factor-2 receptor</td>
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<td>Integrin α-5</td>
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<td>Ephrin A5</td>
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<tr>
<td>Rho/rac guanine nuclear exchange factor</td>
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↑ = Increased gene expression seen
↓ = Decreased gene expression
Chapter Two

Pharmacotherapy in Small Abdominal Aortic Aneurysms

2.1 Introduction

Abdominal aortic aneurysms (AAAs) are present in 5 to 10 percent of men over the age of 65 (Scott et al. 2001), and elective surgical intervention has long been the mainstay of treatment. There is widespread consensus that operative repair is the treatment of choice in larger AAAs, where the risk of rupture increases with the size of the aneurysm (Thompson, 1996; Vardulaki et al. 1998). However, even elective operations carry a significant mortality risk, and the UK small aneurysm trial has shown that for smaller aneurysms (between 4 and 5.5cm) there is no difference in outcome between operation and no intervention (Powell et al. 1996). Currently such patients are treated with best medical therapy, but there has been considerable research into finding a pharmacological treatment to prevent aneurysm expansion and rupture.

2.2 Screening Programmes

A major obstacle to the prevention of mortality and morbidity associated with aneurysms has been the fact that the majority are asymptomatic, and therefore often remain undetected. Abdominal aortic aneurysms have tended to present either as emergencies or as a result of their increasing size, and it has been shown that larger
aneurysms grow more rapidly than their smaller counterparts and are at greater risk of rupture (Vardulaki et al. 1998). These patients would therefore benefit most from operative repair rather than medical intervention. In order for a medical treatment to be of benefit then, it needs to be targeted at aneurysms that are small and asymptomatic. The most obvious way of doing this would be the initiation of a mass screening programme, and indeed, the Multicentre Aneurysm Screening Study (MASS) has shown that as many as 88% of screen-detected aneurysms are below the threshold for surgery.

Screening programmes for AAAs have been causing controversy as far back as 1985 (Collin, 1985; Collin, 1988; Collin, 1993; Scott et al. 1988). Those investigating the feasibility of screening programmes have taken many factors into account. Understandably, a major consideration has been the financial implications. The initial estimate was that every life saved by such a screening programme would cost approximately £9000 (Collin, 1985). Interestingly, only 10% of this estimate related to the cost of the screening programme itself, which is relatively inexpensive. The rest of the expense was an estimate of the cost of operative repair of newly detected AAAs and the associated aftercare.

Conceptually, there may well be an advantage in procrastination (Collin, 1996). Patients with AAAs are more likely than the general population to be hypertensive smokers with severe atherosclerosis affecting carotid, coronary and peripheral arteries. Their mortality risk from all causes other than AAA is approximately twice that of the general population. If the AAA remains undetected then it is highly likely that the patient will die from other causes, and an “unnecessary” operation will have
been avoided. However, this argument assumes that all detected aneurysms would eventually require surgery, whereas pharmacotherapy is intended to prevent this from being the case. In addition, there is both a lead time and a lag time bias in the screening process. The growth rate of small aneurysms is considerably slower than that of larger aneurysms, and many do not show any change in size between six monthly review intervals. By detecting them at this early stage, aneurysms that do require replacement can be operated on whilst the patient is much younger, which therefore gives the patient a greater chance of surviving to the "break-even point" and beyond than patients that would present symptomatically.

On the negative side, screening would by definition involve taking asymptomatic, "well" individuals and converting a number of them into patients with a disease. Considerable psychological morbidity might be expected with this, which would presumably be worsened for the patient with a small aneurysm who is told there is nothing that could be done apart from wait until it becomes large enough to warrant an operation or it bursts. Surprisingly, however, the MASS study did not bear out this hypothesis. This trial involved nearly 70,000 patients and randomised into either screened or non-screened groups. Quality of life was extensively assessed across all the subgroups in the trial by the use of four standardised scales. Anxiety, depression and health-status measurements never differed from sex-matched population norms. There were slight differences in the measurements of health status between those who screened positive and those who screened negative, and also in the postoperative period between those undergoing surgery and those being observed. An effective pharmacotherapy may therefore do much to ease the mental status of the patient, but it would serve two other important functions. The first would be to reduce rupture rates.
The benefit of this is obvious given the massive mortality associated with a ruptured AAA. The second is to reduce the expansion rate, thereby prolonging the time until surgery and perhaps obviating the need altogether.

An approach to developing a suitable pharmacotherapy may be considered from one of two perspectives. Firstly, the drug may be targeted to one of the specific processes that have been shown to influence aneurysm development. These processes can be divided into three distinct areas – matrix degradation, excessive proteolysis and inflammation. Many of the drugs discussed below have been investigated owing to their effects on one or more of these processes. The second approach hinges on newer theories about the nature of arterial disease. Increasingly it has been recognised that arterial disease is neither a matter of simple deranged lipid metabolism nor of isolated local mechanical effects (Berkenboom, 1998; Dupuis, 2001; Heinecke, 1999; Ijem and Granlie, 2000; Weissberg, 1999; Yamada and Topol, 2000). Current belief is that arterial disease represents a low-grade systemic inflammation, which can therefore manifest itself at any point – coronary, carotid, aneurysmal or peripheral vascular disease. The Oxford Heart Protection Study (Anonymous, 2002a) has shown that generalised treatment of arteriopathic patients with statin therapy can reduce their chance of undergoing major adverse events including AAA rupture, regardless of their initial cholesterol level. This approach will be discussed in more detail later.
2.3 Therapeutic Strategies

2.3.a Beta Blockade

Hypertension has been known to be associated with AAAs for many years, and Gadowski et al have shown that hypertension increases the development of aneurysms in the Anidjar/Dobrin rat model (Gadowski et al. 1993). Comparison of aneurysms induced in normal Wistar-Kyoto rats and those in a strain genetically prone to hypertension proved that those in the hypertensive strain had a growth rate twice that of control. Since beta-blockers have been used successfully in the treatment of hypertension, it was not unreasonable to investigate the effect of beta-blockade on aneurysm expansion, as these agents have been shown to slow aneurysm progression in both experimental models (Boucek et al. 1983; Leach et al. 1988) and retrospective studies of patients with AAAs (Brophy et al. 1988) (Englund et al. 1998). Initially this was thought to be purely due to the drugs' effects on blood pressure, but there is considerable evidence to support the theory that beta-blockers exert their beneficial effects on AAAs by another mechanism.

The broad-breasted white turkey is prone to aortic aneurysms, and Boucek examined the effect of propanolol administration upon the collagen content of its aorta (Boucek et al. 1983). After treatment, the tensile strength of the aorta was found to be considerably greater. This was due to stimulation of lysyl oxidase to produce reactive aldehydes for intermolecular cross-links, and stabilisation of cross-linkages between elastin molecules. In addition, the density of cross-linkages between collagen
molecules, which normally increases with age, was reduced. These findings were dose dependent, and unrelated to effects on heart rate or blood pressure.

Studies on another animal model of aneurysmal disease, the lysyl oxidase deficient blotchy mouse, have not been quite so conclusive in their support for the collagen/elastin cross linkage theory. Brophy and Tilson showed that by treating male blotchy mice with propanolol from birth, they could reduce the prevalence of aneurysms at 4 months from 86% to 32% (Brophy et al. 1988). A subsequent experiment by the same group looked at the effect treatment with propanolol had on proteins in the skin of the blotchy rat (Brophy et al. 1989). After 12 weeks treatment with propanolol they found a 147% increase in the content of insoluble elastin and a 54% increase in insoluble collagen. These results suggested that propanolol was exerting its effect via cross-linkage.

By contrast, Moursi looked at three groups; normal siblings of blotchy mice, blotchy mice and blotchy mice treated with propanolol (Hingorani et al. 1998). Blotchy mice showed an increase in aortic diameter at harvest, whilst those treated with propanolol showed no significant increase over controls. Mean heart rate was reduced in the beta-blocked group, but blood pressures were similar in all three groups. Significantly, the lysyl oxidase activity of the blotchy mice was reduced to approximately half that of control and remained low in blotchy mice even when treated with propanolol. Hence the effects seen in this study could not have been attributable to lysyl oxidase activity, nor indeed to reduction in blood pressure.
The paucity of data on the effect of propanolol on aneurysms in humans prompted Tilson to call for a prospective, randomised clinical trial in 1992 (Tilson, 1992). Such a trial was instigated by the Propanolol Aneurysm Trial Investigators and reported in January 2002 (Anonymous 2002b). In this study, 548 patients with asymptomatic aneurysms between 3 and 5 cm in diameter were randomised to receive either placebo (n=272) or propanolol (n=276) and were followed for a mean of 2.5 years. The primary criterion was the mean growth rate of the aneurysm, measured by ultrasound scan every six months. Secondary outcomes also measured included death, surgery, withdrawal from study medication and quality of life measured by the standardised Short Form 36 (SF-36) protocol. There was no significant difference in the growth rates of the two groups, although there was a trend towards more elective operations in the placebo group. There was no difference in death rates, but patients in the treatment arm of the study reported a poorer quality of life, and more of this group stopped taking their medication.

In this robust study it was clear that propanolol has little, if any, effect on the growth rate of AAAs. Crucially, it has also been shown to be poorly tolerated by patients and caused many of them to be non-compliant. Despite promising beginnings and an interesting scientific background, it seems that propanolol has very little potential as a clinical treatment for AAAs.

2.3.b Modification of the Inflammatory Response

With considerable evidence to support the theory that aneurysm expansion and rupture are both mediated by the immune system (Koch et al. 1990) (McMillan and
Pearce, 1997), it is unsurprising that there has been interest in modifying this response as a means of attenuating growth. In the rat model of AAA, the effect of treating the animal with cyclosporin and methylprednisolone was investigated (Dobrin et al. 1996). Aneurysm growth was initiated in rats using the elastase infusion technique (Anidjar et al. 1992). Following initiation, the rats were divided into three groups and treated with cyclosporin, methylprednisolone or saline in the case of the control group. Aortas were harvested and examined, macroscopically and histologically, immediately post perfusion and at five and nine days. Although the diameter of the aorta in all three groups slightly increased immediately following infusion of elastin, there was no significant difference between the groups. Five days post infusion there was still no significant difference in the diameter of the aortas. However, histologically the findings were more varied. The control group showed a marked inflammatory infiltrate throughout all the layers of the aortic wall, consisting of monocytes and macrophages with moderate oedema. This was associated with breakage of the elastic lamellae. None of these features were seen in either of the treatment groups.

Furthermore, at nine days post infusion, a significant difference in the diameters of the aortas was seen for the first time, with the control group having expanded to approximately three times their pre-infusion size, but the treatment groups only grew to around twice their original size. These findings indicate that, at least in this experimental model, preventing the infiltration of inflammatory cells could halt the main spurt of aneurysm growth. Similar results were seen by Ricci et al when using a monoclonal antibody against the macrophage adhesion molecule CD-18 (Strindberg et al. 1996). A third experiment using the same model looked at tumour necrosis
factor binding protein (TNF-BP) and interleukin 1 receptor antagonist (IL-1RA) (Hingorani et al. 1998). Although IL-1RA failed to slow growth, TNF-BP showed similar results to methylprednisolone and cyclosporin.

The one constant factor in these experiments on immune-modification has been that the compounds used have been unacceptable as clinical treatment strategies due to their wide range of action and many side effects. Total immunosuppression is not appropriate, but the use of anti-inflammatories has been examined.

2.3.c Non-Steroidal Anti-Inflammatories

Indomethacin is a powerful anti-inflammatory drug that has been investigated both in the rodent elastase model and in human aortic aneurysmal tissue (Holmes et al. 1996) (Franklin et al. 1999c). Indomethacin reduced both aneurysm growth and MMP-9 activity in the rat and the levels of prostaglandin E2 (PGE-2), interleukin 1 beta (IL-1β) and interleukin 6 (IL-6) in human tissue. However, no effect was seen on MMP-9 in the human explants. Further evidence that it is the reduction in PGE-2 that is the key step here came from Miralles' group (Miralles et al. 1999), who showed that levels of PGE-2 in conditioned media increased with the expansion of the aorta, and that indomethacin prevented both of these events. Furthermore, by in-situ hybridisation of the isoforms of cyclo-oxygenase (COX-1 and COX-2), it was shown that only COX-2 was expressed in the aneurysmal tissue and must therefore be responsible for the inflammation seen.
Importantly, in a retrospective analysis of the large group of patients in the UK small aneurysm trial (Powell et al. 1996), indomethacin was also shown to inhibit aneurysm growth in vivo. The trial was not designed for this purpose and was the result of sub-group analysis, so further trials would be required. Thus we have preliminary evidence that non-selective COX inhibition by indomethacin prevents aneurysm growth, but the side effects of this treatment on the gastrointestinal (Morise and Grisham, 1998), renal (Schlondorff, 1993) and hepatic (Manoukian and Carson, 1996) systems are well known.

2.3.d MMP Inhibition

Many observers have noted an imbalance between MMPs and their naturally occurring inhibitors (TIMPs) in aortic disease (Knox et al. 1997; Mao et al. 1999b), and one of the modes of action of indomethacin is to reduce the activity of matrix metalloproteases. Many other compounds have also been investigated for their anti-MMP properties. BB-94 (or batimastat) has been shown to reduce experimental aneurysm growth in rats (Bigatel et al. 1999) and marimastat is a related drug that prevents elastin degradation and MMP-2 activity in a porcine model of aneurysms (Treharne et al. 1999). Vyavahare’s group have shown that purified elastin, when injected subdermally into rats, is rapidly calcified and that this calcification is associated with intense MMP-2 expression (Vyavahare et al. 2000). Administration of the MMP inhibitor BB-1101 prevented elastin calcification, although the effect was more prominent when the drug was injected at the site of calcification rather than given systemically.
Other experimental drugs have been investigated for their anti-MMP activity. Cowan et al demonstrated that the glycoprotein tenascin-C was induced by MMPs and that this caused hypertrophy of the rat pulmonary artery in vitro (Cowan et al. 2000). Addition of the MMP inhibitor GM-6001 caused a regression of the hypertrophy and suppression of smooth muscle cell proliferation. Systemic treatment with the drug RS132908 reduced aneurysm formation and expansion and preserved elastin in elastase-perfused rat aortas (Moore et al. 1999).

Tetracyclines have long been known to prevent connective tissue breakdown by their inhibitory effect on MMPs (Golub et al. 1991). Petrinec et al demonstrated that doxycycline reduced the growth of degenerative aneurysms and suppressed MMP-9 production in the rat elastase model (Petrinec et al. 1996b). Similar results were obtained with four other chemically modified tetracycline derivatives (Curci et al. 1998; Petrinec et al. 1996a). Using a porcine model of elastase-induced AAA, Boyle also showed that doxycycline reduced elastin degradation and MMP-9 activity (Boyle et al. 1998). Tetracycline itself has been proposed as an MMP antagonist and indeed, when given pre-operatively, has been shown to penetrate the aortic wall (Franklin et al. 1999a). However, these experiments used a concentration of 100μg/ml to produce an effect in vitro, but the concentration following a single bolus of 500mg was 8.3μg/ml in plasma and 2.8 μg/g of aortic tissue. This suggests that in order to reach therapeutic levels the treatment dose would have to be enormous.

Nevertheless, preoperative treatment with doxycycline caused a reduction in both the expression of macrophage MMP-9 mRNA and the activity of MMP-2 in aneurysm tissue (Curci et al. 2000). Also, a small double-blinded, randomised and placebo
controlled pilot study from Finland has shown that treatment with doxycycline for a three month period significantly reduced the rate of aneurysm growth in a cohort of patients as measured by serial ultrasound scans (Mosorin et al. 2001). At six months, there was also a significant reduction in the serum C-reactive protein levels of the treatment group. Although the sample size was small and preoperative confounding effects are not taken into consideration, this trial has provided evidence to support further research into this area. In 1999, Thompson and Baxter initiated a randomised, prospective clinical trial of doxycycline in the treatment of small aneurysms in the USA. This is an ongoing, multicentre trial, the results of which have not yet been reported (Thompson and Baxter, 1999).

2.3.e Anti-Chlamydial Therapy

The hypothesis that atherosclerosis may have an infective aetiology is not new, and it is clear that AAAs and atherosclerosis share many of the same risk factors. Indeed, Lindholt’s group has shown that antibodies against chlamydia are associated with progression of peripheral vascular disease (Lindholt et al. 1999) and that these antibodies may also predict the need for surgery in patients diagnosed with small aneurysms (Vammen et al. 2001a). However, a possible source of error in studies of chlamydial infection has been identified by the same group. Using immunohistostaining to investigate the incidence of chlamydial proteins in the aortas of twenty patients undergoing aneurysm repair, C. pneumoniae was not identified in a single one (Vammen et al. 2002). Nevertheless, what was evident was a high degree of cross-reactivity of non-chlamydial proteins, in particular the human haemoglobin beta chain.
Regardless of this, Mosorin’s group has reported a significant decrease in the rate of expansion of AAAs in patients treated with doxycycline in a prospective, double-blinded, randomised and controlled study (Mosorin et al. 2001). As discussed earlier however, it is unclear whether the effects seen were due to chlamydial eradication or the direct effects of doxycycline as an MMP inhibitor. Another randomised, controlled trial looked at the effect of roxithromycin on aneurysm growth (Vammen et al. 2001b). Patients with small aneurysms were given either roxithromycin or placebo for four weeks, and subsequently followed up for a mean of 1.5 years. Once adjustments had been made for smoking, blood pressure and IgA, there was a significant difference in aneurysm growth between treatment and placebo groups.

Tambiah’s work using rabbit aortas showed that a monocyte chemoattractant protein-1-induced influx of macrophages alone into the aortic wall was insufficient to provoke aortic dilatation (Tambiah et al. 2001). When the macrophages were activated by the presence of chlamydia however, the aortas did undergo dilatation. Moreover, dilatation that occurred in rabbit aortas due to the interaction of macrophages and chlamydial surface antigens was abolished by the administration of azithromycin (Tambiah and Powell, 2002).

