Pathogenesis of emphysema: Molecular mechanisms underlying cigarette smoke-induced cell death.

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ABSTRACT

Cigarette smoking is the major aetiological factor in the development of Chronic Obstructive Pulmonary Disease (COPD), a collection of diseases encompassing chronic bronchitis and emphysema. Emphysema is characterised by enlargement of the distal airspaces in the lungs due to destruction of alveolar walls, and was initially thought to be the result of matrix destruction from a protease-antiprotease and oxidant-antioxidant imbalance, leading to detachment of alveolar cells. However, recently apoptosis has been implicated in alveolar cell loss; increased numbers of apoptotic epithelial and endothelial cells have been observed in the lungs of emphysema patients. Thus the effect of cigarette smoke on apoptotic cell death was investigated.

Unexpectedly, cigarette smoke condensate (CSC) did not induce apoptosis in either an alveolar epithelial type II cell line (A549) or primary human umbilical vein endothelial cells (HUVECs), but instead it induced necrosis and inhibited staurosporine-induced apoptosis. The anti-apoptotic, pro-necrotic, effect of CSC was reproduced in a model system using Jurkat T cells, when either staurosporine or Fas ligation was used as an apoptotic stimulus. Additional studies indicated that these effects might be oxidant-mediated as the antioxidant compounds glutathione and dithiothreitol prevented CSC-mediated apoptosis inhibition, and necrosis. Time course experiments revealed that CSC inhibited an early step in the caspase cascade, whereby caspase-3 was not activated. Moreover, reconstitution of the apoptosome in cytoplasmic extracts from CSC-treated cells, by addition of cytochrome-c and dATP, did not result in activation of caspases-3 or –9. Thus, smoke treatment may alter the levels of pro- and anti-apoptogenic factors downstream of the mitochondria to inhibit active apoptosome formation. Therefore these data demonstrate that CSC treatment did not induce apoptosis as previously reported. More interestingly, CSC inhibited apoptosis by preventing activation of caspases, resulting in necrotic cell death. Thus, cell death in response to cigarette smoke by necrosis, and not apoptosis, may be responsible for the loss of alveolar walls observed in emphysema.

DECLARATION

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous application for candidature for a higher degree. All work presented in this thesis was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

Julie Anne Wickenden

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DEDICATION

This thesis is dedicated to my family

especially

Leonard Tostevin

and

Harry and Eileen Wickenden

If only you could have been here to see it

ABBREVIATIONS

·OH	hydroxyl radical
3-AB	3-aminobenzinamide
4-HNE	4-hydroxy-2-nonenal
7-AAD	7-amino-actinomycin D
ADP	adenosine diphosphate
AIF	apoptosis-inducing factor
ANT	adenine nucleotide translocator
APAF-1	apoptotic protease-activating factor-1
ATP	adenosine triphosphate
BAL	bronchioalveolar lavage
BCA	bicinchoninic acid
Bcl	B cell lymphoma
BH	Bcl-2 homology
BIR	baculovirus IAP repeat
BSA	bovine serum albumin
BSO	butathione sulfoxamine
CAD	caspase-activated DNase
CARD	caspase recruitment domain
CDK	cyclin-dependent kinase
CEB	cell extract buffer
CH-11	anti-Fas activating antibody
CMF-PBS	calcium magnesium free- phosphate buffered saline
COPD	Chronic Obstructive Pulmonary Disease
CSC	cigarette smoke condensate
CSE	cigarette smoke extract
CT	computed tomography
Cyt-c	cytochrome-c
DD	death domain
DED	death effector domain
DIABLO	direct IAP binding with low pI
DISC	death inducing signalling complex
DMEM	Dulbecco's modified eagles medium
DNA	deoxyribose nucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminesence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid

ELF	epithelial lining fluid
ERK	extracellular signal-regulated kinase
E-selectin	endothelial-leucocye adhesion molecule-1/ELAM-1
FACS	fluorescence activated cell sorting
FADD	Fas-associating protein with death domain
FCS	foetal calf serum
FEV_1	forced expiratory volume in 1 second
GPx	glutathione peroxidase
GSH	glutathione
GSSG	glutathione disulphide
GST	glutathione-S-transferase
H_2O_2	hydrogen peroxide
HAT	histone acetyltransferase
HBSS	Hanks balanced salt solution
HDAC	histone deacetylase
HI-FCS	heat-inactivated foetal calf serum
HRP	horseradish peroxidase
HSP	heat-shock protein
HUVEC's	human umbilical vein endothelial cells
IAPs	inhibitor of apoptosis proteins
ICAD	inhibitor of CAD
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
INOS	inducible nitric oxide synthase
ISEL	*in situ* end labelling
IκB	inhibitory protein-κB
IκBK	IκB kinase
LDH	lactate dehydrogenase
L-Glut	L-glutamine
LPS	lipopolysaccharide
LVRS	lung volume reduction surgery
MCP-1	macrophage chemoattractant protein-1
MeH	microsomal epoxide hydrolase
MMP	matrix metalloprotease
NAC	N-aceytl-L-cysteine
NAD	nicotinamide adenosine triphosphate
NF-κB	nuclear factor-κB
NO	nitric oxide
NO_2	nitrogen dioxide

$O_2^{\cdot -}$	superoxide
P/S	penicillin/streptomycin
PAR	poly (adp-ribose)
PARP	poly (adp-ribose) polymerase
PI	propidium iodide
PI3-K	PI3-kinase
PMN	polymorphonuclear leucocyte, neutrophil
PS	phosphatidylserine
PT	permeability transition
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene difluoride
Rb	retinoblastoma protein
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Smac	second mitochondrial activator of caspases
SS	staurosporine
TBE	Tris-borate EDTA
TBS	Tris-buffered saline
TdT	terminal deoxytransferase
TEM	transmission electron microscopy
TIMPs	tissue inhibitors of metalloproteases
TNF-α	tumour necrosis factor- α
TPA	12-O-Tetradecanoylphorbol 13-acetate
TUNEL	terminal deoxytransferase-mediated dUTP nick end labelling
Tween-20	polyethylene glycol sorbitan monolaurate
UV	ultraviolet
VDAC	voltage-dependent anion channel
VEGF	vascular endothelial growth factor
VEGF-R2	vascular endothelial growth factor-receptor 2
XAF1	XIAP-associated factor 1
XIAP	X-linked IAP
$\Delta\psi M$	mitochondrial membrane potential

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Chapter 1: Introduction

1.1. THE RESPIRATORY SYSTEM

The body's requirement for oxygen is absolute, while we can survive a number of days without food or water, a lack of oxygen results in death within a few minutes. The primary function of the respiratory system is to enable oxygen to enter the blood and to give carbon dioxide passage to the external environment.

The conducting airways begin at the larynx and then continue as the trachea, before branching into the left and right primary bronchi. Each bronchus then subdivides into the secondary bronchi, the tertiary bronchi, then the bronchioles, alveolar ducts and alveolar sacs. The bronchi and the small terminal bronchioles form the conducting portion of the lung, purely serving to passage air to the gas exchange portion, which consists of respiratory bronchioles, alveolar ducts and alveolar sacs (Figure 1.1). The acinus is defined as, the respiratory airspaces arising from a single terminal bronchiole (Snider *et al.,* 1985). The bronchi are supported by rings of cartilage, the numbers of which gradually decrease with each successive division, such that airways of less than 1mm have no cartilaginous support. These small airways, and the alveolar sacs, maintain their patency by means of attachments to the underlying connective tissue and other airways and alveoli. This provides outward traction or elastic recoil on surrounding airways, preventing their collapse.

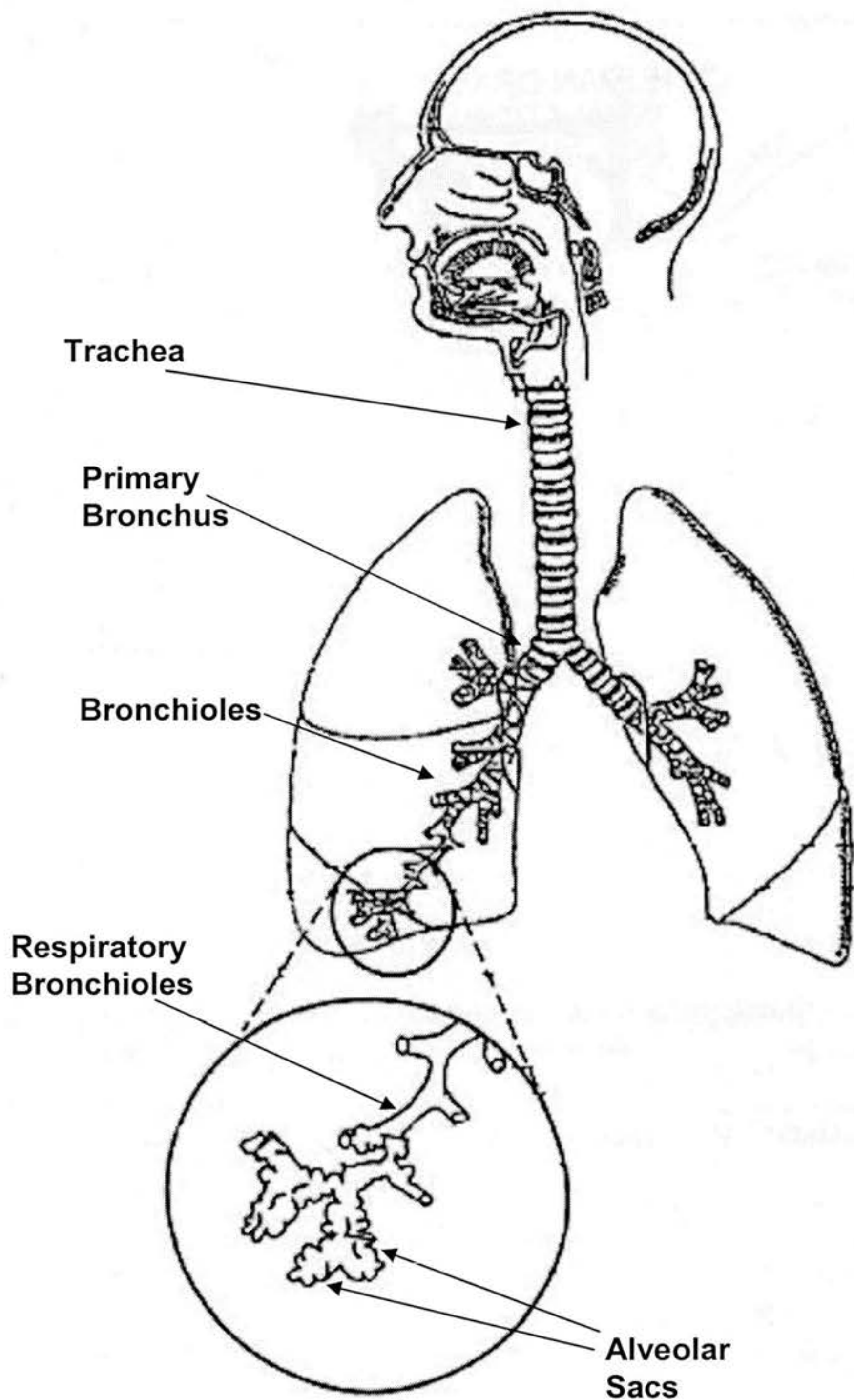


Figure 1.1. Gross structure of the lung.

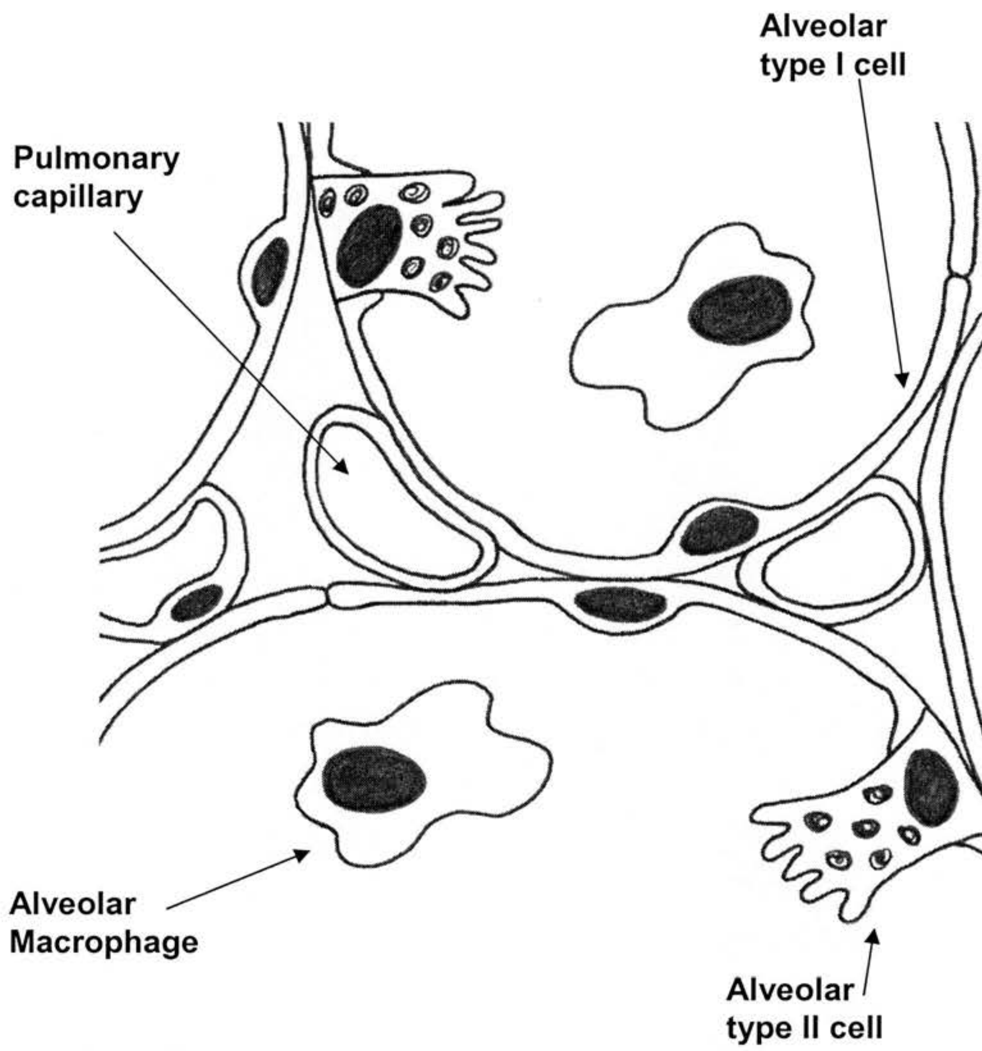


Figure 1.2. Schematic depicting alveolar airspaces.

The epithelial cell population of the bronchiolar region is comprised of Clara cells, ciliated cells, goblet cells, serous cell, endocrine cells and brush cells (Spurzem and Rennard, 2002). The mucous, serous and ciliated cells in this region form the mucociliary escalator, which traps any inhaled particles or microorganisms in the mucus secreted by goblet cells; the movement of the cilia actively transports them upwards towards the trachea, where they are removed by expectoration, or more commonly, by swallowing.

1.1.1. The Alveoli

The alveoli are approximately 200μm in diameter, and through their walls run the capillaries of the pulmonary circulation. The alveoli are supported by connective tissue consisting of fibroblasts, smooth muscle cells and extracellular matrix proteins such as collagen, elastin and fibronectin. Ninety percent of the alveolar surface consists of large, elongated type I pneumocytes (type I alveolar epithelial cells), with the remainder consisting of type II pneumocytes (type II alveolar epithelial cells). The lung epithelium is the first line of defence against inhaled particles, gases and microorganisms, thus tight cell junctions are present in the airway and alveolar epithelium to provide an impenetrable barrier. The alveoli are the gas exchange region of the lung and the thin type I pneumocyte layer lies alongside the thin capillary wall of the pulmonary circulation, to enable maximum gas exchange. The alveolar spaces are patrolled by alveolar macrophages that protect against inhaled micro-organisms and particles, small enough to penetrate beyond the ciliated airways (Figure 1.2)

1.1.1.1. Type I pneumocytes

Type I pneumocytes are the terminally differentiated epithelial cells, which form the main site for gas exchange. Their elongated flattened shape minimises the diffusion distance between the alveolar space and the blood, 0.15μm at its narrowest point (Corrin, 1981). Deoxygenated blood is brought to the lungs from the heart via the pulmonary arteries, which divide to form the capillaries, reform as the post capillary venules and then the oxygenated blood is taken back to the heart by the pulmonary veins. The heart pumps the newly oxygenated blood through the systemic circulation to the tissues.

Gas exchange occurs across the respiratory membrane, which consists of the alveolar fluid, the flattened type I epithelial cell layer, the epithelial basement membrane, interstitial space, the capillary basement membrane and the endothelial cell layer. Inhaled oxygen diffuses from the airway, through the respiratory membrane into arterial blood. Carbon dioxide diffuses from venous blood, across this air-blood barrier into the airway, to be exhaled.

1.1.1.2. Type II pneumocytes

Type II pneumocytes reside in alveolar "corners", are cuboidal in shape, and contain numerous cytoplasmic organelles, including lamellar bodies. Type II cells fulfil a variety of functions with the predominant two being: secretion of pulmonary surfactant (Fehrenbach, 2001), and repopulation of the gas exchange layer by division and

differentiation into type I cells (Adamson and Bowden, 1974). They also maintain the alveolar fluid balance by regulating sodium transport across the epithelial layer and, may contribute to host defence via the production and release of growth factors and inflammatory mediators. Alveolar type II cells also produce, export and help maintain high levels of glutathione (GSH) in lung lining fluid (discussed later).

1.1.2. Epithelial lining fluid (ELF)

The lung epithelium is bathed in the epithelial lining fluid (ELF) consisting of a mixture of proteins and lipids. The ELF protects against invading pathogens, and inhaled chemicals and oxidants. The ELF also aids the clearance of these foreign bodies, and macrophages that have phagocytosed foreign bodies, by trapping them and facilitating their removal from the lung by the mucociliary escalator (section 1.1). The exact composition of ELF is unknown, however it is known to contain, surfactant proteins, antioxidants (glutathione, superoxide dismutase (SOD), catalase, urea, and ascorbate), antiproteases and mucus.

Surfactant

Surfactant is a mixture of approximately 90% lipid and 10% protein, which is synthesised by the type II cell, and stored in cytoplasmic lamellar bodies prior to secretion by exocytosis. The lipid fraction of surfactant consists mainly of phospholipids while the protein fraction contains 50-90% of serum proteins and four apoproteins, surfactant protein (SP)-A, SP-B, SP-C and SP-D. Pulmonary surfactant coats the alveolar surface, reduces surface tension and thereby reducing the effort

needed to inflate the lung. In addition, these proteins play a role in host defence against infection (Frerking *et al.,* 2001).

Antioxidant defences

The lung is continually exposed to reactive oxygen and nitrogen species which, in addition, may form free radicals. Formation of radicals occurs by several mechanisms involving both endogenous and exogenous factors. Superoxide can leak from the mitochondrial electron transport chain or from the NADPH oxidase enzymatic system. The respiratory burst, utilised by inflammatory cells to combat invading pathogens, can also lead to the production of reactive oxygen species, such as superoxide. Superoxide can dismutate to produce hydrogen peroxide a weak oxidising agent that diffuses easily through biological membranes. Nitric oxide is another easily diffusible compound that is synthesised intracellularly by the enzyme nitric oxide synthase (NOS) and is involved in a number of regulatory mechanisms. Nitric oxide can also be converted to nitrogen dioxide (NO_2), peroxynitrate ($OO\text{-}NO_2$) and peroxynitrite (OO-N=O) (section 1.3.3.1). Reactive oxygen and nitrogen species can also derive from inhaled pollutants such as cigarette smoke (discussed further in section 1.3.3.1).

The presence of free radicals at excessive levels may cause damage to cells and tissues. The lung possesses a multitude of antioxidant defences to minimise the damage to biological molecules (Table 1.1). These consist of enzymatic antioxidants (superoxide dismutase, catalase, glutathione peroxidase) and expendable soluble antioxidants (glutathione, vitamin E, vitamin C, and β-carotene). Superoxide dismutase catalyses the dismutation of superoxide to hydrogen peroxide. The hydrogen peroxide produced

must be removed by other enzymes such as catalase or glutathione peroxidase (GPx). Catalase is responsible for the conversion of hydrogen peroxide to water and oxygen, whereas GPx catalyses the oxidation of GSH to GSSG (discussed later) at the expense of hydroperoxides such as hydrogen peroxide. The non-enzymatic antioxidants react directly with radical species and are sacrificed in the process. During this process the antioxidants either themselves become oxidised, or become radical species. In breaking the chain of lipid peroxidation, vitamin E becomes a tocopherol radical that is less damaging than the peroxyl radical (Wang and Quinn, 1999). Vitamin C scavenges $O_2^{\bullet-}$ and ·OH forming the dehydroascorbate radical (Chaudière and Ferrari-Iliou, 1999). In addition to its functions in the GSH system whereby GSH is oxidised to GSSG by GPx, free GSH can scavange radicals such as $O_2^{\bullet-}$ resulting in the formation of the thiyl radical (GS·) and H_2O_2 (Comhair and Erzurum, 2002). Nitrosation of free GSH by ȮNNO- results in the formation of GSNO, which is liberated into free GSH and NO by GPx (Comhair and Erzurum, 2002). The antioxidant network acts in synergy to eradicate radical species and enables regeneration of the reducing compounds. The regeneration of most, if not all, reducing cofactors is coupled to glutathione and/or NADPH metabolism. For example, the tocopherol radical can be reduced back to tocopherol by ascorbate, which can be reduced by GSH, forming GSSG. GSH is then regenerated at the expense of NADPH from the hexose monophosphate shunt.

Table 1.1. Reactive species found in normal pulmonary tissue and the antioxidants that keep them under control.

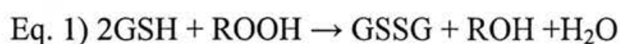
Reactive species	Biological antioxidant defence	Chemical antioxidant
Hydroxyl radical (·OH)	Vitamin C (ascorbate) Glutathione	Mannitol DMSO
Superoxide ($O_2^{\bullet-}$)	Superoxide Dismutase (SOD) Vitamin C (ascorbate) Glutathione	Dithothreitol
Hydrogen peroxide (H_2O_2)	Glutathione Peroxidase (GPx) Catalase Vitamin C (ascorbate) Glutathione	
Singlet oxygen	β-carotene Vitamin C (ascorbate)	
Peroxyl radical (ROO·)	Vitamin E (tocopherol) β-carotene Vitamin C (ascorbate) Glutathione	
Nitric Oxide (NO)		Dithiothreitol
Peroxynitrite (ONOO·)	Glutathione Glutathione Peroxidase	Dithiothreitol

Glutathione (GSH)

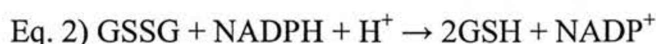
Glutathione (GSH), a tri-peptide (L-γ-glutamyl-L-cysteinylglycine), is present at high levels in lung lining fluid (Cantin *et al.,* 1987). GSH is synthesised from glutamine, cysteine and glycine, by γ-glutamyl cysteine synthetase and GSH synthetase, where γ-glutamyl cysteine synthetase is the rate-limiting step enzyme in this reaction (Figure 1.3). The availability of cysteine also determines the rate of *de novo* GSH synthesis (van Klaveren *et al.*, 1997).

GSH maintains the redox balance in the lung by reacting with oxidising compounds to form glutathione disulphide (GSSG). This reaction may occur spontaneously both intracellularly and extracellularly, however it can also be enzymatically catalysed.

Glutathione peroxidase catalyses the conversion of hydroperoxides or lipid peroxides (ROOH) to less toxic, H_2O or lipid hydroxyl compounds (Eq. 1), with conversion of GSH to GSSG (Meister and Anderson, 1983). GSSG formed intracellularly is reduced by glutathione reductase, at the expense of NADPH (Eq. 2).



Eq. 1) $2GSH + ROOH \rightarrow GSSG + ROH + H_2O$



Eq. 2) $GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$

GSSG can then exchange with protein sulphydryls to form mixed disulphides (protein-SSG), catalysed by protein disulphide isomerase (Eq. 3) (Dickinson and Forman, 2002). Formation of a protein-glutathione mixed disulphide with the active site thiol of an enzyme would presumably result in its inactivation (Dickinson and Forman, 2002).

Eq. 3) $GSSG + Protein\text{–}SH \leftrightarrow Protein\text{–}SSG + GSH$

Furthermore, GSH can protect the lung from xenobiotic compounds by direct conjugation (Figure 1.3), also resulting in the formation of mixed disulphides. Compounds with an electrophilic center readily conjugate with GSH, this reaction may be spontaneous, or catalysed by glutathione-S-transferases (GSTs). These conjugates are typically converted to mercapturic acids before being safely excreted (Meister and Anderson, 1983). Restoration of GSH levels after depletion by conjugation formation requires *de novo* synthesis (Dickinson and Forman, 2002).

In order to maintain a high intracellular GSH to GSSG ratio, GSSG may be reduced enzymatically by glutathione reductase (Dickinson and Forman, 2002). Alternatively, GSSG and low molecular weight protein-SSG may be exported from the cell. Resulting in depletion of intracellular cysteine, the availability of which is limiting in *de novo*

GSH synthesis (stated earlier). To replenish cysteine levels, γ-glutamyltransferase located on the outer surface of the membrane transfers the γ-glutamyl reside from extracellular GSH, GSSG and protein-SSG to other amino acids (Figure 1.3, γ-glutamyl pathway). Cysteinyl-glycine is then degraded to cysteine and glycine by an extracellular dipeptidase. γ-Glutamyl-amino acid, cysteine and glycine are taken up into the cell via an amino acid transporter (Dickinson and Forman, 2002), where GSH is resynthesised.

In the lung, GSH is synthesised in the type II cell and exported to the lung lining fluid (van Klaveren *et al.*, 1987), where it is present at high levels (Cantin *et al.*, 1987). It appears that these high levels are maintained by the limited availability of γ-glutamyl transpeptidase at the type II cell surface (Cantin *et al.*, 1987); the only enzyme capable of breaking the γ-glutamyl bond (Meister and Anderson, 1983). The limited availability of this enzyme likely contributes to decreased GSH catabolism and high extracellular levels. Almost all of the GSH in the lung lining fluid is in the reduced state, which is thought to be due to export of reduced GSH at a rate exceeding that of formation of GSSG (Cantin *et al.*, 1987). It has been shown that a high extracellular thiol concentration stimulates GSH release from cells, whereas a high concentration of disulphides prevents GSH release and promotes uptake and resynthesis (Lu, 1993).

