THE EFFECTS OF OXIDATIVE STRESS
ON VASCULAR SMOOTH MUSCLE CELLS

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by

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To my beloved Father, who is always in my thoughts.
Abstract

Oxidative stress is thought to play an important role in several diseases including coronary heart disease. The effect of oxidative stress on vascular smooth muscle cell (VSMC) function was investigated since these cells are an important participant in atherogenesis, and previous data regarding the effects of oxidative stress on VSMC function are inconclusive or contradictory.

Oxidative stress has been shown to cause both DNA damage and to stimulate cell growth in various cell types, although work in VSMC has been limited. Therefore, the effects of H$_2$O$_2$ on VSMC proliferation were studied using H$_2$O$_2$ as a well defined source of ROS. H$_2$O$_2$ inhibited serum-stimulated $^3$H-thymidine uptake in a dose dependent manner in SMC, by preventing cells from entering S-phase of the cell-cycle.

NFκB appears to play a central role in mediating the cellular effects of oxidative stress, hence the effects of oxidative stress on NFκB regulation and the role IκB plays in mediating these effects in human VSMC were investigated. Using immunofluorescent microscopy, active NFκB was detected in the nucleus of both H$_2$O$_2$ and TNF-α treated cells. Whilst activation of NFκB by TNF-α involved the classical pathway of IκB degradation, this degradation was not observed in H$_2$O$_2$ treated cells. This suggests that H$_2$O$_2$ utilises an alternative pathway of NFκB activation that does not involve IκB degradation.

Finally, the effects of H$_2$O$_2$ on gene expression in SMC were studied using a PCR-based differential display method. A number of differentially expressed genes were identified using this technique following H$_2$O$_2$ treatment. However, subsequent analysis by Northern blotting demonstrated that many of these genes were not differentially expressed. In light of these results, efforts were focused on determining the reasons for such a high level of false positives. A number of modifications to the technique were developed to reduce the generation of non-specific PCR products, reduce the presence of co-migrating cDNA species and improve the efficiency of identifying and screening truly differentially expressed genes associated with oxidative stress.
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2.6 NORTHERN ANALYSIS ................................................................. 89
  2.6.1 RNA Preparation ...................................................................... 89
  2.6.2 Formaldehyde Gel Electrophoresis ...................................... 89
  2.6.3 RNA Transfer ........................................................................ 91
  2.6.4 Probe Synthesis For Northern Analysis .............................. 92
  2.6.5 Hybridisation Of RNA With Labelled Probes ...................... 95
2.7 SCREENING .................................................................................. 96

3. CHAPTER 3 - THE EFFECTS OF HYDROGEN PEROXIDE ON VASCULAR SMOOTH MUSCLE AND ENDOTHELIAL CELL GROWTH ............... 98
  3.1 INTRODUCTION ........................................................................... 99
  3.2 AIM ............................................................................................... 100
  3.3 RESULTS .................................................................................. 102
    3.3.1 Characterisation Of Cell Growth Following Mitogenic Stimulation ............................................................ 102
      3.3.1.1 Quiescence And Synchronicity ............................................. 102
      3.3.1.2 Cell Stimulation By Mitogens .............................................. 104
      3.3.1.3 Determination Of Cell Cycle Characteristics .................... 107
    3.3.2 The Effects Of Hydrogen Peroxide On Cell Growth .......... 111
    3.3.3 The Effects Of Hydrogen Peroxide On Cell Density ............. 134
  3.4 DISCUSSION .............................................................................. 136

4. CHAPTER 4 - THE EFFECTS OF HYDROGEN PEROXIDE ON NFκB ACTIVATION IN VASCULAR SMOOTH MUSCLE CELLS ......................... 145
  4.1 INTRODUCTION ........................................................................... 146
  4.2 AIM ............................................................................................... 147
  4.3 RESULTS .................................................................................. 148
    4.3.1 Western Blot Studies .............................................................. 148
      4.3.1.1 IκB-α Degradation and Regeneration ............................... 148
      4.3.1.2 IκB-β Degradation and Regeneration ............................... 155
    4.3.2 Immunofluorescent Analysis ................................................. 160
      4.3.2.1 p65 Expression ............................................................... 160
      4.3.2.2 Active NFκB Expression ................................................... 160
  4.4 DISCUSSION .............................................................................. 166
Chapter 1

INTRODUCTION
1.1 Atherosclerosis

Atherosclerosis and its associated complications are a major source of morbidity and mortality in men and women of every ethnic group in western civilisations. In the United States alone, more than 5.4 million patients are diagnosed with cardiovascular disease annually and over 500,000 deaths per year are directly attributable to cardiovascular disease (Gotto and Farmer, 1988). In terms of economic costs, cardiovascular disease is associated with at least $8 billion annually in health-care costs and $60 billion annually in lost productivity in the USA (Gotto and Farmer 1988).

Atherosclerosis is a multi-factorial process probably initiated in childhood and becoming clinically manifest in later life. Hyperlipidaemia, hypertension, diabetes mellitus and smoking are well-established risk factors for the development of atherosclerosis, yet to date it has not been possible to link these factors into a common pathogenic mechanism. Although atherosclerotic lesions can be observed throughout the arterial tree, certain regions are predisposed to the development of lesions. These include the aortic arch, the branching sites of larger vessels and the carotid and coronary arteries, i.e. sites subject to high haemodynamic stress. When atherosclerotic disease is present in any one arterial segment, there is a high probability that other arterial beds will be similarly affected (Mitchell and Schwartz, 1965).

1.1.1 Risk factors associated with Atherosclerosis

Until the middle of the 20th Century, atherosclerosis was thought to be a degenerative disease that was an inevitable consequence of ageing, and prevention was not considered. Research in the last three decades has shown that atherosclerosis is neither a degenerative disease nor inevitable. Lesions, particularly early fibro-fatty streaks, can be detected in early adolescence (PDAY study, 1993). Wide variations in cardiovascular morbidity and mortality among countries provided further evidence that atherosclerosis was not inevitable with ageing.

The relationship between cholesterol and atherosclerosis was first described in the Framingham study (Dawber et al., 1957). Since that time, cholesterol and more specifically
LDL cholesterol, has come to play a central role in our concept of the mechanisms of atherogenesis and the progression to atherosclerosis. Several epidemiological studies have shown a relationship between total cholesterol levels and the risk of cardiac events as reviewed by LaRosa et al., 1990, and several intervention studies have provided evidence that lowering the levels of total and LDL cholesterol can reduce cardiac events (Brown et al., 1990; 4 S trial, 1994; WOSCOPS trial, 1996). While the relationship between serum cholesterol and atherogenesis is evident, cholesterol is clearly not the only factor in the aetiology and pathogenesis of atherosclerosis. Additional risk factors which have been identified include hypertension, smoking, diabetes, age and gender. These risk factors appear to act synergistically to result in cardiovascular disease. Although a risk factor such as gender cannot be modified, knowledge of why it predicts the occurrence of cardiovascular disease may suggest other preventative strategies. In more recent searches for risk factors, leukocyte count, fibrinogen levels, homocysteinaemia and even baldness have been proposed.

1.1.2 Future interventions

Studies of animal models have enabled us to define the stages of atherosclerotic lesion development more clearly, and identify cellular and molecular factors that contribute to this process. Clarification of the role of these factors in contributing to atherogenesis will assist in the determination of therapeutic strategies.

1.1.3 Lesion development

An emerging consensus is that the formation of atherosclerotic lesions proceeds through defined stages. The earliest recognisable lesion of atherosclerosis is the ‘fatty streak’, which consists of an aggregation of lipid rich macrophages and T-lymphocytes within the innermost layer of the artery wall, the intima. Fatty streaks have been commonly found in the coronary arteries of autopsy specimens from children (Stary, 1989). Animal studies have shown that fatty streaks precede the development of intermediate lesions, which are composed of smooth muscle cells and macrophages (Rosenfeld et al., 1987; Masuda and Ross, 1990). In turn these lesions develop into more advanced, complex lesions called fibrous plaques. The fibrous plaques increase in size, and by encroaching upon the lumen, impede blood flow. Most sudden deaths from myocardial infarcts are due to plaque
ruptures or fissures, particularly in the margins of the fibrous cap, where there are more macrophages and fewer smooth muscle cells, resulting in haemorrhage into the plaque, thrombosis and occlusion of the artery (Davies and Thomas, 1984).

1.1.3.1 Early lesion-Fatty Streak development

Lesions do not occur at random, but develop preferentially and predictably in sites designated as lesion prone areas. It is likely that such areas are sites of relatively low shear stress associated with domains of reverse blood flow, where the residence time of particles such as lipoproteins and cells are prolonged, facilitating such processes as cellular attachment and endothelial lipoprotein transmigration. Excessive lipid deposition therefore occurs at selected regions of the arterial tree and is particularly prominent at flow dividers (Schwenke and Carew, 1989). This early accumulation of LDL in the sub-endothelial space is mainly a function of altered endothelial cell permeability and not a characteristic of the LDL particle (Schwartz et al., 1991). LDL localised in the sub-endothelial space becomes entrapped by extracellular matrix proteins, and may be subject to oxidative modification by endothelial cells (Morel et al., 1984; Steinbrecher et al., 1984; van Hinsbergh et al., 1986), smooth muscle cells (Heinecke et al., 1986), and resident mononuclear cells (Hiramatsu et al., 1987; Parthasarathy et al., 1986a). Thus LDL accumulation in lesion prone sites may allow for LDL exposure to oxidative stress (Figure 1.1).

Endothelial cells and smooth muscle cells exposed to minimally modified LDL are stimulated to synthesise and secrete adhesion molecules and chemoattractants for monocytes (Cushing et al., 1990). Monocytes adhere to the intact endothelium, extend pseudopodia, migrate through the junctions of adjacent endothelial cells into the sub-endothelial space and become tissue macrophages. Recruitment may also include lymphocytes (McMurray et al., 1993), which have been identified in immunostained sections of atherosclerotic tissue (Arbustini et al., 1991). The accumulation of lipid within macrophages and occasionally by smooth muscle cells gives the lesion its fatty streak appearance. This intracellular lipid deposition seems to be governed by scavenger receptors and not the classic LDL receptor mediated pathway.
Although the scavenger receptor pathway is considered important in the genesis of foam cells, other receptor mediated mechanisms for the macrophage uptake of lipid are evident, including a subclass of Fc receptors, FcγRII-B2 (Stanton et al., 1992); CD32, which recognises mildly oxidised LDL (Endemann et al., 1993); and SR-BI, a CD36 related receptor that recognises native LDL, oxidised LDL, and acetylated LDL (Acton et al., 1994). Mechanisms for oxidised LDL uptake by macrophages have been identified that are distinct from receptor recognition of the modified lipoprotein. One of these derives from the observation that oxidised LDL more readily aggregates than does native LDL. Thus, oxidised LDL uptake may proceed in part, via non-specific phagocytosis of these aggregated forms (Hoff et al., 1992; Steinbrecher and Lougheed, 1992).

### 1.1.3.2 Developed lesion

Not all fatty streaks develop into fibro-fatty lesions. Progression is dependent on the persistence of factors favouring lesion development, and inadequate mechanisms contributing to regression. Two features characterise the progressive transitional lesion, namely, the development of an acellular necrotic lipid core and the infiltration and proliferation of smooth muscle cells in the thickening arterial intima (Figure 1.2). Activation of macrophages within the sub-endothelial space results in the production of various cytokines and growth factors, resulting in smooth muscle cell migration from the intima to the media, followed by proliferation of smooth muscle cells.

Such changes are appropriately considered a reparative response by smooth muscle cells to inflammatory injury. Much of the necrotic core is believed to be derived from the death of macrophage-derived foam cells, presumably because of the cytotoxic components of oxidised LDL (Reid and Mitchinson, 1993). This origin probably accounts for much of the cellular debris, membrane remnants, and laminated bodies found within. Additionally, some core lipid is probably also derived directly from plasma. Plaque mass is substantially increased by the secretory products of the smooth muscle cells, namely, proteoglycans, collagen, and elastin (Reissen et al., 1994).
1.1.3.3 Mature lesion

Unlike the fatty streak, the mature or complex plaque affects all three layers of the arterial wall. The lumen is narrowed, the media is thinned, and lymphocytes are present not only in the thickened intima, but also in the fibrotic vascular adventitia. Glagov et al., (1987) demonstrated that in all species the lesion grows out toward the adventitia until a critical point is reached, at which time the lesion can no longer expand outward at the expense of the normal media and then begins to encroach upon the lumen (Figure 1.3). Monocyte recruitment continues at the edges or shoulders of the plaque where foams cells are most prominent. Smooth muscle cells and collagen are present. Extracellular lipid is prominent, and cholesterol crystals may be present. Calcium deposits may also be seen around the periphery of the lipid core and in the collagen cap resulting in considerable arterial rigidity. Common complications include plaque haemorrhage, endothelial disruption, and mural or occlusive thrombi.
Introduction Chapter 1

Monocyte Adhesion

Figure 1.1. Lipoprotein oxidation and early lesion development. LDL is oxidatively modified by cells of the vessel wall. Modified LDL induces the secretion of adhesion molecules, chemoattractants, cytokines and growth factors. This leads to further infiltration and proliferation of mononuclear cells and foam cell formation, which gives the lesion its fatty streak appearance. EC= endothelial cells, IEL= internal elastic lamina, LDL= low density lipoprotein, MCP-1= monocyte chemotactic protein-1, M-CSF= monocyte colony stimulating factor, MM-LDL= minimally modified LDL, ROS= reactive oxygen species, SMC= smooth muscle cells, X-LAM=adhesion molecules induced by MM-LDL, + = positive induction. Modified from Berliner et al., 1995.
Figure 1.2. Advanced lesion development. MM-LDL stimulates the migration of smooth muscle cells from the media into the intima, resulting in intimal thickening and protrusion into the lumen. Activated smooth muscle cells as well as macrophages secrete PDGF and bFGF, which induce the migration and proliferation of smooth muscle cells. bFGF induces scavenger receptor expression and via these receptors, smooth muscle cells accumulate MM-LDL resulting in foam cell formation. Oxidised LDL (OX-LDL) is cytotoxic and probably accounts for the necrotic core and cellular debris often observed.
Figure 1.3. Mature lesion development. Further infiltration of smooth muscle cells from the media and mononuclear cells from the circulation significantly reduce lumen diameter and impede flow. Extracellular lipid and calcium deposits are prominent. Accumulation of smooth muscle cells strengthens the lesion cap, but the shoulders of the lesions are weakened by the lack of smooth muscles cells and accumulation of macrophages that secrete matrix degrading enzymes. The unstable lesion is susceptible to rupture, and MM-LDL causes endothelial cells to produce a thrombogenic environment, which may lead to thrombus formation and occlusion.
1.1.4 Response to injury hypothesis of Atherosclerosis

Initially proposed in 1973 (Ross and Glomset), the response to injury hypothesis has been continually modified and updated as our knowledge of the pathogenesis of atherosclerosis increases (Ross, 1986,1993). It is generally believed that some form of injury to or abnormal regulatory mechanism of the endothelium is the initiating event in atherosclerosis. Following injury to the endothelium two other principle cells, smooth muscle cells and monocyte/macrophages are believed to be involved in the overall atherosclerotic process. Each of the following cell types have important roles to play in lesion development, therefore each cell type has been discussed in detail with reference to their role in the pathogenesis of atherosclerosis.

1.1.4.1 Endothelial cells

Vascular endothelial cells form a barrier separating deeper layers of the vessel wall and cellular interstitial space from the blood and circulating cells. Since the endothelium acts as a selective permeability barrier to plasma components, it is important to maintain endothelial cell integrity to limit the entry of macromolecules, such as cholesterol rich lipoproteins, into the vessel wall. There is much evidence that suggests the loss of endothelial integrity is one of the important early events in the aetiology of atherosclerotic lesion formation (Ross, 1986).

Factors that may contribute to injury include: mechanical or immunological damage, toxins or viruses, oxidatively-modified LDL lipoproteins and/or their metabolic products, synthesis of growth factors or activation of proteolytic enzymes (Ross, 1993). In addition, reactive oxygen intermediates, e.g. superoxide, and hydroxyl radical, generated by endothelial cells, neutrophils, and other inflammatory cells, possibly coupled to an inadequate cellular antioxidant defence system, may also contribute to the injury process (Warren and Ward, 1986). Endothelial cell injury may be apparent as an alteration in cellular morphology or cell function. Altered endothelial cell properties associated with dysfunction that may be relevant to atherogenesis include: increased expression of leukocyte binding sites on the endothelial surface, altered production of paracrine growth factors, chemoattractants, and vasoreactive molecules; enhanced ability to oxidise LDL and to respond to oxidised lipids and lipoproteins, increased expression of pro- rather than
anticoagulant activities, and the modulation of plasma component levels within the vessel wall through changes in permeability function (DiCorleto and Chisolm, 1986).

1.1.4.2 Macrophage/monocytes

Macrophages are present in all stages of lesion development (Jonasson et al., 1986; Gown et al., 1986). The macrophage is the principal mediator of inflammation in the atheromatous plaque. The involvement of inflammatory cells in human atherosclerosis is now well established. Using cell specific antibodies, several groups have confirmed the presence of macrophages in human lesions (Aqel et al., 1985; Jonasson et al., 1986; Gown et al., 1986). A more unexpected finding was that human lesions also contained T lymphocytes (Hansson et al., 1989). This observation has attracted considerable interest since it suggests the possibility that immune mechanisms may be actively engaged in lesion development.

In the ‘response to injury’ hypothesis, Ross (1993) proposes that monocyte/macrophages are important in the initial stages of lesion development. Loss of endothelial integrity leads to increased production and secretion of chemotactic and growth factors, which in turn stimulates the local accumulation of mononuclear cells, and their migration into the sub-endothelial space. Macrophages within the lesion act as antigen presenting cells to T lymphocytes, and also scavenge and degrade cytotoxic macromolecules such as LDL and its oxidised products from the lesion. Macrophages may also contribute to the oxidative modification of LDL that is trapped within the lesion, through the release of reactive oxygen species, although in vivo evidence is limited. The uptake of modified LDL by the scavenger receptors of macrophages is non-saturable and unregulated leading to foam cell formation and fatty streak development. The presence of oxidatively modified LDL also enhances cell release of chemotactic and growth factors, which leads to additional infiltration of inflammatory cells into the intima, thus initiating a cascade of inflammatory events.

Further to their involvement in the initial response to injury, macrophages may also play an important role in the fibro-proliferative process due to their ability to elaborate numerous growth regulatory molecules. PDGF, IL-1, bFGF, TNF-α, and TGF-β all have the capacity
to modulate the growth of endothelial and smooth muscle cells. Macrophages are also able to synthesise chemotactic factors for each of the cell types. In turn these growth factors and cytokines may induce further expression of mitogenic and chemotactic factors in neighbouring cells by paracrine and autocrine mechanisms. The infiltration and proliferation of macrophages and smooth muscle cells and the deposition of extracellular matrix components are associated with the progression of the lesion to a fibrous plaque or advanced, complicated lesion.

1.1.4.3 Smooth muscle cells

Upon damage to the vascular endothelium, the underlying smooth muscle cells are exposed to a number of mitogenic and chemotactic agents that are released at the site of injury from endothelial cells, macrophages, platelets and smooth muscle cells themselves. These agents include cytokines and growth factors in addition to vasoactive peptides that can act alone or in combination to induce smooth muscle cell migration and or proliferation. If a single factor was solely responsible for the mitogenic action on smooth muscle cells, then finding an inhibitor of this response should be relatively simple. However, animal models of intimal cell damage have shown that multiple growth factors are probably involved (Ferns et al., 1991; Lindner et al., 1991; Rutherford et al., 1997).

**Phenotypic modulation of SMC**

An interesting feature of smooth muscle cells within atherosclerotic lesions, is that they exhibit marked differences in morphology and protein expression patterns as compared to normal medial smooth muscle cells. Vascular smooth muscle cells (VSMC) are the predominant cell type in the media of the normal mammalian artery and as such contribute to its mechanical and contractile properties. Vascular smooth muscle cells perform these functions by maintaining a differentiated contractile phenotype characterised by numerous myofilaments, and a quiescent state of growth.

There is clear evidence demonstrating that smooth muscle cells within human atherosclerotic lesions, and myointimal lesions of experimental animals following vascular injury, show an altered phenotype as compared with normal medial smooth muscle cells (Kocher and Gabbiani 1986; Glukhova et al., 1988; Kocher et al., 1991). This cell
phenotype has been named 'synthetic' in contrast to the 'contractile' phenotype. This process of phenotypic switching is characterised by extensive alterations in the morphological appearance of the cells, including a reduction in myofilaments, and an increase in the formation of secretory protein apparatus such as rough endoplasmic reticulum and Golgi (Campbell et al., 1988). In addition, 'synthetic' intimal smooth muscle cells show reduced levels of a variety of proteins characteristic of differentiated (contractile) smooth muscle cells, including smooth muscle α-actin, smooth muscle myosin heavy chain, caldesmon, vinculin, and desmin (Mosse et al., 1986; Kocher and Gabbiani 1986; Glukhova et al., 1988; Kocher et al., 1991). In animal models of vascular injury, the changes observed in myointimal smooth muscle cells are at least partly reversible in that expression of contractile proteins increases in smooth muscle cells within chronic lesions (Clowes et al., 1988; Kocher et al., 1991).

Therefore, the changes may be an essential part of the repair process, with early decreases reflecting a shift to a less differentiated state with an increased growth capacity (Clowes et al., 1988), and later re-differentiation of the cells and a return of contractile capabilities. Phenotypic changes of medial smooth muscle cells appear to precede their proliferation and migration to the intima. These events are likely to be a consequence of vessel damage, with the release of growth stimulatory and chemotactic factors that may influence the differentiation pattern, proliferation rate and ability to migrate (Schwartz et al., 1990). Cells of a contractile phenotype, respond to vasoactive agents such as nitric oxide (Moncada and Higgs, 1990), prostacyclin (PGI₂), and angiotensin II (Owen, 1986). In contrast, in the synthetic state, they are capable of expressing genes for a number of growth factors and cytokines (Libby et al., 1988; Sjölund et al., 1988), can respond to growth factors by expressing the appropriate receptors, and can synthesise extracellular matrix. In the synthetic phenotype it is presumed that smooth muscle cells can express connective tissue molecules as well as growth factors such as PDGF-AA, and can stimulate themselves as well as neighbouring cells. The synthetic activity of smooth muscle cells will determine the matrix content of the lesion, which in turn could modify their capacity to respond to various agonists. Therefore, smooth muscle cells play a key role in the formation of the fibro-proliferative lesion.
Introduction

SMC Proliferation

Although it is generally considered that smooth muscle cell proliferation is one of the most important events in lesion formation in atherosclerosis in response to injury, and in restenosis after balloon injury, direct evidence of proliferating cells has proven difficult to obtain. In animal models of restenosis, 15-20% of cells found in the neo-intima are PCNA (proliferating cell nuclear antigen) positive. In contrast to the wealth of information that is available on the proliferation kinetics in animal models of arterial injury and repair, little is known about the proliferative profile of human atherosclerotic and restenotic lesions. Plaque progression due to SMC proliferation is probably episodic with periods of slow indolent growth punctuated by brief episodes of greater proliferative activity causing accelerated growth, like the proliferative healing response after angioplasty where severe re-narrowing may evolve over a few months.

The proliferative activity within plaques is usually very low despite high cellularity as evaluated by PCNA staining. No evidence of cell replication could be found in 82% of 118 primary coronary atherectomy specimens, and only a modest number of PCNA-positive cells (usually less than 1%) were found in the majority of the remaining samples (O’Brien et al. 1993). However, a smaller study using the same technique to gauge cell proliferation within plaques found 4% PCNA-positive cells (Pickering et al. 1993). These cells however are not just the expected smooth muscle cells. By use of cell specific immunohistochemical markers and a double labelling technique, PCNA positive cells were identified as smooth muscle cells, macrophages, and endothelial cells.

Approaches to measure proliferation have involved comparing cell phenotypes, immunocytochemical labelling for the proliferating nuclear antigen (PCNA), and tritiated thymidine incorporation and/or bromo-deoxyuridine labelling in normal and diseased tissue. A probable explanation for the discrepancy in the proliferation data is that PCNA is likely to overestimate measurements of cell proliferation (Fairman, 1990; Hall et al., 1990). This problem is highlighted in culture studies of smooth cell proliferation, where PCNA may be a marker of mitogenic activation rather than proliferation (Francis et al., 1992). An increase in intimal thickness is often used as an index of smooth muscle cell proliferation. However, intimal thickening is a combined result of migration of cells into the intima, proliferation, and the laying down of extra-cellular matrix, offset against any cell necrosis.
Nevertheless, the presence of elevated levels of growth factor and cytokine production and their receptors within lesions (reviewed by Ross, 1993), together with evidence that shows neutralising antibodies to these growth factors can significantly reduce lesion progression (Ferns et al., 1991; Rutherford et al., 1997), leaves little doubt that cell proliferation is important in lesion formation and progression in some forms of accelerated atherosclerosis.

An important therapeutic aim, therefore, is to inhibit SMC proliferation. Ideally, inhibition of SMC proliferation must occur selectively, without interference to the important process of endothelial repair or to the normal proliferation of other cells. Although a number of agents have been identified that inhibit SMC proliferation \textit{in vitro} and in certain animal models of SMC proliferation, there has been little success with these agents in the significant inhibition of restenosis or progression of atherosclerosis in man, although some lipid lowering agents can favourably influence the outcome in coronary disease without affecting lesion size (Brown B G, \textit{et al.}, 1993).

\textbf{Plaque stability}

It is important to note, however, that smooth muscle cell proliferation may be an essential reparative process whose complete inhibition may not be desirable. This reparative process may be important in plaque stabilisation (Weissberg \textit{et al.}, 1996). Evidence is accumulating that intimal smooth muscle cells protect plaques from rupturing and render unstable, potentially lethal lesions more stable. Stable plaques have a thick fibrous cap consisting of smooth muscle cells and an extracellular matrix above the lipid core. In the coronary circulation these lesions are clinically silent or cause angina, but rarely rupture or cause life-threatening events. By contrast, unstable plaques are characterised by a thin fibrous cap, with rupture prone areas that contain relatively few smooth muscle cells but abundant macrophages and T cells (Brown K \textit{et al.}, 1993; Davies M J \textit{et al.}, 1993). These plaques are prone to rupture, leading to thrombosis and possibly complete occlusion of the vessel lumen and the life threatening acute coronary syndromes of unstable angina and myocardial infarction (Fuster \textit{et al.}, 1990).

The stability of a plaque seems to be directly related to its smooth muscle cell content, and inversely related to its macrophage content (van der Wal \textit{et al.}, 1994). Since smooth muscle cells are the major cells in the plaque capable of synthesising extracellular matrix
proteins, intimal smooth muscle cells confer stability to the plaque. By contrast, macrophages secrete high concentrations of matrix degrading enzymes that may weaken the fibrous cap (Galis et al., 1994; Moreno et al., 1994).

1.1.5 **Oxidation hypothesis of Atherosclerosis**

Serum cholesterol levels are positively correlated to coronary risk (MRFIT research group, 1982). However, for any given concentration of LDL there is a great individual variation in the extent of atherosclerosis. Many studies have focused on modified LDL as a contributor to the evolution of atherosclerotic lesions to explain this apparent paradox. A hypothesis has developed which proposes that the atherogenicity of LDL is related to the degree of modification.

The so called ‘oxidation hypothesis’ of atherosclerosis (Steinberg et al., 1989; Steinbrecher, et al., 1990; Witztum, 1994) proposes that the oxidative modification of LDL (or other lipoproteins) is important in the pathogenesis of the atherosclerotic lesion, and hence inhibition of LDL modification should theoretically inhibit atherosclerosis. There are several lines of evidence that support the hypothesis.

1) Epidemiological studies have shown an inverse relationship between dietary or plasma concentrations of antioxidants and development of coronary artery disease (CAD) (Riemersma et al., 1989, 1991; Manson et al., 1991, 1992, 1993; Rimm et al., 1993; Stampfer et al., 1993). 2) Immunocytochemistry studies have identified OX-LDL in vivo, and studies with antibodies to various epitopes of OX-LDL have demonstrated intense staining of atherosclerotic lesions, but not of normal arteries (Palinski et al., 1989). 3) LDL extracted from atherosclerotic lesions displayed all the physical, immunological, and biological properties attributed to in vitro preparations of OX-LDL (Daugherty et al., 1988; Ylä-Herttuala et al., 1989). 4) Identification of autoantibodies to OX-LDL in human and animal sera has demonstrated that OX-LDL is immunogenic (Palinski et al., 1989; Salonen et al., 1992). 5) Detection of minimally modified LDL in plasma of human subjects (Hodis et al., 1994). 6) Antioxidant intervention studies have shown prevention or regression of atherosclerosis in animals and non-human primates ranging from 30-70% (Sasahara et al., 1994).
1.1.5.1 **Role of OX-LDL in Atherosclerosis**

*In vitro* studies have provided extensive evidence of the roles OX-LDL may play in the development of atherosclerosis. In addition to the cytotoxic effects of OX-LDL on the endothelium, OX-LDL induces the expression of adhesion molecules, including ELAM-1, ICAM-1 and VCAM-1 from endothelial cells as well the cytokines GM-CSF and M-CSF which may promote cell adhesion, proliferation and differentiation of circulating monocytes (Kume *et al.*, 1992; Berliner *et al.*, 1990; Rajavashisth *et al.*, 1990). Modified LDL can also facilitate monocyte invasion of the intima by eliciting the secretion of monocyte chemoattractants (MCP-1) from smooth muscle cells and endothelial cells (Cushing *et al.*, 1990) and surface expression of monocyte binding proteins. In addition, oxidatively modified LDL is a chemo-attractant for lymphocytes (McMurray *et al.*, 1993). Modified LDL can induce the migration and proliferation of SMC from the media to the intima via its induction of growth factor and cytokine production by macrophages and endothelial cells (Stiko-Rahm *et al.*, 1992; Li *et al.*, 1994a).

Direct effects of OX-LDL on SMC proliferation have also been observed, which possibly involve autocrine growth factor production (Chatterjee *et al.*, 1992; Augé *et al.*, 1995). Finally, OX-LDL uptake via the scavenger receptor pathway can lead to foam cell formation and finally necrosis in macrophages (Brown and Goldstein, 1983). OX-LDL promotes platelet adhesion and aggregation by inhibiting the synthesis of EDRF and stimulating the synthesis of prostaglandins by endothelial cells, and induces tissue factor expression and inhibits protein C synthesis to ultimately provide a thrombogenic environment within the lumen of the vessel wall (Holvoet and Collen, 1994). It is not clear whether oxidised LDL initiates atherosclerosis or contributes to certain stages of lesion development.

There is speculation that modified forms of LDL also exist in plasma and that the lipid hydroperoxides they contain might lead to the initial injury response (Yagi, 1986; Gorog, 1992). This speculation is based on the separation from human and primate LDL of a sub­fraction, referred to as LDL', that has characteristics in common with LDL oxidised *in vitro* (Avogaro *et al.*, 1988; Hodis *et al.*, 1994). There have additionally been reports of increased levels of lipid oxidation products in the plasma of patients at high risk of myocardial infarction (Stringer *et al.*, 1989; Liu *et al.*, 1992), and antibodies that recognise
oxidised LDL but not native LDL have been measured in the plasma of normal humans (Salonen et al., 1992). However, evidence for the presence of oxidised LDL in plasma is lacking, possibly due to its rapid removal from the circulation by hepatic sinusoidal cells, which express scavenger receptors (Steinberg et al., 1989).

A current hypothesis suggests that plasma concentrations of oxidised LDL are derived from LDL that enter the intima from plasma as native LDL, which is subsequently oxidised by the cells in the arterial intima. Loss of endothelial integrity or plaque rupture would then allow the release of oxidised LDL back into the circulation. The idea that a sub-fraction of human LDL is more susceptible to oxidation than the remainder has been demonstrated in humans, and shown to be attributable to a small dense population of LDL, the concentration of which correlates with vascular disease (Chait et al., 1993). Low concentrations of plasma antioxidants such as those found in smokers may permit the formation of a ‘primed’ fraction of LDL that is more susceptible to oxidation.

1.1.5.2 Oxidative stress and Restenosis

The processes involved in atherosclerotic lesion development are different from those involved in restenotic lesion formation after angioplasty. Atherosclerosis takes years to become clinically manifest, whereas clinically evident restenosis can occur a few months after PTCA. Some features of the lesions are very dissimilar, although this may reflect the complexity of atherosclerosis and the time scale in which lesion development occurs. However, some similarities do exist. Lipid and lipoprotein oxidation products are present in both (Ylä-Herttuala et al., 1989; Eber et al., 1994). It is not surprising that phagocytic leukocytes and higher levels of plasma lipoproteins readily enter the ‘wound’ considering the amount of damage that occurs to the endothelium, intima and media by the balloon. Therefore at the site of injury, both lipid substrate and cells known to produce reactive oxygen species are in ample supply, and so the likelihood exists that the resulting lipid peroxidation products could alter cell behaviour locally.
Introduction

Oxidised LDL is known to inhibit endothelial cell migration and proliferation in vitro, whilst it induces migration and proliferation of smooth muscle cells, perhaps through the release of paracrine or autocrine growth factors. It can therefore be hypothesised that OX-LDL has the capacity to retard re-endothelialisation following balloon induced injury and stimulate smooth muscle cell migration and proliferation. In addition, since OX-LDL is a powerful chemoattractant for circulating leukocytes, it also has the capacity to induce inflammatory responses, which could enhance monocyte and smooth muscle cell accumulation, and neo-intimal formation.

1.1.5.3 Modification of LDL

Although it is generally accepted that oxidative modification of LDL occurs in atherosclerotic lesions, the mechanisms and precise sites of these modifications remain unclear. Oxidative modification of LDL is assumed to occur primarily in the arterial intima, in micro-environments sequestered from antioxidants. It is doubtful if LDL is oxidised in plasma because of the many antioxidants present. However, minor degrees of lipid oxidation or protein modifications may occur in plasma. These changes could have occurred elsewhere, or during previous transient passage through the arterial wall. It is estimated that more than 85% of LDL entering the vascular wall re-enters the circulation (Schwenke and Carew, 1989). Such ‘minimally modified’ LDL might then be ‘primed’ for more rapid oxidative modification on subsequent entry into the intima. Upon entry into the intima, lipoproteins bind to connective tissue elements, thereby increasing their residence time, which in turn, increases the probability of oxidation.

The mechanisms of LDL oxidation are unknown. In culture, all the cells of the artery wall can initiate oxidation of LDL, but the relative contribution of endothelial cells, macrophages, or smooth muscle cells to such modification in vivo is unknown. In addition, the mechanisms involved in cellular modification may arise from different pathways. For example, release of superoxide from endothelial or smooth muscle cells might be responsible for initiation of oxidation in some settings or thiols in others (Heinecke et al., 1986). Regardless of the precise mechanisms, numerous in vitro studies have demonstrated that transitional metals are important in cell mediated atherogenic modification of LDL (Heinecke et al., 1984). Cell-free incubation of LDL with cupric ions (Cu^{2+}) also results in
generation of a lipoprotein particle with chemical and physiological characteristics identical with those of cell modified LDL (Gebicki et al., 1991). The transition metal ions commonly used to oxidise LDL in vitro, iron and copper, are effectively bound in vivo by transferrin and ceruloplasmin respectively. Mechanisms have been proposed for the release of transition ions from protein carriers, although the presence of such free metal ions within the intima has not been demonstrated in vivo (Balla et al., 1991; Swain et al., 1994).

1.1.5.4 Minimally Modified LDL

Many of the effects induced by LDL are actually caused by the products of LDL modification. These products may be generated during the early phases of oxidation, before the properties of LDL are changed sufficiently to allow scavenger receptor mediated macrophage uptake. This form of LDL has been termed minimally modified LDL (MM-LDL) (Liao et al., 1991). The biological properties of the lipids in MM-LDL are different from those of fully oxidised LDL (OX-LDL). For example, the lipids in OX-LDL have been shown to be cytotoxic, whereas the lipids in MM-LDL are not in endothelial and smooth muscle cells (Berliner et al., 1990). The activation of a NF-κB-like transcription factor and the induction of genes by MM-LDL, appear to be due to the release of unidentified oxidised lipids as by-products of LDL oxidation (Watson et al., 1994, 1995). With continued oxidation, these bioactive lipids are presumed to be destroyed and new ones formed that account for the different biological activity of OX-LDL.

LDL modification involves a complex chain reaction of events. Initiation can occur in vitro by incubating with cells, copper sulphate, or exposure to UV. Initiation involves depleting the LDL’s natural source of antioxidants that would normally prevent oxidation (minimally modified LDL). Once LDL is depleted of its antioxidants it is susceptible to further oxidation. Continued treatment results in the formation of lipid hydroperoxides which initiate further oxidation of LDL. Conjugated dienes are formed in addition to aldehydes and ketones produced from fatty acid fragmentation, which conjugate to the apoB protein moiety of LDL. Modification to the apoB moiety of LDL allows cellular uptake through the scavenger receptor; not the LDL receptor, which no longer recognises the apoB. Unlike the classic LDL receptor uptake pathway, scavenger uptake of LDL is unsaturable, which leads to unregulated lipid accumulation and foam cell formation. Minimally modified LDL is
still recognisable by the LDL receptor since no modifications have changed the apoB protein. Figure 1.3 shows the three phases of LDL modification; initiation, propagation and decomposition. Features of LDL indicative of oxidative modification include increased oxidation defined by thiobarbituric acid reacting substances (TBARS), decreased particle size, increased electrophoretic mobility, increased unesterified cholesterol content, decreased cholesteryl linoleate content, modified phospholipid content, and fragmentation of apolipoprotein B.
Figure 1.3. LDL modification. Initiation involves a depletion of the natural antioxidant content of LDL with minimal lipoperoxide content. In the propagation phase, copper ions or ROI present in culture medium will generate peroxy radicals leading to chain reactions that result in amplified numbers of lipoperoxides, rearrangement of fatty acid double bonds, and the resulting characteristic formation of ‘conjugated dienes’. Consequent to this propagation, fatty acid fragmentation occurs, leading to the formation of highly reactive intermediates such as ketones and aldehydes, and other oxidised products, such as oxycholesterol derivatives. In part, these reactive intermediates may then complex with the adjacent apoB protein, as well as with phospholipids. The modification of lysine residues of apoB inhibits the ability of LDL to bind to the LDL receptor. Protein modification results in the generation of new epitopes on apoB that are then recognised by macrophage scavenger receptors. This pathway of uptake is unsaturable and leads to foam cell formation. Modified from Witztum, 1993.
1.1.5.5 Detection of LDL modification

The methods available for measuring oxidation are not entirely satisfactory, as they lack accuracy and only give an indirect indication of LDL oxidation. The oxidative modification of LDL is a complex process during which all chemical and functional properties of the lipid and protein moiety of LDL are altered. The onset and progression of lipid peroxidation in LDL solutions can be followed by measuring the formation of MDA, lipid peroxides, conjugated dienes, aldehydes, and fluorescent protein or lipids. Other methods include the measurement of the disappearance of antioxidants and polyunsaturated fatty acids, the fragmentation of apoB, and the increase of the relative electrophoretic mobility. None of the methods give a satisfactory picture of the stages of LDL oxidation. Single measurements of TBARS (MDA) and lipid hydroperoxide content, do not allow conclusions to be made whether oxidation is in its early phase (i.e. before the peroxide and MDA maximum) or late phase (i.e. after the peroxide maximum) when MDA has a constant maximum value, despite further oxidation of LDL. In addition, in different LDL preparations, the rate of formation of lipid hydroperoxides can differ significantly (Esterbauer et al., 1990). To overcome these difficulties, procedures for the continuous monitoring of LDL oxidation were developed.

One method for continuously monitoring the oxidation of LDL is the measurement of the increase in absorption at 234 nm (Esterbauer et al., 1989). This increase develops during LDL oxidation by the conversion of polyunsaturated fatty acids with isolated double bonds (18:2, 20:4) into fatty acid hydroperoxides with conjugated double bonds. Changes in 234 nm absorption with time show three distinct phases (Figure 1.4). (1) A lag phase or induction period, where the absorption does not increase or only slightly increases, indicating that lipid peroxidation is low or absent and antioxidant supplies are depleted. (2) A rapid increase (propagation phase) in \( A_{234} \), which indicates that lipid peroxidation has entered into a propagating chain reaction. (3) A decomposition or terminal phase where hydroperoxides are converted to reactive aldehydes, e.g. malondialdehyde and 4-hydroxynonenal which begins immediately after \( A_{234} \) has reached maximum value. At this period the rate of decomposition of conjugated lipid hydroperoxides exceeds the rate of their formation, and as a consequence, \( A_{234} \) decreases slightly (Esterbauer et al., 1990). This method has the added advantage that only a small amount of valuable LDL is required.
Figure 1.4. The continuous measurement of low density lipoprotein (LDL) oxidation using changes in absorbance at 234 nm. Changes in 234 nm absorption with time show three distinct phases: lag phase, propagation phase, and decomposition phase. The lag time can be calculated and used as an indication of susceptibility of LDL to oxidation, and is particularly suited to clinical studies as an appropriate end measurement of efficacy in antioxidant studies, (Esterbauer et al., 1990).
1.1.6 Antioxidants and Atherosclerosis

Although there is considerable evidence showing that oxidation occurs within atherosclerotic lesions, the presence of oxidative products does not necessarily prove their involvement in the disease process. Consequently, many researchers have devoted their time to define the effects of antioxidants on the extent and nature of lesion formation to determine whether there is a causal role of oxidation in the atherogenic process. The proposal that antioxidants might retard the progression of atherosclerosis is not a new one. Studies examining the effects of vitamin A, C, E, BHT and other antioxidants on experimental atherosclerosis were reported as early as 1940, (reviewed by Chisolm, 1991).

1.1.6.1 Animal studies

The Watanabe Heritable Hyperlipidaemic (WHHL) rabbit, an animal model of human familial hypercholesterolaemia, has proved useful in the study of antioxidant therapy. Two studies simultaneously reported that probucol, an antioxidant drug with lipid lowering properties, markedly reduced fatty streak formation in the WHHL rabbit by unknown mechanisms (Carew et al., 1987; Kita et al., 1987). Both groups suggested that the retardation of fatty streak development was independent of probucol’s lipid lowering property. Many in vitro studies have shown probucol to prevent lipoprotein oxidation under various conditions (Parthasarathy et al., 1986b). Probucol has since been shown to be a successful antiatherogenic agent in other experimental models, including the cholesterol fed rabbit (Daugherty et al., 1989) and non-human primates fed an atherogenic diet (Sasahara et al., 1994). The antioxidant effects of probucol have been attributed to its capacity to scavenge peroxyl radicals and lipid radicals; however, it has numerous other effects on cell function.

Further studies with other antioxidants with peroxyl-radical scavenging properties, such as BHT (butylated-hydroxytoluene) and DPPD (N,N’-diphenylphenylenediamine), neither of which have lipid lowering properties, were also shown to reduce lesion formation in atherogenic rabbit models (Sparrow et al., 1992; Bjorkhem et al., 1991). This work further supported the view that the antiatherogenic role of probucol was due, in part, to its antioxidant properties. Fewer studies have attempted to define the antiatherosclerotic effects of antioxidant vitamins in animal models. The effectiveness of vitamin E against
atherosclerosis is a contentious issue and results of the few studies that have been published are inconsistent. Vitamin E, a scavenger of peroxyl and to a lesser extent alkoxyl radicals, has been reported to retard atherosclerosis progression in certain arteries of non-human primates fed an atherogenic diet (Verlangieri and Bush, 1992) as well as a modified WHHL rabbit model of atherosclerosis (Williams et al., 1992). In contrast, studies have shown no significant benefits of Vitamin E supplementation (Willingham et al., 1993; Kleinveld et al., 1994), whilst others have shown pronounced atherogenic effects (Godfried et al., 1989). There is also limited information available on the antiatherogenic effect of vitamin C. Ascorbate is considered to be the most important antioxidant in human plasma, partly because it usually disappears faster than other antioxidants when plasma is exposed to ROS (Frei et al., 1989). Many studies have been carried out confirming the antioxidant properties of vitamin C in vitro, where its effects are presumably due to maintaining vitamin E in its reduced antioxidant state (Frei et al., 1989). Although there appears to be little beneficial effect of vitamin C supplementation in vivo (Verlangieri et al., 1977; Beetens et al., 1986), significant benefits have been observed after concomitant administration with vitamin E Bocan et al., 1992; Nunes et al., 1993). Interestingly, vitamin C deficiency seems to have proatherogenic effects (Ginter, 1978; Satinder et al., 1987; Kimura et al., 1992).

1.1.6.2 Human studies

Available observational epidemiological and limited randomised trial data support the possibility that antioxidants may have a protective effect in cardiovascular disease, however the current data is promising but not conclusive. The evidence from observational dietary intake and some blood based studies is somewhat stronger for the lipid soluble antioxidants, vitamin E and β-carotene, than for the water soluble antioxidant vitamin C (Gey et al., 1993). This may be due to relatively low rates of vitamin C deficiency in the populations studied or could be due to mechanistic differences. Epidemiological and prospective studies have shown protective effects of vitamin E. Inverse correlations have been found between an increased risk of cardiovascular disease and plasma concentration or dietary intake of vitamin E (Gey et al., 1991; Rimm et al., 1993; Stampfer et al., 1993).
1.1.7 Antioxidants and Restenosis

The limited number of studies carried out on the effects of antioxidants on restenosis show that some of the lipophilic antioxidants known to be successful in inhibiting atherosclerosis progression also appear to be successful in limiting the vascular regrowth after balloon injury. Probucol (Ferns et al., 1992), BHT (Freyschuss et al., 1993), and vitamin E (Lafont et al., 1995) have each been shown to significantly reduce intimal thickening associated with balloon injury in cholesterol fed rabbits. Few studies have been carried out in humans, and data suggests that antioxidant therapy is not as successful in humans as in animal models. Probucol was reported to be of benefit after angioplasty in a limited human study (Lee et al., 1991b). Ebselen, a compound that reduces lipid hydroperoxides, has been reported to limit restenosis after angioplasty in a small group of human subjects (Hirayama et al., 1992). One study showed limited benefit of vitamin E treatment, but in this group of patients, the vitamin E treatment was not offered in advance of angioplasty (DeMaio et al., 1992). Lipophilic substances such as vitamin E and probucol would be expected to require significant time to reach elevated levels in tissue.
1.2 OXIDATIVE STRESS

1.2.1 Introduction

There is good evidence for the involvement of oxidative stress in cardiovascular disease. That antioxidants may be protective, provides further evidence for a role of oxidative stress in cardiovascular disease. Until the 1960s, oxidative stress was not considered relevant in mammalian biology. However, three landmark publications 'radically' changed this perception. The first was the discovery that an enzyme exists in virtually every mammalian cell that has superoxide dismutase activity (McCord and Fridovich, 1969), suggesting that superoxide anions are found constitutively in vivo. A few years later, Babior et al., (1973) showed that the bactericidal activity of neutrophils is associated with production of the superoxide radical, linking oxygen free radicals to inflammatory disease states. The third landmark paper was published in 1981 by Granger et al., in which they propose the hypothesis that reperfusion injury after intestinal ischaemia may be caused by xanthine oxidase-derived oxygen radicals. This paper triggered numerous studies into the diseases associated with ischaemia-reperfusion, myocardial infarction, stroke and shock. The number of diseases in which detrimental oxidation processes have been proposed to play a causative role has grown steadily over the past decade (reviewed by Kehrer, 1993). These include some of the most prevalent diseases in humans in addition to heart diseases, including certain types of cancer, inflammatory-immune injuries, cataract, and some neurodegenerative disorders and epidemiological studies indicate that dietary antioxidants may modify the development of these diseases.