2.3 \textit{HMG CoA Reductase Inhibitors}

The HMG CoA reductase inhibitors (statins) are a group of drugs in which there has been considerable interest recently (Kmietowicz, 2001; Koh, 2000). The statins have been used successfully for their lipid-lowering properties for some time now, but have also exhibited beneficial effects in cardiovascular disease unrelated to this (Palinski,
2001). In laboratory experiments they have been proven to reduce MMP-9 expression by human macrophages (Bellosta et al. 1998) and their anti-inflammatory effects are well documented (Eriksson et al. 2004; Kothe et al. 2000).

2.3.g Drugs Acting on the Renin/Angiotensin Axis

In 1998 a French group reported the effects of angiotensin converting enzyme (ACE) inhibitors and angiotensin II antagonists in a strain of rat prone to rupture of the internal elastic lamina of the aorta (Huang et al. 1998). To ensure any beneficial effects were not due to the antihypertensive properties of the drugs, they were compared to hydralazine and two calcium channel antagonists. Both ACE inhibitor and angiotensin II antagonist prevented rupture of the internal elastic lamina, suggesting this was due to the effect on angiotensin II and not on another part of the renin angiotensin system. However, in all the treatment groups a global reduction of elastin, collagen and cell proteins in the media was noted. This contrasts with the results of Thompson's group, who used both ACE inhibitors and an angiotensin II antagonist in the Anidjar/Dobrin rat model (Liao et al. 2001). In these experiments, the ACE inhibitors prevented aneurysm formation and preserved elastin whilst angiotensin II antagonism had no significant effect. There was no effect on the inflammatory infiltrate seen in the aneurysmal wall, and the reduction in aneurysm formation was independent of blood pressure effects. Clearly further work in this field is needed to elucidate the exact mechanism of action.
2.4 Summary and Hypotheses

From the preceding literature reviews, it can be seen that two of the major processes involved in the development of abdominal aortic aneurysms are inflammation and proteolysis under the action of MMPs. Whilst several drugs have been investigated for potential use as a pharmacotherapy for small aneurysms, none have so far proven ideal. NSAIDs have been shown to reduce both inflammation and aneurysm growth rates but have significant side effects, whilst statins have been shown both to reduce inflammation and to inhibit MMPS. It was therefore hypothesised that either a COX-2 antagonist (rofecoxib) or a statin (simvastatin) would be able to reduce both inflammation and MMP activity within aneurysmal tissue and might be useful as a pharmacotherapy. For this reason, the following experiments were conducted to look at the effect of these drugs on cytokines and MMPs both in vitro and in vivo.
Chapter Three

Materials and Methods

3.1 Human Aortic Explant Culture

In order to investigate the effects of various drugs upon aneurysmal tissue, it was necessary first of all to decide upon a system in which to conduct the experiments. The first set of experiments were conducted using human aneurysmal aortic explants as first described by Franklin et al (Franklin et al. 1999c). This was a method of tissue culture that had already been extensively validated by several methods as will be described later.

Ethics approval from the local ethics board was obtained, and informed consent was sought from each of the subjects. The subjects were patients undergoing elective, open aneurysm repair at the University Hospitals of Leicester NHS Trust. It was explained to the patients that during the procedure, the aneurysm sac would be opened and an inlay graft placed. This would leave a largely redundant sac, part of which was often excised and discarded before the remaining sac was closed over the graft. This redundant sac could therefore easily be used for tissue culture with no adverse effects for the patient.

The operating surgeon was notified of patients who agreed to take part in the study. During their operation, providing the redundant sac was large enough to supply a specimen without compromising safety, a section of the aneurysmal wall was excised.
These full-thickness sections were typically 1.5cm x 3.5cm, taken from below the origin of the inferior mesenteric artery. They were immediately transported in a sterile container of culture medium to the laboratory. Culture medium used was Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with L-glutamine and penicillin and streptomycin to prevent fungal or microbial contamination. Once in the laboratory, the explants were washed in culture medium and cleaned of extraneous blood and fat. When a clean section of aneurysm was left it was divided into four roughly equal segments and each segment was weighed under aseptic conditions.

These were placed in separate compartments of a 6-well culture dish to which culture medium was added. One ml of culture medium was added for every 0.1g wet weight of tissue. The cultures were then transferred to an incubator and allowed to equilibrate for 24 hours at 37°C in standard conditions. The next day, the culture medium was aspirated from the cultures and discarded. The medium was replaced with further DMEM to which known concentrations of selected drugs had been added. The medium added to the control tissue was also treated with any carrier (such as dimethylsulfoxide (DMSO)), in the same concentration as those containing the drug. After a further 48 hours incubation, the conditioned media and the tissue were all harvested and snap frozen in liquid nitrogen. As all conditions were standardised except for the drug being investigated, relatively small numbers were needed to demonstrate significant results should any difference exist.
Fig 3.1 Explant culture. Four explants from the same patient can be seen in the first four wells. The numbers above the explants are the wet weight in grams, and are used to calculate the amount of culture medium to add (1ml per 0.1g).

Franklin's original 1999 paper describes techniques undertaken to validate this system of explant culture (Franklin et al. 1999c). Continuing viability of the explants was recorded for up to twelve days by several different methods. Glucose consumption was measured and was found to continue for up to 12 days, but was at its peak in the first four days. Production of lactate dehydrogenase continued for up to 12 days, and the incorporation of radiolabelled amino acids into cellular proteins confirmed ongoing protein synthesis. Histological appearances at two days were indistinguishable from fresh samples. By four days there was evidence of minor vacuolation within the nuclei, but the overall architecture remained intact. After six
days, however, there was widespread fragmentation of lymphocyte and smooth muscle cell nuclei.

From these experiments it was established that the maximum length of time in which valid observations could be made was approximately four days. As can be seen from the results of early experiments in this thesis, this time period proved to be useful for examining the production of cytokines by the explants, but was probably too short for significant differences in MMP activity to be observed. In order to address this issue, a second series of experiments was initiated, using a different model of aneurysmal disease.

3.2 Porcine Model

The porcine model that was used in these experiments was originally described by Wills et al (Wills et al. 1996). Porcine aortas had already been characterised by Wolinsky and Glagov as being very similar in composition to human aortas (Wolinsky and Glagov, 1967). The principle behind the technique relied upon taking sections of healthy porcine thoracic aorta and pretreating them with elastase for 24 hours. This initiated protein degradation, which continued well after the elastase was washed off. Ongoing proteolysis was confirmed by histological examination of the segments at various time points up to 14 days. In addition, matrix metalloprotease activity measured by gel zymography was markedly raised in the aortic cultures treated with elastase compared to control. Indeed, the levels of MMP activity increased throughout the time period of the experiment, echoing the earlier results of
Anidjar and Halpern (Anidjar et al. 1990; Halpern et al. 1994). Immunoblotting confirmed the presence of MMPs ~1, ~2, ~3 and ~9.

Halpern suggested the principle of latency, hypothesising that further steps beyond the initial injury were required to cause aneurysm formation. Indeed, in his experiments using the isolated rat aortic perfusion model, no exogenous elastase was detectable beyond day two, although the aneurysms did not begin to develop until days 3-6. Crucially, the point of aneurysm development coincided with an increase in endogenous protease activity.

It was also shown that an inflammatory response in the tissue continued well after the elastase was removed. By culturing the tissue in the presence of autologous leukocytes, marked differences were demonstrated between controls and samples pretreated with elastase. In the treated samples, an inflammatory infiltrate consisting of B cells, T cells and macrophages was seen. Elastin-derived peptides are chemotactic for leukocytes (Senior et al. 1980; Senior et al. 1984), which would in part explain the influx of inflammatory cells. Another explanation would be the physical effects of the elastin breaking down tissue integrity to facilitate the entry. Either way, this demonstrated another way in which this model behaves in a similar fashion to aneurysms, as AAAs have been repeatedly shown to display features of inflammation (Holzheimer et al. 1999; Satta et al. 1998). Whether this is a primary feature of aneurysm development or a response to elastin breakdown remains to be proven.
Sections of healthy, porcine thoracic aorta were obtained from a government-approved abattoir (Dawkins, Leicestershire, UK). The aortic segments were washed in sterile saline at the abattoir, a step that dramatically reduced infection rates. They were then transported to the laboratory in culture medium on ice. The culture medium used throughout the porcine experiments was RPMI supplemented with penicillin and streptomycin, L-glutamine and 10% fetal calf serum. As a consequence of needing to supplement the medium with fetal calf serum, no measurements of cytokines could be made in this model.

Under sterile conditions, the aortic segments were cleaned of extraneous tissue and blood clots and divided into 1 cm² sections. These were then pinned out in culture dishes and bathed for 24 hours in culture medium containing porcine pancreatic elastase (Sigma, UK). Wills' experiment characterised the histological changes seen in the tissues when different amounts of elastase were used (1, 10 and 100 U/ml). Using their results, a level of 5 U/ml was shown to be ideal for the purposes of this experiment. At the end of this period the elastase was washed off and replaced with culture medium as before. Depending upon the experiment, drugs were added to the culture medium in sub~, supra- and roughly therapeutic concentrations. The explants were then cultured for 14 days with the medium replaced every 48 hours. The tissue was then harvested and divided in two. Half was immediately fixed in formalin for histological examination whilst the other half was homogenised and used for the determination of matrix metalloprotease activity.
Fig 3.2 Culture dish used for porcine experiments. Note the polyester gauze to support the tissue and pins to prevent the edges from curling out of the medium.

3.3 MMP Extraction

In order to quantify the activity of MMPs within the tissue, the proteases were extracted using methods developed by Vine and Powell (Vine and Powell, 1991). A homogenising buffer was prepared using urea 2 mol/L, TRIS-base 50 mmol/L, sodium chloride 1 g/L, ethylenediamine tetraacetic acid (EDTA) 1 g/L and BRIJ-35 1 g/L (all from Sigma, UK). Phenylmethylsulfonyl urea (PMSF) was added at 0.1 mmol/L to act as a serine protease inhibitor. The resulting solution was brought to pH 7.6 using hydrochloric acid. The tissue was sliced into segments no larger than 1mm x 1mm and placed into homogenising buffer at a ratio of 1ml to 0.1 mg wet weight of tissue. Each sample was homogenised using a tissue homogeniser set at 25,000 rpm
for 5 minutes. Subsequently the samples were spun in an ultracentrifuge at 12,000 rpm and 4°C for 60 minutes. Visking tubing sachets (Fisher Scientific, UK) were prepared and the homogenised supernatants were transferred to these.

Dialysis buffer was prepared using TRIS at 25 mmol/l containing 10 mmol/L calcium chloride (Fisher Scientific, Loughborough, UK), 0.1% BRIJ-35 and 1mmol/L PMSF. The resulting solution was brought to pH 8.5. The homogenates were placed in the Visking tubing sachets into the dialysis fluid and left to dialyse at 4°C for 18 hours.
Fig 3.3 Tissue Homogeniser. The homogeniser shown was used for the preparation of samples and extraction of MMPs. Specimens were first sliced by hand into sections no bigger than 1 cm² and suspended in homogenisation medium. They were then subjected to 5 mins of homogenisation at 25 000 rpm. The homogeniser was used in a fume cabinet as shown to minimise potentially harmful aerosols.
3.4 Protein Standardisation and Gel Zymography

A standard curve of known protein concentrations was constructed using bovine serum albumin at 1, 2 and 3mg/ml, diluted in phosphate buffered saline (PBS). The samples were also diluted in PBS and Bio-Rad protein indicator was added. The resulting concentrations were measured using a colorimeter and the homogenates were then diluted to a concentration of 1 mg/ml using a non-reducing sample buffer. This consisted of 3.6 mls glycerol, 1 ml of 0.5 Molar TRIS solution at pH 6.8, 3.2 mls of 10% sodium dodecyl sulphate and 100 μl of Bromophenol Blue (Sigma, UK) (Bradford, 1976).

The principles of gel zymography were outlined in papers by Heussen and Dowdle (Heussen and Dowdle, 1980) and Granelli-Piperno and Reich (Granelli-Piperno and Reich, 1978). Firstly, gelatin was added to a standard electrophoretic gel, in which it could act as a substrate for proteases. The second key principle was that the proteolytic activity of MMPs was inhibited by reversibly denaturing the enzymes using sodium dodecyl sulphate. In this way, digestion of the gelatin was delayed until after the electrophoresis had run its course. At this point, the SDS was washed off using Triton, a non-ionic detergent, allowing the MMPs to resume their normal configuration and proteolytic activity. The third important principle was that, in these gels, even the inactive form of the enzyme was able to digest gelatine and therefore left a second distinct band on the gel, allowing active and proenzyme forms to be assayed separately. This is due to in-situ activation of the proenzymes by the denaturation/renaturation process and autocatalytic cleavage.
To conduct these experiments, polyacrylamide separating gels were constructed containing collagen 10mg/4mls of water. To this was added a resolving buffer containing 1.5 Molar concentration of TRIS at pH 8.8. 30% Acrylamide/Bis (Protogel, National Diagnostics, Atlanta Georgia) was added, as was a 10% sodium dodecyl sulphate solution (Melford Laboratories, Ipswich, UK). Finally, 50 µl of 10% ammonium persulphate and 5 µl of TEMED (Sigma, UK) were added and the gel allowed to set.

Stacking gels were constructed in the same manner, but with no added gelatine. Also, instead of the pH 8.8 resolving buffer, 0.5 Molar solution of TRIS was used at pH 6.8. This solution was placed on top of the solidified separating gel and a comb with 5mm lanes was positioned.

Once the gels had set, a control was added to each. Conditioned medium taken from cell culture of the human sarcoma line ST 1080 (ECACC No 85111505) was used, as this is known to express large quantities of MMP-2 and MMP-9. The medium was diluted 1:1 with non-reducing sample buffer, as before. The diluted samples were placed in the appropriate lanes and the gels allowed to run for approximately 3-4 hours at 80 mA, using the Mini-Protean II System (Bio-Rad, Hemel Hempstead, UK).

At the end of this time, the gels were washed three times in 2.5% Triton solution for 15 minutes at 37°C. Incubation buffer was prepared with 50 mM TRIS, 50 mM sodium chloride and 0.05 % BRIJ 35 (all Sigma, UK) and 10 mM calcium chloride. There followed an 18-hour incubation period, before the gels were stained with a solution of Coomassie blue (0.1% Coomassie Blue R250 dissolved in 50% methanol,
10% acetic acid and 30% double-distilled water). Finally, the gels were scanned using Imagemaster software to give a semi-quantitative measurement of matrix metalloprotease activity.

Fig 3.4 The Mini-Protean II Electrophoresis Kit. This kit was used for the electrophoresis of all the zymograms in this thesis. Each tank could hold two gels, and the power pack could potentially support four tanks simultaneously. In reality, due to the time taken to prepare the samples for each gel, it was never possible to run more than two gels simultaneously.
3.5 Stereotactic Histological Analysis

In order to quantify the changes in aortic architecture after treatment with elastase, previously described stereotactic techniques were used (Wills et al. 1996). Microscopic sections were prepared by fixing the specimens in formalin. They were then dehydrated in 99% industrial methylated spirits before transferring to xylene (both from Sigma, UK) for four hours and embedding in paraffin. Staining was performed with both haemotoxylin and eosin and elastin Van Gieson.
Fig 3.5 Typical appearance of aneurysmal tissue under light microscopy. Under 400x magnification, the major constituents of the aortic wall can clearly be discerned.

Using haemotoxylin & eosin and elastin Van Gieson staining, collagen appears pink, elastin black and smooth muscle cells yellow. Note the predominance of collagen and the relative paucity of elastin fibres.

The sections were then examined under 400x magnification to assess the relative concentrations of collagen, elastin and smooth muscle. A 100-point test grid was created by using an eyepiece graticule, and points were counted as either being collagen (pink), elastin (black) or smooth muscle (yellow). On each sample, eight random test grids were counted in a blinded manner. Previous work has found this to be a highly reproducible, if somewhat labour intensive, assay. Interobserver limits of
agreement for elastin were found to be $-5.1\%$ to $+6.3\%$, and intraobserver range of agreement was $-3.1\%$ to $+3.6\%$ (Brennan and Silman, 1992).

Figure 3.6 Enzyme linked Immunosorbent Assay. The above diagram represents the double-layer "sandwich" type of ELISA used extensively throughout this thesis. Please refer to the text below.
3.6 Enzyme-Linked Immunosorbent Assay

Throughout the course of this thesis, various commercially available ELISAs have been used. The standard ELISA used was a two-site "sandwich" format. Antibodies \((a)\) to the protein being investigated \((x)\) were bound to the floor of each well on the ELISA plate. Samples or standards were added and incubated on a plate shaker for a set period of time. During this period, any molecules of \(x\) were bound to the antibodies and thus to the floor of the well. At the end of the incubation, excess fluid was washed off.

A second antibody \((b)\) directed against \(x\) was then added, and incubated and washed in a similar manner to before. In this way, antibody \(b\) was indirectly affixed to the floor of the well. Subsequently a third antibody \((c)\), this time conjugated with horseradish peroxidase and directed against antibody \(b\), was added. After incubation and washing, the amount of horseradish peroxidase indirectly bound to the floor of each well was proportional to the amount of \(x\) added in the sample. By adding a set amount of substrate to each well, a colour change was observed which was read on a colorimeter.
3.7 Statistical Methods

The main technique used throughout this thesis was Friedman's Test. This was a nonparametric test that compared three or more paired groups. The test firstly ranked the values in each matched set or row. In the majority of cases, these matched rows were the four separate explants taken from the same aorta. Secondly, Friedman's test added the ranks in each column and compared the columns. If the sums of the columns were very different then the p-value was small. In this way, Friedman's test controlled for variables that would affect all of the values in each row, or in this case, all of the explants from the same aorta. The test looked for differences in each row, and may therefore have shown a significant difference even when graphical representation appeared unremarkable. The p-value gave the probability that random sampling would result in sums of ranks as far apart as were observed if there were no difference between the columns.

One of the weaknesses of this test was that it would only discern whether or not there was a significant difference between the columns, but not where that difference lay. For this reason, wherever a significant difference was shown, a paired Wilcoxon test was also applied between the control column and each of the individual treatments.