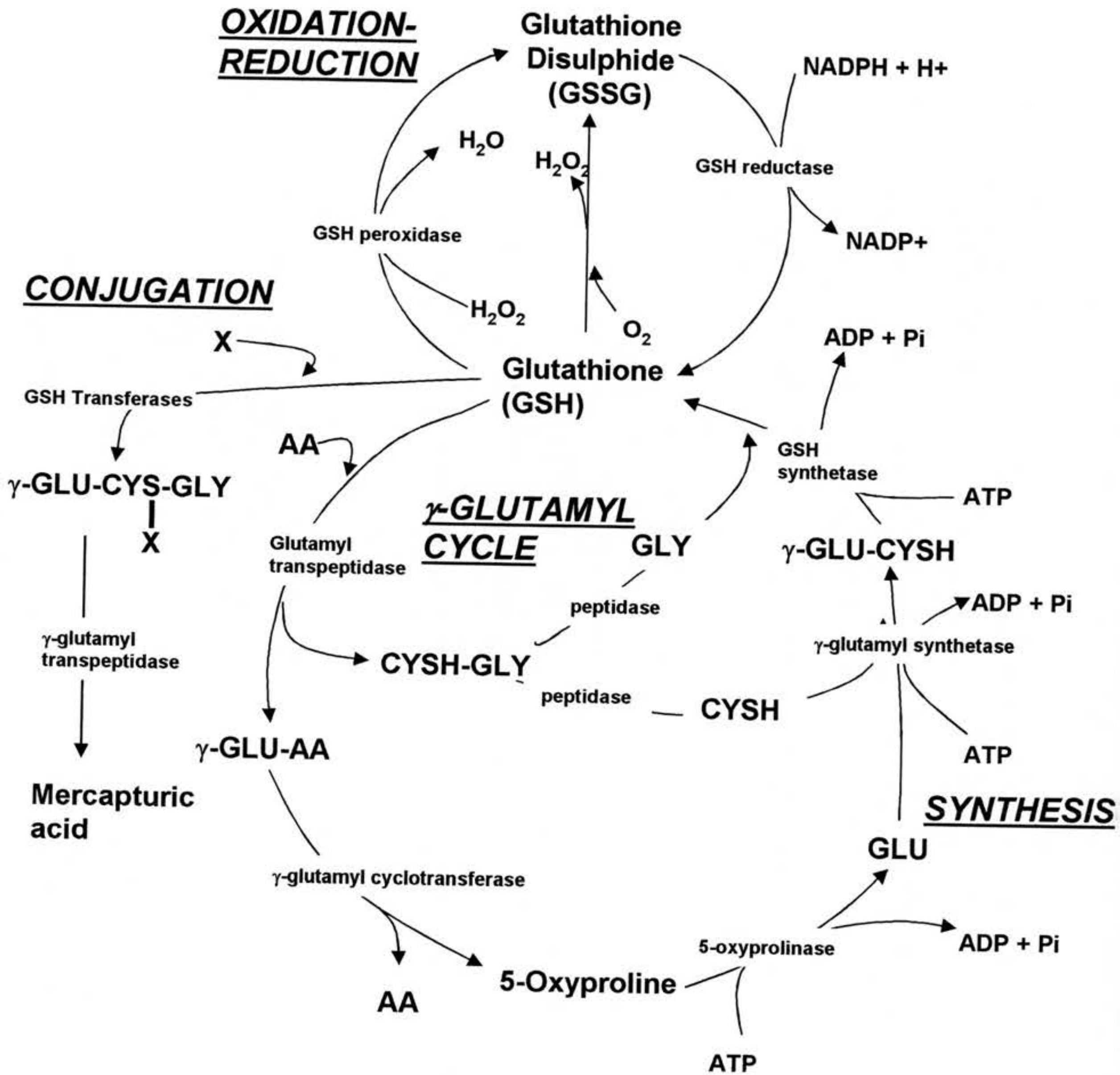


Figure 1.3. Summary of glutathione metabolism.
(Taken from, Meister and Anderson, 1983)

1.2. Chronic Obstructive Pulmonary Disease (COPD)

1.2.1. Overview

Chronic Obstructive Pulmonary Disease (COPD) is a collection of diseases, including chronic bronchitis, and emphysema; defined by "airflow limitation that is not fully reversible, usually both progressive and associated with an abnormal inflammatory response" (Global Initiative for Chronic Obstructive Lung Disease (GOLD), Pauwels *et al.,* 2001). Cigarette smoking is the major aeitiological factor in the development of COPD with 90% of COPD patients being current, or former smokers, however only 15-20% of smokers develop COPD (Turato *et al.,* 2001). The increasing prevalence of COPD means that it will be the third most common cause of death worldwide by the year 2020 (Murray and Lopez, 1996).

1.2.1.1. Chronic Bronchitis

Chronic Bronchitis is defined by "the presence of a chronic productive cough for three months in each of two successive years in whom other causes of chronic cough have been excluded" (Ciba Foundation Guest Symposium, 1959; ATS, 1962). Chronic bronchitis is a disease of the central airways and the symptoms arise as a result of hypersecretion of mucus in the respiratory tract; a consequence of an increase in the volume of submucosal glands and an increase in the number and distribution of goblet cells in the surface epithelium. Squamous metaplasia of bronchial epithelium with loss of cilia occurs (Jeffrey, 2000), predisposing the tissue to cancer, but also impairing the process of mucociliary clearance. In addition, chronic bronchitis is associated with inflammation of the airway wall and mucus secreting apparatus (Mullen *et al.,* 1985).

The production of sputum by COPD patients was originally considered to be irrelevant to the development of airflow limitation and future prognosis (Peto *et al.,* 1983) however, more recent studies by Lange *et al.* (1990) and Vestebo *et al.* (1996) have demonstrated that increased sputum production is associated with a more rapid decline in FEV1 and increased risk of hospitalisation.

1.2.1.2. Emphysema

Emphysema was first described by René Laennec in 1834 after observation of the cut surface of postmortem human lungs that had been dried in inflation (Laennec, 1834). He proposed that the observed emphysematous lesions were due to over-inflation of the lung, which compressed capillaries leading to atrophy of lung tissue. The definition of emphysema has been revised a number of times since, by the Ciba Foundation Guest Symposium (1959), the World Health Organisation (1961) and, most recently, by the American Thoracic Society (ATS, 1962). The current definition is of "abnormal, permanent enlargement of the airspaces distal to the terminal bronchioles accompanied by destruction of their walls and without obvious fibrosis" (Snider *et al.,* 1985).

Two distinct types of emphysema, centriacinar and panacinar, have been identified based on the portions of the acinus involved in the disease. Centriacinar emphysema is characterised by enlarged airspaces found in association with respiratory bronchioles with preservation of the more distal ducts and alveoli, this form of emphysema is most commonly associated with cigarette smoking. Panacinar emphysema, usually associated with α_1-antitrypsin deficiency, refers to abnormally large airspaces across acinar units, and tends to involve all components of the acinus uniformly (Turato *et al.*,

2001). The enlargement of alveolar spaces observed in emphysema (Figure 1.4) results in impaired gas exchange due to decreased surface area. Moreover, collapse of the airways occurs, which is thought to be the result of a loss of alveolar attachments (section 1.1), and loss of elastic recoil due to loss of elastin (section 1.2.4.1.).

1.2.1.3. Systemic effects

COPD is not purely a disease of the airways, a loss of skeletal muscle mass is also observed during the course of the disease. Indeed, exercise limitation is a frequent complaint of COPD sufferers. Elegant studies by Killian *et al.* (1992) demonstrated that COPD patients often stop exercise, not as a result of breathlessness, but because of leg fatigue. The exact mechanism for the loss of muscle mass is not yet clear, however systemic inflammation, increased resting metabolic rate, abnormal skeletal muscle regeneration, and skeletal muscle apoptosis have been proposed to contribute to skeletal muscle atrophy in COPD (Agusti, 2001).

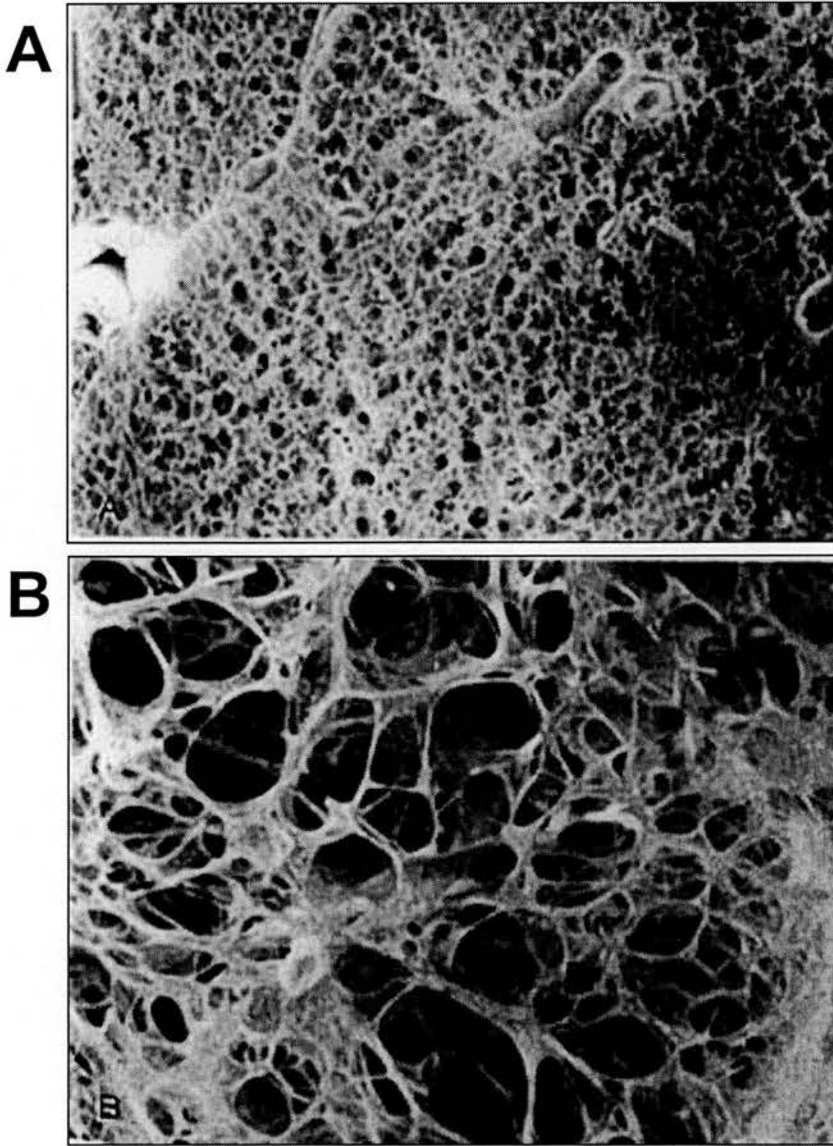


Figure 1.4. Electron micrograph of a normal lung (A) and a lung with emphysema (B). Magnification x 100. Taken from Van Wynsberghe *et al.,* 1990

1.2.2. Inflammation in COPD

Inflammation is the general term for the local accumulation of fluid, plasma proteins, and white blood cells that is initiated by physical injury, infection, or a local immune response (Janeway and Travers, 1997). Inflammation is present in the airways of asymptomatic smokers and is further increased in patients with COPD (Saetta *et al.*, 2001). A connection between these inflammatory changes and the pathophysiology of COPD has been extensively studied; however, it is difficult to find a consensus regarding the types of inflammatory cells and markers in the lung, and the importance of each of these factors in the development of COPD. In many cases this is due to the technique used to gain the information, different techniques sample different portions of the lung, and thus a complete picture is difficult to obtain. Moreover, COPD is a term encompassing a number of diseases, imprecise and variable definitions of the degree of disease mean that categorising patients is complicated, and as a result each set of researchers may study a slightly different sub-group of COPD patients. However, there is a general consensus on the importance of inflammation in COPD and so its characteristics will be described.

1.2.2.1. Inflammatory Cells

Central Airways

In the central airways of smokers with no airflow obstruction, T-lymphocytes and macrophages are predominant in the airway wall, and neutrophils, while present in low numbers in the airway wall, are most prevalent in the airway lumen. In smokers with COPD, airflow limitation is associated with a further increase in T-cells and

macrophages in the airway wall and neutrophils in the lumen. In addition, neutrophilia is more apparent in the epithelium of these individuals (Saetta *et al.,* 1997). A comparison of chronic bronchitic subjects with asymptomatic smokers that have normal lung function, revealed an increase in neutrophil and macrophage numbers and a decreased T-cell $CD4^+/CD8^+$ ratio in the bronchial glands (Saetta *et al.,* 1997). This is in accordance with Lams *et al.,* (2000) who observed increased $CD8^+$ cells in bronchial biopsies from smokers with COPD compared with asymptomatic smokers; however in their study no significant difference in neutrophils was evident. The contrasting views on the occurrence of neutrophilia in the airway wall is possibly due to the ability of neutrophils to migrate rapidly across tissues, thus it is easier to detect neutrophils in the airway lumen than the tissue. The presence of neutrophils in the bronchial glands, lumen and wall may be directly responsible for increased sputum seen in these patients as neutrophil elastase is a potent secretagogue for cultured airway submucosal glands (Nadel, 1991). In severe COPD the numbers of neutrophils in the airway wall increase and this is correlated with increased airflow limitation (Di Stefano *et al.,* 1998).

Peripheral airways

In the seminal study by Hogg *et al* (1968), the peripheral airways were established as a major site of increased airway resistance in smokers, and this has stimulated further study into the pathogenesis of COPD focused on the peripheral airways. Evidence of inflammation has been detected in the peripheral airways of young smokers who do not show any signs of airflow limitation (Niewohener *et al.,* 1974). Although no tissue destruction or fibrosis was evident, mononuclear cells were detected in the airway wall and clusters of macrophages in the respiratory bronchioles. More recent studies have

detected increased numbers of inflammatory cells, such as neutrophils, macrophages and $CD8^+$ T-cells, in the peripheral airway epithelium of smokers as compared with non-smokers (Saetta *et al.,* 2000). In addition, increased numbers of goblet cells have been seen in smokers with chronic bronchitis and airway obstruction when compared with non-smokers; this may also be the cause of increased sputum production in COPD (Saetta *et al.,* 2000). When smokers with and without airflow obstruction are compared, increased lung inflammation, demonstrated by increased total leukocytes (Figure 1.5), occurs in both peripheral airway wall and the airway lumen of smokers with airflow obstruction (Turato *et al.,* 2002). These authors found increased numbers of $CD4^+$ and $CD8^+$ T-cells in the airway wall and increased numbers of macrophages in the airway lumen; however no difference in neutrophils was evident between smoking groups. Comparison of smokers and non-smokers, regardless of the presence of airflow obstruction, has demonstrated a decrease in the $CD4^+/CD8^+$ ratio in the submucosa, however in one study an increased number of neutrophils was only observed in smokers when ex-smokers were excluded from the non-smoking group and only life long non-smokers were taken into account (Lams *et al.,* 1998). When the data was reanalysed comparing obstructed (FEV_1 63 ± 1.6% predicted) with non-obstructed (FEV_1 93 ± 2% predicted) no difference in the presence of inflammatory cells was seen.

Parenchyma

In the parenchyma of the lung, little evidence of tissue destruction or fibrosis is present in healthy smokers (Saetta *et al.*, 2001). However, contrary to definition there is evidence of some alveolar wall fibrosis in otherwise emphysematous lungs (Jeffrey, 2000). The predominant cell type in the parenchyma of COPD patients are $CD8^+$ T-

cells and their presence is correlated with increased airflow limitation (Saetta *et al.*, 1999). It has been shown that $CD8^+$ T-lymphocytes can directly induce alveolar destruction in a transgenic mouse expressing a target antigen on alveolar type II cells (Enelow *et al.,* 1998). This suggests the presence of T-lymphocytes in the alveolar region of COPD patients may contribute to emphysematous lesions in these patients; and also presents the possibility that COPD may indeed be an autoimmune disease.

Table 1.2 Inflammatory cells in the airways of smokers and patients with COPD.

		Smokers				**COPD**			
		PMN	MΦ	$CD4^+$	$CD8^+$	PMN	MΦ	$CD4^+$	$CD8^+$
Central Airways	wall	-	+	+	+	+	++	++	+++
	lumen	+	-	-	-	+	-	-	-
Peripheral Airways	wall	+	+	+	++	+	++	++	+++
	lumen	+	+	+	+	+	++	+	++
Parenchyma /Alveoli		+	+	+	+	++	++	+	+++

PMN- neutrophil, MΦ – macrophage, $CD4^+$ - CD4 T-cell, $CD8^+$ - CD8 T-cell

Systemic circulation

In addition to increased infiltration of inflammatory cells into the lung, increased cell numbers are also detected in the peripheral blood of smokers and COPD patients. Total blood leukocytes are increased in smokers (Bergmann *et al.*, 1998), and COPD patients (Sin and Man, 2003). Moreover, reminiscent of the airways, there is a decreased ratio of $CD4^+$/$CD8^+$ T-cells in peripheral blood of COPD patients (Hodge *et al.,* 2003).

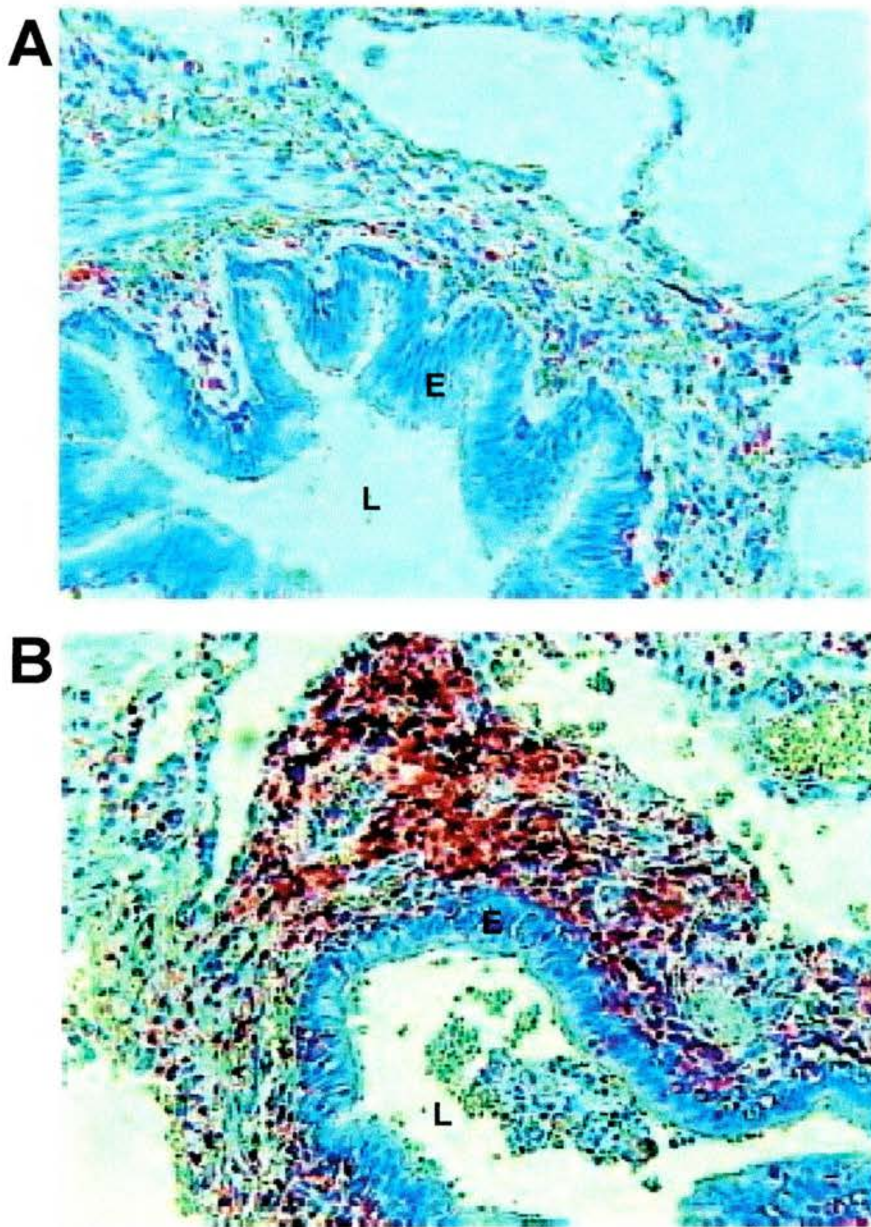


Figure 1.5. Leucocyte infiltration in the small airways. Infiltration of leucocytes stained with anti-CD45 (red) in (A) a smoker with mild COPD and (B) a smoker with severe COPD. Original magnification x400. Taken from Turato *et al*, 2002. L = lumen, E = epithelium

1.2.2.2. Inflammatory Mediators

Many inflammatory mediators including cytokines, chemokines, and cell adhesion molecules, are upregulated in COPD. Although, no consensus on the cytokine profile of COPD patients has been reached, it is generally accepted that COPD patients have increased levels of the pro-inflammatory cytokines, interleukin (IL)-6, IL-8 and tumour necrosis factor-α (TNF-α) in sputum and bronchioalveolar lavage (BAL) fluid, with further increases occurring during exacerbations (Aaron *et al.*, 2001; Bhowmik *et al.*, 2000; Keatings *et al.*, 1996; Pesci *et al.*, 1998; Tanino *et al.*, 2002; Yamamoto *et al.*, 1997). Increased macrophage chemoattractant protein-1 (MCP-1) is also observed in the lungs of COPD patients (de Boer *et al.,* 2000). In addition, systemic inflammation is observed, with increases in plasma IL-6, IL-8 and TNF-α (Eid *et al.*, 2001; Hageman *et al.*, 2003; Yasuda *et al.*, 1998). Moreover IL-10, an anti-inflammatory cytokine was found to be decreased in COPD patients (Takanashi *et al.*, 1999), which may contribute further to inflammation.

Production of many of these cytokines can be induced by exposure to cigarette smoke. Treatment of bronchial epithelial cells, endothelial cells and macrophages *in vitro* with cigarette smoke results in the production of IL-8 and TNF-α (Churg *et al.*, 2003a; Hellermann *et al.*, 2002; Mio *et al.*, 1997; Wang, Ye *et al.*, 2000) and increased levels of cytokines have been detected in the BAL fluid of animals exposed to cigarette smoke (Miller *et al.*, 2002). Moreover, a systemic effect was found with increased plasma TNF-α in mice and guinea pigs after cigarette smoke exposure (Churg *et al.,* 2003b; Wright *et al.,* 2002).

Increased TNF-α levels are thought to be central to the inflammation observed in COPD, which is supported by the observation that mice deficient in the TNF-α receptor are resistant to smoke-induced inflammation and connective tissue breakdown (Churg *et al.,* 2002a). Moreover, emphysematous changes induced by elastase are reduced in TNF-α receptor deficient mice (Lucey *et al.,* 2002). IL-10 inhibits the secretion of TNF-α, thus decreased levels of this protein in COPD patients may intensify TNF-α expression.

Inflammatory cells and resident lung cells secrete cytokines and other inflammatory mediators. IL-8 is expressed by macrophages, neutrophils, endothelium and airway epithelium; macrophages and epithelial cells express TNF-α. MCP-1 is expressed by monocytes, endothelium, and epithelium. These mediators work in a paracrine and autocrine manner activating cells or bringing in additional inflammatory cells. TNF-α activates macrophages, inducing nuclear factor-κB (NF-κB)-dependent transcription of IL-8, which is a strong chemoattractant for neutrophils. As indicated by the name, MCP-1 is a chemoattractant for monocytes. Thus the production of these cytokines in COPD may be responsible for the high numbers of inflammatory cells. Moreover, TNF-α induces expression of intercellular adhesion molecule-1 (ICAM-1) and endothelial-leucocyte adhesion molecule-1 (E-selectin/ELAM-1) on the endothelial cell surface (Pober *et al.*, 1986). Leukocytes pass through the pulmonary circulation, propelled by local blood flow. Cell adhesion molecules, expressed on the endothelium, serve to tether leukocytes, enabling extravasion into the tissues in response to chemoattractants. The lung endothelium in COPD patients displays an increased surface expression of E-selectin, and ICAM-1 (DiStefano *et al.,* 1994; Riise *et al.*,

1994), which may facilitate inflammatory cell influx. This upregulation may be due to the action of cytokines however, a number of *in vitro* studies have demonstrated that cigarette smoke itself induces expression of cell adhesion molecules on endothelial cells. Increased expression of E-selectin (Stone *et al.,* 2002) and ICAM-1 (Kalra *et al.,* 1994; Shen *et al.,* 1996) was observed after exposure to cigarette smoke condensate, which was associated with increased neutrophil and monocyte adhesion.

Thus in response to cigarette smoke, resident lung cells secrete chemotactic cytokines while cell adhesion molecules are displayed on the endothelial surface, resulting in inflammatory cell recruitment. In response to these cytokines, and also the presence of cigarette smoke in the airways, the infiltrating inflammatory cells release further cytokines, inducing additional cell adhesion molecule expression and inflammatory cell influx. Thus in COPD patients, the presence of increased levels of inflammatory mediators may not be responsible for disease pathogenesis, but merely a consequence of increased inflammation.

1.2.4. Hypotheses for the pathogenesis of emphysema

The main theories for the development of emphysema involve loss of extracellular matrix (ECM) components and degradation of elastin. The ECM, as in all tissues, constitutes the structural framework of the lung architecture; it provides attachment for epithelial cells and also affords outward traction for the small airways and alveoli by enabling tissue attachment (section 1.1). Elastin is vital for the elastic recoil of the lung and resistance of the small airways (Takashima and Mead, 1972; Mead, 1971). Thus,

loss of ECM components and/or elastin is detrimental to the compliance of the lung as it results in airway collapse and detachment of alveolar cells.

1.2.4.1. Protease-antiprotease

The hypothesis that emphysema results from an imbalance between the release of proteases from infiltrating inflammatory cells and the antiprotease defence of the lung was first advanced over 40 years ago. Two independent observations laid the foundations for this hypothesis. Firstly, decreased plasma levels of the protease inhibitor, α_1-antitrypsin were observed in individuals who developed premature emphysema (Laurell and Eriksson, 1963). Secondly, emphysematous lesions were observed in rats and hamsters after intra-tracheal administration of the protease papain (Gross *et al.,* 1965). Since then a number of other proteases have been found to induce experimental "emphysema", including the neutrophil serine proteases, elastase and proteinase 3 (Damiano *et al.,* 1986; Kao *et al.,* 1988; Lucey *et al.*, 1985; Senior *et al.,* 1977). A wide variety of proteases, which degrade elastin and collagen, the major components of the ECM, are released by both neutrophils and macrophages (Stockley, 2002; Tetley, 2002). The breakdown products of elastin and collagen, desmosine and hydroxyproline respectively, were increased in BAL fluid after exposure of mice to cigarette smoke (Churg *et al.,* 2002b). In addition to proteolytic destruction of invading pathogens, the function of cellular proteases is to degrade matrix proteins to facilitate migration of the cell through tissue to the site of injury/inflammation (Stockley, 2001). As discussed earlier (section 1.2.2.1.), increases in airway inflammatory cell numbers occurs in cigarette smokers, and neutrophil influx has been observed in mice exposed to cigarette smoke (Churg *et al.,* 2002b). Thus, it is proposed that tissue damage may

result directly from the increased influx of inflammatory cells in cigarette smokers. In addition, proteases and oxidants (discussed later) may be released by these cells into the lung lining fluid, resulting in destruction of matrix proteins. Indeed, increased elastin peptide concentrations are observed in COPD patients (Betsuyaku *et al.,* 1996; Schriver *et al.,* 1992), thus strengthening the hypothesis that emphysema results from lung matrix breakdown. Furthermore, degradation products of elastin and collagen are chemoattractants for monocytes and neutrophils (Hunninghake *et al.,* 1981; Postlethwaite and Kang, 1976; Riley *et al.,* 1988; Senior *et al.,* 1980). Thus matrix breakdown may directly result in recruitment of additional inflammatory cells further exacerbating tissue damage.

Proteases have also been implicated in many of the other pathological features of COPD. Distortion and narrowing of the small airways may be due to, loss of bronchiolar attachments as a result of ECM destruction, and also proteolytic destruction of elastic fibres in respiratory bronchioles (Shapiro, 2002). Instillation of neutrophil elastase resulted in damage to bronchial epithelium, and subsequent airway remodelling (Lucey *et al.,* 1985). Squamous metaplasia of epithelium with loss of cilia was observed, consistent with epithelial changes detected in the bronchi of patients with COPD.

Serine proteases

The serine proteases associated with COPD are, neutrophil elastase, proteinase 3 and cathepsin G. These enzymes are stored as fully mature enzymes in cellular granules, and are mainly limited to neutrophils, mast cells, and a subset of peripheral blood

monocytes, but not tissue macrophages (Shapiro, 2002). Traditionally the serine protease, neutrophil elastase, is viewed as the crucial protease for alveolar matrix destruction and emphysema. As discussed earlier, instillation of elastase results in the development of emphysematous lesions, and increased levels of elastase have also been detected in human subjects with COPD. Elastase-substrate complexes have been detected at sites of lung damage in human emphysema patients (Damiano *et al.,* 1986), and increased neutrophil elastase-α_1-antitrypsin complexes have been detected in individuals with sub-clinical emphysema (Betsuyaku *et al.,* 1999, 2000). In animal models, increases in matrix breakdown products and airspace enlargement induced by cigarette smoke exposure, can be decreased by the administration of exogenous α_1-antitrypsin (Churg *et al.,* 2003b; Dhami *et al.,* 2000), thus further implicating neutrophil elastase in the development of cigarette smoke-induced emphysema.

α1-antitrypsin deficiency

α_1-antitrypsin is a member of the serine protease inhibitor (serpin) superfamily, it is mainly synthesised in the liver and released into the circulation. Its primary function is to protect the lung against neutrophil elastase, although it has been shown that α_1-antitrypsin protects against almost all proteases detected in the lung (Gadek *et al.*, 1981). The majority of the enzyme reaches the lung by diffusion from the plasma, but it is also produced locally by macrophages and bronchial epithelial cells (Parfrey *et al.*, 2003). Approximately 70 variants of α_1-antitrypsin have been identified, classified according to their migration by isoelectric focusing (Pi) (Lomas and Mahadeva, 2002). The two most common deficiency variants, are S (Glu264Val) and Z (Glu342Lys), which result from point mutations within the protein. Individuals who are homozygous

for the more severe Z mutation (ZZ) are rare, and have only 10-15% of the plasma α_1-antitrypsin of normal (MM) individuals. Heterozygotes for S α_1-antitrypsin (MS) results in plasma α_1-antitrypsin levels that are approximately 60% of normal individuals, and is not associated with any clinical symptoms. Individuals with homozygous Z α_1-antitrypsin (MZ) have 40% less plasma α_1-antitrypsin than normal individuals (Sandford and Paré, 2000). Decreased plasma α_1-antitrypsin levels in sufferers result from the formation of periodic acid Schiff-positive (PAS-positive) inclusions, which are retained in the endoplasmic reticulum of hepatocytes (Sharp *et al.*, 1969), reducing α_1-antitrypsin levels in plasma and preventing the usual increase in plasma levels in acute-phase reactions. As a result these individuals develop junior hepatitis, cirrhosis and hepatocellular carcinoma (Eriksson *et al.*, 1986; Sveger, 1976). It is thought that the point mutation in Z α_1-antitrypsin causes the protein to form long chain polymers, which become trapped in the endoplasmic reticulum (Lomas and Mahadeva, 2002). Sufferers also develop early onset panacinar emphysema and chronic bronchitis, which is accelerated further by cigarette smoking (Larsson, 1978). This is a direct consequence of the lowered α_1-antitrypsin levels in sufferers, however in the Z-deficiency the enzyme is also less effective at inhibiting neutrophil elastase (Ogushi *et al.,* 1987). Polymer formation not only prevents secretion but also inactivates the enzyme (Elliot *et al.*, 1996), there is evidence of polymer formation in lung lining fluid (Elliot *et al.,* 1998), which may be induced by the presence of inflammatory mediators or by cigarette smoke-induced acidification of lung lining fluid (Lomas and Mahadeva, 2002).