1.2.1.1 Definitions

Oxidative stress occurs when the rate of formation of reactive oxygen species exceeds the capacity of the antioxidant defence mechanisms within a localised environment. This may be due to an excessive production of ROS or due to a depletion of antioxidants. Reactive oxygen species, many of which are oxygen free radicals and involved in oxidative damage are shown in Table 1.1. Some, such as singlet oxygen (\(^{1}\text{O}_2\)) and hydrogen peroxide (H\(_2\text{O}_2\)), are non-radicals. The half lives of these reactive oxygen species varies greatly. The hydroxyl radical (·OH) is the most reactive and short lived. The peroxyl radical (ROO\(^\cdot\)) is long lived, and those radicals that may be formed from polyunsaturated fatty acids
(PUFAs) could potentially diffuse from their site of generation unless deactivated by antioxidants. The problems faced in the study of reactive oxygen species arise from their short lifetimes, and from their very low steady-state concentrations.

<table>
<thead>
<tr>
<th>Radical</th>
<th>Name</th>
<th>Half-life (37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO$^-$</td>
<td>Hydroxyl radical</td>
<td>$10^9$ sec</td>
</tr>
<tr>
<td>LO$^-$</td>
<td>Lipid alkoxy radical</td>
<td>$10^6$ sec</td>
</tr>
<tr>
<td>LOO$^-$</td>
<td>Lipid peroxyl radical</td>
<td>7 sec</td>
</tr>
<tr>
<td>L$^-$</td>
<td>Lipid carbon-centred radical</td>
<td>$10^8$ sec</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
<td>Minutes</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide anion</td>
<td>$10^5$ sec</td>
</tr>
<tr>
<td>$^1$O$_2$</td>
<td>Singlet oxygen</td>
<td>$10^6$ sec</td>
</tr>
<tr>
<td>HQ</td>
<td>Semiquinone radical</td>
<td>Days</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
<td>1 sec</td>
</tr>
</tbody>
</table>

Table 1.1. An estimate of the different half-lives of different types of radicals and oxidising species that can play a role in biological processes. From Pryor et al., 1986.

1.2.1.2 Sources

A number of endogenous and exogenous sources of radicals and oxidants have been identified (Figure 1.5). They may be derived endogenously as by-products of normal respiration, cytochrome P-450 enzyme activity, and the oxidative burst from phagocytic cells. Exogenous sources may further increase the large endogenous oxidant load. Iron and copper salts promote the generation of oxidising radicals from peroxides (Fenton chemistry). The oxides of nitrogen ($NO_x$) in cigarette smoke (about 1000 ppm) cause oxidation of macromolecules and deplete antioxidant levels. This is likely to contribute significantly to the pathology of smoking. Normal diets contain plants with large amounts of natural phenolic compounds, such as chlorogenic and caffeic acid, that generate oxidants by redox cycling. The importance of each source in any specific disorder is often unknown and the relative role each plays in tissue injury seems certain to vary with the specific
experimental conditions employed. It also appears likely that radical formation is often secondary to the primary disease process. For example, following many types of tissue injury, including trauma, cells will rupture and release their contents. These contents will include transition metals that can rapidly catalyse additional radical-mediated transformations and tissue injury. Phagocytic activation and the disruption of mitochondrial function may also result in the formation of excess ROS. Because these processes routinely accompany tissue injury, separating cause from effect is difficult, particularly when the injury is aggravated and the secondary effect also becomes a cause of injury.

Figure 1.5. Cellular sources of free radicals. All aerobic cells produce free radicals through the action of various membrane-bound and soluble enzymes. The capacity of specific pathways to produce free radicals varies with the cell type. Modified from Kehrre (1993).
1.2.1.3 *Defence mechanisms*

Because of the damaging potential of ROS, cells depend on elaborate defence mechanisms that have evolved to rapidly metabolise the toxic intermediates and to prevent significant free radical injury (Sies, 1993). These systems may be chemical or enzymatic, endogenous or exogenous antioxidants, which work in concert to minimise initiation and propagation of undesirable oxidative reactions within cells (primary defences) and to repair or remove oxidative damage (secondary defences). Some of the antioxidant molecules are synthesised by the body, for example, glutathione or ubiquinol, whereas others have to be provided as micronutrients, for example antioxidant vitamins and trace metals.

At least three cellular processes are involved in the detoxification of oxidants. The first line of defence against superoxide is superoxide dismutase (SOD), which catalyses the diffusion-limited dismutation of superoxide to hydrogen peroxide and oxygen. The cytosolic enzyme contains copper, and the mitochondrial enzyme has manganese as its redox-active metal ion. Hydrogen peroxide formed in peroxisomes is metabolised completely by the high content of catalase in these organelles into $H_2O$ and $O_2$. Because hydrogen peroxide can easily diffuse through membranes, peroxisomal catalase can also participate in the detoxification of hydrogen peroxide generated in other cell compartments. Most important for the metabolism of cytosolic and mitochondrial hydrogen peroxide is the selenium-containing enzyme glutathione peroxidase, which reduces hydroperoxides and $H_2O_2$ to the corresponding alcohols using reduced glutathione as a substrate. Regeneration of reduced glutathione is dependent on glutathione reductase, a reduced NADPH dependant enzyme. Reduction of oxidised NADPH is accomplished by electrons generated by aerobic metabolism through the hexose monophosphate shunt. In addition, lipid-hydroperoxides and products of lipid peroxidation that result from oxidant damage to cellular membranes are detoxified by glutathione-S-transferase using glutathione as a reducing agent. Reduced glutathione is then regenerated via the glutathione redox cycle (Figure 1.6).
Figure 1.6. Cellular defence mechanisms against oxidative stress. All aerobic cells contain a spectrum of chemical and enzymatic antioxidants. These work in concert to minimise the initiation and propagation of undesirable oxidative reactions within cells (primary defences) or to repair oxidative damage (secondary defences). SOD= superoxide dismutase, GSH= reduced glutathione, GSSG= glutathione disulphide. Modified from Kehrer (1993).
There are also many structural defences such as sequestering H₂O₂-generating enzymes in peroxisomes and chelating any free iron or copper salts in transferrin or ferritin or ceruloplasmin to prevent Fenton chemistry. In a case in which all defence mechanisms fail and macromolecules are damaged, repair processes are activated or protein turnover is stimulated to correct the modification or to replace the damaged molecule without affecting cellular functions. Oxidised DNA is repaired by non-specific excision repair enzymes and, more importantly, by a series of glycosylases that are specific for particular oxidised bases. Without cell division these oxidative lesions are dealt with effectively and the mutation rate is kept to a minimum. Oxidised proteins are degraded by proteases. Lipid hydroperoxides are destroyed by glutathione peroxidase. Almost all of these defences appear to be inducible, as are most other types of defences, i.e. the amounts increase in response to use (Halliwell, 1987).

1.2.2 Working with oxidative stress systems

Intact cells can be subjected to oxidative stress by the direct addition of oxidants. This can be as experimentally simple as growing cells at elevated O₂ tensions. Hydrogen peroxide is found to be a convenient reagent as it is commercially available in high purity and is easy to quantify. Redox-active compounds can also be added to cells to generate free radicals. Quinones such as menadione and paraquat have been used extensively to generate free radicals. Enzymatic production of oxygen radicals is commonly achieved using the xanthine (X) xanthine-oxidase (XO) system. While this system is simple and versatile, it must be used with extreme caution when studying proteolysis since commercial preparations of XO are heavily contaminated with proteases.

1.2.2.1 Hydrogen peroxide

Any biological system generating O₂⁻ will produce hydrogen peroxide by the dismutation reaction. Hydrogen peroxide, which is mainly derived from O₂⁻ (1), has been shown to be produced by mitochondria and microsomes in vitro; and the amount produced increases as the surrounding oxygen tension is raised. However, there are also several enzymes that produce hydrogen peroxide without the intermediacy of free O₂⁻ radical. These include glycollate oxidase, D-amino acid oxidase, and urate oxidase. Because the liver has effective mechanisms for disposing of hydrogen peroxide, it has been estimated that the
steady state H$_2$O$_2$ concentration is in the range of $10^7$ to $10^9$ moles per litre, hydrogen peroxide being continuously generated and destroyed. In other animal cells with less effective removal mechanisms, more H$_2$O$_2$ is found, e.g. the lens of the human eye contains 24 μmol per litre H$_2$O$_2$, and rabbit spermatozoa release H$_2$O$_2$ into the surrounding medium, again probably from O$_2^-$. Hydrogen peroxide is known to be produced during phagocytosis, and H$_2$O$_2$ vapour has been detected in human breath. Hydrogen peroxide is a weak oxidising agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can penetrate cell membranes rapidly whereas O$_2^-$ cannot. Once inside the cell it can react with Fe(II) or Cu(I) ions to form the hydroxyl radical, via the Fenton reaction (2), and this is probably the reason for its toxic effects.

\begin{align*}
(1) \quad & 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \\
(2) \quad & H_2O_2 + Fe(II) \rightarrow HO^- + HO^- + Fe(III)
\end{align*}

1.2.3 **Oxidative stress and cells of the vessel wall**

Research carried out since about 1980 has provided persuasive evidence that supports an important role for oxidative damage in the atherosclerotic process (Esterbauer *et al.*, 1992). More importantly, this information has stimulated epidemiological investigations that appear to be supporting the hypothesis that dietary antioxidant supplements, can provide protection against cardiovascular disease (Rimm *et al.*, 1993; Stampfer *et al.*, 1993).

The net oxidative status of the arterial intima is a function of the dynamic balance between the generation of oxidants such as superoxide anions and hydroxyl radicals on the one hand, and the effectiveness and spatial availability of the various intimal antioxidant systems on the other. The cellular sources of reactive oxygen species include activated intimal monocyte-derived macrophages, together with endothelial and smooth muscle cells. However, the relative contributions of these three cell types to the net oxidative stress of the intima have yet to be defined. One is tempted to suggest that macrophages may be the
principal intimal source of pro-oxidant molecules, in keeping with their natural bactericidal and tumouricidal defence roles.

### 1.2.3.1 Oxidative stress and endothelial cells

Oxidative stress can be important in the early stages of lesion development, through endothelial injury or dysfunction. Endothelial cells are both critical targets and sources of reactive oxygen species (Hulsmann and Dubelaar, 1987; Freeman and Crapo, 1982). Endothelial cells of the vasculature possess both xanthine oxidase activity and purine nucleotide phosphorylase activity (Wems et al., 1986). The latter enzyme catalyses the formation of the major xanthine oxidase substrate, hypoxanthine. Therefore oxidation of hypoxanthine by xanthine oxidase, with concomitant formation of the superoxide radical, may be an important mechanism of vascular endothelial cell injury. Rosen and Freeman, (1984) have spin-trapped superoxide and lipid free radicals in suspensions of cultured endothelial cells treated with menadione, a quinone capable of undergoing redox cycling. Peroxides and oxygen radicals generated by microsomal enzymes such as lipoxygenase and cyclooxygenase may also be involved in the initial promotion of oxidative damage in endothelial cells (Mason et al., 1980). A complex balance between exogenous oxidants and endogenous antioxidants such as glutathione and catalase is likely to underlie the fate of reactive oxygen-exposed cells. The glutathione-redox cycle is known to be the major route for scavenging hydrogen peroxide in endothelial cells. TGF-β causes a reduction of GSH levels in EC and a release of hydrogen peroxide.

### 1.2.3.2 Oxidative stress and smooth muscle cells

Oxidative stress continues to be important in lesion development where migration of inflammatory cells into the intima allows the continuous release of free radicals and therefore continued modification of LDL. Reactive oxygen species themselves may have a direct influence on cell activity within the intima and will be discussed later. Several investigators have studied the effects of LDL and modified LDL on smooth muscle cell proliferation. Fless et al., (1982) found that human LDL particles were mitogenic for smooth muscle cells cultured from monkey aorta. Koschinsky et al., (1987) noted a small mitogenic effect of LDL from hypercholesterolaemic, but not from normocholesterolaemic, human blood donors. Others have found that LDL increased PI turnover.
in rat and human arterial SMC implying a stimulatory effect on cellular proliferation (Block et al., 1988) and (Scott-Burden et al., 1989). The latter group also noted an increase in intracellular pH, translocation of PKC to the cell membrane, and proto-oncogene expression, all of which are associated with increased cellular activity. In contrast Augé et al., 1995 showed that highly oxidised LDL was cytotoxic to smooth muscle cells.

1.3 CELL SPECIFIC EFFECTS OF OXIDATIVE STRESS

The importance of oxidative stress in disease processes is reflected in the amount of research now being carried out investigating specific effects of ROS on cells. Data concerning the effects of reactive oxygen species is conflicting: low concentrations may act as cellular messengers to influence many aspects of cell metabolic activity including growth (Burdon et al., 1989; Saran and Bors, 1989), whereas high concentrations may cause cell damage or even cell death. Reactive oxygen species have been traditionally considered to be toxic molecules effecting tissue injury in their local environment. The common mechanism underlying these effects is the destruction of membranes, proteins, and nucleic acids by toxic oxygen metabolites generated by cells in response to various stimuli or signals. However, several lines of evidence suggest that ROS may influence many aspects of cell metabolic activity including growth.

1.3.1 Oxidative stress and cell growth

Growing evidence from many cell types suggests that ROS at low concentrations may serve as second messengers (Schreck et al., 1991; Rao et al., 1993; Schenk et al., 1994). Hydrogen peroxide has been shown to mimic many of the actions of growth factors and hormones in various cell culture systems (Nose et al., 1991; Devary et al., 1991). The production of hydrogen peroxide has been observed upon stimulation of cells with various growth regulatory molecules including insulin, TGF-β, TNF-α, and PDGF (Ohba et al., 1994; Krieger-Brauer and Kather, 1995; Sundaresan et al., 1995). Reactive oxygen species have been shown to stimulate the expression of a wide range of immediate early response genes such as Egr-1 (Ohba et al., 1994; Huang and Adamson, 1995), c-jun, c-fos, and c-myc (Luna et al., 1994). Reactive oxygen species have also been implicated in growth regulation, such as the regulation of Egr-1 expression by TGF-β (Ohba et al., 1994), the modulation of NFκB activity by TNF-α (Muroi et al., 1994) and the induction of c-fos
expression by cytokines and growth factors (Lo and Cruz, 1995). More recently, ROS have been shown to induce phosphorylation of growth factor receptors such as EGFR (Huang et al., 1996) and are thought to mediate many of their effects through redox sensitive kinases and phosphatases. The best example of the second messenger actions of ROS is nitric oxide, which is now widely accepted as having such a role (Crossin, 1991). NO stimulates guanylate cyclase and is known to relax vascular smooth muscle tone and modulates signalling pathways in the developing and adult brain. Reactive oxygen species are found in every cell type and their intracellular levels can be regulated by multiple enzymes such as superoxide dismutase, catalase, glutathione peroxidase/glutathione and peroxidases. Thus ROS fulfil important prerequisites for second messenger molecules: they are small, diffusible and ubiquitous molecules that can be synthesised and destroyed rapidly.

1.3.1.1 Cell cycle

Different growth factors may have either stimulatory or inhibitory effects on smooth muscle cell growth. Indeed the same growth factor may have both stimulatory and inhibitory effects on cell growth depending on their concentration (Battegay et al., 1990). The mechanisms by which such factors alter cell cycle progression have yet to be elucidated, since the control of the cell cycle itself has only recently been understood. To determine the role reactive oxygen species may play in mediating growth factor-induced cell cycle control, further understanding of cell cycle control and its checkpoints are necessary. The main phases of the cell cycle have been classified as M phase, mitosis: G₁, the gap between mitosis and the onset of DNA replication; S phase, the period of DNA synthesis; and G₂, the gap between S and M phases. G₀ is considered to be a phase of quiescence between the M and G₁ phases, from which, cells can exit in the presence of competence and progression factors. Progression of eukaryotic cells through the cell cycle is governed by the sequential formation, activation, and subsequent inactivation of a series of cyclin/cyclin-dependent kinase (Cdk) complexes. These complexes are composed of a kinase subunit (Cdk), and an activating subunit, cyclin, in a complex that is subject to many levels of regulation. In mammalian cells, several Cdks exist; Cdk1 (Cdc2), Cdk2, Cdk3, Cdk4, and Cdk6, that are specialised for different transitions. Cyclins are required for kinase activity and contribute to substrate specificity. These various cyclins have been assigned roles in specific phases of the cell cycle. Thus, cyclin B in association with Cdc2
has been shown to promote G2/M transition (Pines and Hunter, 1989). The expression of cyclin A occurs at the onset of S phase (Figure 1.7), and associates with both Cdc2 and Cdk2 (Pines and Hunter, 1990; Dulic et al., 1992). Cyclins D and E are defined as G1 cyclins, as their expression occurs mainly in G1. The expression of cyclin E occurs with sudden onset in mid/late G1, in parallel with their appearance, cyclin E-Cdk2 complexes having strong kinase activity. In contrast, cyclin D shows rather constant expression through a cell cycle (Lew et al., 1991). The activation of these kinases is carefully controlled at multiple levels. Concentrations of the activating cyclin subunits are modulated both transcriptionally and through periodic ubiquitin-dependent proteolysis, and the kinase subunits are subject to both activating and inhibiting phosphorylation.

The first stage in initiating cell cycle progression is the synthesis of the D-type cyclins, (Figure 1.8) and their transcription is absolutely dependent on the presence of growth factors. This has led to the idea that D-type cyclin synthesis acts as a growth factor sensor, linking cell cycle progression to external cues. As cells enter the cycle from quiescence (G0), one or more D-type cyclins (D1, D2, and D3) are induced as part of the early response to growth factor stimulation, and both their synthesis and assembly with their catalytic partners (Cdks), depend on mitogenic stimulation (Sherr, 1994). D-type cyclins bind to several different Cdks, Cdk2, Cdk4, Cdk5 and Cdk6 of which the most important appear to be Cdk4 and Cdk6 (Matsushime et al., 1992; Bates et al., 1994). Only two substrates are known for D-type cyclin-Cdks, the E2F family of transcription factors, that are thought to regulate the cell-cycle dependent synthesis of proteins required for S phase such as DNA polymerase-α and thymidine kinase (La Thangue, 1994). The other substrate is the retinoblastoma tumour suppressor protein (Rb). Rb and Rb-like proteins (p130 and p107) are under-phosphorylated throughout G1 phase, phosphorylated at the G1/S transition, and remain phosphorylated until late mitosis (DeCaprio et al., 1992).

The cyclin E-Cdk2 complex is the next to appear in the mammalian cell cycle after the D-types, in late G1 phase, and is also a potent regulator of Rb. Cyclin E synthesis is regulated such that there is a burst of cyclin E transcription only in late G1 and early S phase. The cyclin E-Cdk2 complex is essential for the cell to begin DNA replication (Dulic et al., 1992). Cyclin E-Cdk2 is associated with E2F and p107 and p130. Once cells enter S phase cyclin E is degraded and Cdk2 forms complexes with cyclin A and E2F trans-activation
activity decreases. The onset of cyclin A synthesis late in G$_1$ is important for the G$_1$-S transition, because inhibition of cyclin A function in cultured cells can also inhibit S phase entry. Complexes of Cdc2 (Cdk1) with cyclins A and B become active in G$_2$ and are required for mitosis. Since Cdc2 is constitutively present throughout the cell cycle, it is the accumulation and periodic destruction of cyclin B that determines the pattern of mitosis.

![Diagram](attachment:image.png)

**Figure 1.7. Expression of cyclins E, A, B (mitotic cyclin), and p27 is periodic,** (Sherr, 1996). D-type cyclins are expressed throughout the cycle in response to mitogen stimulation (the period indicated by the top bar). The cyclins assemble with more stably expressed Cdk's to temporally regulate their activities. D-type cyclins form complexes with Cdk4 and Cdk6; cyclin E with Cdk2; cyclin A with Cdk2 (in S-phase) and Cdc2 (Cdk1) (in late S and G$_2$); and cyclin B with Cdc2. The holoenzymes can be negatively regulated by phosphorylation, so that even though cyclin B-Cdc2 complexes progressively assemble as B cyclins accumulate, their catalytic activity is restricted to mitosis. p27 levels are high in quiescent cells, fall in response to mitogenic stimulation, remain at lower threshold levels in proliferating cells, and increase again when mitogens are withdrawn. In proliferating cells, most p27 is complexed with cyclin D-Cdk4 complexes.
Figure 1.8. G₁ progression. D-type cyclins, Cdk4 (or Cdk6), p21<sup>Cip1</sup>, E2F and PCNA are induced as part of the delayed early response to mitogens in cells entering the cycle from quiescence, whereas p27<sup>KIP1</sup> levels fall. The cyclin D-Cdk4 complex titrates p27 and also enters into complexes with p21 and PCNA. Once the p27 threshold is exceeded, cyclin D-bound Cdk4 is phosphorylated by a Cdk-activating kinase (CAK) and becomes enzymatically active. The stoichiometry of p21 (and possibly p27) in the complexes determines the state of the enzyme. Cyclin E is expressed later in G₁ phase than the D cyclins and enters into quaternary complexes containing Cdk2. Activation of the G₁ Cdk2 triggers pRb phosphorylation and the release of pRb-bound E2F. Cyclin A is first synthesised near the G₁-S boundary and accumulates in complexes with Cdk2 as cyclin E undergoes periodic degradation in S-phase. Modified from Sherr, (1994).
1.3.1.2 Cell cycle checkpoints

Although cell cycle transition depends on the underlying CDK cycle, superimposed check point controls help ensure that certain processes are completed before others begin. Many checkpoint pathways have now been identified. Among these are checkpoints that co­ordinate cell size and cell cycle progression, inhibit mitosis while in $G_1$, restrict DNA replication to once per cell cycle, and make DNA synthesis dependant upon $G_1$ cyclins. One of the most studied checkpoint is the DNA damage control point, which induces a number of physiological responses thought to facilitate DNA repair processes. Among these responses are cell cycle arrest in $G_1$, S-phase, and $G_2$, a slowing of DNA replication, increased transcription of genes encoding proteins that participate in DNA replication and repair, and if necessary, induction of apoptosis (Hartwell and Weinert, 1989). Cell cycle arrest caused by damage or incompletion of earlier cell cycle events can be caused not by damage or incompletion per se, but by specific surveillance mechanisms that detect mistakes and induce inhibitors of key cell cycle transitions.

Cyclin D, E and A dependent kinases are negatively regulated by a distinct family of Cdk inhibitors including $p21^{cip}$, $p27^{kip}$, p15 and p16. $p21$ is a potent inhibitor of Cdk activities that are required for progression from the $G_1$ phase of the cell cycle into S phase. $p21$ binds to cyclin-Cdk complexes, including cyclin D-Cdk4 and cyclin E-Cdk2 and is thought to act as a Cdk-cyclin assembly factor. Cyclin-Cdk complexes containing a single $p21$ molecule are still catalytically active, those containing multiple $p21$ molecules are not, and changes in the stoichiometry of $p21$ are sufficient to account for the conversion (Zhang et al., 1994). $p21$ can also directly inhibit DNA synthesis by binding to and blocking PCNA function (Waga et al., 1994) without interfering with PCNA dependent nucleotide excision repair (Li et al., 1994). Transcription of the $p21$ gene is upregulated as cells become senescent or quiescent, and after serum stimulation of quiescent cells, and decreases as cells enter S phase (Noda et al., 1994). $p21$ is also induced following DNA damage as a consequence of the accumulation of $p53$, and DNA damage induced growth arrest is mediated primarily by $p53$ induction of $p21$ (El-Deiry et al., 1993). In addition, $p21$ is thought to be induced independently of $p53$ by as yet unknown mechanisms.
Another regulator of G₁ progression, p27\textsuperscript{kip}, exhibits a region of sequence identity to p21 and can also curb the activity of Cdk's, although p27 has a higher binding affinity with Cdk4-cyclin D than Cdk2-cyclin E complexes (Toyashima and Hunter, 1994). Like p21, the stoichiometry of p27 in cyclin-Cdk complexes may determine whether it acts as an inhibitor or not. However, p27 and p21 respond to different signals, and their levels change reciprocally as cells progress through G₁, in quiescent cells levels of p27 are high, but once cells enter the cell cycle, levels fall (Kato et al., 1994). Studies have shown that p27 mRNA levels do not differ between cycling and non-cycling cells, and suggests that a decline in levels observed upon entry into G₁ is the result of increased binding of p27 to Cdk4-cyclin D complexes which effectively titrates p27 and reduces the total amount of 'free' p27 to further inhibit Cdk2-cyclin E (Polyak et al., 1994). Again, the stoichiometry of p27 may determine whether Cdk activity is inhibited or not. When cells are deprived of serum, synthesis of p27 not only increases, but the inhibitor is released from Cdk4-cyclin D complexes as cyclin D is degraded. The loss of cyclin D-dependent kinase activity coupled with p27 mediated inhibition of cyclin E-Cdk2 induces arrest in G₀/G₁ within a single cycle. TGF-β also induces cell cycle arrest by inhibiting Cdk4 synthesis therefore raising the effective level of 'free' p27. Research indicates a role for hydrogen peroxide in mediating the effects of TGF-β-induced cell cycle inhibition (Shibanuma et al., 1991, 1992a). In addition, hydrogen peroxide release is observed after UV irradiation and is thought to play a role in mediating signalling responses including the activation of transcription factors NFκB, AP-1, and Egr-1 and activation of growth factor receptors in response to UV (Mohan and Meltz, 1994; Huang et al., 1996).

The ability of reactive oxygen metabolites to activate some of the major signalling pathways, used otherwise by growth factors to transduce signals from the cell membrane to the nucleus, may be useful to cells subjected to oxidative stress, providing a means by which adaptive responses such as increased antioxidant activities, repair processes or enhanced homeostatic mechanisms might be induced. Little is known about how these adaptive responses are mediated in eukaryotic cells. Information on adaptation to oxidative stress in bacteria supports the speculation that parallel responses may occur. In enteric bacteria, various transcription factors have been identified that control gene expression in response to an increased concentration of ROS (Farr and Kogoma, 1991). One such factor is oxyR, which mediates the expression of at least eight genes involved in protecting the
bacterial cells from oxidative damage in response to H$_2$O$_2$. A conformational change of oxyR upon direct oxidation of the protein appears to unleash its transcriptional activating potential.

1.3.2 Oxidative stress and NFkB activation

NFkB is a transcription factor that has attracted widespread interest among researchers due to its unusual nature including, the diverse nature of molecules capable of NFkB activation, the complexity of its subunits, its rapid regulation, its unusual control mechanisms, the wide range of genes that it controls, its pivotal role in immunological processes, and its seeming involvement with so many disease processes. In non-stimulated cells, NFkB resides in the cytoplasm in an inactive complex with an inhibitor protein called IkB. A diverse range of stimuli cause a release of IkB, allow NFkB to enter the nucleus and bind to DNA control elements, and activate transcription of genes encoding proteins involved with immune or inflammatory responses and with cell growth control.

Several lines of evidence have indicated that NFkB may play an important role in atherosclerosis (Witzum and Steinberg, 1991; Berliner et al., 1992; Collins, 1993). NFkB has been detected in many cells of the vessel wall including endothelial cells, smooth muscle cells, macrophages and T cells and has been shown to be activated in these cells in response to various inflammatory or proliferative stimuli found within the lesion area (Müller et al., 1993; Collins, 1993; Baueuerle and Henkel, 1994; Qwarnström et al., 1994). Recently the presence of activated NFkB has also been detected in human atherosclerotic lesions. Immunofluorescent and immunohistochemical techniques using a specific antibody to activated NFkB, demonstrated the presence of NFkB in the fibrotic thickened intima/media and atheromatous areas of the atherosclerotic lesion in both smooth muscle cells and macrophages. In addition activated NFkB was also identified in endothelial cells in less advanced lesions, suggesting a role for NFkB in the initiation of atherosclerosis (Brand et al., 1996). The fact that NFkB activation can occur by a wide variety of stimuli, including oxidative stress, raises the possibility that the diverse risk factors associated with the initiation of atherosclerosis, may be linked to a final common pathway of induced gene expression. The NFkB family of transcription factors have been implicated in the transactivation of a number of genes, which include those mediating control of cell growth,
expression of receptors, adhesion molecules, and components of the cytoskeleton. Furthermore, this family also regulates gene expression of a number of viruses, including CMV (Baeuerle, 1991), which have been implicated in the formation of plaques (Ross, 1993; Speir et al., 1994).

To date, five members of the mammalian NFκB/Rel family have been cloned and characterised (Figure 1.9). These are c-Rel, NFκB1 (p50/p105), NFκB2 (p52/p100), RelA (p65), and RelB (Baldwin, 1996). Members of the Rel family have in common a highly conserved region, the Rel homology domain (RHD), which functions in DNA binding, dimerisation, and interactions with IkB. Active forms of NFκB exist as heterodimeric complexes, classic NFκB (the dimer of p50 and RelA) has been intensively studied being the most abundant dimeric complex and existing in almost all cell types. Other dimeric combinations exist and although little is known of their role, they are thought to be cell type specific and have different gene targets.

Recent evidence confirms the existence of multiple forms of IkB that appear to regulate NFκB by distinct mechanisms. The known IkB proteins include IkB-α, IkB-β, Bcl-3, p105, p100, and the product of the Drosophila gene cactus. IkB-α, IkB-β, Bcl-3, and cactus are each the sole product of a dedicated gene, whereas p105 and p100 are full-length NFκB precursor proteins of p50 and p52 respectively, which have several of the activities ascribed to IkB proteins (Figure 1.9). Two other IkB proteins are known, IkB-γ contains sequences from the carboxy terminus of p105, which has so far only been detected in mouse lymphoid cells (Inoue et al., 1992). IkB-δ, which consists of the carboxy terminal sequences of p100, has been predicted to exist on the basis of analogy with IkB-γ, but has not yet been detected in cells.
Introduction

Chapter 1

Rel homology domain

- p65 (RelA)
- c-Rel
- RelB
- Dorsal
- Dif
- p105/p50 (NF-κB1)
- p100/p52 (NF-κB2)
- IκB-α
- IκB-β
- Bcl-3
- cactus

Figure 1.9. The NFκB/Rel and IκB families of proteins. The NFκB/Rel family is characterised by the presence of the Rel homology domain. The IκB proteins contain ankyrin repeats. NFκB1 and NFκB2 are proteins that contain both the Rel homology domain and the ankyrin repeats. Dorsal, Dif and cactus are Drosophila proteins. Taken from Baldwin, (1996).
IkB-α has been studied extensively, and cloning studies have revealed that IkB-α contains ankyrin repeats and exhibits homology with a protein encoded by the gene Bcl-3 (Baeuerle and Henkel, 1994; Siebenlist et al. 1994). Very little is known at present about IkB-β, however the 46 kD protein has recently been purified to homogeneity, and a cDNA clone derived (Thompson et al., 1995). Like IkB-α, IkB-β contains ankyrin repeats and is associated with NF-κB forms in the cytoplasm of various cells and preferentially binds/interacts with dimers that contain c-Rel or RelA. Where IkB-α is targeted by a signalling pathway initiated by TNF-α, IL-1β, LPS, and PMA, IkB-β reportedly is targeted only by pathways initiated by LPS or by IL-1β at least in the 70Z/3 pre-B cell line and the Jurkat T cell line (Thompson et al., 1995). It is not clear whether targeting of IkB-β occurs in all cell types.

The IkB interaction with NFκB subunits occurs via residues in the Rel homology domain, and presumed contact in or around the nuclear localisation sequence appears to play a critical role in inhibiting nuclear localisation of NFκB (Baeuerle and Henkel, 1994). In addition, data indicate that a single IkB protein targets the NFκB dimer (Ernst et al., 1995). Progress has been made toward identifying the mechanisms whereby NFκB is activated to allow its translocation to the nucleus in response to numerous stimuli (Figure 1.10). Within minutes after cellular stimulation, IkB-α is completely degraded, thus permitting the rapid nuclear import of NFκB (Beg and Baldwin, 1993; Gilmore and Morin, 1993). More recent studies have shown that destabilisation of IkB-α appears to involve signal-dependent phosphorylation at Ser-32 and Ser-36 (Brockman et al., 1995; Brown et al., 1995); phosphorylation at these sites leads to its subsequent ubiquitination, which renders IkB-α susceptible to breakdown by the 26S proteosome (Chen et al., 1995).

However, the biochemical mechanisms for coupling these modification steps remain unclear, and specific kinases responsible for signal-induced phosphorylation of IkB-α remain to be identified. Identification of similar serine residues in IkB-β suggest that activation mechanisms are similar for the two IkB proteins and that a kinase that is identical or very similar to the IkB-α kinase would target IkB-β (DiDonato et al., 1996). Similar mechanisms involving phosphorylation, ubiquitination and degradation are also responsible for controlling the processing of the NFκB precursor p105 (Palombella et al.,
1994; Orian et al., 1995) (Figure 1.10). Initial studies that demonstrate the correlation of \( \text{IkB-} \alpha \) loss with the appearance of nuclear NFkB also indicate that \( \text{IkB-} \alpha \) levels recover within an hour or so. Promoter analysis has identified the presence of multiple NFkB binding sites on the \( \text{IkB-} \alpha \) gene, so that activation of NFkB would induce further synthesis of \( \text{IkB-} \alpha \) (Baeuerle and Henkel, 1994; Siebenlist et al., 1994). These results indicate that NFkB and \( \text{IkB} \) are components of a mutual regulatory system in which the levels of one regulatory component control the activity or quantity of the other. The rapid accumulation of \( \text{IkB-} \alpha \) following its loss is apparently important in re-establishing cytoplasmic pools of NFkB/ \( \text{IkB} \) complexes. Additionally, this reaccumulation appears to repress the activity of NFkB activity following induction because resynthesised \( \text{IkB-} \alpha \) enters the nucleus, interacts with NFkB forms and inhibits DNA binding (Arenzana-Seisdedos et al., 1995).

A puzzling feature of NFkB is that its activation is triggered by a great variety of agents. A recent review listed over 60 agents including cytokines, mitogens, bacterial and viral products, chemical agents, and physical and oxidative stress which were found to activate NFkB in many cell types (Siebenlist et al., 1994). Recent studies have shown that low concentrations of hydrogen peroxide can also activate NFkB and that various antioxidant drugs inhibit the activation of NFkB by oxidative stress. More interestingly, most of the antioxidants also inhibited the activation of NFkB induced by various agents including IL-1\( \beta \), TNF-\( \alpha \), LPS, phorbol esters, viral proteins, and double stranded RNA as reviewed by Schreck et al., 1992. This suggests that although the inducers may have different effects on cells and use distinct signalling pathways within cells, they also funnel into a common pathway with regards to the activation of the NFkB/IkB complex in the cytoplasm. \( \text{IkB-} \alpha \) seems to be the major target for all of these signals, according to degradation studies. On the one hand, it is possible that all of these diverse signals converge on an effector molecule upstream of \( \text{IkB-} \alpha \) to induce activation, for example an \( \text{IkB-} \alpha \) kinase. Alternatively, \( \text{IkB-} \alpha \) may be the common target of multiple independent pathways. Little is known of the mechanisms involved in ROS mediated NFkB activation. NFkB is sensitive to redox variations in vitro; yet general oxidation of the transcription factor inactivates the complex in intact cells (Toledano and Leonard, 1991). This suggests that the factor is selectively modified by specific mechanisms and not by the general cellular redox potential, although the activating mechanisms themselves may be redox sensitive.
Figure 1.10. **Mechanisms of NFκB activation.** Following stimulation of the cell, IκB or a precursor protein (p105) becomes phosphorylated (step 1), by a presently unknown kinase. The IκB form becomes ubiquitinated (step 2) and degraded by the proteosome (step 3). In the case of p105, the degradation is partial (generating p50). NFκB then translocates to the nucleus (step 4) where it activates a variety of genes (step 5), including IκB-α and p50/p105 (step 6). Alternatively, phosphorylation of IκB is thought to induce its dissociation from NFκB (step A), which, due to the unstable nature of IκB leads to proteolysis (step B).
1.3.3 Oxidative stress and gene expression

A number of genes are now known to be regulated by NFκB including genes encoding for TNF-α (Barath et al., 1990), IL-1β (Hiscott et al., 1993), IL-6, IL-8 (DeForge et al., 1993), PDGF (Montisano et al., 1992), M-CSF (Rosenfeld et al., 1992; Clinton et al., 1992, GM-CSF, MCP-1 (Ylä-Herttuala et al., 1991), tissue factor (Mackman et al., 1991), adhesion molecules VCAM-1 (Neish et al., 1992)), ICAM-1 (Lo et al., 1993), and c-myc (Duyao et al., 1990). These gene products are thought to play important roles in the initiation and progression of atherosclerosis. They are released from various cells of the vessel wall and infiltrating leukocytes, stimulate SMC proliferation and migration, and attract further inflammatory cell infiltration. Several in vitro studies have demonstrated the presence of a number of growth factors and cytokines that can be released from cells under similar conditions to those found within the atherosclerotic plaque, and the resulting effects they have on cell adhesion, proliferation, and migration. Immunohistochemical studies have now confirmed the presence of such factors in vivo (Majesky et al., 1990, 1991; Moyer et al., 1991; Arbustini et al., 1991; Barath et al., 1991; Clinton et al., 1992). The recent detection of activated NFκB in the cells of human plaques, and evidence that antioxidants can inhibit or prevent further progression of atherosclerosis in animal models further supports the role of oxidative stress mediated gene expression in atherosclerosis.

Changes in gene expression by cells of the arterial intima include increased or decreased expression of those also expressed by the tunica media and genes that are expressed uniquely by the intima. Such differences in expression include proteins of varied function (reviewed by Schwartz et al., 1995). 1) Adhesion; integrins, ICAM, VCAM. 2) Matrix proteins; fibronectin, laminin, osteopontin. 3) Cytoskeletal proteins; smooth muscle cell actin, vinculin, calponin, cytokeratin 8, 18. 4) Growth factors; bFGF, PDGF-A, MCSF-1, GMCSF. 5) Cytokines; TNF-α, IL-1, IL-2, IL-6, IL-8.

Loss of expression is also evident for a number of genes associated with the ‘contractile’ phenotype of smooth muscle cells, when they switch to a ‘synthetic’ phenotype, including smooth muscle myosin, desmin, caldesmon, and α-actin (Glukhova et al., 1988; Kocher and Gabbiani, 1986). In addition, the production of various matrix-degrading enzymes is found to be induced in vulnerable regions of lesions (Galis et al., 1994). The over-
expression or loss of expression of certain molecules within the lesion may simply reflect the phenotypic modulation of resident cells. More importantly, plaque cells may show over-expression or repression of certain genes as a result of specific mediators present in the plaque environment. Particularly important among such mediators are oxidation products or more traditional inflammatory mediators (Steinberg, 1993; Libby and Hanson, 1991). It has been suggested that NFκB is the common factor linking inflammation and oxidation (Collins, 1993), but more recently it is evident that a common effector exists upstream of NFκB activation and is thought to involve the intracellular production of free radicals (Baeuerle and Henkel, 1994).

1.3.3.1 Identifying gene expression changes

Changes in gene expression define developmental as well as pathological aspects of cells. Of the 100,000 or so genes carried in mammalian cells, only a small fraction of these (10-15%) is expressed in any individual cell. Identifying the set of active genes involved in any stage of development or pathophysiology would provide important information for understanding the mechanisms of disease and provide potential targets for therapeutic intervention. The development of a technique to identify and isolate genes that are differentially expressed in various cells, or in cells under different conditions, has been the aim of many molecular biologists for the past 20 years. To be of practical use, such a technique needs to satisfy several criteria.

1) It should be possible to analyse most or all the 15,000 mRNA species in a cell including rare mRNA species
2) The reproducibility of this technique should be good
3) Ideally, the method should allow comparisons of mRNA from different cells or between cells of altered states
4) The technique should allow the identified mRNAs to be used for subsequent identification and isolation of the corresponding genes, mRNAs or cDNAs
5) The method ideally should be fast, cheap, and easy
Until 1992, there were only two methods available for the identification of differentially expressed genes. Subtractive hybridisation (Zimmerman et al., 1980) or differential hybridisation (St. John and Davis, 1979), requires the construction of a cDNA library from a study tissue (for example tumour tissue), which is subsequently hybridised with mRNA from a control tissue, to leave only those cDNA species unique to the study tissue. Although this technique has been used successfully in the identification of a number of important genes, it is a time consuming procedure, technically difficult to reproduce, and has a requirement for a large amount of cells or tissue to generate sufficient mRNA (Lee et al., 1991a). This last prerequisite makes this technique difficult when tissue is scarce. Additionally, the method allows only the qualitative detection of differentially expressed genes and therefore gives no quantitative indication of differences in expression. Secondly, nuclear run-on transcription is applied to analyse changes in the level of expression of genes (Strauss et al., 1990). However, this method can only be applied to detecting changes in expression of known genes.

**1.3.3.2 Differential Display**

Differential display was first described by Liang and Pardee (1992), and is based upon the assumption that most cells express some 15,000 genes. In principle, every individual mRNA molecule is capable of being reverse transcribed and amplified by the polymerase chain reaction (PCR) provided suitable primers are available. The technique uses sets of random primers to create cDNA generated by reverse transcription (RT) of mRNA, which can be further amplified by PCR. Pairs of primers are selected so that each will amplify DNA from about 50 to 100 mRNAs because this number is optimal for display on a sequencing gel. Selection of 3’ primers takes advantage of the polyadenylated (polyA) tail present on most eukaryotic mRNAs (Jackson and Standart, 1990) to anchor the primer at the 3’ end of the mRNA, plus two additional 3’ bases. A primer such as 5’-TnCA would allow anchored annealing to mRNA containing TG bases located just upstream of their polyA tails (Khan et al., 1991). By probability, this primer will recognise one twelfth of the total mRNA population because there are 12 combinations of the last 3’ bases. In each of the 12 primer fractions, one would expect approximately 1250 individual mRNA species to be present. However, the average number of bands that can be visualised on a polyacrylamide gel is 50 to 100 amplified mRNA species, which is optimal for the reaction conditions chosen. As a consequence more primer combinations are needed to cover the
total mRNA population. Recently, it has been calculated that at least 300 primer combinations are necessary to display all the possible cDNAs as bands on polyacrylamide gels (Bauer et al., 1993).

Differential display fits most of the criteria described above for the successful isolation and identification of differentially expressed genes. The method allows numerous side by side comparisons of RNA from different samples, and the visualised RNAs can be isolated relatively easily for further analysis. However, the technique is not completely reproducible, the method has a tendency to identify high copy number mRNA species, and although differential display allows a rapid identification of differentially expressed genes, the subsequent analysis is labour intensive and expensive. Nevertheless, as differential display becomes more widely used, the methodology and the success rate will undoubtedly improve.
1.4 OBJECTIVES

Alterations in vascular SMC function are thought to play a key role in human atherogenesis. It is possible that exposure to oxidative stress is responsible in part for initiating these changes. This hypothesis was tested in several ways.

1. Firstly, the effect of oxidative stress on smooth muscle cell proliferation was investigated since data regarding proliferative effects of oxidative stress are not conclusive and contradictory observations have been reported. In particular the effects of oxidative stress on cell cycle parameters have been assessed. Oxidative stress may cause DNA damage or stimulate cell growth, hence studies were carried out to elucidate a possible effect of oxidative stress in modulating cell cycle progression.

2. Secondly, reactive oxygen species are increasingly thought to act as second messenger molecules, and their presence is required in the activation of certain transcription factors. The transcription factor NFκB in particular is thought to play an important role in mediating oxidative stress effects. Therefore, the effects of oxidative stress on activation of NFκB in smooth muscle cells were investigated, and attempts were made to determine the mechanisms that lead to its activation.

3. Finally, the ultimate role of transcription factor activation is to induce expression of genes. NFκB is involved in the induction of a large number of genes, and especially those involved in mediating inflammatory events. Using the relatively new technique of differential display, studies were carried out to identify changes in gene expression in smooth muscle cells subjected to oxidative stress to identify novel oxidative stress-specific genes. Such genes may provide new insight into the mechanisms of oxidative stress-associated disease processes.
Chapter 2

METHODS
2.1 CELL CHARACTERISATION

2.1.1 Cell Culture

Human umbilical artery smooth muscle cells were dissected from 1-2 day old umbilical cords. The adventitia was carefully removed and 2 mm$^2$ sections were cut from the dissected artery. The explants were then placed in 25 cm$^2$ tissue culture flasks, with 5 ml of Waymouths/Hams-F12 (without phenol red) medium containing 16.6% (v/v) FCS, 2 mM Glutamine, 50 μg/ml penicillin/streptomycin (5000 U/ml, 5 mg/ml) at 37°C in a 7% CO$_2$/93% air environment. Bovine aortic smooth muscle cells were isolated from freshly dissected bovine aorta. The aorta was opened by longitudinal dissection and the endothelium was carefully removed by scraping the luminal surface with a scalpel blade and rinsing 4 times with Hanks Buffered Salt Solution (HBSS). 2 mm$^2$ sections were cut from the opened aorta. To minimise contamination with adventitial tissue and therefore fibroblast growth, care was taken not to cut the sections deeper than 1.5 mm. Sections were cultured in Dulbecco’s Modified Essential Medium (DMEM) (without phenol red) containing 16.6% (v/v) FCS, 2 mM Glutamine, 50 μg/ml penicillin/streptomycin. Smooth muscle cell growth was distinguished by its “hills and valley” growth pattern and α-actin staining. Rat aortic smooth muscle cells were kindly provided by Elizabeth Wood (William Harvey Research Institute, London) and cultured in DMEM as described above. Bovine aortic endothelial cells were obtained by collagenase digestion. The cells were then cloned to remove contaminating fibroblasts or smooth muscle cells, and grown in DMEM as above. Cells were used for experimental analysis between passages 4 and 10.

2.1.2 Thymidine Incorporation Assay

Cells were plated at $1 \times 10^4$ cells/ml on to 24 well plates in Waymouths/Hams-F12 medium containing 16.6% (v/v) FCS. To allow cells to become synchronous, cells were then washed to remove excess serum then grown in medium containing 0.1% (v/v) FCS. After 48 h, cells were treated with various concentrations of FCS or H$_2$O$_2$. At various time points after treatment cells were pulse labelled with H$^3$-thymidine (1 mCi/ml) for 2 h. The medium was then removed and plates washed twice with ice cold HBSS before storing at -20°C. For the assay, plates were thawed at room temperature and washed twice in ice cold HBSS, twice with ice cold 5% (w/v) TCA, rinsed once with ethanol and allowed to air dry.
Proteins were either harvested onto filters or dissolved in 0.3N NaOH solution for 1 h at 37°C, before being added to scintillation fluid. Plates were counted on either a 2500 TR liquid scintillation analyser (Packard) or a Topcount Microplate reader (Packard).