In Chapter Seven, unpaired data were compared using a standard Mann-Whitney test. Comparisons of two methods of assay were performed using two techniques. The first was to use Spearman's correlation, although this technique was not the most appropriate, as discussed in the Chapter. The other method used was an Altman-Bland plot, which is discussed in more detail in Section 7.4.d.
All data analyses were performed using Graphpad Prism software, and the techniques used were reviewed by a Professor of Statistics.
Chapter Four

The Role of Anti-Inflammatories in the Prevention of Aneurysm

Development

4.1 Introduction

The natural history of abdominal aortic aneurysms is one of abnormal proteolysis, intramural inflammation and aortic dilatation (Grange et al. 1997; Halloran and Baxter, 1995). It would be natural for a medical treatment for aneurysms to attempt to target these processes. There is a huge amount of data available on the processes involved in inflammation and a vast range of anti-inflammatory drugs in the pharmacopoeia. Originally used to reduce pain, swelling and fever, it has become clear that anti-inflammatory agents have a diverse range of effects on a diverse number of systems. One of the commonest side effects of non-selective anti-inflammatory drugs has been gastro-intestinal haemorrhage (Gabriel et al. 1991). This illustrates well the different systems affected by these drugs. Aspirin is used as a platelet inactivator, and works in this function by irreversibly inhibiting the cyclo-oxygenase capability of prostaglandin G/H synthase (Patrono, 1994). Aspirin irreversibly acetylates a single serine residue at position 259 of cyclo-oxygenase 1, which is the only form of the enzyme expressed in platelets. Hence, as an anti-platelet agent, the effect of aspirin lasts as long as the life span of the platelet – up to 30 days. By inhibiting platelet function in this way, bleeding is made more likely. In addition, aspirin blocks the production of prostaglandin E2, which is essential for the
production of protective mucinous secretions from the gastric mucosa. Lack of this protective mucous layer can lead to ulceration, bleeding and perforation.

For years, the main thrust of research in this area has been to develop drugs that have the desired anti-inflammatory properties without unwanted side effects. This led to the identification of two different isoforms of the prostaglandin synthase enzyme, known as cyclooxygenase-1 and -2 (COX-1 and COX-2). Whilst COX-1 was found to remain at fairly constant levels despite external stimulation, COX-2 expression could be upregulated by a variety of stimuli, and was therefore suggested to be responsible for the changes seen in acute inflammation (Masferrer et al. 1990; Pash and Bailey, 1988; Sebaldt et al. 1990). Another major difference between these isoforms is the site at which they are found. Crucially, the inducible form of COX was found in macrophages, whereas it was the non-inducible, COX-2 form that predominated in normal tissues. These two enzymes represent the main site of action of all anti-inflammatory drugs to a greater or lesser degree. The enzymes convert the common precursor, arachidonic acid into the intermediates prostaglandin G2 and prostaglandin H2, but the end product of this pathway is determined by tissue-specific isomerases. Thus it was perceived that by inhibiting the isoforms selectively, the resultant cytokine cascades could be manipulated.

Cytokine cascades can be both pro- and anti-inflammatory, and appreciation of this is fundamental to both cytokine biology and clinical medicine (Dinarello, 2000). Cytokines are small proteins with molecular weights ranging from 8 to 40,000 daltons. The term cytokine is now used preferentially to lymphokine or monokine since nearly all cells are capable of synthesising these proteins and not just
lymphocytes and monocytes. They are classified by their biological activities rather than any structural similarities, and are mainly involved with the body's response to insult rather than "housekeeping" activities.

Dinarello argued that cytokines are different to hormones for several reasons (Dinarello, 2000). Firstly, hormones are expressed by a select group of tissues whereas cytokines can be produced by almost any cell. Secondly, hormones are the main product of the cells that synthesise them, whereas cytokines form a very minor part of the cellular output. Thirdly, hormones are constitutively expressed as part of the routine homeostatic mechanisms, whilst cytokines tend to be produced as a response to noxious stimuli.

As mentioned earlier, cytokines can be pro- or anti-inflammatory. Classification into these two categories, however, is not always straightforward. Whilst it is fairly universally accepted that IL-1 and TNF-alpha are proinflammatory, some of the other cytokines are more controversial. IL-4, IL-10 and IL-13, for example, are potent activators of B lymphocytes. Nevertheless, because they are capable of suppressing genes for IL-1 and TNF-alpha, they are regarded as anti-inflammatory. IL-6 is another controversial cytokine. It has consistently been shown to correlate with clinical severity in inflammatory, autoimmune, and infectious disease more closely than either IL-1 or TNF-alpha (Dinarello, 1997). Yet unlike IL-1 or TNF-alpha, there is no evidence that IL-6 is, itself, proinflammatory. Infusion of IL-1 and TNF-alpha produced responses that mimic septic shock, but IL-6 infusion failed to induce hypotension in humans even in very high doses. IL-6 did not induce PGE-2 (Dinarello et al. 1991), and has been shown to suppress IL-1 induced cyclo-oxygenase
(Hauptmann et al. 1991). Whatever its primary actions are *in vivo*, due to its consistent correlation with clinical severity, IL-6 can be regarded as a reliable marker of inflammation.

4.1.a COX-2 Selectivity – Drugs and Assays

The degree to which a specific drug may selectively inhibit COX-1 or COX-2 may be measured at a number of levels. At the most basic level, direct assays can be performed *in vitro*, and are relatively rapid and simple. The drawback here, however, is that these relatively simple tests do not reflect the complex interactions between drug and enzyme that occur *in vivo*.

At the next level, assays have been developed which measure the activity of the enzyme in whole blood (Patrignani et al. 1994; Patrono et al. 1980). These assays rely on basic premises. Firstly, COX-1 activity is inferred from the inhibition of thromboxane B2 production. Thromboxane B2 is produced during the clotting cascade by platelets, and reflects platelet COX-1. Secondly, COX-2 activity is inferred by the production of prostaglandin E2, which reflects the activity of COX-2 from monocytes.

The third level at which COX selectivity can be measured is the clinical outcome. Whilst this is probably the most relevant in terms of the actual use of the drug, there are many obvious problems here. To gain an accurate picture, a large number of patients would have to be recruited and followed up for a long period of time. The effect on the gastric mucosa would be hard to quantify without regular endoscopy,
which would not be acceptable to many patients. The effect on platelets would only be apparent clinically by an increased frequency of haemorrhage, which may be difficult to detect and certainly has ethical implications. It is perhaps unsurprising, then, that the second type of assay has become the most commonly used to establish selectivity. Using these assays it has been possible to rank the various COX inhibitors in order of their selectivity (FitzGerald and Patrono, 2001).

Franklin et al. have shown that the use of anti-inflammatory drugs can reduce the expression of PGE-2, IL-1 beta and IL-6 by aneurysmal tissue in culture, and more importantly that patients taking indomethacin had a reduced aneurysm growth rate (Franklin et al. 1999b). However, indomethacin has a relatively poor side effect profile and for the purpose of this thesis, a COX-2 selective inhibitor was sought which might demonstrate the same benefits without the harmful side-effects.

Rofecoxib was found to be the most selective for COX-2 of the anti-inflammatories tested in several assays and was therefore chosen to be the drug used as a COX-2 antagonist. For a non-selective COX antagonist, naproxen was chosen. This was a commonly used NSAID, and had the advantage of being an almost equal inhibitor of COX-1 and COX-2. Another good reason for choosing these two drugs was the readily available data from clinical trials in the literature. The Vioxx Gastrointestinal Outcomes Research (VIGOR) study (Bombardier et al. 2000) had yielded some controversial results. This study compared the use of rofecoxib 50mg once daily to naproxen 500mg twice a day in 8076 patients with rheumatoid arthritis for a median of nine months. The end-points measured were the incidence of gastrointestinal haemorrhage, gastrointestinal perforation or symptomatic peptic ulcer.
Unsurprisingly, the incidence of these side-effects were much lower in the rofecoxib group than the naproxen group – 2.1 per 100 patient-years as compared to 4.5 (p<0.001).

More controversial were the differing effects on cardiovascular outcomes. The VIGOR study showed that the relative risk of a confirmed thromboembolic cardiovascular event (myocardial infarction, unstable angina, cardiac thrombus, resuscitated cardiac arrest, sudden or unexplained death, ischaemic stroke, and transient ischaemic attacks) was 2.38 compared to naproxen (p=0.002). Many explanations have been offered for this apparent difference. It was not a primary endpoint in the trial, and the overall numbers are relatively small (less than 70) and may therefore not be representative. Also, up to 4 per cent of the patients in the VIGOR trial met the FDA criteria for use of aspirin as secondary prevention of major cardiovascular events, yet all patients on aspirin were excluded from the trial. Nevertheless, as a direct result of the increase in thrombotic events, rofecoxib has recently been withdrawn from clinical use.

These results were not echoed in a major study of celecoxib versus NSAIDs (the CLASS study) (Silverstein et al. 2000). There may well be explanations for this. Patients on aspirin were not necessarily excluded from the CLASS study, which may have given a more representative sample than VIGOR. Also, the majority of patients in the CLASS study had osteoarthritis as opposed to rheumatoid arthritis in VIGOR. It is easily conceivable that a group of patients with a systemic inflammatory disease may well have an increased susceptibility to cardiovascular events, whereas such a link to osteoarthritis would be harder to explain. It is possible that naproxen had a
cardioprotective effect that was not shared by the variety of NSAIDs used in the
CLASS study, although there has been no convincing epidemiological evidence to
back this up.

Mukherjee argued that in both the VIGOR and the CLASS study, the annualised
myocardial infarction rates were higher in the COX-2 antagonist groups than in
placebo in a meta-analysis of 23 407 patients in primary prevention trials (Mukherjee
et al. 2001). The rate in VIGOR was 0.74% compared to 0.52% from the meta­
analysis (p=0.04) and in CLASS the rate was 0.80% (p=0.02).

Could there be a scientific basis for an increased risk of cardiovascular events in
patients treated with COX-2 selective inhibitors? Fitzgerald pointed out that COX-2
may well be important in physiological conditions and that the concept of COX-1
being purely physiological and COX-2 purely pathological is an oversimplification.
For example, COX-2 antagonists reduce the urinary excretion of prostacyclin, and by
inference, the production of prostacyclin in normal subjects (Catella-Lawson et al.
1999; McAdam et al. 1999). Prostacyclin is thought to be part of a homeostatic
mechanism, which limits the consequences of platelet activation in vivo (FitzGerald et
al. 1984). If this were the case, then it might explain the increase in thrombotic events
seen in patients treated with COX-2 antagonists. Proving the hypothesis goes beyond
the scope of this thesis, but the debate certainly provides a further dimension to the
backdrop of the experiments conducted herein.
4.2 Plan of Investigation

In order to investigate the effect of COX inhibition on aneurysmal tissue, four separate series of experiments were initiated. Two drugs were chosen; naproxen for inhibition of COX-1 and COX-2, and rofecoxib for selective COX-2 inhibition. Within each set of experiments there was a control group to which no drug was added, as well as a low, medium and high dose of the drug. In each case, the medium dose was equivalent to a therapeutic dose in vivo.

Two different models of aneurysm disease were used. Human aneurysmal aortic explants were cultured in the presence of the drug to be investigated and the conditioned culture medium was subsequently analysed for cytokine content using ELISAs. In parallel experiments, cultures of porcine aortas that had been pre-treated with elastase were used. After 14 days in culture with the drug under investigation, the tissue was harvested and analysed for matrix metalloprotease activity using gel zymography. Between six and nine explants were used in each experiment.

4.3 Methods

4.3.a Abdominal Aortic Explants

Tissue cultures of human abdominal aortic explants were established using techniques described in the Materials and Methods chapter. The explants were cultured in media containing rofecoxib, naproxen or control. The amount of culture medium used was titrated to the weight of each explant at 0.1 mls/mg. In this way, the media harvested
from each explant were comparable. After 48 hours in culture, the conditioned medium was harvested and analysed for cytokine content.

4.3.b Porcine Model

The elastase-perfused porcine aortic model of aneurysms was utilised as described earlier. The aortic segments were incubated for 14 days in medium containing rofecoxib, naproxen or control. At the end of the incubation period, the tissue was harvested and protein standardised. This allowed comparable samples to be assayed for matrix metalloprotease activity.

4.3.c Drug Preparation

4.3.c.1 Rofecoxib

At room temperature, rofecoxib is a yellow/white powder with very poor solubility. The chemical name is 4-[4-(methylsulfonyl)phenyl]-3-phenyl-2(5H)-furanone, and the empirical formula C_{17}H_{14}O_{4}S. The molecular weight is 314.46 and the chemical structure is shown in Figure 4.1.
In order to create working solutions that could be added to the culture medium, it was necessary to find a suitable solvent. Rofecoxib is insoluble in water and only very slightly soluble in ethanol and acetone. In methanol it is slightly soluble, and this proved to be the best solvent. Care was taken to avoid adding more than 0.5% methanol to the culture medium, as this would be harmful to the explants. Dosages of the drug used were chosen to reflect the steady state plasma concentration reached in clinical practice. The maximum plasma concentration reached after multiple dosages of 25mg in healthy adults was 321 (+/- 114) ng/ml (data from Merck, Sharpe and Dome). This proved to be roughly equivalent to 1mM and therefore the explants were cultured in media containing 0.1mM, 0.3mM and 1 mM rofecoxib. All three of these and the control medium contained the same amount of methanol at a concentration of 0.5%.

Fig 4.1 Chemical structure of rofecoxib.
4.3.c.2 Naproxen and Naproxen-sodium

Naproxen is a white, crystalline powder that is insoluble in water. Although it is freely lipid soluble, there was difficulty in preparing working solutions at neutral pH. However, the sodium salt of naproxen is available as a modified release preparation, and the sodium salt is easily soluble in water. The chemical name of the compound is (+)-6-methoxy- alpha-methyl-2-naphthaleneacetic acid and the empirical formula C$_{14}$H$_{14}$O$_3$. The structure of the molecule is shown in Figure 4.2.

![Chemical structure of naproxen](image)

Fig 4.2 Chemical structure of naproxen

The maximum plasma concentrations reached with administration of 500mg per day of naproxen in divided doses, or of 500mg of naproxen sodium once daily were similar, at around 50 µg/ ml (data from Roche product monograph). To reflect this, the concentrations used in the culture media were 1, 10 and 100 µg/ ml of naproxen sodium. As the drug was soluble in water, there was no need to add carrier to the control medium.
4.3.d Matrix Metalloprotease Activity

At the end of the 14 day incubation period, the porcine tissue was harvested and immediately snap-frozen in liquid nitrogen. The stored tissue was later homogenised and used for the determination of MMP activity as previously described.

4.3.e Cytokine Analysis

Conditioned media were harvested from the human explant cultures after 48 hours and snap frozen. They were later analysed for cytokine content using commercially available ELISAs.

4.3.f Statistical Analysis

Each experiment gave comparable paired data that showed the effect of different concentrations of the drug used. Analysis was performed using Friedman’s test, as a Gaussian distribution was not assumed and the sample sizes were too small to meaningfully test for normality. In the case of missing data (one porcine explant from the naproxen experiments became infected and had to be discarded), Friedman’s test was applied to intact columns and Wilcoxon’s matched pairs test used to compare the column containing the missing datum. These results were corroborated by imputation of the missing datum using the column mean. All analyses were performed using Graphpad Prism software.
4.4 Results

4.4.a Cytokines

Some of the results that were found in these experiments were surprising. From early experiments it had already been established that the human aortic explant cultures were not suitable for examining the effect of drugs on MMP activity, as the time scale over which the explants had been validated was too brief. However, these explants provided a useful system in which to examine the effects on cytokines. The first surprise came with these results (Figs 4.3 and 4.4). Although indomethacin had previously been shown to reduce interleukin-6 concentration in human aortic explants, the same was not seen with rofecoxib or naproxen.

![Graph showing the effect of administering naproxen to human aortic explant cultures for 48 hours](image)

Fig 4.3 The effect of administering naproxen to human aortic explant cultures for 48 hours (p=0.506, n=9).
Fig 4.4 The effect of rofecoxib administration on human aortic explant cultures (p=0.615, n=7).

In the naproxen experiments, the median value of IL-6 measured in the control medium was 187 ng/ml, whilst the 95% confidence interval was 66 – 516 ng/ml. For the increasing doses of naproxen, the medians were 285 (144 – 458) ng/ml, 201 (82 – 401) ng/ml and 165 (56 – 532) ng/ml respectively.

In the rofecoxib experiments, the values were 165 (93 – 207) ng/ml, 115 (42 – 230) ng/ml, 99 (0 – 355) ng/ml and 140 (30 – 328) ng/ml from control up to the highest dose group.

The effect of rofecoxib on both TNF-α and IL-1β were also measured and again, no significant trend was seen when analysed using Friedman’s test. In the control group, the median level (95% confidence interval) for TNF-α was 0.56 (0.37 – 0.94) pg/ml.
For the treatment groups, the values were 0.56 (0.26 – 0.85) pg/ml, 0.41 (0.18 – 1.07) pg/ml and 1.01 (0.53 – 1.27) pg/ml (see Fig 4.5). For IL-1β, the values were very low.

In the control group, the median was 7.4 (0 – 27.69) pg/ml and the values for the treatment groups were 1.0 (0 – 24.02) pg/ml, 10.0 (0 – 45.62) pg/ml and 7.50 (0 – 35.07) pg/ml respectively (see Fig 4.6). Although there was a significant difference when analysed by Friedman’s test, none of the columns were significantly different from control.
Fig 4.5 The effect of rofecoxib on the secretion of TNF-α in human explants (p=0.319, n=7).

Fig 4.6 The effect of rofecoxib on IL-1β produced by human explants (p<0.001, n=7).
4.4.b Matrix Metalloproteases

Gel zymography was performed to demonstrate MMP activity in the porcine aortic explant model. MMP activity was quantified using a scanner linked to Imagemaster software. This gave an optical density measurement that is recorded on the y-axis. Separate bands on the gels represented the secreted, proenzyme forms and the active forms of the enzymes MMP-9 and MMP-2 respectively.