Matrix metalloproteases (MMPs)

Matrix metalloproteases (MMPs) are a large family of Zn^{2+} and Ca^{2+}-dependent proteases, which can degrade the extracellular matrix proteins collagen and elastin (O'Connor and FitzGerald, 1994). Macrophages are the major source of MMPs, although they can be expressed by epithelial cells and fibroblasts. Neutrophils contain MMP8 and MMP9 in secondary and tertiary granules, which are released upon activation (Tetley, 2002). MMPs are secreted as inactive proenzymes and/or are bound to endogenous inhibitors, the tissue inhibitors of metalloproteases (TIMPs). MMPs can be activated directly by proteolysis (Nagase, 1997); also degradation of associated proteins by other proteases can also influence MMP activation, for example neutrophil elastase can degrade TIMPs (Tetley, 2002). Once activated MMPs degrade extracellular matrix proteins and can also cleave a variety of non-matrix proteins. MMPs also inactivate inhibitors of other proteases, such as α_1-antitrypsin (Gronski *et al.*, 1997; Sires *et al.*, 1994; Tetley, 2002), thus amplifying the proteolytic burden on the lung. In addition MMPs act upon inflammatory mediators, thereby regulating inflammation. They proteolytically activate transforming growth factor-β (TGF-β) and TNF-α, whereas they inactivate interleukin-1β by degradation (Tetley, 2002).

An association has also been observed between the development of COPD and presence of MMPs. Increased MMP2, MMP8 and MMP9 have been detected in the BAL fluid of COPD patients (Betsuyaku *et al.,* 1999; Segura-Valdez *et al.,* 2000). In addition, macrophages from patients with COPD release more MMP-9 compared with non-smokers and healthy smokers (Lim *et al.,* 2000; Russell *et al.,* 2002a,b). Increased MMP1 expression by the alveolar epithelium has also been detected in patients with

emphysema (Imai *et al.,* 2001). Investigation of the role of MMPs in emphysema has been aided by the use of transgenic technology. Increased expression of MMP1 in the lungs of mice results in the development of emphysematous lesions (D'Armiento *et al.,* 1992; Foronjy *et al.,* 2003; Shiomi *et al.,* 2003). Moreover, MMP12 expression appears to be critical to the development of emphysema in mice; exposure of MMP12 deficient (MMP12-/-) mice to cigarette smoke resulted in decreased neutrophil and monocyte recruitment and decreased matrix breakdown products (Churg *et al.,* 2002b). Moreover, MMP12-/- mice were resistant to cigarette smoke-induced emphysema (Hautamaki *et al.,* 1997).

1.2.4.2. Oxidant-antioxidant hypothesis

It has been suggested that an oxidant-antioxidant imbalance is an important feature in the pathogenesis of COPD. In addition to antiproteases, the lung lining fluid contains a multitude of antioxidants (MacNee, 2000). In smokers, the lung is exposed to oxidants, both directly from cigarette smoke and released from infiltrating inflammatory cells. The presence of free iron in the lung lining fluid enables the production of additional ROS by Fenton chemistry (section 1.3.3.1 and appendix 1). Oxidative stress results when an increased oxidative burden in the lungs of smokers and/or depletion of antioxidant defences shifts the balance in favour of oxidants. Oxidative stress has many effects; it can directly degrade the components of the lung matrix and induce epithelial injury, inactivate antiprotease defences, induce sequestration of neutrophils in the microvascular circulation and induce the expression of proinflammatory mediators. Evidence of lung oxidative stress has been reported in COPD patients and smokers; local oxidative stress can be detected as increases in exhaled nitric oxide (Ansarin *et al.,*

2001; Montuschi *et al.*, 2001; Silkoff *et al.*, 2001), hydrogen peroxide (Dekhuijzen *et al.*, 1996) and the lipid peroxidation markers 8-isoprostane (Montushi *et al.*, 2000) and ethane (Paredi *et al.*, 2000). Moreover, increases in plasma and urinary F_2-isoprostane (Morrow *et al.*, 1995; Pratico *et al.*, 1998) indicate that systemic oxidative stress also occurs.

Effects on antioxidant defences

The lung epithelial lining fluid contains a well-developed antioxidant system, which includes the enzymes superoxide dismutase, catalase, glutathione peroxidase and the antioxidants, ascorbate, urate, vitamin E and glutathione. This system serves to protect the components of the lining fluid and the underlying airway cells from oxidative injury (MacNee, 2000; Van der Vliet *et al.*, 1999). However, inhaled oxidants, and those released by infiltrating inflammatory cells may deplete lung antioxidants resulting in oxidative stress. Although GSH, the major antioxidant present in lung lining fluid, is increased in the BAL fluid of chronic smokers (Morrison *et al.*, 1999), it is depleted during acute cigarette smoking. A dose- and time- dependent decrease in GSH levels was observed after instillation of CSC into rat lungs *in vivo*, and after CSC treatment of epithelial cells *in vitro*, which returned to above control levels within 12-24 hours (Rahman *et al.*, 1995). As outlined previously (section 1.1.2), GSH is oxidised to GSSG to maintain a redox balance, and often inactivates electrophilic compounds by the formation of GSH conjugates. GSH-protein conjugates have been recovered from the lungs of rats exposed to cigarette smoke, and after *in vitro* exposure of lung epithelial cells (Rahman *et al.*, 1995). Thus, cigarette smoking may deplete GSH by the formation of GSH conjugates, rather than direct oxidation of GSH to GSSG by free

radicals. The increased GSH levels in chronic cigarette smokers may be attributed to increased expression of γ–glutamyl cysteine synthetase (section 1.1.2). *In vitro* exposure of alveolar epithelial cells results in increased expression of mRNA transcripts for this enzyme (Rahman *et al.,* 1996). Thus increased GSH in the lungs of chronic cigarette smokers may act as a protective mechanism, however the deleterious effects of cigarette smoking may occur during and immediately after cigarette smoking when these defences are depleted.

Decreased levels of vitamin-E are observed in the lungs of smokers compared to non-smokers (Pacht *et al.,* 1986), which was attributed to increased oxidative metabolism of vitamin-E to the quinone product. Vitamin-E is a naturally occurring lipophilic compound that preferentially inserts into cell membranes. Moreover it is a chain breaking antioxidant that scavenges peroxyl radicals and thus protects against lipid peroxidation (Ricciarelli *et al.,* 2001; Wang and Quinn, 1999). Increased markers of lipid peroxidation are seen in COPD patients when compared to unaffected individuals (Corradi *et al.,* 2003; Paredi *et al.,* 2000; Pratico *et al.,* 1998; Rahman *et al.,* 2002), and also in healthy smokers (Euler *et al.,* 1996; Fahn *et al.,* 1998; Hoshino *et al.,* 1990; Lepenna *et al.,* 1995; Mezzetti *et al.,* 1995; Morrow *et al.,* 1995; Petruzzelli *et al.,* 1998; Steinberg and Chait, 1998). These effects can be prevented by vitamin-E supplementation (Hoshino *et al.,* 1990; Steinberg and Chait, 1998). Lipid peroxidation may occur as a direct consequence of smoke exposure, exposure of rat tracheal epithelium to cigarette smoke results in increased levels of conjugated dienes (Churg and Cherukupalli, 1993).

Inactivation of antiprotease defences

Cigarette smoking amplifies the protease-antiprotease imbalance by inactivating endogenous antiproteases. Decreased α_1-antitrypsin activity is detected in the lungs of smokers when compared with non-smokers (Carp *et al.,* 1982; Gadek *et al.,* 1979), and in the lungs of rats exposed to cigarette smoke (Janoff *et al.,* 1979). α_1-antitrypsin has an active site methionine, which is susceptible to oxidation. Oxidation of this residue results in decreased inhibitory activity (Johnson and Travis, 1979). It has been shown that cigarette smoke is capable of oxidising this enzyme *in vitro* (Evans and Pryor, 1992), and α_1-antitrypsin recovered from the lungs of smokers was oxidised (Carp *et al.,* 1982). Thus, it is thought that cigarette smoking contributes to the pathogenesis of COPD by causing a "functional α_1-antitrypsin deficiency". However, numerous other proteases and antiproteases are present in the lung; therefore the perception that inactivation of one single antiprotease results in emphysema may be an oversimplification.

Degradation of lung matrix/epithelial injury

Cigarette smoking results in damage to the airway epithelium, demonstrated by an increase in airway permeability after acute cigarette smoking (Morrison *et al.,* 1994). This may be a consequence of epithelial cell detachment, which has been observed *in vitro* in response to cigarette smoke (Lannan *et al.,* 1994). Although GSH prevented these effects, implying an oxidant-mediated process, detachment may be due to proteolytic degradation of lung matrix as a result of cigarette smoke-induced oxidative

inactivation of antiprotease defences. Acute cigarette smoking results in inflammatory cell infiltration (Saetta *et al.,* 2001), thus the recruited and activated cells may exacerbate antiprotease inactivation. Moreover, oxidants are capable of directly degrading matrix components, such as collagen (Riley and Kerr, 1985), and therefore cigarette smoke-induced oxidative stress, may have a direct effect on matrix integrity.

Expression of pro-inflammatory mediators

Many pro-inflammatory genes, upregulated in COPD patients, are under the control of the redox-sensitive transcription factor NF-κB, including IL-6, IL-8, TNF-α and ICAM-1. NF-κB is sequestered in the cytoplasm in an inactive form by inhibitory protein-κB α (IκBα). Upon stimulation, by oxidants or cytokines, IκB kinase (IκK) phosphorylates IκBα resulting in ubiquitination and degradation of IκBα and release of NF-κB. NF-κB rapidly translocates to the nucleus, binds to its target motif in the promoter region of key genes and activates transcription. After NF-κB dependent resynthesis, IκBα enters the nucleus and facilitates the removal of NF-κB from the DNA and back to the cytoplasm (Janssen-Heininger *et al.,* 2000).

Cigarette smoke has been shown to increase NF-κB activation and DNA binding in a number of cell types (Anto *et al.,* 2002; Shen *et al.,* 1996). Also, acute *in vivo* exposure of guinea pigs to cigarette smoke resulted in increased NF-κB nuclear translocation with an increase in DNA binding potential in the lung (Nishikawa *et al.,* 1999). Moreover, lipopolysaccharide (LPS) stimulation of BAL cells from smokers resulted in more rapid activation of NF-κB than cells from non-smokers (Mochida-Nishimura et al., 2001).

It has been shown that cigarette smoking can affect gene expression via modulation of chromatin, the complex of DNA and histone proteins, which in an inactive state is tightly wound. To enable transcription, chromatin is unwound, opening up the DNA structure, enabling increased accessibility for transcription factors. DNA unwinding can be promoted by acetylation of histone proteins (Gregory *et al.*, 2001), which may be induced by oxidative stress and is modulated by histone acetyltansferases (HATs) and histone deacetylases (HDACs). HATs induce acetylation of histones, whereas HDACs promote deacetylation (Figure 1.6). Repression of HDAC with the inhibitor Trichostatin A leads to increased histone acetylation and increased inflammatory cytokine gene expression (Ito *et al.,* 2001). Decreased HDAC expression was observed in macrophages after cigarette smoke exposure *in vitro* (Ito *et al.,* 2001), which was correlated with increased cytokine expression. As these results could be mimicked with H_2O_2 exposure the authors proposed that modulation of chromatin by acetylation could be induced directly by oxidative stress. Thus cigarette smoke, via imposing oxidative stress, may increase the expression of proinflammatory cytokines, by increased histone acetylation in conjunction with activation of transcription factors.

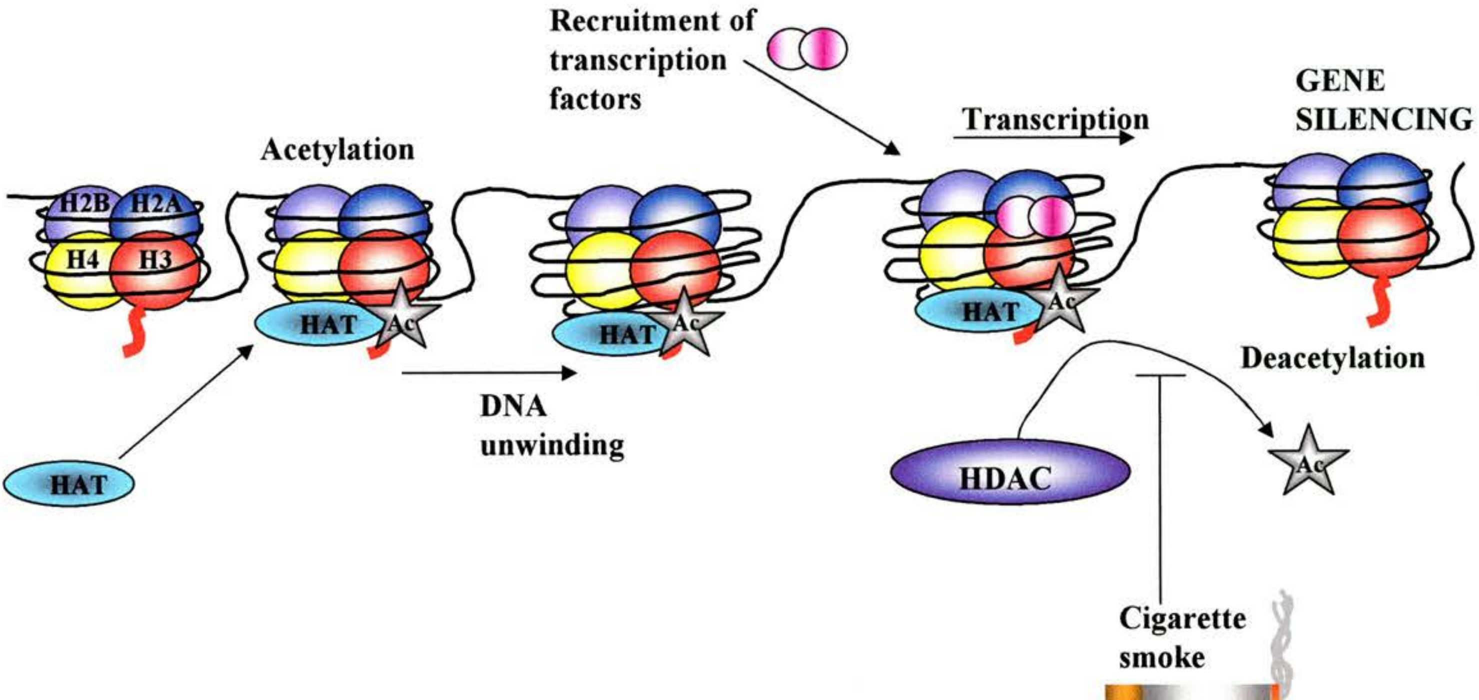


Figure 1.6. Inflammatory gene expression can be influenced by histone acetylation. Chromatin is a complex of DNA wound around a histone core. Histone acetyltransferases (HATs) induce histone acetylation resulting in DNA unwinding and increased accessibility for transcription factors, after transcription histone deacetylases (HDACs) promote deacetylation of histones returning chromatin to its inactive, silent state. Cigarette smoke decreases the expression of HDACs thus promoting histone acetylation (Ito *et al*, 2001).

1.2.4.3. Apoptotic destruction of alveoli

It has long been recognised that loss of alveolar epithelial cells occurs in emphysema, however this was thought to be the consequence of detachment as a result of matrix degradation. Recently apoptosis has been suggested as a mechanism for the loss of alveolar cells in emphysema. Indeed, instillation of active caspase-3 into mouse lungs, in combination with a protein transfection reagent, results in alveolar wall destruction and airspace enlargement, as early as 2 hours after instillation (Aoshiba *et al.,* 2003). Increased numbers of apoptotic epithelial and endothelial cells have been detected in the lungs of emphysema patients (Kasahara *et al.,* 2001; Segura-Valdez *et al.,* 2000), associated with decreases in the expression of vascular endothelial growth factor (VEGF) and VEGF-receptor (VEGF-R) (Kasahara *et al.,* 2001). Moreover, instillation of a VEGF receptor inhibitor induces lung cell apoptosis and the development of emphysematous lesions (Kasahara *et al.,* 2000). Also, high levels of VEGF were measured in BAL fluid from non-smokers but were undetectable in healthy smokers (Koyama *et al.,* 2002), thereby implicating cigarette smoking in the downregulation of VEGF and VEGF-R. VEGF is a growth factor required for the survival of endothelial cells (Neufeld *et al.*, 1999), and withdrawal of this factor results in apoptosis *in vitro* (Gerber *et al.*, 1998a, b) and *in vivo* (Alon *et al.*, 1995). Thus decreased expression of VEGF and its receptor by cigarette smoking, resulting in lung cell apoptosis, may be responsible for the development of emphysematous lesions.

Exposure to cigarette smoke *in vitro* has been reported to induce apoptosis in many cell types (Aoshiba *et al.,* 2001; Hoshino *et al.,* 2001; Ishii *et al.,* 2001; Vayssier *et al.,* 1998; Wang 2001). Moreover, exposure of rats to cigarette smoke results in apoptosis

in sites away from the lung, such as the gastric mucosa (D'Agostini *et al.,* 2001; Ma *et al.,* 1999; Wang, Ma *et al.,* 2000), the skin (D'Agostini *et al.,* 2000), and the testis (Rajpurkar *et al.,* 2002). Although decreases in VEGF levels have been implicated in cigarette smoke-induced apoptosis, the factors that mediate cigarette smoke-induced cell death are yet to be elucidated. As discussed earlier, increased oxidative stress occurs in cigarette smokers, and increased products of lipid peroxidation, such as 4-hydroxynonenal (4-HNE) (Rahman *et al.,* 2002), have been detected in COPD patients. Increased apoptosis is evident after stimulation of cells with 4-HNE (Bruckner and Estus, 2002; Herbst *et al.,* 1999; Ji *et al.,* 2001; Kalinich *et al.,* 2000; Liu, Kato *et al.,* 2000; Reuf *et al.,* 2001; Zhang *et al.,* 2001), and oxidative stress has been demonstrated to induce apoptosis in many cell types (reviewed in Chandra *et al.*, 2000). Thus many factors could be responsible for cigarette smoke-induced apoptosis, requiring further investigation within this area.

1.2.5. Prevention of repair

The lung has a substantial capacity to mediate repair responses (Rennard, 2001). Fibroblasts play an important role in tissue repair; they proliferate at sites of injury, produce matrix proteins and remodel the matrix via contraction. However in the presence of cigarette smoke, both the migration and proliferation of fibroblasts is inhibited (Nakamura *et al.,* 1995). Also, cigarette smoke impairs fibroblast-mediated wound contraction in an *in vitro* system, via inhibition of fibronectin release from fibroblasts (Carnevali *et al.,* 1998). The effects of cigarette smoke condensate (CSC) on collagen gel contraction and fibronectin production was attenuated by the addition of N-acetyl-L-cysteine (NAC), a precursor of GSH, and amplified by the addition of

butathione sulfoxamine (BSO), an inhibitor of γ-glutamyl cysteine synthetase (Kim *et al.,* 2002). These data demonstrate the effects of cigarette smoke on wound repair could be prevented by GSH, thus oxidative stress may be responsible for the inhibition of repair mechanisms by cigarette smoke.

Cigarette smoke has also been shown to significantly decrease elastin resynthesis both *in vitro* and *in vivo*. Cigarette smoke exposure resulted in approximately 85% inhibition of elastin resynthesis, assessed by the formation of desmosine, the final step in the synthesis of mature elastin (Laurent *et al.,* 1983). Osman *et al* (1985) instilled elastase into the lungs of hamsters and monitored subsequent elastin resynthesis by incorporation of ^{14}C into desmosine. Animals exposed to cigarette smoke for 1 week after elastase exposure showed 40% reduction in cross-link formation when compared with animals left to recover in room air for an equivalent time period. Thus, in addition to inducing lung damage, cigarette smoke appears to exacerbate this damage by preventing regenerative processes.

1.2.6. The effector cells of COPD

As discussed earlier, both neutrophils and macrophages are present in the lung of smokers and COPD patients, so it is unlikely that there is a single effector cell that is responsible for the development of COPD. The cells of the immune system work in concert with each other; each cell type releasing mediators that modify the behaviour of others. For instance, the proteases released by inflammatory cells can affect the proteases of another, as in the case of MMPs, which can inactivate α_1-antitrypsin (Gronski *et al.*, 1997; Sires *et al.*, 1994; Tetley, 2002) making the lung more susceptible

to neutrophil elastase-mediated attack. In addition neutrophil elastase can activate MMPs by proteolysis (Gronski *et al.*, 1997; Sires *et al.*, 1994), increasing the macrophage-mediated protease burden in the lung. Mice deficient in the macrophage metalloelastase, MMP12, did not develop emphysema (Hautamaki *et al.,* 1997) so implicating the macrophage in emphysematous tissue destruction. However the presence of normal levels of MMP12 was not sufficient to develop emphysema, elevated lung neutrophil numbers were also required (Churg *et al.,* 2002b). Administration of an anti-neutrophil antibody to smoke exposed mice prevented neutrophil influx, but not macrophage numbers, and attenuated smoke-mediated increases in the matrix breakdown products, desmosine and hydroxyproline (Dhami *et al.,* 2000). Thus the development of COPD is therefore most likely due to combined effects of a number inflammatory cell types.

1.2.7. Susceptibility

Only a small proportion, approximately 10-20%, of smokers develop COPD, suggesting an interaction between genetic and environmental factors. Many studies have investigated specific "susceptibility factors" for COPD, but with little success so far. Increased reactivity of inflammatory cells, gene polymorphisms, and adenoviral infection, have all been implicated in the susceptibility to COPD.

The process of neutrophil migration results in damage to the extracellular matrix thus, smoke-induced neutrophil migration in susceptible individuals may be higher than in non-susceptible individuals. A study by McCrea *et al* (1994) studying IL-8 levels observed no overall difference between non-smokers and healthy smokers, however a

small subset of smokers had levels above that of healthy controls, and the BAL fluid had a greater chemotactic potential for neutrophils. Thus these smokers may represent a susceptible population. It has also been shown that neutrophils from patients with emphysema are more sensitive to chemoattractants, and have an increased potential to digest connective tissue (Burnett *et al.,* 1987). Migration of neutrophils is facilitated by expression of endothelial cell adhesion proteins, which bind to proteins on the surface of the neutrophil, capturing them from the circulation and enabling extravasation into the tissues. COPD patients have increased endothelial cell expression of E-selectin, and ICAM-1 (DiStefano *et al.,* 1994; Riise *et al.*, 1994). These proteins capture circulating neutrophils to enable extravasation into the tissues. Thus the enhanced inflammatory response in these individuals may be due to the enhanced neutrophil numbers, or rate of neutrophils recruited, compared with non-susceptible smokers.

Polymorphisms in the TNF-α promoter have been identified and implicated in the pathogenesis of COPD but are associated with different ethnic populations. A guanine to adenine substitution at position –308 (TNF-α308G/A) has been associated with chronic bronchitis in Japanese (Sakao *et al.,* 2001) and Taiwanese (Huang *et al.*, 1997) populations, but appears to have no association with the development of COPD in Caucasian subjects (Higham *et al.,* 2000). This polymorphism is thought to increase TNF-α production (Thomas, 2001), and could be the mechanism for the increased TNF-α levels observed in COPD patients.

Polymorphisms in enzymes that are involved in detoxification of xenobiotic agents present in cigarette smoke (discussed in section 1.3.3.2) have also been linked to COPD

pathogenesis. Glutathione-S-transferases (GSTs) catalyse the conjugation of GSH with electrophilic compounds. Six isoenzymes of GST have been identified; alpha, mu, pi, theta, sigma and kappa. GSTpi-1 (GSTP1) and GSTmu-1 (GSTM1) are expressed in alveoli, alveolar macrophages and respiratory bronchioles (Sandford and Paré, 2000). Homozygous deletion of the GSTM1 gene results in a complete absence of the protein and is associated with emphysema in individuals with lung cancer (Harrison *et al.*, 1997) and chronic bronchitis in heavy smokers (Baranova *et al.*, 1997). Polymorphisms in GSTP1 have also been associated with COPD; substitution of valine for isoleucine at position 105 (GSTP1-105Ile/Val) is increased in these individuals (Ishii *et al.*, 1999), which results in decreased activity of the enzyme (Johansson *et al.*, 1998; Sundberg *et al.*, 1998). However, this appears to have no association with COPD in Korean (Yim *et al.*, 2002) populations. Microsomal epoxide hydrolase (mEH) is a xenobiotic-metabolising enzyme that also aids detoxification of highly reactive intermediates in cigarette smoke. Two polymorphisms have been detected in this gene, which encode for fast or slow enzyme activity (Hassett *et al.*, 1994). A higher occurrence of homozygosity for slow activity mEH was detected in COPD patients than normal individuals (Smith and Harrison, 1997). Individuals with any of these polymorphisms may have a decreased ability to metabolise harmful compounds in cigarette smoke. A number of other polymorphisms have also been associated with the pathogenesis of COPD, such as in matrix metalloproteases (reviewed in Wallace and Sandford, 2002), surfactant proteins (Guo *et al.*, 2001), hemeoxygenase-1 (Yamada *et al.*, 2000), and IL-13 (van der Pouw Kraan *et al.*, 2002).

It has also been proposed that susceptibility to COPD may result from childhood adenoviral infection. Hogg and colleagues have shown that adenoviral DNA persists in the lungs after infection, and have shown that individuals with COPD possess more adenoviral DNA, specifically the E1A gene, than control subjects (Matsuse *et al.*, 1992). Moreover, expression of E1A protein was detected in lung epithelial cells lining the airways, alveoli, and submucosal glands (Elliott *et al.,* 1995). A guinea pig model of latent adenoviral infection displayed increased inflammatory cell influx when exposed to cigarette smoke than uninfected animals (Vitalis *et al.,* 1998), indicating that adenoviral infection may exacerbate the local inflammatory response and subsequent lung damage. This was reinforced by further studies that demonstrated that emphysematous changes were increased in smoke-exposed E1A infected animals compared to uninfected animals (Meshi *et al.,* 2002). Moreover, E1A protein expression increased with the severity of emphysema in human subjects, which was also associated with increased inflammation (Retamales *et al.,* 2001). Increased inflammation by E1A infection may be related to increased production of chemotactic agents. Transfection of lung epithelial cells with E1A resulted in increased production of IL-8 in response to LPS stimulation (Keicho *et al.,* 1997).

As outlined above, individual susceptibility to COPD may be due to a variety of reasons, which makes study of the disease difficult. Treatment of cells in culture, or animal models, with cigarette smoke produces a range of effects, however these studies with normal systems may not aid our understanding of susceptibilty. This is emphasised by the fact that it is difficult to induce emphysema in animal models using cigarette smoke as the stimulus, without using susceptible strains (Mahadeva and

Shapiro, 2002). Thus, elucidation of the susceptibility factor for COPD would greatly aid our understanding of, and research into, this disease.

1.3. CIGARETTE SMOKING

1.3.1. History of tobacco use

Tobacco was brought to Europe from the Americas in the 15th century and nearly 100 years after its introduction, the smoking of tobacco in pipes was widespread. This gave way to the consumption of tobacco as snuff, followed by smoking of cigars and cigarettes. Cigarette smoking became the dominant form of tobacco consumption in the developed world during the 20th century. Although initially hailed for its medicinal properties, the detrimental effects of tobacco consumption were soon realised. In the early 20th century many articles were published addressing the health effects of smoking. In 1912 Dr I Adler made the first strong association between cigarette smoking and lung cancer (Adler, 1912), however it wasn't until 1930 that a statistical correlation was made. A number of studies in the 1950's and 1960's demonstrated an association between smoking and the symptoms of COPD; breathlessness, cough and sputum production (reviewed in Burney, 1964). The publication of the 1964 report of the US Surgeon General highlighted the deleterious health effects of smoking to the general public (Burney, 1964), resulting in a dramatic decline in the numbers of smokers. This has continued to the present day, for example in 2002, 25% of the British population were current smokers (Lader and Meltzer, 2003), compared with 41% in 1976 (Office of National Statistics, 1976).