2.1.3 Hoechst 33258 fluorescence Assay

This assay can be used as an alternative method for studying DNA synthesis and therefore cell growth. The assay involves the measurement of fluorescence at 460 nm following excitation at 360 nm that occurs when the dye bis-benzimide (Hoechst 33258) binds to adenine-thymine (A-T) rich sequences of DNA. This method has the advantage over [³H]-thymidine incorporation in that DNA levels can be measured in quiescent cells and proliferating cells. This allows a distinction to be made between dead and quiescent cells, whereas measuring [³H]-thymidine incorporation levels does not. Data can be presented as DNA content (ng/well) using a standard curve of known amounts of DNA against fluorescence (Figure 2.1a). Data can also be presented as cell number per well by making a calibration curve of known cell number against fluorescent intensity/DNA content (Figures 2.2a and 2.2b). Standard curves show that the assay is linear between 100 and 10,000 cells per well. Cell numbers are often underestimated due to plating efficiencies and growth conditions. Cell lysates are a more accurate measurement of cell number than those plated for the purpose of standard curves. 96 well plates were washed twice with Dulbecco’s PBS-A and stored frozen. The plates were thawed and air dried. To solubilise the cells 50 µl of 0.02% (w/v) SDS in 1 x SSC was added to each well. The plates were incubated at 37°C for 20 min and then 50 ml of 1 x SSC was added to each well followed by 100 ml of Hoechst 33258 dye (1 µg/ml). Plates were incubated for 20 min to allow binding of Hoechst dye to the DNA. Following excitation at 355 nm, fluorescence emission was read at 460 nm using a cytofluor 2350 plate reader (Millipore). DNA standards were prepared from a 1 mg/ml stock of salmon sperm DNA using serial doubling dilutions to give a final concentration of 15.6 ng/well, 31.2 ng/well, 62.5 ng/well, 125 ng/well, 250 ng/well and 500 ng/well.
Figure 2.1. Standard curves of (A) DNA concentration against fluorescence units and (B) protein concentration against OD_{620} units. DNA standards were serially diluted from a 1 mg/ml stock of salmon sperm DNA to give final concentrations of 15.625 ng/well, 31.25 ng/well, 62.5 ng/well, 125 ng/well, 250 ng/well and 500 ng/well. Protein standards were serially diluted from a 1 mg/ml stock of BSA solution to give final concentrations of 1 mg/ml, 500 μg/ml, 250 μg/ml, 125 μg/ml, 62.5 μg/ml, 31.2 μg/ml, and 16.6 μg/ml.
Figure 2.2. Comparison of standard curves of DNA content against cell number (a) and cell lysates (b). (a) HASMCs were plated at various dilutions onto 96 well culture plates and grown for 24 h before being assayed with Hoechst 33258. (b) Cells were added onto 96 well culture plates at various dilutions and assayed immediately in 0.02% (w/v) SDS, 1 x SSC under the same conditions.
2.1.4 Cell Cycle analysis using flow cytometry

Flow cytometry was used to assess the cell cycle characteristics of HASMC before and after treatment with FCS or H$_2$O$_2$. Cells can be analysed with respect to their DNA content and size. Using only a single DNA specific fluorochrome to measure DNA content does not allow the discrimination between cells in G$_0$ and G$_1$ or G$_2$ and M, which contain equivalent amounts of DNA. The cell population that falls between the two peaks is calculated to be those cells in S-phase. Various software packages are available to estimate the number of cells in S-phase using curve fitting models. Human aortic smooth muscle cells (HASMC) were cultured in six well plates at a concentration of $5 \times 10^4$ cells per well and incubated with the appropriate treatments. Cells were trypsinised with 0.25% (w/v) trypsin, 0.05% (w/v) EDTA and centrifuged for 1 min at 14,000 rpm to produce a pellet. The pellet was resuspended in 70% ethanol in PBS and left for 30 min at 4°C, washed once with PBS then finally resuspended in 1 ml PBS. 2 ml of RNase A (50 mg/ml) was added to the cell suspension followed by 10 ml of propidium iodide (400 ng/ml). Cells were mixed and incubated for 15 minutes at 37°C, then washed again by centrifugation with PBS, and again resuspended in 1 ml PBS. Cell aggregates, observed under the microscope were broken up by drawing the cell suspension through a 25 gauge needle and filtered through cell sieves < 100 μm (Falcon). Flow cytometry analysis was carried out on the FACScan flow cytometer (Becton Dickinson) using LYSIS II Version 1.1 software and was set up for FL2 detection with DDM (doublet discrimination mode) to distinguish between single cells and cell aggregates. After acquiring cell information, cell cycle analysis was carried out using the Cell-FIT software, version 2.01.2 (Becton Dickinson).

2.1.5 BIORAD protein assay

A 5-fold dilution of Bradford reagent (Biorad) was made in dH$_2$O. 980 μl of the diluted reagent was dispensed into a cuvette containing 20 μl of sample. After 10 min incubation at room temperature, the OD$_{595}$ was taken and the protein concentration determined from a standard curve prepared from a 1 mg/ml stock of BSA.
2.1.6  Lowry assay

Protein concentration was measured by the method of Lowry et al., (1951). This method was adapted for use on a 96 well microtitre plate. The assay measures the total protein content within each well and can therefore be used to measure cell death. Any dead cells will be non-adherent and will be removed after washing. The cells were lysed and total protein levels calculated. The following solutions were prepared:

Solution A -  
(i) 2% (w/v) Na₂CO₃ in 0.4% (w/v) NaOH  
(ii) 1 % (w/v) CuSO₄  
(iii) 2% (w/v) Potassium sodium tartrate

Solution A was prepared according to the following ratio - 50:1:1 (i):(ii):(iii)

Solution B -  Folin's reagent : dH₂O (1:7)

Supernatant from cells were removed, and each well washed once with PBS. Cells were lysed with 40 μl of 0.3M NaOH which was added to each well containing cells for 30 min at 37°C then for a further 30 min at room temp. 150 μl of solution A was added to each well and incubated for 15 min at room temp. 100 μl of solution B was then added to each well and incubated at room temp for 30 min. 96 well plates were read in a Thermomax microplate reader (Molecular Devices) at 620 nm. Protein concentrations were calculated using a standard curve of OD₆₂₀ against protein standards (made from a 1 mg/ml stock of BSA) (Figure 2.1b).

2.1.7  MTT assay

A tetrazolium salt has been used to develop a quantitative colourimetric assay for cell survival and proliferation (Mosmann, 1983). The assay differs from the above in that it detects living but not dead cells and the signal generated is dependent on the degree of activation of the cells. This assay measures dehydrogenase enzyme activity (Slater et al., 1963). The tetrazolium ring is cleaved in active mitochondria, and so the reaction occurs only in living cells. MTT (3-(4,5-dimethyliazol-2-yl)-2,5-diphenyl tetrazolium bromide) was dissolved in PBS (5 mg/ml) and filter sterilised. After the appropriate treatments, cells were incubated with 10 μl of the MTT stock solution (in 0.1 ml of growth medium (w/o phenol red)). After 4 h at 37°C for MTT cleavage, the formazan product was solubilised by
the addition of 100 μl of 0.04 N HCl in isopropanol. The plate was then read at 570 nm (ref. 630 nm). Viability was assessed as percentage change from control.

2.1.8 Hydrogen peroxide concentration determination

Hydrogen peroxide was always made up from a fresh 30% (v/v) (8.8 M) stock solution and diluted appropriately. The peroxide content was verified by titration with potassium permanganate (Bernt et al., 1965).

2.2 LDL PREPARATION

2.2.1 LDL isolation

A fast and small-scale method for isolating LDL from human plasma was employed based on that of Chung et al. (1986). 570 μl of plasma was added to Eppendorf tubes containing 186 mg KBr. The samples were then transferred to ultracentrifuge tubes (Beckman). The tubes were then carefully layered with 1.35 ml of 0.9% (w/v) NaCl solution containing EDTA (1 mg/ml) (density 1.006) using a 1 ml syringe to avoid air bubbles. Each tube was heat sealed and centrifuged in a TL100 benchtop ultracentrifuge (Beckman) using a TV100 rotor, with the following settings, time-30 min; speed-100,000 rpm; temp-7°C; acceleration-5; deceleration-5. After ultracentrifugation, the crude LDL band was removed from each tube. A hole was pierced in the centre of each band using a 21’ gauge needle attached to a 1 ml syringe and approximately 0.66 ml was withdrawn.

A second isolation was required to further purify the LDL fraction from albumin. 570 μl of crude LDL was added to Eppendorf tubes containing 186 mg KBr, and dissolved (final density 1.21 g/ml). All samples were ultracentrifuged as previously described. After centrifugation, each LDL band was removed from the tube using a syringe as previously described.

LDL was desalted by either dialysis or a PD10 gel exclusion column. The column was firstly equilibrated with 25 ml of 0.01 M PBS. Duplicate LDL fractions were combined and made up to a final volume of 3 ml in 0.01 M PBS. LDL was applied to the column, followed by a wash with 1 ml PBS, this fraction was discarded. The LDL fraction was
eluted with 2.5 ml of 0.01 M PBS, collecting into a 5 ml tube. Dialysis resulted in a less dilute sample, but took considerably longer. Dialysis tubing was boiled in 3 washes of dH₂O. One end of the tubing was sealed with plastic sealers. The LDL fraction was transferred to the dialysis tubing and the other end sealed. The dialysis tubing was then immersed in 10 mM PBS (>100 ml buffer per 1 ml sample) and left at 4°C on a magnetic stirrer. The buffer was changed twice over the course of 36 h. Using a glass Pasteur pipette, the top of the dialysis tube was pierced and the dialysed LDL transferred to an Eppendorf tube. LDL was stored at 4°C under nitrogen.

The protein content of the isolated LDL was determined by the method of Lowry as described previously. The purification of the LDL fraction was monitored by agarose gel electrophoresis, carried out using a Corning electrophoresis unit (Corning Medical). 1 μl samples of serum, crude LDL and purified LDL were applied to the cathode side of 10 x 12 cm universal agarose gels (Corning Medical), and subjected to electrophoresis in 0.05 M sodium barbital buffer (pH 6.8), for 35 min with a 90V direct current potential. Lipoproteins were stained for 5 min in 0.3% (w/v) Fat Red B, and the gel was de-stained overnight with 50% (v/v) methanol.

### 2.2.2 LDL modification

After dialysis, LDL protein concentration was reassessed using the Lowry assay as previously described. To obtain a minimally modified form of LDL, 1 mg/ml LDL was incubated with varying concentrations of Cu²⁺SO₄ (5-20 μM) for different times at 37°C. Modification was assessed by measuring the formation of conjugated dienes at 234 nm on a spectrophotometer every 5 min. After equilibration of the sample, the reaction was stopped when the OD₂₃₄ reached 0.1, which was usually when conjugated dienes were beginning to form (beginning of the propagation phase). The reaction was stopped using 1 mM EDTA. Samples were stored at 4°C under nitrogen. Modification was also assessed using agarose gel electrophoresis as described previously. Using samples of native (unmodified), minimally oxidised and fully oxidised LDL, electrophoretic mobility was compared. Minimally modified LDL migrates at the same rate as native LDL, whereas fully modified LDL, which has a more negative overall charge moves further towards the anode.
2.3 WESTERN BLOT ANALYSIS

2.3.1 Preparation of whole cell lysates

Cultured cells were grown in 6 well plates at a concentration of $5 \times 10^4$ cells per well. After appropriate treatments, the supernatant was removed and the plates were washed twice with ice cold HBSS. Plates were placed on ice and 1 ml of 1 x SDS sample buffer (see appendix II) was added to each well. Cells were scraped off the plate with a cell scraper and the cell suspension transferred into 1.5 ml Eppendorfs which were immediately frozen at -20°C until required. After thawing, the protein samples were briefly sonicated on ice (Branson sonifier 250) to shear DNA. Immediately prior to use, the samples were heat treated for 5 min at 100°C then placed on ice for a further 5 min. The protein samples were microfuged for 1 min at 14,000 rpm to pellet any insoluble material. The supernatant was then transferred into clean Eppendorf tubes.

2.3.2 SDS-PAGE analysis of proteins

SDS-PAGE was carried out using a discontinuous buffer system (Ornstein,) in which the buffer in the reservoirs is of a different pH and ionic strength from the buffer used to cast the gel. The gel was made up as two layers, an upper stacking gel and a lower resolving gel. The stacking gel is at a lower percentage of acrylamide than the resolving gel and at pH 6.8, whereas the resolving gel and running buffer are at pH 8.8. This ensured that the proteins ran through the stacking gel as tight bands and were only separated when they began to migrate through the higher pH of the resolving gel. The percentage of acrylamide used depends on the size range of the proteins of interest.

Generally, 7.5% (w/v) resolving and 4% (w/v) stacking gels were used. The resolving gel was mixed from a stock solution of 30% (w/v) bis-acrylamide (acrylamide 37.5:1) containing a final concentration of 375 mM Tris-base (pH 8.8) and 0.1% (w/v) SDS. Polymerisation was initiated by the addition of 100 µl of freshly prepared 10% (w/v) ammonium persulphate and 20 µl tetramethylethylenediamine (TEMED). Approximately 4.5 ml of the acrylamide solution was poured between the glass plates and immediately overlaid with 50:50 water:methanol to prevent oxygen from diffusing into the gel and inhibiting polymerisation and to ensure a flat gel surface. When the gel was set, the
methanol was poured off, the gel was rinsed well with water and excess water removed with filter paper.

The stacking gel was prepared in the same way but with Tris-base pH 6.8 instead of pH 8.8. The stacking gel was layered over the resolving gel and the 15 tooth Teflon comb (1 mm) inserted. Following polymerisation, the comb was removed and the gels inserted into the electrophoresis tanks which were filled with Tris-glycine electrophoresis buffer (25 mM Tris base, 250 mM glycine (pH 8.3), 0.1% (w/v) SDS. Samples and molecular markers were added to a volume of 20 μL and the gels were typically run at 20 mA per gel, depending on the size of the protein. After electrophoresis, the gel was either immunoblotted as described below, or stained by soaking in Coomassie blue stain overnight (50% (v/v) methanol, 10% (v/v) acetic acid, 0.25% (w/v) Coomassie blue) followed by destaining in 20% (v/v) methanol, 7% (v/v) acetic acid. The gel was then soaked in 0.5% (v/v) glycerol in destaining solution for 10 min prior to drying using the Easy Breeze gel dryer (Hoeffer Scientific Instruments).
2.3.3 Transfer-Hybridisation of proteins

Proteins from the acrylamide gel were blotted onto nitrocellulose film. The gel was immersed in transfer buffer (20% (v/v) methanol, 40 mM glycine, 50 mM Tris-base) and covered with a piece of Hybond ECL Nitrocellulose membrane pre-soaked in transfer buffer. The gel/nitrocellulose was sandwiched between several layers of postslip filter paper pre-soaked in transfer buffer, two porous pads and two plastic supports (Figure 2.3). The entire construction was then immersed in an electrophoresis tank (Hoeffer scientific instruments) equipped with standard platinum electrodes, that contained Tris-glycine buffer (transfer buffer) at pH 8.3. The nitrocellulose filter was placed toward the anode. The proteins were transferred from the gel onto the nitrocellulose membrane by applying a constant current of 80 mA overnight. The gel was stained as before, to confirm that the transfer was complete. Proteins were then detected with specific antisera. Figure 2.4a shows a Coomassie blue staining of proteins isolated from human SMC, and the efficiency of transfer of proteins onto nitrocellulose filter. Some high molecular weight proteins remained on the gel (>200 kDa) after transfer (Figure 2.4b), subsequent experiments were not affected since proteins investigated were no larger than 65 kDa.

![Figure 2.3. Electrophoresis apparatus for transfer of proteins from a polyacrylamide gel to a nitrocellulose filter.](image-url)
Figure 2.4. SDS PAGE gels showing Coomassie Blue staining of proteins isolated from Human SMC before (A) and after (B) transfer onto nitrocellulose filter.
2.3.4 Immunoblotting with specific antisera

All incubations were carried out at room temperature. All antibody incubations were performed in 100 mm$^2$ square petri-dishes with slow agitation on an orbital shaker (Sorvall). The membrane was washed with PBS-T (0.05% (v/v) Tween-20 in PBS) for 5 min, then blocked with 10 ml blocking solution (5% (w/v) milk solid in PBS-T) for 30 min. After blocking, the membrane was washed twice in PBS for 5 min each, and 10 ml primary antibody was added at the appropriate dilution for each particular antibody (see below) diluted in 0.5% (w/v) milk solid in PBS-T. Membranes were incubated for 1 h with the primary antibody then washed three times in PBS before addition of the relevant secondary antibody conjugated to HRP. The secondary antibody was diluted to the appropriate concentration in 0.5% (w/v) milk solid in PBS-T. 10 ml of the secondary antibody was added to the membrane and incubated for a further hour. After incubation of the secondary antibody, the membrane was washed three times with PBS-T. Antibody binding was detected using an ECL detection system (Amersham), which involved mixing equal amounts of reagents 1 and 2 and incubating with the membrane for 1 min. Excess ECL reagent was removed, the membrane was wrapped in Saran Wrap and exposed to autoradiographic hyperfilm ECL (Amersham) for 1 - 5 min, then developed in an automatic Curix 60 developer (AGFA).

All antibodies were for human proteins and were diluted appropriately for western analysis.

<table>
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<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Species</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
<th>Species</th>
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<td>Anti-rabbit HRP</td>
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<tr>
<td>Anti-IkB-β IgG</td>
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<tr>
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<td>Anti-mouse HRP</td>
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<tr>
<td>Anti NF-κB p65 sub-unit</td>
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<td>goat</td>
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2.4 IMMUNOFLUORESCENT STAINING OF CELLS

HASMC were cultured on 8 well chamber slides (Nunclon) on either plastic or fibronectin coated glass at a concentration of $1 \times 10^4$ cells per chamber. Cells were incubated with appropriate treatments and then fixed with 50:50 methanol:acetone at -20°C for 10 min. Slides were air dried for 5 min, rinsed in PBS and were either stored in PBS at 4°C, or used immediately for antibody staining. Slides were initially blocked to reduce non-specific binding using 1:30 dilution in PBS-A of whole serum from the same species as the secondary antibody was derived. Slides were then rinsed three times with PBS. Generally, 100 ml of primary antibody was added to each well at a dilution of 1:50 or 1:100. Slides were incubated for 1 h at 37°C or overnight at 4°C then washed three times with PBS. Secondary antibody was added at an appropriate dilution for 1 h at 37°C in the dark, then washed three times with PBS. For nuclear staining, slides were incubated with 1:1000 dilution of propidium iodide (PI) (10 mg/ml stock) for 5 min then rinsed three times with PBS. Slides were mounted with coverslips in Vectamount with antifade and sealed using rubber cement. Slides were stored at 4°C in the dark. Fluorescence was detected using a fluorescent microscope (Axioplan microscope, Zeiss) at 494 nm (excitation) and 518 nm (emission) for FITC and 536 nm (excitation) and 617 nm (emission) for PI.

<table>
<thead>
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<th>Species</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
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2.5 DIFFERENTIAL DISPLAY

2.5.1 RNA preparation

Isolation of RNA

Total RNA is preferred over poly (A) RNA because of its cleaner background signal, ease of purification and integrity verification. To obtain good preparations of RNA it is necessary to minimise the activity of RNases from either cellular sources after lysis, or those introduced from laboratory equipment and solutions. A number of precautions were therefore routinely taken. All glassware and plasticware were autoclaved for 15 min at 15 p.s.i. on liquid cycle or baked at 180°C for at least 8 h. Electrophoresis tanks were cleaned with detergent solution, rinsed in water, dried with ethanol, and filled with either 3% (v/v) H$_2$O$_2$ or RNase away™ for 10 min or overnight respectively, then finally rinsed thoroughly in diethyl pyrocarbonate (DEPC) treated dH$_2$O. All solutions were prepared using RNase free glassware and DEPC treated dH$_2$O and wherever possible were autoclaved for 15 min as described previously. All reactions, unless stated, were carried out on ice to minimise RNase activity.

The RNAzol™ B method of RNA isolation is a modification of the single step procedure of Chomaczynski and Sacchi (1987), which uses guanidium thiocyanate to lyse the cells. The properties of RNAzol™ B promote formation of complexes of RNA with guanidium and water molecules, and abolish hydrophilic interactions of DNA and proteins, which allows their efficient removal from the aqueous phase leaving only RNA remaining.

Cells were plated at $1 \times 10^6$ cells per T175 cm$^2$ fibronectin-coated culture flask and grown to confluency. Cells were incubated with the appropriate treatments and then rinsed twice with cold HBSS. Flasks were kept on ice and cells were lysed directly in the culture flask by adding 4 ml RNAzol™ B. The lysed cells were scraped and passed through a 21 gauge needle 4-5 times to homogenise the mixture. To obtain greater yields, the homogenate was divided between 2 ml Eppendorf tubes instead of glass corex tubes, which reduced the volume wastage when removing the aqueous phase and allowed better visibility of RNA pellets. 0.2 ml of chloroform was added to each 2 ml of homogenate, mixed by shaking and left on ice for 5 min. The tubes were centrifuged at 4°C in an Eppendorf 5402 centrifuge at 12,000 rpm (10,000 g) for 15 min. The upper aqueous layer was carefully removed and
transferred to clean 2 ml tubes. An equal amount of isopropanol was added to each tube (1 ml), mixed, and left on ice for 15 min to precipitate RNA. Tubes were centrifuged for 15 min at 12,000 rpm, 4°C to pellet the RNA. The resulting supernatant was removed and pellet washed with 75% (v/v) EtOH by vortexing and subsequent centrifugation at 8,000 rpm (7,500g) for 8 min. The RNA pellet was air dried briefly and resuspended in 100 µl of 0.5% (w/v) SDS in DEPC H₂O. An incubation at 60°C for 15 min was sometimes required to dissolve RNA. The RNA concentration was determined from the OD₂₆₀ using the formula:

\[
[ \text{[RNA]} \, (\mu\text{g/\mu l}) = \text{OD}_260 \times 40 \times 100 \text{ (dilution)} + 1000 ]
\]

so that a solution whose OD₂₆₀ equals 1 contains approximately 40 µg of RNA per ml.

The final preparation should be free of DNA and proteins, and have a 260/280 ratio of greater than 1.9. RNA integrity was confirmed by running 1 µl of sample on a 1% (w/v) agarose gel and staining with ethidium bromide. Two major bands should be clear which correspond to the 18S and 28S subunits of rRNA. Smearing of the bands was taken as an indication of RNA degradation.

**DNAse treatment of RNA**

Success of the Differential Display technique depends on the integrity of the RNA and on it being free of chromosomal DNA contamination (discussed in Chapter 6). Total RNA was cleared of any DNA contamination using the message clean kit (Genhunter). 50 µl of RNA (1 µg/µl) was mixed with 5.5 µl of 10 x buffer and 1 µl of DNAs1 and incubated at 37°C for 30 min. 40 µl of 3:1 phenol:chloroform was added, mixed and left for 10 min on ice. The sample was centrifuged for 5 min at 14,000 rpm and the supernatant collected and transferred to a clean tube. The RNA was precipitated with 5 µl 3M Sodium acetate (pH 5.4) and 200 µl 95% (v/v) EtOH and left at -70°C for at least 1 h. The RNA was then pelleted and washed with 70% (v/v) EtOH. Finally, the pellet was air dried and resuspended in 25 µl of DEPC dH₂O. The RNA concentration and integrity was confirmed by spectrophotometry and agarose gel electrophoresis. Yield was usually 50% of the original starting material.
Methods Chapter 2

Reverse transcription of mRNA

Reverse transcription of the mRNA’s were carried out using the RNAmap™ and RNAimage™ kits (Genhunter). The following components were thawed and set on ice. Three reverse transcription reactions were set up (20 µl final volume) for each RNA sample in 0.5 ml Eppendorf tubes, and each contained one of three different one-base-anchored H-TnM primers (where M may be G, A or C).

9.4 µl dH₂O  
4.0 µl 5 × RT buffer  
1.6 µl dNTP (250 mM)  
2.0 µl Total RNA (DNA-free) (0.1 mg/ml, freshly diluted)  
2.0 µl H-TnM (2 mM)

The reaction tubes were incubated in a thermocycler (Omnigene, Hybaid) which was programmed to 65°C, 5 min → 37°C, 60 min → 75°C, 5 min → 4°C. After 10 min at 37°C, 1 µl MMLV reverse transcriptase was added to each tube, mixed quickly by flicking and incubation continued. At the end of the reverse transcription, the tubes were spun briefly to collect condensation. Tubes were stored on ice for PCR or were stored at -20°C for later use.

2.5.2 PCR

The following components from the RNAmap™ or RNAimage™ kits were thawed and then set on ice. All PCR reactions were set up on ice to a final volume of 20 µl.

10.0 µl dH₂O  
2.0 µl 10 × PCR buffer  
1.6 µl dNTP (25 µM)  
2.0 µl H-AP primer (2 µM)  
2.0 µl H-TnM (2 µM)  
2.0 µl RT-mix from step I  
0.2 µl α[³²P]dATP (3000 Ci/mmole)  
0.2 µl AmpliTaq™ (Perkin-Elmer)

The reactions were mixed well by pipetting up and down and placed in a Thermowell thin walled polycarbonate 96 microwell plate (Costar). 25 µl of mineral oil was overlaid to prevent evaporation of the reaction mixture. The thermocycler was programmed to run with block control at:

<table>
<thead>
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<th>Step No.</th>
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<th>Extension</th>
<th>No. of cycles</th>
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<td>72°C for 30 sec</td>
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<tr>
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<td></td>
<td></td>
<td>72°C for 5 min</td>
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2.5.3 Denaturing Polyacrylamide Electrophoresis

A 6% (w/v) denaturing polyacrylamide gel was prepared with 100 ml of 6% (w/v) polymerising gel solution (19:1), 266.6 µl of 10% (w/v) APS and 53.3 µl of TEMED. The gel solution was carefully poured between two secured glass plates to ensure no air bubbles were present. 2 x 24 shark-tooth combs were placed upside down between the glass plates. The gel was allowed to set for 2 h at room temperature. Once the gel was set the combs were removed, inverted and inserted so that they just touched the gel surface. The gel tank was then filled with 1 x TBE buffer. The gel was pre-run for 30 min at 2000 Volt, 50 mA, 60 Watt. Frozen PCR samples were thawed and 15 µl of the PCR mixture was transferred from under the mineral oil layer to a clean well. Of the 15 µl sample, 3 µl was added to 2 µl loading dye and denatured by heating to 80°C for 2 min prior to loading. To remove urea build up wells were flushed with buffer before loading. 5 µl sample was loaded into each well (a maximum of 40 samples could be loaded per gel). The gel was run at 60 Watts as before, for 3 - 3.5 h until the xylene dye reached the bottom of the gel. The gel was then blotted onto 3M paper and covered with saran wrap. The gel was dried in a slab gel dryer (Geldry Sr., Hoeffer Scientific Instruments) for 2 h at 80°C. The dried gel was then placed in an autoradiograph cassette with Hyperfilm MP and left overnight at room temperature.

2.5.3.1 Isolation of cDNA from Gels

Luminescent labels were attached to the dried gel before exposing to X-ray film. After developing the film (overnight to a 72-h exposure), the autoradiogram was orientated with the gel. Bands of interest were located by punching through the film with a needle at the four corners of each band, and then removed with a clean razor blade. The gel slice and 3M paper were then soaked in 100 µl dH2O for 10 min. The tubes were sealed with parafilm and boiled for 15 min, before centrifugation to collect condensation and to pellet the gel and paper debris. Supernatants were transferred to clean Eppendorf tubes. 10 µl of 3M sodium acetate (pH 5.4) plus 5 µl of glycogen (10 mg/ml) and 450 µl of 100% EtOH were added to each tube to precipitate the DNA. The tubes were then left on dry ice or in a -80°C freezer for 30 min. The tubes were spun for 10 min at 4°C to pellet the DNA. The resulting supernatant was removed and pellet rinsed with 200 µl ice-cold 85% (v/v) EtOH. A further brief spin ensured removal of residual ethanol. The pellet was dissolved in 10 µl of dH2O and 4 µl was used for re-amplification. The remainder was stored at -20°C.
2.5.3.2 Re-amplification of cDNA probe

Re-amplification was carried out using the same primer set and PCR conditions except the dNTP concentration was 20 μM (not 2 to 4 μM) and no isotope was added.

\[
\begin{align*}
20.4 \mu l & \text{ dH}_2\text{O} \\
4.0 \mu l & 10 \times \text{PCR buffer} \\
3.2 \mu l & \text{dNTP (250 μM)} \\
4.0 \mu l & \text{H-AP primer (2 μM)} \\
4.0 \mu l & \text{H-T}_{11}\text{M (2 μM)} \\
4.0 \mu l & \text{cDNA template from step 8} \\
0.4 \mu l & \text{AmpliTaq}^\text{TM} \text{ DNA polymerase}
\end{align*}
\]

30 μl of PCR samples were run on a 1.5% (w/v) agarose gel and stained with ethidium bromide. If re-amplification was unsuccessful in the first round of PCR, 4μl of 1:100 dilution of the first-round PCR sample was used as a template for another 40-cycle amplification. The remaining PCR samples were stored at -20°C for cloning. The size of the re-amplified PCR products were checked to see if they were consistent with their size on the DNA sequencing gel.

2.5.4 Cloning of cDNA

Cloning of cDNA into \textit{E. coli} allows sufficient amplification of the chosen cDNA to enable sequencing and Northern analysis to be conducted. Cloning was carried using Invitrogen’s Original TA cloning vector kit which provides a quick, one-step cloning strategy for the direct insertion of a PCR product into a plasmid vector.

2.5.4.1 Ligation of PCR products into plasmid \textit{pCR}^{TM}II

The following components were thawed on ice. Ligation reactions were prepared for each of the PCR products re-amplified from the sequencing gel to a final volume of 10 μl. The ligation reactions were incubated overnight at 14°C then stored at -20°C or used immediately.

\[
\begin{align*}
5 \mu l & \text{ dH}_2\text{O} \\
1 \mu l & \text{ ligation buffer} \\
2 \mu l & \text{pCR}^{TM}\text{II vector (25 ng/μl)} \\
1 \mu l & \text{chosen PCR product} \\
1 \mu l & \text{T4 DNA ligase (4 U/μl)}
\end{align*}
\]
2.5.4.2 Transformation of plasmids into competent cells

Ligation mixtures and competent cells were thawed on ice. 2 μl of 0.5 M β-mercapto-ethanol was added to each vial of One Shot™ (INVαF') competent cells and mixed by gentle stirring with a pipette tip. 1 μl of each ligation reaction was then added to the cells and gently stirred. The vials were incubated on ice for 40 min to allow the plasmid DNA to absorb onto the cells. The cells were then heat shocked for approximately 30 sec at 4°C to allow the DNA to enter the cells. The vials were then placed on ice for a further two minutes after which time 450 μl of SOC medium (at room temp) was added to each vial. The vials were left at 37°C in a shaking incubator at 225 rpm for 1 h. Transformed cells were plated gently at 50 μl and 100 μl onto pre-dried agar plates treated with the relevant antibiotic selection plus X-gal. After 15 min the plates were inverted and incubated at 37°C overnight to allow colonies to develop. The plates were then moved to 4°C for a few hours to allow colour to develop. Agar plates were prepared by melting LB Agar (see appendix II), once the molten agar had cooled down to approximately 55°C. Carbenicillin was added to a final concentration of 50 μg/ml. Agar was poured onto 88 mm² round petri dishes to a depth of 5 mm, and allowed to set for 1 h. Plates were then treated with 40 μl X-gal (40 mg/ml stock) ensuring complete coverage of the plates, and allowed to dry for 15 min. Competent cells were then added to the dried plates.

2.5.5 Colony Selection

α-Complementation

This method of selection involves the use of host bacteria which are Lac⁻ i.e. part of the regulatory region of the gene for β-galactosidase (lac Z) is missing which is provided by insertion of a plasmid vector containing the missing regions. Although neither the host-encoded nor the plasmid encoded fragments are themselves active, they may associate to form an enzymatically active protein. The Lac⁺ colonies formed from this type of complementation are easily recognised because they form blue colonies in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) due to the production of β-galactosidase. However, insertion of a cDNA fragment into the plasmid almost invariably disrupts this complementation and therefore bacteria containing recombinant plasmids form white colonies. Some colonies were found to be white with blue centres, these colonies were not generally chosen since they may represent partial
inactivation (i.e. incomplete cDNA species were inserted or full cDNA’s were too small), however, colony PCR (see below) showed that some of these colonies contained the correct size insert.

Single selected colonies were picked with inoculating loops under sterile conditions and added to 3 ml LB broth containing carbenicillin in a 15 ml Falcon tube (one tube for one colony), or 25 ml LB broth in a 50 ml Falcon tube depending on the plasmid DNA isolation method. Tubes were left overnight in a shaking incubator at 37°C at 225 rpm to produce a saturated culture of *E. coli* containing plasmid DNA. Of the 3 ml or 25 ml cultures, 1 ml was removed and mixed with 0.5 ml glycerol, placed in cryotubes (Nunc) and stored at -20°C.

**Colony PCR**

Further to discovering that multiple cDNA species existed within one cDNA band from the sequencing gel and hence within transformation reactions, this method was chosen as a fast way of checking insert presence and size of colonies before growing up and isolating the plasmid DNA. Colonies were toothpicked for replating at the same time as inoculating culture medium, so that if inserts were present, the cultures were already growing. Restriction analysis after plasmid isolation would confirm these results. 25 µl reaction mixtures comprised the following:

<table>
<thead>
<tr>
<th>2.5 µl 10 × PCR buffer</th>
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<tbody>
<tr>
<td>1.0 µl dNTP’s (200 µM final) of each (dATP, dCTP, dGTP)</td>
</tr>
<tr>
<td>1.25 µl primer Sp6</td>
</tr>
<tr>
<td>1.25 µl primer T7</td>
</tr>
<tr>
<td>15.9 µl dH₂O</td>
</tr>
<tr>
<td>0.125 µl Amplitaq™</td>
</tr>
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</table>

The PCR reaction mixture was pipetted into a 96 well plate and a toothpick of each colony was added to each well, then overlaid with 30 µl of mineral oil and placed in the thermocycler on block control at:

<table>
<thead>
<tr>
<th>Step No.</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>No. of cycles</th>
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<tbody>
<tr>
<td>1</td>
<td>94°C for 5 min</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>94°C for 1 min</td>
<td>55°C for 1 min</td>
<td>72°C for 30 sec</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>72°C for 5 min</td>
<td></td>
<td></td>
<td>1</td>
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</tbody>
</table>
6 μl PCR samples were mixed with 1 μl loading buffer and 3 μl dH₂O and run on a 4% (w/v) Nusieve gel with relevant markers. Sp6 and T7 primer sites are positioned approximately 97 bp and 88 bp respectively from the insertion site, therefore, cloned inserts will be 185 bp longer than the actual size (see Figure 2.5 for plasmid map).

2.5.6 Isolation of plasmid DNA

All of the methods described below for purifying plasmid DNA involve the alkaline lysis method of isolation.

Minipreparation of plasmid DNA

This protocol is a modification of the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). This method was initially carried out to confirm plasmid presence and correct size of insert, before a more expensive but cleaner preparation was carried out. 2 ml cultures were transferred to 2 ml Eppendorf tubes and DNA pelletted in a microcentrifuge for 2 min. The DNA pellet was then kept on ice for 5-15 min after which time 100 μl of solution I (see appendix II) was added, vortexed briefly and kept on ice for a further 5 min. 200 μl of solution II was then added to each tube, vortexed briefly and incubated on ice for 5 min. Finally 150 μl of ice cold solution III was added to each tube, vortexed and incubated on ice for a further 5 min. The cell debris was pelleted by centrifuging for 1.5 min. The supernatant was transferred to clean tubes and DNA extracted with a (1:1) phenol:chloroform solution. The plasmid DNA was ethanol precipitated, washed with 70% ethanol, dried under vacuum and resuspended in 25 μl dH₂O and 1 μl RNase A (5 μg/ml).

QIAGEN plasmid midi kit

The manufacturer’s instructions were followed for the isolation of plasmid, this method gave a sufficient yield of DNA for sequencing and a purer preparation. Briefly, 10 μl samples were removed from the frozen cultures, or colonies were selected and added to 50 ml Falcon tubes containing 15 ml L-broth with carbenicillin and incubated overnight at 37°C in a shaking incubator. Plasmid DNA was pelleted by spinning tubes in a CPK R centrifuge (Beckman) at 3000 rpm for 20 min. The pellets were resuspended in 4 ml of cold buffer P1 and transferred to glass corex tubes. 4 ml of buffer P2 was then added, mixed by vortexing and left for 5 min at room temp. 4 ml of chilled P3 buffer was added,
mixed and left on ice for 15 min. The tubes were then centrifuged at 4°C for 30 min at 30,000 g (15,800 rpm) in a RC5C Sorvall centrifuge (Dupont). Supernatants were removed and placed onto pre-equilibrated Qiagen-tip 100 columns. The columns were then washed with $2 \times 10$ ml QC buffer and DNA eluted with $5$ ml of QF buffer. DNA was precipitated with $0.7$ volumes of isopropanol, centrifuged at $15,000$g (12,000 rpm) at $4°C$ for $30$ min, and washed with $70\%$ ethanol. After drying under vacuum the DNA pellet was resuspended with $100 \mu l$ dH$_2$O for sequencing.

HYBAID RECOVERY™ plasmid midi prep kit
This method was employed for the isolation of multiple plasmid preparations, the protocol significantly reduced the time required to isolate plasmid DNA and was used for plasmid preparations of 20 or more. 25 ml cultures were centrifuged to pellet DNA, as before, washed in dH$_2$O to remove excess culture medium, resuspended in $200$ μl pre-lysis solution, and mixed by vortexing. $400$ μl alkaline lysis solution was then added to lyse the cells, degrade RNA and denature proteins and chromosomal DNA. Tubes were gently inverted 15 times then incubated at room temp for 5 min before $300$ μl of ice cold neutralising solution was added to each tube. Tubes were vortexed for 5 sec at full speed to produce a uniform white precipitate. The tubes were then left on ice for 5 min and spun for 5 min in an Eppendorf micro-centrifuge at 14,000 rpm. The supernatants were removed and placed into clean 2 ml tubes. Plasmid DNA was purified by allowing the DNA to bind to binding buffer which was then washed and eluted with $100 \mu l$ dH$_2$O or TE buffer.
Figure 2.5. TA Cloning vector map
DNA concentration measurements

DNA concentrations were determined using a DU-64 spectrophotometer (Beckman), by measuring OD at 260 and 280 nm. Samples were diluted 1:100 in dH₂O, and concentrations were calculated using the formula:

\[
[\text{[DNA]} \, (\mu g/\mu l) = \text{OD}_{260} \times 50 \times 100 \, \text{(dilution)} + 1000]
\]

For sequencing, DNA was diluted to 250 ng/ml and 4 µl sample used per sequencing reaction.

2.5.7 Restriction enzyme analysis

The TA cloning vector has many restriction sites incorporated into the plasmid so that calculation of insert length and orientation may be confirmed. Two EcoRI sites are positioned exactly either side of inserted cDNA so that the total size of EcoRI fragments are only a few bp longer than the actual cDNA insert (Figure 2.5). For EcoRI digest, 3 µl of sample DNA was mixed with 2 µl of one-phor-all buffer, 5 µl dH₂O and 0.5 µl EcoRI enzyme (0.5 U). The mixture was incubated for 2 h at 37°C. Restriction fragments were separated on a 4% Nusieve gel (3% (w/v) Nusieve, 1% (w/v) agarose) or 1.8% (w/v) agarose gel depending on the expected size of restriction fragments.

2.5.8 Agarose Gel electrophoresis

DNA and RNA molecules were separated according to size on agarose gels. Generally 1% (w/v) agarose, 1.8%(w/v) agarose or 4% Nusieve gels were used according to the size of the fragments. 4% (w/v) Nusieve gels gave particularly good resolution of smaller DNA bands and were especially useful when extracting DNA from gels for PCR labelling. The appropriate volume of agarose or Nusieve in TBE buffer was made and boiled to dissolve the agarose (see Appendix II). The agarose solution was cooled to 60°C and ethidium bromide added to a final concentration of 0.5 µg/ml before pouring onto a horizontal slab mini-gel casting block. 1 µg DNA samples were made up to 10 µl with dH₂O and 0.1 × volume of loading buffer, and electrophoresed in TBE at 5V/cm against standard molecular weight markers (ϕX174 and 1 Kb markers (BRL)). DNA bands were detected under UV
light. For RNA analysis, DEPC treated TBE buffer was used to make and run the gels which were usually 1% (w/v) agarose.

2.5.9 Sequencing

Sequencing was carried out on an ABI 373 DNA sequencer (Perkin Elmer) using the Dye terminator labelling method. Sequences were compared to those of EMBL and Genbank databases for homology to known sequences.

2.5.10 Oligonucleotide synthesis of primers

After sequences of cDNA's were analysed, primers of 20 bp were designed which corresponded to the 3' and 5'-most ends of the cDNA, or to a region which would give 200-300 bp fragments if the insert size was shorter than this based on full length sequences from the database. Primers were designed to give an approximate 50:50 G:C content to ensure efficient reannealing with cDNA templates. Primers were kindly synthesised by Rosie Chatfield (YRI) on a 392 DNA/RNA synthesiser (Applied Biosystems) After synthesis was complete, the protected oligonucleotide was cleaved from the solid support by treatment (4 x 0.5 ml for 15 min) with concentrated ammonium hydroxide (>35%). The oligonucleotide was deprotected by incubation in the same solution at 55°C for 12 h in a sealed tube, then dried down for 6 h under vacuum at 25°C. DNA pellets were resuspended in 200 μl dH2O and the concentration determined at OD_{260}. Alternatively, primers were synthesised to order from Perkin Elmer.

2.5.11 Confirmation of sequence with new primers (Re-PCR)

PCR was carried out on reverse transcription (RT) mixes from original differential display using the newly synthesised primers to confirm presence of differentially expressed cDNA and to provide a template for radiolabelled probes for Northern analysis. 25 μl reactions of the following were made:
13.9 µl dH₂O
2.5 µl 10 × PCR buffer
4.0 µl dATP (200 µM)
4.0 µl dCTP (200 µM)
4.0 µl dGTP (200 µM)
4.0 µl dTTP (200 µM)
1.25 µl Primer 1
1.25 µl Primer 2
0.125 µl AmpliTaq™ DNA polymerase
2.0 µl RT mix

A positive and negative control were routinely added. This involved using genomic DNA which should give a product providing primers do not span intron/exons, and RNA which should give no product without reverse transcription. Samples were overlaid with 30 µl mineral oil, placed in thermocycler (Omnigene, Hybaid) and run at:

<table>
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<tr>
<th>Step No.</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>No. of cycles</th>
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<tbody>
<tr>
<td>1</td>
<td>94°C for 1 min</td>
<td>55°C for 1 min</td>
<td>72°C for 30 sec</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>94°C for 1 min</td>
<td>55°C for 1 min</td>
<td>72°C for 5 min</td>
<td>32</td>
</tr>
</tbody>
</table>

Samples were run on a 4% (w/v) NuSieve gel. In some cases cDNA’s were not successfully re-amplified from RT reactions because the original RT reaction from differential display may not have extended 5’ enough and therefore would not contain the primer sites. Two methods were employed to solve this, which involved reverse transcription of the original mRNA samples.

1. mRNA was reverse transcribed with the specifically designed downstream primer and then PCR was carried out with both the upstream and downstream primers.

2. mRNA was reverse transcribed with random hexamers as the downstream primer which would bind to many different sites along the length of the mRNA to produce different length cDNA’s. Further PCR was carried out with the specific up- and down-stream primers.
2.5.12 Reverse transcription PCR (RT-PCR)

20 μl reactions of the following were prepared using components from the RT-PCR kit (Perkin Elmer):

- 4.0 μl 25 mM MgCl₂ solution
- 2.0 μl 10 × PCR buffer
- 2.0 μl DEPC dH₂O
- 2.0 μl dATP (1 mM)
- 2.0 μl dCTP (1 mM)
- 2.0 μl dGTP (1 mM)
- 2.0 μl dTTP (1 mM)
- 1.0 μl RNase inhibitor (1 U/ml)
- 1.0 μl MMLV reverse transcriptase (2.5 U/ml)
- 1.0 μl Down stream primer (random hexamer or oligo) (1 μM)
- 1.0 μl RNA (PAW109 control) ≤ 1 μg total RNA

Samples were overlaid with 30 μl mineral oil and run at:

<table>
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<tr>
<th>Step</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>No. of cycles</th>
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<tbody>
<tr>
<td>1</td>
<td>95°C for 2 min</td>
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<td></td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>95°C for 1 min</td>
<td>60°C for 1 min</td>
<td></td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>72°C for 7 min</td>
<td>1</td>
</tr>
</tbody>
</table>

The following components were mixed to give a final volume of 78 μl:

- 4.0 μl 25 mM MgCl₂ solution
- 8.0 μl 10 × PCR buffer
- 65.5 μl dH₂O
- 0.5 μl AmpliTaq™ DNA polymerase (final concentration 2.5 U/100 μl)

78 μl master mix was added to each RT tube, mixed gently and spun briefly to allow the mineral oil to settle on the top of the tube of the mix. 1 μl of each primer was subsequently added to make the final volume to 100 μl. Samples were run at:

Controls included a negative control (no RNA added) and a positive control (PAW109 control RNA) which is RNA transcribed from plasmid PAW109 DNA. The plasmid contains primer sites for numerous lymphokine genes. Samples were run on a 4% (w/v) Nusieve gel.
2.6 NORTHERN ANALYSIS

By using RNA from the same treatments as for differential display, confirmation of
differential expression of bands isolated from the gel may be carried out using Northern
blot hybridisation. Clones or DNA templates can be used to probe total RNA from the cell,
which will indicate if the expression pattern is identical to that observed on the gel and
provide information about the size of the RNA species if not already known.

2.6.1 RNA preparation

Total RNA was isolated as described previously. RNA from different conditions were
diluted to approximately 4 μg/μl in DEPC dH₂O. If samples were too dilute, they were re­
precipitated by adding 0.1 × volume of 3M sodium acetate (pH 5.4) and 2.5 × volume of
Absolute EtOH to the RNA solution and incubated overnight at -70°C. DNA was pelleted
by centrifuging at 14,000 rpm at 4°C for 15 min in an Eppendorf 5402 centrifuge. The
DNA was washed with 70% (v/v) EtOH and allowed to air dry to remove residual ethanol,
rather than dried using the DNA 100 Speed Vac (Savant) (used for DNA) which may over­
dry the pellet and make re-suspension difficult. If re-suspension was not successful, the
RNA solution was incubated at 65°C for 15 min then placed on ice. RNA pellets were re­
dissolved in a smaller volume of DEPC dH₂O to give a final RNA content of 20 μg or 4
μg/μl.