4.4.b.1 MMPs and Naproxen

For the proenzyme form of MMP-9, the optical density readings were 0.97 (0.68 – 1.13), 0.82 (0.52 – 1.18), 0.32 (0.11 – 0.67) and 0.34 (0.15 – 0.49) respectively. Although there was a missing data point in the 10 μg/ml group, when the column median was imputed and the data analysed by Friedman’s test, there was a significant difference with a p-value of <0.001. This was confirmed by comparing the individual columns using Wilcoxon’s signed rank test (p-values are shown above the graph, see Fig 4.7).
Fig 4.7 After two weeks in culture, porcine aortic explants treated with naproxen expressed significantly lower levels of MMP-9 activity than controls (Friedman’s test, p<0.001, n=6).

For the active form of MMP-9, the results were 0.17 (0.06 – 0.34), 0.17 (0.11 – 0.22), 0.12 (0.07 – 0.22) and 0.09 (0.07 – 0.14). This just failed to reach significance when analysed by Friedman’s test with a p-value of 0.066. There was, however, a significant difference between control and the highest dose group with a p-value of 0.031 (see Fig 4.8).
Fig 4.8 The effect of naproxen on the active form of MMP-9. There was no significant difference when analysed by Friedman's test, although there was a difference between control and the highest dose group (p=0.031, n=6).

For the proenzyme form of MMP-2, the results with naproxen were 0.40 (0.30 – 0.50), 0.53 (0.44 – 0.59), 0.46 (0.32 – 0.60) and 0.34 (0.21 – 0.52). There was no significant difference (see Fig 4.9).
Fig 4.9 The effect of naproxen on the proenzyme form of MMP-2. No significant effect was demonstrated (p=0.163, n=6).

Finally for naproxen, the effect on the active form of the enzyme MMP-2 was measured. For the control group, the optical density reading was 0.34 (0.29 – 0.44), whilst increasing the concentration of naproxen gave results of 0.41 (0.32 – 0.48), 0.30 (0.22 – 0.43) and 0.24 (0.11 – 0.37). These results are shown in Fig 4.10.
Fig 4.10 The effect of naproxen in the active form of the enzyme MMP-2. No significant difference was demonstrated ($p=0.156$, $n=6$).

4.4.b.2 MMPs and Rofecoxib

Whilst naproxen was able to reduce the levels of MMP-9 in the aortic aneurysmal cultures, the same was not true of rofecoxib. For the proenzyme form of MMP-9, the optical density recorded in the control group was 0.34 (0.22 – 0.40) and in the treatment groups the results were 0.30 (0.25 – 0.48), 0.30 (0.24 – 0.40) and 0.33 (0.27 – 0.45). Clearly there was no significant difference here (see Fig 4.11).
Fig 4.11 The effect of rofecoxib on the proenzyme form of MMP-9. No significant
effect was seen (p=0.247, n=7).

For the active form of MMP-9, the results were very low. The median optical density
in the control group was 0.03 (0.02 – 0.05), whilst the results when rofecoxib was
added in increasing doses were 0.05 (0.03 – 0.07), 0.04 (0.01 – 0.07) and 0.04 (0.02 –
0.06). No significant difference was demonstrated, although it would be hard to draw
any conclusions from such a low range of values (see Fig 4.12).
Fig 4.12 The effect of rofecoxib on the expression of the active form of MMP-9. The values are all very low, but no significant difference is demonstrated (p=0.405, n=7).

For the proenzyme form of MMP-2, the results were 0.24 (0.15 – 0.37), 0.25 (0.18 – 0.34), 0.22 (0.14 – 0.31 and 0.23 (0.15 – 0.28). These (non-significant) results are shown in Fig 4.13.

Fig 4.13 The effect of rofecoxib on the proenzyme form of MMP-2 (p=0.116, n=7).
Finally, the effect of rofecoxib on the expression of the active form of MMP-2 was measured. The results were 0.03 (0.01 – 0.08), 0.04 (0.02 – 0.05), 0.04 (0.01 – 0.06) and 0.03 (0.01 – 0.04, see Fig 4.14).

Fig 4.14 Effect of rofecoxib on the active form of MMP-2. All the values were very low, but there was no significant difference seen between control and treatment groups (p=0.801, n=7).
4.4.c Specific COX Assays

In order to prove that the rofecoxib was having any effect at all on the explant culture, levels of both prostaglandin E2 (PGE-2) and thromboxane B2 (TBX-2) were also measured. PGE-2 was reduced in a dose dependent manner by rofecoxib. The median level in the media of the controls was 4632 (4124 – 4952) ng/ml, and with the increasing doses of rofecoxib the results were 3635 (1943 – 4203) ng/ml, 1806 (1111 – 3825) ng/ml and 1699 (362 – 3368). These results are displayed graphically in Fig. 4.15.

Fig 4.15 The effect of rofecoxib on the expression of prostaglandin E2. The drug reduced the expression of PGE-2 in a dose-dependent manner. Overall variability was proven using Friedman’s test (p=0.002, n=7). The columns were then compared with control using Wilcoxon’s signed rank test and the individual p-values are given above the graph.
Rofecoxib caused a slight reduction in the levels of thromboxane B2, although this was not significant when analysed by Friedman's test \((p=0.116)\). The median and 95% confidence intervals for the control group were 405 (309 – 544) ng/ml, whilst the respective treatment group results were 336 (194 – 495) ng/ml, 233 (124 – 464) ng/ml and 227 (69 – 587) ng/ml \((n=7, \text{ see Fig 4.16})\).

![Graph](image)

Fig 4.16 Thromboxane B2 production reflects the activity of COX-1. As can be seen in the graph above, there was a slight, dose-dependent reduction but this was not statistically significant \((p=0.116, \text{ n=7})\). This is also very much in keeping with a drug that has anti-inflammatory properties but is highly selective for COX-2.
4.5 Discussion

The results that were observed during the course of these experiments were not in keeping with initial expectations. It must be remembered that the primary aim of this work has always been focussed on finding a medical therapy that would prevent aneurysm growth and rupture. Any such treatment would have to be safe in long term use and acceptable to the patient, and would have to target the twin processes of inflammation and matrix degradation. Clearly powerful, non-selective, non-steroidal anti-inflammatories would not be appropriate, but it was hoped that COX-2 selectivity might provide a suitable alternative by reducing the expression of MMPs by the aortic explants. However, it is clear from section 4.4.b.2 that no such effect was seen.

As the results from the experiments using rofecoxib failed to show any effect on MMP-9 or cytokine expression, it was suspected that the rofecoxib might not have been having any effect at all in this system. It was conceivable that some part of the drug delivery process had been in error, or that this COX-2 antagonist simply had no effect on the tissues in culture. Further tests were therefore performed to see if this had been the case. The conditioned media from the human explant experiments were assayed for levels of prostaglandin E2, and also thromboxane B2. The results are shown in figs 4.15 and 4.16. A reduction in prostglandin E2 would suggest inhibition of COX-2, whereas decreased levels of thromboxane B2 suggest a reduction in the activity of COX-1. As can be seen in Fig 4.15, there was a dramatic reduction in PGE-2 in explants treated with rofecoxib, demonstrating strong activity against COX-2. Fig 4.16 shows a much smaller decrease in TBX-2 which was not statistically
significant, as would be expected in a drug which is highly selective for COX-2. Clearly, the drug was thereby demonstrated to be active in this system.

The results with regards to interleukin-6 were particularly interesting, as it closely reflects inflammation within the tissue. It had already been shown to be reduced by the action of indomethacin on aortic explants (Franklin et al. 1999c), and therefore should have been reduced at least by naproxen, if not by rofecoxib. That it was reduced by neither of these drugs was somewhat unexpected, but may reflect the controversial role of IL-6. As discussed earlier, at the outset of this work, IL-6 was presumed to be a pro-inflammatory cytokine, but evidence from the literature suggests that it may actually be anti-inflammatory (Dinarello, 1997; Dinarello et al. 1991; Hauptmann et al. 1991). This controversy may explain why the relationship between IL-6 and inflammation is perhaps not as linear as previously supposed. In addition, one paper has shown that increased levels of IL-6 are associated more with aorto-occlusive disease than AAA, casting some doubt on its relevance to these experiments (Reilly et al. 1999).

Other cytokines measured were interleukin 1β and tumour necrosis factor alpha (Figs 4.5 and 4.6). Although both of these were present in small concentrations, neither showed a consistent reduction in response to either drug. Indeed, measurement of these two cytokines in human explants throughout this thesis gave extremely variable results, unrelated to drug administration. This was in spite of the fact that standard curves were produced as expected, indicating the assays were working well. For this reason, the assays were not repeated for the explants treated with naproxen. Subsequent work in our laboratories has shown that there was a degree of degradation
of cytokines kept in the -70° C freezer, and this was especially true of IL-1β and TNF-α. This may explain the inconsistent results found in this thesis.

The effect on MMP-9 activity was the most important finding in these experiments. It was demonstrated that the addition of naproxen to explants in culture caused a reduction in both the proenzyme form and active MMP-9 as measured by gel zymography. This had not been demonstrated previously, although Thompson’s group showed that indomethacin reduced MMP-9 activity in the rat elastase perfusion model (Holmes et al. 1996). Interestingly, none of the rats treated with indomethacin then went on to develop aneurysms, whereas five out of six of the control animals did. In Holme’s experiments, the dramatic prevention of aneurysm development by indomethacin was clearly thought to be due to inhibition of MMP-9. By contrast, the aneurysmal aortic explants treated with indomethacin by Franklin et al showed a reduction in IL-6, IL-1β and PGE-2, but no effect on MMP-9 was seen (Franklin et al. 1999c). Why the difference? By looking at the two experimental systems one explanation springs to mind. The main sources of MMP-9 in Holme’s work were macrophages, of which there was an effectively endless supply in the living rat subjects. Macrophages in tissue culture were limited in number, and it is quite possible that differences in washing and cleaning the explants between Franklin’s experiments and those in this thesis may have resulted in different numbers of macrophages remaining in the culture.

Prostaglandin E2 has been postulated as a major contributor to aneurysm development, and has been shown to be vastly increased in aneurysmal aortas when compared to controls (Holmes et al. 1997). In addition, this study showed that PGE-2
was related to macrophage-like cells in the inflammatory infiltrate, and these findings have been echoed several times (Franklin et al. 1999b; Franklin et al. 1999c; Hakamada-Taguchi et al. 2003; Hong et al. 2000; Miralles et al. 1999; Reilly et al. 1999; Walton et al. 1999). Indeed, Walton et al went on to demonstrate that PGE-2 was harmful to aortic smooth muscle cells in culture, caused cell death with oligonucleosome production, and inhibited growth in mixed smooth muscle and monocyte culture from aneurysms. Holmes showed that PGE-2 was associated with high concentrations of COX-2 from tissue macrophages in aneurysmal tissue that were not seen in normal aortas. These papers formed significant evidence that selective inhibition of COX-2 would reduce the concentration of harmful PGE-2 and could potentially prevent MMP-9 mediated matrix degradation.

The evidence gathered from the experiments in this chapter clearly does not support that hypothesis. COX-2 selective inhibition had no effect on MMP-9 activity nor on IL-6 expression. This was made all the more striking by the fact that naproxen did reduce MMP-9 activity. These results were so unexpected that at first experimental error, or a peculiarity of the drug preparation was expected. For this reason, specific assays of COX-1 and COX-2 activity were conducted on the cultures treated with rofecoxib. These behaved exactly as predicted, suggesting that the COX-2 selective inhibitor was doing what it was supposed to, but nevertheless having no effect on MMP activity.

Why, then, was combined COX-1 and COX-2 inhibition able to reduce MMP activity whilst inhibition of COX-2 did not? There is strong evidence that COX-2 is the only significant isoform of the enzyme in aneurysmal tissue. Again, it is conceivable that
the effect of these drugs in vitro may be different to in vivo, whether due to the absence of macrophages or some other cellular interaction. Whether the small amounts of COX-1 expressed in aneurysms were enough to trigger a sequence of events leading to increased MMP activity has not been proven, but one finding is clear.

In the models used for these experiments, COX-2 selective inhibition was not sufficient to prevent the processes involved in aneurysm formation. This was an important negative finding, and implied that the coxibs would not be useful as a medical treatment for small aneurysms. The next step was therefore to investigate whether simvastatin would be more effective.
Chapter 5

The Role of HMG CoA Reductase Inhibitors (Statins) in the Prevention of Aneurysm Development

5.1 Introduction

The results of the experiments using COX-2 antagonists suggested that they would not be suitable as a medical therapy for small abdominal aortic aneurysms, and therefore further drugs would need to be investigated. The most exciting prospect was undoubtedly the group of drugs known as statins, or HMG CoA reductase inhibitors. These drugs had been used for many years for the control of hypercholesterolaemia, and are well tolerated (Feher et al. 1995). Various side effects have been reported, ranging from myositis and rhabdomyolysis to neuropathy and raised liver function tests. The overall risk of rhabdomyolysis has been reported as 0.1 - 0.2% (Hamilton-Craig, 2001), but the risk of less serious events such as myositis may be much higher, up to 1 - 5% (Thompson et al. 2003). The risk of rhabdomyolysis and other adverse effects with statin use can be exacerbated by several factors, including compromised hepatic and renal function, hypothyroidism, diabetes, and concomitant medications. Antibiotics such as clarithromycin which affect the cytochrome P450 system should be avoided in patients undergoing statin therapy as they increase the risk.

Concomitant administration of fibrates increases the plasma concentration of statins but has been shown to be safe in a study of 102 patients (Feher et al. 1995). No patient in this randomised, controlled trial suffered with rhabdomyolysis, although
four had mildly raised creatinine kinase and fourteen showed borderline disturbances in aspartate transaminase.

Peripheral neuropathy has also been reported in association with statin use both in epidemiological studies and case reports, but not in a randomised, controlled trial (Backes and Howard, 2003). The only treatment that has been recommended for any of these side-effects was cessation of statin treatment, which was at least partially effective in all cases.

The evidence suggesting that statins might be of benefit in the treatment of small aneurysms had been mounting in the literature. HMG CoA reductase inhibitors had been developed and marketed for the lowering of plasma cholesterol, and studies have shown that they are very effective at this, with reductions in low density lipoproteins (LDLs) of between 19 and 60% (Takemoto and Liao, 2001). It also became clear that they had other beneficial effects, which may or may not have been related to their cholesterol-lowering properties. The West of Scotland Coronary Outcomes Prevention Trial (WOSCOP) provided strong evidence that treatment with statin therapy reduced the incidence of myocardial infarction and death from coronary heart disease (Shepherd et al. 1995). However, subgroup analysis of this trial showed that even when cholesterol levels in the statin-treated and placebo groups were comparable, patients taking a statin had a significantly lower risk of coronary heart disease than age matched control subjects taking placebo. Moreover, meta-analysis of trials of cholesterol-lowering therapy have suggested that the risk of myocardial infarction in patients treated with statins is lower than that in patients whose cholesterol is lowered by other means, despite similar cholesterol levels in both groups (Pekkanen et al. 1990).
Earlier work of Brown et al in the Familial Atherosclerosis Treatment Study (Brown et al. 1993a; Brown et al. 1993b) had shown a very large reduction (75%) in coronary events despite a modest reduction in the degree of stenosis seen (<1%). This was attributed to a major decrease in the number of lipid-rich foam cells in selected plaques that displayed a "high-risk" morphology, i.e. those plaques with a large lipid core and high numbers of intimal macrophages. Looking at these findings in the light of subsequent research, it might be suggested that the clinical benefits of statin therapy were not simply due to cholesterol-lowering, but that some other mechanism or mechanisms must be involved.

5.1.a Statins and Vascular Inflammation

The concept of vascular events as a product of inflammation within the atherosclerotic wall rather than as a direct result of atherosclerosis was relatively new, but by the late 1990s, laboratory work had begun to show that statins could reduce inflammatory markers. Pahan et al looked at the effect of lovastatin on astrocytes, microglia and macrophages derived from rats, and demonstrated a reduction in the levels of TNF-α, IL-1β and IL-6 produced in response to lipopolysaccharide (Pahan et al, 1997). Inoue et al described experiments using statins on cultured human umbilical vein endothelial cells (HUVECs) (Inoue et al, 2000). They demonstrated that 4 different statins all caused a reduction in the inflammatory cytokines IL-1β and IL-6, as well as a reduction in COX-2. The observed reductions were both at the transcription level (mRNA) and in the expression of the molecules in the culture supernatant. Kothe’s group took human lung macrophages from bronchoscopies and cultured them in the presence of Chlamydia, which had previously been linked to vascular inflammation.
(Kothe et al, 2000). Co-incubation in the presence of cerivastatin resulted in lower levels of IL-8 and of monocyte chemattractant protein (MCP-1), although the levels of TNF-α were not affected. Similar effects were seen when the experiments were repeated using HUVECs.

Clinically, the Cholesterol and Recurrent Events (CARE) study examined the effect of statin therapy on patients who had had previous myocardial infarcts (MIs) (Ridker et al. 1998). In a case-control study, these patients were randomised to receive either pravastatin or placebo and followed by serial measurements of C-reactive protein (CRP) and serum amyloid-A (SAA), both markers of inflammation. 391 patients subsequently had a further myocardial infarct, and these 391 were compared to an equal number of age- and sex-matched controls who had no further coronary event. The highest risk group from this study was the group that showed signs of persistent inflammation but had been randomised to receive placebo. In the placebo group, the association between inflammation and risk was significant in the control group, but not in the treatment group. The same group of investigators also showed that long-term treatment with pravastatin in patients who had had an MI reduced the level of CRP (Ridker et al. 1999). In subjects treated with placebo, the plasma CRP tended to increase over the course of 5 years, whilst in those treated with a statin, CRP tended to fall.

The Pravastatin Inflammation/CRP Evaluation study consisted of two separate branches (Albert et al. 2001a; Albert et al. 2001b). In the first part of the study, patients who had already had an MI were given pravastatin and followed up for 24 weeks. In the second part of the study, healthy volunteers were randomised to receive
either pravastatin or placebo, and followed for the same length of time. CRP levels were measured in all patients at baseline, 12 and 24 weeks. In both arms, pravastatin therapy was shown to reduce CRP independently of measurements of LDL-cholesterol.