1.3.2. Diseases caused by cigarette smoking

In addition to COPD over 40 different diseases have been associated with cigarette smoking. In 2000 cigarette smoking resulted in 114,000 deaths in the United Kingdom,

25% of which were the result of COPD (Peto *et al.*, 1994). A number of cancers, not just of the lung, are connected with smoking as well as correlations with other fatal diseases, such as heart disease, stroke, pneumonia and the development of aortic aneurysm. Moreover, many non-fatal diseases such as ulcers, cataracts and periodontal disease have a positive correlation with smoking history. Smoking during pregnancy also poses risks for the unborn child, such as low birth weight and limb defects (Wald and Hackshaw, 1996).

1.3.3. Constituents of cigarette smoke

1.3.3.1. Free Radicals

Cigarette smoke contains a number of reactive oxygen and nitrogen species with the potential to generate additional radicals by a number of reactions, in total cigarette smoke contains 10^{14-16} free radicals per puff. One of the most interesting, and highly studied, radicals in smoke is the semiquinone. Semiquinones (QH•) exist in equilibrium with quinones (Q) and hydroquinones (QH_2) in the tar phase of cigarette smoke. Redox cycling by these quinone radicals produces superoxide ($O_2^{\bullet-}$), which can further dismutate to form hydrogen peroxide (H_2O_2) (appendix 1A). During the process of smoking the tar fraction of cigarette smoke is deposited in the lung where water-soluble components dissolve into the pulmonary fluids. These water-soluble compounds include components of the quinone system, thus $O_2^{\bullet-}$ and H_2O_2 can form within lung lining fluid. Moreover, both cellular fluids and cigarette tar contain free iron, which can catalyse the production of hydroxyl radicals (•OH) from hydrogen peroxide by Fenton chemistry (Pryor and Stone, 1993) (appendix 1B).

Nitric oxide (NO) is present in cigarette smoke, at levels as high as 250ppm (Chow *et al.*, 1993), which can be oxidised to form reactive nitrogen dioxide (NO_2) (Beckman and Koppenol, 1996). Additional reactive molecules, peroxynitrate (OO-NO_2) and peroxynitrite (OO-N=O), can be formed by reaction of $O_2^{\bullet-}$ with NO_2, or NO respectively (appendix 1C) (Augusto *et al.*, 2002).

Free radicals can attack lipid membranes inducing lipid peroxidation (appendix 1D), which results in the production of peroxyl radicals (ROO•) (Halliwell and Chirico, 1993). NO_2, OH• or $O_2^{\bullet-}$, the initiator radicals (In•), can react with polyunsaturated fatty acids (PUFA's; RH) to form carbon-centred radicals (R•), which react with oxygen to form peroxyl radicals (Porter *et al.,* 1995). Peroxyl radicals then attack an additional PUFA molecule, which enables the process to continue in a chain reaction, attack other membrane proteins, or two peroxyl radicals may combine resulting in termination of the reaction (Halliwell and Chirico, 1993).

1.3.3.2. Chemical components

Cigarette smoke is a complex mixture of over 4000 chemicals, a number of which are listed in appendix 2 (Heseltine, 1987). Many of these chemicals arise from combustion of the tobacco leaf itself, however a number of chemicals are added during cigarette manufacture (Action on Smoking and Health, 2001). Most of the additives serve an unknown purpose, but many others appear to be added to enhance "flavour" or augment the uptake of specific components, such as nicotine (Action on Smoking and Health, 2001). From the identified list of chemicals, 47 have been classified as carcinogens by

the International Agency for Research on Cancer (IARC, 2000), however this may be an underestimation, as many have not yet been tested.

Many of the individual components of cigarette smoke have been implicated in the pathogenesis of COPD. For example, in addition to its addictive properties encouraging continuation of the smoking habit, nicotine is chemotactic for human neutrophils and enhances responsiveness to chemotactic agents (Totti *et al.,* 1983); therefore it may contribute to the increased inflammatory response observed in COPD (Section 1.2.2). Acrolein and acetaldehyde are the major α,β-unsaturated aldehydes present in cigarette smoke. As electrophilic components they conjugate with GSH (Section 1.1.2) and thus may deplete lung antioxidants as observed in cigarette smokers (described in section 1.2.4.). In addition, acrolein is an initator of, as well as a product of, lipid peroxidation (Esterbauer *et al.*, 1991). Therefore, acrolein in cigarette smoke may propagate the lipid peroxidation observed in response to cigarette smoke (section 1.2.4.). Moreover, these compounds have pro-apoptotic properties; they induce apoptosis in a number of cell types (Li, Hamilton *et al.*, 1997; Menegola *et al.*, 2001), and thus may be implicated in the cell loss observed in the pathogenesis of emphysema. Acrolein and acetaldehyde in cigarette smoke may also inhibit repair processes, discussed in section 1.2.5. The inhibitory effects of cigarette smoke on fibronectin production and fibroblast migration can by mimicked by the addition of either acrolein or acetaldehyde (Carnevali *et al.,* 1998; Nakamura *et al.,* 1995).

1.4. CELL DEATH

1.4.1. Historical Perspective

Cell death has been recognised since the nineteenth century. Initially, cell death was considered to be a degenerative phenomenon produced by injury and termed necrosis. However, the occurrence of a physiological, naturally occurring cell death was documented in Lecture XV of Virchows Cellular Pathology (1859) "death brought on by (altered) life – a spontaneous wearing out of living parts", and was termed necrobiosis. It has been suggested recently that Virchow may have documented the first observations of apoptosis as he clearly states that necrobiosis was very different from necrosis (Gerschenson and Geske, 2001). The development of staining methods in the late 1800's, and more elaborate and powerful microscopes, enabled cell death to be studied in more detail (Majno and Joris, 1995). In 1885 Walther Flemming documented that the epithelial lining of regressing ovarian follicles was littered with cells, the nuclei of which were "breaking up". His drawings show cells with pyknotic chromatin typical of apoptosis and many free apoptotic bodies, he termed this death "chromatolysis" (Flemming, 1885). Since then many striking examples of apoptosis have been documented (for review see Majno and Joris, 1995).

However it wasn't until John Kerr joined forces with Andrew Wyllie and Sir Alastair Currie that the term "apoptosis" was coined, Kerr had previously called the phenomenon "shrinkage necrosis", describing a characteristic cell death with distinct morphological features (Kerr *et al.,* 1972). The name apoptosis was taken from the Greek word used to describe the "dropping off" or "falling off" of petals from flowers,

or leaves from trees. The cell death they described was characterised morphologically by structural changes involving "nuclear and cytoplasmic condensation and breaking up of the cell into a number of membrane-bound, ultrastructurally well-preserved fragments" (Kerr *et al.,* 1972). However, since then a number of biochemical and morphological markers to enable identification of apoptotic cells have surfaced. However the discovery that the DNA of apoptotic cells is fragmented into discrete oligonucleosomal fragments, easily detected by gel electrophoresis was a significant finding (Arends *et al.,* 1990; Wyllie, 1980). The discovery of this marker has lead to an enormous increase in the volume of published papers on apoptosis.

Development of *Caenorhabditis elegans* as a tool for genetic research revealed that exactly 131 of 1090 somatic cells in the *C.elegans* embryo die during development (Sulston and Horvitz, 1977; Sulston *et al.,* 1983). More interestingly, individual cells of developmental cell lineages die at specific, apparently pre-determined stages of development. Further study revealed the existence of the *Ced* genes, which controlled essentially all the somatic deaths in *C.elegans* (Horvitz *et al.,* 1983). Thus it was thought that cell death in other species may result from the activation of a specific death program. Sequencing of *Ced-3* revealed that it was related to a family of cysteine proteases, called caspases due to their requirement to cleave after an aspartate residue (Yuan *et al.,* 1993). The name caspase denotes that they are cysteine-dependent aspartate-specific proteases (Alnemri *et al.,* 1996). In many cases, activation of caspases is seen as an essential marker, alongside morphological changes, to identifiy apoptosis; cell death that assumes any other form is labelled necrotic. However, the caspases are not the only death effector pathway; a number of caspase-independent cell

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death mechanisms, many with characteristics of apoptosis, necrosis, or a mixture of the two have been documented, making the field of cell death highly complex!

1.4.2. Apoptosis

1.4.2.1. The Caspases

The caspases are a family of cysteine proteases, which can be further divided into two sub-families. One sub-family is an essential part of the cell death machinery; the other plays a role in inflammation (Nicholson and Thornberry, 1997). Fourteen caspases have been identified so far, including the murine caspases –11 and –12 which as yet have no identified human homolog (Thornberry and Lazebnik, 1998). These proteases are highly specific, recognising a four amino acid sequence, designated P_1-P_4. They possess an absolute requirement for aspartate at the P_1 position. The caspases can be divided into three groups based on their substrate specificity: group one recognises the consensus sequence WEHD (caspase-1, -4, and -5), the second group DEXD (caspase-2, -3, and –7) and the third group (L/V)EXD (caspase-6, -8, and –9) (Table1.3). The pro-apoptotic *C.Elegans* caspase Ced-3, the most closely related to caspase-3, recognises a DEXD consensus sequence (Table 1.3).

Table 1.3 Specificities and biological functions of caspases

		P_4-P_1 specificity	Function
Group I (WEHD)	Caspase-1	WEHD	Inflammatory
	Caspase-4	(W/L)EHD	
	Caspase-5	(W/L)EHD	
Group II (DEXD)	CED-3	DETD	Effector
	Caspase-3	DEVD	
	Caspase-7	DEVD	
	Caspase-2	DEHD	
Group III ((L/V)EXD)	Caspase-6	VEHD	Initiator
	Caspase-8	LETD	
	Caspase-9	LEHD	

Adapted from Nicolson and Thornberry, 1997

The division of caspases by their substrate specificity correlates with their function. Group I caspases are predominantly involved in inflammatory processes. Group II caspases recognise a sequence often found in non-caspase proteins cleaved during apoptosis. These caspases are designated "effector caspases" and they are responsible for cell disassembly. The caspases belonging to group III are all "initiator caspases", responsible for initiation of the caspase cascade. (Nicholson and Thornberry, 1997).

Activation of the caspases

The caspases exist in normal cells as inactive zymogens consisting of three domains: an NH_2 terminal domain, a large subunit and a small subunit (Thornberry and Lazebnik, 1998). Both subunits contain essential components of the catalytic machinery and activation occurs via proteolytic cleavage within a short linker segment that connects the large and the small subunits of the proenzyme (Salvesen and Dixit, 1999; Thornberry and Lazebnik, 1998). This linker domain may play a role in preventing spontaneous autoactivation of the proenzyme, as it is too short to enable heterodimer

formation (Chang and Yang, 2000). Following cleavage, the large and small subunits associate to form a heterodimer, after which, two heterodimers associate to form a tetramer with two independent catalytic sites (Thornberry and Lazebnik, 1998). Cleavage of the linker domain results in a partially active intermediate caspase, which processes itself to yield the active caspase. This self-cleavage removes the short inhibitory NH_2 prodomain from the large subunit, which enhances the activity of the caspase (Thornberry and Lazebnik, 1998). The NH_2 domain plays a role in regulating activation of the proenzyme, as recognition of at least four amino acids in this region is a necessary requirement for efficient catalysis (Thornberry and Lazebnik, 1998).

Caspases are proteolytically activated by other caspases, either by an upstream caspase or by "induced proximity". The effector caspases are activated by an upstream caspase, however concentrating the zymogens results in activation of the initiator caspases. Unprocessed enzymes have limited proteolytic capability, yet it is thought that bringing them into close proximity enables activation of either themselves, or adjacent caspases (Salvesen and Dixit, 1999; Donepudi and Grütter, 2002). Execution of apoptosis by caspases occurs via two specific pathways, namely the extrinsic and intrinsic pathways.

1.4.2.2. Death-receptor mediated apoptosis: the extrinsic pathway

The "extrinsic" pathway of cell death is initiated by the binding of a death receptor to its cognate ligand (Figure 1.7). A number of death receptor pathways exist, such as Fas and TNF, however the basic mechanism of activation for all pathways is the same. Binding of a ligand to a cell-surface death-receptor results in trimerisation and recruitment of a death domain (DD)-containing adaptor molecule to the cytoplasmic DD

of the receptor. In the case of the Fas pathway the adaptor molecule is FADD (Fas-associating protein with death domain). This complex is termed the death-inducing signaling complex (DISC). The N-terminal of FADD contains a death effector domain (DED), which binds to two DED domains present in caspase-8, bringing multiple molecules together resulting in autoactivation as discussed above; from this point, death receptor signalling diverges in different cells (Scaffidi *et al.*, 1998). In type I cells[1], such as lymphoid cells, receptor ligation leads to a massive activation of caspase-8, which then directly cleaves and activates procaspase-3 (Budihardjo *et al.*, 1999). However, in type II cells[2], caspase activation is generally insufficient to activate procaspase-3. In this case caspase-8 cleaves the cytoplasmic protein Bid to form truncated Bid (tBid), which activates the "intrinsic" mitochondrial pathway (Kaufmann and Hengartner, 2001).

[1,2] The nomenclature of type I and type II cells here refers to the ability of the extrinsic pathway to activate capase-3, this is not to be confused with the type I and type II pneumocytes of the lung

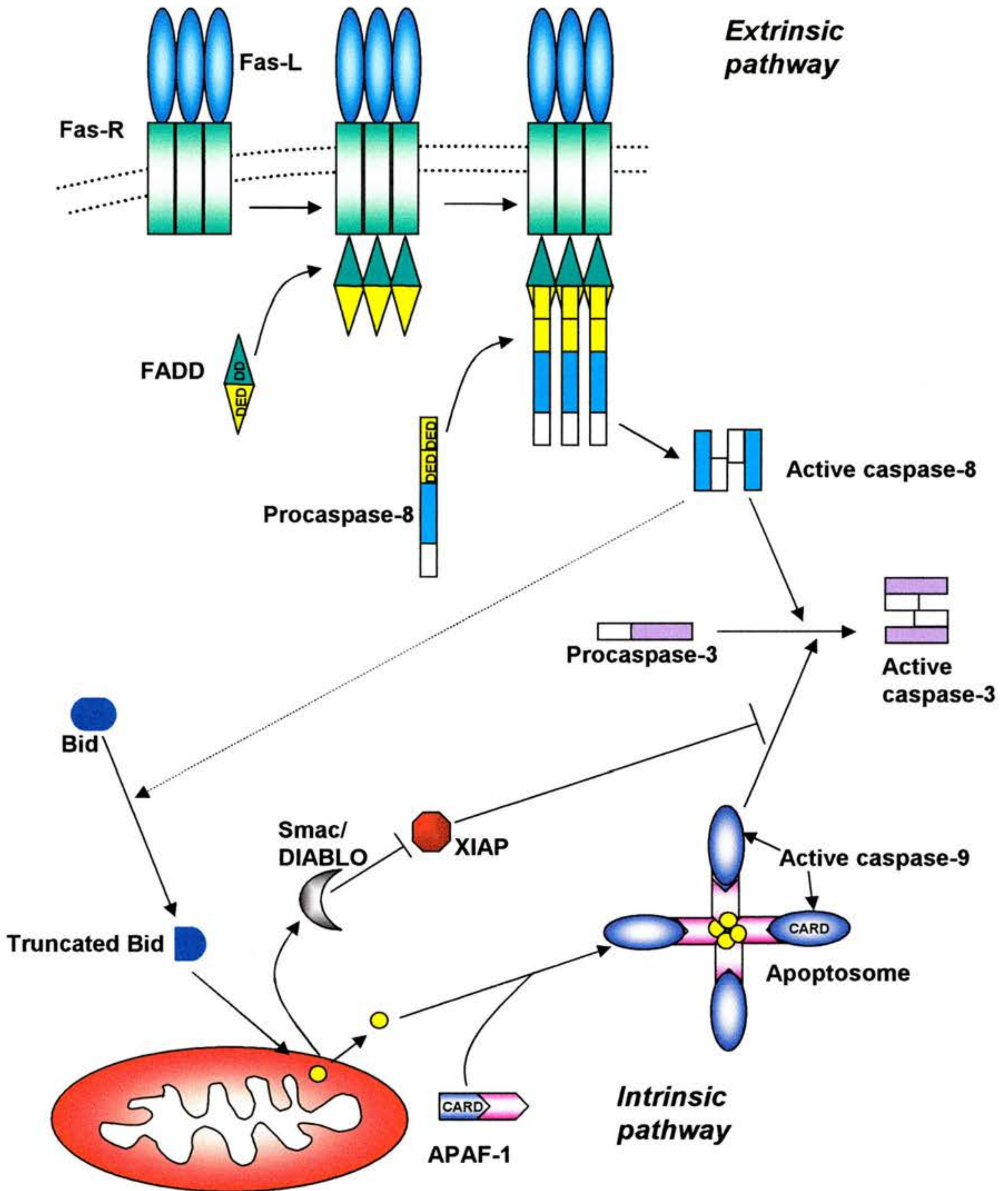


Figure 1.7. Basic schematic illustrating the extrinsic and intrinsic pathways of caspase activation. *The extrinsic pathway*: Ligation of death receptors, results in autoactivation of caspase-8 and subsequent activation of the effector caspase, caspase-3. Cleavage of Bid by caspase-8 induces cytochrome-c release from the mitochondria, which can also be induced by environmental or chemical induced stress. *The intrinsic pathway*: cytochrome-c, dATP, APAF-1, and procaspase-9 associate to form the apoptosome, resulting in autoactivation of caspase-9, which also activates caspase-3.

1.4.2.3. The apoptosome

The "intrinsic" pathway is controlled by the mitochondria and is activated by environmental stresses, such as growth factor withdrawal, or via the action of truncated Bid from the extrinsic pathway (Figure 1.7). Originally mitochondria were not thought to play a role in apoptosis, as few noticeable changes of this organelle were seen. However, this changed after Xiadong Wang and colleagues identified three factors essential for activation of the intrinsic pathway, cytochrome-c, dATP and a new protein apoptotic protease-activating factor-1 (Apaf-1) (Liu *et al.,* 1996; Yang *et al.,* 1997; Zou *et al.,* 1997). During apoptosis cytochrome-c is released from the mitochondria (Kluck *et al.,* 1997; Scaffidi et al., 1998; Yang *et al.,* 1997) and forms a complex with APAF-1 and dATP, which is termed the apoptosome. The apoptosome complex is formed by a mechanism that is not clearly understood, however it has been proposed that dATP binds to the consensus nucleotide-binding domain of APAF-1 and is hydrolysed to dADP inducing a transient conformational change in APAF-1. This conformational change is stabilised by the recruitment of cytochrome-c, which promotes multimerisation of the APAF-1-cytochrome-c complex enabling recruitment of procaspase-9 via the caspase recruitment domains (CARDs) of the molecules (Figure 1.7). Activation of procaspase-9 occurs via autocatalysis as a result of zymogen clustering, after which the active enzyme activates downstream caspases (Li, Nijhawan *et al.,* 1997; Zou *et al.,* 1999). It was initially believed that APAF-1 was only required transiently for caspase-9 activation after which active enzyme was released, however it is now thought that the complex represents the true, active form of caspase-9 (Rodriguez and Lazebnik, 1999)

1.4.2.4. How caspases result in the demise of the cell

The caspases play a key role in the self-degradation of complex cellular material to enable safe and efficient removal of the cell "corpse". Once activated by initiator caspases, the effector caspases cleave a distinct set of proteins enabling disassembly of the cell (Nicholson and Thornberry, 1997). In most cases, proteolytic cleavage results in loss of function of the target protein; however gain of function can be obtained, either by removal of a negative regulatory domain, or by inactivation of a regulatory subunit (Hengartner, 2000). There are over 100 identified substrates of the caspases, although not all are cleaved during the process of apoptosis (Fischer *et al.,* 2003). In most cases the consequences of cleavage are poorly understood, however, in a few cases, proteolysis of certain components can be linked to discrete morphological and biochemical changes of cell death.

Caspases activate the nuclease that mediates oligonucleosomal DNA fragmentation, caspase-activated DNase (CAD, Enari *et al.,* 1998). In healthy cells CAD is complexed with its inhibitor, inhibitor of CAD (ICAD) and is functionally inactive. In apoptotic cells, caspases-3 and –7 cleave ICAD, releasing CAD and enabling DNA degradation (Sakahira *et al.,* 1998). DNA is an immunogenic molecule, it has been proposed that break down of DNA into oligonucleosomal fragments not only aids removal of the corpse, but also prevents inappropriate immune responses *in vivo* (Nagata *et al.*, 2003).

Poly (ADP-ribose) polymerase (PARP) is inactivated by caspases during the process of apoptosis (Kauffman *et al.,* 1993; Lazebnik *et al.,* 1994; Tewari *et al.,* 1995). PARP is a nuclear enzyme that catalyses the ADP-ribosylation of histones and other nuclear

proteins in order to facilitate DNA repair. During apoptosis PARP is cleaved at a single site by caspase-3, which separates the DNA-binding and catalytic domains, inactivating the enzyme activity. It was originally thought that cleavage of PARP occurred to prevent DNA repair during the breakdown of the nucleus. However, PARP-mediated DNA repair is energetically expensive. During apoptosis DNA fragmentation by CAD can cause significant activation of PARP and thus deplete cellular energy stores, energy that is required to execute apoptosis. In some cases an inability to cleave PARP during apoptosis results in depletion of the ATP pool and enhanced cell death, possibly by necrosis (Herceg and Wang, 1999) (see section 1.4.4). However, it has been documented that, in some cases, PARP cleavage is not essential for apoptotic cell death (Leist *et al.,* 1997; Wang *et al.,* 1997).

Pyknosis and fragmentation of the nuclei, along with shrinkage of the cell, is one of the most profound and striking morphological changes observed during apoptosis. Major cytoskeletal changes occur during this process, many of which are caspase-mediated. Lamins are nuclear envelope proteins that are cleaved by caspases (Takehashi *et al.,* 1996) resulting in nuclear fragmentation during the final stages of apoptosis execution (Lazebnik *et al.,* 1995). That this process was caspase-mediated was demonstrated by the use of lamins with mutated caspase cleavage sites. The presence of these mutants delayed the onset of apoptosis and prevented classical nuclear condensation; instead the nuclear envelope collapsed while the nuclear lamina remained intact (Rao *et al.,* 1996). Thereby implicating caspase-dependent lamin cleavage as an important stage in nuclear pyknosis. Disassembly of the cell requires breakdown of actin filaments. Neutrophils deficient in gelsolin display delayed membrane blebbing, indicating that actin reorganisation is an important factor for this hallmark of apoptosis (Kothakota, 1997).

Gelsolin is cleaved by caspases during apoptosis to produce a constitutively active fragment, which induces F-actin depolymerisation (Kothakota, 1997).

1.4.2.5. Regulation of cell death

Given the irreversible nature of proteolytic degradation of cellular proteins, caspase activation and activity have to be tightly regulated. The conversion of zymogens to their active forms comprises the initial level of caspase regulation (see section 1.4.2.1). However, additional levels of regulation exist in the form of endogenous caspase inhibitors that act either at the level of cytochrome-c release or by specific inhibition of active caspases.

The Bcl-2 Family

One of the most prominent families of apoptosis regulators is the Bcl-2 family, which consists of approximately 16 proteins in humans. Bcl-2, an apoptosis-suppressor, was originally discovered in B-cell lymphoma as a proto-oncogene located at the breakpoints of t(14;18) (q32;q31) chromosomal translocations (Bakhshi *et al.,* 1985). In these translocations the *Bcl-2* gene is moved into juxtaposition with powerful enhancer elements of the immunoglobulin heavy chain (IgH) locus resulting in the overexpression of Bcl-2. It was shown in early experiments that exogenous overexpression of this gene conferred increased cell survival of pre-B-cells after withdrawal of essential growth factors (Vaux *et al.,* 1988). In addition, the use of anti-sense technology has shown that reduction of the levels of Bcl-2 results in accelerated cell death after growth factor withdrawal (Reed *et al.,* 1990a,b). Thus Bcl-2 emerged as

the first example of an intracellular apoptosis suppressor and also the first identified proto-oncogene that affected tumour development via cell death regulation rather than cell division. The Bcl-2 family of proteins seem to act upon the mitochondria to either facilitate, or prevent, cytochrome-c release (Adams and Cory, 1998), and therefore they mostly confer protection upon cells undergoing apoptosis via the "intrinsic" pathway. However, they may protect cells acting via the death-receptor induced "extrinsic" pathway depending on whether the cells require a mitochondrial amplification step to achieve sufficient activation of effector caspases (Scaffidi *et al.,* 1999).

Anti-apoptotic

Bcl-2 subfamily

Bcl-2
Bcl-x_L
Mcl-1
Bcl-w
CED-9

A1/Bfl-1

Pro-apoptotic

Bax subfamily

Bax
Bak
Bok

BH3-only subfamily

Bik/Nbk
Hrk/Dp5
Bim/Bod
Blk

BNIP3

Bad
Bid
EGL-1

Figure 1.8. The Bcl-2 family. Three sub-families exist in the Bcl-2 family, the Bcl-2 cohort promote cell survival whereas the Bax and BH3-only subfamilies facilitate apoptosis. All contain between at least one Bcl-2 homology (BH) regions, many contain hydrophobic transmembrane (TM) domains.

The Bcl-2 family of proteins contain at least one of four Bcl-2 homology (BH) domains, termed BH1-4. The family can be sub-divided into three sub-families, Bcl-2, Bax and BH3-only (Figure 1.8). The pro-survival family members (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1, NR-13, BHRF1 and LMW5-HL) contain at least three domains, BH1, BH2 and BH4 (Zha *et al.,* 1996), however those most similar to Bcl-2 (Bcl-w and Bcl-x_L) contain all four regions. The members of the Bax sub-family (Bax, Bak and Bok), are also very similar to Bcl-2 in that they contain the domains BH1-3. The third sub-family contains only the BH-3 domain and thus bears little resemblance to any other family members, and is named the BH3-only sub-family (Bik, Blk, Hrk, BNIP3, Bim_L, Bad, Bid and EGL-1). Most anti-apoptotic Bcl-2 family members also contain a hydrophobic stretch of amino acids at the C-terminus that anchors them in membranes (Figure 1.8), most commonly the outer mitochondrial membrane, the endoplasmic reticulum and the nuclear envelope, whereas the pro-apoptotic members are mainly located in the cytosol or associated with the cytoskeleton (Hsu *et al.,* 1997; Krajweski *et al.,* 1993; Puthalakath *et al.,* 1999; Wolter *et al.,* 1997). However, the localisation of Bcl-2 family members to the mitochondrial membrane is important in their pro-/anti-apoptotic action (Zamzami *et al.,* 1996). The regulation of apoptosis by the Bcl-2 family remains poorly defined. These proteins are multifunctional and at least three general functions have been defined, a) dimerisation with other family members, b) binding to non-homologous proteins, and c) formation of ion channels.

The formation of dimers is thought to play a key role in maintaining a balance between the pro- and anti-apoptotic members of the family, dictating the relative sensitivity or resistance to apoptotic stimuli. It has been shown that Bcl-2 is able to form both

homodimers with itself and also form heterodimers with Bax (Yin *et al.,* 1994). Similarly Bcl-x_L can form homodimers, and heterodimerises with Bax and Bak, (Diaz *et al.*, 1997). The mechanism of dimerisation was elucidated after analysis of the crystal structure of Bcl-x_L, which contains two central hydrophobic α-helices surrounded by five amphipathic helices (Muchmore *et al.,* 1996). The α-helices representative of the BH1, BH2 and BH3 domains form the borders of an elongated hydrophobic cleft, to which the hydrophobic face of a BH3 amphipathic α-helix can bind (Muchmore *et al.,* 1996; Sattler *et al.,* 1997). A model has been proposed that some Bcl-2 family members may exist in two conformations: one in which the BH domains form a pocket, and the other in which the amphipathic α-helix of BH3 rotates to expose its hydrophobic surface so that it can bind to the receptor-like crevice on its dimerisation partner (Sattler *et al.,* 1997). Expression of a synthetic peptide representing the BH3 domain of Bax prevented dimer formation between endogenous Bax and Bcl-x_L, indicating the importance of this domain in dimer formation (Jürgensmeier *et al.,* 1998). Thus, heterodimers are formed when there is an equal balance of pro- and anti- apoptotic family members, however when either are in excess, homodimers form between either two anti-apoptotic members, or two pro-apoptotic members, and cells are either protected from, or susceptible to, apoptosis respectively (Oltvai *et al.,* 1993; Oltvai and Korsmeyer, 1994). It is thought that the BH3-only tribe, with the exception of Bid, induce apoptosis solely by binding and inactivating their pro-survival cousins (Cory and Adams, 2002).