2.6.2 Formaldehyde Gel Electrophoresis

This method is adapted from those of Lehrach et al. (1977) and Goldberg (1980).
10 × Formaldehyde gel-running buffer was prepared to make a final concentration of 0.1 M
MOPS (pH 7.0), 40 mM sodium acetate and 5 mM EDTA (pH 8.0) (see appendix II). A
2% (w/v) agarose gel was prepared by adding 1.5 g agarose to 75 ml DEPC dH₂O and
dissolved by heating. When the temperature of the gel was below 50°C, 17 ml of
formaldehyde and 9.2 ml of 10 × formaldehyde gel-running buffer were added to give final
concentrations of 2.2 M and 1 ×, respectively. The gel was cast in a fume hood and allowed
to set for at least 30 min. Samples were prepared by mixing the following:
Methods

Chapter 2

12.5 µl deionised formamide
5.0 µl formaldehyde
2.5 µl 10 x running buffer
2.5 µl DEPC dH₂O
5.0 µl RNA sample (4 µg/µl) or RNA markers

The samples were incubated for 15 min at 65°C, chilled on ice then spun briefly to collect condensation. 2.5 µl of formaldehyde gel-loading buffer was then added to each sample. Before loading the samples, the gel was pre-run for 5 min at 5V/cm. 30 µl samples were loaded into the lanes of the gel along with RNA markers 0.16-1.77 Kb and 0.24-9.5 Kb. The gel was submerged in 1 x formaldehyde gel-running buffer and run at 115V until the bromophenol blue had migrated approximately 8 cm. The marker lanes could then be cut, and stained with ethidium bromide or transferred with the RNA onto a nylon membrane and stained with methylene blue. Ethidium bromide staining of gels containing RNA samples was avoided, since intercalation of the dye with the RNA appeared to reduce the efficiency of transfer.

2.6.2.1 Ethidium Bromide staining of markers

The appropriate marker lanes were cut from the gel, stained with ethidium bromide (1 µg/ml in DEPC dH₂O) for 5 min, and rinsed in DEPC dH₂O for 30 min, then viewed under UV light. A photograph was taken of the marker lanes against a ruler.

2.6.2.2 Methylene blue staining of markers

After transfer and fixing, the marker lanes were cut away and soaked in 5% (v/v) acetic acid for 15 min at room temp. The filter was then transferred to a solution of 0.5 M sodium acetate (pH 5.2) and 0.04% (w/v) methylene blue for 5-10 min then rinsed in dH₂O for 5-10 min. The standard molecular weight RNA bands should appear as sharp, well resolved, bands.

2.6.2.3 Calculating molecular weights of RNA samples

By plotting the log₁₀ of the size of the fragments of RNA against the distance migrated, the resultant curve was used to calculate the sizes of RNA species detected by hybridisation after transfer from the gel to a solid support.
2.6.3 RNA Transfer

Transfer was carried out by capillary elution, where buffer is drawn from a reservoir and passes through the gel into a stack of paper towels. The nucleic acid is eluted from the gel by the moving stream of buffer and is deposited on a nylon membrane. A weight applied to the top of the paper towels helps to ensure a tight connection between the layers of material used in the transfer system. Before transfer, one small corner of the gel was removed to help orientation, and the gel was then rinsed in several changes of DEPC dH₂O to remove the formaldehyde. A glass support, that is longer and wider than the gel, was placed inside a deep tray and covered with a piece of Whatman 3MM paper so that the sides of the paper overlapped the support onto the bottom of the tray (Figure 2.6). The tray was then filled with 20 x SSC and when the 3MM paper was thoroughly wet, any air bubbles were smoothed out.

Figure 2.6. Capillary transfer of nucleic acids from agarose gel to a nylon membrane. The nucleic acid is eluted from the gel by the moving stream of buffer (capillary action) and is deposited on a nylon membrane. A weight applied to the top of the paper towels ensures a tight connection between the layers of material used in the transfer system.
The gel was placed onto the support in an inverted position so that it was centred on the wet 3MM paper, again making sure there were no air bubbles between the gel and the paper. A piece of Hybond-N nylon membrane was cut to approximately the same size as the gel, with a 1 mm overlap, and a corner of the membrane was removed to match the corner cut from the gel. The membrane was then immersed in 20 × SSC to wet the filter. The gel was surrounded (but not covered) with Saran Wrap, which prevents liquid from flowing directly to the paper towels. The wet nylon membrane was placed on top of the gel so that the cut corners were aligned. Two pieces of 3MM paper cut to the same size as the gel were soaked in 2 × SSC and placed on top of the wet nylon membrane, smoothing out any air bubbles. A stack of paper towels were cut just smaller than the 3 MM papers and placed on top. A 500 g weight was placed on top and transfer allowed to proceed overnight. As the paper towels became wet, they were replaced with new towels.

After transfer, the paper towels and 3MM paper from the top of the gel were removed, and the gel turned over so that the nylon membrane was now on the bottom of the gel. The positions of the wells of the gel were marked on the membrane with a soft leaded pencil. The gel was peeled from the membrane and stained with ethidium bromide, as before, to check that complete transfer of RNA had occurred. DNA was fixed to membranes by UV cross-linking at 254 nm, in a UV Stratalinker 2400 (Stratagene). The membrane was covered in Saran Wrap and stored in an air tight container at room temp until required for hybridising.

2.6.4 Probe synthesis for Northern analysis

Immobilised RNA can be probed with DNA, RNA or single stranded oligonucleotides. Probes can either be known sequences of cDNA’s which were isolated from the original gel, or groups of unknown sequences isolated from a single band from the original gel which may contain one or more cDNA species. Templates were provided from either RT-PCR of the original RNA samples, PCR from genomic DNA, cloned inserts i.e. after enzyme digestion of plasmid DNA, or cDNA eluted from the differentially expressed band from the original gel. Whichever template was used, the sample was purified by running on an agarose gel and elution using QIAquick elution kits.
Methods

Chapter 2

QIAquick Gel extraction of cDNA

cDNA probes can be purified from agarose gels either after re-amplification or after cloning into plasmid. Qiagen have produced a suitable protocol for recovery and purification of DNA from agarose gels which enables DNA between 100 bp and 10 kb to be isolated free of contaminants which may inhibit enzyme reactions. The negatively charged DNA is bound to anionic matrix and the contaminants are washed through the column. The DNA is then eluted from the matrix by raising the ionic strength of the buffer. Low melting agarose gels are preferred. The DNA fragment of choice was prepared from either re-amplification by PCR or an EcoRI digested plasmid preparation and separated on 2% (w/v) agarose gel. The band of interest was excised from the agarose gel with a clean, sharp scalpel blade. After the gel slice had been weighed, 3 volumes of buffer QX1 were added to one volume gel (100 mg ~ 100 μl) and incubated at 50°C for 10 min.

To help dissolve the gel, the tubes were mixed by inverting every 2-3 min during the incubation. 1 gel volume of isopropanol was added, mixed and placed in a QIAquick spin column with a 2 ml collection tube and centrifuged for 1 min at 13,000 rpm. The flow through was discarded. To wash, 0.75 ml of buffer PE was added to the column and centrifuged for a further minute. The flow through was discarded and the column spun for 1 min to remove residual wash buffer. Finally, DNA was eluted with 50 μl H$_2$O by spinning for 1 min. The presence of DNA was confirmed by measuring OD$_{260}$ and gel electrophoresis.

Random prime labelling of cDNA probe

A method modified from Feinberg and Vogelstein (1983). Random prime labelling of probes was carried out whenever the sequence of the probe was unknown, or if primers had failed to produce a correct template for whatever reason. This method was extremely useful for labelling cDNA’s directly from excised gels which had not been re-amplified, and allowed labelling of multiple cDNA species that would have been selected out by re-amplification. However, the disadvantage of this method is that a considerable amount of DNA is required, and without further PCR, starting material is sparse. cDNA of less than 200 bp are not recommended for labelling by this method. The main consideration in interpreting results from this method is that the specific activity of probes are greatly reduced therefore sensitivity is lower, and multiple bands may arise from either splice variants or multiple cDNA’s.
Random prime labelling was carried out using Rediprime™ labelling kit (Amersham). DNA to be labelled was purified using QIAquick gel extraction kit and used at a concentration of 2.5-25 ng in 45 μl in dH2O. The DNA was denatured by heating to 95-100°C for 5 min and was briefly spun to collect condensation. The denatured DNA was then added to the labelling mix by gently flicking the tube until the blue colour from the labelling mix was distributed evenly. After a brief spin, 5 μl of Redivue™ α-[32P]dCTP (3000 Ci/mmol) (Amersham) was added and mixed by gentle pipetting. The tube contents were briefly microfuged and incubated at 37°C for 10 min. The reaction was stopped by adding 5 μl of 0.2 M EDTA to the tube. The DNA reaction was then added to a pre-equilibrated nick spin column (Pharmacia Biotech), and spun for 5 min at 1,500 rpm. Immediately prior to hybridising, the labelled DNA was collected and denatured by heating to 95-100°C for 5 min and then placed on ice.

**Primer specific labelling of cDNA probe**

This method ensures specific labelling of chosen cDNA using carefully selected primers. The technique uses PCR to amplify cDNA and incorporates labelled dCTP, and it is therefore important to design a template which has at least 30% Cytosine content. PCR was carried out on chosen cDNA with specific primers to give a band of at least 150 bp. 1 μg of sample was run on a 4% (w/v) Nusieve gel for 30 min whilst the following components were thawed, mixed and kept on ice:

- 2.0 μl dH2O
- 1.0 μl 10 × PCR buffer
- 0.6 μl dATP, dGTP, dTTP (200 μM)
- 0.5 μl primer 1
- 0.5 μl primer 2
- 0.2 μl AmpliTaq™

Using a 10 μl pipette, 5.0 μl of the agarose gel containing the cDNA was withdrawn. By checking the pipette tip under the UV light, the cDNA should light up in the presence of ethidium bromide, care was taken not to expose the PCR mix to UV light. The fragment of cDNA in agarose was added to the PCR mix along with 5 μl of Redivue™ α-[32P]dCTP (3000 Ci/mmol) and mixed carefully. The sample was overlaid with 30 μl of mineral oil and programmed at:

<table>
<thead>
<tr>
<th>Step No.</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>No. of cycles</th>
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<tr>
<td>1</td>
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<td>72°C for 5 min</td>
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Immediately after PCR, 60 µl of TE buffer was added under the oil layer to prevent the mixture setting when cool due to the agarose content. 70 µl of the PCR mix was then removed from under the oil layer and placed on a pre-equilibrated nick spin column and spun for 5 min at 1,500 rpm. The purified labelled cDNA was then denatured at 95-100°C for 5 min the placed on ice prior to hybridising.

2.6.5 Hybridisation of RNA with labelled probes

Pre-hybridisation buffer was made to give a final concentration of 5 × SSC, 5 × Denhardt’s reagent, 0.5% (w/v) SDS and 20 µg/µl salmon sperm DNA (heated to 100°C before placing on ice). The pre-hyb buffer was heated to 60°C and the filter pre-hybridised for 1-2 h at 60°C in a hybridising oven (Hybaid). The denatured radiolabelled probe was added directly to the pre-hybridisation fluid, and incubation continued at 60°C for 16-24 h. Excess probe was removed and the filters were placed in a plastic sandwich box and washed in pre-warmed 2 × SSC, 0.1% (w/v) SDS buffer (low stringency) three times in a shaking incubator at 60°C for 10 min each followed by two washes of 15 min each in 0.2 × SSC, 0.1% (w/v) SDS at the same temperature. Finally, the filter was rinsed in 2 × SSC at room temp to remove any residual SDS, covered in Saran Wrap and exposed to an Instant Imager (Packard) for 1-12h or an autoradiographic film (Hyperfilm MP) at -70°C with an intensifying screen for 24-48 h.

For shorter hybridisation times, Rapid-Hyb™ buffer (Amersham) can be used as an alternative to the usual hybridisation buffer. Pre-hybridisation was carried out at 65°C for 15 min, the filter was then incubated with labelled probe for 2 h at 65°C. The filter was washed once at room temp with 2 × SSC, 0.1% (w/v) SDS for 15 min then washed twice for 10 min at 65°C in 0.2 × SSC 0.1% (w/v) SDS. The disadvantages associated with the rapid hybridisation technique are higher background, and reblotting with a different probe is difficult since the old probe is difficult to remove.
2.7 SCREENING STRATEGIES

2.7.1 Screening by hybridisation

This method allows the simple screening of many hundreds of thousands of colonies simultaneously, and is especially important for differential display, as it allows the selection of colonies containing a specific target sequence. Either colonies themselves are lysed directly onto a nylon membrane, or the products of colony PCR or EcoRI digested plasmids can be transferred to a nylon membrane by the method of Southern (1975). Filters can then be hybridised to a labelled probe with either DNA, RNA or small oligonucleotides.

2.7.2 Colony transfer

Transformants were screened by the method of Grosveld et al. (1982). 100 white positive clones for each transformation were replated onto a fresh agar plate with antibiotics and X-Gal. At the same time, samples were taken for colony PCR. Colonies were grown overnight at 37°C then moved to 4°C to allow colour to develop. Those colonies which were still white after replating were taken as true positives. 82 mm² round nylon membranes were pre-wet by placing on clean agar plates. The filters were then laid gently on top of the plates containing positive clones until they became saturated. The filters and agar were then positioned with an 18-gauge needle. Using blunt-ended forceps the filters were peeled off the agar and placed colony side up onto clean agar plates and incubated for 2-3 h at 37°C. When colonies became visible, the filters were removed from the incubator and prepared for lysis.

2.7.3 In-Situ Lysis of colonies and binding of DNA to nylon membranes

Four pieces of Whatmann 3MM paper were cut to fit into the bottom of four plastic trays. Each piece of paper was saturated with one of the following solutions and any excess liquid removed.

-10% (w/v) SDS
-Denaturing solution (0.5 M NaOH, 1.5 M NaCl)
-Neutralising solution (1.5 M NaCl, 0.5 M Tris-Cl (pH 7.4))
- 2 × SSC
Methods

Chapter 2

The nylon membranes were lifted from the agar plates and placed colony side up on the SDS impregnated 3M paper. After the first filter had been exposed to the SDS solution for 3 min, the filter was transferred to a second sheet of 3M paper saturated with denaturing solution for 5 min. The filters were then transferred to a third sheet of 3M paper containing neutralising solution for 5 min. The filters were finally transferred to the last sheet of paper soaked in 2 x SSC and soaked for 5 min. The filters were allowed to dry by placing, colony side up, on a clean sheet of dry 3M paper for at least 30 min at room temp. The DNA bound to the filters was then fixed by UV cross linking at 254 nm, in a UV Stratalinker 2400 (Stratagene), and stored in foil at room temperature under vacuum until required for hybridisation.

2.7.4 Southern transfer of DNA from colony PCR and Plasmid digestion

Plasmid DNA was digested with EcoRI or colony PCR was carried out as described previously. The DNA fragments were separated on a 2% (w/v) agarose gel stained with ethidium bromide (0.5 μg/μl). After electrophoresis, the gel was photographed under UV light with a ruler so that the distance any band has migrated can be read directly from the photograph. DNA fragments were transferred to a nylon membrane by capillary transfer in a similar way to RNA transfer.

After electrophoresis, the gel was soaked for 2 x 15 min in several volumes of 1.5 M NaCl, 0.5 N NaOH, with constant, gentle agitation to denature the DNA. The gel was rinsed briefly in dH2O, then neutralised by soaking for 2 x 15 min in several volumes of 1.5 M NaCl, 0.5 M Tris (pH 7.2), at room temperature with constant, gentle agitation then rinsed once more in dH2O. DNA was transferred in the same way as for Northern Hybridisation as previously described, by capillary transfer using a nylon membrane (Hybond N) to immobilise the DNA. Transfer was carried out for 2-3 h, DNA was fixed to the membrane by UV cross-linking at 254 nm, in a UV Stratalinker 2400 (Stratagene). Membranes were stored in foil at room temperature under vacuum until required for hybridisation.
Chapter 3

THE EFFECTS OF HYDROGEN PEROXIDE ON VASCULAR SMOOTH MUSCLE AND ENDOTHELIAL CELL GROWTH
Chapter 3

3.1 Introduction

Intimal cell accumulation is one of the most critical processes in atherogenesis and appears to result from a combination of cell migration and proliferation (Ross, 1993). In contrast to the wealth of information that is available on the proliferation kinetics in animal models of arterial injury and repair, little is known about the proliferative profile of human atherosclerotic and restenotic lesions. Although in both animal and human lesions the proliferating cells represent only 1-3% of total cells within the lesion, as many as 30% of the cells within a localised area of a lesion were shown to be positive for markers of proliferation (Rekhter and Gordon, 1995). By use of cell specific immunohistochemical markers and a double labelling technique, PCNA positive cells have been identified as smooth muscle cells, macrophages and endothelial cells (Gordon et al., 1990). A number of factors released following injury to the vascular wall from platelets, the endothelium, and infiltrating leukocytes, are thought to be influential in cellular migration and proliferation. However, it remains unclear which growth promoting and growth inhibiting actions dominate in the injured vessel wall of experimental animals and humans.

Recent *in vivo* data suggest that reactive oxygen species may play a role in atherosclerosis and restenosis following balloon angioplasty. Epidemiological studies show a correlation between antioxidant therapy and a decreased incidence of coronary events in both men and women (Rimm et al., 1993; Stampfer et al., 1993). Antioxidants such as vitamins C, E, and probucol have been demonstrated to induce a beneficial effect on vessel remodelling in animal models of balloon injury (Schneider et al., 1993; Nunes et al., 1995), which may suggest a role for oxidative stress in the vessel response to injury.

Data concerning the effects of reactive oxygen species on cells is conflicting: low concentrations may act as cellular messengers to influence many aspects of cell metabolic activity including growth (Burdon et al., 1989; Saran and Bors, 1989), whereas high concentrations may cause cell damage or even death. Active oxygen species are, in general, considered to be cytotoxic and damaging to cells. Deleterious effects of oxidants on cells include scission of DNA strands, oxidation of DNA bases and damage to the cytoskeleton (Hinshaw et al., 1986; Halliwell, 1987). However, accumulating evidence suggests that reactive oxygen species are involved in some physiological function including cell growth.
Previous reports have shown that oxidants stimulate growth in various cell types (Rudland et al., 1977; Ellem and Kay, 1983; Crane et al., 1985; Murrell et al., 1990; Brightman et al., 1992).

A role for oxidative stress-induced mitogenesis is further strengthened by work which shows that antioxidants can reverse these mitogenic effects. Antioxidants have been shown to reverse the inhibitory effects on proliferation caused by oxidative stress, or by inducing apoptosis as a means of controlling cellular proliferation (Tsai et al., 1996).

3.2 Aim

Relatively few studies have been undertaken in smooth muscle cells to establish the role of oxidative stress, despite the involvement of these cells in many disease processes. Of the few studies carried out, a definitive role of oxidative stress remains to be determined. Proliferation and cytotoxic studies reported in the literature are very often based on single or inappropriate time points i.e. too early to establish an effect or too late when cells may have recovered. Longer and more detailed studies are therefore required to understand the overall effect of oxidative stress on cells, since the effects of oxidative stress are apparently complex and may be time dependant. The paradoxical effects of oxidative stress on cells have posed a problem for investigators. With so many different sources of reactive oxygen species being used, it is not surprising that proliferative or cytotoxic effects are not always consistent. Studies carried out with oxidatively modified lipoproteins are the most contradictory. The heterogeneic nature of oxidised LDL has posed many problems to investigators, and even the use of pure sources of ROS does not completely solve the problem. Different cells cope with oxidative stress in different ways, so that the metabolism and possible production of reactive oxygen intermediates may vary between cell types.

The effects of oxidative stress have been investigated in this study using hydrogen peroxide as a source of reactive oxygen species, whose properties and characteristics have been well defined. Most of the work reported in the literature use either the superoxide anion or hydrogen peroxide, since these are the first sources of reactive oxygen species released intra- or extra-cellularly. After its passive diffusion through the cell membrane, hydrogen
peroxide can be converted into more reactive oxygen compounds such as the superoxide anion, $O_2^-$, and the hydroxyl radical $OH^-$ (Halliwell and Gutteridge, 1989). The growth stimulatory and inhibitory effects of hydrogen peroxide have been investigated in vascular smooth muscle cells and endothelial cells, to determine the importance of oxidative stress in contributing to lesion development through smooth muscle cell proliferation and endothelial cell death or dysfunction. The effects of hydrogen peroxide on cell cycle analysis were investigated to assess in more detail the effects of oxidative stress on cell growth.
3.3 Results

3.3.1 CHARACTERISATION OF CELL GROWTH FOLLOWING MITOGENIC STIMULATION

Growth studies were carried out on both smooth muscle cells and endothelial cells since both may be exposed to oxidative stress within the vessel wall. Comparisons of their different responses to oxidative stress may further our understanding of the pathophysiological role of oxidative stress within the vessel wall and atherosclerosis as a whole. A number of studies have shown conflicting results of oxidative stress effects, which seems to depend on the species of cells investigated. Therefore bovine, rat and human cells were used in this study. Knowledge of differences between species may also aid our explanations for their observed differences in response to oxidative stress.

3.3.1.1 Quiescence and synchronicity

Typically, in a population of growing cells that are all proliferating rapidly but asynchronously, about 30% will be in S-phase at any instant. Therefore, to optimise the sensitivity of the $[^3H]$-thymidine incorporation assay, the cells were rendered quiescent and were synchronised in Go/G1. The simplest method to achieve this was by serum starving the cells for 48-72 h. Chemical treatment may block the cells in a particular phase of the cell cycle, but may not necessarily be reversible. Rat Aortic Smooth Muscle Cells (RASMC), which rapidly proliferate, were less sensitive to serum deprivation than human and bovine smooth muscle cells and hence took longer to make quiescent (3 days or more in serum free medium). RASMC had a higher basal rate of incorporation suggesting that some cells were still not quiescent after this time. Human cells were particularly sensitive to reductions in serum levels and quiescence had to be achieved by incubating the cells in 0.1%(v/v) serum containing medium rather than serum free medium for 48 h. FACS analysis was used to determine the proportion of cells in each phase of the cell cycle after quiescence. Figure 3.1 shows that in Human Aortic Smooth Muscle Cells (HASMC), 93.5% of cells were in Go/G1, 2.8% in S-phase and 3.7% in G2/M after serum deprivation for 48 h compared to 44.3%, 42.4% and 13.4% respectively in exponentially growing cells. How these values change with FCS stimulation and H$_2$O$_2$ treatment will be discussed later.
Figure 3.1. Distribution of cell cycle phases in quiescent (A) and exponentially growing HASMC (B). HASMCs were (A) made quiescent by culturing in 0.1% (v/v) FCS for 48 h, or (B) restimulated with 16% (v/v) FCS. Cells were detached with 0.25% trypsin, 0.05% EDTA, and resuspended in 70% (v/v) ethanol in PBS with propidium iodide (400 ng/ml). DNA content was analysed on a FACScan flow cytometer (Becton Dickinson) using LYSIS II software. Cell cycle compartments were estimated using S-Fit analysis. Representative distributions from four independent experiments are shown.
3.3.1.2 Cell Stimulation by mitogens

Thymidine uptake measurements were carried out to assess mitogenesis. Pulse-labelled thymidine uptake was measured in time-windows over a specific period. This provides more information than simple total uptake measurements since rates of incorporation can be observed within a cell cycle. This can be used to determine the boundaries of the S-phase of the cell cycle, where DNA synthesis is maximal and therefore incorporation of $[^3]$H-thymidine is observed at a maximum rate.

In order to investigate the inhibitory or stimulatory effects of H$_2$O$_2$ on cell growth, a suitable mitogen was required which would stimulate $[^3]$H-thymidine incorporation. FCS is the standard mitogen applied and was always included in culture medium to sustain growth. More specific growth factors have also been used to stimulate proliferation by several investigators, since FCS is a heterogenous mixture of mitogenic factors. The effects of both FCS and PDGF were assessed for their ability to stimulate cell proliferation. Both FCS and PDGF produced a dose dependant stimulation of $[^3]$H-thymidine uptake in HASMC (Figure 3.2). Since FCS stimulated proliferation more markedly than PDGF (10-fold increase), FCS was chosen for further studies. The optimum concentration of FCS for sustaining growth is 10-16\% (v/v). However, to observe further increases above those induced by FCS due to H$_2$O$_2$ treatment, a lower concentration of FCS was used in thymidine incorporation assays (5\%) (v/v), which would still stimulate incorporation, but to a lesser extent. Human smooth muscle cells grown on fibronectin-coated-plastic responded to serum or PDGF stimulation more than cells on plastic alone (Figure 3.3) in $[^3]$H-thymidine incorporation assays. As a result, all further experiments were performed on fibronectin coated culture flasks and plates. Human smooth muscle cells grown on plastic differed in phenotype and tended to grown in clumps, whereas those grown on fibronectin spread more evenly over the culture flask surface. Bovine and Rat smooth muscle cells as well as Human and Bovine endothelial cells grew well on plastic alone.
Figure 3.2. Dose response effects of PDGF (A) and FCS (B) on $[^3]$H-thymidine incorporation in HASMC. Cells were plated at $1 \times 10^4$ cells/ml on to fibronectin coated 96 well plates in 16% (v/v) FCS. Cells were synchronised in 0.1% (v/v) FCS for 48 h then restimulated with varying concentrations of (A) PDGF (5-30 ng/ml), or (B) FCS (0.1-16%) for 36 h. [3H]-thymidine was added (1 µCi/ml) for the last 3 h of the experiment. Incorporated isotope was measured by liquid scintillation counting of trichloroacetic acid-insoluble material in the cell layer.
Figure 3.3. Comparison of dose response curves to PDGF (A) or FCS (B) grown on plastic or fibronectin coated culture plates. HASMC were plated at $1 \times 10^4$ cells/ml on to either plastic (■) or fibronectin coated 96 well plates (▲) in 16% (v/v) FCS. Cells were synchronised in 0.1% (v/v) FCS for 48 h then restimulated with varying concentrations of (A) PDGF (5-30 ng/ml), or (B) FCS (0.1-16%) for 36 h. [3H]-thymidine was added at a concentration of 1 μCi/ml for the last 3 h of the experiment. Incorporated isotope was measured by liquid scintillation counting of trichloroacetic acid-insoluble material in the cell layer. * p<0.05, ** p<0.01, n.s. not significant (p>0.05). Unpaired t-test of cells grown on plastic versus fibronectin coated plates.
3.3.1.3 Determination of cell cycle characteristics

Thymidine incorporation was measured in 2 hour windows at various time points after serum stimulation. Incorporation of $[^3]$H-thymidine began around 12 h after serum addition and increased with time, corresponding to the beginning of S-phase. Maximum DNA synthesis occurred at 20 h ± 2 h after serum stimulation in Bovine Aortic Endothelial Cells (BAEC) (Figure 3.4a) and 24 h ± 4 h in Bovine (BASMC) (Figure 3.4b), Rat and Human aortic smooth muscle cells (Figure 3.5), after which time incorporation fell to basal levels. The proportion of cells undergoing DNA synthesis, and the time taken for maximum incorporation to occur varied considerably among experiments. This may be due to differences in passage number of the cells and the state of the cells, e.g. extent of synchronicity. Studies from other laboratories have indicated a considerable range of variability in cell-cycle parameters in BAEC as a function of propagation in vitro (Goldsmith et al., 1984). The increase in incorporation of radio-labelled thymidine after serum stimulation occurred at least three fold, and often up to 6-7 fold increases could be observed. Different mitogens may induce S-phase at different times, for instance PDGF has been reported to induce maximum incorporation much later than serum (up to 80 h after treatment) (Grainger et al., 1994). In similar experiments PDGF failed to stimulate thymidine uptake in HASMC by 36 h (Figure 3.6). This is probably due to secondary effects of PDGF stimulation, e.g. autocrine production of mitogenic factors and/or their receptors. Differences in cell passage number or phenotype may also produce different growth curves. It has been shown that freshly isolated smooth muscle cells respond very differently to mitogens compared to cells in later passages (Grainger et al., 1991; Kemp et al., 1991). In addition, the method employed for the isolation of cells from the vasculature may be important. For example, explant or enzyme dispersal techniques may favour the growth of particular phenotypes, which have been reported to respond differently to mitogens with respect to cell-cycle characteristics and the degree of proliferation (Kirschenlohr et al., 1995).
Results

Figure 3.4. $[^3H]$-thymidine incorporation assay showing typical DNA synthesis in BAEC (A) and BASMC (B). Cells were plated at $1 \times 10^4$ cells/ml on to 24 well plates for 24 h in 16% (v/v) FCS. Cells were made quiescent in 0.1% (v/v) FCS for 2-3 days. Cells were stimulated with 5% (v/v) FCS (○) or left untreated in 0.1% FCS (▲). $[^3H]$-thymidine incorporation was measured in 2 h windows at various time points after stimulation, by liquid scintillation counting of trichloroacetic acid-insoluble material in the cell layer.
Figure 3.5. [\textsuperscript{3}H]-thymidine incorporation assay showing typical DNA synthesis in HASMC (A) and RASMC (B). Cells were plated at $1 \times 10^4$ cells/ml on to 24 well plates for 24 h in 16\% (v/v) FCS. Cells were made quiescent in 0.1\% (v/v) FCS for 2-3 days. Cells were stimulated with 5\% (v/v) FCS. [\textsuperscript{3}H]-thymidine incorporation was measured in 2 h windows at various time points after stimulation, by liquid scintillation counting of trichloroacetic acid-insoluble material in the cell layer.
Figure 3.6. [\textsuperscript{3}H]-thymidine incorporation assay comparing FCS and PDGF induced DNA synthesis in HASMC. Cells were plated at $1 \times 10^4$ cells/ml on to fibronectin coated 24 well plates in 16% (v/v) FCS. Cells were synchronised in 0.1% (v/v) FCS for 48 h then restimulated with 16% (v/v) FCS (\lambda) or 30 ng/ml PDGF (\mu). [\textsuperscript{3}H]-thymidine incorporation was measured in 2 h windows at various time points after stimulation, by liquid scintillation counting of trichloroacetic acid-insoluble material in the cell layer.
3.3.2 THE EFFECTS OF HYDROGEN PEROXIDE ON CELL GROWTH

To investigate the effects of hydrogen peroxide on mitogenesis, quiescent cells were treated with various concentrations of hydrogen peroxide in the presence or absence of FCS. Hydrogen peroxide was always added 30 minutes before FCS treatment to ensure incorporation into the cell before the addition of any mitogenic factors. It was hoped this would reduce the possibility of oxidative modifications to FCS, which may cause additional effects and prevent any interactions of \( \text{H}_2\text{O}_2 \) with antioxidants present in the serum. Cells were incubated under these conditions from 4 to 36 hours before pulse labelling with \( \text{H}^3\)-thymidine to build up a picture of DNA synthesis that occurs after stimulation, and to observe any alteration in the distribution of DNA synthesis after hydrogen peroxide treatment. The effects of hydrogen peroxide whether alone, or with FCS varied considerably between cell types and species and therefore each cell type will be dealt with separately.

3.3.2.1 Bovine and Human Aortic endothelial cells

Effects of hydrogen peroxide on FCS stimulation

Hydrogen peroxide reduced the number of BAEC passing through S-phase (18 h after FCS stimulation), in a dose dependant manner. Thymidine uptake was inhibited by 37.9%, 57% and 60% 18 hours after FCS treatment at hydrogen peroxide concentrations of 10, 25 and 50 µM respectively. This inhibition occurred in the absence of cell toxicity as assessed by trypan blue exclusion. Similar observations were found in HAEC, with significant inhibitory effects at 18 h (Figure 3.8a). However, this inhibition was transient, in that hydrogen peroxide had less effect on FCS induced DNA synthesis at later time points (24 hr) (Figure 3.7b and Figure 3.8b). The longer the cells were incubated, the more hydrogen peroxide treated cells recovered from this inhibition. This is possibly due to increased resistance, e.g. faster turnover or metabolism of hydrogen peroxide from the cell or induction of defence mechanisms such as antioxidant enzymes, catalase, GSH etc. However, by examining the whole time course of hydrogen peroxide treatment, a clearer picture of events was obtained (Figure 3.9).
These results suggest that the effect of hydrogen peroxide on endothelial cell proliferation is to delay cell entry into S-phase, rather than inhibit cell entry into S-phase completely. Hydrogen peroxide appeared to delay the peak of FCS induced DNA synthesis by 2-4 h. This delay was dose dependant with higher concentrations of hydrogen peroxide further delaying cell cycle transition into S-phase, after which time cells underwent peak rates of incorporation in a similar pattern to control cells. This explains the apparent increase in DNA synthesis observed after H$_2$O$_2$ treatment in BAEC by 24 hours (Figure 3.7b). However, in the case of endothelial cells, the maximum amount of DNA synthesis achieved by H$_2$O$_2$ plus FCS treated cells, failed to reach levels induced by FCS alone. This suggests that some cells failed to be stimulated at all, or had been damaged/killed by hydrogen peroxide treatment.
Figure 3.7. \[^{3}H\]-thymidine incorporation assay showing dose response to H\(_{2}\)O\(_{2}\) at 18 h (A) and 24 hr (B) in BAEC. Cells were plated at 1 \times 10^4 cells/ml on to 24 well plates for 24 h in 16% (v/v) FCS. Cells were made quiescent in 0.1% (v/v) FCS for 2-3 days. Cells were stimulated with 5% (v/v) FCS and treated with 0, 10, 25 or 50 \(\mu\)M H\(_{2}\)O\(_{2}\) 30 min prior to FCS addition. \[^{3}H\]-thymidine incorporation was measured in 2 h windows as described previously for (A) 18 h and (B) 24 h after stimulation. * \(p<0.05\) ** \(p<0.01\), *** \(p<0.005\) to 5% (v/v) FCS.
Fig 3.8. $[^{3}H]$-thymidine incorporation assay showing dose response to $H_2O_2$ at 18 h (A) and 24 hr (B) in HAEC. Cells were plated at $1 \times 10^4$ cells/ml on to 24 well plates for 24 h in 16% (v/v) FCS. Cells were made quiescent in 0.1% (v/v) FCS for 2-3 days. Cells were stimulated with 5% (v/v) FCS and treated with 0, 10, 25 or 100 μM $H_2O_2$ 30 min prior to FCS addition. $[^{3}H]$-thymidine incorporation was measured in 2 h windows as described previously for (A) 18 h and (B) 24h after stimulation.* p <0.05, ** p <0.01 compared to 5% (v/v) FCS.
Fig 3.9. [³H]-thymidine incorporation assay showing time course response to H₂O₂ in BAEC. Cells were plated at 1 x 10⁴ cells/ml on to 24 well plates for 24 h in 16% (v/v) FCS. Cells were made quiescent in 0.1% (v/v) FCS for 2-3 days. Cells were stimulated with 5% (v/v) FCS and treated with 0, 10, 25 or 50 μM H₂O₂ 30 min prior to FCS addition. [³H]-thymidine incorporation was measured in 2 h windows at various time points after stimulation, by liquid scintillation counting of trichloroacetic acid-insoluble material in the cell layer.
Results

Effects of hydrogen peroxide after FCS stimulation

Experiments were carried out to identify time points at which endothelial cells were most responsive to hydrogen peroxide. Growth arrested BAEC were stimulated with 5% (v/v) FCS, 10 μM or 25 μM H₂O₂ was then added at specific time points afterwards. Incorporation was measured 20 h after FCS stimulation, which corresponded to the maximum peak of DNA synthesis. Maximum inhibition (75%) of incorporation occurred when hydrogen peroxide (25 μM) was added at the same time as FCS, and inhibition continued within the first 12 h of the G₁ phase of the cell cycle (Figure 3.10). After 12 h, H₂O₂ was less effective in reducing DNA synthesis in these cultures. Similar kinetics were observed with 10 μM H₂O₂ although incorporation of isotope was inhibited to a lesser extent.

Species Differences

There were no observed differences between human or bovine endothelial cells in their response to H₂O₂. Human endothelial cells grew more slowly than bovine endothelial cells and were more sensitive to serum deprivation. Higher basal rates of [³H]-thymidine incorporation were often observed for human cells. This may be a result of the method used to render them quiescent (0.1% (v/v) FCS rather than serum free), and that human endothelial cells take longer to become quiescent under low serum conditions. Human endothelial cells had higher basal rates of proliferation than human smooth muscle cells. This may be accounted for by the fact that endothelial cell growth is affected by contact inhibition and in these experiments were plated at sub-confluent densities to allow further stimulation by FCS. When hydrogen peroxide was added to unstimulated endothelial cells in similar experiments, significant inhibition of basal levels of incorporation could still be observed (34%-62%) at early incubation periods. This suggests that hydrogen peroxide may have additional effects separate to its interference with the cells’ ability to respond to mitogen, or may inhibit cell cycle progression of those cells that were not already quiescent from serum deprivation, or is possibly toxic to cells that are further stressed by low serum conditions.
Figure 3.10. Effect of H$_2$O$_2$ on $[^3]$H-thymidine incorporation in BAEC after FCS stimulation. Cells were plated at $1 \times 10^4$ cells/ml on to 24 well plates for 24 h in 16% (v/v) FCS. Cells were made quiescent in 0.1% (v/v) FCS for 2-3 days. Cells were stimulated with 5% (v/v) FCS for 20 h. Cells were treated with 10 μM (▼) or 50 μM (■) H$_2$O$_2$ at various times after FCS stimulation, or FCS alone (●). $[^3]$H-thymidine incorporation was measured for the last 2 h of each time point as described previously. Values are plotted as a percentage of the maximum level of incorporation (FCS).
Results

Chapter 3

Cell Viability

Although 10, 25 and 50 μM hydrogen peroxide inhibited FCS induced $[^{3}\text{H}]-\text{thymidine}$ incorporation, these experiments do not allow a clear differentiation between possible cell death or inhibition of cell proliferation. The Hoechst assay quantifies total DNA within a population, and if reductions in DNA content are observed, they can be accounted for by a loss of cells after washing since non-viable cells are not adherent. This assay was utilised in later experiments involving smooth muscle cells.

Protein assays can be used similar to the Hoechst assay to compare the total cell number of treated and untreated cells. However, this assay does not give an indication of cell viability, rather a simple difference in total protein content due to loss of dead cells after washing.

Protein concentrations were also measured to assess the toxic effects of hydrogen peroxide on endothelial cells. Protein content can be used to determine cell number like the Hoechst assay, using standards of known cell number against OD values. In this experiment, protein content was assessed as a percentage change in OD from control, (untreated cells in 0.1% (v/v) FCS) for each time point (Figure 3.11). Serum stimulation caused a 50% increase in total protein content after 24 h. Pre-treatment with hydrogen peroxide up to a concentration of 50 μM did not affect the FCS induced increase in protein content. Since hydrogen peroxide did not significantly reduce the amount of protein in each well compared to control levels, it is likely that concentrations of $\text{H}_2\text{O}_2$ below 50 μM were not toxic to cells. Concentrations of $\text{H}_2\text{O}_2$ above 50 μM may have been toxic, since endothelial cells exposed to this concentration were unable to incorporate $[^{3}\text{H}]-\text{thymidine}$ after FCS treatment. 200 μM hydrogen peroxide certainly appeared to be toxic to endothelial cells since no recovery of the proliferative response was observed by 26 h (Figure 3.12a). Some papers suggest toxicity at greater than 50 μM (Spragg, 1991), others suggest 100 μM and above in other cell types (Barchowsky et al., 1987, 1989), however, sensitivity may indeed be cell and species specific.
Figure 3.11. Effect of H$_2$O$_2$ treatment on protein content in BAEC. Cells were plated at $1 \times 10^4$ cells/ml on to 24 well plates for 24 h in 16% (v/v) FCS then made quiescent in 0.1% (v/v) FCS for 2-3 days. Cells were stimulated with 5% (v/v) FCS and treated with 0, 10, 25 or 50 μM H$_2$O$_2$ 30 min prior to FCS addition. At various time points after FCS stimulation, plates were washed in PBS and assayed for total protein content using the method of Lowry et al., (1951), n.s.- not significantly different (p>0.05) compared to 5% (v/v) FCS. 100% was taken as the protein concentration of quiescent cells at time 0h.
Results

Chapter 3

A.

- 500-ftas a 5% FCS
- FCS + H2O
- 0.1% FCS

B.

- 5000-
- 4000-
- 2000-
- 1000-

Figure 3.12. Effects of 200 μM H2O2 on [3H]-thymidine incorporation following stimulation with FCS in BAEC (A) and BASMC (B). Cells were plated at 1 x 10^4 cells/ml on to 24 well plates for 24 h in 16% (v/v) FCS. Cells were made quiescent in 0.1% (v/v) FCS for 2-3 days. Cells were stimulated with 5% (v/v) FCS, treated with 200 μM H2O2 30 min prior to FCS addition, or left untreated in 0.1% (v/v) FCS. [3H]-thymidine incorporation was measured in 2 h windows as described previously at 18 h, 24 h, 36 h, and 40 h after stimulation. ** p<0.01, *** p<0.005, H2O2 treatment compared to 5% (v/v) FCS.
3.3.2.2 Smooth Muscle cells

Bovine aortic smooth muscle cells

It has previously been reported that smooth muscle cells respond differently to oxidative stress than endothelial cells. Rao and Berk (1992) showed that 200 μM hydrogen peroxide caused a significant increase in proliferation (a concentration that is toxic to endothelial cells) in rat aortic smooth muscle cells. Results of the work of Rao and Berk could not be confirmed in this present study using bovine aortic smooth muscle cells. Treatment with 200 μM H$_2$O$_2$ did not induce proliferation in the absence or the presence of FCS (Figure 3.12b), but inhibited the mitogenic response to FCS by 99%. This raises concern over the possible toxic effects of hydrogen peroxide at 200 μM. Work carried out using bovine aortic smooth muscle cells demonstrated that hydrogen peroxide at 200 μM inhibited proliferation in response to 5% FCS. However, hydrogen peroxide appeared to have a bi-phasic effect (Figures 3.13 and 3.14) on smooth muscle cells with a stimulatory effect at lower concentrations (10 μM). Experiments on bovine smooth muscle cells proved variable. Stimulation of proliferation (above serum levels) was seen in early passage of cells (p1-10) treated with low concentrations of hydrogen peroxide. In later passages (10-20), most concentrations of hydrogen peroxide tested were found to inhibit proliferation, and no stimulation of proliferation could be observed. Because the effects of hydrogen peroxide on smooth muscle cells may be species dependent, smooth muscle cells from other species were also investigated.

Rat and Human aortic smooth muscle cells

Rat and Human SMC were used to investigate whether hydrogen peroxide stimulates SMC proliferation in cells derived from different species. Again the importance of examining the events within a wide time frame was evident. Early time points indicated a dose dependant inhibition of FCS induced proliferation by hydrogen peroxide (Figures 3.15a and Figure 3.16a) similar to the effects observed in endothelial cells. However, from later time points (24 h), it was evident that the extent of thymidine uptake in cells pre-treated with H$_2$O$_2$ was similar to, or greater than, those treated with FCS alone (Figures 3.15b and 3.16b). These apparently discordant results were due to a delayed entry into S-phase as previously observed in endothelial cells. Figure 3.17 shows the time course of events for hydrogen peroxide treated RASMC’s. 5% (v/v) FCS stimulated S phase entry by 12 h after initial
treatment and maximum DNA synthesis occurred by 18 h, however, 100 µM hydrogen peroxide delayed this FCS induced S-phase entry by 8 h, with no incorporation of isotope until 20 h post addition. As a consequence, hydrogen peroxide shifted the peak of FCS induced DNA synthesis to 26 h. The delay seemed to be more marked in smooth muscle cells than in endothelial cells. Unlike endothelial cells, the delay was not accompanied by an overall reduction in thymidine incorporation for each concentration of hydrogen peroxide. At certain concentrations of hydrogen peroxide, peak uptake exceeded levels reached by FCS alone.
Figure 3.13. [³H]-thymidine incorporation assay showing dose response to H₂O₂ at 18 h (A) and 24 hr (B) in BASMC. Cells were plated at $1 \times 10^4$ cells/ml on to 24 well plates for 24 h in 16% (v/v) FCS. Cells were made quiescent in 0.1% (v/v) FCS for 2-3 days. Cells were stimulated with 5% (v/v) FCS and treated with 0, 10, 25 or 50 μM H₂O₂ 30 min prior to FCS addition. [³H]-thymidine incorporation was measured in 2 h windows as described previously after (A) 18 h and (B) 24 h after stimulation. * p < 0.05, *** p < 0.005 compared to 5% (v/v) FCS.
Results

Fig 3.14. $[^3]$H-thymidine incorporation assay showing time course responses to $\text{H}_2\text{O}_2$ in BASMC. Cells were plated at $1 \times 10^4$ cells/ml on to 24 well plates for 24 h in 16% (v/v) FCS. Cells were made quiescent in 0.1% (v/v) FCS for 2-3 days. Cells were stimulated with 5% (v/v) FCS and treated with 0, 10, 25 or 50 $\mu$M $\text{H}_2\text{O}_2$ 30 min prior to FCS addition. [3H]-thymidine incorporation was measured in 2 h windows at various time points after stimulation, by liquid scintillation counting of trichloroacetic acid-insoluble material in the cell layer.
Figure 3.15. [3H]-thymidine incorporation assay showing dose response to H2O2 at 18 h (A) and 24 hr (B) in RASMC. Cells were plated at 1 x 10^4 cells/ml on to 24 well plates for 24 h in 16% (v/v) FCS. Cells were made quiescent in 0.1% (v/v) FCS for 2-3 days. Cells were stimulated with 5% (v/v) FCS and treated with 0, 5, 50 or 100 μM H2O2 30 min prior to FCS addition. [3H]-thymidine incorporation was measured in 2 h windows as described previously after (A) 18 h and (B) 24 h after stimulation. * p<0.05, ** p<0.01, *** p<0.005 compared to 5% (v/v) FCS.
Figure 3.16. \[^{3}H\]-thymidine incorporation assay showing dose response to \( \text{H}_{2}\text{O}_{2} \) at 16 h (A) and 24 hr (B) in HASMC. Cells were plated at \( 1 \times 10^{4} \) cells/ml on to fibronectin coated 24 well plates for 24 h in 16% (v/v) FCS. Cells were made quiescent in 0.1% (v/v) FCS for 48 h. Cells were stimulated with 5% (v/v) FCS and treated with 0, 10, 25, 50 or 100 \( \mu \text{M} \) \( \text{H}_{2}\text{O}_{2} \) 30 min prior to FCS addition. \[^{3}H\]-thymidine incorporation was measured in 2 h windows as described previously after (A) 16 h and (B) 24 h after stimulation. ** \( p < 0.01 \), *** \( p < 0.005 \) compared to FCS control.
Fig 3.17. [³H]-thymidine incorporation assay showing time course responses to H₂O₂ in RASMC. Cells were plated at 1 × 10⁴ cells/ml on to 24 well plates for 24 h in 16% (v/v) FCS. Cells were made quiescent in 0.1% (v/v) FCS for 2-3 days. Cells were stimulated with 5% (v/v) FCS and treated with 0, 5, 50 or 100 µM H₂O₂ 30 min prior to FCS addition. [³H]-thymidine incorporation was measured in 2 h windows at various time points after stimulation, by liquid scintillation counting of trichloroacetic acid-insoluble material in the cell layer.
**FACS analysis**

To confirm the delay effects observed with $[^3]$H-thymidine incorporation studies, cell-cycle analysis was performed using flow cytometry to assess the percentage of cells in $G_0/G_1$ and S-phase after hydrogen peroxide and FCS treatment. Table 3.1 shows the DNA profiles for each phase of the cell cycle. In quiescent cells, the percentage of cells held in $G_0/G_1$ was 90% of the cell population and 3.5% were in S-phase. After 18 hours of FCS stimulation, the percentage of cells in the resting phase $G_0$ had decreased to 43%, and those in S-phase had increased to 42% while the percentage of cells in $G_2/M$ was not significantly changed. Treatment of the growing culture with hydrogen peroxide and FCS delayed S-phase transition of the cells, 63.3% of cells were still in $G_0$ and only 24.5% of cells were in S-phase after 18 hours. After 36 hours of FCS stimulation, the percentage of cells in S-phase had decreased from 43% to 27% and those in $G_0$ were 64%. However, when hydrogen peroxide was added before FCS stimulation the number of cells in S-phase after 36 hours had increased to 35% and the number in $G_0$ had decreased to 52%.