These experiments have given convincing evidence that statins were able to reduce vascular inflammation, one of the key elements of aneurysm pathogenesis.

**5.1.b Statins and Matrix Metalloproteases**

In Bellosta’s elegant paper both murine peritoneal macrophages and human monocyte-derived macrophages from the blood of healthy volunteers were cultured (Bellosta et al. 1998). Firstly, the cultures were treated with either fluvastatin or placebo, and the levels of MMP-9 activity in the conditioned media were measured by gel zymography. The drug inhibited the activity of MMP-9 in both murine and human macrophages by around 30%. This reduction was confirmed by both Western blotting and ELISA. The addition of phorbol esters to the cultures caused an increase in MMP-9 activity by 50%. In cultures treated with fluvastatin, however, this increase was cut by half. These effects were reversed by mevalonate, the product of the action of HMG CoA reductase (see Chapter 6). Finally, in order to prove that the drug was not having a direct effect on the activation of MMP-9 after secretion, macrophages were cultured in the absence of the drug. After 24 hours, the conditioned media were collected and separated from the cells. The media were then cultured for a further 24 hours in the presence of the drug at the same doses as before. In this case, no effect
on MMP-9 activity was seen, proving that the action of the drug was occurring at a cellular level.

A more clinical paper randomised 32 hypercholesterolaemic patients with coronary artery disease to receive either 20mg simvastatin daily or placebo for 14 weeks (Son et al, 2003). Plasma MMP-9 levels were measured before and after the treatment period, and in the simvastatin group there was a significant reduction. This reduction correlated well with a similar reduction seen in plasminogen activator inhibitor 1 (PAI-1).

Further laboratory work has been undertaken to examine the effect of statins on the development of intimal hyperplasia on organ cultures of human saphenous vein explants taken from patients undergoing coronary artery bypass grafting (Porter et al. 2002). These experiments showed that not only did simvastatin reduce the formation of neointimal hyperplasia, but that MMP-9 activity was also inhibited.

More recently, Nagashima reported the effects of cerivastatin on the production of MMP-9 on organ cultures of aneurysmal aortas (Nagashima, 2002). The statin reduced the levels of both active and total MMP-9 in a dose dependent manner, although these measurements were all performed by ELISA rather than zymography.

Consistent evidence exists then, that statins can reduce matrix metalloprotease activity, another key feature of aneurysm pathogenesis.
**5.1.c Further Pleiotropic Effects of Statins**

Pathogenesis of AAAs has been shown to be associated with disruption of the architecture of the aortic wall, much of which is due to the activity of MMPs. Changes in the ratios of collagen:elastin:smooth muscle cells have been reported. Several studies have shown that statins affect smooth muscle cell migration or proliferation, although the role in aneurysm pathogenesis is unclear. Porter showed that proliferation as well as migration of human smooth muscle cells across a modified Boyden chamber was inhibited by simvastatin (Porter et al. 2002). Fukumoto demonstrated that fluvastatin reduced the number of smooth muscle cells within the intima of a strain of hypercholesterolamic rabbits (Fukumoto et al. 2001). Vincent described a reduction in angiogenesis due to statin-mediated inhibition of endothelial cell migration (Vincent et al., 2001). The mechanism by which this occurred will be discussed in more detail in Chapter 6.

New research has looked at the potential of T-helper cells to display pro-inflammatory (TH-1) or anti-inflammatory (TH-2) properties. Kato’s group showed that in CD4 cells cultured *in vitro*, the statins promoted TH-1 polarisation, and in that way could be considered anti-inflammatory (Hakamada-Taguchi et al. 2003). They confirmed their findings *in vitro* by parallel work in a murine model.

The literature detailed above provided excellent justification for the proposal of statins as a suitable treatment for small abdominal aortic aneurysms, leading to the following experiments in this chapter.
5.2 Plan of Investigation

Two systems were used to examine the effects of simvastatin on aneurysmal tissue. Firstly, the porcine model described in the Materials and Methods chapter was used to look at the effect of simvastatin on MMP activity, as assessed by gel zymography. One of the advantages of using this model was the uniformity of the tissue cultures, which were all subjected to the same amount of proteolysis in the first 24 hours. Because of this, meaningful interpretations of histology could be made in a way that was simply not possible using human aneurysmal tissue, as the latter was very heterogeneous in nature.

Human aneurysmal explants were also cultured and treated with simvastatin in order to examine the effects of the drug on inflammation and cytokine production.
5.3 Methods

5.3.a Porcine Model

Porcine explants were established using the techniques described earlier. After treatment for 24 hours with elastase, the explants were cultured in a control solution or one of three doses of simvastatin for 14 days. The tissue was subsequently harvested and divided into two. Half was used for histological analysis, whilst half was used for the determination of MMP activity.

5.3.b Human Aneurysmal Explants

As before, explants of human aneurysmal aortas were established in the laboratory. These were divided into 4 and either control fluid or one of three doses of simvastatin was added to the culture medium. After 48 hours in culture, the conditioned medium was harvested and analysed for cytokine content using ELISA.
5.3.c Drug Preparation

Simvastatin is a white crystalline powder that is derived from a fermentation product of *Aspergillus terreus*. It is practically insoluble in water, but freely soluble in methanol and ethanol. The chemical name is butanoic acid, 2,2-dimethyl-, 1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)-ethyl]-1-naphthalenyl ester and the empirical formula is C\textsubscript{25}H\textsubscript{38}O\textsubscript{5}. The molecular weight is 414.57, and the structural formula is shown in Fig. 5.1.

![Chemical structure of simvastatin](image)

In this state, the drug is a lactone, which is biologically inactive. *In vivo*, the drug is readily hydrolysed to the corresponding hydroxy acid, which is a potent inhibitor of HMG CoA-reductase, and the hydrolysation needed to be performed artificially before adding the drug to the explants.
To activate the drug, 8 mg of simvastatin were diluted in 0.4 mls of ethanol. To this was added 0.6 ml of sodium hydroxide (NaOH) at a concentration of 0.1 Molar. This mixture was subsequently heated at 60° C for 2 hours and then brought to pH 7.4 by the addition of 0.1 M hydrochloric acid. The final volume was brought to 2 mls with distilled water, giving a stock solution of 10 mM concentration activated simvastatin. From this, working solutions of 10 μM, 30 μM and 1 μM and a control solution were derived, all containing a carrier, 1% dimethyl sulfoxide (DMSO). When added to the culture medium, these gave final concentrations of 0, 0.1 μM, 0.3 μM and 1 μM simvastatin, each with the same concentration of carrier, 0.01% DMSO (DMSO may be toxic to tissue cultures at levels above 0.05%). The middle concentration of 0.3 μM is approximately equivalent to the plasma levels achieved in a patient taking 20 mg simvastatin once daily.

5.3.d Histological Analysis

After 14 days in culture, the porcine explants were harvested and cut in half. Whilst half was frozen for MMP estimation, the other half was preserved in formaldehyde for histological examination. The slides were stained for elastin and collagen, and the relative proportions of elastin, collagen and smooth muscle cells were measured using stereotactic techniques described earlier.

5.3.e Matrix Metalloprotease Activity

This was assayed using gel zymography (see Materials and Methods chapter)
5.3.f Cytokine Analysis

Again, this was performed using commercially available ELISA kits as described earlier.

5.3.g Statistical Analysis

All statistical analyses were carried out using Graphpad Prism software. As each experiment consisted of a single explant that had been divided into four and treated with different concentrations of simvastatin, the data were treated as paired samples and analysed for intercolumn variability using Friedman's test. If variability was proven, then the individual columns were analysed using Wilcoxon matched pairs. In any case of missing data points, the columns were analysed as given, and the results corroborated by imputation of the column mean.
5.4 Results

5.4.a Human Aortic Explants and Cytokines

The results of the assays for interleukin-6 showed significant intercolumn variability when analysed by Friedman’s test (p=0.023, n=6). The medians (95% confidence intervals) were 108.3 (73-139.5) ng/ml, 51.25 (20.34-98.46) ng/ml, 35 (13.03-72.64) ng/ml and 71.75 (30.59-89.91) for the control and increasing dosage groups respectively. Interestingly, whilst the middle dose group showed a significant difference from control when tested using Wilcoxon’s signed rank test, the higher dose group did not (see Fig 5.2).

The results for TNF-α did not show any significant variation between the columns (see Fig 5.3). The medians (95% confidence intervals) were 1.70 (0.56-3.39) pg/ml, 1.78 (0.57-3.41) pg/ml, 1.87 (0.52-3.74) pg/ml and 1.87 (0.27-4.86) pg/ml respectively (n=7).

Measured levels of IL-1β were much lower than anticipated, with several samples registering none at all (Fig 5.4). This was despite the assays giving good standard curves, and a repeated ELISA gave similar results. The levels were 0 (0-4.5) pg/ml in the control group and 2.2 (0-6.49) pg/ml, 1.25 (0-3.33) pg/ml and 1.65 (0-4.38) pg/ml in the treatment groups (n=6).
5.4. b Porcine Model and MMP Activity

Gel zymography to demonstrate MMP activity in the porcine model gave a number of significant results. The MMPs were analysed in both the secreted, proenzyme form and the activated form (see Fig 5.5). The proenzyme form of MMP-9 demonstrated a significant difference between the groups (Friedman’s test, p=0.013, n=8). The medians (95% confidence intervals) were 0.90 (0.49-1.37), 0.61 (0.49-0.71), 0.43 (0.25-0.59) and 0.30 (0.15-0.55). The p-values are given in Fig. 5.6.

The active form of MMP-9 was also significantly reduced by simvastatin (p=0.016). The optical densities were as follows; 0.21 (0.03-0.56), 0.18 (0.08-0.31), 0.11 (0.02-0.26) and 0.13 (0.05-0.23) for the respective groups (n=8, see Fig 5.7).

Similarly, the proenzyme form of MMP-2 showed significant variation when treated with simvastatin (p=0.027, n=8). The medians and 95% confidence intervals were 0.50 (0.32-0.70), 0.35 (0.26-0.63), 0.31 (0.19-0.48) and 0.32 (0.12-0.55). The results are shown graphically in Fig 5.8.

Finally, the active form of MMP-2 showed a highly significant reduction in response to simvastatin (Friedman’s test, p<0.01, n=8). The results for the control and treatment groups respectively were 0.18 (0.14-0.23), 0.12 (0.06-0.17), 0.13 (0.08-0.15) and 0.09 (0.07-0.15) and individual p-values are shown in Fig 5.9.
5.4. c Histology

Histological data are expressed as percentage elastin in the treated aorta, as described in Chapter 3. Of note, the aortas treated with simvastatin tended to show preservation of the elastin lamellae towards the centre of the specimen when compared to those treated with elastin only (see Figs 5.10-5.12). The positive control group was treated with elastin for 24 hours and then cultured for 14 days in exactly the same way as the treatment specimens. The percentage elastin (95% confidence interval) for this group was 23.5 (11.8-31.9)%, compared to 25.4 (17.5-30.0)%, 42.2 (28.8-50.6)% and 42.9 (33.0-49.5)% for the treatment groups. There was a significant variation as demonstrated by Friedman’s test (p=0.019, n=9, see Fig 5.13). Negative controls were also established which were treated in exactly the same way as the others except no elastase was added at all. These had a median elastin content of 62.8% with a 95% confidence interval of 58.4-67.9%, n=3.
Fig 5.2 The effect of simvastatin on the production of interleukin-6 by human aneurysmal aortic explants. There was a significant variation between the columns as shown by Friedman’s test (p=0.034, n=6). The individual columns were compared with control using Wilcoxon’s signed rank test and the resulting p-values are shown above the corresponding columns.
Fig 5.3 The effect of simvastatin on the production of tumour necrosis factor α by human aneurysmal aortic explants. As can be seen in the graph, no significant difference was demonstrated (Friedman’s test, p=0.844, n=6).
Fig 5.4 The effect of simvastatin on the production of interleukin 1-β by human aneurysmal aortic explants. The levels of IL-1β found in the aortic explants were much lower than expected, with several explants displaying none at all. Despite this, the assays produced good standard curves (p=0.844, n=6).
Fig 5.6 The effect of simvastatin on the expression of the proenzyme MMP-9 by porcine aortic explants. There was a significant variation between the columns as shown by Friedman’s test (p=0.013, n=8). Although the medians decreased in a dose-dependent manner, only the middle dose group was significantly different to control, as the spread of the high dose group was too large.
Fig 5.7 The effect of simvastatin on the expression of active MMP-9 by porcine aortic explants. Although the graph is not very striking, there is a significant downward trend (Friedman, p=0.016). The medium and high dose are significantly lower when compared to control using Wilcoxon's signed rank test (p-values are shown above the graph).
Fig 5.8 The effect of simvastatin on the expression of the proenzyme MMP-2 in porcine aortic explants. There was a significant variation between the columns (Friedman’s test, $p=0.027$, $n=8$). The individual dose groups were compared to control using Wilcoxon’s signed rank test and the resulting $p$-values are given above the graph.
Fig 5.9 The effect of simvastatin on the expression of the active form of the enzyme MMP-2 in porcine aortic explants. There was significant intercolumn variation (Friedman's test, p=0.007, n=9). The individual columns were compared with control using Wilcoxon’s matched pairs test and the resulting p-values are shown above the graph.
Fig 5.10 Photomicrograph of a porcine aortic segment which has been cultured for 14 days. This segment was not pretreated with elastase and shows excellent preservation of the normal architecture throughout the whole specimen. Note the organised elastin lamellae which show up blue with the elastin Van Gieson staining.
Fig 5.11 Photomicrograph of a porcine aortic segment pretreated with elastase and then cultured for 14 days. In this specimen, the elastin lamellae (shown in blue) are very poorly preserved throughout the entire segment.
Fig 5.12 Photomicrograph of a porcine aortic segment treated with elastase and then cultured in medium containing simvastatin at 1 μM concentration. The top half of the picture shows a more central portion of the segment in which the elastin lamellae are relatively well-preserved, whilst the bottom half of the picture shows the periphery of the segment in which the elastin has been almost completely lost. This may be due to the action of exogenous elastase on the outermost parts of the explants, whereas the central elastin lamellae have been preserved due to suppression of endogenous MMPs (see text).
Fig 5.13 Histological analysis of porcine aortic segments treated with elastase and simvastatin. The x-axis shows the dose of simvastatin in which the aortic segments were cultured, whilst the y-axis shows the percentage of elastin in the segments at the end of the time in culture. As can be seen from the graph, there was a significant trend towards preservation of elastin in the higher dose treatment groups (Friedman’s test, p=0.10, n=6). The individual columns were compared to control using Wilcoxon’s signed rank test and the resulting p-values are displayed above the columns.
5.5 Discussion

The results of these experiments echo the results of other work, particularly that of Bellosta (Bellosta et al. 1998) and Nagashima (Nagashima et al. 2002). In the first of these studies, Bellosta showed that statins reduce the secretion of MMP-9 by human macrophages in culture, although here we were looking at organ culture rather than cell culture. Earlier experiments in our laboratories had failed to show any reduction in MMP activity in human aortic explants under the action of simvastatin, and at the time we hypothesised that this was due to the short time in culture (48 hours compared to two weeks for the porcine model). However, it was also possible that this apparent lack of response may have been due to a low concentration of macrophages in the organ culture system.

In the porcine model, the results have shown a consistent reduction in MMP activity throughout the proenzyme and active forms of MMP-2 and MMP-9 in response to treatment with simvastatin. The results for MMP-9 were as expected, bearing in mind the earlier results of Bellosta. Similar results for MMP-2 have not previously been described. Publication of Nagashima’s work in July 2002 made it more difficult to explain the failure of simvastatin to inhibit MMP activity in our pilot experiments on human explants. Nagashima also experimented on human aneurysmal aortic explants, this time using cerivastatin (a drug whose licence for use in humans has recently been withdrawn). This series of experiments showed a dose-dependent reduction in both active and total MMP-9, although the protease levels were only assayed using ELISA. This paper also localised the production of MMPs to neutrophils and macrophages within the aortic wall.
Interestingly, the medium and low doses of simvastatin both caused a significant reduction in the levels of interleukin-6, whereas no effect was seen with the higher dose (see Fig 5.2). It is not clear whether this apparent lack of effect was due to the higher dose being in some way toxic to the cultures, or whether it was simply a result of the relatively small sample sizes. This effect has not been reported previously, whereas there have been reports of statins causing reduction in the secretion of IL-6. Pahan's group looked at cultured astrocytes, microglia and macrophages from rats, in which inflammation had been induced by lipopolysaccharide (Pahan et al. 1997). They demonstrated that lovastatin not only caused a reduction in IL-6, but also in IL-1β and TNF-α. Inoue's group experimented with cultured human umbilical vein endothelial cells (Inoue et al. 2000). Using four different HMG CoA reductase inhibitors, they showed that all of the drugs were able to reduce the expression of mRNA for IL-6 and IL-1β. Moreover, the levels of the cytokines in the culture supernatants were also reduced.

Once again, in these experiments the levels of IL-1β and TNF-α were both extremely low, with many cultures registering zero (see Figs 5.3 and 5.4). This was despite the fact that the assays themselves were shown to be working well by producing good standard curves.

The results of histological analysis overall showed a preservation of elastin in organ cultures treated with simvastatin. Due to the extremely heterogeneous nature of the aneurysmal arterial wall, it was not possible to obtain comparable data from the human cultures, but the porcine experiments showed consistent results. The positive
controls were treated with elastase for 24 hours before this was washed off and replaced by culture medium for a further 14 days. As has been previously described by Wills et al (Wills et al. 1996), the initial elastolysis induced further protein degradation by MMPs which lasted well after the elastase had been removed. These positive controls were found to have very little elastin left throughout the entire specimen at the end of the two-week culture period (see Fig 5.11). The segments treated with simvastatin, however, had a different appearance. The periphery of these tissues appeared very similar to that in the positive controls, with almost total loss of elastin lamellae. In the centre of the tissue, the lamellae were well-preserved, with appearances similar to the negative controls to which no elastase had been added (see fig 5.12). One explanation for this would be that the peripheral tissue was degraded directly by the action of the exogenous elastase, whereas degradation of the elastin towards the centre of each segment relied upon activation of endogenous MMPs. Treatment with higher doses of simvastatin blocked the action of these MMPs, and therefore the architecture was preserved.