Binding of Bcl-2 family members to non-homologous proteins may explain activities previously associated with Bcl-2 members. For example, Bcl-2 can bind with Raf-1 via

the BH4 domain, pulling the kinase from the cytosol to the mitochondrial membrane where it induces phosphorylation of Bad (Wang *et al.,* 1996), a pro-apoptotic family protein that antagonises both Bcl-2 and Bcl-x_L by heterodimer formation (Yang *et al.*, 1995). However, phosphorylation of Bad interferes with its ability to bind Bcl-2/Bcl-x_L (Zha *et al.,* 1996) and as Bad contains no membrane-anchoring domain, it moves into the cytosol and forms a complex with 14-3-3 scaffold proteins (Zha *et al.,* 1996). Liberation of Bcl-2/Bcl-x_L from Bad results in increased cell survival as these proteins are able to perform their anti-apoptotic function (Wang *et al.,* 1996).

The translocation of cytochrome-c from the mitochondrial intermembrane space to the cytoplasm plays a crucial role in apoptosis, and it is thought that the Bcl-2 proteins act by either facilitating or preventing this release. Currently two models have been proposed as possible mechanisms for cytochrome-c release, each supported by substantial evidence. Elucidation of the structure of Bcl-x_L indicated that the Bcl-2 family members may act by forming pores in membranes; Bcl-x_L, in particular the $\alpha 5$ and $\alpha 6$ helices, shares striking similarity to the pore-forming domains of some bacterial toxins, such as diptheria toxin (Muchmore *et al.,* 1996). As predicted, Bcl-2, Bcl-x_L, and Bax form ion channels when added to synthetic membranes (Antonsson *et al.,* 1997; Minn *et al.,* 1997; Schendel *et al.,* 1997; Schlesinger *et al.,* 1997) and can lyse a suspension of erthrocytes when added extracellularly (Antonsson *et al.,* 1997). Moreover, deletion of the $\alpha 5$ and $\alpha 6$ helices abolishes channel formation by Bcl-2 and Bax (Schendel *et al.*, 1997). The pro-apoptotic protein Bax provides the most convincing argument for pore formation by Bcl-2 proteins. Bax is located in the cytosol of healthy cells, however upon apoptotic stimulation it dimerises and translocates to the mitochondria, inserting into the outer membrane (Gross *et al.,* 1998). It is thought that

this may create a channel in the outer mitochondrial membrane and facilitate release of cytochrome-c (Jürgensmeier *et al.,* 1998). Whether the pore formed by Bax is of a sufficient size to enable release of cytochrome-c and other intermembrane proteins from the mitochondria is unclear; however it may be that larger channels are formed by oligomerisation of identical family members, although evidence is not forthcoming (Newmeyer and Ferguson-Miller, 2003). Antagonism by heterodimer formation may play a direct role in regulating the formation of pores; Bcl-2 can prevent dimerisation and channel formation by Bax (Antonsson *et al.,* 1997; Gross *et al.,* 1998).

Alternatively, release of cytochrome-c from the intermembrane space may result from rupture of the outer mitochondrial membrane. In some apoptosis scenarios, loss of inner mitochondrial membrane potential occurs, indicating the opening of a large conductance channel known as the mitochondrial permeability transition (PT) pore (Zamzami *et al.,* 1996). Although the actual architecture of the PT pore is not yet known, it is thought to be formed from a combination of proteins normally located in the inner and outer mitochondrial membranes, such as the voltage-dependent anion channel (VDAC), Cyclophilin-D, and the adenine nucleotide translocator (ANT), which operate in concert to form a pore with a diameter of approximately 1.5kDa (Crompton, 1999). The PT pore is thought to form at contact sites between the outer and inner membranes (Zoratti *et al.,* 1994). Formation of the pore results in equilibration of ions within the intermembrane space and the matrix resulting in disruption of the inner mitochondrial membrane potential ($\Delta\psi m$), uncoupling of oxidative phosphorylation and matrix swelling. As the inner mitochondrial membrane has a larger surface area than the outer membrane, matrix swelling can eventually lead to outer membrane rupture and

release of proteins from the intermembrane space (Green and Reed, 1998). Bcl-2 family proteins have been reported to associate with the PT pore and regulate its function (Zamzami *et al.*, 1996). Overexpression of Bcl-2, or Bcl-x_L, inhibits mitochondrial permeability transition (Shimizu *et al.*, 1996c; Single *et al.*, 2001; Zamzami *et al.*, 1996), whereas Bax overexpression induces it (Xiang *et al.*, 1996). However the importance of the PT pore in apoptosis has not yet been fully established; it is still unclear as to whether PT pore formation occurs prior to caspase activation, as some studies have shown that cytochrome-c release and caspase activation can occur prior to any detectable loss of $\Delta\psi m$ (Yang *et al.*, 1997).

In contrast to the hypopolarisation associated with PT pore opening, hyperpolarisation of the inner membrane has been observed prior to cytochrome-c release (Vander Heiden *et al.*, 1997, 1999). This is thought to result from the continued creation of the H^+ ion gradient despite loss of the ability to exchange mitochondrial ATP for cytosolic ADP, reflecting dysfunction of the VDAC, ANT or both. Ion channel formation by Bcl-x_L prevents hyperpolarisation of the inner mitochondrial membrane, subsequent matrix swelling and outer membrane rupture (Vander Heiden *et al.*, 1997), by preventing VDAC closure (Vander Heiden *et al.*, 2001) and maintaining ATP/ADP exchange (Vander Heiden *et al.*, 1999).

Inhibitor of apoptosis proteins (IAPS)

While the Bcl-2 proteins act to prevent caspase activation, an additional level of regulation acts to prevent integration of the caspase signal, once activated. Inhibitor of apoptosis proteins (IAPs) are a family of proteins that contain at least one baculovirus

IAP repeat (BIR) domain. The BIR domain is a ~70kDa-residue zinc-binding domain, of which, between one to three copies are found in the IAP proteins. Seven family members have been identified in humans, NIAP, c-IAP-1/HIAP-2/MIHB, c-IAP-2/HIAP-1/MIHC, XIAP/hILP/MIHA, ML-IAP/Livin/KIAP, Survivin/TIAP and Apollon/BRUCE (Deveraux and Reed, 1999). These IAPs have also been found in organisms such as yeast, which neither contain caspases nor undergo apoptosis (Uren *et al.,* 1998); indicating that the IAPs have functions in addition to caspase inhibition. More recently it has arisen that the IAPs are also involved in a number of diverse cellular processes including, cell cycling, ubiquitination and receptor-mediated signalling (Holcik, 2002). Nevertheless, they regulate caspase activity by binding to, and inhibiting, active caspase-3, -7 and –9 (Datta *et al.,* 2000; Deveraux *et al.,* 1998; Roy *et al.,* 1997). The BIR domains are responsible for inhibiting specific caspases; in particular BIR2 specifically targets caspases-3 and –7 whereas BIR3 targets caspase-9.

The IAPs act upon active caspases and processing of the proenzyme is essential for their action. This is demonstrated by overexpression of X-linked IAP (XIAP) in U937 cells, which did not inhibit caspase-9 processing, yet inhibition of the mature enzyme was observed (Datta *et al.,* 2000). Moreover, XIAP does not associate with or inhibit a catalytically active unprocessed form of caspase-9, XIAP associates, via the BIR3 domain, with a segment of the small subunit which becomes exposed after proteolytic processing (Srinivasula *et al.,* 2001). The N-terminus of the p12 subunit of caspase-9 fits in a conserved groove on the BIR3 domain of XIAP. The close proximity of the N-terminus to the catalytic active site suggests that XIAP may hinder the entry of the substrate to the active site (Srinivasula *et al.,* 2001). However, more recent studies have

shown that binding of XIAP traps caspase-9 in a monomeric state and thus prevents formation of the catalytically active dimer (Shiozaki *et al.,* 2003).

The BIR region is less critical during IAP inhibition of caspase-3 and –7. The linker sequence between BIR1 and 2 possesses higher affinity for the caspase than the BIR domain (Huang *et al.,* 2001). This linker occupies the active site of the caspase preventing entry of the substrate (Chai *et al.,* 2001; Huang *et al.,* 2001; Riedl *et al.,* 2001; Sun *et al.,* 1999). Interestingly, the linker sequence occupies the active site in a reverse orientation to the substrate (Chai *et al.,* 2001; Huang *et al.,* 2001; Riedl *et al.,* 2001). The BIR2 region, with its weaker affinity for the caspase, subsequently latches onto the caspase to stabilise the interaction (Huang *et al.,* 2001). Although the linker sequence is the essential component for caspase inhibition it cannot bind, or inhibit, either caspase on its own (Chai *et al.,* 2001; Sun *et al.,* 1999). However the BIR2 domain can be replaced with either glutathione-S-transferase (GST) or BIR1 without significant loss of caspase inhibition (Chai *et al.,* 2001; Huang *et al.,* 2001; Sun *et al.,* 1999). It has been proposed that the presence of BIR2, or BIR/GST, may prevent the linker sequence forming a "non-productive conformation" due to an intramolecular interaction or possibly by preventing aggregate formation due to the hydrophobicity of the linker sequence (Chai *et al.,* 2001; Huang *et al.,* 2001).

In much the same way as the Bcl-2 family have anti-apoptotic factors to keep the pro-apoptotic factors in check, endogenous antagonists of the IAP family exist. The first identified inhibitor of the IAPs was discovered simultaneously in humans and in mice and was named Smac/DIABLO (second mitochondrial activator of caspases (hu)/ direct IAP binding with low pI (ms)) (Du *et al.,* 2000; Verhagen *et al.,* 2000). This protein

resides in the mitochondrial intermembrane space (Du *et al.,* 2000; Verhagen *et al.,* 2000), and is released alongside cytochrome-c upon induction of apoptosis (MacFarlane *et al.,* 2002). Once released, Smac/DIABLO eliminates the caspase inhibitory effect of many IAPs (Chai *et al.,* 2000; Du *et al.,* 2000; Verhagen *et al.,* 2000) by binding to either the BIR2 or BIR3 domain, liberating the bound caspase (Chai *et al.,* 2000, 2001; Huang *et al.,* 2001; Liu *et al.,* 2000; Srinivasula *et al.,* 2001). More recently another IAP antagonist has been identified, XIAP-associated factor 1(XAF1) (Liston *et al.,* 2001). XAF1 neutralises the IAPs in a different manner to Smac/DIABLO, XAF1 interacts directly with XIAP resulting in its sequestration in the nucleus where it is unable to interact with caspases in the cytosol (Liston *et al.,* 2001). In addition to quenching IAP proteins by direct binding, it has been demonstrated that the IAP antagonists can affect IAP protein levels by suppressing global protein translation, resulting in depleted intracellular levels (Holley *et al.,* 2002; Yoo *et al.,* 2002).

Differential expression of the IAP proteins is detected *in vivo*, which may provide a mechanism for controlling the presence or absence of IAPs in response to particular stimuli. However, regulation of the IAPs also occurs as a result of post-translational modification. Decreased levels of IAPs have been detected after the induction of apoptosis, which can be prevented by the addition of proteosome inhibitors (Yang *et al.,* 2000). In addition to the BIR domains, the IAPs possess a RING domain that is responsible for self-ubiquitination and degradation (Yang *et al.,* 2000). Moreover it has been demonstrated that the IAPs also ubiquitinate associated proteins such as Smac/DIABLO (MacFarlane *et al.,* 2002), and caspases (Huang *et al.,* 2000; Suzuki *et al.,* 2001) resulting in their degradation. The degradation of caspases may serve to

prevent release of active enzyme from the XIAP:caspase complex by Smac/DIABLO. Thus in addition to direct degradation of Smac/DIABLO this may provide an additional mechanism by which IAP counteracts its antagonists. However, it has been elegantly demonstrated that the antagonists can also provide a pro-apoptotic counterbalance to the anti-apoptotic effects of ubiquitination of proteins by IAPs, by promoting the self-ubiquitination and degradation of the IAPs themselves (Holley *et al.,* 2002; Yoo *et al.,* 2002). Thus, depending on the target, ubiquitination by the IAPs serves to both promote and prevent apoptosis.

Thus, the IAPs and their antagonists act as an apoptotic rheostat, working against one another to either lower or increase the threshold of activation for the execution of the apoptotic pathway by caspases.

Heat-shock proteins (HSPs)

Heat shock proteins (HSPs) are a family of highly conserved proteins that are induced in response to an array of physiological and environmental stresses (For review see Beere, 2001). HSPs are classified according to their molecular weights, HSP-25, HSP-47, HSP-60, HSP-70/72, HSP-90, HSP-110. These proteins fulfil a variety of roles; in unstressed cells they function as molecular chaperones to facilitate the correct folding of polypeptide chains. However HSPs-27, -70 and –90, also interfere with both death-receptor mediated and mitochondria-mediated apoptosis.

Each of these proteins have been shown to prevent formation of a functional apoptosome. HSP-70 and -90 act by binding APAF-1, HSP-70 prevents recruitment of

procaspase-9 (Saleh *et al.*, 2000), HSP-90 prevents the oligomerisation of APAF-1 (Pandey *et al.*, 2000). HSP-27 binds and sequesters cytochrome-c after its release from the mitochondria (Bruey *et al.*, 2000). Therefore expression of these proteins can confer protection against apoptotic cell death.

Other mechanisms of caspase regulation

Post-translational modification by either phosphorylation or nitrosylation, in addition to ubiquitination described above, is a mechanism employed to regulate the caspases. Caspases contain an essential cysteine thiol group, which is susceptible to *S*-nitrosylation resulting in its inactivation (Dimmeler *et al.*, 1997; Li, Billiar *et al.*, 1997; Mannick *et al.*, 1999; Melino *et al.*, 1997; Rössig *et al.*, 1999; Tenneti *et al.*, 1997; Török *et al.*, 2002; Zeigler *et al.*, 2003). Substitution of the active site cysteine for alanine renders the caspase resistant to *S*-nitrosylation (Dimmeler *et al.*, 1997; Mannick *et al.*, 1999; Rossig *et al.*, 1999). Inhibition of caspase activity by *S*-nitrosylation has been shown to prevent apoptotic cell death (Li *et al.*, 1999; Melino *et al.*, 1997; Rössig *et al.*, 1999; Tenneti *et al.*, 1997; Török *et al.*, 2002; Zeigler *et al.*, 2003). In addition to inhibiting the activity of caspases, nitric oxide a potent nitrosylating agent, prevents their activation (Li *et al.*, 1999; Török *et al.*, 2002; Zech *et al.*, 2003). It has been demonstrated that nitric oxide prevents functional oligomerisation of APAF-1, and recruitment of caspase-9 (Zech *et al.*, 2003). Nitric oxide prevented apoptosome formation only in the presence of additional cytosolic factors, indicating there may be another, yet unknown, target for caspase regulation by nitrosylation. Caspase nitrosylation has been shown to have a role in physiological regulation of the apoptotic pathway. In healthy cells caspase-3 can be detected in a nitrosylated state, whereas

upon induction of apoptosis it becomes denitrosylated, freeing the active site thiol (Mannick *et al.,* 1999, 2001). More recently it has been shown that a higher percentage of caspase-3 and –9 compartmentalised in the mitochondria is nitrosylated, compared to that present in the cytosol (Mannick *et al.,* 2001), implying that nitrosylation acts to prevent activation of caspases in the mitochondrial intermembrane space where zymogens are kept in close proximity.

Activation of either the phosphatidylinositol 3-kinase (PI3-K) pathway (Cardone *et al.,* 1998), or the extracellular signal regulated kinase (ERK) pathway (Allan *et al.,* 2003), can induce phosphorylation of procaspase-9 preventing its activation by the apoptosome. Moreover, the activity of active caspase-9 can be affected by phosphorylation (Cardone *et al.,* 1998). Caspase-9 does not need to be processed to be enzymatically active (Stennicke *et al.,* 1999). Phosphorylation of either form may act as a regulator of enzymatic activity, preventing both autoactivation of pro-caspase-9 as a result of induced proximity, and activation of downstream caspases by processed caspase-9. Alternatively, it has been demonstrated that the activity of processed caspase-9 is low in the absence of cytochrome-c and dATP, addition of these factors induced a 2000-fold increase in caspase activity (Stennicke *et al.,* 1999). As discussed earlier, binding to a cytosolic factor, Apaf-1, is not only essential for the activation of procaspase-9 but also appears to represent the active form of caspase-9 (Rodriguez and Lazebnik, 1999). Therefore, like nitrosylation, phosphorylation of caspase-9 may prevent assembly of the apoptosome resulting in an inability to activate itself, or prevent activated caspase-9 from processing downstream caspases. Thus, phosphorylation and nitrosylation may act as cellular survival signals; to prevent induction of the apoptotic

pathway by spontaneously activated caspases, possibly via a requirement for the activation of a denitrosylation/dephosphorylation pathway as part of the death-inducing signal.

1.4.3. Caspase-independent cell death

Recently, cell death that displays many of the morphological and biochemical markers of apoptosis, but without the activation of caspases, has been identified (Belmokhtar *et al.*, 2001; Brown *et al.*, 2000; Carmody and Cotter, 2000; Clarke *et al.*, 2003; Larmonier *et al.*, 2002; Mathiasen *et al.*, 2002; Quignon *et al.*, 1998; Sperandio, *et al.*, 2000; Susin *et al.*, 2000; Wolf *et al.*, 1999). Moreover, inhibition of caspases does not necessarily result in survival but rather changes the form of cell death (Chautan *et al.*, 1999; Liu *et al.*, 2003; Lemaire *et al.*, 1998; McCarthy *et al.*, 1997; Oppenheim *et al.*, 2001; Sané and Bertrand, 1999; Uzzo *et al.*, 2001; Volbracht *et al.*, 2001; Xue *et al.*, 2001). As discussed earlier, caspases are directly responsible for the hallmarks of apoptosis. Thus additional pathways that enable disassembly of the cell, possibly by cleaving the same substrates, must exist. A number of alternative death pathways have been described, many mediated by proteases. Cathepsins, calpains, and granzyme B have the ability to cleave caspase substrates and are activated in many of the alternative death pathways (reviewed in Leist and Jäättelä, 2001). Cell disassembly in many of these alternative cell deaths, although appearing to mimic classical apoptosis, contain subtle differences, *e.g.* often chromatin condensation is less compact. These types of cell death may also only possess some apoptotic markers and not others, or may display some necrotic-like markers. The propensity of some authors to label cell death as apoptotic after detection of only one or two morphological or biochemical markers makes determination of the

frequency of alternative cell deaths in the literature problematic. However, the increasing recognition of alternative death pathways may enable more detailed analysis in the future.

One, well characterised caspase-independent cell death pathway is that of apoptosis inducing factor (AIF). Like many classical apoptotic components, AIF is normally contained in the mitochondrial intermembrane space (Daugas *et al.,* 2000a; Susin *et al.,* 1999), however after a death stimulus AIF is liberated through the permeabilised outer membrane. Once released AIF translocates to the nucleus (Cande *et al.,* 2002; Daugas *et al.,* 2000a,b) where it induces peripheral chromatin condensation and high molecular weight DNA fragmentation, where the DNA is fragmented into 50kbp fragments (Wang *et al.,* 2002). Cell death induced by AIF can be both caspase-dependent and caspase–independent, according to a number of pieces of evidence. Translocation of AIF to the nucleus occurs in response to certain stimuli, in the presence of chemical caspase inhibitors (Ferri *et al.,* 2000; Susin *et al.,* 1999). Moreover, translocation of AIF has also been documented in cells in which there is no caspase activation owing to knockout of *Apaf-1*, *caspase-3* or *caspase-9* (Susin *et al.,* 2000). Microinjection of knockout cells with exogenous AIF also induces cell death without caspase activation but with an apoptotic morphology (Susin *et al.,* 2000). Interaction between AIF and the caspase pathway also occurs, with AIF triggering release of cytochrome-c from the mitochondria and thus inducing activation of the caspase cascade (Daugas *et al.,* 2000b; Susin *et al.,* 1999; Yu *et al.,* 2002). However, it has been demonstrated that release of AIF can occur after caspase activation (Arnoult *et al.,* 2002; Lassus *et al.,* 2002;

Robertson *et al.,* 2002; Susin *et al.,* 1997), indicating involvement in caspase-dependent pathways.

1.4.4. Necrosis

Necrotic cell death is characterised by osmotic swelling of the cell and organelles, cytoplasmic vacuolisation and eventually cell lysis (Wyllie *et al.*, 1980). Traditionally this form of cell death has been though of as a passive "accidental" cell death, however inhibition of caspases, or induction of apoptosis in a caspase-defective cell type, can result in a caspase-independent cell death with a necrotic phenotype (Chautan *et al.,* 1999; Hirsch *et al.,* 1997; Matsumura *et al.,* 2000). In many cases necrotic cell death can be prevented by inhibition of a pathway or overexpression of a protein. Thus necrotic cell death may represent an active cell death additional to the traditional caspase-dependent pathway.

If the energy status of the cell is compromised, apoptotic stimulation results in necrosis (Formigli *et al.,* 2000). The main ATP-dependent steps in the apoptotic pathway are, active nuclear transport to enable nuclear apoptosis (Yasahura *et al.,* 1997), and formation of the apoptosome (Eguchi *et al.,* 1999). Thus, it is not surprising that ATP depletion prevents execution of the apoptotic pathway. However prevention of apoptosis in this situation does not promote cell survival, instead the cell dies by necrosis (Eguchi *et al.,* 1999; Lee and Shacter, 1999; Leist *et al.,* 1997; Samali *et al.,* 1999; Single *et al.,* 2001). Artificial maintenance of the cellular ATP level prevents necrosis and enables apoptosis to occur (Leist *et al.,* 1997; Samali *et al.,* 1999). In situations of DNA damage, the DNA repair enzyme PARP is activated, which as

discussed earlier (Section 1.4.2.4), results in rapid ATP depletion. This leads to inhibition of apoptosis and induction of necrotic cell death, which can be prevented by the use of PARP inhibitors (Lee and Shacter, 1999; Palomba *et al.,* 1996). In some cases opening of the mitochondrial PT pore, with associated loss of $\Delta\psi m$, is responsible for ATP depletion and necrosis (Qian *et al.,* 1997; Single *et al.,* 2001). Opening of the PT pore prevents ATP synthesis for as long as the pore is open (Bernardi *et al.,* 1999), thus prolonged opening can lead to depletion of intracellular ATP stores. During apoptosis, not all mitochondria in a cell undergo permeability transition at the same time, enabling intact mitochondria to supply the energy required to execute the apoptotic program (LeMasters, 1999). However, rapid and profound opening of the PT pore is thought to occur during necrosis, resulting in energy depletion (Denecker *et al.,* 2001a). Cells can be protected from necrosis by overexpression of Bcl-2, or Bcl-x_L, (Denecker *et al.,* 2001b; Fukuda and Yamamoto, 1999; Guénal *et al.,* 1997; Shimizu *et al.,* 1996a,b,c; Subramanian *et al.,* 1995), which has been associated with delaying, or preventing, the of loss $\Delta\psi m$ (Guénal *et al.,* 1997; Shimizu *et al.,* 1997c; Single *et al.,* 2001) possibly by prevention of PT pore opening.

Autophagy, a mechanism for degradation and recycling of cytosolic proteins, is employed during starvation, differentiation and aging to enable cell survival, however autophagy is also involved in the cells demise. Autophagic (type II) cell death is characterised by the formation of large cytoplasmic vacuoles, a morphology reminiscent of necrosis. Autophagic vacuoles, formed with the aid of a distinct set of proteins, engulf cytoplasmic organelles and fuse with lysosomes, degrading their contents (Mizushima *et al.,* 2002). During autophagic cell death mitochondria are engulfed and

destroyed (Tolkovsky *et al.,* 2002; Xue *et al.,* 2001), a process initiated by mitochondrial permeability transition (Elmore *et al.,* 2001), and prevented by Bcl-2 (Tolkovsky *et al.,* 2002). It has been proposed that death ensues as a result of ATP depletion however, cells without mitochondria can produce ATP by glycolysis, although temporarily (Tolkovsky *et al.,* 2002). An inability to synthesise proteins has been observed, possibly as a result of autophagic degeneration of ribosomes, endoplasmic reticulum and Golgi (Bursch *et al.,* 2000). Ultimately, autophagic cell death results in fragmentation and phagocytosis of the cell corpse (Kitikana and Kuchino, 1999). Autophagic cell death has been observed during development (Clarke, 1990), however it also occurs when apoptosis is induced via the mitochondria in the presence of caspase inhibitors (Tolkovsky *et al.,* 2002), thus it may be another form of caspase-independent cell death.

1.4.5. Apoptosis vs. Necrosis

The original description of apoptosis by Kerr, Wyllie and Currie (1972) identified apoptosis and necrosis as "two distinct patterns of morphological change", thus separating them as mutually exclusive phenomenon. However, it has become clear that apoptosis and necrosis more likely represent two extremes of a near continuum of cell death, emphasised by the fact that inhibition of one form of cell death does not result in survival but enables another form of cell death to predominate. In most situations it is only possible to study these alternative cell deaths when caspases are inhibited, indicating that in most cell types caspase-dependent cell death is dominant. However, this does indeed demonstrate that other death pathways exist. Moreover, there appears to be enormous overlap and shared signalling pathways among the different death

programs. Depending on the conditions and/or cell type different steps are activated in response to a death stimulus, resulting in different degrees and combinations of the features of cell death. In order to study these alternative cells death adequately, the propensity to label cell death as apoptotic on the basis of one or two positive markers must be discouraged. Instead detailed analysis of the biochemical and morphological changes occurring during cell death must be undertaken. Thus, it may be that we should categorise cell death as either "active" or "passive", with the apoptotic-like and necrotic-like characteristics being merely an observation not a classification. The frustration felt by many researchers when classifying death as apoptotic or necrotic, is summed up in an eloquent discussion by Robert Sloviter (2002). He proposes, "that the direct description of entities is preferable to the confusing use of names that do not accurately describe the entities they purport to represent", which he surmises in the following statement "By analogy, the presence of teeth easily differentiates a dog from a bottle of wine, but it does not define a dog. If it did, a cat would also be a dog".

1.5. AIMS

- Determine whether cigarette smoke condensate induces apoptosis of an alveolar epithelial type II like cell line (A549) or primary human umbilical vein endothelial cells (HUVECs).
- Investigate the molecular mechanisms involved in cigarette smoke-induced apoptosis

Chapter 2: Materials and Methods

2.1. CELL LINES

2.1.1. Human alveolar epithelial type II cells (A549)

The human alveolar type II-like epithelial cell line (A549) were obtained from the European Collection of Animal Cell Cultures (ECACC, Porton Down, UK) and grown in Dulbecco's Modified Eagles medium (DMEM, Sigma, Poole, UK) supplemented with 10% heat-inactivated foetal calf serum (HI-FCS, LabTech International, Ringmer, UK) 100U/ml Penicillin, 1μg/ml Streptomycin (P/S, Invitrogen Life Technologies, Paisley, UK) and 2mM L-Glutamine (L-Glut, Invitrogen Life Technologies). A549 cells were grown in 162cm^2 tissue culture flasks (CoStar, Corning, NY) and subcultured 1 in 4 every two days. Cells were seeded into tissue culture plates at 3x10^4 cells/cm^2, incubated overnight and treated at approximately 70% confluency in media containing 2% HI-FCS. For serum-free treatments cells were seeded at 2x10^4 cells/cm^2 and incubated overnight. The media was removed, the cells washed with calcium/magnesium-free phosphate-buffered saline (CMF-PBS) and serum-free media was applied. Cells were further incubated overnight and treated at 70% confluency in serum-free media.

2.1.2. Human leukaemic T cell line (Jurkat)

The human leukaemic T cell line (Jurkat) was obtained from the European Collection of Animal Cell Cultures (ECACC) and grown in continuous culture in RPMI (Invitrogen Life Technologies) supplemented with 10% HI-FCS, P/S and L-Glut. Jurkat cells were grown in 75cm^2 tissue culture flasks (CoStar) and subcultured 1 in 3 every two to three

days. Cells were treated at a density of $1x10^6$ cells/ml in either full media or media containing 1% HI-FCS

2.1.3. Human umbilical vein endothelial cells (HUVEC)

Primary Human Umbilical Vein Endothelial Cells (HUVEC's) were donated by Dr Peter Henriksen of the Rayne Laboratory, University of Edinburgh. Cells were grown in EBM-2 media (BioWhittaker, Verviers, Belgium) with supplements supplied by the manufacturer. Cells were seeded into 24 well plates at a density of $0.05x10^6$ cells/well or 96 well plates at $0.01x10^6$ cells/well and cultured overnight prior to treatment. Cells were treated in full media between passages 4 and 6.

2.2. CIGARETTE SMOKING

2.2.1. Preparation of Cigarette smoke condensate (CSC)

Cigarette smoke condensate was prepared fresh at a concentration of 1 cigarette/ml CMF-PBS. The smoke from a medium tar cigarette (Regal) was drawn up into a glass syringe and passed into a tonometer agitating CMF-PBS (Figure 2.1). Each puff consisted of approximately 35ml of cigarette smoke, with 20 puffs consisting one cigarette. The condensate was sterile filtered using a 0.22μm syringe filter prior to use.