**3.3.2.3 Cell Viability**

**Cell Number**

To assess the toxic effects of hydrogen peroxide, total cell counts were measured after treatment with 100 µM and 200 µM H$_2$O$_2$ for 24 hours in the presence or absence of serum. Table 3.2 shows HASM cell number and percentage change from control (cells cultured in 0.1% (v/v) FCS containing medium for 24 h). FCS treatment caused a small increase in cell number over 24 h. It was not expected that a large increase in cell number would be observed, since it is known from earlier studies that S-phase occurs around 24 h in these cells. Cell division (M-phase) would occur later (up to 30). In the presence of serum, cell numbers after 100 µM H$_2$O$_2$ treatment did not differ from control, whilst 200 µM H$_2$O$_2$ treatment resulted in a small reduction in cell number (12.1%). Cell numbers decreased with both concentrations of H$_2$O$_2$ without serum by 6.9% and 20.7% respectively compared to controls. The reduction in cell number associated with H$_2$O$_2$ treatment in the absence of serum may be due to toxicity, or further inhibition of cell division. If a small percentage of cells had undergone cell division even in the absence of serum (those cells that had already passed G$_0$/G$_1$), then hydrogen peroxide may have delayed these cells from further division. Serum appeared to afford some protection to the inhibitory effects of H$_2$O$_2$ or simply
allowed more cells to proliferate. Treatment with 200 μM H$_2$O$_2$ did reduce cell number, which was somewhat expected, since in all proliferation studies this concentration failed to stimulate DNA synthesis when incubated with FCS.

**Hoechst Assay**

To investigate the possible toxic effects of H$_2$O$_2$ on SMC, and to confirm that increases in thymidine uptake were not due to DNA repair mechanisms, Hoechst assays were performed. Treatment with 100 μM H$_2$O$_2$ did not affect total DNA content for up to 56 h after treatment (Figure 3.18). No significant increases in DNA content were observed in either FCS or FCS plus H$_2$O$_2$ treated cells until 40 h after treatment. This suggests that the increase in thymidine uptake was due to mitogenesis and not DNA repair, since increases in DNA synthesis occur within 3 h of DNA damage (Cattley et al., 1988).

**MTT Assay**

Further assessments of cellular viability were made using the MTT assay which measures mitochondrial activity (Methods, Chapter 2). A range of concentrations of hydrogen peroxide (1-50 μM) were added to cells before FCS stimulation. Activity was measured after 3 h treatment to assess any early toxic effects and again 24 h later. Hydrogen peroxide caused a significant dose dependant increase in MTT activity compared to control (5% (v/v) FCS), after 3 h of treatment (Figure 3.19) in BASMC. By 24 h, MTT activity was similar to control values. In RASMC (Figure 3.20) H$_2$O$_2$ plus FCS treated cells had a slightly lower MTT activity than FCS alone, but still achieved a 20% increase in activity over control cells (0.1% (v/v) FCS). It is unlikely that the increase in activity observed, was due to an increase in cell number between 3 and 24 h. An increase in mitochondrial activity is possibly due to greater energy metabolism. These results show that 1-50 μM H$_2$O$_2$ treatment in BASMC and RASMC does not impair mitochondrial function.
Table 3.1. **Cell cycle analysis of quiescent, FCS and \( \text{H}_2\text{O}_2 \) treated HASMC using FACS.** Cells were plated at \( 5 \times 10^4 \) cells per well in 6 well plates and made quiescent with 0.1% (v/v) FCS for 48 h. Cells were then treated with 100 μM \( \text{H}_2\text{O}_2 \) and/or 5% (v/v) FCS for 18 and 36 h. After treatment, cell nuclei were isolated and stained with propidium iodide immediately before FACS analysis. Cell cycle analysis was assessed using Cell-FIT software (Becton Dickinson).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>18 Hours</th>
<th>36 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2 )</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{G}_0\text{G}_1 )</td>
<td>90.0</td>
<td>43.0</td>
</tr>
<tr>
<td>S</td>
<td>3.5</td>
<td>42.3</td>
</tr>
<tr>
<td>( \text{G}_2\text{+M} )</td>
<td>6.5</td>
<td>13.7</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3.2. **Effect of \( \text{H}_2\text{O}_2 \) on cell number in HASMC after 24 h treatment.** Cells were plated at \( 5 \times 10^4 \) cells per well in 6 well plates. Cells were made quiescent by incubating with 0.1% (v/v) FCS for 48 h. After treatment for 24 h, cells were rinsed twice with HBSS and trypsinised with 0.25% (w/v) trypsin, 0.05% (w/v) EDTA, and cell number assessed after staining with trypan blue dye using a haemocytometer. Control (0.1% (v/v) FCS).

<table>
<thead>
<tr>
<th>24 h Treatment</th>
<th>24 h Treatment</th>
<th>24 h Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>16% FCS</td>
<td>118</td>
<td>Control</td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2 ) 100 μM</td>
<td>100</td>
<td>( \text{H}_2\text{O}_2 ) 100 μM</td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2 ) 200 μM</td>
<td>87.9</td>
<td>( \text{H}_2\text{O}_2 ) 200 μM</td>
</tr>
</tbody>
</table>
Figure 3.18. Hoechst assay showing the effects of FCS and H$_2$O$_2$ on DNA content over time in HASMC. Cells were plated at $1 \times 10^4$ cells/ml on to fibronectin coated 96 well plates for 24 h in 16% (v/v) FCS. Cells were made quiescent in 0.1% (v/v) FCS for 48 h. Cells were treated with either 5% (v/v) FCS (▲), or 5%(v/v) FCS with 100 μM H$_2$O$_2$ added prior to FCS addition (■), or left untreated (0.1% (v/v) FCS) (▼). Cells were treated for various time points before being assayed for DNA content with Hoechst 33258.
Figure 3.19. MTT assay showing the effects of H$_2$O$_2$ on mitochondrial activity after 3 h (A) and 24 h (B) treatment in BASMC. Cells were plated at $1 \times 10^4$ cells/ml on to 6 well plates for 24 h in 16% (v/v) FCS. Cells were made quiescent in 0.1% (v/v) FCS for 2-3 days. Cells were treated with 5% (v/v) FCS alone (control) or with a range of concentrations of H$_2$O$_2$ added 30 min prior to FCS addition. Cells were treated for either (A) 3 h or (B) 24 h before being assayed for MTT activity. Values are plotted as the percentage change from control values.
Figure 3.20. MTT assay showing the effects of H₂O₂ on mitochondrial activity after 24 h treatment in RASMC. Cells were plated at 1 × 10⁴ cells/ml on to 6 well plates for 24 h in 16% (v/v) FCS. Cells were made quiescent in 0.1% (v/v) FCS for 2-3 days. Cells were treated with 5% (v/v) FCS alone or with 100 μM H₂O₂ added 30 min prior to FCS addition or left untreated (0.1% (v/v) FCS). Cells were treated for 24 h before being assayed for MTT activity. Values are plotted as the percentage change from control values (0.1% (v/v) FCS). * p< 0.05 compared to control.
3.3.3 THE EFFECTS OF HYDROGEN PEROXIDE ON CELL DENSITY

The effects of increasing cell densities on hydrogen peroxide treatment were investigated in both endothelial and smooth muscle cells. Confluent cells were trypsinised and plated into 24 well culture dishes at a range of cell densities. Cells were stimulated for 16 hours with 5\% (v/v) FCS then treated with 50 μM hydrogen peroxide for a further 6 hours. [3H]-thymidine incorporation was measured for the last 2 hours of the experiment.

3.3.3.1 Endothelial Cells

As expected, endothelial cells showed a density dependant response to stimulation by FCS (Figure 3.21a). As the density of the cells increased, the extent of incorporation also increased up to $6 \times 10^4$ cells/ml. Further increases in the number of cells plated were not accompanied by further increases in thymidine incorporation. A decrease in thymidine uptake was observed at $25 \times 10^4$ cells/ml, possibly because these cells may have been partially contact inhibited. Hydrogen peroxide caused a density related inhibition of [3H]-thymidine incorporation. The greatest inhibitory effects were observed at the lowest cell densities. This may have been because cells plated at lower densities were not affected by density dependant inhibition and continued to proliferate at a high rate and hence were more susceptible to the effects of hydrogen peroxide.

3.3.3.2 Smooth muscle cells

In smooth muscle cells, FCS produced a linear increase in cell proliferation with increasing densities of cells, since these cells are not influenced to such a great extent by contact inhibition (Figure 3.21b). Hydrogen peroxide caused a reduction in proliferation at all cell densities except for the highest density ($25 \times 10^4$ cells/ml). Possible reasons for cell mediated protection from hydrogen peroxide include: Diluted effects of hydrogen peroxide on increasing numbers of cells. There are more cells to produce catalase and secrete it into the medium, therefore hydrogen peroxide stability in the medium is reduced and results in less cell damage.
Results Chapter 3

A.

![Bar graph showing the effects of H₂O₂ on different cell densities in BAEC (A) and BASMC (B).](image)

B.

![Bar graph showing the effects of H₂O₂ on different cell densities in BAEC (A) and BASMC (B).](image)

Figure 3.21. [³H]-thymidine incorporation assay showing the effects of H₂O₂ on different cell densities in BAEC (A) and BASMC (B). Cells were plated at different densities on to 24 well plates for 16 h in 5% (v/v) FCS. Cells were treated for a further 6 h with or without 50 μM H₂O₂. [³H]-thymidine (1 μCi/ml) was added for the last 2 h of the experiment and measured as described previously.
3.4 Discussion

Endothelial cell injury and smooth muscle cell migration and proliferation are considered to be essential events in the development of atherosclerosis. Many factors including oxidative stress have been postulated to cause endothelial injury. Concomitant with endothelial injury is the migration and proliferation of smooth muscle cells from the underlying media into the intima. Oxidative stress may have direct effects on smooth muscle cells, or indirect influences e.g. from endothelial injury and release of growth stimulating factors, or the influence of neighbouring cells such as macrophages resulting in the induction of proliferation and migration of smooth muscle cells. Studies were carried out to determine the growth stimulatory and inhibitory effects of hydrogen peroxide on vascular smooth muscle and endothelial cells.

The effects of hydrogen peroxide on vascular smooth muscle and endothelial cells were investigated. Little evidence of cell proliferation or indeed gross cytotoxicity was found. Inhibition of \(^{3}\text{H}\)-thymidine incorporation was observed in all cell types tested. This inhibitory effect was dose dependant. Both human and bovine endothelial cells were more sensitive to the effects of \(\text{H}_2\text{O}_2\) than smooth muscle cells of the same species.

**Endothelial Cells**

When complete time courses of \(\text{H}_2\text{O}_2\) treatment were carried out, it became evident that the inhibition of \(^{3}\text{H}\)-thymidine incorporation that was initially observed, was not due to inhibition of DNA synthesis but rather a delay in transition through the cell cycle, compared to controls. Hydrogen peroxide treatment reduced DNA synthesis by up to 60% by 24 h, however this inhibition was transient since incorporation was then observed 2-4 h later in a similar pattern to control cells. The delay was dose dependant with increasing concentrations of \(\text{H}_2\text{O}_2\) further delaying cell cycle transition into S-phase. In endothelial cells, this delay was accompanied by an overall reduction in DNA synthesis for each concentration of \(\text{H}_2\text{O}_2\), which suggests that some cells failed to be stimulated at all, or had become damaged.

Experiments were carried out to investigate if there was a particular time interval at which endothelial cells were most responsive to \(\text{H}_2\text{O}_2\). Maximum inhibition occurred when \(\text{H}_2\text{O}_2\)
was added before FCS stimulation. Inhibition continued within the first 12 h after stimulation, although the extent of inhibition was reduced with time. After 12 h, hydrogen peroxide was less effective in inhibiting DNA synthesis. This suggests that hydrogen peroxide is inhibiting the cell cycle in G1, since S-phase starts soon after 12 h in these cells and by this time hydrogen peroxide is less effective.

Cell viability was assessed, and no significant changes in total protein content were observed for H$_2$O$_2$ concentrations between 10 and 50 μM. Concentrations above 50 μM are thought to be toxic to endothelial cells. 200 μM certainly showed toxic effects since cells failed to incorporate labelled thymidine within the 26 h time course. This is in agreement with previous reports which have shown toxicity at greater than 50 μM or 100 μM in endothelial cells (Spragg, 1991).

**Smooth Muscle Cells**

Hydrogen peroxide failed to stimulate proliferation in smooth muscle cells. Despite numerous attempts to repeat the work of Rao and Berk, (1992), hydrogen peroxide did not stimulate DNA synthesis alone or in combination with FCS. Low levels of stimulation above FCS levels were observed in BASMC at low concentrations of hydrogen peroxide, but this failed to reach significance. Intermediate concentrations had little effect on DNA synthesis and higher concentrations caused inhibition of DNA synthesis. Again this inhibition was caused by a delay in the ability of cells to enter S-phase and not simply a reduction in the number of cells undergoing proliferation. Like endothelial cells, the effects were dose dependant.

Smooth muscle cells were less sensitive to H$_2$O$_2$ and could tolerate concentrations up to 150 μM without any noticeable toxicity. In RASMC, 150 μM H$_2$O$_2$ treatment delayed DNA synthesis until 26 h after FCS stimulation compared to 18 h in cells treated with FCS alone. At this concentration, hydrogen peroxide delayed the cell cycle transition into S-phase by 8 h, without any deleterious effects to the cells. Stimulation of DNA synthesis was then observed comparable with control levels.

Unlike endothelial cells, the delay observed with smooth muscle cells was not accompanied by an overall reduction in DNA synthesis, and in some cases exceeded levels
Discussion

reached by FCS alone. This again reflects the reduced sensitivity of smooth muscle cells to hydrogen peroxide. Cell cycle analysis using flow cytometry confirmed hydrogen peroxide effects on delaying cell cycle transitions. After 24 h of FCS stimulation, 42% of cells were in S-phase compared to 26.8% when H$_2$O$_2$ was added before stimulation. The contrasting reports in the literature regarding the effect of hydrogen peroxide on cell growth, may be due to the conditions employed, which are not always similar. Hydrogen peroxide effects vary depending on the type, species and density of cells, the concentration and duration of H$_2$O$_2$ exposure (Wiese et al., 1995), as well as the type of culture medium, and whether the cells are quiescent or proliferating.

Such variations in the cellular response to oxidative stress leads to the question as to what determines whether cells are sensitive to damage by hydrogen peroxide or are induced to proliferate? One would expect that poor antioxidant defence mechanisms i.e. an inability to remove hydrogen peroxide efficiently from the cell would result in increased sensitivity to damage. It might be inferred that the balance between the concentration of oxidant present and the ability of the cell to deal with this stress through antioxidant enzymes or free radical scavengers, is critical in determining whether cell proliferation occurs, or indeed, if cytotoxicity becomes manifest.

The concentration required to affect cellular activities in different cell types can vary considerably. For example, to induce expression of competence gene family mRNAs and subsequent cell proliferation, H$_2$O$_2$ concentrations from 0.1 to 0.2 mM have been reported to be sufficient (Shibanuma et al., 1990). Higher concentrations (1 to 3 mM) have been found to mimic the effects of insulin on protein tyrosine phosphorylation in hepatocytes and other cell lines (Heffetz et al., 1990; Hadari et al., 1992). Maximal activation of the transcription factor NFkB and c-jun mRNA induction in some cell lines requires 0.1-0.25 and 0.25 mM H$_2$O$_2$, respectively (Schreck et al., 1991; Devary et al., 1991; Meyer et al., 1993). This variation is most likely due to differences in persistence of H$_2$O$_2$ in the culture medium, and is governed principally by the rate of intracellular H$_2$O$_2$ metabolism (Schraufstatter et al., 1988), the release of antioxidants from the cell into the medium, and the amount or composition of serum components (Link and Riley, 1988). The rate at which H$_2$O$_2$ is removed from the medium reduces the effective concentration of H$_2$O$_2$ and possibly explains the reported differences in concentrations needed for maximum effects.
Discussion

Chapter 3

(Barker et al., 1994). The half-life of H$_2$O$_2$ in the presence of cells is 35 minutes in PBS (Chubatsu and Meneghini, 1993). The presence of more complex culture media may alter the turnover rate of hydrogen peroxide.

Although several investigators have observed proliferative effects of hydrogen peroxide on smooth muscle cells, there are a number of possible reasons for the observed discrepancies in results. The increase in proliferation observed in RASMC after H$_2$O$_2$ treatment by Rao and colleagues was only two-fold. Curiously, the addition of catalase to these cells did not reverse H$_2$O$_2$ effects but instead increased the proliferative response. Shibanuma et al., (1990) reported that H$_2$O$_2$ alone does not induce DNA synthesis, but when co-incubated with insulin will synergistically enhance the rate of proliferation. Many investigators have used cell number as an index of proliferation and continued studies for up to 7 days after treatment, but this is impractical for incorporation experiments. Further experiments including later time points may indeed show stimulation of H$_2$O$_2$ treated cells above those of FCS. However, upon stimulation, cells become less synchronous with time, and incorporation assays require a synchronised population of cells to be effective in measuring proliferation rates. Other investigators have used much higher concentrations of H$_2$O$_2$ to demonstrate a stimulatory effect, yet these concentrations were toxic to the cells used in the present studies. This may reflect subtle differences in culture conditions, passage number, or cell isolates.

Wiese et al., (1995) have previously shown that toxic effects of hydrogen peroxide are dependent on cell density, the culture media and the duration of exposure using HA-1 cells. At cell concentrations below $10^6$ cells/60 mm dish, H$_2$O$_2$ toxicity was inversely related to cell density, whereas cell density was not an important factor at cell concentrations of $10^6$ cells/60-mm dish and above. The effects of increasing cell density on H$_2$O$_2$ treatment were investigated to see if increasing numbers of cells would offer some protection to the whole population against H$_2$O$_2$ treatment. Endothelial cells were more susceptible to hydrogen peroxide at lower densities, where cells would be proliferating. However, as the number of cells increased, the rate of proliferation was reduced through density dependant inhibition, and the effects of hydrogen peroxide were not as dramatic. Smooth muscle cells, which are not affected by density dependant inhibition, were susceptible to hydrogen peroxide induce inhibition of DNA synthesis at all cell densities except $25 \times 10^4$ cells/ml. This is most
Discussion

probably due to a dilution in the amount of hydrogen peroxide per cell, and many
investigators discuss concentrations of H\textsubscript{2}O\textsubscript{2} in relation to cell number. An increase in the
total amount of catalase secreted into the medium will account for a faster metabolism of
H\textsubscript{2}O\textsubscript{2} from the medium and enhanced protection. In bacteria, similar findings have been
reported. Ma and Eaton, (1992) noted that catalase offered the greatest amount of
protection at higher bacterial density, and concluded that at increased cellular density, H\textsubscript{2}O\textsubscript{2}
has poor access to individual cells.

Previous investigators have reported that very low concentrations of H\textsubscript{2}O\textsubscript{2} (3-15 \textmu M) can
stimulate growth and proliferation, low concentrations (120-150 \textmu M) induce a transient
adaptive response, whereas intermediate concentrations (250-400 \textmu M) cause permanent
loss of replicative or divisional competence, and high concentrations (1 mM) result in cell
death (Wiese \textit{et al.}, 1995). The results in this present study are in general agreement with
respect to low and intermediate concentrations, where low concentrations (25-150 \textmu M)
cause a transient compromise in cell division, and intermediate concentrations (200 \textmu M
and above) cause a permanent loss of replicative function. However, stimulation of growth
and proliferation of endothelial and smooth muscle cells with very low concentrations
could not be unambiguously confirmed in the present studies.

Although reactive oxygen species can cause DNA damage and stimulate DNA repair
mechanisms, this is unlikely to explain the H\textsubscript{2}O\textsubscript{2} induced \[^{3}\text{H} \text{-thymidine incorporation in}
cells after the initial delay. Firstly, hydrogen peroxide caused no decrease in MTT activity
or an increase in the number of cells that failed to exclude trypan blue. Secondly, hydrogen
peroxide has been shown to induce short patch DNA repair (Pero \textit{et al.}, 1990) and unlike
agents such as N-acetoxy-2-acetylaminofluorene, causes only small increases in
unscheduled DNA synthesis (DNA synthesis due to repair not replication). Thirdly,
although hydrogen peroxide has been shown to increase unscheduled DNA synthesis
(e.g. in hepatocytes) concentrations above 1 mM were required, and DNA synthesis
occurred within 3 h (Cattley \textit{et al.}, 1988) compared to 12-18 h in these studies. Thus,
delayed induction of \[^{3}\text{H} \text{-thymidine incorporation appears to be primarily due to FCS
induced cell proliferation rather than DNA repair mechanisms.
Wiese et al. (1995) also observed that hydrogen peroxide appeared to cause a delay in cell cycle transition, the duration of which was related to hydrogen peroxide concentration and could be diminished by pre-conditioning. They observed an 18 h cell cycle for HA-1 cells, 4 h S-phase, 3 h G2 phase, 1 h M phase, 10 h G1. Treatment of the cells with H$_2$O$_2$, caused a lengthening of the cell cycle to approximately 26 h, i.e. 8 hr lag phase. Emerging from H$_2$O$_2$ induced growth arrest, the cells immediately appeared to re-establish their normal cell cycle and division time.

Hydrogen peroxide may be toxic to mammalian cells, but at the concentrations used in the current study (1 to 150 μM), cytotoxicity manifests itself as a transient inhibition of cell growth and division, rather than a necrotic cell destruction. Until recently, this selective compromise of cell division had only been reported in E. coli (McKenna and Davies, 1988) and yeast (Davies et al., 1995). Clearly, if one exposes mammalian cells to extremely high concentrations of H$_2$O$_2$ (1 mM), particularly at low cell densities, necrotic cell death will occur, but the physiological significance of such effects is not yet clear. Exposure of BASMC to very low concentrations of H$_2$O$_2$ resulted in stimulation of cell growth above control values, although this failed to reach statistical significance. However the main finding of this study was that relatively low concentrations of H$_2$O$_2$ causes a transient delay of cell growth and proliferation. Studies have shown that this transient delay is important in the cell’s adaptive response and can prevent further insults of hydrogen peroxide from being damaging (Wiese et al., 1995). Thus adaptation to the oxidative stress caused by hydrogen peroxide appears to be an important mechanism by which mammalian cells can cope with fluctuating levels of oxidants generated by their own respiration, by auto-oxidation, by the oxidative burst of neighbouring cells, or by metabolism of a wide variety of H$_2$O$_2$ producing drugs and toxins.

Although our understanding of bacterial adaptation to oxidative stress is far from complete, it is well advanced in comparison with eukaryotic studies. Limited information is available on adaptation to oxidative stress in yeast (Collinson and Dawes, 1992; Davies et al., 1993a, 1994), and few published investigations appear to have studied possible adaptation mechanisms to oxidative stress in mammalian cell lines (Spitz et al., 1987; Lu et al., 1993). What are the adaptive responses observed in H$_2$O$_2$ induced cell cycle delay in vascular
smooth muscle cells and endothelial cells, and what is their relevance to the pathogenesis of atherosclerosis and restenosis?

Transient cell cycle delay has previously been reported in endothelial and smooth muscle cells following treatment with TGF-β. Cell cycle analysis indicated that after 24 hours of exposure to TGF-β, the cells were blocked from entering S-phase, and the fraction of cells in G1 was increased (Heimark et al., 1986). Results of recent studies have suggested that TGF-β may also have delayed effects on smooth muscle cell growth. Stouffer and Owens (1994) have shown that TGF-β delayed the mitogenic effects of EGF, bFGF, and PDGF-BB. After the delay, TGF-β synergistically enhanced proliferation when co-incubated with the other mitogens. Studies by Kim et al (1993) show that the delayed cell cycle progression by TGF-β is correlated with altered regulation of growth-related gene expression when compared to EGF induced proliferation. These studies suggest that several adaptive responses are involved: 1) possible interference with receptor signalling systems, 2) interference with cell cycle mechanisms, 3) secondary stimulation by the autocrine/paracrine production of growth factors and/or growth factor receptors.

Transient cell cycle delay is a common response to DNA damage, where DNA replication is prevented until all repair mechanisms have been instituted. Cell cycle check points allow the detection and subsequent repair processes to be carried out after DNA damage. Further check points will detect when repair is complete and will allow the cell to re-enter the cycle. However, hydrogen peroxide and TGF-β treatment of cells, do not induce DNA damage, yet still cause a delay in the transition from G1 to S-phase, only recently has the precise mechanisms of this inhibition of S-phase transition been understood. By identifying the mechanisms involved in TGF-β induced cell cycle delay, we may also understand the mechanisms by which hydrogen peroxide induces cell cycle delay.

Several observations related to TGF-β effects on growth restriction led many researchers to hypothesise that reactive oxygen species (ROS) may have a role in TGF-β and other growth factor-mediated responses. Several studies have shown an increase in H₂O₂ production by cells treated with TGFβ, (Das et al., 1991; White et al., 1992; Thannickal et al., 1993). Shibanuma et al. (1991), proposed that hydrogen peroxide release by mouse osteoblastic cells is a signal for cell cycle dependent inhibition of DNA synthesis by TGF-
Insulin has been shown to activate a membrane-bound NADPH oxidase enzyme capable of generating hydrogen peroxide and contributing to signal transduction (Krieger-Brauer and Kather, 1992). PDGF and the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) also caused release of H$_2$O$_2$, whilst catalase inhibited the stimulation of DNA synthesis induced by PDGF and TPA (Shibanuma et al., 1990). Finally, TNF-α and IL-1β have also been shown to stimulate cells to produce active oxygen species (Beutler and Cerami, 1988; Meier et al., 1989).

As well as detecting their presence in response to growth factor treatment, other investigators have demonstrated that active oxygen species stimulate growth factor-related intracellular signals, such as Na$^+$-H$^+$ exchange (Shibanuma et al., 1988), and c-fos and c-myc mRNA expression (Crawford et al., 1988). Quiescent mesangial cells are thought to produce the equivalent of 10$^{-8}$ M H$_2$O$_2$, which may rise to 10$^{-6}$ M (or higher) during inflammation (Baud et al., 1981). That 10$^{-6}$ M is able to modulate cell growth suggests that it acts as a signal to affect various cellular pathways. It is also tempting to speculate that hydrogen peroxide may induce other growth factors such as PDGF. Montisano and colleagues have shown that hydrogen peroxide can induce growth factor production, they showed that PDGF mRNA expression was increased in pulmonary endothelial cells after exposure to hydrogen peroxide (Montisano et al., 1992).

It now seems reasonable to believe that reactive oxygen species could function as physiological second messengers in physiological conditions. Nitric oxide is known to be a messenger molecule regulating immune function and blood vessel dilatation. It also serves as a neurotransmitter in the brain and the peripheral nervous system (Lowenstein and Snyder, 1992). Evidence now suggests that hydrogen peroxide could be another active oxygen species that operates as a second messenger in growth factor signalling.
3.5 Summary

Direct effects of \( \text{H}_2\text{O}_2 \) treatment in vascular cells have been investigated and it has been confirmed that hydrogen peroxide plays an important yet complex role in modulating cell proliferation. Hydrogen peroxide treatment appears to inhibit mitogen induced proliferation in both smooth muscle cells and endothelial cells. However, upon closer investigation, this inhibition is due to a delay in the FCS-induced cell cycle initiation. This delay is transient since cells recover and continue to progress through the cell cycle and incorporate thymidine. The recovery of endothelial cells through the cell cycle is slightly less than for smooth muscle cells, which suggests that endothelial cells are more sensitive to hydrogen peroxide effects than smooth muscle cells. The mechanisms involved in \( \text{H}_2\text{O}_2 \) induced cell cycle delay are unknown, but are likely to play an important role in adaptation responses to stress. The following chapter describes experiments in which a possible role for hydrogen peroxide as a second messenger and its involvement in the activation of NFkB, an important transcription factor known to be a key component in the regulation of cell growth and proliferation was investigated.
Chapter 4

THE EFFECTS OF HYDROGEN PEROXIDE ON NFKB ACTIVATION IN VASCULAR SMC
4.1 Introduction

Eukaryotic cells have developed various mechanisms to deal with oxidative stress. The mechanisms controlling this adaptation to oxidative stress remain unclear, but probably resemble mechanisms observed in bacterial cells. The previous chapter presented data showing that hydrogen peroxide induces a temporary delay in the FCS-induced cell cycle progression of smooth muscle cells. Evidence suggests that this delay may be important in the cells adaptation to oxidative stress (Wiese et al., 1995).

A number of growth factors have been shown to result in the generation of hydrogen peroxide and other reactive oxygen species and several growth factors appear to require the presence of ROS in order to elicit their growth factor responses (Beutler and Cerami, 1989; Shibanuma et al., 1991; Sundaresen et al., 1995). This chapter reports on experiments designed to investigate the possible role of reactive oxygen species as second messengers, specifically the effects of hydrogen peroxide on transcription factor activation.

There is good evidence suggesting that reactive oxygen species may mediate the activation of NFkB. Treatment of some cells with H2O2 can activate NFkB (Schreck et al. 1992a, 1992b) and this can be blocked by treatment with certain antioxidants such as NAC or PDTC which block the signal induced phosphorylation of IkB (Beg et al., 1993; Henkel et al., 1993; Sun et al., 1993). In addition, cells induced to overexpress antioxidant enzymes such as glutathione peroxide also prevent IkB phosphorylation and subsequent degradation (Kretz-Remy et al., 1996).

The majority of investigations into NFkB activation have been carried out in haematopoietic lineage cells, particularly in mature B cells, thymocytes, and macrophages, since NFkB is primarily noted for its involvement in immune and inflammatory responses. Investigations of H2O2 induced NFkB activation have so far only extended to studies in endothelial cells (Barchowsky et al., 1995), Jurkat T cells (Schreck et al., 1991), and the human epithelial carcinoma cell line HeLa (Meyer et al., 1993), without a suggestion of NFkB activation mechanisms. Likewise no evidence has been obtained for the presence of IkB in smooth muscle cells, or IkB activation by H2O2. Only indirect evidence exists showing that antioxidant treatment inhibits TNFα-induced NFkB activation by inhibiting
IkB-α phosphorylation and degradation (Beg et al., 1993; Henkel et al., 1993; Sun et al., 1993; Kretz-Remy et al., 1996). Using electrophoretic mobility shift assays, a constitutively expressed NFκB-like activity was identified in both bovine and human smooth muscle cells (Lawrence et al., 1994). This constitutive expression could be reduced with antioxidant treatment (Bellas et al., 1995). Hitherto, this is the only direct evidence that NFκB activation occurs in smooth muscle cells, but it does not elucidate the mechanisms involved in NFκB activation, or to the nature of the heterodimer.

4.2 Aim

No evidence has been presented to date of NFκB activation in smooth muscle cells by extracellular stimuli such as TNF-α and reactive oxygen species. In addition, there is little evidence to suggest that IkB-α or any other inhibitory proteins exist in smooth muscle cells, let alone what regulatory function they may possess. Therefore, evidence for NFκB activation and for a role of IkB regulation in human umbilical artery smooth muscle cells was sought. The effects of hydrogen peroxide and TNF-α on NFκB activation was investigated using immunoblotting and immunofluorescent techniques using specific antisera to IkB-α and IkB-β. Western blot analysis was performed to detect the presence and investigate the regulation of IkB-α and IkB-β in smooth muscle cells by monitoring their degradation and regeneration upon induction by TNF-α and H₂O₂.
4.3 Results

4.3.1 WESTERN BLOT STUDIES

4.3.1.1 \(\text{IkB-}\alpha\) degradation and regeneration

Activation of NF\(\kappa\)B from cytoplasmic pools occurs temporally with proteolytic degradation of the inhibitory protein IkB-\(\alpha\). Nearly complete degradation of IkB-\(\alpha\) occurs within minutes after administration of extracellular stimuli such as TNF-\(\alpha\) or PMA in many cell types, an observation that has now been reported by many laboratories (Beg et al., 1993; Brown K, et al., 1993, Sun et al., 1993). Studies were carried out to investigate the role of IkB-\(\alpha\) in smooth muscle cells and to compare the effects of hydrogen peroxide and TNF-\(\alpha\) on IkB-\(\alpha\) degradation. All cell lysates were applied to SDS-polyacrylamide gels in duplicate (5 \(\mu\)l and 10 \(\mu\)l samples), and equal loading was assessed using anti-\(\alpha\)-actin antibody staining.

Effects of TNF-\(\alpha\) on IkB-\(\alpha\) degradation and re-synthesis

Human aortic smooth muscle cells were stimulated with 10 ng/ml of TNF-\(\alpha\) for various time intervals, after which cells were harvested and proteins solubilised in SDS sample buffer (see appendix II) at 4 °C, and used in Western blot analysis (Chapter 2). Figures 4.1a and 4.1b show typical representative immunoblots using an antibody directed against the 37 kDa IkB-\(\alpha\). Initially treatments were carried out for 0, 15 min, 30 min, 1 h, 2h after stimulation with TNF-\(\alpha\) (Figure 4.1b), however it became evident that IkB-\(\alpha\) degradation began as little as 5 minutes after treatment, therefore, a 5 minute incubation was incorporated into further experiments. IkB-\(\alpha\) was completely degraded by 15 minutes in all experiments. Interestingly, accumulation of newly synthesised IkB-\(\alpha\) could be observed 30 minutes after stimulation and levels were comparable to control by 2 hours. Staining of the same blot with an antibody to smooth muscle cell \(\alpha\)-actin, showed equal loading of samples.
**Figure 4.1. Western blot analysis of IκB-α after TNF-α treatment.** HASMC were treated with 10 ng/ml TNF-α (A) for 0, 5, 15, 30 min, 1, 2 h, (lanes 1-6, 5 µl sample), (lanes 7-12, 10 µl sample), and (B) for 0, 1, 5, 15, 30 min, 1, 2 h (lanes 1-7, 5 µl sample). Western blots were probed with anti-IκB-α antibody (1:1000), stripped and re-probed with anti-α-actin antibody (1:1000), and detected using ECL detection system.
Results

Chapter 4

Effects of IL-1β on IkB-α degradation and re-synthesis

Treatment of smooth muscle cells with 1 ng/ml IL-1β gave similar results to those observed with TNF-α, however complete degradation was not observed until 30 minutes or more after treatment compared to 15 minutes after TNF-α treatment (Figure 4.2). Levels of IkB-α began to accumulate after 1 hour but had not reached levels comparable to controls by 2 hours.

Effects of H_2O_2 on IkB-α degradation and re-synthesis

Hydrogen peroxide treatment of smooth muscle cells failed to reproduce the effects observed by TNF-α and IL-1β. Treatment of cells with 100 μM H_2O_2 did not alter the levels of IkB-α up to 2 h after treatment (Figures 4.3 and 4.4). Degradation was not observed, in fact, a small gradual increase in IkB-α levels could be seen in all experiments. To confirm that loss and re-appearance had not occurred within 15 minutes of treatment, a 5-minute time point was included in further experiments (Figure 4.4).

Effect of single or continuous doses of H_2O_2

Experiments were carried out to determine whether the increase in IkB levels were due to a continued presence of H_2O_2 or whether a single one minute pulse of H_2O_2 was sufficient to induce the observed increase. Cells were either treated for various times indicated in Figure 4.4 and harvested, or were treated for 1 min, rinsed and harvested after the indicated times for protein isolation (Figure 4.4). No notable changes were apparent between a single or continued treatment of H_2O_2 when compared to duplicate samples and loading controls.

Effects of co-incubation of H_2O_2 and TNF-α

Hydrogen peroxide and TNF-α were co-incubated with smooth muscle cells to determine if H_2O_2 would enhance or interfere with TNF-α mediated IkB-α degradation and re-synthesis. Hydrogen peroxide failed to block NFκB activation caused by TNF-α since results from co-treatment of H_2O_2 and TNF-α did not differ from TNF-α treatment alone (Figure 4.5). IkB-α degradation and reappearance was observed with no delay.
Figure 4.2. Western blot analysis of IkB-α after IL-1β treatment. HASMC were treated with 1 ng/ml IL-1β for 0, 5, 15, 30 min, 1, 2 h, (lanes 1-6, 10 μl sample), (lanes 7-12, 5 μl sample). Western blots were probed with anti-IkB-α antibody (1:1000), and detected using ECL detection system.
Figure 4.3. Western blot analysis of IκB-α after H$_2$O$_2$ treatment. HASMC were treated with 100 μM hydrogen peroxide for (A) 0, 5, 15, 30 min, 1, 2 h, (lanes 1-6, 10 μl sample), (lanes 7-12, 5 μl sample), and (B) 0, 5, 15, 30 min, 1, 2 h (lanes 1-6, 7.5 μl sample). Western blots were probed with anti-IκB-α antibody (1:1000), and detected using ECL detection system.
Figure 4.4. Western blot analysis of IκB-α after H$_2$O$_2$ treatment. (A) HASMC were treated with 100 μM hydrogen peroxide for 0, 5, 15, 30 min, 1, 2 h, (lanes 1-6, 5 μl sample), (lanes 7-12, 10 μl sample) or (B) HASMC were treated with 100 μM hydrogen peroxide for 1 min, then medium replaced and incubated for 0, 5, 15, 30 min, 1, 2 h, (lanes 1-6, 5 μl sample), (lanes 7-12, 10 μl sample). Western blots were probed with anti-IκB-α antibody (1:1000), stripped and re-probed with anti-α-actin antibody (1:1000), and detected using ECL detection system.
Results

Chapter 4

Figure 4.5. Hydrogen peroxide does not affect TNF-α induced IκB-α degradation. HASMC were treated with either (A) 10 ng/ml TNF-α alone (B) or hydrogen peroxide (100 μM) and TNF-α (10 ng/ml), for 0, 5, 15, 30 min, 1, 2 h, (lanes 1-6, 5 μl sample), (lanes 7-12, 10 μl sample). Western blots were probed with anti-IκB-α antibody (1:1000), stripped and re-probed with anti-α-actin antibody (1:1000), and detected using ECL detection system. C - Control (RAW cells).
4.3.1.2 *IkB-β degradation and regeneration*

*Effects of TNF-α on IkB-β degradation and re-synthesis*

Immunoblots were subsequently probed with specific antisera to IkB-β. A 50 kDa protein was detected in both control and TNF-α treated samples (Figure 4.6A). Whilst TNF-α caused a rapid degradation and regeneration of IkB-α, no significant change in IkB-β expression was observed. This suggests that TNF-α stimulation of NF-κB must be primarily targeted through the IkB-α pathway. Co-incubation of cells with TNF-α and H₂O₂ did not alter the effects of TNF-α treatment on IkB-β levels (Figure 4.6B).

*Effects of H₂O₂ on IkB-β degradation and re-synthesis*

A 50 kDa band was detected in control and H₂O₂ treated samples (Figure 4.7A). However, no change in IkB-β levels was observed up to 2 hours after H₂O₂ treatment after comparing duplicate samples and loading controls. This suggests that either H₂O₂ fails to activate NFκB in these cells, or mechanisms of NFκB activation do not involve the inhibitory proteins IkB-α or IkB-β. Several other IkB molecules have been discovered, including IkB-γ and IkB-δ. However, since antibodies to these were not available, it was not possible to determine the role of these inhibitory proteins in the activation of NFκB. In agreement with the previous experiments using hydrogen peroxide, no difference in effects could be observed on IkB-β expression between single and continuous doses of hydrogen peroxide (Figure 4.7B).
Figure 4.6. Western blot analysis of $\text{IkB-}\alpha$ (37 kDa) and $\text{IkB-}\beta$ (50 kDa). HASMC were treated with either (A) 10 ng/ml TNF-\(\alpha\) alone (B) or hydrogen peroxide (100 \(\mu\)M) and TNF-\(\alpha\) (10 ng/ml), for 0, 5, 15, 30 min, 1, 2 h, (lanes 1-6, 5 \(\mu\)l sample), (lanes 7-12, 10 \(\mu\)l sample). Western blots were probed with anti-\(\text{IkB-}\alpha\) antibody (1:1000), stripped and re-probed with anti-\(\text{IkB-}\beta\) antibody (1:1000), and detected using ECL detection system.
Figure 4.7. Western blot analysis of IκB-α (37 kDa) and IκB-β (50 kDa) comparing (A) single and (B) continued doses of H₂O₂. (A) HASMC were treated with 100 μM hydrogen peroxide for 0, 5, 15, 30 min, 1, 2 h, (lanes 1-6, 5 μl sample), (lanes 7-12, 10 μl sample) or (B) Cells were treated with 100 μM hydrogen peroxide for 1 min, then medium replaced and incubated for 0, 5, 15, 30 min, 1, 2 h, (lanes 1-6, 5 μl sample), (lanes 7-12, 10 μl sample). Western blots were probed with anti-IκB-α antibody (1:1000), stripped and re-probed with anti-IκB-β (1:1000), antibody, and detected using ECL detection system.
4.3.1.3 *p65 expression*

The levels of the p65 subunit of NFκB were measured to confirm that IκB degradation was not due to a general instability of proteins following the respective treatments and to confirm equal loading of samples. A band of 65 kDa was detected which corresponded to the NFκB subunit RelA (Figure 4.8). Levels of p65 were not significantly affected by either H₂O₂ or TNF-α treatment suggesting treatment did not result in non-specific effects on protein stability, or deficient synthesis, emphasising the selectivity of IκB degradation observed in TNF-α treated cells. This observation is in agreement with previously published data suggesting that treatment of cells with TNF-α or other NFκB activating conditions does not result in the induction of p65 protein or mRNA (Sun et al., 1993 and Ueberla et al., 1993).

4.3.1.4 *Active NFκB expression*

Direct evidence was needed to confirm that NFκB activation occurred in H₂O₂ treated cells as suggested by many investigators (Bauerle and Henkel 1994; Schmidt et al., 1995), despite no apparent IκB-α degradation. Immunoblots were probed with an antibody which specifically recognises part of the nuclear localisation signal (NLS) on the p65 subunit of NFκB (Kaltschmidt et al., 1995). For the antibody to recognise the NLS epitope, IκB-α must be dissociated from NFκB to reveal the NLS region. Therefore, the antibody specifically recognises only activated NFκB. Despite using a number of antibody concentrations, a band of 65 kDa corresponding to the active form of NFκB could not be detected. Neither TNF-α nor H₂O₂ induced the expression of this protein sufficiently to be detected on a Western blot. TNF-α was shown to induce IκB-α degradation and re-accumulation on the same blot which indicated that NFκB activation had occurred, but this was not detectable using this antibody.
Figure 4.8. Western blot analysis of p65. HASMC were treated with either (A) 10 ng/ml TNF-α alone (B) or hydrogen peroxide (100 μM), for 0, 5, 15, 30 min, 1, 2 h, (lanes 1-6, 10 μl sample), (lanes 7-12, 10 μl sample). Western blots were probed with anti-p65 antibody (1:1000), and detected using ECL detection system. C - Control (U9137 cells), M - marker.
4.3.2 IMMUNOFLUORESCENT ANALYSIS

4.3.2.1 p65 expression

In a bid to confirm activation of NFκB in smooth muscle cells in response to TNF-α and H₂O₂, immunofluorescent studies were undertaken using specific antibodies to the p65 subunit of NFκB, to provide direct evidence of NFκB activation. Since the p65 subunit of the NFκB dimer translocates to the nucleus upon release of IκB, an increase in nuclear staining of the protein should be observed upon stimulation. Previous reports have shown that NFκB is activated 1 hour after initial stimulation with H₂O₂, reaches a peak between 1 to 4 h, and returns to control levels by 24 hours in endothelial cells (Barchowsky et al., 1995). In Jurkat cells, similar observations have been made with NFκB returning to control levels by 16 hours after treatment with H₂O₂ (Schreck et al., 1991). Smooth muscle cells were treated with H₂O₂ for 2 and 24 hours. Neither 2 or 24 hour treatments showed a significant accumulation of staining within the nucleus.

4.3.2.2 Active NFκB expression

It was hoped that the recent purification of a specific antibody to the active component of p65 (Boehringer Mannheim), would improve sensitivity. This antibody does not recognise the majority of p65 present in cytoplasmic pools, hence it was expected to facilitate the study of the activation and subsequent translocation of NFκB into the nucleus following stimulation. Smooth muscle cells were treated for 2 and 24 hours with a range of concentrations of H₂O₂. Investigators have previously shown that concentrations of H₂O₂ in excess of 200 μM are required to activate NFκB (Ares et al., 1995). A 200 μM H₂O₂ treatment was therefore included in these experiments.

Smooth muscle cells treated with increasing concentrations of H₂O₂ showed an increase in p65 immunoreactivity, and nuclei were preferentially stained after 2 hours (Figures 4.9, 4.10, and 4.11). The greatest nuclear staining was observed with 200 μM H₂O₂ after 2 and 24 hour treatments, although nuclear staining was evident at all concentrations used. After 24 hours, the intensity of nuclear staining had reduced somewhat. FCS treatment for 24 hours did not induce translocation of NFκB into the nucleus, since no nuclear staining was observed. A 200 μM H₂O₂ treatment for 24 hours appeared toxic to the cells, since the cells...
began to round up, which may have accounted for a higher intensity of staining in these cells. After 2 hours, no apparent toxicity was observed with 200 μM, the extent of NFκB activation was greatest in these cells which raises speculation as to the role of NFκB activation in cell death.

As a positive control, cells were stimulated with TNF-α for 2 and 24 hours (Figure 4.12). TNF-α treatment also resulted in the translocation of the active subunit to the nucleus. Figure 4.12 shows nuclear localisation of active NFκB after 2 and 24 hours treatment with 10 ng/ml TNF-α. A small amount of p65 immunoreactivity was still observed in the cytoplasm, this could be p65 which had been freed from IκB but not yet taken up into nuclei. Unstimulated control cells gave a considerably weaker cytoplasmic as well as nuclear staining, showing that this antibody to active p65 is well suited to monitor the activation state of NFκB.
Figure 4.9. Immunofluorescent analysis of active NFκB in control cells. HASMC were incubated in 0.1% (v/v) FCS containing medium (control) or 10% (v/v) FCS containing medium for 24 h. Cells were stained with anti-NFκB (active p65 subunit) antibody (1:50) using a FITC conjugated secondary antibody (1:100). Magnification x 40.
Figure 4.10. Immunofluorescent analysis of active NFkB after 2 h H$_2$O$_2$ treatment. HASMC were treated with 50, 100 or 200 μM H$_2$O$_2$ for 2 hours. Cells were stained with anti-NFkB (active p65 subunit) antibody (1:50) using a FITC conjugated secondary antibody (1:100). Magnification × 40.
Figure 4.11. Immunofluorescent analysis of active NFκB after 24 h H₂O₂ treatment. HASMC were treated with 50, 100 or 200 μM H₂O₂ for 24 hours. Cells were stained with anti-NFκB (active p65 subunit) antibody (1:50) using a FITC conjugated secondary antibody (1:100). Magnification x 40.
Figure 4.12. Immunofluorescent analysis of NFκB after TNF-α treatment. HASMC were treated with TNF-α (10 ng/ml) for 2 or 24 hours. Cells were stained with anti-NFκB (active p65 subunit) antibody (1:50) using a FITC conjugated secondary antibody (1:100). Magnification × 40.
4.4 Discussion

Studies were undertaken to investigate the effects of oxidative stress on NFκB activation in smooth muscle cells. Little is known of the mechanisms involved in the activation of NFκB by various inducers of IκB degradation. TNF-α and H$_2$O$_2$ were compared for their effects on NFκB activation, and the evidence for IκB mediated regulation was investigated.