From the evidence obtained from these experiments, it became apparent that simvastatin was able to reduce both matrix metalloprotease activity and the secretion of interleukin-6 (a cytokine linked with inflammation) in tissue cultures. These represented two highly important findings, but there remained two further steps. The first was to elucidate the pathway by which these effects were mediated, and the second was to see whether the same beneficial effects could be demonstrated in vivo. Chapter Six will deal with the first of these questions, and Chapter Seven the second.
Chapter Six

The Role of Isoprenoid Metabolites

6.1 Introduction

The experiments in Chapter Five demonstrated that simvastatin was able to reduce both MMP activity and the secretion of interleukin-6, a cytokine closely related to inflammation. What was not made clear in that chapter was the mechanism by which these effects were mediated. As discussed in earlier chapters, there has been considerable debate about the so-called “pleiotropic effects” of the statins, and whether they were due to a reduction in cholesterol, or whether some other pathway was involved (Farmer, 2000; LaRosa, 2001; Takemoto and Liao, 2001). The key to this question lies in an understanding of the metabolic pathways that are inhibited by the statins. Fig 6.1 shows a simplified pathway including the isoprenoid intermediates geranylgeraniol and farnesyl (which are usually conjugated in vivo with pyrophosphate), along with the relevant enzymes. It is these intermediates that will form the focus of this chapter.

6.1.a Mevalonate and Inflammation

Mevalonate is the metabolite whose synthesis is directly blocked by the action of HMG CoA reductase inhibitors as can be seen in Fig 6.1. It would be logical to
assume that any effect seen in a biological system that is directly attributable to the action of a statin would be reversed by the addition of mevalonate. Indeed, Bellosta showed just that in his work with human macrophage cultures (Bellosta et al. 1998). Whereas the secretion of MMP-9 was blocked by the addition of fluvastatin or simvastatin to the culture media, this effect was reversed by the addition of mevalonate.

Diomede treated mice with statins over a short time period such that blood cholesterol levels were not reduced (Diomede et al. 2001). By creating a pouch and using carrageen and lipopolysaccharide to induce inflammation, they were able to demonstrate that the statins inhibited the recruitment of leukocytes and the secretion of inflammatory mediators into the exudate, including IL-6 and MCP-1. The administration of mevalonate reversed the effect on leukocyte recruitment. Intriguingly, they repeated the experiments using squalestatin, a drug that only inhibited the production of sterol metabolites. This had no effect on inflammation, suggesting that the observed anti-inflammatory properties of statins were due to the inhibition of non-sterol derivatives of mevalonate rather than any effect on cholesterol.
Fig 6.1 The cholesterol synthesis pathway. Enzyme systems are shown in red.
6.1.6 Oxidative Stress

Hattori investigated the production of nitric oxide and inducible nitric oxide synthase (iNOS) in vascular smooth muscle cells in response to interleukin 1-β with or without interferon γ (Hattori et al. 2002). They found that the production was inhibited by statins, and that this inhibition was significantly prevented by the addition of mevalonate. Moreover, by adding geranylgeranyl pyrophosphate to the cultures, the inhibition was completely reversed. Similarly, Pahan et al examined the effect of statins on nitric oxide and iNOS in rat astrocytes, microglia and macrophages, as well as the effect on the cytokines IL-1β, IL-6 and TNF-α (Pahan et al. 1997). They also demonstrated an inhibitory effect and, importantly, showed that it was not due to any effect on the end products of the pathway, i.e. cholesterol and ubiquinone. This time, the effect on iNOS was reversed by the addition of either mevalonate or farnesyl pyrophosphate, suggesting that it is the farnesylation rather than geranylation of messenger proteins that plays a key step. By contrast, treating cultured SMCs with IL-1β, Finder demonstrated an increase in NOS-2 (Finder et al. 1997). When a statin or GGTI-298 were added, this resulted in a further increase, or superinduction. Interestingly, the addition of a FTI blocked the IL-1β-induced increase in NOS-2. The addition of geranylgeraniol reversed this superinduction, whereas the addition of farnesol did not. He concluded that a farnesylated protein mediated the induction of NOS-2, whereas a geranylgeranylated protein suppressed it.

Sebti’s group also showed that rat pulmonary artery SMCs, cultured in the same way as before, produced superoxide in response to IL-1β or PDGF (Boota et al. 2000). This superoxide production was blocked by both FTI and GGTI administration.
6.1.c Oncogenesis and the Cell Cycle

The activity of these metabolites has been investigated in many different areas, including oncogenesis. Sebti’s group has looked at the G-protein Ras (Bredel et al. 1998; Lerner et al. 1995; Pollack et al. 1999; Sun et al. 1998). This cellular signalling molecule is readily transformed into one of several oncogenic forms, which leads to a loss of the normal control of proliferation and hence tumour growth. Ras needs to be prenylated before it can be incorporated into the cell membrane, and Carboni et al looked at the effect of blocking this prenylation in both Ras and another cell-signalling molecule, R-Ras2/TC21 (Carboni et al, 1995). The effect on cell function was measured by growth, which was both anchorage-dependent and independent, the restoration of the cytoskeleton and the reversal of transformed morphology. They inhibited prenylation by using a farnesyl transferase inhibitor (FTI), and this completely blocked the function of oncogenic Ras although it had no effect on R-Ras2/TC21.

Another group of cell signalling molecules is the GTPase family, Rho. These enzymes also require prenylation in order to be activated, although this time by the addition of geranylgeraniol. Golovchenko et al showed the effect of excess insulin on this system (Golovchenko et al. 2000). They demonstrated that insulin increased the activity of geranylgeranyl transferase (GGT) and thereby increased the availability of geranylgeranylated Rho-A (GGRho-A). This in turn doubled the activation of NF-κB in cultured vascular smooth muscle cells. They went on to demonstrate that an inhibitor of GGT (GGTI-286) blocked these effects of insulin, showing another significant activity of the isoprenoid metabolites.
Other work on vascular smooth muscle cells has been aimed at showing different effects of specific FTIs when compared to GGTIs. Stark et al. used serum-dependent cell growth as measured by thymidine uptake and cell counts, as well as apoptosis (measured by DNA-staining and flow cytometry) in serum-free cultures (Stark et al. 1998). They found that, as expected, the drug GGTI-298 suppressed protein geranylgeranylation, but that it also prevented cell growth and promoted apoptosis. This contrasted markedly with farnesyl transferase inhibition. Using FTI-277, they inhibited protein farnesylation but had little effect on cell growth and none on apoptosis. A similar reduction in growth and increase in apoptosis was seen in human glioma cells treated with either GGTI or FTI (Bredel et al. 1998). A combination of both an FTI and a GGTI was able to inhibit cell proliferation by up to 80% in adrenocortical cells transformed with the ki-RAS subtype (Mazet et al. 1999). However, optimal inhibition was seen at concentrations of 10 µM, whereas levels of GTI-298 as low as 15µM caused cell lysis. Similarly, a combination of GGTI and FTI was needed to prevent ki-RAS prenylation in two models of neoplasia (Lobell et al. 2001). Lobell examined the effects in both a rat pancreatic tumour and ki-RAS transgenic mice with mammary tumours. Again it was shown that an infusion of GGTI was toxic; continuing the infusion for 72 hours caused death.

Considerable work has been performed investigating the importance of protein prenylation in the cell cycle. GGTI-298 has been shown to arrest tumour cells in the G1 phase of the cycle in human lung carcinoma cultures (Sun et al. 1999). FTI-277, however, was suggested to cause cell cycle arrest in the G2-M phase (Miquel et al. 1997). Chappell also showed that the stimulation of cell-cycle progression in breast
cancer cells caused by insulin was reversed by the addition of a GGTI (Chappell et al. 2001).

6.1.d Osteoclast Activity

There has been limited research into the role of the isoprenoid metabolites in osteoclast activity. Bisphosphonates have been shown to activate the enzyme caspase-3 in osteoclasts and cause apoptosis, thereby preventing bone resorption (Benford et al. 2001). This process was mimicked by the action of GGTI-298, whereas FTI-277 had no such effect. Bisphosphonates and GGTI-298 have also been shown to inhibit osteoclast formation and function by preventing the incorporation of radiolabelled mevalonate (Coxon et al. 2000). Again, FTI-277 had little effect on osteoclast morphology or function.

6.1.e Angiogenesis and Leukocyte Adhesion

One interesting paper looked at the effect of statins and GGT inhibition on angiogenesis, a feature of several disease processes but especially tumour growth (Park et al. 2002). It was found that statins were able to attenuate angiogenesis when examined in three systems. In the first, chick chorioallantoic membranes were stimulated with vascular endothelial derived growth factor (VEGF), and in the second, murine corneas were stimulated with fibroblast growth factor. The third system looked at the formation of tube-like structures in cultured human endothelial cells. GGTI-298 was effective in the cell culture and was thought to act by preventing the geranylgeranylation of Rho and thereby its membrane localisation.
Liu looked at the adhesion of leukocytes to fibronectin (Liu et al. 1999). It was found that statins prevented this adhesion by preventing garanylgeranylation of Rho. The effect was mimicked by the use of GGTI-298 and reversed by the addition of geraniol. Neither farnesol nor FTI demonstrated this activity.

6.2 Plan of Investigation

Using human aneurysmal aortic explants, the effect of simvastatin or a combination of simvastatin and mevalonate on the production of the cytokine IL-6 were examined. Due to the poor results from IL-1β and TNF-α in previous experiments, these assays were not repeated. Using the porcine model, the same combinations of drugs were investigated for their effect on MMP activity.

The second series of experiments looked at the effect of inhibiting the two isoprenoid transferases using the drugs GGTI-298 and FTI-277. Once again, the effect on MMP activity was investigated with the porcine model, whilst the effect on IL-6 was investigated using human aneurysmal aortic explants.
6.3 Methods

6.3.a Porcine Model

Porcine explants were established using the techniques described earlier. After treatment for 24 hours with elastase, the explants were cultured in a control solution or the drugs under investigation for 14 days. The tissue was subsequently harvested and used for the determination of MMP activity.

6.3.b Human Aneurysmal Explants

As before, explants of human aneurysmal aortas were established in the laboratory. These were divided into 3 and either control fluid, simvastatin or a combination of simvastatin and mevalonate were added in the first series, and control, GGTI-298 or FTI-277 in the second series. After 48 hours in culture, the conditioned medium was harvested and analysed for cytokine content using ELISA.

6.3.c Drug Preparation

6.3.c.1 Mevalonate

Mevalonate was supplied as a white powder (Sigma). The chemical formula is C_{6}H_{12}O_{4} and the molecular weight is 148.16. The chemical structure is shown in Fig 6.2. It was used in excess in the culture medium, in concentrations of 100\mu M.
Fig 6.2 The chemical structure of mevalonate – an intermediate in the cholesterol synthesis pathway.

6.3.c.2 GGTI-2133

GGTI-2133 (Calbiochem) was supplied as a pale yellow powder. Its chemical formula is C\textsubscript{27}H\textsubscript{28}N\textsubscript{4}O\textsubscript{3}, and the molecular weight is 456.5. The chemical name is 4-[[N-(Imidazol-4-yl)methyleneamino]-2-(1-naphthyl)benzoyl]leucine, and the structural formula is shown in Fig 6.3. It is a peptidomimetic and inhibits GGT with a 140-fold selectivity over FT. Its IC\textsubscript{50} (the concentration needed to inhibit 50% of the target enzyme activity) is 38 nM, and the concentration used was 200 nM. It was poorly soluble in water, and therefore was dissolved in DMSO. The final concentration of
DMSO in the culture media was 0.01%, which is below the level of toxicity. The same concentration of DMSO was used in the control cultures.

Fig 6.3 The chemical structure of GGTI-2133 – a selective inhibitor of geranylgeranyl transferase.

6.3.c.3 FTI-277

FTI-277 (Calbiochem) was supplied as an off-white powder. Its chemical formula is C_{22}H_{29}N_{3}O_{3}S_{2}, and the molecular weight is 447.6. The structural formula is shown in Fig 6.4. It is a selective inhibitor of farnesyl transferase and does not inhibit geranylgeranyl transferase even at micromolar concentrations. It was also dissolved in DMSO with a final DMSO concentration of 0.01%. The IC-50 is 50 nM, and the final concentration in the culture medium was 250 nM.
6.3. Matrix Metalloprotease Activity

The technique of gel zymography described in the Materials and Methods Chapter was used.

6.3. Cytokine Analysis

Again, this was performed using commercially available ELISA kits as described earlier.

6.3. Statistical Analysis

All statistical analyses were carried out using Graphpad Prism software. As each experiment consisted of a single explant that had been divided into three and treated
with different drugs or control media, the data were treated as paired samples and
analysed for intercolumn variability using Friedman’s test. If variability was proven,
then the individual columns were analysed using Wilcoxon matched pairs.

6.4 Results

6.4.a Mevalonate

In the mevalonate series of experiments, the explants were divided into three equal
parts and then cultured either in control medium, medium supplemented with
simvastatin or a combination of simvastatin and mevalonate. As expected, the
simvastatin reduced the activity of MMP-2 and MMP-9. A typical gel is shown in Fig
6.5. For the proenzyme form of MMP-9, the median optical density of the zymogram
bands and 95% confidence intervals were 0.74 (0.58-1.26) for the control group, 0.26
(0.09-0.76) for the simvastatin group and 0.62 (0.44-1.16) in the simvastatin and
mevalonate group (see fig 6.6).

For the active enzyme MMP-9, the results for control were 0.23 (0.14-0.35), for
simvastatin 0.08 (0.05-0.13) and for the combined mevalonate and simvastatin 0.20
(0.10-0.31) (see Fig 6.7).

For the proenzyme MMP-2, the results were 0.50 (0.28-0.57) for control, 0.29 (0.18-
0.40) for simvastatin and 0.47 (0.25-0.55) for the combined group (see Fig 6.8).
For the active MMP-2 enzyme, the results were 0.40 (0.25-0.49), 0.24 (0.16-0.33) and 0.45 (0.22-0.55) for the control, simvastatin and mevalonate groups respectively (see Fig 6.9).

When IL-6 was measured by ELISA, the results were 363 (222-545) ng/ml in the control group, 231 (107-454) ng/ml in the simvastatin group and 339 (176-426) ng/ml in the combined simvastatin and mevalonate group (See Fig 6.10).

6.4.b GGTI-2133 and FTI-277

For the proenzyme form of MMP-9, the measured optical density in the control group was 0.46 (0.31-0.84), in the group treated with GGTI-2133 it was 0.50 (0.35-0.85) and in the group treated with FTI-277 it was 0.53 (0.36-0.72) (see Fig 6.11).

Active MMP-9 gave the following results; 0.19 (0.11-0.38) for control, 0.23 (0.19-0.28) for GGTI and 0.20 (0.15-0.30) for FTI (see Fig 6.12).

For proenzyme MMP-2, the median in the control group was 0.35 (0.24-0.55), in the GGTI treated group it was 0.30 (0.21-0.54) and in the group treated with FTI it was 0.32 (0.23-0.48) (Fig 6.13).

The active form of MMP-2 measured 0.26 (0.18-0.41) in control, 0.31 (0.22-0.43) in the GGTI group and 0.26 (0.20-0.35) in the FTI group (see Fig 6.14).
Finally, when IL-6 was assayed by ELISA, the amount in the conditioned media from the control group was 118 (0-391) ng/ml, 40 (19-126) ng/ml in the GGTI group and 48 (0-260) ng/ml in the FTI group (see Fig 6.15).

Fig 6.5 A typical mevalonate gel. Lane 1 was loaded with conditioned medium from culture of HT1080, a human sarcoma line known to produce large quantities of MMP-9 and MMP-2. Lane 2 was loaded with homogenate of control tissue, and demonstrates significant activity of both MMP-9 and MMP-2. Lane 3 was loaded with homogenate of tissue treated with simvastatin, and shows a considerable reduction in the activity of both MMPs. The tissue in lane 4 was treated with a combination of simvastatin and mevalonate, and it is clear the mevalonate has reversed the inhibitory effect of simvastatin.
Fig 6.6 The effect of simvastatin and mevalonate on the activity of proenzyme MMP-9 in porcine aortic cultures. The middle (simvastatin) group were treated with 100µM simvastatin, whilst the right hand (S & M) group were treated with a combination of simvastatin 100µM and mevalonate 100µM. The columns were significantly different when analysed by Friedman’s test (p=0.002, n=8). The individual columns were also analysed using Wilcoxon’s matched pairs and the p-values are given above the graph. Simvastatin caused a significant decrease, which was reversed by the addition of mevalonate.
Fig 6.7 The effect of simvastatin and mevalonate on the activity of the enzyme MMP-9 on porcine aortic cultures. There was a significant difference between the columns (Friedman’s test, p=0.030, n=8) and the differences between the individual columns were analysed using Wilcoxon matched pairs and the p-values given above.
Fig 6.8 The effect of simvasatin and mevalonate on the activity of the proenzyme MMP-2 in porcine aortic cultures. Analysing the columns for variability using Friedman’s test gave a p-value of 0.005 (n=8). Individual intercolumn differences were analysed and the p-values given above.
Fig 6.9 The effect of simvastatin and mevalonate on the activity of the enzyme MMP-2 in porcine aortic cultures. There was significant difference between the columns as analysed by Friedman’s test (p=0.038, n=8). Wilcoxon’s matched pairs test was used to compare the columns and the p-values given above.
Fig 6.10 The effect of simvastatin and mevalonate on the secretion of IL-6 by human aneurysmal aortic explants. Although Friedman’s test did not show a significant variation between the columns, the levels of IL-6 in the group treated with simvastatin tended to be lower than either control or mevalonate and simvastatin combined.
Fig 6.11 The effect of isoprenoid transferase inhibitors on the activity of the proenzyme MMP-9. As can be seen from the graph, neither of the transferase inhibitors GGTI-2133 and FTI-277 had any effect on MMP activity (Friedman’s test, \( p=0.956, n=6 \)).