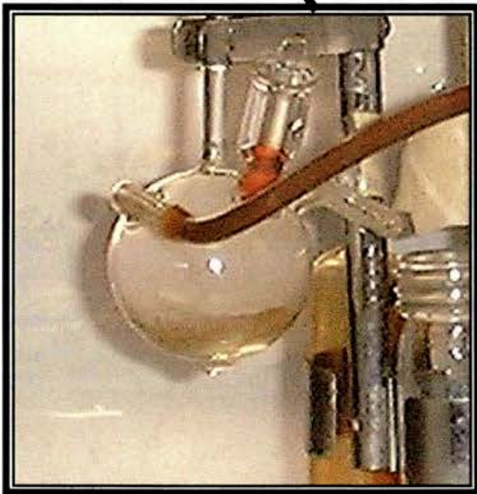


Figure 2.1. The Cigarette smoking machine and tonometer.

2.3. GENERAL METHODS

2.3.1. Cell Morphology

Cytospins of Jurkat cells, or cells grown on coverslips were fixed for one minute in methanol (BDH, Lutterworth, UK). Cytoplasm was stained for one minute in DiffQuick solution 1 and nuclei were stained for one minute with DiffQuick solution 2.

2.3.2. Protein Assay

Protein concentration of samples was measured using the bicinchoninic acid method (BCA, Pierce, Rockford, IL). This method utilises the reduction of Cu^{2+} to Cu^{+} by protein and is detected by a unique reagent containing bicinchoninic acid. Two molecules of bicinchoninic acid chelate one molecule of Ca^{+} forming a purple reaction product. This product exhibits a strong absorbance at 562nm, which is nearly linear with increasing protein concentration.

A standard curve was prepared of Bovine Serum Albumin (BSA, Sigma) ranging from 0.6 to 0.05mg/ml. 10µl of standards and samples were applied to wells of a 96-well plate (Costar) and incubated at 37°C with 200µl of assay reagent for 30 minutes. The absorbance was read at 570nm and the concentration of the samples was determined by linear regression.

2.3.3 Assay for total glutathione (GSH)

Intracellular GSH was measured by a method adapted from Tietze (1969) and Vandeputte *et al.* (1994) Briefly, cells grown in 6-well plates were treated, washed twice in CMF-PBS and harvested by adding 0.5ml trypsin-EDTA (Invitrogen Life Technologies) and incubating at 37°C until cells had detached. The trypsin was neutralised with 1ml of culture medium and the suspension was transferred into 1.5ml eppendorfs. Cells were then pelleted by centrifugation at 2000rpm (Heraeus Biofuge Pico) for 4 minutes. The supernatant was removed and the cells were washed in 1ml CMF-PBS prior to repelleting. The CMF-PBS was removed and the pellets were stored at –70°C until required.

Pelleted cells were lysed in 1ml of ice-cold extraction buffer (see appendix 3) and homogenised with a teflon pestle. The lysates were then sonicated in icy water for 2-3 minutes with regular vortexing. To ensure that the cells were properly lysed two freeze/thaw cycles were performed at -70°C defrosting the samples on ice. The lysates were centrifuged at 3000 rpm for 4 minutes at 4°C prior to transferring the supernatant to pre-chilled eppendorfs. All samples and solutions were kept on ice for the assay.

GSH (Sigma) stock solution was prepared at 1mg/ml in KPE (appendix 3) and a standard curve was prepared ranging from 8μg/ml (23.85nmoles) to 0.125mg/ml (0.37nmoles). 20μl of standard or samples were added to a 96-well plate (CoStar) in singular, filling no more than 32 wells of the plate. 120μl of 0.66mg/ml DTNB and 13.3μl/ml glutathione reductase were added to each well with a multichannel pipette.

Conversion of GSSG to GSH was allowed to occur for 30 seconds when 60µl of 0.66µg/ml β-NADPH was added to each well. The absorbance at 410nm was determined immediately in a microplate reader and then every 30 seconds for 2 minutes. This process was repeated twice more on the same plate to provide replicates. Linear regression was used to determine the GSH content in nmoles/ml of each the samples and was expressed as nmoles GSH/mg protein after determining protein concentration as described earlier.

2.4. Assays to detect apoptosis

2.4.1. Flow cytometry analysis of phosphatidylserine exposure

Externalisation of phosphatidylserine (PS) is an early marker of apoptosis, which can be detected by binding of annexin-V. Viable cells do not bind annexin-V or take up PI and were detected in the bottom left hand corner of the FACS dot-plot (Figure 2.2, green), cells in the early stages of apoptosis display PS on the outer membrane leaflet yet retain cell membrane integrity thus they bind annexin-V but are not permeable to PI; these cells were detected in the bottom right hand corner of the FACS dot-plot (Figure 2.2, blue). Using this method it is not possible to differentiate late apoptotic cells (secondary necrosis) from necrotic cells. Late apoptotic cells display PS on the outer membrane leaflet and are permeable to PI, PS remains on the inner membrane leaflet in necrotic cells yet as they have lost their cell membrane integrity annexin-V can enter the cell along with PI and bind the intracellular PS. Therefore these cells fluoresce in both FL-1 (annexin) and FL-2 (PI) and were detected in the top right hand corner of the

FACS dot-plot (Figure 2.2, pink). In all experiments apoptotic and necrotic controls of 2μM staurosporine (SS) and 10mM H_2O_2 were included (Figure 2.2. B and C).

Treated cells were trypsinised and collected in eppendorf tubes. The cell pellets were washed sequentially with CMF-PBS and annexin-V binding buffer (2mM $CaCl_2$ in Hanks Balanced Saline Solution HBSS, Sigma). Cells were stained in Annexin-V (Molecular Probes, Leiden, Holland) diluted 1/600 and PI (Molecular Probes) at 0.3μg/ml in Annexin-V binding buffer, at a concentration of $1x10^6$/15μl. Cells were analysed immediately on a Becton Dickinson FACSComp using CellQuest software.

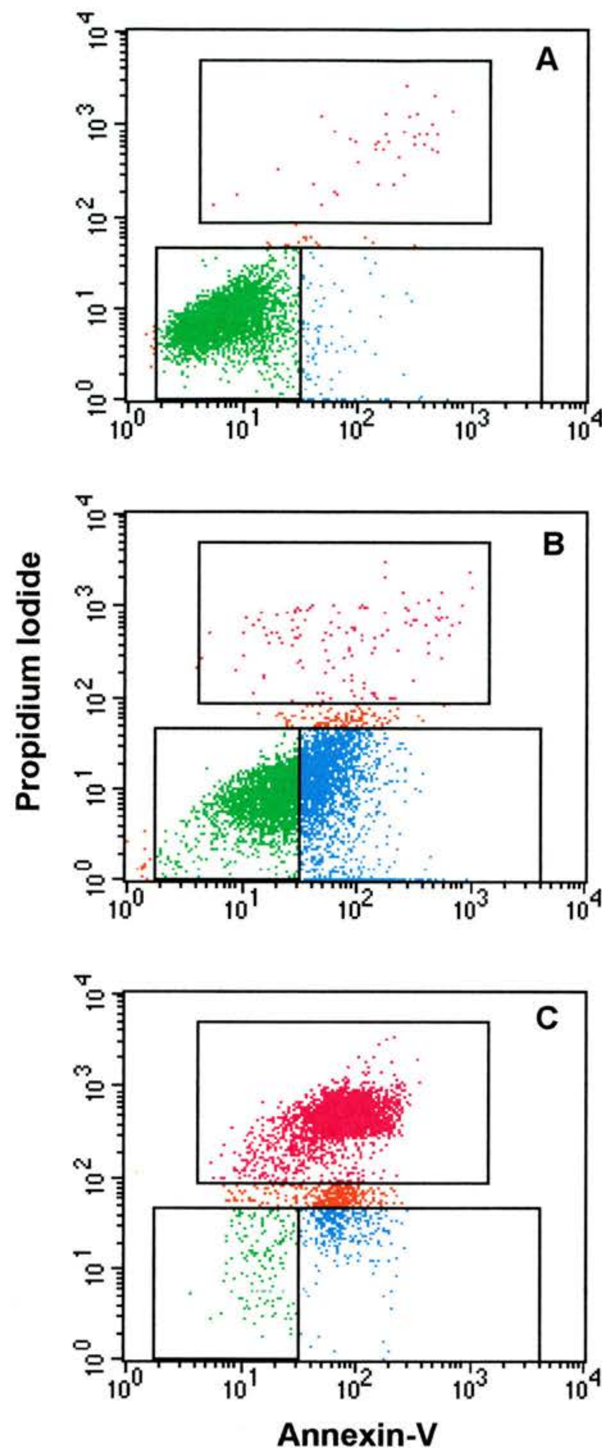


Figure 2.2. Detection of PS externalisation by annexin-V binding in A549 cells. A549 cells were incubated with either media alone **(A)**, 2μM SS **(B)**, or 10mM H_2O_2 **(C)**. Cells were stained with Annexin-V and PI and analysed by flow cytometry to determine the percentage of viable (Annexin –ve, PI –ve, *green*), apoptotic (Annexin +ve, PI –ve, *blue*) and necrotic (Annexin +ve, PI +ve, *pink*) cells.

2.4.2. Oligonucleosomal DNA fragmentation (DNA laddering)

2.4.2.1. Genomic DNA extraction protocol

Initially DNA fragments were isolated using a genomic DNA extraction kit (Promega). Briefly, 600μl nuclei lysis buffer was added to the cell pellet, 5μl RNase A was added and tubes were incubated at 37°C in a dri-block (Stuart Scientific, Tilling Drive Stone, Staffs, UK) for 30 minutes. Samples were cooled to room temperature and 200μl protein precipitation solution was added. Samples were vortexed, chilled on ice for 5 minutes and the precipitate was pelleted by centrifugation at 13000 rpm (Heraeus Biofuge Pico) for 4 minutes. Supernatant was removed, placed in 1.5ml eppendorf, and 600μl of room temperature iso-propyl alcohol was added. Samples were mixed by inversion until DNA was visible, which was pelleted by centrifugation at 13000 rpm (Heraeus Biofuge Pico) for 1 minute. Supernatant was decanted and 600μl of room temperature 70% ethanol was added. Samples were inverted gently to wash the DNA and pelleted at 13000 rpm (Heraeus Biofuge Pico) for 1 minute, and supernatant was gently aspirated. Pellets were air-dryed for 10-15 minutes prior to rehydration. 100μl of DNA rehydration solution was added to the DNA and incubated at 65°C for 1 hour. DNA was analysed by agarose gel electrophoresis on a 1% gel at 50V as in section 2.4.2.4.

2.4.2.2. "CURRENT PROTOCOLS IN IMMUNOLOGY" protocol

Adapted from Current Protocols in Immunology, Section 5, basic protocol 3. Floating cells were harvested from the media by centrifugation. Adherent cells were washed and 250μl TTE solution (see appendix 3) was added per well (500μl total) and cells were

scraped from the well with a Teflon scraper. Cell lysates was added to 1.5ml eppendorf containing floating cells, labelled tube "B". Tubes were centrifuged at 13000 rpm for 10 minutes at 4°C (Heraeus Biofuge Fresco) and supernatant was placed into fresh eppendorfs labelled "T". The pellet in tube "B" was resuspended in 0.5ml TTE solution and 0.1ml ice-cold 5M NaCl (see appendix 3) added to the tubes labelled B and T. Tubes were vortexed vigorously, 0.7ml ice-cold isopropanol was added to each tube and vortexed once more. The DNA was precipitated and collected by placing the tubes at -20°C for 30 minutes and centrifuging at 13000 rpm for 10 minutes at 4°C (Heraeus Biofuge Fresco). Supernatant was carefully aspirated and excess alcohol was removed with the corner of a sterile tissue. Ice-cold ethanol (1ml) was added to each tube and samples were centrifuged at 13000 rpm at 4°C for 10mins (Heraeus Biofuge Fresco). Supernatant was removed as previously, and pellets were allowed to air-dry. TE buffer (50µl, see Appendix 3) was added to the DNA pellet and incubated for approximately 1-2 hours at 37°C. DNA loading buffer (see appendix 3) was added to a 1x final concentration and the samples heated for 10 minutes at 65°C. Electrophoresis was performed on a 1% agarose gel at 50 volts for approximately 2 hours as in section 2.4.2.4.

2.4.2.3. Mini-prep protocol

Approximately $1x10^6$ cells were lysed in 500µl 7M Guanidine hydrochloride (see appendix 3) and applied to Wizard SV miniprep columns (Promega, Madison, WI) pre-wetted with column wash solution (Promega). Columns were centrifuged at 13,000 rpm for 2 minutes before flowthrough was disposed of and 750µl wash solution was applied

to the column and re-centrifuged, this was repeated with 250μl wash solution before eluting DNA with 50μl TE/RNase (50μg/ml). Samples were analysed by electrophoresis on a 1.8% agarose gel at 50 volts for 2 hours as in Section 2.4.2.4.

2.4.2.4. Agarose gel electrophoresis

Agarose gel electrophoresis was performed on extracted DNA to determine the presence of oligonucleosomal DNA fragments. Agarose (Seakem, Rockland, ME, USA) was dissolved in Tris-borate EDTA (TBE) buffer (see appendix 3) by melting in a microwave. Once cooled to approximately 50°C, 0.5μg/ml ethidium bromide (Sigma) was added and the gels were poured. The DNA was visualised using a ultraviolet (UV) light source (UVP Laboratory Products, Upland, CA, USA), images were captured with a digital camera (UVP Laboratory Products).

2.4.3. Acridine Orange and Ethidium Bromide Staining for Apoptosis

This assay utilises the ability of ethidium bromide and acridine orange to intercalate into the DNA and fluoresce when viewed by epilumination. All cells will take up acridine orange, when intercalated it makes DNA appear green, and RNA red. Only non-viable cells take up ethidium bromide, which makes DNA appear orange. The ethidium bromide will overwhelm the acridine, enabling distinction between the two dyes. Apoptosis and necrosis can be monitored by observing the dye taken up by the cell in combination with nuclear morphology. Viable cells have normal green nuclei, apoptotic cells have condensed green nuclei and necrotic cells have normal orange nuclei. Cells that have undergone secondary necrosis have orange condensed nuclei.

Dye containing 100 μg/ml acridine orange (Sigma) and 100 μg/ml ethidium bromide (Sigma) was prepared in CMF-PBS and added to the cell media after treatment to provide a working concentration of 4μg/ml of both dyes. Cells were visualised immediately by epifluorescent microscopy and the number of viable cells with normal nuclei (VN), viable cells with condensed nuclei (VA), non-viable cells with normal nuclei (NVN) and non-viable cells with condensed nuclei (NVA) were determined. A total of 200 cells were counted per well with each treatment being performed in duplicate. The apoptotic/necrotic index was determined by the use of the following equations:

$$\% \text{ apoptotic cells} = \frac{VA+VN}{VN+VA+NVN+NVA} \times 100$$

$$\% \text{ necrotic cells} = \frac{NVN}{VN+VA+NVN+NVA} \times 100$$

2.4.4. Detection of Hypodiploid cells (Sub G_0)

During the process of "classical" apoptosis, caspase-activated DNase (CAD) cleaves DNA into 200bp fragments, which can be gently washed from fixed, permeabilised cells and the DNA content can be analysed, by flow cytometry. The cells are analysed using a method termed "pulse processing", when a cell passes through the excitation laser of the flow cytometer there is a gradual increase in fluorescence, which increases to a peak when the whole cell is in the laser beam, and then decreases as the cell leaves the laser beam (Figure 2.3.B). The area and width of this curve is proportional to the amount of fluorescence, and hence DNA, within the cell. Plotting this data enables the different stages of the cell cycle to be determined. During the first gap phase (G_1) human cells contain 46 chromosomes (diploid), which are replicated during S-phase so that cells in

the second gap phase of the cell cycle (G_2) contain twice as much DNA. However, apoptotic cells that have lost their fragmented DNA after washing will contain less than the normal amount of DNA (hypodiploid). These cells are detected as a peak to the left of the G_1 peak as they emit less fluorescence (Figure 2.3.D).

Analysis of apoptosis by flow cytometry can be combined with electrophoretic analysis of fragmented DNA eluted from fixed cells. This enables conformation of apoptosis as DNA eluted from apoptotic cells displays classical DNA laddering while DNA fragmented during necrosis is detected as a smear.

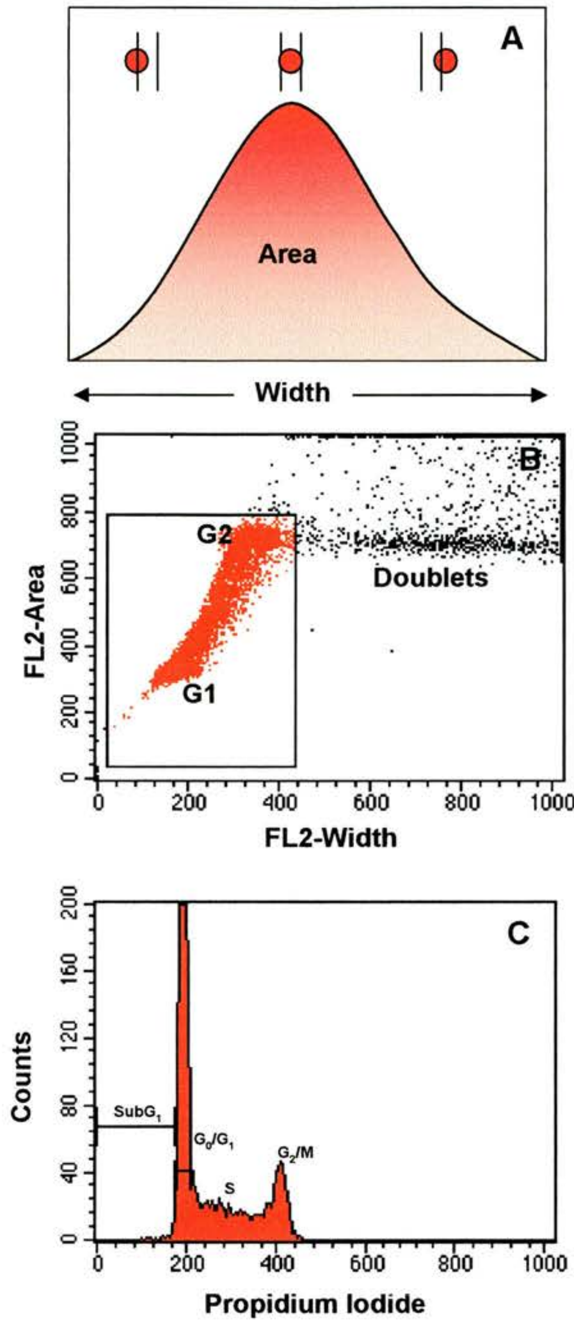


Figure 2.3. $SubG_1$ in A549 cells. Apoptosis was determined by monitoring the number of hypodiploid cells. Fragmented DNA was gently washed from cells, remaining DNA was stained with propidium iodide and the cells were analysed by flow cytometry. Apoptotic cells were identified by pulse processing where DNA content is determined as the cell passes through the excitation laser **(A).** Plotting area vs. width **(B)** enables the identification of cells in each stage of the cell cycle and those with with less than the normal (G_1) amount of DNA (Sub G_1) **(C)**

Method adapted from "Current Protocols in Flow Cytometry, section 7.5.8, basic protocol 5". Briefly, the cell cycle was synchronized by culturing overnight without serum prior to treatment. Cells were harvested by trypsinisation, centrifuged at 1000 rpm for 5 minutes (MSE Mistral 3000) and transferred to 1.5ml eppendorfs. Cells were pelleted once more by centrifugation at 2000 rpm for 4 minutes (Heraeus Biofuge Pico). Replicate cell pellets were resuspended in 1ml CMF-PBS (approximately $0.5x10^6$ cells/pellet). Cells were fixed by admixing with a pastette, 1ml of cell suspension with 10mls ice-cold 70% ethanol and placed on ice for 2 hours. Cells were collected by centrifugation at 200 g for 10 minutes (MSE Mistral 3000), and ethanol was decanted and the remainder allowed to evaporate. Cell pellets were resuspended in 50µl DNA extraction buffer (see appendix 3), transferred to 1.5ml eppendorfs and incubated at 37°C for 1 hour in a shaking incubator (Stuart Scientific). Cells were centrifuged for 10minutes at 1500 g (Heraeus Biofuge Pico) and supernatant containing low-molecular weight DNA was removed and stored at 4°C. Cells were resuspended in 1ml PI staining solution (see appendix 3) and incubated for 30 minutes in the dark. Flow cytometry was utilized for analysis by pulse processing on the FL-2 channel. Low molecular weight DNA was analysed by agarose gel electrophoresis. 40µl of supernatant was placed in a 1.5ml eppendorf, 5µl of DNase-free RNase A (Sigma) was added and tubes were incubated at 37°C for 30 minutes. 5µl of 1mg/ml proteinase K (Sigma) was added and tubes were returned to the incubator for 30 minutes. DNA was electrophoresed in a 0.8% agarose gel as in section 2.4.2.4.

2.4.5. Immunoblotting

2.4.5.1. Preparation of lysates

Cells were lysed in either RIPA lysis buffer or Cell Extract Buffer (CEB, appendix 3; $1x10^6$ cells/15μl) and placed on ice for 15 minutes. Lysates were passed through a 21 gauge needle approximately 10 times prior to centrifugation at 13000 rpm for 15 minutes at 4°C. The supernatants were removed and stored at -70°C until needed.

2.4.5.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS reducing buffer (see appendix 3) was added to cell lysates and boiled at 96°C for 5 minutes. Denatured samples were applied (controlled for protein concentration) to either 10% or 12% acrylamide gels with 4% stacking gels (see appendix 3), and electrophoresed at 125 volts for approximately 1 hour. Protein was then blotted onto polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham, Little Chalfont, UK) or nitrocellulose (Hybond-ECL, Amersham) in an electroblotting apparatus (BioRad, Hemel Hempstead, UK). Nitrocellulose membrane was equilibrated in transfer buffer (see appendix 3), PVDF membrane was pre-wetted in methanol for 10 seconds before being equilibrated in transfer buffer. The gels were placed onto the membrane and 2 pieces of filter paper, also soaked in transfer buffer, were placed on either side. Fibre pads soaked in transfer buffer were placed either side of the filter paper and the entire sandwich was placed into the gel holder cassette with the gel closest to the grey side of the cassette (see Figure 2.4). The process was repeated for the second gel. The cassettes were placed in the electrode module, the tank filled with

cooled 1x transfer buffer and the cooling block added. Transfer was performed at 80mA per gel for 90 minutes.

2.4.5.3. Caspase-3

Blots were blocked for 1 hour in CMF-PBS containing 2% caesin (Sigma) at room temperature on a gyrorocker (Stuart Scientific). Blots were subsequently probed with a polyclonal rabbit antibody to caspase-3 (Pharmingen, Oxford, UK) diluted 1/1000 in blocking buffer overnight at 4°C, washed three times in CMF-PBS and detected with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Santa Cruz, Wembley, UK) diluted 1/5000 in 2% casein/CMF-PBS. Blots were washed three times in CMF-PBS and the signal was detected by enhanced chemiluminesence (ECL, Amersham) and X-ray film (MR-1, Kodak, Rochester, NY).

2.4.5.4. Caspase-9

Blots were blocked for 1 hour in CMF-PBS containing 5% non-fat milk powder (Marvel) at room temperature on a gyrorocker (Stuart Scientific) prior to probing overnight at 4°C with a polyclonal rabbit antibody to caspase-9 (Pharmingen) diluted 1/2500 in 5% Marvel/CMF-PBS containing 0.05% Tween-20 (Sigma). Blots were washed three times in CMF-PBS/Tween and detected with an HRP-conjugated goat anti-rabbit antibody (Dako, Ely, UK) diluted 1/5000 in 5% Marvel/CMF-PBS-Tween. Membranes were washed three times in CMF-PBS/Tween and once in CMF-PBS. The signal was detected using ECL (Amersham) and X-ray film (MR-1, Kodak).

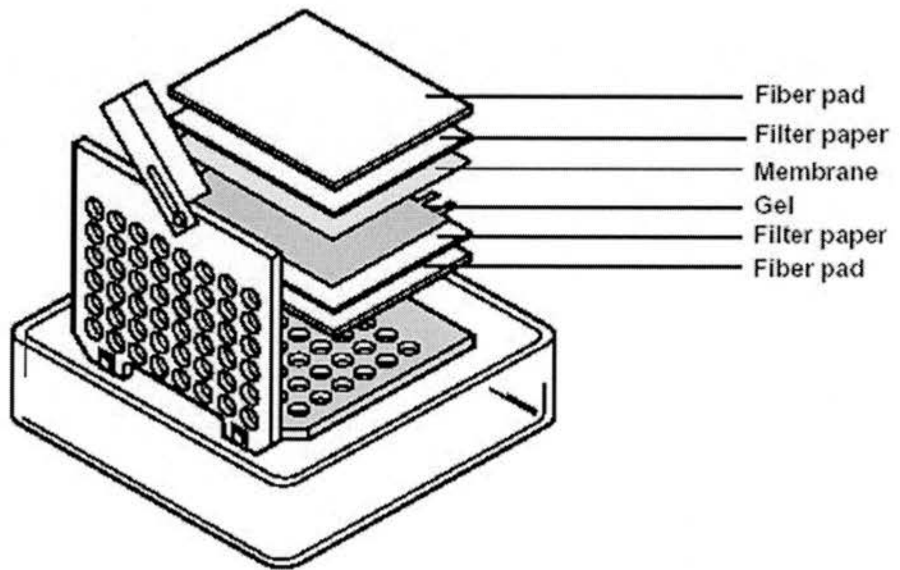


Figure 2.4. Assembly of the gel/membrane sandwich and the gel holder cassette (From BioRad)

2.4.5. Assay for caspase-3 activity

The effect of CSC and hydrogen peroxide on caspase-3 activity was determined using the caspase-3 assay kit (Calbiochem, Nottingham, UK) as per manufacturers instructions. Briefly, active caspase-3 (30U) was placed into a half-volume 96 well plate prior to the addition of the inhibitor supplied with the kit, 1, 5 and 10% CSC and 1mM H_2O_2. The plate was then incubated at 37°C for 1 hour before the addition of the caspase-3 substrate conjugated to p-nitroalanine. Caspase-3 activity was determined by measuring the change in absorbance at 405nm over 3 hours.

2.4.6. Release of lactate dehydrogenase (LDH)

The presence of lactate dehydrogenase (LDH) in the media after cell treatment was used as a marker of necrosis. LDH was detected using the cytotoxicity detection kit (Roche, Lewes, UK) which, detects LDH in two stages, in the first stage LDH reduces NAD^+ to NADH + H^+ by oxidation of lactate to pyruvate; the second stage a formazan salt is formed by the transfer of H/H^+ to tetrazolium salt by a diaphorase catalyst. The formazan salt can be detected spectrophotometrically at 490nm.

Briefly, cells treated in 96 well plates were centrifuged at 250 *g* for 10 minutes. 100μl of the supernatant was removed with a multichannel pipette, placed in a fresh 96 well plate and stored at 4°C. LDH reagent was prepared by adding reagent 1 to reagent 2 in a 1:45 ratio and adding 100μl to the cell supernatants. The plates were incubated in the dark for 30 minutes and the absorbance at 490nm with a 730nm reference filter was determined. In order to ensure plating densities were equivalent in all assays

supernatants were diluted in fresh media to represent a lesser plating density (section 3.5.1). The percentage cytotoxicity was determined by:

$$\text{Cytotoxicity (\%)} = \frac{\text{experimental value - low control}}{\text{high control - low control}} \times 100$$

2.5 ASSAYS TO STUDY STRESS SIGNALLING PATHWAYS

2.5.1. Western blotting for phosphorylated and native p38

Cells were lysed in RIPA lysis buffer (see section 2.4.5.1) and the protein concentration was determined using the BCA protein assay (section 2.3.2). 20μg of protein was applied to a 10% acrylamide gel with 4% stacking gel (see appendix 3) and electrophoresed and electroblotted as in sections 2.4.5.2. Blots were blocked in 4% Marvel in Tris-buffered saline (TBS, appendix 3) overnight at 4°C. Membranes were probed with anti-phosphorylated p38 [pTpY180/182] (BioSource, Camarillo, CA, USA) diluted 1/2000 in 5% BSA/TBS-0.05%Tween for 2 hours at room temperature. Blots were washed three times in TBS/Tween before being probed with HRP-conjugated goat anti-rabbit antibody (Santa Cruz) diluted 1/5000 in 5% Marvel/TBS-Tween for 1 hour at room temperature. Unbound antibody was removed by washing three times with TBS-Tween and once with TBS. The signal was detected using ECL (Amersham) and X-ray film (MR-1, Kodak). To detect native p38, antibodies were stripped from the blot by incubating with strip buffer (see appendix 3) at 60°C for 45 minutes. Excess strip buffer was removed by washing three times with TBS-Tween and blots were blocked in 5% Marvel/TBS-Tween overnight at 4°C. Membranes were probed with anti-native p38 (Sigma) diluted 1/40000 in 5% Marvel/TBS-Tween for 3 hours at room temperature. Subsequent steps were as for phosphorylated p38.