IκB regulation

Using specific antisera to IκB-α, a 37 kDa protein corresponding to the inhibitory molecule, was detected in both control and TNF-α treated smooth muscle cells using Western blot analysis. Treatment of cells with 10 ng/ml TNF-α resulted in the rapid degradation of IκB-α. Within 5 minutes, IκB-α protein levels began to decline and complete degradation was observed after 15 minutes. This was in accord with previous reports of IκB-α degradation by TNF-α in other cell types (Beg et al., 1993; Sun et al., 1993; Brown K, et al., 1993). Previous studies which have demonstrated the relationship between IκB-α loss and the appearance of NFκB in the nucleus, also indicated that IκB-α levels recovered within an hour or so (Scott et al., 1993; Read et al., 1994). The reappearance of IκB-α depends on protein synthesis and correlates with the rapid induction of IκB-α mRNA. In these experiments rapid reappearance of IκB-α was also observed. IκB-α reappeared 30 minutes after TNF-α treatment and regained levels comparable to control by two hours. It has previously been reported that upon activation of NFκB and degradation of IκB, the trans-activating NFκB dimers potently induce the expression of their own inhibitor, IκB-α, which presumably is destined to restore the inhibited state (Brown K, et al., 1993). Therefore, the observed re-synthesis of IκB-α is part of a built-in feedback mechanism, which assures a transient response once the initiating signal fades. This is an essential feature for the regulation of genes whose functions may be harmful if expressed unchecked. The rapid synthesis of IκB-α following its loss is apparently important in re-establishing cytoplasmic pools of NFκB/IκB-α complexes. Importantly, this re-synthesis also appears to repress NFκB activity following induction because re-synthesised IκB-α enters the nucleus, interacts with NFκB forms, and inhibits DNA binding (Arenzana-Seisdedos et al., 1995). Additionally, IκB has been found to displace bound p50/RelA from its cognate recognition sites on DNA in vitro (Zabel and Baueuerle, 1990).
The observed degradation and re-accumulation effects were proven not to be due to unequal loading. Immunostaining of the same blot with α-actin showed that protein samples were loaded equally. Levels of p65 were measured in the samples to show that IκB-α degradation was not due to a general instability of proteins due to treatment. No significant changes in p65 levels could be observed, thus emphasising the selectivity of IκB degradation observed in TNF-α treated cells.

Regulation of IκB-α levels could also be observed in smooth muscle cells upon treatment with IL-1β. A similar pattern of degradation and re-accumulation of IκB-α was observed, although the time scale of events was slightly longer. Complete degradation was not observed until 30 minutes after initial stimulation. Reappearance of IκB-α was observed 1 hour after treatment, however, IκB-α levels had not returned to control by 2 hours. Experiments involving later time points may have demonstrated levels of IκB-α returning to normal. The delayed responses to IL-1β suggests that the pathways leading to TNF-α and IL-1β induced IκB-α degradation and subsequent NF-κB activation are dissimilar, possibly through mechanisms upstream as well as downstream of IκB-α degradation. These results agree with previous reports, for example, Thompson et al. (1995), showed that TNF-α, IL-1β, LPS and PMA led to the stimulation of NFκB, degradation and subsequent re-accumulation of IκB-α in pre-B cells or Jurkat cells. They showed that both TNF-α and PMA induced IκB-α degradation much quicker than LPS and IL-1β, and levels of IκB-α protein recovered over a shorter time period. Interestingly, although LPS and IL-1β were found to induce IκB-β degradation, neither TNF-α nor PMA induced a concurrent degradation of IκB-β.

Although it is evident both cytokines target IκB-α, the mechanism involved in IκB-α degradation appears to differ, e.g. TNF-α and IL-1β may induce different kinases to phosphorylate IκB-α, or the same kinase may be differentially activated by the two inducers and likewise for the degradation machinery involved. In addition, the mechanisms involved in the reaccumulation of IκB-α may differ, since reaccumulation times were delayed by at least one hour in IL-1β treated cells. This may involve mechanisms allowing more time for NFκB to remain bound to DNA, for example, by controlling IκB-α mRNA
stability and subsequent protein synthesis. Identification of kinases responsible for phosphorylation, and proteases or proteosomes involved in the degradation of IkB-α will provide more information about the mechanisms of NFκB activation by TNF-α and IL-1β.

Evidence of hydrogen peroxide induced NFκB activation has been observed in a number of cell types, including endothelial cells, and Jurkat T cells. However, the role of IkB has not been established. With the knowledge that IkB-α is present in smooth muscle cells and that TNF-α and IL-1β can activate NFκB through IkB-α release in these cells, it was hypothesised that H2O2 would activate NFκB in a similar manner. H2O2 treatment of smooth muscle cells failed to reproduce the effects observed by TNF-α and IL-1β. A 37 kDa protein was detected in both control and H2O2 treated cells. 100 μM H2O2 failed to induce IkB-α degradation for up to 2 hours after initial treatment. It is unlikely that degradation and reaccumulation had occurred within 5 minutes, since this process entails translocation of NFκB to the nucleus, and synthesis of mRNA and protein. Despite the lack of induction of IkB-α degradation by H2O2, co-treatment of smooth muscle cells with H2O2 and TNF-α induced IkB-α degradation in a similar manner to TNF-α alone. IkB-α degradation and reappearance was observed, with no delay in degradation or reappearance. Hydrogen peroxide therefore neither inhibits nor enhances TNF-α mediated NFκB activation.

A possibility exists that other IkB proteins are involved in NFκB activation or are used as alternative mechanisms for regulating NFκB activation. A number of IkB proteins have now been identified, and the possibility that other IkB proteins are involved in NFκB modulation in smooth muscle cells cannot be ruled out. As yet, the only other IkB with an appropriate antibody to detect it is IkB-β. IkB-β has only recently been isolated and cloned (Thompson et al., 1995), and was found to have a number of similarities to IkB-α. A 50 kDa protein was detected in smooth muscle cells and in both TNF-α and H2O2 treated samples. Whilst TNF-α induced a rapid degradation and reaccumulation of IkB-α, no change in expression of IkB-β was observed after TNF-α treatment. This suggests that the primary target for degradation in TNF-α mediated NFκB activation is IkB-α, not IkB-β. This agrees with reports for other cell types which suggest that IkB-α degradation is controlled by TNF-α, PMA, LPS and IL-1, whilst IkB-β degradation plays a role in LPS
and IL-1 activation but not that due to TNF-α or PMA (Thompson et al., 1995). Hydrogen peroxide treatment of cells also did not effect the expression of IκB-β, whether applied as a single or continuous dose, and co-incubation of cells with TNF-α and H₂O₂ did not alter this effect.

An unexpected finding reported by Thompson and his colleagues was that levels of IκB-α and IκB-β proteins in cells are present in equivalent levels, even though IκB-α is the primary inhibitory molecule involved in NFκB activation. Unlike IκB-α, NFκB activation does not lead to the induction of transcription of IκB-β mRNA, suggesting that NFκB released from IκB-β is not down regulated by an auto-regulatory feedback mechanism. The lack of induction of new IκB-β synthesis ensures that active NFκB is persistent in the nucleus for longer (up to 24 hours after stimulation), than when activated by IκB-α release. Whilst the IκB-α response is used for rapid, transient activation of NFκB, IκB-β is involved in the persistent activation of NFκB. Different signalling mechanisms may induce NFκB activation to reflect the type of stress incurred by the cell, e.g., transient or persistent chronic stress. IκB-β may also be important in constitutive activation of NFκB, where IκB-α is absent e.g. testis, and no feedback inhibition mechanism is present. Although the signalling pathways leading to the activation of IκB-α or β must be distinct, and their degradation kinetics are different, it is thought they are released by similar mechanisms e.g. similar kinases and proteases, since both IκB-α and IκB-β possess serine residues in homologous positions which are thought to be targets for phosphorylation and subsequent degradation (Baldwin, 1996).

The failure of H₂O₂ to induce IκB-α or β degradation and reaccumulation in smooth muscle cells suggests that either (1) NFκB is not activated by H₂O₂, (2) activation occurs via mechanisms other than IκB-α or IκB-β degradation, e.g., IκB-γ, IκB-δ, or (3) methods employed were not sensitive enough to detect any changes. Evidence does exist to show that H₂O₂ can induce NFκB activation in other cells. In endothelial cells, 25 μM was sufficient to increase NFκB activity in 1 hour using EMSAs (Barchowsky et al., 1995), whilst others have reported 500 μM was necessary (Shono et al., 1996). Treatment of Jurkat T cells with 30-50 μM H₂O₂ increased NFκB activity, which was maximal 4 hours after treatment (Schreck et al., 1991). One recent paper has reported NFκB activation in
human vascular smooth muscle cells by \( \text{H}_2\text{O}_2 \), however, concentrations from 200 \( \mu\text{M} \) to 1000 \( \mu\text{M} \) were required to stimulate NF\( \kappa \)B activity (Ares et al., 1995). However, previous studies have clearly shown that concentrations above 200 \( \mu\text{M} \) are toxic to smooth muscle cells (Chapter 3).

To determine whether hydrogen peroxide would activate NF\( \kappa \)B, western blot analysis was carried out using an antibody to both the p65 subunit and the active NF\( \kappa \)B (NLS region of p65, see below). Protein levels of p65 did not differ between control and TNF-\( \alpha \) or \( \text{H}_2\text{O}_2 \) treated cells. It was later discovered that although I\( \kappa \)B-\( \alpha \) levels fluctuate upon NF\( \kappa \)B activation due to feedback mechanisms, NF\( \kappa \)B activation does not induce the synthesis of further NF\( \kappa \)B subunits (Sun et al., 1993). Therefore, p65 levels would not differ upon NF\( \kappa \)B activation, and only its translocation to the nucleus would be apparent. Comparisons of nuclear versus cytoplasmic extracts may therefore be a more useful study.

Experiments carried out using an antibody specific to activated NF\( \kappa \)B proved inconclusive. The antibody failed to detect a 65 kDa protein corresponding to the active p65 subunit of NF\( \kappa \)B. The samples used in the Western blots were prepared from whole cell lysates. Since the active form of NF\( \kappa \)B is most likely to be found within the nucleus, nuclear extracts may have improved the success of this antibody in detecting the active form of NF\( \kappa \)B. However, this antibody proved more useful in immunofluorescent studies as described later. In the original paper describing the antibody, the only evidence that the antibody was suitable for Western blotting came from an immunoblot of cells transfected with a p65 expression vector, hence, the antibody may not be suitable for Western blot analysis in other cell types with lower levels of expression of NF\( \kappa \)B. The majority of work carried out using this antibody involved immunofluorescent and supershift assays (Kaltschmidt et al., 1995).

**Activation of NF\( \kappa \)B in SMC**

Because the activation of NF\( \kappa \)B is regulated at a post-translational level, by the dissociation of a pre-existing cytoplasmic complex, the activation of the factor cannot be directly monitored by detection of new mRNA or protein. In intact cells, the activation of NF\( \kappa \)B can be monitored by various techniques. The electrophoretic mobility shift assay (EMSA), which relies on the *de novo* appearance of \( \kappa \)B-specific DNA binding activity in nuclear or total cell extracts, is still the most frequently used technique. The availability of
antibodies has greatly facilitated the analysis of protein-DNA complexes, which form with DNA probes containing high affinity NFkB binding sites in EMSAs. Another method is the analysis of sub-cellular fractions of cells, using cytosolic and nuclear extracts in combination with Western blotting as previously described. With this technique, a redistribution of NFkB subunits from cytosol to nucleus is indicative of the activation of the factor. An indirect method to assess NFkB activation is to monitor for an increased expression of target genes but this approach is limited in two respects. Firstly, the appearance of the reporter gene product may not accurately reflect the rapid activation of NFkB; nor may the stability of the reporter protein allow the detection of a subsequent down regulation of NFkB activity. Secondly, the expression of most target genes usually relies on other unrelated transcription factors as well. The direct detection of NFkB in immunofluorescent studies using specific antibodies provides the investigator with additional information including the cellular localisation of the targeted protein, and in vivo analysis of its presence using tissue sections.

To establish whether H2O2 activated NFkB in smooth muscle cells, immunofluorescent studies were undertaken. Using an antibody to the p65 subunit, experiments were carried out to follow the translocation of NFkB to the nucleus following H2O2 treatment. The antibody to p65 did not clearly distinguish between cytoplasmic and nuclear NFkB following H2O2 treatment. This antibody does not distinguish between active and non-active NFkB and since the p65 subunit is ubiquitous throughout the cytoplasm and nucleus, general staining of the cell was noted and translocation of p65 into the nucleus could not be observed. The majority of p65 complexes are reported to remain in the cytoplasm upon activation despite complete degradation of IkB-α (Sun et al., 1994), it is therefore not surprising that differences in nuclear and cytoplasmic staining failed to be detected.

A potentially more discriminating tool is the antibody designed by Kaltschmidt et al. (1995), which binds to the NLS of NFkB that is revealed when IkB dissociates. This antibody does not bind to the majority of p65 in the cytoplasm and is therefore more sensitive to measuring nuclear translocation of p65-containing dimers. Human smooth muscle cells were treated with H2O2 at various concentrations for 2 and 24 hours, evidence of NFkB activation and nuclear translocation was assessed. Hydrogen peroxide treatment resulted in the activation of NFkB as observed by the increase in immunoreactivity of the
p65 antibody and the preferential staining of nuclei. Increasing concentrations of H$_2$O$_2$ resulted in increasing intensity of the fluorescent probe after 2 hours. Activation of NFkB in cells could still be observed after 24 hours of treatment although staining intensities were. Treatment with 200 μM H$_2$O$_2$ gave the greatest degree of activation after 2 hours, however, by 24 hours this concentration proved to be toxic and caused the cells to round up. This agrees with results reported in the third chapter of this thesis which shows that H$_2$O$_2$ is toxic to these cells at this concentration, especially in the absence of serum. This suggests that NFkB activation may have a role in oxidative stress induced cell death. A recent study has suggested such a role for NFkB in apoptosis, Grimm et al. (1996), reported that serum starvation of 293 cells caused cell death accompanied by activation of RelA-containing NFkB. It is thought that RelA can modulate apoptosis in diverse ways depending on the cell type and the nature of the stimulus. Neither control cells nor serum stimulated cells were strongly stained with this antibody, suggesting that nuclear staining in these cells was specifically due to H$_2$O$_2$. TNF-α treatment of smooth muscle cells for 2 and 24 hours also resulted in activation of the NFkB complex and translocation to the nucleus, which was also greatest in 2 hour treated cells. The increased immunofluorescence observed after H$_2$O$_2$ and TNF-α stimulation did not appear to result from new synthesis of the p65 protein, but from increased accessibility of the NLS epitope for two reasons. Firstly, p65 expression is not induced by TNF-α or other inducers of NFkB activation (Sun et al., 1993; Ueberla et al., 1993). Secondly, Western blotting confirmed that control and TNF-α or H$_2$O$_2$ stimulated cells contained equal amounts of p65 protein.

Although control cells did not show specific nuclear staining, a general non-specific staining of the cytoplasm was observed, and background staining was still considerable in those cells stimulated with H$_2$O$_2$ or TNF-α. The weak staining of these cells could indicate that the antibody was not entirely specific for the IκB-released p65. Alternatively, it is possible that p65 was detected which had transiently lost IκB but could be recaptured by newly synthesised IκB (Rice and Ernst, 1993). A third possibility is that the antibody can to some extent displace IκB from p65.

**Alternative Mechanisms of NFkB Activation**

There is strong evidence supporting the notion that H$_2$O$_2$ activates NFkB in many cell types, fewer reports discuss possible mechanisms for its activation (Schreck et al., 1992;
Discussion

Kretz-Remy et al., 1996). Despite showing that NFκB could be activated in smooth muscle cells upon treatment with H₂O₂ using immunofluorescent studies, efforts to determine the mechanisms involved in its activation by monitoring IκB protein degradation proved unsuccessful. How can NFκB activation occur without observing degradation of IκB proteins? The possibility exists that other IκB proteins may be important in controlling NFκB activation, however, to date there are no suitable methods for measuring their activity. It may for example be possible that NFκB activation could occur without IκB degradation: Treatment with hydrogen peroxide may cause IκB to dissociate from NFκB and allow translocation into the nucleus, without IκB degradation. However, IκB in uncomplexed forms has been reported to be considerably unstable and prone to degradation (Beg and Baldwin, 1993).

It may be possible that IκB is partially dissociated from the NFκB complex, sufficient to reveal the nuclear localisation signal, but insufficiently dissociated for phosphorylation and/or degradation to occur (Figure 4.15a). Oxygen radicals can covalently modify many different amino acid residues in proteins, thus altering their activity (Pacifici and Davies, 1990). IκB may be modified slightly by oxidation to reveal the NLS yet remain complexed to NFκB, or hydrogen peroxide may inhibit degradation possibly by masking serine residues involved in phosphorylation or lysine residues involved in ubiquitination and degradation (Scherer et al., 1995). NFκB would still induce further IκB-α synthesis and may explain the increase in IκB-α levels observed with H₂O₂ treatment. The idea that IκB may still be partially complexed to NFκB within the nucleus may be strengthened by evidence that shows that IκB-α can be found in the nucleus whilst complexed to NFκB (Cressman and Taub, 1993), however more recently, it has been speculated that IκB found in the nucleus is the newly synthesised IκB-α, and serves to inhibit further NFκB-DNA binding (Arenzana-Seisdedos et al., 1995).

Alternatively, NFκB may be the target of oxidative modification (Figure 4.15b), this must selectively disturb the p65-IκB interaction because p65 has to remain functional in DNA binding and transcription activation. However, the role of direct oxidation on the NFκB complex has previously been investigated, these studies showed that direct oxidation actually inhibits DNA binding of NFκB (Toledano et al., 1991). This does not rule out the
role of redox-regulation in NFkB activation completely. Changes in the redox state of cells are thought to induce modifications of cellular signalling molecules other than transcription factors, such as protein kinases, protein phosphatases (Figure 4.15c).

That hydrogen peroxide on its own is unable to activate a purified NFkB/IkB complex (Schreck et al., 1991), suggests that H₂O₂ may act on an effector molecule upstream of IkB phosphorylation and degradation such as a redox-sensitive kinase or phosphatase. Signalling components that may function down-stream of H₂O₂ in NFkB activation have not yet been identified, but the use of kinase and phosphatase inhibitors in reactive oxygen species mediated NFkB activation should further elucidate target molecules of reactive oxygen species.

4.5 Summary
Experiments were carried out to investigate the role of hydrogen peroxide and TNF-α on NFkB activation in smooth muscle cells using immunoblotting and immunofluorescent techniques. With the use of specific antisera to IkB-α and IkB-β, Western blot analysis was performed to detect the presence and possible regulation of IkB-α and IkB-β. Smooth muscle cells contained both the IkB-α and IkB-β forms of the inhibitory molecules that control NFkB activation. However, only IkB-α seems to be involved in NFkB activation in these cells since its rapid degradation was observed upon treatment with TNF-α and IL-1β. The levels of IkB-α recovered with time because active NFkB induces further transcription of the inhibitory molecule as part of a negative control mechanism. The levels of IkB-β did not alter with TNF-α treatment. Although IkB-α would appear to be the main target for controlling NFkB activation in smooth muscle cells, it did not play a part in the activation of NFkB in response to hydrogen peroxide. Neither IkB-α or IkB-β protein levels were affected after treatment of smooth muscle cells with hydrogen peroxide despite immunofluorescent studies showing that NFkB activation does occur. It is possible that other IkB regulatory molecules are involved in hydrogen peroxide mediated NFkB activation, or that other activation pathways exist in addition to the serine phosphorylation and subsequent degradation of IkB-α frequently reported in the literature, for example a redox regulated pathway which does not involve IkB degradation.
Figure 4.15. Models to explain NFκB activation by H₂O₂. IκB bound to p65 masks the nuclear localisation signal (NLS) and prevents NFκB from being active. (a) H₂O₂ induces activation of a cellular factor which induces a conformational change in the complex, and reveals the NLS region without IκB release. (b) H₂O₂ may also directly cause a conformation change to IκB to reveal the NLS region. H₂O₂ induces the release of IκB by direct oxidant attack, thus revealing the NLS region and allow DNA binding. IκB release is followed by degradation by proteosomes. (c) H₂O₂ may induce the activation of a cellular factor e.g. a kinase which facilitates IκB removal and subsequent NFκB activation. Based on a model of ROS activation of iron regulatory protein 1 (IRP1) (Hentze, 1996).
Chapter 5

THE USE OF DIFFERENTIAL DISPLAY TO INVESTIGATE THE EFFECTS OF OXIDATIVE STRESS ON GENE EXPRESSION IN VASCULAR SMC
5.1 Introduction

Cellular responses to oxidative stress such as hydrogen peroxide and oxidised lipoproteins, involve changes in the expression of many genes including those involved in repair, and those thought to protect the cell against additional oxidative damage (Devary et al., 1992; Shimm et al., 1992). Many of the genes induced by oxidative stress are regulated by NFκB, these include cytokines, growth factors, and adhesion molecules, which are all thought to play an important role in the initiation and progression of atherosclerosis as discussed in Chapter 1. Induction of these genes have been demonstrated in lesions, using immunohistochemical studies, and the detection of activated NFκB in lesion cells further supports a role for oxidative stress mediated gene expression in atherosclerosis. Phenotypic modulation of smooth muscle cells is thought to occur in response to these growth regulatory factors which are abundant in the lesion, in addition, oxidative stress which is also prevalent in the lesion may directly influence smooth muscle cell behaviour.

Changes in expression of certain genes by intimal smooth muscle cells might be the result of two different mechanisms. It has been suggested that smooth muscle cells of the intima belong to a unique lineage of cells derived from a common precursor or differentiated from typical smooth muscle cells (Benditt and Benditt, 1973). Alternatively, and more obviously, intimal smooth muscle cells may show over- or under-expression of certain molecules in response to mediators already present in the lesion. Particularly important among such mediators are oxidation products or more traditional inflammatory mediators. Collins (1993), has suggested that a common factor linking inflammation and oxidation is the role of NFκB as a trans-activating factor induced by oxidising radicals and by many cytokines. Many of the genes shown to be over-expressed in the lesion might be explained as an inflammatory response. By identifying gene markers associated with smooth muscle cell phenotypes, and the factors which induce their change in expression, we may gain a better understanding of the regulation of smooth muscle cell differentiation and its role in atherosclerosis and restenosis. In addition, new markers of altered phenotype may provide novel targets for the inhibition of smooth muscle cell migration and proliferation that is involved in lesion progression.
In 1992, Liang and Pardee first described a novel approach to identify differentially expressed genes in various cells or altered conditions known as Differential Display (see Chapter 1, Section 3.4.2). Since then, this technique has been used by investigators from very different scientific fields to approach a whole range of disease processes from plant pathology to chronic cardiac rejection. Differential display is now well established as a method to analyse changes in expression of genes associated with disease. Its main advantages over previous techniques are that relatively small amounts of starting material are required since differential display is PCR based, and it allows the side by side comparison of multiple samples of RNA. As with any new technique, methodology has evolved and improved. However, a number of major obstacles remain with the method including, the lack of reproducibility, the redundancy and under representation of certain mRNA species, the isolation of PCR artefacts which lead to a high number of ‘false positives’, and the production of cDNAs of less than 300 bp, which makes further screening and sequence analysis difficult. Nevertheless, differential display has the potential of being a powerful technique in identifying differential expression of genes associated with disease progression.

5.2 Aim

Differential display was employed to study the differential expression of genes associated with oxidative stress. One advantage of this technique over the alternative subtractive hybridisation method is that differential display allows parallel comparisons to be carried out of more than two conditions at any one time. To take full advantage of this, a comparison of not only hydrogen peroxide treatment against control cells was carried out, but also the effects of oxidatively modified LDL and native unmodified LDL. This would provide further information regarding the suitability of hydrogen peroxide as a model of oxidative stress within atherosclerosis, how similar the two sources of oxidative stress are in their actions, and if oxidation of LDL is important or whether merely the presence of LDL is sufficient. With time, the aims of this thesis extended to determining ways of improving the differential display technique to reduce the number of false positives that became apparent. This aspect is discussed in Chapter 6.
5.3 Results

5.3.1 EXPERIMENTAL DESIGN

5.3.1.1 Modification of LDL

As discussed in Chapter 1 (Section 1.3.4), minimally-modified LDL seems to be more biologically active than oxidised-LDL, which tends to be more toxic (Berliner et al., 1990; Watson et al., 1995). Since LDL would undergo further modification when cultured with cells, it was decided to use minimally modified LDL in this study. Therefore to investigate and compare gene expression associated with different oxidative stresses, minimally-modified LDL was used for culture with smooth muscle cells. Common problems associated with working with modified LDL include the heterogeneity of the LDL fraction, and quantifying the extent of modification. Whilst it is impossible to improve on the heterogeneity problem, except to use the same source of LDL for each study, and the same method of isolation, there are a number of solutions to make sure the extent of modification is uniform. Unfortunately, the methods available for measuring oxidation are not entirely suitable, as they lack specificity and give indirect indications of LDL oxidation. A suitable method for continuously monitoring the oxidation of LDL developed by Esterbauer et al. (1989), involves measuring increases in absorption at 234 nm as discussed in Chapter 1, Section 1.1.5.5. This method allows determination of the initiation stage and other stages of LDL modification, and has the added advantage that only a small amount of valuable LDL is required.

LDL was minimally modified in the presence of copper sulphate, oxidation was carefully monitored using absorption at 234 nm (see Chapter 2). Oxidation was stopped immediately after the initiation phase and before the beginning of the propagation phase (i.e. after the lag phase) and dialysed to remove copper and EDTA before use on cells. Figure 5.1 shows the monitoring of copper oxidation of LDL. Increasing the copper concentration increased the rate of conjugated diene formation and hence the rate of oxidation. In addition, reducing the concentration of LDL increased the rate of conjugated diene formation. Addition of 1mM EDTA at 30 min prevented further increases in conjugated diene formation and therefore further oxidation.
**Figure 5.1. Copper sulphate oxidation of LDL.** Monitoring of isolated human LDL was carried out by measuring changes in absorption of $A_{234}$ nm. (A) Effects of copper sulphate concentration on LDL oxidation rates. (B) Effects of differing concentrations of LDL on copper sulphate induced LDL oxidation. ▼ 50 μM EDTA was added 30 minutes after initiation of oxidation.
3.1.2 Sample preparation

Human aortic smooth muscle cells (passage 8) were treated with either 100 μM H₂O₂ or 20 μg/ml Native or MM-LDL in 0.1% FCS containing medium for 18 hours. Controls were cultured in 0.1% FCS only. RNA was isolated and made free of contaminating DNA. Figure 5.2 shows that the RNA was free from contamination and undegraded. Differential display was carried out using the four different RNA samples. Bands were displayed on a polyacrylamide gel and compared. Typical differential display gels using the four RNA samples with different primer combinations are shown in Figures 5.3-5.5. Samples were loaded in triplicate and only those bands of which two or more were present were used for further analysis.

Figure 5.2. RNA integrity. RNA from Control (1), H₂O₂ (2), Native-LDL (3), or MM-LDL (4) treated cells were isolated and DNase treated. Samples were run on a 1% (w/v) Agarose gel and stained with x% Ethidium Bromide. Marker φX 174.
Figure 5.3. Typical differential display gel. PCR was carried out using AP6 and T₁₁ A or T₁₁ C primers. C - control, N - native-LDL, M - minimally modified-LDL, H - H₂O₂. Samples were loaded in triplicate.
Figure 5.4. **Typical differential display gel.** PCR was carried out using AP1 and $T_{11}$ A or $T_{11}$ G primers. C - control, N - native-LDL, M - minimally modified-LDL, H - $H_2O_2$. Samples were loaded in triplicate.
Figure 5.5. Typical differential display gels. PCR was carried out using AP2 with $T_{11}$A, $T_{11}$C or $T_{11}$G primers or AP10 with $T_{11}$A. C - control, N - native-LDL, M - minimally modified-LDL, H - $H_2O_2$. Samples were loaded in triplicate.
5.3.2 EXPRESSION PATTERNS AND DISTRIBUTION OF cDNA BANDS

Differential display was carried out using RNA samples from untreated (control) cells, hydrogen peroxide treated cells, and native (N-LDL) and minimally modified LDL (MM-LDL) treated cells, to take full advantage of the benefits of differential display in allowing multiple comparisons. Comparisons could therefore be made between MM-LDL treated cells and hydrogen peroxide treated cells, N-LDL and MM-LDL treated samples, N-LDL and hydrogen peroxide treated samples along with comparisons against untreated cells. This would indicate whether oxidative stress induced any changes in gene expression, whether hydrogen peroxide and MM-LDL were similar models of oxidative stress, and whether MM-LDL induced effects were due to increased oxidative stress or merely the presence of lipoproteins.

Tables 5.1 and 5.2 show typical patterns of expression of cDNA bands identified on polyacrylamide gels after differential display. Samples were PCR amplified in triplicate to improve reproducibility and limit the chances of picking up PCR artefacts, only cDNAs which produced two or three bands were used for further analysis. The number of crosses represents the number of bands observed for each set of triplicates. Table 5.3 summarises the patterns of expression observed between the treated cells and control cells, by summating the occurrences of bands found exclusively to each treatment, and the occurrences of bands found common to the different treatments.

Table 5.3 shows similarities and differences in gene expression that exist between the four treatments. Surprisingly, no cDNAs were expressed which were common to MM-LDL and H\textsubscript{2}O\textsubscript{2} treatments. This would indicate that they were indeed very different sources of oxidative stress and have different effects on gene expression. Only 1 band was found unique to MM-LDL and 2 bands to N-LDL, whereas 12 were found common to both treatments. In contrast, 8 bands were found uniquely expressed in control cells, and 7 bands uniquely expressed in H\textsubscript{2}O\textsubscript{2} treated cells. The differences between control and H\textsubscript{2}O\textsubscript{2} treatment accounted for 86% of total differences observed. No bands were found common to both treatments, and of the 34 bands that were analysed, 14 were repressed or expressed exclusively with H\textsubscript{2}O\textsubscript{2} treatment.
### Table 5.1. Differential Display expression patterns

This table shows patterns of expression from two primer combinations AP1 and AP2 and their identity. PCR was carried out in triplicate and crosses represent bands as seen on sequencing gel. See Figures 5.4 and 5.5 for pictures of the bands on sequencing gels.

<table>
<thead>
<tr>
<th>ID.</th>
<th>Control</th>
<th>N-LDL</th>
<th>MM-LDL</th>
<th>H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1.1</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>A1.2</td>
<td>+++</td>
<td>+++</td>
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<td>+ +</td>
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<td>+ +</td>
</tr>
<tr>
<td>G2.15</td>
<td>+ +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2.16</td>
<td>+ +</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 5.2. Differential Display expression patterns.

This table shows patterns of expression using the AP6 primer. PCR was carried out in triplicate and crosses represent bands as seen on sequencing gel. See Figure 5.3 for a picture of the bands on a sequencing gel.

<table>
<thead>
<tr>
<th>ID.</th>
<th>Control</th>
<th>N-LDL</th>
<th>MM-LDL</th>
<th>H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6.1</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>A6.2</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>A6.3</td>
<td></td>
<td></td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>A6.4</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A6.5</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A6.6</td>
<td></td>
<td></td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>C6.1</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6.2</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6.3</td>
<td>++</td>
<td></td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>C6.4</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>C6.5</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>C6.6</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Expression in:</td>
<td>AP-1</td>
<td>AP-2</td>
<td>AP-6</td>
<td>Total</td>
</tr>
<tr>
<td>---------------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>-------</td>
</tr>
<tr>
<td>1 only</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>2 only</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3 only</td>
<td></td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>4 only</td>
<td>6</td>
<td>1</td>
<td></td>
<td>7</td>
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<tr>
<td>1 + 2</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1 + 3</td>
<td>1</td>
<td></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1 + 4</td>
<td></td>
<td>2</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>2 + 3</td>
<td>2</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>2 + 4</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>3 + 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1 + 2 + 3</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>7</td>
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<tr>
<td>1 + 2 + 4</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 + 3 + 4</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2 + 3 + 4</td>
<td>2</td>
<td></td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 5.3. Typical distributions of bands observed between treatments using three different arbitrary primers AP-1, AP-2, and AP-6. 1) Control, 2) N-LDL, 3) MM-LDL, 4) H₂O₂ treatment. Combination 1 + 2 + 3 + 4 was not included since bands were only identified which were different in at least one of the treatments.
5.3.3 CONFIRMATION OF DIFFERENTIAL EXPRESSION

Because most of the differences in expression occurred between hydrogen peroxide treated cells and untreated cells (Table 5.3), further differential display reactions and confirmatory experiments were carried out using only H₂O₂ treated and untreated cells. This minimised the number of PCR conditions for further differential display experiments and reduced the amount of RNA needed for further analysis using Northern blots. Once differences had been confirmed with hydrogen peroxide or control treatment, the analysis could be extended to look at further conditions such as LDL treatment and possibly antioxidant treatment.

Despite differential expression being observed between treatments when compared on a sequencing gel, true differences in expression can only be confirmed using Northern analysis. This would show if any of the cDNA bands were artefacts of PCR. Confirmation of differential expression is labour intensive, involving the isolation of cDNA from the sequencing gel, re-amplification of the cDNA, cloning of the cDNA into an appropriate vector, sequencing and identification of the sequence, and preparation of cDNA to use as a probe to detect RNA species in a Northern blot.

Initially, 23 bands from a polyacrylamide gel of six primer combinations were isolated, purified, and amplified using identical primers to the original RT-PCR reaction. 21 bands were successfully isolated and amplified from the polyacrylamide gel to give the same sized products as identified on the gel. (Figure 5.6). These cDNAs were then successfully cloned into a TA vector and DNA isolated and purified which enabled sequences to be identified for each band. A summary of the sequence data is presented in Table 5.4. Two bands failed to amplify after the first 40 rounds of PCR. No further attempts to re-amplify the bands were made since further PCR reactions may introduce artefacts into the PCR mixture and would lead to the generation of false positives during subsequent analysis.
Figure 5.6. cDNA after excision from differential display gel and amplification. Bands of interest were excised and PCR carried out using the same primers as the original PCR. Samples were run on a 4% Nusieve gel.
Of 21 cDNAs isolated from AP-1 and AP-2 primers, 10 were found to be more than 95% homologous to known sequences present in the EMBL/Genbank sequence databases. Of the remaining 11 cDNA species, no homology could be found in the database to these sequences. The sizes of all bands isolated and sequenced varied between 74 bp and 800 bp, the average being 293 bp. 21 cDNAs were found to be homologous to genes in the database; these genes varied in size between 165 bp and 7.7 Kb, averaging 2.52 Kb. In almost all cases, the cDNAs isolated represented the 3' most region of the mRNA, this most often being in the 3'-untranslated region (UTR), (Figures 5.7 and 5.8). This is expected since differential display depends upon amplification of RNA from the 3' end using poly (T) primers. On one occasion, poly (T) primers annealed further upstream, which generated a cDNA of 133 bases with sequence homology to collagen (pro-α1 V) mRNA, being such a large message (6.3 Kb), the chances of primer annealing upstream of the 3' region is increased. The problem of cDNAs frequently being derived from the 3'-untranslated region has been highlighted by many investigators and makes characterising the cDNAs more difficult, since UTR sequences of certain genes are often not included in databases, or different species or different genes may share similar UTR sequences.
<table>
<thead>
<tr>
<th>ID.</th>
<th>Size (bp)</th>
<th>Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1A</td>
<td>500</td>
<td>Laminin B1 chain</td>
</tr>
<tr>
<td>1.2A</td>
<td>386</td>
<td>ORF of mRNA (KIAA0093)</td>
</tr>
<tr>
<td>1.3G</td>
<td>800</td>
<td>Novel</td>
</tr>
<tr>
<td>1.5G</td>
<td>531</td>
<td>ORF of mRNA (PIGHEP3 homologue)</td>
</tr>
<tr>
<td>1.6G</td>
<td>214</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>1.8G</td>
<td>150</td>
<td>Novel</td>
</tr>
<tr>
<td>1.9G</td>
<td>113</td>
<td>Novel</td>
</tr>
<tr>
<td>1.10G</td>
<td>390</td>
<td>Novel</td>
</tr>
<tr>
<td>1.11G</td>
<td>120</td>
<td>Novel</td>
</tr>
<tr>
<td>2.1A</td>
<td>490</td>
<td>ADP/ATP Translocase</td>
</tr>
<tr>
<td>2.2A</td>
<td>443</td>
<td>Ribosomal protein S3a (v-fos)</td>
</tr>
<tr>
<td>2.5A</td>
<td>376</td>
<td>Novel</td>
</tr>
<tr>
<td>2.6A</td>
<td>290</td>
<td>Novel</td>
</tr>
<tr>
<td>2.7A</td>
<td>255</td>
<td>Fork Head transcription factor (FKHR)</td>
</tr>
<tr>
<td>2.9C</td>
<td>204</td>
<td>HepG2 cDNA (hmd3f06m5)</td>
</tr>
<tr>
<td>2.10G</td>
<td>413</td>
<td>Novel</td>
</tr>
<tr>
<td>2.11G</td>
<td>391</td>
<td>Nrf2 (nfE2 related)</td>
</tr>
<tr>
<td>2.12G</td>
<td>218</td>
<td>Vinculin/Metavinculin</td>
</tr>
<tr>
<td>2.14G</td>
<td>130</td>
<td>Novel</td>
</tr>
<tr>
<td>2.15G</td>
<td>127</td>
<td>Novel 2.15 and 2.15 same sequence</td>
</tr>
<tr>
<td>2.16G</td>
<td>127</td>
<td>Novel</td>
</tr>
</tbody>
</table>

**Table 5.4.** Sequence identity of cDNAs isolated showing differential expression. cDNAs produced using AP-1 and AP-2 primers in conjunction with the relevant H-T11A, C or G primers. ORF-open reading frame.
Figure 5.7. Most cDNAs isolated are homologous to the very 3’ end of mRNA species.
Figure 5.8. Most cDNAs are homologous to the very 3' end of mRNA species.
Using the sequence data, primers were designed of sequences 20 bp upstream and downstream of the cDNA. If sequences were known for each cDNA species, primers were made to give PCR products of 200-300 bp, ideally within the translated region, to avoid sequences such as repeats, polyadenylation sites and polyadenylation signals. Primers were designed for all but two sequences, which were novel and A-T rich (1.8 and 1.11).

All cDNAs were used as probes in Northern analysis except 1.5G, 1.8G, 2.6A, and 2.9C, which were not present in either H₂O₂ treated or control cells. Initially, clones were used to probe Northern blots of control and hydrogen peroxide treated cells. 8 cDNAs were successfully radiolabelled either by primer specific PCR labelling or random prime labelling. Of the 8 cDNAs that were used as probes, 4 produced signals on Northern blots (1.2A, 2.2A (Figure 5.9), 2.5A (Figure 5.10), 2.7). However, 1.2A, 2.5A and 2.7A were weakly expressed and were difficult to detect. One cDNA probe bound non-specifically to all RNA, i.e. ribosomal, transfer RNA, etc. (2.12G, Figure 5.10), and 3 did not produce a signal (2.10G, 2.11G, 2.15G). None of the signals attained were differentially expressed between treatments.

Despite a lack of RNA expression apparent on a number of Northern blots, clones were confirmed to be present in original RNA samples after RT-PCR. Using positive and negative controls, RT-PCR of the four original RNA samples produced fragments of the expected size. 12 of 13 cDNAs were successfully amplified, however, not all cDNAs showed the same expression observed on the differential display gel. In most cases, the specific RNA was found in all 4 treatments, with the exception of metavinculin/vinculin, which was present in only 3 (Control, N-LDL, MM-LDL samples but never in H₂O₂ treatment) (Figure 5.11). These patterns of expression are not consistent with those observed on the differential display gel.
Figure 5.9. Northern Analysis of clone 2.2 (Fte-1). Control (C), and H$_2$O$_2$ (H) treated RNA was electrophoresed on a 1.75% formaldehyde gel, and transferred to nitro-cellulose filter. The filter was hybridised with a PCR $^{32}$P-labelled probe (Fte-1) overnight.
Figure 5.10. Northern Analysis of clones 2.12G (Vinculin/Metavinculin) and 2.5A (unknown). Control (C), and H$_2$O$_2$ (H) treated RNA was electrophoresed on a 1.75% formaldehyde gel, and transferred to nitro-cellulose filter. The filter was hybridised with the appropriate PCR [$^{32}$P]-labelled probe overnight.
2.5A

M 1 2 3 4 5 6

Key:
1 - H₂O₂
2 - N-LDL
3 - MM-LDL
4 - Control
5 - (-ve) control
6 - (+ve) control

271 bp

2.12G (Metavinculin/Vinculin)

M 1 2 3 4 5 6

Key:
1 - H₂O₂
2 - N-LDL
3 - MM-LDL
4 - Control
5 - (-ve) control
6 - (+ve) control

350 bp

Figure 5.11. Examples of RT-PCR showing presence of differentially expressed bands in original RNA samples. RT-PCR was carried out using the original RNA samples with primers designed from the original differential display bands. (M) marker φX 174. (-ve control) RT-PCR mix, with primers, no RNA. (+ve control) RT-PCR mix, with primers, plus control DNA/RNA: PAW109 control (IL-1α RNA) (Perkin-Elmer), or genomic DNA.
5.4 Discussion

Differential display has provided an alternative method to study changes in gene expression in cell systems. The primary advantage of differential display over current techniques is the possibility of making multiple comparisons between tissue or cell samples. Side by side comparisons can be made for example of numerous developmental stages or samples of different patients. In this chapter, the use of differential display as a potential tool to investigate changes in gene expression associated with different sources of oxidative stress, has been explored.

Comparisons were carried out between hydrogen peroxide and minimally modified-LDL (MM-LDL) treated cells and included a native LDL (N-LDL) treatment and no treatment as suitable controls. RT-PCR was carried out using RNA from each of the four samples. Patterns of expression of cDNAs were compared by looking for similarities or differences among the four conditions, after display of the cDNAs on a sequencing gel. Tables 5.1 and 5.2 represent expression of cDNAs among the four treatments after RT-PCR, Table 5.3 summarises the similarities and differences.

The data suggests that no particular pattern is predominant. There were no incidences of bands present in both oxidative stress conditions (MM-LDL and H$_2$O$_2$ only), which suggests that the two treatments have fundamentally very different effects on gene expression. Expression in both N-LDL and MM-LDL treatments (but not control or H$_2$O$_2$) was observed on only two occasions, suggesting that expression of these bands was due to the presence of lipoproteins irrespective of their oxidative state. This may also suggest that the different states of oxidative modification between native and minimally modified LDL was insufficient. This may be due to either further modification of the native LDL upon cell incubation or storage, or due to insufficient modification of the minimally modified LDL.

Expression of cDNA bands unique to each treatment were also identified. Of 34 events, only one cDNA band was up-regulated uniquely by MM-LDL treatment and 2 bands up-regulated uniquely by N-LDL treatment. In contrast 7 cDNA bands were up-regulated in response to H$_2$O$_2$ treatment, and 8 cases appeared where only bands in control cells were
observed. Very often, cDNA bands were either expressed in all conditions except H$_2$O$_2$ treatment, or only present in H$_2$O$_2$ treatment. The differences between control and hydrogen peroxide treatment accounted for 86% of total differences observed. No bands were found common to both conditions. This may suggest that hydrogen peroxide treatment causes significant changes in gene expression associated with oxidative stress. Considering the last observation, further work was carried out using only hydrogen peroxide treated samples and control samples. This minimised the number of PCR reactions necessary for further differential display experiments and reduced the amount of RNA needed for further analysis using Northern blots. Reducing the number of samples to be analysed also seemed to improved reproducibility of PCR products when carried out in triplicate.

To confirm differential expression of the excised cDNAs, sequence information was obtained which would permit the designing of primers that could be subsequently used in RT-PCR reactions or PCR labelling experiments. 50% of the sequences obtained were found to be homologous to sequences that had previously been identified by others and entered into the gene databases GENBANK and EMBL. The other 11 sequences were not homologous to any sequences in the databases and therefore represent novel sequences. Novel sequences are of greater interest since they represent unknown markers of disease processes, and provide novel targets of therapeutic intervention. However, the identification of familiar gene sequences is also of interest in determining processes or pathways involved in disease processes or in linking disease processes with common well-defined mechanisms.

Of the known sequences identified, all represented mRNA sequences, thus confirming the use of differential display as a method of detecting mRNA from cells and proving that cDNAs from the gel were not artefacts of PCR or from contaminating chromosomal DNA. Over 40 different sequences were obtained, the length of which varied considerably, from 74 bp to 800 bp, the average being 293 bp. Of the 21 recognised genes to which the cDNAs were homologous, mRNA lengths varied between 165 bp and 7.7 Kb averaging 2.52 Kb. In almost all cases, the cDNAs isolated represented the 3' most region of the mRNA, this most often being in the 3'-untranslated region (Figures 5.7 and 5.8). This suggests that
although there is a tendency towards the amplification of cDNAs at the 3' region of mRNA, there is no bias in the length of the cDNA amplified, or the length of the mRNAs from which the cDNAs were amplified. It has been suggested that differential display is biased in terms of amplifying high copy number mRNAs and raises concern over the identification of rare mRNA species (Bertioli et al., 1995).

After obtaining sequencing information, primers were designed to obtain longer length cDNAs, which would aid further characterisation or for use in RT-PCR. RT-PCR using new primers were carried out on the original RNA samples to produce the same cDNAs as those derived using the original oligo dT and arbitrary primers. This would confirm that the cDNAs were not PCR artefacts and represented true messages. RT-PCR can also be used to check for the presence of the particular cDNA between samples as a quick but non-quantitative way of confirming differential expression.

mRNA that appeared to be differentially expressed on a differential display gel did not seem to be differentially expressed when message specific primers were used in RT-PCR. The non-specific amplification of cDNAs from RT-PCR in all four conditions suggests that either PCR artefacts have been introduced into the RT-PCR reaction, or the band represented on the original sequencing gel was not truly unique to one or more experimental conditions. However, since both positive and negative controls were consistently included in RT-PCR experiments it is unlikely that the former explanation is responsible for the lack of reproducibility in expression being observed. This highlights one of the main problems of differential display, it lacks reproducibility, and hence the need for further confirmatory experiments.