Fig 6.12 The effect of isoprenoid transferase inhibitors on the activity of the enzyme MMP-9. Again, no effect is seen (Friedman’s test, \( p=0.956, n=6 \)).
Fig 6.13 The effect of isoprenoid transferase inhibitors on the activity of the proenzyme MMP-2. Once again, no effect is seen (p=0.252, n=6).

Fig 6.14 The effect of isoprenoid transferase inhibitors on the activity of the enzyme MMP-2. As in the previous cases, no effect was seen (p=0.072, n=6).
Fig 6.15 The effect of isoprenoid transferase inhibitors on the secretion of IL-6 by human aneurysmal aortic explants. Whilst the levels of IL-6 in those explants treated with GGTI-2133 and FTI-277 appear lower than controls, this is not statistically significant (Friedman's test, p=0.486, n=6).

6.5 Discussion

It had already been demonstrated in Chapter 5 that simvastatin fulfilled the two criteria set out at the start for providing a pharmacotherapy for small aneurysms, namely the inhibition of MMP activity and anti-inflammatory properties, at least in vitro. The experiments in this chapter were designed to investigate the pathways by which these effects were mediated. As mevalonate is the intermediate whose production is immediately inhibited by statins, it was expected that its addition would reverse any effects caused by simvastatin. Indeed, this was exactly what happened as can be seen in Figs 6.5-6.10.
Further investigation of the pathway was undertaken using inhibitors of isoprenoid transferases, GGTI-2133 and FTI-277. Fig 6.1 shows how these transferases are important in prenylating cell-signalling proteins such as Ras and Rho. It was thought that this might be the pathway by which simvastatin affects MMP activity and inflammation, and therefore at least one of the drugs should have mimicked the action of HMG CoA reductase inhibitors. However, as can be seen in Figs 6.11-6.15, neither of the drugs demonstrated any effect. There may be several reasons for this.

Firstly, for some reason the drugs may not have been active in the experimental models used. The only real way to check whether the drugs were active would have been to investigate their effects on protein prenylation, something that falls outside the scope of this thesis.

Secondly, the active metabolite may have been further down the cholesterol synthesis pathway, perhaps a sterol compound such as squalene or ubiquinone. One way to test this theory would be to use squalestatin, a drug that only inhibits the synthesis of sterol metabolites in the cholesterol synthesis pathway. Indeed, Diomede has performed experiments on inflammation in a rat model and found that mevalonate, but not sterols, was the key mediator in the anti-inflammatory effects of statins (Diomede et al. 2001).

The third hypothesis as to why GGTI-2133 and FTI-277 had no effect involves a pathway directly from mevalonate that has not, as yet, been fully elucidated. Further work would be necessary to prove which of these three hypotheses are correct.
There is no isoprenoid transferase inhibitor that is licensed for use in humans, and the geranylgeraniol transferase inhibitors are known to be toxic (Lobell et al. 2001; Mazet et al. 1999). The statins, however are safe and well tolerated, and the next next step was to investigate whether the beneficial effects seen *in vitro* would also be demonstrated *in vivo*.
Chapter 7

Randomised, Controlled Trial in Human Subjects

7.1 Introduction

From the outset, the aim of this thesis was to lead towards a pharmacotherapy for small abdominal aortic aneurysms. The first chapter explored the pathogenesis of aneurysms to identify potential therapeutic targets and the second chapter summarised previous work investigating pharmacotherapy. The preceding experimental chapters investigated the suitability of various non-steroidal anti-inflammatories, COX-2 antagonists and statins. Non-steroidals have been shown in other research to reduce cytokine secretion (Franklin et al. 1999b) but have an unacceptable side-effect profile (Gabriel et al. 1991). COX-2 antagonists have far fewer side-effects, but the experiments in Chapter 4 demonstrated that they did not possess the inhibitory effect on MMP activity that was hoped for.

Statins have been shown in other work to reduce matrix metalloprotease activity (Bellosta et al. 1998), and have also been shown to reduce inflammation. Diomede showed that lovastatin reduced the secretion of IL-6 into exudate in a murine model of inflammation in a dose-dependent manner (Diomede et al. 2001), whilst Ni et al showed a reduction in monocyte chemo-attractant protein 1 (MCP-1) in a rat model in response to both pravastatin and cerivastatin (Ni et al. 2001). These results were
echoed by the experiments in Chapter Five using both porcine and human tissue cultures.

The aim of Chapter Seven then, was to investigate whether the same beneficial effects were seen in aneurysmal tissue when the statins were administered \textit{in vivo}. In order to do this, a randomised and controlled, double-blinded clinical trial of simvastatin versus placebo was established.

\textbf{7.2 Plan of Investigation}

Patients undergoing elective abdominal aortic aneurysm repair were recruited into the trial and randomised to receive either simvastatin or placebo for three weeks prior to their operation. Blood samples were taken at the time of recruitment and then again immediately before induction of anaesthesia for the AAA repair. These were analysed using ELISA to determine whether the statin had any effect on systemic concentrations of various cytokines.

During the operation, a section of the redundant aneurysm sac was harvested and taken to the laboratory. Part of the sac was homogenised for the later analysis of MMP activity, whilst part was cultured for 48 hours. The culture supernatant was subsequently used to analyse cytokine secretion by the aortic tissue.

Finally, once the assays had been completed, the blinding code was broken and the results were statistically analysed.
7.3 Methods

7.3.1 Randomised, Controlled Trial

Local ethics approval was sought and obtained. A power calculation was performed to establish the number needed to recruit to achieve 90% power. Patients undergoing elective abdominal aortic aneurysm repair were identified through the vascular outpatient clinics. These patients were invited to participate in the trial, and those who agreed were visited at home and the protocol explained. Informed consent was obtained and the trial medication was given to the subjects, along with information sheets and contact numbers. Trial medication in the form of simvastatin 40mg or placebo were kindly provided by Merck, Sharpe and Dome, and the randomisation schedule was provided in sealed envelopes by a third party randomisation centre. The patients were advised to take their trial medication each morning for three weeks prior to their surgery. Three weeks was chosen as a realistic time between identification of suitable patients and surgery, which was typically performed within a month of the clinic appointment.

At the first visit, a health questionnaire was filled in and blood samples were taken and transported to the laboratory. C-reactive protein measurements were made by the pathology labs, and serum was frozen for later analysis. When the patients arrived in the hospital for their operation, their trial medication bottles were collected and the remaining pills counted to ensure compliance. Immediately prior to induction, further blood samples were taken. During the procedure, a $1cm^2$ section of aortic sac was
excised and transported to the laboratory. Half was immediately frozen whilst half was cultured.

7.3.b Tissue Culture

In order to investigate cytokine expression, tissue culture was performed in a similar manner to that described in the Materials and Methods Chapter. The aortic explants were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with L-glutamine, penicillin and streptomycin. No drugs were added to the culture medium in this experiment. After 48 hours, the culture supernatant was harvested and analysed for cytokine content using ELISAs.

7.3.c Gel Zymography

Half of the aortic segment was immediately transported to the lab and snap frozen. This segment was subsequently thawed and used for MMP analysis by gel zymography as previously described. Briefly, the segments were homogenised and the homogenates were dialysed to increase the protein concentration before being protein standardised. They were then loaded onto an SDS-polyacrylamide gel containing gelatin as a substrate. After the gel had run, it was stained to show bands of gelatinase activity which were read using Imagemaster software.

7.3.d Cytokine Analysis

All cytokine analyses were carried out using commercially available ELISAs.
7.3.e Statistical Analysis

Non-parametric analyses were used throughout and differences between control and treatment groups were investigated using Mann-Whitney tests. Comparisons of assays were performed using both Spearman’s correlation and Altman-Bland plots. The merits and problems with these methods are discussed further in section 7.4.d. All statistical analysis was performed using Graphpad Prism software.
7.4 Results

7.4.1 Power Calculation

A power of 90% was aimed for, with the main criteria being change in MMP-9 and IL-6. By coincidence, the standard deviations of both of these variables were found to be 0.32 from earlier experiments. Assuming a difference of 25% to be significant, the following equation was used:

\[ m = \frac{2(z_a + z_{2b})^2 \sigma^2}{\delta^2} \]

Where:
- \( m \) = the number in each arm of the study,
- \( z_a \) = first ordinate from the normal distribution table,
- \( z_{2b} \) = second ordinate from the normal distribution table,
- \( \sigma \) = standard deviation of population, and
- \( \delta \) = expected difference in measured variable

The values were as follows:

\[ m = \frac{2 \times 10.507 \times 0.32 \times 0.32}{0.0625} \]

Which gave the final value, \( m = 34.4 \).

Thus at least 35 patients were needed in each arm of the study, and we aimed for 40.
For reasons that will be discussed later, recruitment had to be abandoned earlier than anticipated and recruitment targets were not reached. The study population reflected the typical aneurysm patient – 21 patients were recruited, of which all but 2 were male, giving a male to female ratio of 9.5:1. All of the subjects were white Caucasian in racial origin, despite being drawn from a population with a very high proportion of Asians, reflecting the genetic nature of the disease. The majority of the subjects (12/21) were in their 8th decade. There were no diabetics in the study, and although the majority had smoked in the past, all agreed to stop smoking in the run up to their operation. When the randomisation codes were broken, it was found that 10 patients were on placebo and 11 were taking simvastatin, as shown in table 7.1.

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Simvastatin</th>
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</thead>
<tbody>
<tr>
<td>Total number</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Male</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>Female</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Age (Median ± S.D.)</td>
<td>74 ± 4.6</td>
<td>71 ± 7.5</td>
</tr>
</tbody>
</table>

Table 7.1 Demographics of the study population

Of these patients, one went into renal failure prior to his operation and the results were excluded. One had his aneurysm fixed endovascularly and sac was also not available for technical reasons in two further patients.
7.4.1 MMP Assays

7.4.1.1 MMP-9

MMPs were assayed both by gel zymography and by ELISA. As described previously, gel zymography relies upon the gelatinase activity of the enzyme to produce bands upon the gel which represent both the active and proenzyme form of the drug. Although there are commercially available ELISAs to measure both active and inactive MMP-2 and -9, results using these kits in our laboratories have been poor, with both poor standard curves and reproducibility. For this reason, the ELISAs used in this chapter only measured total MMP quantity.

When the proenzyme form of MMP-9 was measured by gel zymography in the placebo subjects, the median value (and 95% confidence interval) was 0.45 (0.34-0.73, n=10). In the treatment group, the value was 0.31 (0.21-0.40, n=7). When compared using a Mann-Whitney test, there was a significant difference between treatment and control, with a p-value of 0.007 (see Fig 7.1).
Fig 7.1 Comparison of the levels of the proenzyme MMP-9 as measured by gel zymography. The levels in samples of aortic tissue were measured and compared using a Mann-Whitney test. There was a significant difference between the placebo and control group with a p-value of 0.007.

A similar comparison was performed for the levels of the active form of MMP-9 as measured by gel zymography. The median value for the placebo group was 0.70 (0.31-1.44, n=10). For the group taking simvastatin, the values were 0.94 (0.23-2.01, n=7). There was no significant difference between the groups (see Fig 7.2).
Fig 7.2 Comparison of active MMP-9 in the control and treatment groups as measured by gel zymography. There was no significant difference between the two groups (Mann-Whitney, p=0.887).

In addition, the numeric value of the MMP-9 concentration in tissue homogenates was measured using ELISA. In this case, the median amount of MMP-9 in the aortic segments from the control group was 213.5 ng/ml, with a 95% confidence interval of 134-396 ng/ml (n=10). In the treatment group the values were 129 (36-317, n=7) ng/ml. Again, there was no significant difference between the groups (see Fig 7.3).
Fig 7.3 Concentration of MMP-9 in aortic segments from the trial subjects, as measured by ELISA. Although the apparent trend is for a reduction in MMP-9 levels in patients taking simvastatin, there was no statistical significance (p=0.364).

7.4.c.2 MMP-2

Matrix metalloprotease-2 was also measured in active and proenzyme forms by gel zymography, and in total by ELISA. Gel zymography for the proenzyme form of MMP-2 demonstrated no significant difference. The median and 95% confidence interval for the placebo group was 0.41 (0.25-0.91, n=10). For the group taking simvastatin the results were 0.51 (0.36-1.04, n=7) (see Fig 7.4).
Fig 7.4 Comparison of the levels of the proenzyme form of MMP-2 in the control group and the group taking simvastatin as measured by gel zymography. No significant difference was demonstrated ($p=0.475$).

For the active form of the enzyme, the results were 3.17 (1.31-6.82, n=10) for the control group and 5.13 (1.77—11.74, n=7). The graph is shown in Fig 7.5.

Fig 7.5 Comparison of the active form of MMP-2 in the control group and those taking simvastatin. There was no significant difference between the columns ($p=0.270$).
When the total enzyme MMP-2 was measured by ELISA, the median value in the placebo group was 195 ng/ml and the 95% confidence interval was 130-281 ng/ml (n=10). In the treatment group, the result was 171 ng/ml (132 – 237 ng/ml, n=7). There was no significant difference when compared by Mann-Whitney test (see Fig 7.6).

![Graph showing comparison of total MMP-2 as measured by ELISA in the aortic wall of the trial subjects.](image)

Fig 7.6 Comparison of total MMP-2 as measured by ELISA in the aortic wall of the trial subjects. Once again, there was no significant difference between the two groups (p=0.740).
7.4.d Comparison of Assays

As both MMP-2 and MMP-9 had been measured in the same tissue using two very different methods, it was decided to compare the results of the two methods. This was done in two ways. Firstly, the two sets of results were plotted on a single graph and a line of best fit was calculated to give a graphical representation. Using these data, the two methods could be compared using Spearman's correlation. However, there are problems in applying this statistical technique to this kind of data.

Firstly, the correlation coefficient given by the test only measures association rather than agreement between the two sets of data. They will correlate if the points lie on any straight line, but will only show agreement if the points lie on the line y = x. This is made more complicated by the fact that gel zymography measures enzyme activity and gives a value in units of optical density, whereas ELISA measures enzyme content, and gives values in ng/ml. For these reasons, simple correlation is probably not the best statistical technique for comparing these two methods. Perhaps more suitable would be the Altman-Bland plot.

Using this technique, data are ranked within their group. The mean of the two ranks of the paired samples is then plotted on the x-axis, whilst the difference between the ranks is plotted on the y-axis. Any outlier is more obvious using this method. In addition, the mean difference gives an estimate of bias, whilst the 95% "limits of agreement" are given by the mean difference ±2 x the standard deviation of the difference.
7.4.d.1 MMP-9 assays

The results from the MMP-9 ELISA were compared first of all to the optical densities of the proenzyme form, then the active form and finally to the added optical density of both forms.

Correlating proenzyme MMP-9 activity with the total MMP-9 as measured by ELISA gave a p-value of 0.018, although as discussed earlier, this figure must be viewed with caution (see Fig 7.7). More importantly, the mean difference in rank was 0.4, and the 95% limits of agreement were −8.3 to 8.1. The Altman-Bland plot is shown in Fig 7.8.

Fig 7.7 Despite the apparently poor fit of the points on this graph, Spearman’s test gave a significant p-value of 0.018. As discussed in the text, however, this value has little meaning.
Fig 7.8 Altman-Bland plot of proenzyme MMP-9 zymography versus total MMP-9 ELISA. See text for fuller explanation.

The same two analyses were performed with active MMP-9 (zymography) versus total (ELISA). The p-value for Spearman’s test was 0.25, with the same proviso as previously (Fig 7.9). From the Altman-Bland data, the mean difference in rank was 0.1, and the 95% “agreement limit” was −10.8 to 11.0 (see Fig 7.10).
Fig 7.9 There was poor correlation between the zymographic measurement of active MMP-9 and the total MMP-9 measured by ELISA.

Fig 7.10 Altman-Bland plot of the data for active MMP-9 against total MMP-9.
Also, the optical densities of both proenzyme and active MMP-9 were added together to give a zymographic representation of total MMP-9, and this was compared to the total MMP-9 measured by ELISA. Using Spearman’s test gave a p-value of 0.025, but as discussed previously, this figure has little meaning (see Fig 7.11). Of more relevance was the mean difference in rank and the 95% “agreement limits” which were 0 ± 12.9. The Altman-Bland plot is shown in Fig 7.12.

![Graph showing correlation between total MMP-9 as measured by gel zymography and ELISA.](image)

Fig 7.11 Correlation between total MMP-9 as measured by gel zymography and ELISA. Although Spearman’s correlation gave a significant p-value of 0.025, this is of dubious relevance, as explained earlier.
7.4.d.2 MMP-2 Assays

Comparing proenzyme MMP-2 measured by zymography to the total MMP-2 content measured by ELISA using Spearman’s correlation gave a p-value of less than 0.001 (Fig 7.13). Moreover, the mean difference in the ranks was 0 with a 95% agreement limit of ± 7.1. The Altman-Bland plot is given in Fig 7.14.
Fig 7.13 Relationship between proenzyme MMP-2 measured by zymography and total MMP-2 measured by ELISA. Spearman’s test gave a p-value of <0.001, but as always, this has to be viewed in context.

Fig 7.14 Altman-Bland plot of the ranks of the measurements of proenzyme MMP-2 by zymography and total MMP-2 by ELISA.
The results for active MMP-2 were a p-value of 0.799 using Spearman's correlation, whilst the mean difference in rank was 0 ± 13.8. These data are shown in Fig 7.15 and 7.16 respectively.

Fig 7.15 Relationship between active MMP-2 on zymography and total MMP-2 measured by ELISA. The almost flat line shows that there is no agreement.

Fig 7.16 Altman-Bland plot of active MMP-2 zymography against total MMP-2 ELISA.
Finally, the added optical densities of proenzyme and active MMP-2 zymograms were compared to total MMP-2 measured by ELISA (Figs 7.17 and 7.18). The p-value from Spearman's correlation was 0.814, whilst the mean difference in rank and 95% agreement limits were 0 ± 13.4.

Fig 7.17 Relationship between total MMP-2 as measured by gel zymography or by ELISA. Very poor agreement is shown by the graph.