2.5.2. Western blotting for phosphorylated and native ERK

Cells were lysed in RIPA lysis buffer (see section 2.4.5.1) and the protein concentration was determined using the BCA protein assay (section 2.3.2). 10μg of protein was applied to a 10% acrylamide gel with 4% stacking gel (see appendix 3) and electrophoresed and electroblotted as in sections 2.4.5.2. Non-specific binding sites were blocked by incubation with 5% Marvel/TBS for 4 hours at room temperature or overnight at 4°C. Membranes were probed with anti-phosphorylated p44/42 (New England BioLabs, Hitchin, UK) at 1/2000 in 5% Marvel/TBS-Tween overnight at 4°C, washed three times in TBS-Tween and probed with HRP-conjugated goat-anti rabbit antibody (New England BioLabs) diluted 1/4000 in 5% Marvel/TBS-Tween for 1 hour at room temperature. The signal was detected and the blots stripped of antibodies as for p38. Blots were blocked overnight at 4°C in 5% Marvel/TBS-Tween and probed with anti-native ERK (Santa Cruz) diluted 1/2000 in 5%Marvel/TBS-Tween for 3 hours. Subsequent steps were as for native p38

2.5.3. Western blotting for heat-shock proteins (HSP)-27, -70 and -90

Cells were lysed in RIPA lysis buffer (see section 2.4.5.1) and the protein concentration was determined using the BCA protein assay (section 2.3.2). 10μg of protein was applied to a 10% acrylamide gel with 4% stacking gel (see Appendix 3) and electrophoresed and electroblotted as in sections 2.4.5.2. Non-specific binding sites were blocked by incubation with 5% Marvel in TBS for 1 hour at room temperature with rocking prior to incubation with anti-HSP-27 (StressGen) diluted 1/5000, anti-

HSP-70 (StressGen, Victoria, Canada) diluted 1/15000, and/or anti-HSP-90 diluted 1/35000 in 5% Marvel/TBS-Tween overnight at 4°C. Blots were washed three times in TBS-Tween and probed for 1 hour at room temperature with HRP-conjugated goat-anti rabbit antibody (StressGen) diluted 1/5000 in 5% Marvel/TBS-Tween. Subsequent steps were as for phosphorylated p38.

Chapter 3: Cigarette Smoke Condensate induces epithelial and endothelial cell necrosis

3.1. INTRODUCTION

Emphysema is characterised by enlargement of distal airspaces due to destruction of alveolar walls. Apoptosis of alveolar epithelial and endothelial cells has been implicated in the pathogenesis of emphysema (section 1.2.4.3.).

To determine whether cigarette smoke condensate (CSC) induced apoptosis in epithelial and endothelial cells *in vitro*, an alveolar epithelial type II cell line (A549) and primary human umbilical vein endothelial cells (HUVECs) were used. The data presented in this chapter shows that CSC does not induce apoptosis in either cell type, but instead induces necrosis.

3.2. CIGARETTE SMOKE CONDENSATE APPEARS TO INDUCE APOPTOSIS IN A549 CELLS

3.2.1. Cigarette smoke condensate induces a morphological change in A549 cells.

A549 cells grown on coverslips, and treated with CSC, were stained with DiffQuick. Cells treated with doses of 1% and 5% CSC retained normal morphology (Figure 3.1.B and C), whilst cells treated with 10% CSC underwent a morphological change. The cells decreased in overall size and the nuclei appeared smaller and more darkly stained (Figure 3.1.D). Condensation of nuclei is a hallmark of apoptotic cell death and thus the smaller, more darkly stained nuclei may represent apoptotic cells. However, condensed nuclei may also indicate cells that are transcriptionally inactive.

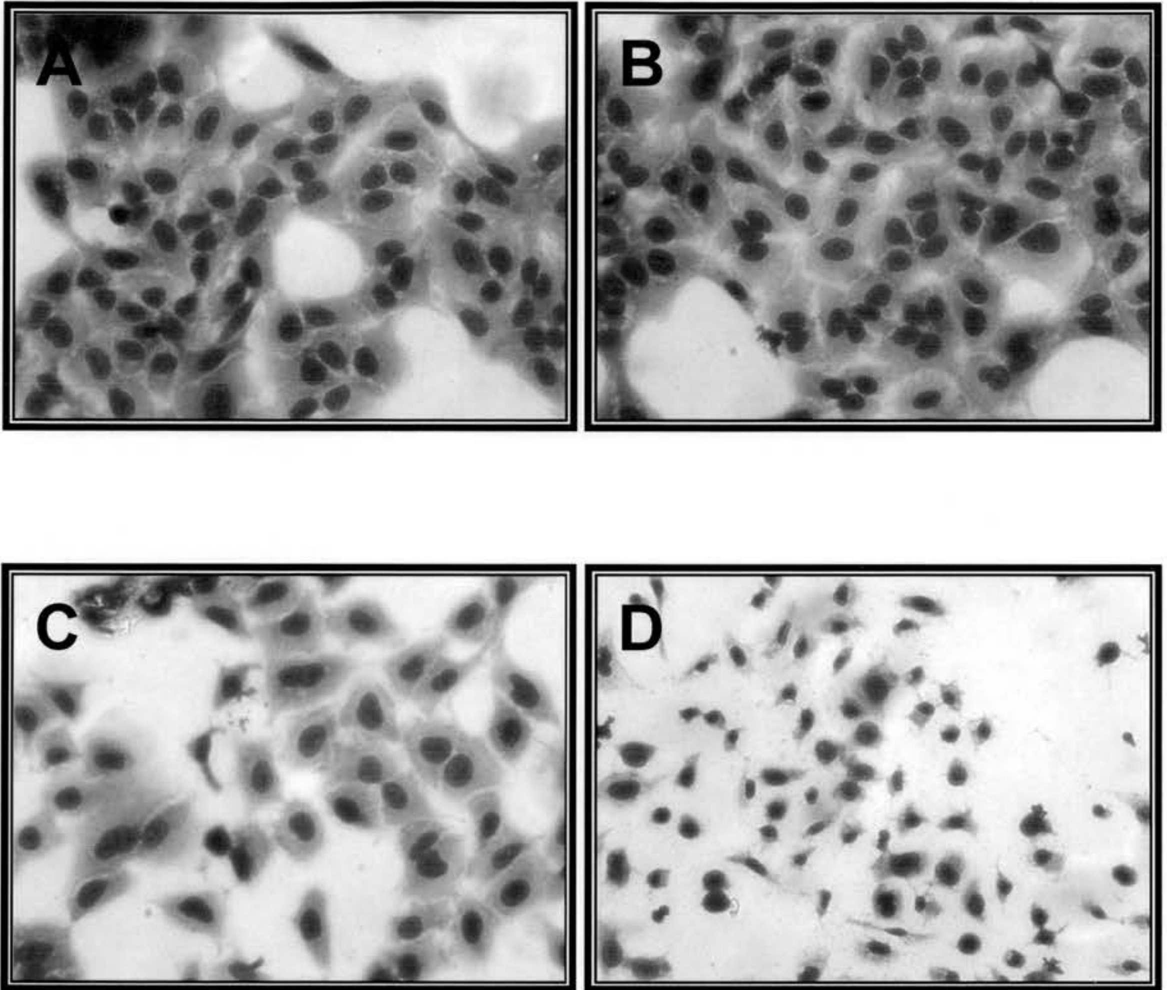


Figure 3.1. Cigarette smoke condensate induces morphological changes in A549 cells. Cells were grown on sterile glass coverslips, treated for 24 hours with either media alone **(A)**, 1% CSC **(B)**, 5% CSC **(C)**, or 10% CSC **(D)** and stained with DiffQuick. Magnification x34

3.2.2. Cigarette smoke condensate induces phosphatidylserine exposure in A549 cells.

Using annexin-V and propidium iodide (PI) staining, cell death was studied in A549 cells after CSC treatment for 4, 8 and 24 hours. An increase in annexin-V positive cells was detected after exposure to 5% and 10% CSC (Figure 3.2.C and D), this was evident as a shift to the right in the FL-1 channel. Moreover, a slight increase in PI uptake (FL-2) was detected (Figure 3.2.C and D), however this increase was not significant (Figure 3.3.B). After further analysis of the results it was evident that no apoptosis was detected after treatment with 1 and 5% CSC, but that a significant level of apoptosis was seen after treatment with 10% CSC at all three time points (Figure 3.3.A). Thus, CSC appeared to be inducing apoptosis in A549 cells. As outlined in section 1.4.5, apoptosis represents one extreme of a near continuum of cell death, a stimulus may result in display of some markers but not others. Thus to characterise cell death accurately, it is necessary to determine the presence of additional hallmarks of cell death. PS exposure is an early marker of apoptosis and in order to ascertain whether CSC induced "classical" apoptosis the presence of more advanced markers of apoptosis were studied.

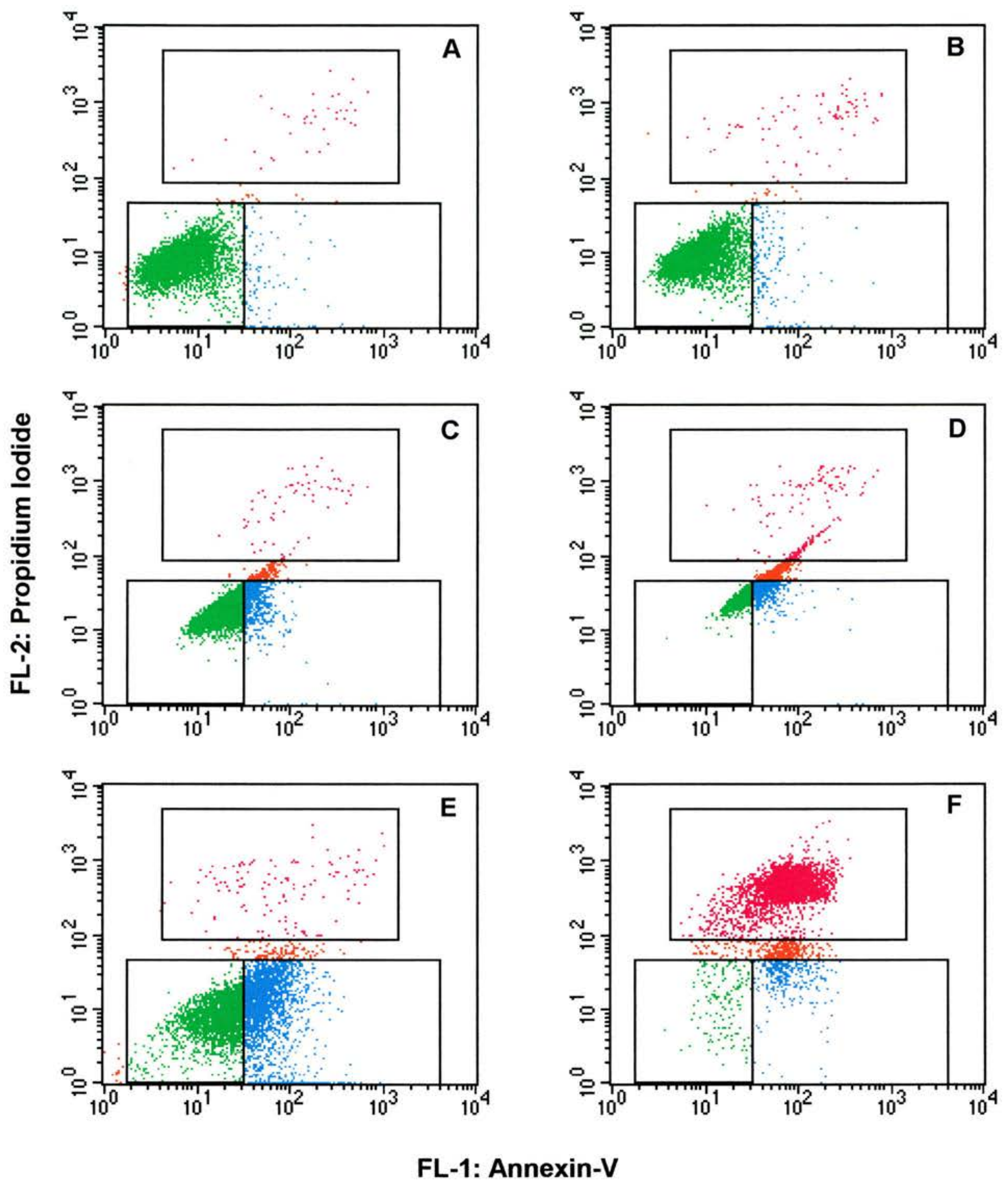


Figure 3.2. Cigarette smoke condensate induces PS externalisation and annexin-V binding in A549 cells. A549 cells were incubated with either media alone **(A)**, 1% CSC **(B)**, 5% CSC **(C)**, 10% CSC **(D)** 2μM SS **(E)** or 10mM H_2O_2 **(F)**. Cells were stained with Annexin-V and PI and analysed by flow cytometry to determine the percentage of viable (*green*), apoptotic (*blue*) and necrotic (*pink*) cells. Representative of 3 experiments.

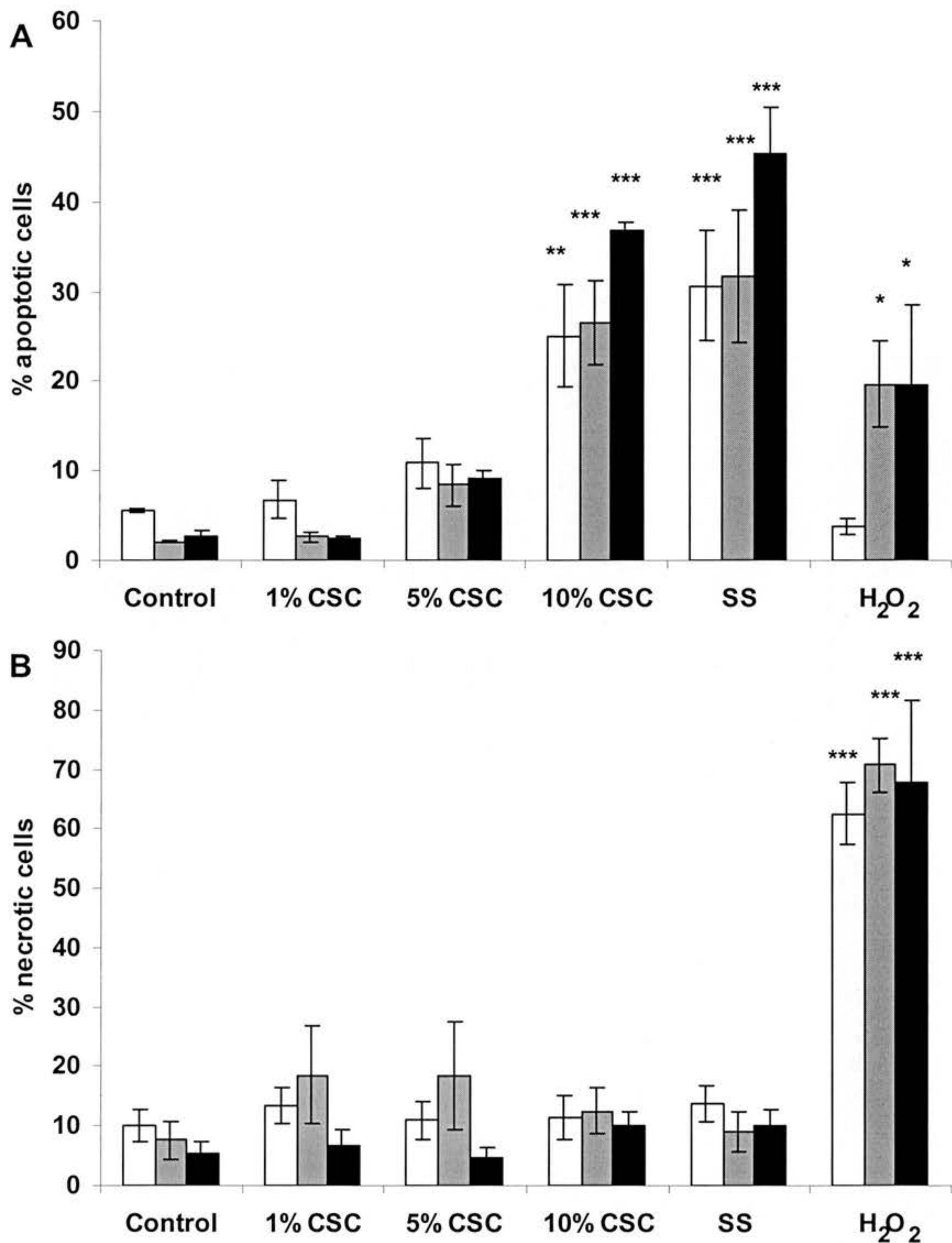


Figure 3.3. Cigarette smoke condensate induces apoptosis in A549 cells detected by annexin-V/propidium iodide staining. Cells were treated for 4 hours (*open bars*), 8 hours (*grey bars*) and 24 hours (*closed bars*) with either media alone or 1, 5 or 10% CSC. Treatment with 2μM staurosporine (SS) or 10mM H_2O_2 acted as positive controls for apoptosis and necrosis respectively. Apoptotic **(A)** and necrotic **(B)** cells were determined by flow cytometry after annexin-V and propidium iodide staining. Results expressed as mean of two experiments ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.3. A549 CELLS DO NOT DISPLAY ADDITIONAL MARKERS OF APOPTOSIS AFTER CSC EXPOSURE

3.3.1. Cigarette smoke condensate does not induce apoptosis detected by Sub G_1

When the data were analysed, the cell cycle profiles of cells treated with 1-10% CSC (Figure 3.4.B-D) did not differ from untreated cells (Figure 3.4.A). The apoptotic control (Figure 3.4.E) had a similar profile to untreated cells, but only the main G_0/G_1 peak was evident in the necrotic control (Figure 3.4.F). This may be due to selective depletion; fragile cells may have been destroyed during the process of necrosis or, during the fixation, permeabilisation, and staining method. Few hypodiploid cells were detected with any of the treatments (Figure 3.5) although staurosporine (SS) treatment resulted in a slightly more cells in the sub-G_1 range than any other treatment. The presence of only a small peak in the apoptotic control (Figure 3.4.E) was of some concern; however cells that have undergone apoptosis in the late stages of the cell cycle may not be detected as a sub-G_1 peak as they would contain more DNA than a diploid cell. Therefore these cells would shift to the left but would be detected in the S phase of the plot. Synchronising cells in the same stage of the cell cycle prior to stimulation can overcome this. Therefore, synchronisation of A549 cells by incubation overnight with mimosine (Wu *et al.,* 1994) was attempted.

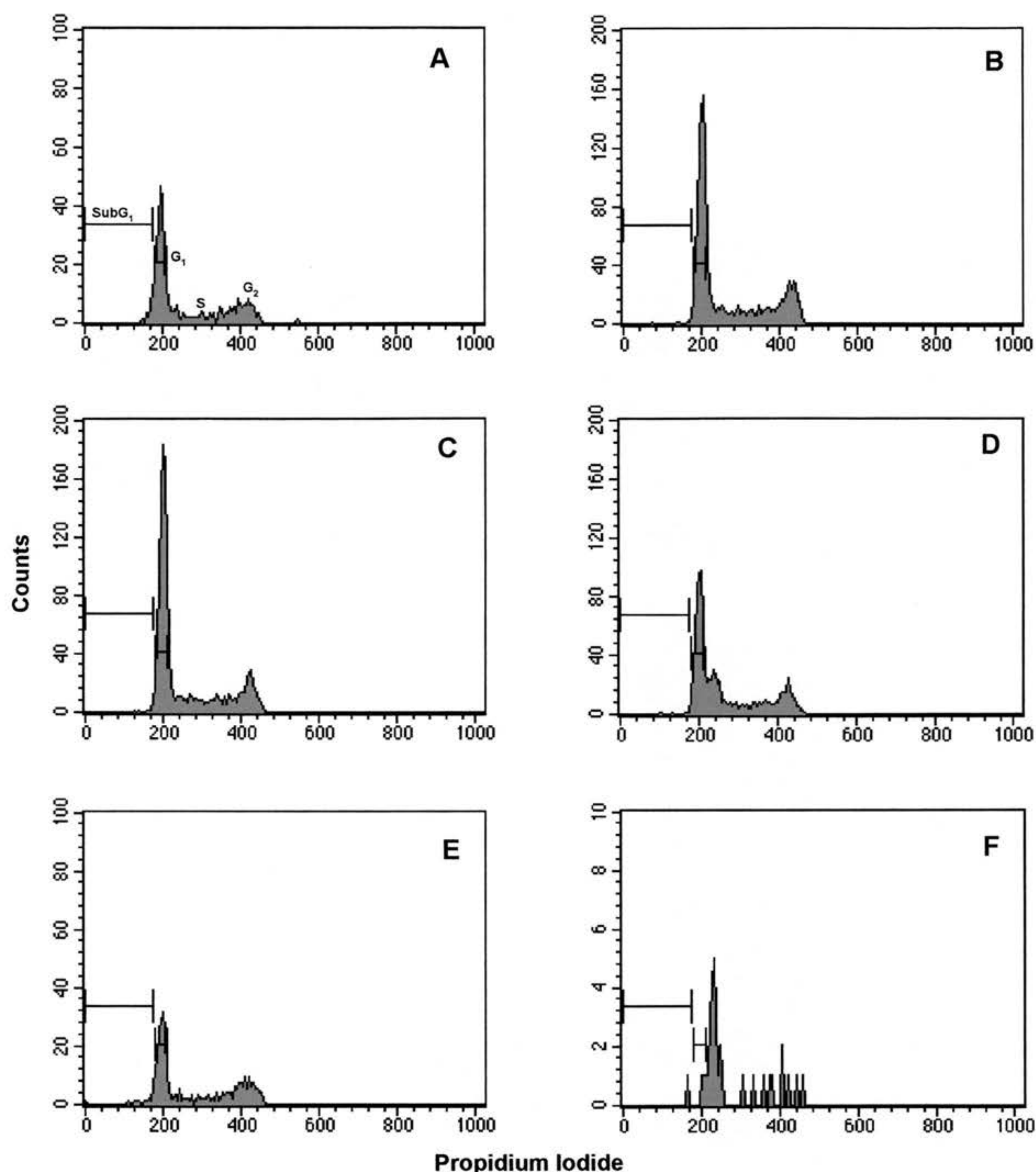


Figure 3.4. Effect of CSC on the cell cycle and determination of apoptosis by $SubG_1$. Cells were incubated overnight in serum-free media prior to treatment for 4, 8 and 24 hours with either media alone **(A)**, 1% CSC **(B)**, 5% CSC **(C)**, 10% CSC **(D)**, 2μM SS **(E)** or 10mM H_2O_2 **(F)**. Cells were fixed, permeabilised and fragmented DNA was eluted. Remaining DNA was stained with propidium iodide and cells were analysed by flow cytometry. Fluoresence intensity vs. number of cells was plotted to determine the stages of the cell cycle and to identify the sub G_1 peak. Representative plots of 8 hour exposure are shown.

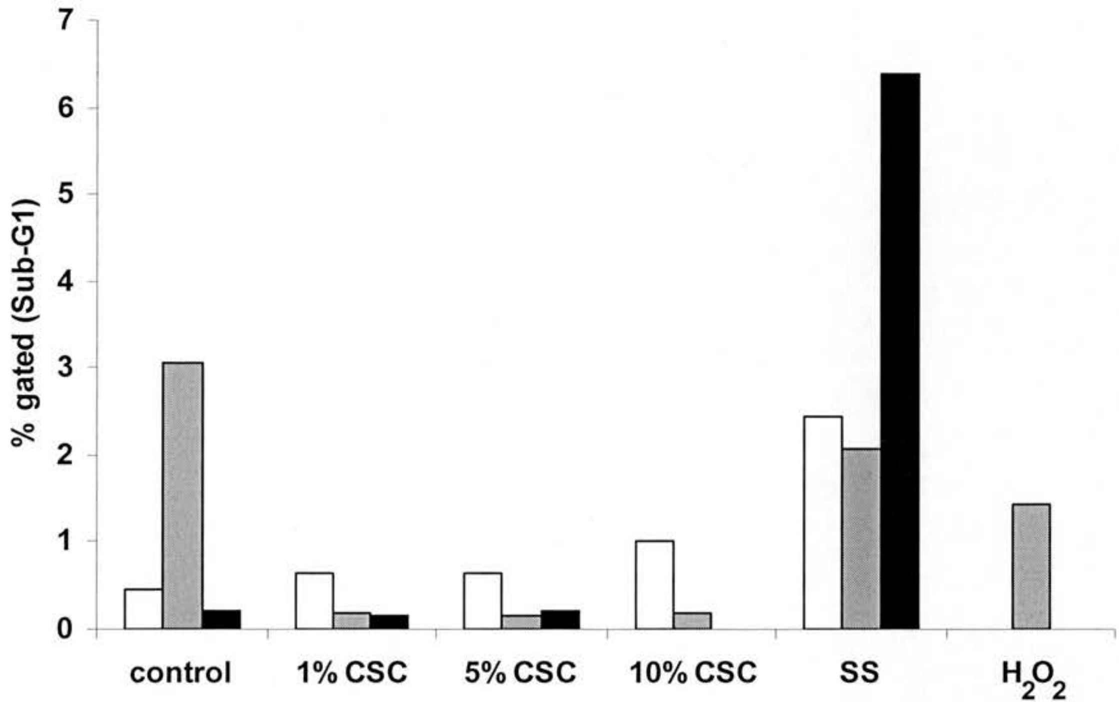


Figure 3.5. Effect of CSC on apoptosis in A549 cells, detected by SubG$_1$. Cells were incubated overnight in serum free media prior to treatment for 4 (*open bars*), 8 (*grey bars*) and 24 hours (*closed bars*). Cells were permeabilised and fragmented DNA was eluted prior to staining with PI and analysis by flow cytometry. Apoptosis was detected as a peak to the left of the G_0/G_1 peak. Results are mean of one experiment performed in triplicate.

Synchronisation with mimosine resulted in cell cycle profiles that contained more cells in G_2/M and less in G_1/G_0 (Figure 3.6.A) than cells incubated in serum-free media alone (Figure 3.4.A), and an almost identical profile was obtained from cells treated with 1% CSC after synchronisation with mimosine (Figure 3.6.B). Mimosine arrests cells at the G_1/S boundary of the cell cycle and the presence of most of the cells in G_2/M 8 hours after mimosine was removed indicates that there is an 8 hour transit time through S-phase. The profile obtained from cells treated with 5% CSC showed more cells in the DNA synthesis stage of the cell cycle than in G_2/M (Figure 3.6.C), indicating that these cells are either progressing slower through S-phase or, more likely, were held up for longer at the G_1/S boundary while the cells affected DNA repair before engaging cell cycle progression. Almost all cells were retained in G_1/G_0 after treatment with 10% CSC (Figure 3.6.D). Suggesting that exposure to 10% CSC had blocked DNA synthesis and cell proliferation (Figure 3.6D).

A small percentage of cells were detected in the sub-G_1 range with all treatments after synchronisation with mimosine. However, only treatment with 1% CSC for 24 hours and SS for 8 hours induced an increase in sub-G_1 cells compared to the control, albeit very small (Figure 3.7). A concern was that DNA fragmentation was not occurring in response to any treatment, therefore the DNA washed from the cells after fixation was analysed by electrophoresis. No DNA was detected on the gels (data not shown), indicating that fragmentation was not occurring after any treatment. Therefore, additional methods were used to determine whether oligonucleosomal fragmentation was occurring.