True differences in gene expression associated with experimental conditions requires confirmation using Northern blot analysis. Despite little RNA being required to carry out differential display, considerable amounts of RNA are needed for these confirmatory steps. For this reason, Northern analysis was carried out using only control and hydrogen peroxide treated cells. cDNA probe synthesis was carried out using the PCR labelling method or random prime labelling method. PCR labelling is more sensitive, and hence low copy number RNA’s should also be detected on Northern blots. The random prime labelling method was much faster and easier. However, the method gave higher
background signals, but was useful for cDNAs whose sequence did not allow suitable primers to be designed (e.g. A-T rich sequences), or for cDNAs whose sequence was unknown. The random prime labelling method also allowed labelling of multiple cDNA species in the same reaction. All templates for probes were derived from cloned inserts that had been re-amplified or excised, ensuring that only one cDNA species was labelled.

Using both control and hydrogen peroxide treated RNA, Northern analysis was carried out on various isolated cDNAs to determine if their expression was truly different. Not all cDNA species were successfully labelled, but of those that were, only 3 hybridised to messages and one hybridised non-specifically. However, none of the messages were differentially expressed between treatments.

This highlights a further problem with differential display, a high false positive rate. These false positives can arise with a frequency as high as 70% (Sun et al., 1994). Identification and confirmation of other differentially expressed cDNAs from these experiments may improve the current success rate. The following chapter investigates in more detail the problems of differential display. It provides possible explanations for the high false positive rate, and possible mechanisms to improve the detection and identity of truly differentially expressed genes.
5.5 Summary

1. Using differential display allows simultaneous comparisons to be made of various RNA samples. Experiments investigating similarities and differences in gene expression between smooth muscle cells treated with hydrogen peroxide and MM-LDL, showed that the two sources of oxidative stress are very different in the pattern of genes that they induce. Treatment with N-LDL did not significantly change the pattern of gene expression observed with MM-LDL suggesting that these two treatments are quite similar in their effects. The majority of differences in gene expression observed came from comparing hydrogen peroxide treated cells to control cells. 86% of differences found on a number of differential display gels were due to the over- or under-expression of genes associated with hydrogen peroxide treatment, and therefore a number of these differentially expressed products were further analysed.

2. cDNAs were isolated, cloned and sequence data obtained. Almost all cDNAs isolated were derived from the very 3' end of the original mRNA, and varied in length from less than 100 bp to 800 bp. 50% of the cDNAs were found to be homologous to genes already identified and submitted in gene databases. The remaining cDNAs found to be differentially expressed were novel and remain unidentified.

3. None of the cDNAs that appeared differentially expressed on the differential display gel were shown to be differentially expressed using Northern analysis. Many of the cDNAs failed to hybridise to any message at all, suggesting that the relevant mRNA has an extremely low copy number, or that the cDNA probe used was not derived from RNA at all, but an artefact of PCR or cloning. Three differential display products did hybridise to messages of the correct size, which confirms that those isolated products were derived from mRNA sequences, but were not under- or over-expressed uniquely to hydrogen peroxide treatment as the original display might suggest.
Chapter 6

DIFFERENTIAL DISPLAY: PROBLEMS AND IMPROVEMENTS
6.1 Introduction

Although differential display has been used to successfully isolate and sequence differentially expressed genes associated with various diseases including cancer (Sun et al., 1994), heart disease (Russell et al., 1994), and diabetes (Nishio et al., 1994), two problems remain inherent with the technique. Firstly, the pattern of differential expression initially observed on a sequencing gel often cannot be confirmed on Northern blot analysis (Liang et al., 1992, and Sun et al., 1994). Secondly, the cDNAs obtained usually represent the extreme 3' end of the mRNA, often in the 3' untranslated region which makes further characterisation of cDNAs difficult, since this part of the mRNA is frequently not included in the sequence data submitted to gene databases. It became clear quite early in the project that lack of reproducibility was a serious problem, and considerable time was spent trying to improve the reliability of the method.

6.2 Aim

Previous experiments were carried out to identify novel targets of oxidative stress using differential display. Although the technique yielded numerous sequences of both known and novel genes possibly regulated by oxidative stress, the importance of these genes was unclear without knowledge of their differential expression between samples. As the technique of differential display clearly needed refining, efforts were concentrated on finding reasons for such a high frequency of false positives and improvements were considered that would streamline the process of identifying true positives. To reduce the number of false positives, it was first necessary to understand the reason for such effects, then to suggest ways of optimising reproducibility and improving techniques.
6.3 Results

The initial focus of improving differential display’s inherent problems was to improve the quality of the starting material. Secondly, having confirmed that new mRNA samples were derived from cells exposed to oxidative stress, they were used to probe some of the cDNAs isolated from differential display experiments described in chapter 5. Thirdly, experiments showed that multiple cDNAs were present within a single band on a differential display gel, which may account for such high false positive rates. Finally, measures were taken to improve the resolution of bands on a differential display gel to reduce the number of co-migrating cDNAs, and to improve the efficiency of screening and identifying differentially expressed cDNAs.

6.3.1 STARTING MATERIAL

6.3.1.1 Sample quality

It is essential that the sample RNA is undegraded and free from contaminating DNA. The integrity of RNA was routinely checked on a 1% agarose gel stained with ethidium bromide (Figure 5.2). Chromosomal DNA was removed by DNase 1 treatment of the RNA samples to ensure that mRNA and not genomic DNA products were amplified and displayed. DNA contamination is probably the main source of false positives or negatives. Since these measures were already incorporated into the original methods employed they are not considered as a further improvement, although their importance must be stressed. In subsequent experiments an additional control was included, an RNA sample that had not been reverse transcribed in the first half of the reaction, but had then been PCR amplified along with other RT samples, i.e. RNA was the template for PCR not cDNA. If any bands were present in this lane, they were due to the PCR amplification of contaminating DNA sequences and were excluded in consideration of differential expression of bands. Only 1% of bands were observed in this lane, however, they were frequently less than 100 bp in length, only bands of greater than 100 bp were excised for further analysis.
6.3.1.2 Sample choice

Although it appears obvious, it is important to ensure that samples are sufficiently different to enable detection of changes in gene expression. Considerations are required into the appropriate time points to compare, or the correct dosage of a treatment that will induce a change in gene expression. Ideally a suitable marker should be used to confirm differences have occurred, such as the production of a particular protein, changes in appearance, or growth rates for example.

6.3.1.3 Sequential samples

It became apparent that differential display could be dramatically improved by using sequential samples, such as a time course of a particular treatment or different stages of development (Liang et al., 1993). This could reduce the chance of a single difference in expression occurring as a result of PCR artefacts. If real changes were to occur, they would most likely be present in increasing or decreasing amounts throughout the set of samples, rather than being present or absent.

After having obtained a number of Northern blots with no apparent difference in expression, it was feared that an 18 hour treatment with hydrogen peroxide may not be significantly different from untreated cells. In addition, Wiese et al., (1995) had suggested that by 18 hours the cell would have recovered from its treatment. During an 18 hour H$_2$O$_2$ treatment in HA-1 cells, Wiese showed that proteins were synthesised in three phases; early (0-4 h), middle (4-8 h), and late (8-15) h. No other H$_2$O$_2$ induced proteins were synthesised beyond 18 hours after treatment, by which time adaptation had already maximised.

A logical progression was therefore to obtain RNA from H$_2$O$_2$ treated cells after different lengths of incubation. HASMC were treated with 100 μM H$_2$O$_2$ for 30 min, 2 h, 6 h, 12 h, and 18 h, or left untreated. RNA was isolated and used to make Northern blots that could be probed with the original group of cDNAs. The time course treatment of RNA was also used to carry out further differential display reactions to improve on the original conditions and possibly reduce the presence of high false positives.
6.3.1.4 *Exclusion of loading differences*

Before using the ‘time-course’ Northern blots, GAPDH levels were first analysed to confirm equal loading and to avoid time wasting with poor, uneven blots, (GAPDH blots have been shown when appropriate).

6.3.1.5 *Confirmation of oxidative stress*

To confirm that oxidative stress was apparent among an 18 hour incubation of hydrogen peroxide, a suitable marker of oxidative stress was required to probe the Northern blots. In choosing a suitable marker of oxidative stress, a first choice would be one of the antioxidant defence mechanisms found within the cell for example, catalase, or superoxide dismutase, or glutathione peroxidase. However, Wiese *et al.* (1995), showed that cells had recovered from \( \text{H}_2\text{O}_2 \) treatment by 18 hours, and reported that Northern blot and enzymatic activity analyses revealed no significant increases in the transcription or translation of the classical antioxidant enzymes catalase, glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase, copper and zinc superoxide dismutase or manganese superoxide dismutase in \( \text{H}_2\text{O}_2 \) treated HA-1 cells. Therefore the marker chosen was GADD153 whose RNA levels had previously been reported to change in response to oxidative stress.

**GADD153**

Although low in normal proliferating cells, the expression of the mammalian gene GADD153 (named by the fact that it is induced by Growth Arrest and DNA Damage) is dramatically increased in response to a variety of stress stimuli. Such stresses include nutrient depletion, oxidative stress, prostaglandin A\(_2\) and calcium ionophore. A member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, GADD153 is thought to be involved in the control of cell growth and differentiation in response to cellular stress signals (Zhan *et al.*, 1994; Barone *et al.*, 1994). A variety of oxidants have been shown to induce GADD153 mRNA (Guyton *et al.*, 1996) in HeLa cells, therefore the effect of hydrogen peroxide on GADD153 mRNA expression in smooth muscle cells was investigated. The human GADD153 gene was cloned by Park *et al.* (1992). Primers were designed to span part of the coding region of the 895 bp mRNA. GADD153 cDNA was
made by PCR of human genomic DNA using the specific primers designed to give a product of 340 bp (Figure 6.1) and labelled with $[\alpha^{-32}P]dCTP$ by random prime labelling. When RNA from $H_2O_2$ treated cells was probed with GADD153 cDNA a rapid induction of GADD153 mRNA was observed 30 minutes after $H_2O_2$ treatment (Figures 6.2 and 6.3). Maximum induction occurred 2 hours after $H_2O_2$ treatment with a 3 fold induction observed. Levels of GADD153 mRNA then fell with time until after 12 hours where an increase in levels was again observed. The same blots were subsequently probed with GAPDH cDNA to confirm equal loading and transfer of mRNA. After normalising, the level of induction of GADD153 mRNA was 2.25 fold by 2 hours (Figure 6.4).

**Figure 6.1. GADD153.** GADD153 cDNA was made by PCR of human genomic DNA using the specific primers designed to give a product of 340 bp and PCR labelled using $[32P]-dCTP$. Lane 2 shows PCR reaction without template (control).
Figure 6.2. Northern Blot of GADD153 and GAPDH. RNA from H$_2$O$_2$ treated HASMC was electrophoresed, and transferred to nitro-cellulose filter. The filter was hybridised with a random-prime-labelled GAPDH probe, washed, and subsequently hybridised with a random-prime-labelled GADD153 probe. Blots were read on a digital image analyser (Canberra Packard).
Figure 6.3. Expression analysis of Northern blots. (A) GADD153 mRNA. (B) GAPDH mRNA. Message levels were measured with the image analyser (Canberra Packard).
Figure 6.4. Adjusted GADD153 mRNA levels. Message levels were measured with the image analyser (Canberra Packard). Adjustments were calculated by dividing GADD153 mRNA levels by GAPDH mRNA levels.
6.3.2 NORTHERN BLOT ANALYSIS USING mRNA FROM A TIME COURSE EXPERIMENT

Having shown that GADD153 was differentially expressed in the new time course RNA samples, the RNA could be used on Northern blots to confirm differential expression of the early differential display products. A further 13 probes were made from the AP1/AP2 differential display products against the new time course RNA. Despite many attempts, a number of probes did not hybridise to any RNA on Northern blots, and neither random prime nor PCR labelling could improve the outcome. Interestingly, 2.2, which had previously hybridised to a 1.2 Kb transcript (Figure 5.9), hybridised successfully to a 1.2 Kb transcript from time course RNA (Figure 6.5). Clone 2.2 was 85% homologous to two mRNA sequences encoding for ribosomal protein S3a and v-fos transformation effector protein (Fte-1). On closer inspection, ribosomal protein S3a and v-fos transformation effector protein (Fte-1) possessed the same sequence. Both are involved in increased activity of the cell. Clone 2.2 was found only to be present in H$_2$O$_2$ treated cells on the original differential display gel.

Northern analysis using time course RNA, showed that Fte-1 mRNA is induced rapidly, within 30 minutes of H$_2$O$_2$ treatment, then declines to below control levels by 2 hours (Figures 6.5 and 6.6). After normalising the data for loading and transferring efficiencies, it was evident that a further increase in Fte-1 mRNA was observed after 6 and 12 hours of treatment (Figure 6.7).
Figure 6.5. Northern Blot of 2.2 (Fte-1) and GAPDH. RNA from H₂O₂ treated HASMC was electrophoresed, and transferred to nitro-cellulose filter. The filter was hybridised with a random-prime-labelled GAPDH probe, washed, and subsequently hybridised with a random-prime-labelled 2.2 (Fte-1) probe. Blots were read on a digital image analyser (Canberra Packard).
Figure 6.6. Expression analysis of Northern blots. (A) 2.2 mRNA. (B) GAPDH mRNA. Message levels were measured with the image analyser (Canberra Packard).

215
Figure 6.7. Adjusted 2.2 (Fte-1) mRNA levels. Message levels were measured with the image analyser (Canberra Packard). Adjustments were calculated by dividing 2.2 mRNA levels by GAPDH mRNA levels.
Of the other probes tested, only 2.12 hybridised to transcripts from the time course RNA. Probe 2.12 was 100% homologous to metavinculin/vinculin. Metavinculin differs from vinculin by a 200 bp insertion into the vinculin sequence. Since the region of homology of 2.12 was found to be at the very 3’ most end of the sequence it was not possible to determine if 2.12 was identical to vinculin or metavinculin. Although metavinculin mRNA is 200 bp longer, Northern analysis would not be able to distinguish between the two transcripts of approximately 5 Kb. Previous experiments to label the 200 bp metavinculin-specific insert, resulted in non-specific hybridisation (Chapter 5, Figure 5.10). Therefore, Northern blot analysis was carried out using the original differential display product. The cDNA was excised and re-amplified using the same arbitrary primers as the original RT-PCR reaction then random prime labelled. This probe, which may contain a number of cDNAs (see later) hybridised to at least three transcripts on the Northern blot. Bands of 9.5 Kb, 4.4 Kb, and 1.8 Kb were observed, the strongest signal being from the 4.4 Kb transcript (Figure 6.8).

To investigate further if vinculin or metavinculin was regulated by H$_2$O$_2$ treatment, western analysis was carried out on whole cell lysates from cells treated with H$_2$O$_2$ for 0, 30 min, 2 h, 6 h, 12 h, and 18 h. An antibody that binds to vinculin and metavinculin was used to detect the 130 kDa and 150 kDa proteins. Figure 6.9 shows that only vinculin at 130 kDa was detected in HASMC, hydrogen peroxide treatment had no effect on the expression of vinculin.
Figure 6.8. Northern Blot of 2.12G (Metavinculin/Vinculin) and GAPDH. RNA from H₂O₂ treated HASMC was electrophoresed, and transferred to nitro-cellulose filter. The filter was hybridised with a random-prime-labelled GAPDH probe, washed, and subsequently hybridised with a random-prime-labelled 2.12G (Metavinculin/Vinculin) probe. Blots were read on a digital image analyser (Canberra Packard).
Figure 6.9. Western analysis of Vinculin/Metavinculin. HASMC cells were treated with H$_2$O$_2$ for the appropriate times and duplicate samples of cell lysates were electrophoresed on an 10% SDS-PAGE gel. The proteins were transferred to nitro-cellulose film and blotted with an antibody to Vinculin (1:10,000) and detected using ECL detection system.
6.3.3 MULTIPLE cDNAs MAY CO-MIGRATE

The next consideration in determining reasons for such high false positive rates was the possibility that multiple cDNAs may exist of similar size, which would co-migrate on a polyacrylamide gel. Re-amplification of an excised band can often result in the production of multiple bands of almost identical size. Thus in subsequent steps the re-amplified material contains a mixture of DNA products, some of which are not differentially expressed. Multiple products may derive from PCR artefacts during amplification, or may genuinely occur as a result of primer specific RT-PCR.

Co-migrating cDNAs may misrepresent differentially expressed bands in two ways (Figure 6.10). Firstly, if a differentially expressed cDNA in RNA population X co-migrates with a cDNA that is not differentially expressed, it may be missed during PCR amplification, cloning and sequencing (A). Secondly, if a cDNA expressed uniquely to population X is the same length as a cDNA unique to population Y, migrating together, they will appear as a constitutively expressed band and be missed from analysis (B).

![Figure 6.10](image)

**Figure 6.10. Multiple cDNAs may exist within one band.** Differently expressed cDNAs may co-migrate to give misinterpretations of differentially expressed bands on a sequencing gel. This may occur in two ways to either enhance (A) or mask (B) the appearance of differentially expressed bands on a gel.
Experiments were carried out to investigate the presence of multiple cDNAs within a single excised band and to what extent this occurred (Figure 6.11). A number of bands were excised which appeared to be differentially expressed. These bands were then PCR amplified using the same primers as used in the original differential display experiment, and cloned in the usual manner. Multiple positive colonies were then selected and PCR was carried out on the colonies to determine the size of each insert. Immediately, variations could be observed in the size of inserts from a single band. These variations must arise from PCR or cloning artefacts, since the limit of resolution of the differential display gel is only a few base pairs. This method is a much faster way of determining insert size than by amplifying plasmid DNA and restriction enzyme digestion, although this step is necessary for sequencing. A number of clones were then selected for sequencing to compare sequence data.

Figure 6.11. Selection of multiple cDNAs from single bands. Bands of interest were excised and PCR amplified. Amplified products were then cloned and a number of positive clones were used in colony PCR as a quick way of comparing insert size. Clones were then selected for sequencing to compare sequence data.
Results

Figure 6.12 shows the variation in insert size of 7 differential display products after colony PCR. Five or 10 colonies were selected for each band and toothpicked for colony PCR. After sequencing, comparisons can be made to determine if the inserts are from the same cDNA or are different cDNAs. Tables 6.1 and 6.2 show that of the 7 bands excised, not once was a cDNA found exclusively to a band, showing that multiple cDNAs do exist in every band excised. Considerable variation could be found in the number of different cDNAs found within a single band. In one band (A1) 4 out of 4 inserts tested contained different sequences. In further experiments using differential display carried out with new time course RNA, similar findings were made (Table 6.3). Four bands were excised which showed transient increases or decreases in expression over a time course of treatment, and multiple clones were selected for sequencing. Two of the bands did not contain multiple cDNAs, however, if more colonies had been selected for sequencing, other cDNAs may have appeared. In one particular band (4.6), of 15 colonies selected, 6 contained different cDNAs.

The implications of these findings is that improvements are needed not only in the resolution of co-migrating bands, but also in finding ways to select a differentially expressed cDNA out of the multiple pool of cDNAs that exists.
Figure 6.12. Different sized cDNAs exist in one migrating band. (A) Colony PCR of cloned differential display products A2, A4, A5, A6, C6, 10 colonies were tooth-picked for each differential display product. (B) Colony PCR of cloned differential display products A1, C1 and C6, five colonies were tooth-picked for each. PCR was carried out using SP6 and T7 primers. (M) Marker φX 174. Samples were run on a 4% Nusieve gel.
### Table 6.1. Multiple cDNAs exist in one migrating band.

Four differentially expressed bands were excised from the AP6 gel, amplified by PCR, and cloned into TA vector. Ten positive colonies were selected for each band. After colony PCR to size the inserts, 5 colonies were selected; 4 of which were similar in size, one being different. Plasmid DNA was then isolated, purified and sequenced using SP6 and T7 primers. Sizes refer to the length of sequence obtained.

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<thead>
<tr>
<th>ID</th>
<th>Size (bp)</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
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<td>Novel (same)</td>
</tr>
<tr>
<td>A2.2</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>Nicotinamide Methyl Transferase</td>
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<tr>
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<td>A6.3</td>
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Table 6.2. Multiple cDNAs exist in one migrating band. Four ‘differentially’ expressed bands were excised from the AP6 gel, amplified by PCR, and cloned into TA vector. Five positive colonies were selected for each band. After colony PCR to size the inserts, 4 colonies were selected. Plasmid DNA was then isolated and purified and sequenced using SP6 and T7 primers. Sizes refer to the length of sequence obtained.

<table>
<thead>
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</tr>
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<tr>
<td>C6.4</td>
<td>78</td>
<td>Novel</td>
</tr>
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</table>
### Table 6.3. Multiple cDNAs exist in one migrating band.

Four ‘differentially’ expressed bands were excised from the AP4 gel, amplified by PCR, and cloned into TA vector. A number of colonies were selected for each band. After colony PCR to size the inserts, plasmid DNA was then isolated and purified and sequenced using SP6 and T7 primers. Sizes refer to the length of sequence obtained.

<table>
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<th>Band</th>
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<th>Identity</th>
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<td>506</td>
<td>Ornithine decarboxylase antizyme</td>
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</tr>
<tr>
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<td>610</td>
<td>STM-2/S182 homologue</td>
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<td>4.1.19</td>
<td>612</td>
<td>60 KDa Ribonucleoprotein (Ro)</td>
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<tr>
<td></td>
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6.3.4 IMPROVING THE RESOLUTION OF DIFFERENTIAL DISPLAY cDNAs

An inherent problem associated with denaturing polyacrylamide gels, is that although denaturing gels generally produce much sharper bands than non-denaturing gels, which may aid the detection and recovery of differentially expressed bands, they are also associated with a larger number of doublets and triplets especially at the lower molecular weight range (<200 bp). Non-denaturing gels also allow easier processing of the cDNA fragments, since a further ethanol precipitation step is not necessary. However, although non-denaturing gels simplify the band complexity in the lower half of the gel, double stranded DNA, as well as hetero-duplexes (formed as a result of Taq DNA polymerase errors) for the same gene may be resolved as different bands on the non-denaturing gel. In all differential display experiments, only bands greater than 100 bp were excised, which also aided characterisation of the DNA fragments, and excisions of bands found within doublets and triplets were avoided where possible.

Experiments were carried out to improve resolution of not only doublets and triplets but of all bands on a sequencing gel to reduce the number of co-migrating cDNAs. Previously, differential display products were run on a 6% polyacrylamide gel for 3 hours at 60 W constant power. The resulting gels displayed products ranging in size from 10 to 500 bp, the majority of bands being 200-300 bp in size. Recently, a gel electrophoresis system was developed (Becton Dickinson), which accommodates longer sequencing gels and allows control of the temperature of the gel when power is applied. It is thought that fluctuations in temperature and temperatures exceeding 50°C may interfere with resolution, therefore, by specifically controlling the temperature of the gel, resolution should be enhanced. The gels are also 50% longer than the original gels, which should allow either a larger range of product sizes to be displayed on the gel, or allow greater resolution between a particular subset of cDNAs.
Samples were applied simultaneously to both the original gel and the new longer gel under the following conditions:

<table>
<thead>
<tr>
<th>Old method</th>
<th>New method</th>
</tr>
</thead>
<tbody>
<tr>
<td>6% denaturing polyacrylamide gel</td>
<td>6% denaturing polyacrylamide gel</td>
</tr>
<tr>
<td>3 hr run</td>
<td>6 hr run</td>
</tr>
<tr>
<td>1700 V, 60W</td>
<td>1800 V, 100W</td>
</tr>
<tr>
<td>Varied temperature</td>
<td>Constant 50°C</td>
</tr>
<tr>
<td>Standard length sequencing gel (40 cm)</td>
<td>Longer gel (60 cm)</td>
</tr>
</tbody>
</table>

Figures 6.13 and 6.14 show comparisons between the two methods of display. These figures showed that a single band could be further resolved into 2 bands. In addition, more than 4 bands became apparent after improved resolution of a single intense band.

The longer resolving gel also allows a greater range of cDNA lengths to be displayed, and enhances resolution of larger sized cDNAs, which often do not travel far enough down a gel to be observed clearly. This method permits the isolation of cDNAs of up to 1 Kb in size, which would aid further characterisation of the cDNA, and partly solve the problem of cDNAs only matching the 3’ UTR of mRNAs.

Because this gel system is not bound to filter paper and dried, recovery of bands is improved, since a further ethanol precipitation step is not required to remove paper debris. PCR amplification can be directly carried out on the reconstituted band of interest.
Figure 6.13. Improving the resolution of co-migrating cDNAs. Differential display products from primer combination AP4 and T₁₁A were run on a normal 6% polyacrylamide gel under normal conditions (A) or were run on a 4.5% polyacrylamide gel (Becton Dickinson) according to the manufacturer's instructions to improve resolution of co-migrating cDNAs (B).
Figure 6.14. Improving the resolution of co-migrating cDNAs. Differential display products from primer combination AP4 and T_{11}C were run on a normal 6% polyacrylamide gel under normal conditions (A) or were run on a 4.5% polyacrylamide gel (Becton Dickinson) according to the manufacturer's instructions to improve resolution of co-migrating cDNAs (B).
3.5 REAMPLIFICATION OF EXCISED BANDS

Current methods of reamplification can result in poor yields, and even fail to amplify some products, especially larger products, after 40 cycles. Table 6.4 shows that a second round of amplification does not significantly improve yields (80 cycles). In addition, the generation of non-specific products was routinely observed with an extended number of PCR cycles giving rise to false positives. Figure 6.15 shows that PCR amplification of excised bands can produce non-specific products after further rounds of PCR. One approach to limit further PCR of non-specific products is to cut out the specific band of the correct size from the gel and purify it, using the Qiagen kit, Qiaex. Further PCR can then be carried out on the single PCR product.

<table>
<thead>
<tr>
<th>Primer</th>
<th>No of bands after 40 cycles</th>
<th>No of bands after 80 cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP1</td>
<td>9 of 9</td>
<td>—</td>
</tr>
<tr>
<td>AP2</td>
<td>13 of 14</td>
<td>13 of 14</td>
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<tr>
<td>AP4</td>
<td>4 of 6</td>
<td>4 of 6</td>
</tr>
<tr>
<td>AP6</td>
<td>4 of 12</td>
<td>6 of 12</td>
</tr>
</tbody>
</table>

Table 6.4. A second round of PCR amplification (80 cycles) of excised bands does not significantly improve yields.

Figure 6.15. Further rounds of PCR can generate non-specific products. Bands were excised from a differential display gel and PCR amplified for (A) 40 cycles, or (B) after the first round of PCR, a further 40 cycles of PCR were carried out using a 1:100 dilution of template. Samples were run on a 2% agarose gel. M - φX174 marker.
6.3.6 IDENTIFYING DIFFERENTIALLY EXPRESSED cDNAs

With the knowledge that multiple cDNAs exist within a single band, and that PCR artefacts may be introduced during re-amplification of the bands, a suitable strategy was required to isolate and detect true differential expression of cDNAs with minimal effort. Use of the random prime labelling method may allow bands of interest to be confirmed for differential expression, whilst limiting the introduction of PCR and cloning artefacts, since cDNA is labelled directly after excision from the sequencing gel without further PCR amplification, cloning or sequencing. The disadvantage of random prime labelling is that if multiple cDNAs are present within a single band, all will be labelled and many bands may appear on a Northern blot. Further cloning and sequencing would then be necessary to determine which cDNA corresponds to bands present on a Northern blot. However, if no bands appear on a Northern blot, or bands do not appear to be differentially expressed, then the differential display product can be eliminated from further studies and new differential display products be sought. Using the alternative method, a number of clones would have been isolated and sequenced before discovering that the cDNA failed to produce or signal or failed to show differential expression.

With the above in mind, differential display was carried out using the new time course RNA covering hydrogen peroxide treatments from 0 to 18 h with primers AP4 and T_{11}A and T_{11}C. Figures 6.16 and 6.17 show examples of differentially expressed bands present after displaying on a sequencing gel using time course RNA.
Figure 6.16. Differential display gel using time course RNA. RT-PCR was carried out using AP4 and T$_{11}$A and T$_{11}$C primers.
Figure 6.17. Differential display gel using time course RNA. RT-PCR was carried out using AP5 and T_{11}A and T_{11}C primers.
A number of bands were recovered from the gel, and without further PCR amplification were used as probes for Northern analysis. Bands were labelled by the random prime method. Of 4 bands isolated, all were successfully labelled, however, only one of these hybridised to mRNA on a Northern blot (Figure 6.18). Probe 4.6 produced a number of signals on a Northern blot of various sizes (Figure 6.19). Using the image analyser, lanes were scanned to show how many bands were present and if any changes in expression were evident (Figure 6.20). At least 5 bands were detected (one being a doublet), most of which showed changes in expression, although the patterns of expression were not similar for each band.

Since it was clear that at least one of the bands showed differential expression, band 4.6 was amplified by PCR and cloned to determine which cDNA was responsible for at least one of the bands present on the Northern blot. Twenty positive clones were selected for colony PCR. Figure 6.21 shows the insert size for each colony, showing only 2 or 3 bands of varying size. One clone was used as a probe for both Northern and Southern blots. cDNA from clone 12 was amplified and isolated and labelled using the random prime labelling method. Figure 6.21 shows that clone 12 hybridised to a 7-9 Kb transcript and contained the same DNA sequence as clones 1, 2, 4 and 10, and 11 to 20. Clone 12 may represent band A of the original Northern of 4.6 (Figure 6.19) since both bands are of similar size. Due to the high background of clone 12, it is difficult to determine if differential expression on the Northern blot is apparent. Using the image analyser to measure total counts, it was evident that a progressive decrease in expression occurred with time. By 18 hours of hydrogen peroxide treatment, mRNA levels were 50 % less than control levels. Clone 12 contained 280 bp of sequence that was not homologous to any known sequences in GENBANK and EMBL sequence databases.
Figure 6.18. Northern analysis of band 4.6 after random prime labelling. Differential display product 4.6 was excised from the gel and labelled without further PCR amplification. RNA from H2O2 treated HASMC was electrophoresed and transferred to nitro-cellulose filter. The filter was hybridised for 2 h with a random prime labelled probe of 4.6 cDNA. A to E represent bands visible after 1 week exposure to autoradiograph.
Figure 6.19. Image analysis of probe 4.6. Data was acquired on image analyser (Canberra Packard) for (A) 2 h and (B) 4 h 47 min. (C) GAPDH control, data was acquired for 2 h. (D) Vertical analysis of bands.
Figure 6.20. Differential expression of bands A to E from probe 4.6. Lanes were scanned on an image analyser and counts measured for each band. Graphs represent total counts for each lane.
Figure 6.21. Northern and Southern blots with probe 12. Band 4.6 was PCR amplified and cloned into TA vector. Twenty colonies were toothpicking for colony PCR using T7 and SP6 primers, 1 to 10 (B) and 11 to 20 (C). Colony 12 was labelled with $^{32}$P by random prime labelling and hybridised with Northern (A) and Southern blots (B) and (C). Colony PCR samples were run on 2% agarose gel. M - φX174 marker.
Results

Chapter 6

Since clone 12 possibly represented one of the signals on the Northern blot of differential display product 4.6, other clones were selected to discover the identity of the bands. Since clone 12 cDNA did not hybridise to cDNA from clones 3, 5, 6, 7, 8, and 9 of the 20 selected, these clones would be subsequently used as probes for Northern analysis. Clone 3 was chosen since the size of its insert differed most from the other clones. Clone 3 cDNA was isolated and labelled as before and used as a probe for Northern analysis of time course RNA. Probe 3 produced a weak signal that took 60 hours to acquire on the image analyser, and suggested that probe 3 bound to a very weakly expressed mRNA species of 1.5 Kb (Figure 6.22). A progressive increase in expression of mRNA is observed with time, in contrast to the decrease observed with time for probe 12. By 18 hours of hydrogen peroxide treatment, mRNA levels are 50% greater than control levels (Figure 6.23). Clone 3 also contained 280 bp of sequence that again did not show homology to sequences found in GENBANK/EMBL, and might therefore represent a novel sequence.
Figure 6.22. **Northern analysis of clone 3.** Clone 3 was selected from a number of colonies from the differential display product 4.6. RNA from H$_2$O$_2$ treated HASMC was electrophoresed and transferred to nitro-cellulose filter. The filter was hybridised for 2 h with a random prime labelled GAPDH probe, washed, and subsequently hybridised with a random prime labelled probe of clone 3 cDNA. Image shows acquisition of data after 68 hours for probe 3, and 4 hours for GAPDH.
Figure 6.23. Differential expression of bands from probes 12 and 3. Clones 3 and 12 were selected from colonies of differential display product 4.6. Northern blots were scanned on an image analyser and counts measured for each lane. Graphs represent percentage change from control.
6.4 Discussion

Although in principle, the technique of differential display is simple and elegant, in practice it is difficult. Recent reports on refinements and methods suggest that the involved procedures are technically challenging (Bauer et al., 1993; Liang et al., 1993). Differential display has been used successfully to identify many differentially expressed genes, yet a number of problems remain intrinsic to the technique, which have limited its usefulness. The problems encountered with differential display are summarised below:

1. The generation of non-specific, non-reproducible PCR products (noise level) at a rate comparable to the number of real differences.
2. Failure to reproduce the pattern of expression observed on the differential display gel.
3. Failure to clone cDNAs that would reconfirm differences of mRNA levels.
4. Frequently no signal or no difference in message levels is detected on Northern blots when probed with cloned cDNA fragments.
5. The extent of differential expression as revealed by Northern blotting is frequently less dramatic than that suggested from the intensities of the original PCR.
6. cDNAs obtained often represent only ~300 bp of sequence.
7. cDNAs mostly represent the extreme 3' end of mRNA (usually in the 3'UTR) making further characterisation of the cDNA difficult.
8. Bias towards detection of high copy number mRNA species.
9. Considerable quantities of mRNA are required for confirmatory steps.

In summary, the main problem of differential display is the rather high rate of 'false positives' among cloned genes, which cannot be confirmed by Northern analysis. A major problem is that a single band on the gel may not be composed solely of one cDNA species. Even though there are reports that one band represents one gene (Guimares et al., 1995), it becomes increasingly evident in the literature that single bands displayed on a gel are additive products of distinct cDNA fragments. Some of these cDNAs may be derived from differentially expressed genes; some may not.
Discussion

IMPROVEMENTS TO THE TECHNIQUE

Earlier attempts at differential display did not identify genes that were differentially expressed (Chapter 5). Improvements were therefore necessary in not only the reproduction of RT-PCR, but also in selectively identifying the positive clones that would confirm differential expression. The first step in seeking reasons for failure and possible ways of improvement was to consider the starting material.

Sample quality

Whilst every effort was taken to ensure minimal DNA contamination, an additional control was included to the PCR reactions containing sample RNA without reverse transcribing. Any cDNA products resulting after PCR would have derived from DNA sources. A small percentage of bands were visible in this sample, which tended to be less than 100 bp, therefore any differences in expression between treatments less than 100 bp were excluded from further analysis.

Multiple samples are more informative

It is evident in differential display that the noise level, i.e. the number of non-reproducible bands, can be quite comparable to the number of real differences. It is therefore necessary to repeat PCR reactions to determine which differences can be confirmed. Routinely, triplicate samples have been used. The alternative approach, is to compare several samples side by side, which is possible with differential display. Differentially expressed genes that are unique to a process such as cell transformation, can be more surely isolated. Multiple displays, therefore, provide an internal control to minimise choosing false positives that randomly vary between lanes or individual cell specific genes that may not be relevant to the system under study. These multiple samples can represent RNA's isolated from different patients with the same disease, from successive developmental stages, or from different time points after a certain treatment. Further RNA samples were therefore isolated from HASMC after treatment with hydrogen peroxide over various time points. HASMC were incubated with 100 μM H₂O₂ for 30 min, 2 h, 6 h, 12 h, or 18 h, or left untreated.
Discussion

Chapter 6

Confirmation of sample differences

After proving that numerous differential display products from previous experiments were not differentially expressed on Northern blots (Chapter 5), it was feared that the treatment of hydrogen peroxide for 18 hours was not sufficient to detect changes in gene expression. To confirm that this treatment was representative of oxidative stress conditions and would be sufficient to detect changes in gene expression, a suitable marker was used. GADD153 was chosen since it is known to be upregulated in cells under various stress-conditions including oxidative stress. Sequence was obtained and primers designed to produce a suitable template for probing Northern blots of the new time course RNA (Figure 6.1).

Hydrogen peroxide treatment caused a rapid increase in expression of GADD153 mRNA after 30 minutes (Figures 6.2 and 6.3) in HASMC. This increase was maximal by 2 hours of treatment, showing a 2.25 fold induction after normalising for GAPDH, after which time levels declined (Figure 6.4). Interestingly, levels of GADD153 mRNA increased again by 18 hours of H₂O₂ treatment. Guyton et al., (1996), also showed that H₂O₂ induced GADD153 mRNA in a biphasic manner in HeLa cells. They reported a 5 fold-induction that was maximal after 2 hours of H₂O₂ treatment with a second induction occurring at 8 h. The greater fold induction is likely accountable by the concentration of H₂O₂ used, in Guyton’s work, 50 µM H₂O₂ was used compared to 100 µM used in this study. They showed that increasing amounts of H₂O₂ reduced the extent of induction of GADD153 mRNA. Differences in the time for the second increase in induction may also be due to different cell cycle times of the two cell types.

Northern analysis of old differential display products

Differential display products from previous experiments were then hybridised to Northern blots containing time course RNA. One product that did hybridise to RNA on the Northern blots was clone 2.2, which was found to be 100% homologous to v-fos transformation effector protein (Fte-1). Fte-1 may play a role in transport of mammalian proteins into the mitochondria. Studies have suggested a role for altered mitochondrial function in production of a transformed phenotype (Kho and Zarbl, 1992). A rapid induction of Fte-1 mRNA could be observed after 30 minutes of H₂O₂ treatment and declined to control levels by 2 hours (Figure 6.6). Further increases in expression could be observed at 6 and 12 hours of treatment. Fte-1 may well be cell cycle regulated.
Another differential display product that previously produced a signal on a Northern blot was 2.12, however, clone 2.12 which showed 100% homology to both vinculin and metavinculin, hybridised non-specifically. Therefore, random prime labelling of the original differential display product (which may contain other cDNA species) was carried out without further cloning. This resulted in hybridisation to 3 transcripts on a Northern blot containing time course RNA. Bands of 9.5 Kb, 4.4 Kb, and 1.8 Kb were identified, the 4.4 Kb transcript showing the strongest signal (Figure 6.8). However, no distinct changes in gene expression could be observed between samples, further highlighting the lack of true positives identified with differential display.

Further experiments were carried out using western analysis to determine if changes were apparent in protein expression. An antibody that recognises both metavinculin and vinculin could only detect the presence of vinculin in both control and hydrogen peroxide treated cells (Figure 6.9). In addition, no differences in protein levels of vinculin were apparent between treatments. It was therefore impossible to conclude what effects, if any, hydrogen peroxide had on metavinculin, at either the level of gene expression or protein synthesis.

Summary of results
Over 30 hybridisations were carried out on old differential display products using new time course RNA. Approximately 6 contained bands that were of the correct size if known. Only one of these showed slight differential expression (clone 2.2, Fte-1). One showed non-specific binding, and the rest did not produce a signal despite probes being successfully labelled. This suggests that either the messages contained in the Northern blots are of very low copy number and are unable to be detected, or that the probes were not derived from mRNA templates in the original differential display, possibly deriving from chromosomal DNA contamination, or PCR or cloning artefacts.

Differential display with time course RNA improves reproducibility
Further differential displays were carried out, using time course RNA. The reproducibility of bands was significantly improved using time course RNA samples (Figures 6.16 and 6.17). It is far more likely that changes in expression are due to real changes and not PCR artefacts. Using time course RNA also appeared to reduce the number of non-specific PCR
products, but as a consequence, the number of differences evident were reduced. In
addition, using sequential samples of RNA reduced the number of bands containing
multiple cDNA species. Bands were chosen which represented progressive increases or
decreases in expression.

Mismatching of primers may generate non-reproducible bands
It is thought that many false positives are due to imperfect annealing of primers to
sequences within the mRNA pool, thereby producing the non reproducible bands found on
a gel. Evidence has shown that often 2-3 bases are mismatched between arbitrary primers
and original cDNAs. G-C bases are more prone to mismatching than A-T bases and most
mismatching occurs at the 5' end (Liang et al, 1995). Certain primers were found to
produce more bands than others on a differential display gel, which were considered to be
more successful, however, these extra differential display products are a result of non-
specific priming, and the more mismatches that occur, the more bands are produced (Mou
et al., 1994). It is questionable, what the definition of successful primer combinations is:
those that produce many bands of which a few are certain to be non-specific products, or
those that produce few bands but which are more likely to be true representations of
differential expression.

After analysing the sequence data of 93 differential display products, only one clone
showed signs of mispairing to an arbitrary primer. None of the one-base-anchored oligo dT
primers were shown to mispair. The mispair involved a single substitution of a G to a C on
a cDNA made from AP6 and T11A primers. Since mismatching occurred only once in 93
reactions, it is unlikely that the high false positive rate in these experiments was due to
mispairing of primers.

\[ \text{primer AP6 - 5'}-\text{AAGCTTGACCACAT-3'} \]
\[ \text{clone 5.4 - 5'}-\text{AAGCTTCCACCACAT-3'} \]

Multiple cDNAs may co-migrate
One possible reason for such high 'false positives' is that DNA recovered from what
appears to be a unique band, contains additional undetectable overlapping, or unresolved
cDNAs (Callard et al., 1994). Li et al., (1994b) showed each band recovered from a
sequencing gel to contain 3 or more different cDNA fragments. However, Liang et al.,
(1995), consider the occurrences of multiple cDNAs to be less frequent. They showed that 50% of bands on a sequencing gel were confirmed as being differentially expressed on Northern blots. Of these bands, 30% contained only one species (as determined by cloning and sequencing of 6 clones of each), 40% contained two species, and 30% contained more than 2 species, where only one species turned out to be differentially expressed. If two thirds of bands confirmed as being differentially expressed contained more than one cDNA, it is likely that those bands that did not show differential expression contain a greater fraction of multiple cDNAs.

Experiments were therefore carried out to determine the likelihood of multiple cDNAs existing within a single differentially expressed band, and the frequency at which they occurred. Eleven differential display bands were analysed and it was immediately evident that multiple cDNAs exist within a single band (Tables 6.1, 6.2, and 6.3). Only 19% of bands contained one cDNA, the remainder containing multiple cDNAs: 36% of bands contained 2 cDNA species, 18% contained 3 cDNA species, and 27% contained 4 or more cDNA species. All 7 bands isolated from the original differential display experiment contained more than one cDNA species (Tables 6.1 and 6.2). From the differential display experiment using time course RNA, only 2 of 4 bands isolated, contained only one cDNA species (Table 6.3). This confirms that time course RNA does represent an improvement in reproducibility and generation of non-specific PCR products.

It was also clear from the data that considerable variation occurs in the sizes of these cDNAs. For co-migration on a polyacrylamide denaturing gel, one would expect the cDNAs to be of similar size plus or minus a few base pairs. However, variations in insert size were evidently much greater than this (Figure 6.12). This may be accounted for by insufficient PCR amplification of a cDNA, for example clone A4.1 was half the size of A4.2 (Table 6.1) despite containing the same sequence.

Improving resolution
A common problem of denaturing polyacrylamide gels is that although they generally produce much sharper bands than non-denaturing gels, they are frequently associated with a larger number of doublets and triplets, especially at lower molecular weights, which makes isolating single bands difficult. Bands in the lower molecular weight range, as well
as obvious doublets and triplets were avoided, although this reduces the number of possible differentially expressed cDNAs. Instead, efforts were made to improve the resolution of not only doublets and triplets, but of all the bands on a gel, to reduce the number of co-migrating cDNAs, and hence reduce the number of false positives. A recently developed gel electrophoresis system marketed by Becton Dickinson, featured a longer sequencing gel and controlled temperature changes that occur during the running of the gel. Fluctuations in temperature and overheating of the gel can result in poor resolution of bands. It was anticipated that bands would be separated farther, in addition to holding more bands per gel. Differential display samples were run on both gels to compare resolution.

The longer gel significantly improved resolution of bands (Figures 6.13 and 6.14). What appeared to be a single band could be further resolved into 2 or 4 bands. Whatever number of cDNAs are contained within a single band, evidently this method of improving resolution of differential display products should at least half the proportion of multiple cDNAs. It is not clear, however, if bands that have been further resolved represent the same or different cDNA species. This would determine whether improved resolution helps the problem of co-migration of multiple cDNA species. The longer resolving gel permits the isolation of cDNAs up to 1 Kb in size. This may help the characterisation of cDNAs, which often fall outside of the 3' UTR of mRNAs.

Reamplification of excised bands generates non-specific products

To clone or radio-label cDNAs excised from a gel, they first need to be amplified by PCR to enhance cloning efficiency and to ensure rare mRNA species are detected. However, it is speculated that a number of multiple cDNAs present in a single band may be a result of non-specific PCR products or cloning artefacts. Further rounds of PCR of bands resulted in the generation of non-specific products (Figure 6.15). Most bands re-amplified after the first round of PCR. A second round of PCR did not significantly improve yields (Table 6.4). Therefore, those bands that failed to be re-amplified after the first round of PCR were excluded.

Whilst further PCR may introduce non-specific products, it is also believed that PCR amplification of rare cDNA species would be lost though competition. It would be interesting to compare Northern blots of random prime labelled cDNA products before and
Discussion

after further PCR amplification. Would PCR incorporate further non-specific band production, or compete out less frequently found cDNAs, which would not be amplified?

cDNA labelling- Random prime v PCR labelling
There are two pathways to take in determining true differential expression of bands once displayed on a sequencing gel. One method involves directly labelling the DNA fragment of interest using random prime labelling, which avoids PCR competition and loss of rare mRNA species, and reduces the addition of non-specific fragments often associated with PCR. However, all cDNA species within a single band will be radiolabelled and may produce multiple bands on a Northern blot, which can not be attributable to one cDNA species. Cloning and sequencing are then necessary to determine the identity of the differentially expressed cDNA.

Alternatively, PCR labelling requires amplifying the differential band of interest, cloning and obtaining sequence information to then use as a probe on a Northern blot. This method can seem a long way round to determine whether a cDNA product on a gel is truly differentially expressed. However, for products less than 200 bp, random prime labelling is not suitable and therefore this approach is necessary.

Having discovered that a number of cDNAs existed within a single band, the random prime labelling method was chosen for further experiments. If no messages were detected on a Northern blot (or if messages were not differentially expressed), a new clone could then be tested. If more than one message was detected, then suitable screening strategies were required to find the cDNA attributable to the differentially expressed message with minimal effort.

Screening and re-confirmation of clones
After cloning the differential display product of interest, the inserts need to be amplified, purified, and checked for size before sequencing or labelling. A rapid method to check for insert presence and size is to PCR directly from the colonies rather than restriction enzyme digestion of plasmid DNA. This provides a quick screening method to check if multiple cDNAs exist within a single transformation reaction. Specific colonies can then be selected to amplify and purify plasmid DNA before labelling or sequencing. Colony PCR is also
useful for Southern blot analysis in which multiple inserts can be hybridised to a cDNA probe to identify similar or different inserts (Figure 6.21).