Fig 7.18 Altman-Bland plot of the ranks of the measurements of total MMP-2 by gel zymography and by ELISA.
7.4.e Difference in C-Reactive Protein Measurements

C-reactive protein (CRP) was measured both at the time of recruitment and immediately before induction. As the plasma levels of CRP in each patient varied considerably before the experiment began, the main point of interest was any difference between the two measurements in each individual, and whether the simvastatin had any effect on this. Laboratory measurements of less than 5 ng/ml are not accurate, and are therefore simply recorded as <5. In order to be able to perform meaningful comparisons, <5 was taken as zero, and 5 was subtracted from all other readings. From this, any reduction in CRP over the time period was analysed and the two groups compared using a Mann-Whitney test (Fig 7.19). Although there was a slightly greater reduction seen in the simvastatin group, this was not significant, with a p-value of 0.579. This may have been due to the large number of "zero" readings. The median reduction in the placebo group was 0 ng/ml (-0.5 to +2.5 ng/ml, n=10), and in the simvastatin group it was 1 ng/ml (-0.4 to 3.8 ng/ml, n=10)
Fig 7.19 Net reduction in plasma CRP levels between recruitment after three weeks of taking the trial medication. The slightly greater reduction seen in the simvastatin group was not significantly significant (p=0.579).
7.4.f Tissue Inhibitors of Metalloproteases (TIMPs)

TIMP-1 and TIMP-2 were measured both in the plasma at the start and end of the experiment, and in that part of the aortic segment that was immediately frozen. Of relevance was any change in the plasma level over the course of this experiment, as well as the levels in the tissue following administration of the trial medication.

There was little difference in the individual plasma levels of TIMP-1 over the course of the experiment. For the simvastatin group, the median increase in TIMP-1 was 31.5 ng/ml, but there was a very wide confidence interval (-224 to +205 ng/ml, n=8). In the placebo group there was a median decrease of -60 ng/ml (-262 to +13 ng/ml, n=9) (see Fig 7.20).

Fig 7.20 Change in TIMP-1 levels over the course of the experiment. Although the simvastatin group tended to be more positive than the controls, there was no significant difference (p=0.167).
Levels of TIMP-1 measured in the tissues tended to be slightly higher in the simvastatin group. The median value and confidence limits were 601 ng/ml (526-639 ng/ml, n=9), compared to 510 ng/ml (414-587 ng/ml, n=6) in the placebo group. This was not significant (see Fig 7.21).

Fig 7.21 Levels of TIMP-1 in aneurysmal tissue extracted from the trial patients (p=0.088).

There was minimal change in the plasma levels of TIMP-2 at the start and end of the trial. For the placebo group, the median increase was 1 ng/ml (-3.8 to + 9.4 ng/ml, n=9). The simvastatin group showed a median increase of 2.5 ng/ml (-17 to + 9.6 ng/ml, n=7, see Fig 7.22)
Fig 7.22 Change in the plasma levels of TIMP-2 from start to end of the trial (p=0.681).

The absolute levels of TIMP-2 in the aortic segment were also measured. The median in the placebo group was 34 ng/ml (24 – 42 ng/ml, n=9), whilst that in the simvastatin group was 24.5 (13.5 – 33 ng/ml, n=7, see Fig 7.23).

Fig 7.23 TIMP-2 levels in the aortic sac. There was no significant difference between placebo and simvastatin (p=0.145).
7.4.g Monocyte Chemo-attractant Protein 1 (MCP-1)

MCP-1 was measured in the aneurysmal sac by ELISA. The median value (and 95% confidence interval in the placebo group) was 13.3 pg/ml (8 – 18.6 pg/ml, n=10), whilst in the simvastatin group the values were 9.7 pg/ml (6 – 24 pg/ml, n=7, see Fig 7.25).

Fig 7.25 Levels of monocyte chemo-attractant protein 1 in the aneurysm sac (p=0.813).

7.4.h Interleukin-6

Finally, the levels of interleukin-6 were measured in the culture supernatant using ELISA. For the control group, the median level was 214 ng/ml, and the 95% confidence interval 111- 321 ng/ml (n=10). The result from the group taking simvastatin was 180 ng/ml (71 – 312 ng/ml, n=7, see Fig 7.26).
Fig 7.26 The levels of interleukin-6 measured in the culture supernatant using ELISA (p=0.740).
7.5 Discussion

7.5.a The Oxford Heart Protection Study

From the results it can be seen that far fewer patients were recruited for this trial than was originally intended. The main reason for this was the publication of the Oxford Heart Protection Study (Anonymous2002a). It appeared in The Lancet in 2002, early on during recruitment. It was the largest study of lipid-lowering therapy in the world, with over 20,000 patients recruited into a double-blinded, placebo-controlled and randomised trial, and it made some very striking findings. There was a significant reduction in mortality from all causes in patients allocated to receive simvastatin (12.9% compared to 14.7%, p<0.001), but perhaps even more remarkable was the effect on major vascular events. These included myocardial infarction, stroke or revascularisation, and in high-risk groups, the rates of these major vascular events were reduced by around a third. In 1000 patients whose only risk factor was peripheral vascular disease, treatment with a statin for five years would prevent around 70 major vascular events. The data from such a large trial was extremely convincing, and as a result of this, nearly all of the patients that were referred to the vascular clinic with an aortic aneurysm were already taking a statin, and were therefore not eligible for recruitment into the study. In addition, it became clear that to withhold statin therapy from the patients in the placebo group was no longer ethical. For this reason, recruitment was abandoned.
7.5. b Results of the Clinical Trial

Unsurprisingly, the results from this clinical trial were not as conclusive as expected. Perhaps what is surprising is that there were any significant results at all, given that only around a quarter of the planned numbers were recruited. The proenzyme form of MMP-9 was significantly reduced in the simvastatin group, and the total amount of MMP-9 as measured by ELISA was reduced, although not to such a great extent. A reduction in MMP-2 was not proven in this study.

The comparison of assays gave some interesting results. Neither Spearman’s correlation nor the Altman-Bland analysis were perfect instruments to measure the agreement between the two techniques, but as the mean difference in each case was approximately zero, it was clear there was no consistent bias. However, the agreement limits were wide in most cases, indicating a relatively poor match. However, in the case of the total MMP-9 measurements, the match was much better. Interestingly, the ELISA measuring MMP-2 correlated very well with the gel zymography for active MMP-2, but not at all with either proenzyme or total MMP-2.

The degree to which the levels of C-reactive protein changed during the treatment period was the same for both groups. Previously, however, statin therapy has been shown to reduce plasma CRP levels in a large, randomised controlled trial (Albert et al. 2001a; Albert et al. 2001b). Other studies have also confirmed a reduction in both CRP and high sensitivity CRP (hs-CRP) (Jialal et al. 2001; Musial et al. 2001). The fact that no reduction was seen in this trial may either be due to the small sample size or to the shorter treatment period (three weeks compared to 24 weeks in the
PRINCE trial). As discussed earlier, however, a treatment period of longer than three weeks would not have been feasible in this study population.

There were no significant results from any of the measurements of TIMPs. This may have been a result of the small sample sizes, or it may be that simvastatin had no effect on either TIMP-1 or TIMP-2. The figures that we do have suggest a slight increase in TIMP-1 levels in the treatment group and a slight decrease in TIMP-2. This would be interesting, as it has been shown that TIMP-2 levels are increased in AAA (Elmore et al. 1998; Knox et al. 1997), and that TIMP-2 levels increase in relation to MMP-9 (Hong et al. 2000). TIMP-1, on the other hand, has been associated with lower levels of MMP-9, preservation of the vascular architecture and prevention of rupture (Allaire et al. 1998).

In this trial, neither IL-6 nor MCP-1 were shown to be decreased by statin therapy. IL-6 had been one of the key targets at the outset of this thesis, but as discussed in Chapter Four, the role of IL-6 as a pro- or anti-inflammatory cytokine has subsequently become less clear. However, a reduction in IL-6 was shown in the in vitro experiments in Chapters Five and Six, and it must be hoped that a larger clinical trial would demonstrate similar results. Statins have been shown to both reduce (Bustos et al. 1998; Kothe et al. 2000) and increase (Kiener et al. 2001) MCP-1, so the lack of effect in these experiments is perhaps unsurprising.
7.5.c Conclusions

The aims of this thesis were firstly to identify targets for pharmacotherapy in small abdominal aortic aneurysms, and secondly to find a drug which would address those targets. Chapter One detailed how the matrix metalloproteases and in particular MMP-2 and MMP-9 were crucial to aneurysm development. In addition, the literature also showed that inflammation played a key role and would also need to be tackled. Chapter Two dealt with previous attempts to find a pharmacotherapy, which had various degrees of success. Chapter Three detailed the scientific techniques which would be used in the experimental chapters, whilst Chapter Four showed that COX-2 inhibitors were unlikely to be useful in this role.

Chapter Five involved in vitro work and showed that statins were able to reduce both MMP activity and markers of inflammation, at least in tissue cultures. Chapter Six went on to elucidate the pathway by which these effects were mediated. The fact that all the effects were reversible by the addition of mevalonate showed that this was the key pathway, but that the effects were not mediated by either farnesylated or geranylated proteins.

It is difficult to draw too many conclusions from Chapter Seven. The numbers involved were far fewer than necessary, but despite this, beneficial effects on MMP-9 were seen.
Summary

The important positive findings of this thesis were that simvastatin was able to reduce both inflammation and matrix metalloprotease activity within aneurysmal aortic tissue. This was seen in both human and porcine tissue, and the results were echoed to a certain degree in vivo. In addition, simvastatin preserved the normal architecture of the aortic wall when analysed histologically. These effects were mediated through the mevalonate pathway. Important negative results include the lack of response to isoprenoid transferases, suggesting that prenylation of messenger proteins did not play a significant role. In addition, the failure of a COX-2 antagonist to prevent these processes was important and at the present time, several COX-2 antagonists have been withdrawn due to an increased risk of adverse cardiovascular events.

Areas of interest for future work include the effect of statins on oxidative stress within the aortic wall and using drugs such as squalestatin to further elucidate the pathway. Although a randomised, controlled trial would no longer be possible, an observational study of aneurysm growth rates in patients taking a statin compared to historical data would be of great interest.

It would not be true to say that this thesis alone has provided enough evidence to alter clinical practice. However, it has added to the wealth of evidence showing the benefits of statin therapy. Since starting this work, prescribing practices for statins have drastically altered such that all patients with vascular disease are now eligible, not just those with elevated cholesterol. Indeed, in the summer of 2004, statins have
been licensed for over-the-counter sales to the public, making them more widely available than ever before.
Appendix 1

Acknowledgements

I am deeply grateful to Professor Matt Thompson and Professor Janet Powell, without whom none of this work would have been possible. For day to day supervision and encouragement my thanks must again go to Matt Thompson and also to Professor Nick London. Many thanks to Professor Peter Bell and the Royal College of Surgeons of England and the British Vascular Foundation for awarding me a Research Fellowship in order to carry out this research.

Laboratory work was carried out within the Department of Surgery at the University of Leicester and I must thank all of the staff within the department for their help. Particular thanks to Dr Stephen Goodall and Dawn Crowston for their patience and assistance. Thanks to the vascular surgeons of the University Hospitals of Leicester for their help in providing human tissue and particular thanks to Professor Ross Naylor, Mr Rob Sayers and Mr Martin Dennis. Thanks to Professor Keith Abrahams for statistical advice.

Thanks to Roche and Merck, Sharpe and Dome for providing many of the drugs used in these experiments, and to the staff at Dawkins for their help in obtaining porcine tissue.

Finally, my thanks to Mairead both for statistical advice as well as for all the other support she has given me over the course of this work.
Appendix 2

Presentations Arising from this Thesis

“Simvastatin Reduces The Levels of Active MMP-2 and Interleukin-6 in Human Aneurysmal Tissue”
Shortlisted for the BJS Prize at the Vascular Surgical Society meeting 2001

“Normal Architecture of The Aneurysmal Wall is Preserved by Simvastatin”
Midlands Vascular Society 2002

“Simvastatin Preserves Elastin and Reduces The Inflammatory Response in Abdominal Aortic Aneurysms”
Association of Surgeons of Great Britain and Ireland 2002

“MMP-9 May Be a Useful Marker of Endoleak in Endovascular Aneurysm Repairs”
Vascular Surgical Society 2002

“Potential Use of Anti-inflammatories in the Treatment of Small Aneurysms”
Aneurysm Congress, Stockholm 2003

“Mevalonate Reverses the Beneficial Effects of Simvastatin on Abdominal Aortic Aneurysms”
Society of Academic and Research Surgery 2003

“Simvastatin Reduces MMP Activity in the Aortic Wall of Patients with Abdominal Aortic Aneurysms”
Association of Surgeons of Great Britain and Ireland 2004

“Is Cox-2 Inhibition Alone Enough to Reduce Abdominal Aortic Aneurysm Formation?”
Society of Academic and Research Surgery Leeds 2002 (Poster)

“Simvastatin Reduces the Expression of MMP-9 and Interleukin-6 In Aortic Aneurysmal Tissue”
Aneurysm Congress, Stockholm 2003 (Poster)

“Serum MMP-9 May Be a Useful Marker of Endoleak in Endovascular Aneurysm Repair”
Aneurysm Congress, Stockholm 2003 (Poster)
Publications

“Management of Intermittent Claudication – The Role of Statins”
**J Evans**, N Bhardwaj, IM Loftus, MM Thompson
British Journal of Surgery 2002, 89, 1323

“Simvastatin Reduces the Levels of MMP-2 and Interleukin-6 in Human Aneurysmal Aortic Tissue”
(Abstract)
**J Evans**, PRF Bell, JT Powell and MM Thompson
British Journal of Surgery 2002, 89, 529-531

“Simvastatin Preserves Elastin and Reduces the Inflammatory Response in Aortic Aneurysmal Tissue”
(Abstract)
**J Evans**, IM Loftus, PRF Bell, JT Powell and MM Thompson
British Journal of Surgery 2002, 89 Issue s1, 22
## Appendix 3

### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>8-Ohdg</td>
<td>8-hydroxy 2-deoxyguanosine</td>
</tr>
<tr>
<td>AAA</td>
<td>Abdominal Aortic Aneurysm</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetyl Choline</td>
</tr>
<tr>
<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>BRIJ-35</td>
<td>Polyoxyethylene monolauryl ether</td>
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<tr>
<td>CARE</td>
<td>Cholesterol and Recurrent Events</td>
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<tr>
<td>CD-18</td>
<td>Cluster of Differentiation 18 (leukocyte membrane protein)</td>
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<td>CLASS</td>
<td>Celecoxib Long-term Arthritis Safety Study</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclo-oxygenase</td>
</tr>
<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetra-acetic Acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>FTI</td>
<td>Farnesyl Transferase Inhibitor</td>
</tr>
<tr>
<td>GGTI</td>
<td>Geranylgeraniol Transferase Inhibitor</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
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<tr>
<td>HLA</td>
<td>Human Leukocytic Antigen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>HMG CoA</td>
<td>Hydroxy Methylglutarate Coenzyme A</td>
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<td>HUVEC</td>
<td>Human Vascular Endothelial Cell</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>IgA/G</td>
<td>Immunoglobulin A/G</td>
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<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>kDa</td>
<td>KiloDalton</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>MAGP</td>
<td>Microfibril-Associated Glycoprotein</td>
</tr>
<tr>
<td>MASS</td>
<td>Multicentre Aneurysm Screening Study</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemoattractant Protein-1</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarct</td>
</tr>
<tr>
<td>mm-LDL</td>
<td>Malondialdehyde modified Low Density Lipoprotein</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloprotease</td>
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<tr>
<td>MoxLDL</td>
<td>Mildly oxidised Low Density Lipoprotein</td>
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<tr>
<td>MT-MMP</td>
<td>Membrane Type Matrix Metalloprotease</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide Dinucleotide</td>
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<td>NHS</td>
<td>National Health Service</td>
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<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
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<tr>
<td>NSAID</td>
<td>Non-Steroidal Anti-Inflammatory Drug</td>
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<tr>
<td>PAF-AH</td>
<td>Platelet Activating Factor Acetylhydrolase</td>
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<td>PAI-1</td>
<td>Platelet Activator Inhibitor 1</td>
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<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
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<tr>
<td>PGE-2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl Flouride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PRINCE</td>
<td>Pravastatin Inflammation/CRP Evaluation</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute (culture medium)</td>
</tr>
<tr>
<td>rt-PCR</td>
<td>Reverse transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SAA</td>
<td>Serum Amyloid A</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>SF-36</td>
<td>Short Form 36</td>
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<td>SMC</td>
<td>Smooth Muscle Cell</td>
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<td>TBX-2</td>
<td>Thromboxane 2</td>
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<td>TEMED</td>
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</tr>
<tr>
<td>TH-1/2</td>
<td>T-Helper Cell Type 1/2</td>
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<td>TIMP</td>
<td>Tissue Inhibitor of Metalloprotease</td>
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<td>TNF-α</td>
<td>Tumour Necrosis Factor Alpha</td>
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<tr>
<td>TRIS</td>
<td>(Hydroxymethyl) aminomethane</td>
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<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
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<td>VEGF</td>
<td>Vascular Endothelial-derived Growth Factor</td>
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<tr>
<td>VIGOR</td>
<td>Vioxx Gastrointestinal Outcomes Research</td>
</tr>
<tr>
<td>WOSCOP</td>
<td>West of Scotland Coronary Outcomes Prevention</td>
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</tbody>
</table>
Appendix 4

Reference List

Notes: CORPORATE NAME: Heart Protection Study Collaborative Group.

Notes: CORPORATE NAME: Propanolol Aneurysm Trial Investigators.


Notes: CORPORATE NAME: PRINCE Investigators.

Notes: CORPORATE NAME: PRINCE Investigators.


Notes: CORPORATE NAME: VIGOR Study Group.


Notes: CORPORATE NAME: Aneurysm Detection and Management Veterans Affairs Cooperative Study.


Sun, J., Qian, Y., Hamilton, A.D. and Sebti, S.M. (1998) Both farnesyltransferase and geranylgeranyltransferase I inhibitors are required for inhibition of oncogenic K-Ras prenylation but each alone is sufficient to suppress human tumor growth in nude mouse xenografts. *Oncogene* 16, 1467-73.