Once sequence information is obtained, one can decide whether to use as probes depending on whether the cDNAs are novel or not (some investigators are only interested in looking for novel genes). Primers can be designed with the knowledge of cDNA sequence to provide longer templates to use as probes, or to provide templates that fall within the coding region. Probes are then made by specific PCR labelling to reconfirm differential expression. Because PCR labelling is more specific, this method tends to be more sensitive since only one cDNA is amplified. This usually provides much cleaner bands with lower background, and if multiple bands are found, these must be due to multiple splice variants, and not multiple cDNA contamination.

**Northern analysis of time course differential display products**

Further differential display reactions were carried out, using time course RNA, which gave increasing or decreasing changes in expression on a differential display gel. Only one of 4 bands excised, produced a signal on a Northern blot (4.6), despite all bands being successfully labelled. As expected, this probe hybridised to a number of bands on the Northern blot, because multiple cDNA species were likely to exist within the band (Figure 6.18). The image analyser not only reduces the acquisition time required to observe bands (4 hours instead of two weeks), but also counts the radioactivity in each lane and permits quantified measurements of gene expression changes. More than five bands were detected which ranged in size from 0.4 to 9 Kb and varied in patterns of expression (Figures 6.19 and 6.20).

Band 4.6 was cloned to determine the identity of the cDNAs that created the expression patterns observed. From previous data, it was established that a number of positive colonies were needed to be selected to ensure all possible cDNA species would be detected. 20 colonies were therefore initially selected, one of which would be used to probe a Northern blot and probe Southern blots containing DNA sequence from the other colonies to eliminate similar clones. Clone 12 hybridised to a 7-9 Kb message and contained the same DNA sequence as clones 1, 2, 4, and 11-20 (Figure 6.21).
expression of the 7-9 Kb band declined with time. By 18 hours of H$_2$O$_2$ treatment, mRNA levels were 50% less than control.

The second clone isolated and used as a probe, clone 3, hybridised weakly to a 1.5 Kb transcript (Figure 6.22). This mRNA species increased in expression with time, by 18 hours of H$_2$O$_2$ treatment, mRNA levels were 50% greater than control levels. Both clones 3 and 12 represented different sequences of 280 bp and neither clone showed homology to sequences found in Genbank/EMBL databases. Despite the differences in expression being only two-fold (Figure 6.23), clones 3 and 12 may represent novel targets of oxidative stress in HASMC. This emphasises another problem of differential display, in that expression differences on a Northern blot are very often less than the observed differences on the sequencing gel. Typically, no greater than 5 fold differences are observed by those investigators fortunate enough to obtain differentially expressed genes. In these experiments, however, when bands could be observed on Northern blots, changes in mRNA levels never exceeded 2 fold.

Since other cDNA species clearly existed within band 4.6, more clones would have been used to probe Northern blots if time had allowed. Further characterisation of the novel sequences would be carried out, to obtain full length sequences, and to determine the distribution patterns and importance of the novel genes.
6.5 Summary

In identifying reasons for such high false positives associated with differential display, a number of problems were observed.

1. PCR re-amplification of excised bands can generate non-specific products.
2. Mismatching of primers to cDNA templates can generate non-specific products.
3. cDNA's can co-migrate in a single differential display band, so that what appears to be a single band may contain four or more different cDNAs.

The generation of non-specific products or PCR artefacts, can reduce the reproducibility of differential display, and increase the chances of isolating a 'false positive'. A number of measures were taken to increase the reproducibility of RT-PCR reactions, decrease the generation of non-specific products, and increase the efficiency of identifying differentially expressed genes.

1. DNAse treatment of RNA ensured minimal DNA contamination.
2. RNA control lanes were run alongside samples on differential display gel.
3. Use of sequential samples, i.e. a time course of H$_2$O$_2$ treatment up to 18 hours.
4. Confirmation of treatment, i.e. a marker of oxidative stress was used to confirm that H$_2$O$_2$ treatment was sufficient to induce changes in gene expression.
5. New primers were used which were longer, had a lower GC content, and contained a restriction site to aid cloning and sequencing.
6. Improvements were made to the resolution of bands on a denaturing polyacrylamide gel with a gel electrophoresis system that was longer and controlled temperature fluctuations. Resolved 4 bands from 1.
7. Use of random prime labelling of differential display products without PCR amplification reduced the generation of non-specific bands, and ensured all cDNA present in a single differential display product would be labelled.
8. Colony PCR and Southern analysis provided a fast method of detecting insert presence and size, and identification of positive clones.
9. cDNAs were identified in this manner that showed increasing and decreasing patterns of expression with time. Both cDNAs have been sequenced and remain novel.
Chapter 7

GENERAL DISCUSSION
7.1 Introduction

Oxidative stress has been implicated in a number of disease processes and is thought to be a contributing factor to the pathogenesis of atherosclerosis. The oxidation hypothesis of atherosclerosis extends from the response to injury hypothesis to suggest that oxidative modification of lipoproteins influences the cellular function of resident cells as well as stimulating inflammatory processes including the migration and infiltration of inflammatory cells, and the induction of various growth factors and cytokines. The migration and activation of these inflammatory cells further increases the oxidative imbalance within the vessel wall. The generation of reactive oxygen species by cells present in the lesion further enhances LDL modification and exacerbates the response to injury. The migration of smooth muscle cells from the media into the intima, their subsequent proliferation and extracellular matrix production, the infiltration of macrophages and the production of lipid laden foam cells are all part of a natural wound healing response to injury. Aberrant control of these repair responses leads to the formation of the characteristic atherosclerotic lesion.

Oxidised lipids and ROS have direct and indirect effects on vascular smooth muscle cells. The production of growth regulatory molecules by activated monocytes provide chemotactic and mitogenic signals for smooth muscle cells. The direct effects of oxidative stress sources on smooth muscle cells have been less studied and observations seem to be contradictory. Therefore, experiments were carried out to investigate what influences, if any, oxidative stress (in the form of hydrogen peroxide) has on vascular smooth muscle cell growth. Experiments were also carried out to identify mechanisms by which oxidative stress may influence cell activity and to identify changes in gene expression associated with oxidative stress. Identification of genes specifically associated with oxidative stress may provide new insights into oxidative stress mediated disease processes.

7.1.1 EFFECTS OF OXIDATIVE STRESS ON CELL GROWTH

The response to injury hypothesis proposes that injury to the endothelium causes vascular smooth muscle cells to migrate from the media into the intima, where they proliferate and lay down extracellular matrix as a normal repair mechanism. However, the continued presence of growth factors, cytokines and modified lipoproteins within the intima seems to maintain this repair response, and consequently substantial intimal thickening can occur.
Hydrogen peroxide has been shown by many investigators to stimulate vascular smooth muscle cell proliferation associated with an induction of several growth-related proto-oncogenes, including c-myc and c-fos. However, other investigators have shown contradictory effects of hydrogen peroxide including DNA damage, impaired cell function or cell death. Hydrogen peroxide effects vary depending on the type, species and density of cells, the concentration and duration of $\text{H}_2\text{O}_2$ exposure (Wiese et al., 1995), as well as the type of culture medium, and whether the cells are quiescent or proliferating. Experiments were therefore carried out to determine what effects oxidative stress has on smooth muscle cell proliferation and mitogen-induced entry into the cell cycle.

Hydrogen peroxide treatment alone does not stimulate smooth muscle cell proliferation, in contrast to reports by Rao and Berk (1992). They showed that 200 $\mu$M hydrogen peroxide significantly stimulated proliferation in rat smooth muscle cells. To determine if this effect was species dependant, the effect of hydrogen peroxide on bovine, rat and human smooth muscle cells was examined. Hydrogen peroxide treatment failed to stimulate proliferation in any of the species tested at concentrations up to 200 $\mu$M. More detailed experiments were carried out to determine if hydrogen peroxide would enhance or inhibit proliferation of smooth muscle cells stimulated by serum. Hydrogen peroxide pre-treatment did not enhance the serum induced entry into S-phase and subsequent incorporation of radiolabelled thymidine, rather, pre-treatment inhibited serum induced entry into S-phase. Treatment of smooth muscle cells with hydrogen peroxide did not inhibit entry of cells into S phase, but delayed their entry. This response was determined to be dose dependant with increasing concentrations of hydrogen peroxide increasing this delay. Upon entry into S phase, proliferation rates were comparable to those from cells treated with serum alone. This delayed effect was observed in all species tested.

Similar experiments were carried out with bovine and human endothelial cells. Treatment of endothelial cells with hydrogen peroxide alone did not induce proliferation. Hydrogen peroxide treatment again delayed serum induced stimulation of cells. Unlike smooth muscle cells, the delayed entry observed in endothelial cells was associated with an overall reduction in proliferation rates compared to serum treated cells. Endothelial cells were more susceptible to hydrogen peroxide’s toxic effects than smooth muscle cells. Whilst hydrogen peroxide concentrations up to 150 $\mu$M were not toxic to smooth muscle cells,
concentrations over 50 μM were toxic to endothelial cells. The difference in susceptibility between smooth muscle cells and endothelial cells may reflect their ability to cope with oxidative stress. The antioxidant defence capacity of each cell type has been reported to be different (Verkerk and Jongkind, 1992). This will determine how quickly they can metabolise different oxidants. For example, the Tₜₕ of 1.0 mM H₂O₂ in macrophage and lymphocyte cultures containing 2 × 10⁶ cells/ml is 0.8 and 24.0 min respectively (Schraufstatter et al., 1988). If endothelial cells are less effective at removing hydrogen peroxide from the culture medium, they will be exposed to oxidative stress for prolonged periods and may be subjected to more oxidative damage than smooth muscle cells.

Studies have shown that TGF-β shifts the peak of PDGF or EGF-induced DNA synthesis by 8 hours, and hydrogen peroxide release was suggested as a signal for this cell cycle-dependant inhibition of DNA synthesis by TGF-β (Shibanuma et al., 1991; Kim et al., 1993). By looking at the mechanisms involved in TGF-β induced cell cycle delay we may be able to understand the mechanisms involved in hydrogen peroxide induced cell cycle delay. Shibanuma and his colleagues have focused on identifying the mechanisms of cell cycle delay induced by TGF-β and hydrogen peroxide. They have found that hydrogen peroxide induces the phosphorylation of a 30 kDa protein (Shibanuma et al., 1991), which was identified as the heat shock protein HSP28 (Shibanuma et al., 1992a), which is involved in the differentiation-mediated cell cycle arrest (Spector et al., 1993). This suggests that hydrogen peroxide may be involved in differentiation pathway of smooth muscle cells. HSP28 may however be involved in more than one type of cell cycle delay not necessarily leading to cell cycle arrest. TGF-β is known to be involved in cell differentiation as well as stimulation and inhibition of proliferation. Since hydrogen peroxide production is observed in cells following TGF-β treatment (Thannickal et al., 1993; Ohba et al., 1994) hydrogen peroxide may play a role in this pathway. Shibanuma also discovered a novel gene associated with hydrogen peroxide treatment, TSC-22, which is a new member of the early response genes, and encodes a small polypeptide (18 kDa) that is a putative transcriptional regulator, based on its leucine zipper structure (Shibanuma et al., 1992b). Another novel clone identified was hydrogen peroxide-inducible clone-5 (hic-5) which encoded putative zinc finger motifs and was reported to have a role in the growth-inhibitory pathway associated with in vitro senescence. Down regulation of hic-5 was also found to contribute to tumourigenesis (Shibanuma et al., 1994).
TGF-β-induced cell cycle delay has been well investigated, and many factors have now been identified that control cell cycle transition. TGF-β treatment of cells leads to the induction of p27 and p21 which are potent inhibitors of cyclin-dependant kinases (El-Diery et al., 1993; Polyak et al., 1994; Toyoshima and Hunter, 1994), as well as inhibition of cdk2 and cdk4 and cyclin E production, all of which are essential for S-phase transition (Geng and Weinberg, 1993; Hannon and Beach, 1994). Oxidative stress has recently been shown to cause cell cycle arrest in Chinese hamster ovary cells. Treatment of cultured cells with hydrogen peroxide caused cells to arrest in G₁ without any signs of DNA damage or loss of metabolic activity (Clopton and Saltman, 1995). Specific effects of hydrogen peroxide on cell cycle components have not yet been determined, although oxidative stress has been shown to inhibit cell cycle transition through a p53 independent inhibition of p21 (Russo et al., 1995).

Hydrogen peroxide treatment of human smooth muscle cells induced the expression of GADD153. Hydrogen peroxide induced cell cycle delay may involve the Growth-arrest-and-DNA-damaging (GADD) family of genes, since they are known for their effects on growth arrest and differentiation, and are induced by a variety of cellular stresses including oxidative stress. GADD45 protein binds proliferating cell nuclear antigen (PCNA), and is involved in both DNA repair and cell cycle arrest. GADD45 is regulated by p53, which acts as a transcription factor to control levels of its gene expression (Smith et al., 1994). GADD153 protein is a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors and may therefore modulate expression of C/EBP-regulated genes. GADD153 has been reported to be induced by hydrogen peroxide treatment and appears to contain an upstream AP-1 binding element that may regulate its expression (Guyton et al., 1996).

7.1.2 EFFECTS OF OXIDATIVE STRESS ON NFκB ACTIVATION

Oxidative stress has been reported to play important role in intracellular signalling (Schreck et al., 1991; Schenk et al., 1994). As previously discussed, in addition to mediating the effects of TGF-β, ROS are produced in response to TNF-α, PDGF and insulin treatment, and the use of antioxidants have shown that this production is necessary to elicit the effects of these growth regulatory molecules responses (Beutler and Cerami, 1989; Shibanuma et al., 1991; Sundaresen et al., 1995). The role of oxidative stress in
mediating the activation of the transcription factor NFκB by various stimuli has been the subject of in-depth investigation. Three lines of evidence support the idea that NFκB is an oxidative-stress responsive factor: firstly, its activation in response to low concentrations of hydrogen peroxide; secondly, the suppressing effect of a variety of antioxidants on its activation in response to a variety of stimuli; thirdly, observations showing that several NFκB-activating agents can stimulate the production of ROS (reviewed by Schreck et al., 1992). Little is known of the mechanisms involved in ROS mediated NFκB activation. NFκB is sensitive to redox variations in vitro, however, general oxidation of the transcription factor inactivates the complex in intact cells (Toledano and Leonard, 1991).

The importance of NFκB activation in smooth muscle cells and other cells of the vessel wall has been emphasised in immuno-histochemical studies that have detected the presence of active forms of NFκB in resident cells of the atherosclerotic lesion, including smooth muscle cells, macrophages and endothelial cells (Brand et al., 1996). NFκB may be important in smooth muscle cell function, since antisense oligonucleotides to the p65 subunit of NFκB inhibited human smooth muscle cell adherence and proliferation, and prevented neo-intima formation in balloon angioplasty treated rat carotid arteries (Autieri et al., 1995). It was not known if hydrogen peroxide has any effect on NFκB activation in smooth muscle cells. Therefore, the effects of hydrogen peroxide on NFκB activation were examined in human smooth muscle cells. The mechanisms of NFκB activation and the presence and role of inhibitory proteins (IκB) in smooth muscle cells were also investigated.

Under normal circumstances IκB is present in the cytoplasm at high levels complexed to NFκB to render the transcription factor inactivate and prevent its translocation to the nucleus. Upon cell stimulation IκB-α degradation occurs concomitantly with NFκB activation. Once NFκB enters the nucleus and binds to DNA, the synthesis of further IκB protein is observed, which acts as feed back control mechanism to further inhibit NFκB activation (Arenzana-Seisdedos et al., 1995). In smooth muscle cells, TNF-α treatment activated NFκB, since loss of IκB-α protein was observed within minutes of addition to cells. In agreement with this theory of NFκB activation and its control, protein levels of IκB-α were seen to accumulate after 30 minutes, reaching control levels by one hour of
treatment. No change in IκB-β levels were observed following TNF-α treatment of cells. This suggests that IκB-α is the main target involved in NFκB activation following TNF-α treatment.

Hydrogen peroxide treatment did not appear to activate NFκB, since IκB-α levels did not alter following treatment of smooth muscle cells with hydrogen peroxide. Hydrogen peroxide treatment did not inhibit NFκB activation since, co-treatment of cells with TNF-α and hydrogen peroxide still induced IκB-α degradation and re-accumulation.

A more direct measurement of NFκB activation was carried out using immuno-fluorescent studies with an antibody specific to the p65 subunit of active NFκB. TNF-α treatment of smooth muscle cells resulted in specific immunostaining of cell nuclei, further confirming activation of NFκB and its subsequent translocation into the nucleus by TNF-α. In addition, treatment of smooth muscle cells with hydrogen peroxide also resulted in specific immunostaining of the nucleus. This suggests that activation and translocation of NFκB to the nucleus does occur in hydrogen peroxide treated cells even though IκB degradation could not be observed.

It may be possible that activation of NFκB by hydrogen peroxide involves other IκB proteins. Until recently only IκB-δ and IκB-γ had been discovered as additional members of the IκB family, to which no antibodies are available. S. Whiteside and A. Israel (Pasteur Institute, Paris) at a recent NFκB meeting, reported on the characterisation of IκB-ε, a novel protein with six ankyrin repeats belonging to the IκB family. IκB-ε was found to be a major IκB protein exclusively associated with RelA and c-Rel in all cell lines investigated. In THP-1 cells it constituted more than 90% of the IκB proteins. The presence of IκB-ε in SMC and its degradation following hydrogen peroxide treatment needs to be determined. There have been no reports of hydrogen peroxide-induced IκB degradation as yet. If it becomes apparent that the newly identified IκB proteins do not play a role in hydrogen peroxide induced NFκB activation in smooth muscle cells, then alternative mechanisms need to be sought. One suggestion mentioned in chapter 4 was that hydrogen peroxide induces NFκB activation without interfering with IκB binding. It may possible that hydrogen peroxide treatment causes a conformational change thus releasing the NLS region.
without IκB removal. Alternatively, hydrogen peroxide may induce a factor which competes for binding with NFκB and by binding to IκB, releases the inhibitory molecule, thus revealing the NLS region and activating NFκB, and because IκB is bound to the factor, it is not susceptible to proteolytic degradation.

Further support for a theory that NFκB activation may occur without IκB degradation comes from a new report identifying a non-classical pathway of NFκB activation (Imbert et al., 1996). The authors show that tyrosine phosphorylation of IκB as opposed to serine phosphorylation (the classical pathway), represents a proteolysis-independent mechanism of NFκB activation. Mutational analysis suggests that tyrosine 42 is the target of phosphorylation in IκB-α, which is located in close proximity to serines 32 and 36, which regulate degradation of IκB-α. Tyrosine phosphorylated peptides can be specifically recognised by SH2 domains, which are structural motifs found in numerous cytoplasmic signalling proteins (Pawson, 1995). Phosphorylated tyrosine 42 in IκB-α may bind an SH2 domain-containing protein and it may be this interaction that releases the inhibitor from an equilibrium binding with NFκB. The authors also suggest that ROS could be inducers of tyrosine phosphorylation of IκB-α, since hydrogen peroxide is a potent inhibitor of tyrosine phosphatases leading to the activation of protein tyrosine kinases (PTK) (Heffetz et al., 1992). All protein tyrosine phosphatases have reactive cysteine residues in their active site, a feature that makes some of these enzymes oxidant sensitive. H₂O₂ has been shown to activate the tyrosine kinases Ick, fyn, zap-70, and syk in T cells (Schieven et al., 1993, 1994). It will be interesting to see which of the over a hundred conditions known to activate NFκB, rely on tyrosine phosphorylation. Good candidates are those activating tyrosine kinases, such as PDGF, Insulin, UV, IL-1, TNF-α, and TGF-β, all of which interestingly require the intracellular production of hydrogen peroxide to mediate their effects.

Tyrosine phosphorylation therefore seems to be a plausible mechanism through which the redox environment can regulate signal flow through the NFκB pathway. Tyrosine kinase activity can be increased by oxidants and decreased by antioxidants. In contrast, tyrosine phosphatase activity, which dephosphorylates the protein tyrosine residues that are phosphorylated by tyrosine kinases, is inhibited by oxidants and protected by antioxidants (Bauskin et al., 1991; Fischer et al., 1991; Devary et al., 1992). It seems possible that
hydrogen peroxide and other oxidants may activate NFκB by inducing tyrosine phosphorylation of IκB. Whether hydrogen peroxide induces a kinase or inhibits a phosphatase to elicit this effect is unknown, as is the identification of suitable kinases and or phosphatases. However, the use of selective kinase and phosphatase inhibitors will allow rapid identification of such suitable candidates.

7.1.3 GENE EXPRESSION

The aim of many researchers in atherosclerosis is to find inhibitors of SMC proliferation. These inhibitors must selectively inhibit SMC proliferation without interference of proliferation in other cells. Many eukaryotic cells share a common or very similar pathway of mitogenic stimulation which involves initiation of a cascade of events ultimately leading to DNA synthesis and cell division. Unfortunately, this commonality is may preclude the identification of selective inhibitors of SMC proliferation. Many researchers are now studying changes in gene expression which are unique and necessary for SMC proliferation in order to identify potential new targets of inhibition.

Experiments were carried out to investigate changes in gene expression in smooth muscle cells associated with oxidative stress. Such genes which are likely to be induced following oxidative stress include those involved in protecting the cell from further damage, e.g. antioxidant enzymes; those involved in repair, e.g. removal of damaged DNA bases, proteases to remove damaged proteins; new synthesis of damaged molecules; and as a last resort, the cell may induce genes involved in the induction of apoptosis. If NFκB is part of an antioxidant stress response system as suggested, the factor should also be able to activate genes with protective functions such as antioxidants. One such candidate could be the inducible Mn-dependant, mitochondrial form of SOD. The transcription of the MnSOD gene is activated when cells are stimulated with TNF-α, LPS and PMA (Wong and Goeddel, 1988; Fujii and Taniguchi, 1991). Another candidate, is the gene encoding for the human form of thioredoxin, an enzyme involved in the reduction of cysteines in oxygen-damaged proteins (Holmgren, 1989). The transcription of the thioredoxin gene is also induced by a spectrum of agents known to induce NFκB. The identification of such genes associated with oxidative stress may provide new insights into the mechanisms of oxidative stress-associated disease processes, and may provide suitable markers of oxidative stress.
In addition to studying the effects of hydrogen peroxide treatment on gene expression, experiments included the study of both oxidatively modified LDL and native LDL treated cells. Since differential display allows simultaneous comparisons of multiple samples, the patterns of gene expression associated with the three experimental conditions (in addition to a control) could be compared side-by-side. This would indicate whether the two different sources of oxidative stress would induce the same set of genes, it would also show whether native and oxidatively modified LDL were similar or very different in their effects on gene expression.

It was apparent that the set of genes induced by hydrogen peroxide were different to those induced by modified LDL. Modification of LDL results in the production of various reactive oxygen species of which hydrogen peroxide may account for a very small proportion. Therefore it is likely that the effects of oxidised LDL on smooth muscle cell gene expression is different to hydrogen peroxide effects. In addition their routes of entry into the cell are very different. Hydrogen peroxide enters the cell directly whereas oxidised LDL enters the cell via scavenger receptors or phagocytosis, and is thought to associate with the membrane, thereby initiating lipid peroxidation reactions with membrane lipids. Hydrogen peroxide is thought to enter the cell immediately whereas LDL uptake is much slower, and although the half life of hydrogen peroxide is longer than for lipid peroxides, alkyl and peroxyl radicals, the fact that lipid peroxidation is a chain reaction of events suggests that oxidative stress is more persistent in a cell treated with oxidatively modified LDL than hydrogen peroxide.

The differential display gel patterns of expression between native and oxidatively modified LDL were very similar. This suggests that either minimally modified LDL was insufficiently modified to elicit any effects in addition to native LDL, or that native LDL had undergone some degree of modification before or during cell incubation. The majority of differences in gene expression were observed between control and hydrogen peroxide treated cells. No cDNAs expressed were found common to both treatments. These two conditions were analysed further since they represented the clearest differences in gene expression. A number of cDNAs were isolated, amplified, and sequenced. To confirm that these cDNAs were truly differentially expressed, probes were made from each cDNA to
hybridise with RNA from the two treatments. Many probes failed to hybridise to RNA on Northern blots, and of those that did, none were found to be differentially expressed.

That none of the cDNAs initially identified in differential display were in fact differently expressed was a major cause for concern. Previous reports have proved this technique to be successful, although many admit that a number of false positives were obtained before they identified differentially expressed genes, whilst others did not mention their success rate. It was necessary to investigate further why differential display had proved unsuccessful in identifying differentially expressed genes associated with oxidative stress, and how the technique could be improved to increase the success rate.

The first approach to improving the differential display technique was to improve the quality of the starting material. The most obvious questions arising from this work were “Although 100 μM hydrogen peroxide may be sufficient to induce cell cycle delay in smooth muscle cells, is it sufficient to observe gene expression changes and what is the appropriate time point that should be studied to observe these changes?”. These questions were therefore addressed. To confirm that hydrogen peroxide treatment did induce gene expression, a suitable marker was required which could be used as a probe in Northern analysis to show a change in gene expression associated with the treatment. GADD153 was chosen as the marker since its expression is known to be induced under stress conditions such as oxidative stress, including hydrogen peroxide (Guyton et al., 1996). A probe of GADD153 was designed to hybridise to the RNA samples from control and hydrogen peroxide treated cells. Hydrogen peroxide caused a 2.25 fold induction in GADD153 mRNA expression, thereby confirming that treatment led to the induction of a known marker of oxidative stress.

It was possible that by 18 hours cells had recovered from hydrogen peroxide treatment and that changes in gene expression would no longer be observed. Such a possibility was supported by the report that in HA-1 cells, hydrogen peroxide treatment induced a number of changes in gene expression and protein synthesis which were completed by 18 hours. After 18 hours, the cells had recovered from the oxidative stress situation (Weise et al. 1995). To answer the second part of the question (what is the appropriate time point of treatment that should be studied), advantage was again taken of the ability to compare
multiple samples in differential display. Cells were treated with hydrogen peroxide for various times to build up a time course of RNA samples which should cover all changes in gene expression within an 18 hour period. The advantage of using samples of a time course of treatment for differential display is that any changes in gene expression are easier to observe, since a progressive increase or decrease in gene expression would be apparent as opposed to an 'all or nothing' situation when comparing treatment versus control. This would also reduce the chances of isolating apparent differences which were in fact PCR artefacts.

Having ensured that hydrogen peroxide treatment was a good model of an oxidative stress situation and that most changes would be accounted for in an 18 hour period, the time course RNA was used initially to confirm differential expression of those cDNAs isolated from early differential display experiments. One product that hybridised to the time course RNA was clone 2.2 which was found to be 100% homologous to v-fos transformation effector protein (Fte-1). Fte-1 is thought to play a role in mitochondrial import of proteins, and studies show that it may be important in altered mitochondrial function associated with a transformed phenotype (Kho and Zarbl, 1992). Fte-1 mRNA was rapidly induced in hydrogen peroxide treated cells, with levels being maximal at 30 minutes and declining by 2 hours. Further increases in expression were also observed 6 and 12 hours after treatment. A second clone that hybridised to time course RNA was clone 2.12 which was 100% homologous to the 3' end of the vinculin/metavinculin gene. Again, there were no apparent changes in gene expression. Of 30 hybridisation experiments only 6 cDNAs hybridised to time course RNA. The remainder did not hybridise, this suggests that either the copy number of the messages contained in the RNA samples were so low they could not be detected, or that the cDNAs were not derived from mRNA templates but from contaminating DNA or PCR cloning artefacts.

The second approach to improving differential display success was to investigate why false positives occur. Liang et al. (1995) had discovered that imperfect annealing of primers to sequences within the RNA pool could lead to the production of non-reproducible bands on a sequencing gel and make comparisons of samples misleading. Certain primer combinations produce more non-specific bands than others, often 2-3 bases are mismatched and seem to involve G-C bases more than A-T bases. However, after
comparing 93 sequences of differential display products, mismatching does not seem to be the underlying fault in the poor success of these experiments. Only one clone showed signs of mispairing to an arbitrary primer, involving a single base substitution of a G to a C.

Further experiments were then carried out to determine other reasons for the high false positive rate of differential display. The possibility existed that cDNA recovered from a single band, contained additional undetectable overlapping or unresolved cDNAs which would interfere with the identity of differentially expressed bands. Early sequencing and Northern blotting experiments analysed only one colony for each isolated band. If more than one cDNA species were present in that band, they would not be detected. Experiments were therefore carried out to determine the likelihood of multiple cDNA species existing within a single differentially expressed band, and the frequency at which this occurred. It was evident that a single differential display band could contain more than one cDNA. In fact, 82% of bands isolated from differential display contained two or more different cDNA species. In one band, there were 6 different species identified. Interestingly, all the bands isolated from early differential display experiments were found to contain more than one cDNA species, which could explain why none of the clones showed differential expression. It would therefore be necessary to find the cDNA responsible for the pattern of expression observed on the differential display gel amongst the multitude of other cDNAs which have co-migrated.

The problem of co-migration was addressed by trying to improve resolution of cDNAs on the differential display gel. A gel electrophoresis system was used which was 50% longer and controlled temperature fluctuations to help resolution and to improve the sharpness of bands. The longer gel enabled more bands to be displayed on a gel and allowed greater separation of bands. The longer gel electrophoresis system significantly improved resolution of bands when compared to the original electrophoresis system. Single bands that appeared on the original gel were resolved into a further 2 or 4 bands. Although there may be a high number of co-migrating cDNAs within a single band, if resolution is at least doubled using this method, then the screening required to find the differentially expressed cDNA should be at least halved. The longer gel also permitted the isolation of larger cDNAs which often do not separate very well. This should aid characterisation of the
cDNA since more sequence information can be obtained that is more likely to fall within the coding region of an mRNA.

Other improvements to the technique involved labelling cDNAs isolated from the gel without further re-amplification, which was shown to generate non-specific PCR products in addition to the cDNA(s) of interest. Radiolabelling of the total differential display product was carried out rather than a single clone, to ensure that all cDNA species present in the differential display band were accounted for. If multiple bands were detected after Northern blotting, further experiments were then necessary to identify the cDNA attributable to the differentially expressed message.

By incorporating all the improvements discussed above, further differential display reactions were carried out using the time course RNA which gave clear increases or decreases in expression on a differential display gel. One isolated band was radiolabelled and used in Northern analysis. This differential display product hybridised to more than five messages of varying length and expression. The differential display product was cloned and a number of colonies screened using colony PCR and Southern analysis to identify which colonies hybridised to which messages. Clone 12 hybridised to a 7-9 Kb message, the expression of which declined with time. By 18 hours of treatment mRNA levels were 50% less than control. A second clone (clone 3) hybridised weakly to a 1.5 Kb message. This mRNA species increased in expression with time, by 18 hours of treatment, mRNA levels were 50% greater than control levels. That both clones were derived from the same differential display product yet showed opposite patterns of expression, further confirms the presence of multiple cDNA species in a single differential display band. The two clones represented different sequences of 280 bp and neither clone showed sequence homology to any genes found in Genbank/EMBL databases. Clone 3 and 12 thus represent novel genes induce in response to oxidative stress.

Although the changes in gene expression observed in these differential display experiments never exceed more than 2.5 fold, it is important to note that any change in RNA expression may be sufficient to elicit cellular responses. RNA expression is only one of several mechanisms of gene regulation. Post transcriptional regulatory mechanisms are almost always involved which could not be investigated using this methodology. The oxidative
stress response may also induce changes in RNA transport and processing control, translational control, mRNA degradation control or even protein activity control. For example, aconitase, an iron responsive regulatory protein, controls the translation and stability of ferritin mRNA and transferrin receptor mRNA depending upon the cytosolic iron concentration. Therefore oxidative stress may influence post-translational control of mRNA in addition to transcriptional control.

Since the introduction of differential display in 1992, over a 100 reports regarding improvements and/or successful applications have been published (Liang and Pardee, 1995). However, success has not been achieved by all investigators, or at least not without considerable effort. A recent review (Debouck, 1995), summarised the attitude of many who have experienced differential display, entitled ‘Differential Display or Differential Dismay?’ Yet in spite of its limitations, there have been numerous reports of the successful use of the differential display technique, as well as the continuing evolution of the original technique and supporting methodologies, which have increased its speed and reproducibility, and therefore its use in the elucidation of biological targets. The improvements described here should increase the efficiency of identifying differentially expressed genes. Although not differentially expressed, novel mRNA species that are found highly expressed may serve as useful markers of oxidative stress.

7.2 Future work

At the start of this thesis, the effects of oxidative stress on smooth muscle cells were investigated because it was hypothesised that ROS may have a proliferative effect on smooth muscle cells and may contribute to the pathogenesis of atherosclerosis and restenosis. By identifying the mechanisms involved in this proliferative effect, it was anticipated that antioxidants could be used to inhibit smooth muscle cell proliferation and control the pathogenesis of atherosclerosis and restenosis. However, it was apparent that the proliferative effects of oxidative stress in cells were complex. This was dependant not only on the concentration used, but the cell type, species and passage number. Unexpectedly, hydrogen peroxide induced a delay in the cell cycle as opposed to initiation of the cell cycle. Future studies would involve identifying the mechanisms involved in this cell cycle delay and the reasons for such an effect, by starting with the knowledge of the
effects of TGF-β on cell cycle delay, to understand hydrogen peroxide mediated effects. What role does this cell cycle delay have? Is the cell cycle momentarily paused to enable the cell to decide which path to follow following oxidative stress e.g. proliferation, differentiation, or apoptosis? The delay may simply be a mechanism to ensure all repair mechanisms are carried out before further DNA replication. As discussed, it is increasingly apparent that ROS may act as second messengers to elicit the effects of various growth regulatory molecules. The use of ROS as an effector molecule involved in NFκB activation is an interesting area to pursue. Future studies should be aimed at identifying kinases or phosphatases involved in hydrogen peroxide mediated NFκB activation to determine if hydrogen peroxide is involved in the alternative or classical pathway of activation. Further work would elucidate the purpose of an alternative pathway of activation, e.g. is the alternative pathway cell cycle specific? Are specific NFκB heterodimers involved? Is the pathway involved in mediating different responses e.g. apoptosis or proliferation? Finally studies have implicated a role of NFκB in apoptosis as well as cell growth. What role does hydrogen peroxide have in this response since it is known to induce both apoptosis and proliferation? Finally, having made a number of improvements to the differential display technique further experiments would be carried out to identify any genes that are specific to an oxidative stress situation. There is still room for further improvements, especially in screening of isolated clones in Northern analysis. Such alternatives may include RNAse protection assays which may be more sensitive to detecting rare mRNA species; reverse Northern analysis, which enables a number of cDNAs to be simultaneously isolated and screened using either minimal RNA; affinity capture, which allows the differentially expressed cDNA to be directly isolated from the Northern blot. The problem of differential display may not lie in the technique itself but how it is used. Subtle changes in gene expression may not be apparent following a mild treatment of oxidative stress. It would be more useful to compare normal versus diseased states of cells or tissues where clear differences are more likely to be apparent, and the genes will be more pathologically relevant. Challenges that would then lie ahead include, how to characterise the large number of genes emerging from the technique, to realise their biological importance, and to discover ways of controlling their effects in vivo.
APPENDIX I

MATERIALS & SUPPLIERS
Appendix I - Materials and Suppliers

Manufacturers of equipment are stated where appropriate in the text.

**Antibodies**
- Anti Cellular Fibronectin antibody (monoclonal mouse IgM) (Sigma)
- Anti Laminin antibody (monoclonal mouse IgG1) (Sigma)
- Anti Mouse IgG1 FITC conjugate antibody (Sigma)
- Anti Mouse IgG1 HRP conjugate antibody (Sigma)
- Anti NFκB p65 active subunit (mouse) antibody (Boehringer Mannheim)
- Anti p65 subunit antibody (rabbit) (Sigma)
- Anti Rabbit IgG1 FITC conjugate antibody (Sigma)
- Anti Rabbit IgG1 HRP conjugate antibody (Sigma)
- Anti Vinculin antibody (monoclonal mouse IgG1) (Sigma)
- Anti-IκB-β (C20) (rabbit IgG) antibody (Santa Cruz)
- Anti-IκBα MAD-3 (C21) (rabbit IgG) antibody (Santa Cruz)
- Anti-SM-α-actin (mouse IgG2a) antibody (Sigma)

**Chemicals and Solutions**
- (37%) (v/v) Formaldehyde (Sigma)
- 10 × React 3 buffer (Gibco BRL)
- 10% (w/v) Ammonium-persulphate solution (Bio Rad)
- 10% (v/v) SDS solution (Bio Rad)
- 20 × SSC (BDH)
- 50 × Denhardt’s solution (BDH)
- Acetic acid (BDH)
- Bis-acrylamide 30% (w/v) (37.5:1) (Anachem)
- Bis-acrylamide 6% (w/v) (19:1) (Anachem)
- Chloroform (Fisons)
- DEPC (diethyl pyrocarbonate) (BDH)
- EDTA (Sigma)
- Ethanol (Fisons)
- Formamide (Sigma)
- Isopropanol (Fisons)
- Methanol (Fisons)
- One-phor-all buffer (Pharmacia Biotech)
- PBS tablets (Oxoid)
- RNAzol B (TEL-TEST)
- Sodium Acetate (BDH)
- Sodium chloride (Sigma)
- Sodium Hydroxide (BDH)
- TEMED (tetramethylethylenediamine) (Bio Rad)
- Tris-base (Sigma)
- Tris-HCl (Sigma)
- Xylene (BDH)

**Tissue culture**
- 0.25% trypsin/0.05% EDTA solution (Gibco BRL)
- 100 mm² square petri-dishes (Sterilin)
- Carbenicillin (Sigma)
- Cell sieves (100 μm nylon) (Falcon)
- Cryotubes (Nunclon)
Appendix I - Materials and Suppliers

- Fibronectin pre-coated tissue culture flasks (175 mm²) (Falcon)
- Foetal calf serum (Gibco BRL)
- Glutamine (Gibco BRL)
- Ham’s F-12 Medium (Gibco BRL)
- Hanks balanced salt solution (Gibco BRL)
- Human recombinant TNF-α (DGRO80B) (Bachem Pharmacia)
- Penicillin/Streptomycin (Gibco BRL)
- Plasma Fibronectin (Sigma)
- Tissue culture flasks 25 mm², 75 mm², 175 mm² (Nunclon)
- Tissue culture plates 6, 24 and 96 wells (Nunclon)
- Waymouth’s medium (Gibco BRL)

Enzymes
- Amplitaq DNA polymerase (Perkin Elmer)
- EcoRI (Gibco BRL)
- RNase A (Promega)
- T4 poly nucleotide kinase (Boehringer Mannheim)

Radiolabelling
- ³²P-dATP, ³²P-dCTP (Redivue) (Amersham)
- Hybond ECL nitrocellulose membrane (Amersham)
- Hybond nitrocellulose membrane (Amersham)
- Hyperfilm cassette (Amersham)
- Hyperfilm ECL autoradiographic film (Amersham)
- Hyperfilm MP autoradiographic film (Amersham)
- Rapid Hyb buffer (Amersham)
- Rediprime DNA labelling system (Amersham)

Miscellaneous
- 3 MM Chromatography paper (Whatman)
- Agar (Lab M)
- Agarose Ultrapure (Gibco BRL)
- Bacto-yeast extract, bacto-tryptone (Difco)
- Bromophenol blue (Sigma)
- DNA markers φX174 HaeIII and 1 Kb (Gibco BRL)
- Gelslick (AT biochem)
- Hoechst 33258 (Sigma)
- Message clean kit (GenHunter)
- Mineral oil (Sigma)
- MTT (3-(4,5-dimethyliazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma)
- Nusieve (electrophoresis grade) (FMC Bioproducts)
- Plasmid midi kit (Qiagen)
- Pre-stained protein molecular weight standards (Sigma)
- Propidium Iodide (10 mg/ml) (Sigma)
- QIAquick gel extraction kit (Qiagen)
- Recovery plasmid midi prep kit (Hybaid)
- RNA markers 0.16-1.77 Kb and 0.24-9.5 Kb (Gibco BRL)
- RNAimage kit (GenHunter)
- RNAmap kit (GenHunter)
- RNase away (Molecular Bio-products)
Appendix I - Materials and Suppliers

Salmon sperm DNA (Sigma)
Saran wrap (The Dow Chemical company)
TA cloning kit (Invitrogen)
X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) (Sigma)
Appendix II - Buffers and Solutions

1 × SDS sample buffer:
50 mM Tris-base (pH 6.8), 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol.

1 × SSC:
150 mM NaCl, 15 mM Tri-sodium citrate

1% Ethidium bromide solution:
1 g ethidium bromide in 100 ml dH2O

1% Agarose mini-gel:
0.25 g agarose in 25 ml 1×TBE buffer. Microwave to dissolve, cool.

1.5% agarose/formaldehyde gel:
1.5g agarose in 74 ml 1×TBE, dissolve in microwave. 17 ml 37% (v/v) formaldehyde added when molten agarose reaches 65°C, and 9.5 ml of 10 × MOPS poured into RNase free Horizon 11:14 casting block.

1.8% Agarose mini-gel:
0.45 g agarose in 25 ml 1×TBE buffer. Microwave to dissolve, cool

3.7% Formaldehyde solution:
37% (v/v) Formaldehyde 5 ml, 10 × PBS 5 ml, dH2O 40 ml.

4% Nusieve minigel:
0.75 g Nusieve, 0.25 g agarose in 25 ml 1×TBE buffer. Microwave to dissolve, cool

5 × RT buffer:
125 mM Tris-Cl (pH 8.3), 188 mM KCl, 7.5 mM MgCl2, 25 mM DTT.

10 × formaldehyde gel-running buffer:
MOPS 41.2g, 3M sodium acetate 16.6 ml, 500 mM EDTA 20 ml in 1 litre dH2O.

10 × labelling buffer:
1 M Safe TdT buffer, BSA (RIA grade) 0.5 mg/ml, 0.6 mM 2-Mercaptoethansulphonic acid

10 × PCR buffer:
100 mM Tris-Cl (pH 8.4), 500 mM KCl, 15 mM MgCl2, 0.01% (w/v) gelatine.

10 × stop buffer:
0.1 M EDTA (pH 8.0)

10 × TBE:
Tris-base 108.8g, boric acid 55.6g, EDTA disodium salt 9.3g in 1 litre dH2O (pH 8.0).

Agarose gel loading buffer:
30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF.
DNA loading dye:
95% (v/v) formamide, 10 mM EDTA (pH 8.0), 0.09% (w/v) xylene cyanol FF,
0.09% (w/v) bromophenol blue.

Formaldehyde gel loading buffer:
50% (v/v) glycerol, 1 mM EDTA, 0.25% (w/v) bromophenol blue, 0.25% (v/v) xylene
cyanol FF.

LB medium:
bacto-tryptone 10g, bacto-yeast extract 5g, NaCl 10g in 1 litre dH2O. pH adjusted to 7.0
with 5N NaOH. Autoclave for 20 min at 15 p.s.i. on liquid cycle.

LB Agar:
Agar 15g to 1 litre of LB medium, autoclave.

PBS-T:
NaCl 8 g, KCl 0.2 g, Na2HPO4 1.44 g, KH2PO4 0.2 g, 0.5 ml Tween-20 in
1 litre dH2O, (pH 7.4).

Pre-hybridisation buffer:
6.25 ml 20 x SSC, 2.5 ml 50 x Denhardt’s reagent, 1.25 ml 10% (w/v) SDS, 15 ml dH2O
0.5 ml salmon sperm DNA (1 mg/ml).

SOC medium:
2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO4,
20 mM glucose.

Solution I:
50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0).

Solution II:
0.2 N NaOH, 1% (w/v) SDS.

Solution III:
60 ml 5 M potassium acetate (3 M final), 11.5 ml glacial acetic acid (5 M final), 28.5 ml
dH2O, (pH 4.8).

TE buffer:
10 mM Tris-Cl (pH 7.4), 1 mM EDTA (pH 8.0).

Transfer buffer:
20% (v/v) methanol, 40 mM glycine, 50 mM Tris-base.

Tris-glycine electrophoresis buffer:
Tris-base 3 g, glycine 18.8 g, 10% (w/v) SDS 10 ml in 1 litre dH2O.

X-Gal Stock Solution (40 mg/ml):
400 mg X-Gal in 10 ml dimethylformamide. Store at -20°C in the dark.
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### ABBREVIATIONS

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>BAEC</td>
<td>Bovine aortic endothelial cells</td>
</tr>
<tr>
<td>BASMC</td>
<td>Bovine aortic smooth muscle cells</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>Cu²⁺</td>
<td>Cupric ion</td>
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<tr>
<td>Cu₂SO₄</td>
<td>Copper sulphate</td>
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<tr>
<td>dATP</td>
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<tr>
<td>dCTP</td>
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<td>Diethyl pyrocarbonate</td>
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<td>DNA</td>
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<tr>
<td>GAPDH</td>
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<tr>
<td>GMCSF</td>
<td>Granulocyte/macrophage colony stimulating factor</td>
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<td>Glutathione (reduced form)</td>
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<td>Human aortic smooth muscle cells</td>
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<td>HBBS</td>
<td>Hanks buffered salt solution</td>
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<td>HCl</td>
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<tr>
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<td>Messenger RNA</td>
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304
µg  Microgrammes
µl  Microlitre
µM  Micromolar
mA  Milliamp
mg  Milligrammes
ml  Millilitre
mm  Millimetre
mM  Millimolar
MCP-1  Monocyte chemotactic protein-1
MCSF  Macrophage colony stimulating factor
MDA  Malondialdehyde
MgCl₂  Magnesium chloride
MM-LDL  Minimally modified LDL
MOPS  3-(N-morpholino)propanesulphonic acid
M  Molar
MTT  3-(4,5-dimethyliazol-2-yl)-2,5-diphenyl tetrazolium bromide
Na₂CO₃  Sodium carbonate
NaCl  Sodium chloride
NADP  Nicotinamide adenine diphosphate
NADPH  Nicotinamide adenine diphosphate (reduced form)
NaOH  Sodium acetate
NFκB  Nuclear factor κB
N-LDL  Native LDL
NLS  Nuclear localisation signal
ng  Nanogrammes
nm  Nanometre
nM  Nanomolar
NO  Nitric oxide
OD  Optical density
ORF  Open reading frame
OX-LDL  Oxidised LDL
PBS  Phosphate buffered saline solution
PCNA  Proliferating cell nuclear antigen
PCR  Polymerase chain reaction
PDGF  Platelet derived growth factor
PGI₂  Prostacyclin
PI  Propidium iodide
PKC  Protein kinase C
PMA  Phorbol 12-myristate 13-acetate
PTCA  Percutaneous transluminal coronary angioplasty
RASMC  Rat aortic smooth muscle cells
RNA  Ribonucleic acid
ROI  Reactive oxygen intermediates
ROS  Reactive oxygen species
rpm  Revolutions per minute
RT  Reverse transcription
RT-PCR  Reverse transcription-PCR
SDS  Sodium salt
SMC  Smooth muscle cells
SOD  Superoxide dismutase
<table>
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<tr>
<td>SSC</td>
<td>Saline-sodium citrate buffer</td>
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<tr>
<td>TBARS</td>
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</tr>
<tr>
<td>TBE</td>
<td>Tris-borate EDTA buffer</td>
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<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
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<td>TEMED</td>
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<td>Untranslated region</td>
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<td>Ultra violet</td>
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<td>Volt</td>
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<td>v/v</td>
<td>Volume to volume ratio</td>
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