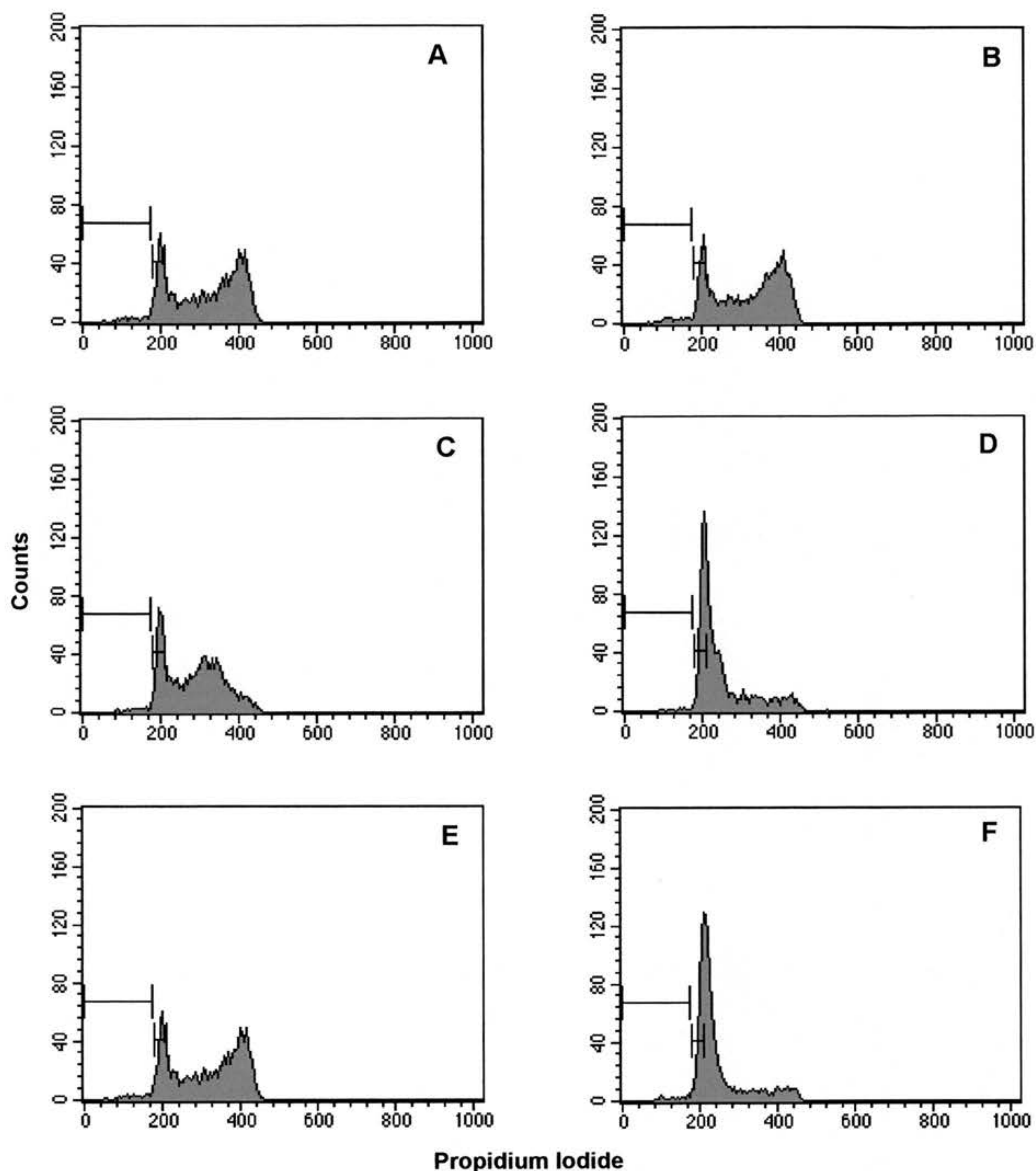


Figure 3.6. Effect of synchronisation on the cell cycle and determination of apoptosis by SubG$_1$. Cells were synchronised overnight with mimosine in serum-free media prior to treatment for 4, 8 and 24 hours with either media alone **(A)**, 1% CSC **(B)**, 5% CSC **(C)**, 10% CSC **(D)**, 2μM SS **(E)** or 10mM H_2O_2 **(F)**. Cells were fixed, permeabilised and fragmented DNA was eluted. Remaining DNA was stained with propidium iodide and cells were analysed by flow cytometry. Fluoresence intensity vs. number of cells was plotted to determine the stages of the cell cycle and to identify the sub G_1 peak. Representative plots of 8 hour exposure are shown.

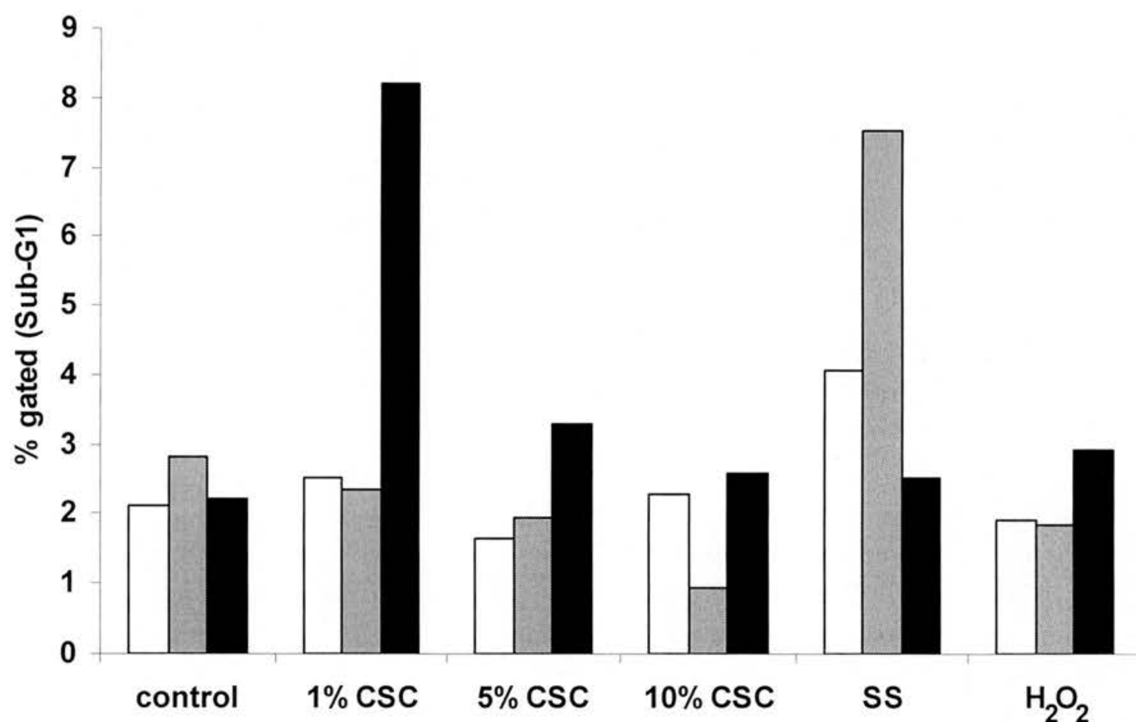


Figure 3.7. Effect of CSC on apoptosis in A549 cells after synchronisation with mimosine, detected by $SubG_1$. Cells were incubated overnight in mimosine prior to treatment for 4 (*open bars*), 8 (*grey bars*) and 24 hours (*closed bars*). Cells were permeabilised and fragmented DNA was eluted prior to staining with PI and analysis by FACS. Apoptosis was detected as a peak to the left of the G_0/G_1 peak. Results are mean of one experiment performed in triplicate.

3.3.2. Development of method to monitor oligonucleosomal DNA fragmentation in A549 cells

Total DNA was extracted from CSC-treated cells and analysed for DNA laddering. Initially, genomic DNA was extracted after CSC treatment using a genomic DNA extraction kit (Promega). However, no ladders were detected after any treatment (Figure 3.8). This could have been due to fragments produced being small, since the protocol was originally developed for extraction of complex genomic DNA. Therefore, a protocol developed specifically for the identification of oligonucleosomal fragments, was used ("Current Protocols in Immunology"). Again no DNA ladders were observed with any treatment (Figure 3.9). Finally, a protocol that uses mini-prep spin columns to retain the fragmented DNA was tried. No DNA ladders were detected (Figure 3.10). This protocol had originally been developed for use with apoptotic neutrophils; therefore neutrophils were aged for 20 hours in Teflon to induce apoptosis prior to DNA extraction. Electrophoresis of extracted DNA showed that DNA ladders could be detected by this method when aged neutrophils were used (Figure 3.10. PMN).

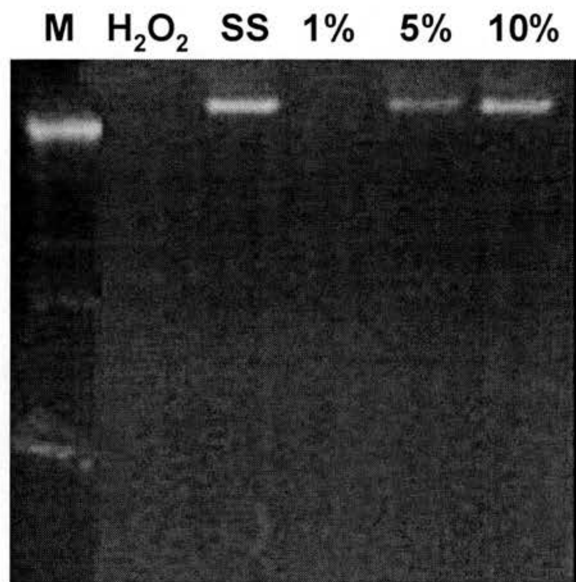


Figure 3.8. Isolation of oligonucleosomal fragments using the Promega Wizard genomic DNA isolation kit. A549 cells were treated with either media alone, 10mM H_2O_2, 2μM SS, 1% CSC, 5% CSC or 10% CSC for 24 hours prior to lysis and extraction of DNA.

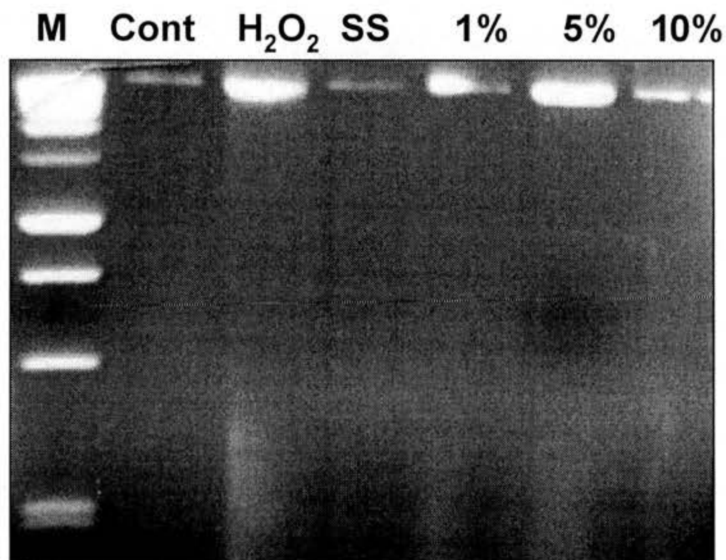


Figure 3.9. Isolation of oligonucleosomal fragments using the "Current Protocols in Immunology" method. A549 cells were treated with either media alone, 10mM H_2O_2, 2μM SS, 1% CSC, 5% CSC or 10% CSC for 24 hours prior to lysis and extraction of DNA.

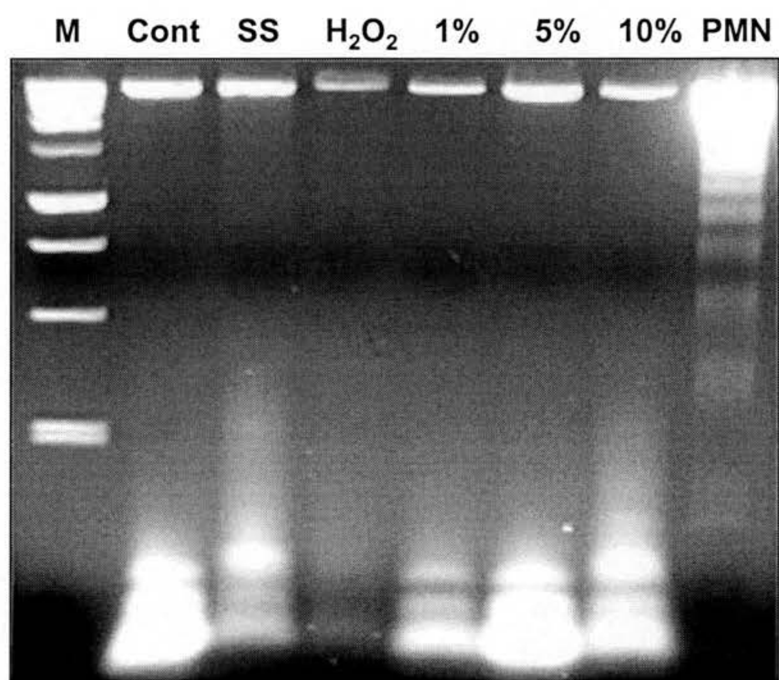


Figure 3.10. Isolation of oligonucleosomal fragments using the miniprep protocol. A549 cells were treated with media alone, 2µM SS, 10mM H_2O_2,1%, 5% or 10% CSC for 24 hours prior to lysis and isolation of DNA. Neutrophils aged overnight in teflon (PMN) were used as a positive control for DNA laddering.

3.3.3. CSC does not induce morphological changes in A549 cells as detected by transmission electron microscopy (TEM)

A549 cells treated with 1, 5 and 10% CSC for 24 hours were analysed by transmission electron microscopy (TEM) to look for apoptotic changes. No changes indicative of apoptosis were evident in cells treated with 1 and 5% CSC (Figure 3.11.B,C), such as chromatin margination or condensation. Moreover, these cells retained morphological characteristics similar to untreated cells (Figure 3.11.A), except that cells treated with 5% CSC appear to possess swollen endoplasmic reticulum (Figure 3.11C), an early marker of necrosis (see discussion). Cells treated with 10% CSC contained cytoplasmic vacuoles (Figure 3.11.D), which occurs during necrosis (section 1.4.4.), and it was difficult to identify individual organelles. As described in section 1.4.4 cytoplasmic vacuoles are also characteristic of a necrotic-like or autophagic cell death. Moreover, during autophagy, autophagolysosomes engulf and degrade intracellular organelles. This may be what is occurring in cells treated with 10% CSC (Figure 3.11). Thus these data further indicate that necrotic-like cell death not apoptosis occurs in response to cigarette smoke exposure.

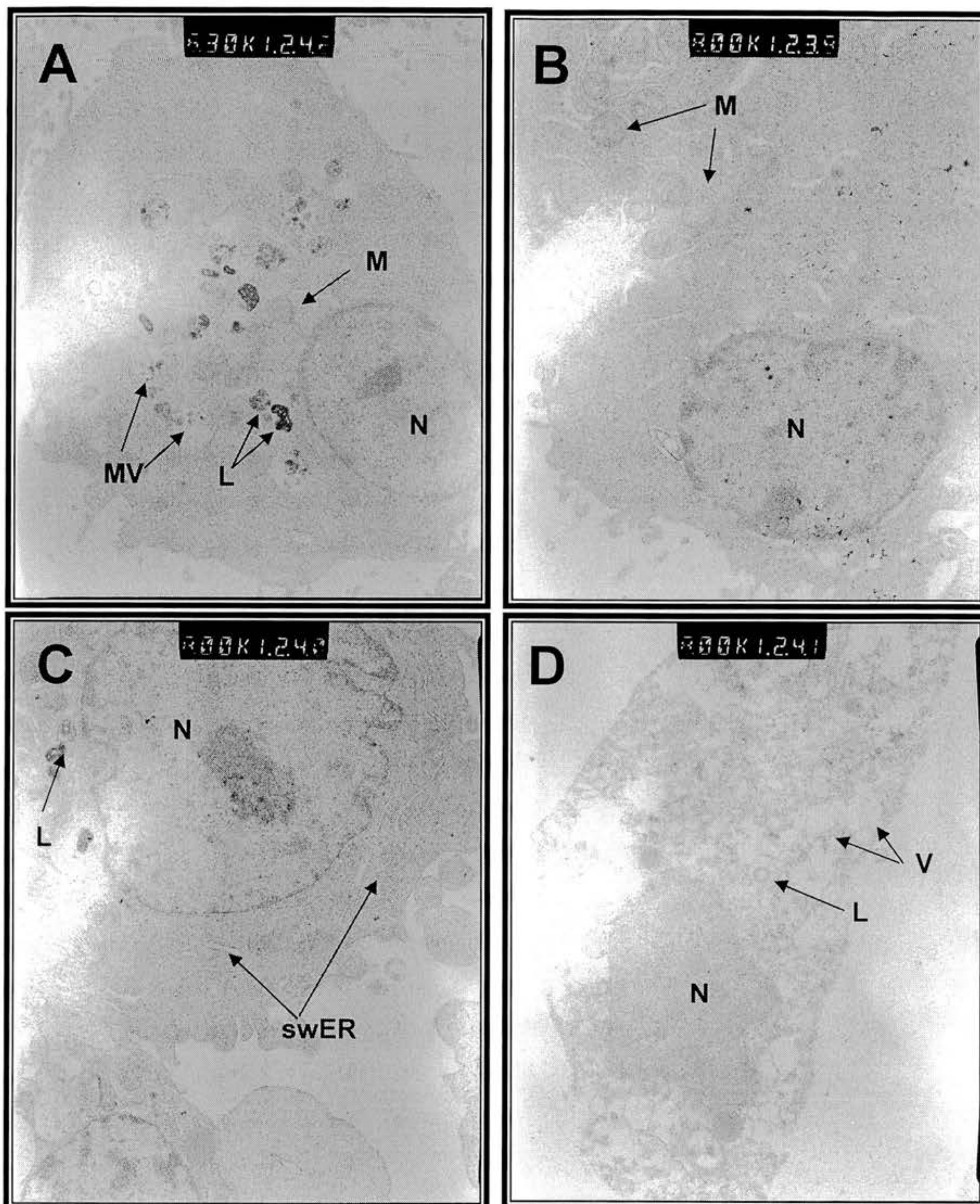


Figure 3.11. Cigarette smoke condensate induces morphological changes in A549 cells detected by transmission electron microscopy (TEM). A549 cells were treated for 24 hours with either media alone **(A)**, 1% CSC **(B)**, 5% CSC **(C)** or 10% CSC **(D)**. Cells were trypsinised and pelleted prior to being fixed in gluteraldehyde and processed for TEM. Nuclei (N), mitochondria (M), Lamellar bodies (L), multivesicular bodies (MV), swollen ER (swER) and vacuoles (V) are marked. Magnification, A: x6300, B-D: x8000

3.4. PHOSPHITIDYLSERINE EXPOSURE IN RESPONSE TO CIGARETTE SMOKE CONDENSATE IS THE RESULT OF AUTOFLUORESCENCE

Previous studies have shown that cigarette smoke treatment causes cells to autofluoresce (Skold *et al.,* 1989, 1992, 1993; Streck *et al.,* 1994). As we could not detect any markers of apoptosis in A549 cells in response to CSC, with the exception of annexin-V binding the possibility that these results could be due to autofluorescence was investigated. A549 cells were incubated with media alone or 1-10% CSC for 4, 8 or 24 hours and the cells were stained with annexin-V alone as described in the materials and methods. The cells were analysed using flow cytometry and FL-1 versus counts was plotted. A dose-dependent increase in FL-1 was detected in the absence of annexin-V at all time points (Figure 3.12.A, C, E and Figure 3.13.B), which mimicked the fluorescence profiles of cells stained with annexin-V (Figure 3.12.B, D, F and Figure 3.13.A). Moreover, no significant difference in FL-1 was detected between each treatment, whether stained or unstained. Attempts to quench the autofluorescence with crystal violet were unsuccessful as the cells were permeabilised during the procedure, resulting in internalisation of both dyes, the consequence of which was increased fluorescence in both channels after all treatments (data not shown).

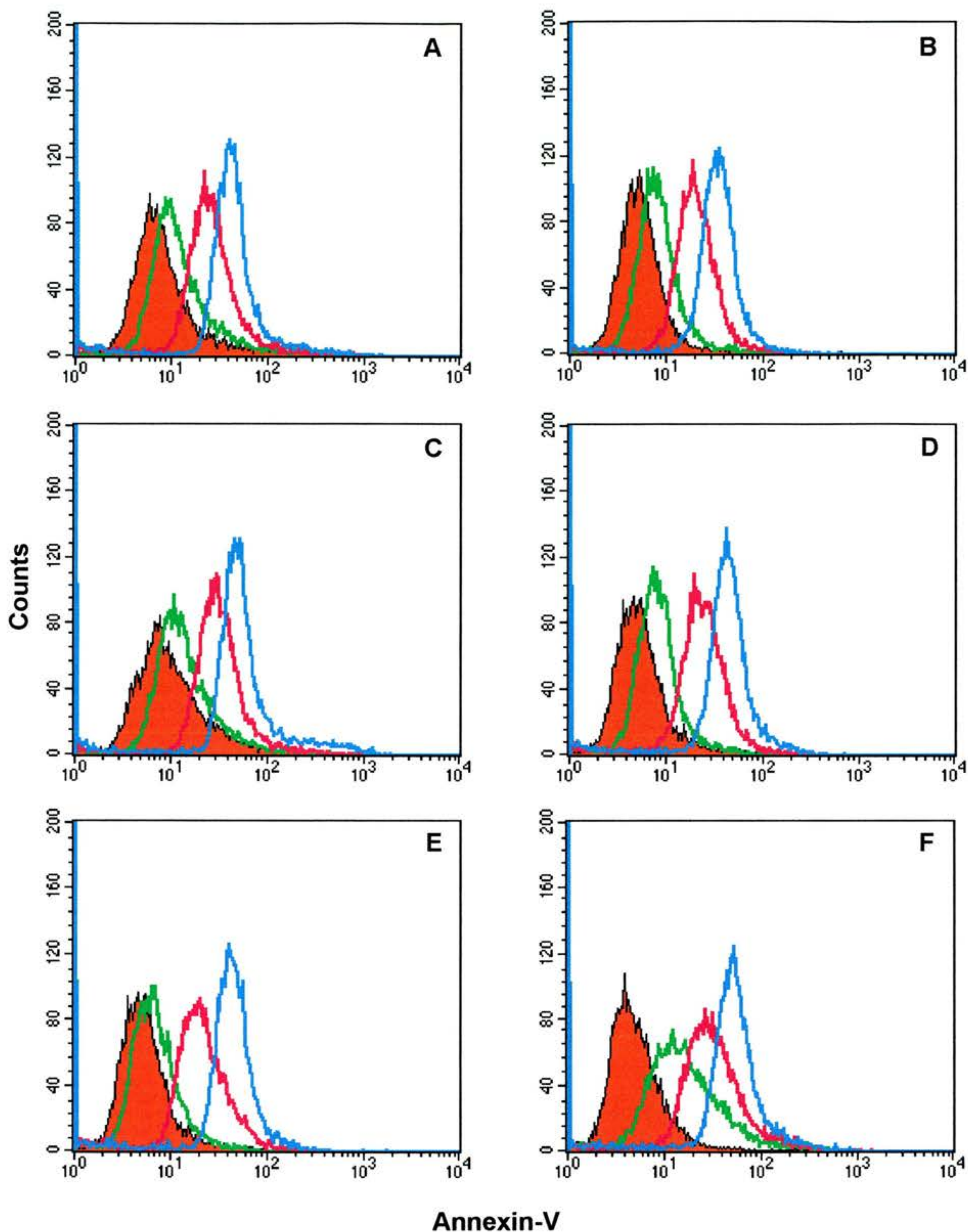


Figure 3.12. Cigarette smoke condensate induces autofluoresence in A549 cells. A549 cells were incubated with either media alone (*red*), 1% CSC (*green*), 5% CSC (*pink*) or 10% CSC (*blue*) for 4 **(A and B)**, 8 **(C and D)**, or 24 hours **(E and F)**. Cells were either left with no stain **(A,C,E)** or stained with annexin-V **(B,D,F)** prior to analysis by flow cytometry.

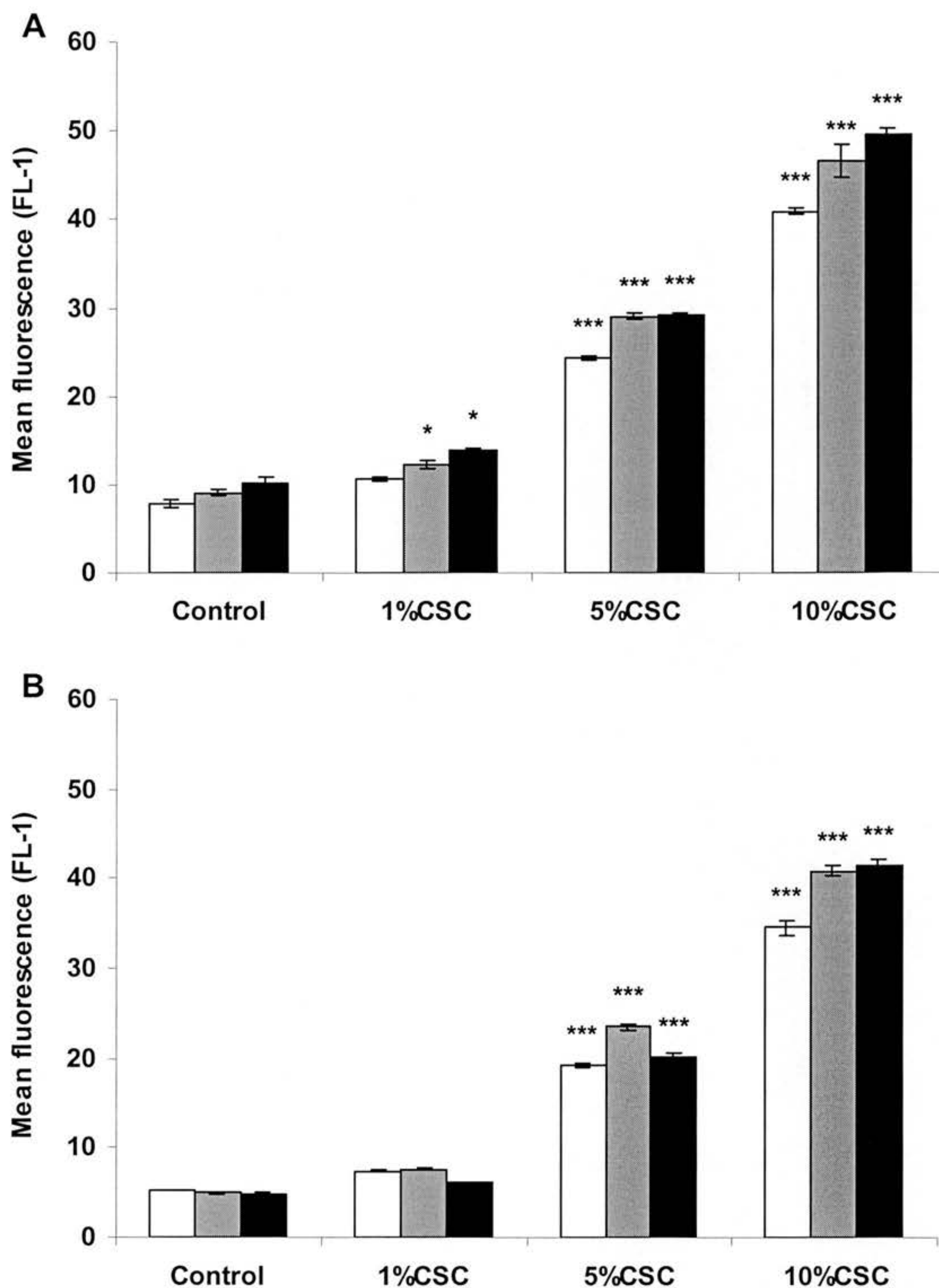


Figure 3.13. Cigarette smoke condensate treatment of A549 cells results in autofluoresence detected by FL-1. Cells were treated with either media alone, 1, 5 or 10% CSC for 4 hours (*open bars*), 8 hours (*grey bars*) and 24 hours (*closed bars*) after which cells were either stained with annexin-V **(A)** or left unstained **(B)**, and analysed by flow cytometry. Results expressed as mean of one experiment performed in triplicate ± SEM, * $p<0.05$, *** $p<0.001$. No significant difference was seen between stained and unstained cells for each treatment at each time point.

3.5. CIGARETTE SMOKE CONDENSATE INDUCES NECROSIS IN EPITHELIAL CELLS

3.5.1. Cigarette smoke condensate induces necrosis in A549 cells as detected by acridine orange / ethidium bromide staining

A549 cells treated with 1, 5 or 10% CSC for 6 and 24 hours were studied for evidence of apoptosis and necrosis using the acridine orange and ethidium bromide staining method. Cells treated with 1% and 5% CSC (Figure 3.14 and Figure 3.15.B and C) remained viable, yet a significant increase in necrotic cell death was seen when cells were treated with 10% CSC for 24 hours (Figure 3.14 and Figure 3.15.D). This technique relies on identification of nuclear morphology to distinguish between viable, apoptotic, necrotic and secondary necrotic cells. The necrosis seen as a result of CSC exposure was not secondary necrosis, as nuclear pyknosis was not seen prior to loss of cell membrane integrity (Figure 3.15.D). Nuclear swelling is a well-documented phenomenon of necrosis (Wyllie *et al.,* 1980), which was not evident in cells that displayed membrane permeability (Figure 3.15D). However, this mimicked the morphology seen after incubation with the hydrogen peroxide, the necrotic control (Figure 3.15F). Treatment of A549 cells with 2μM SS demonstrated that apoptotic nuclear pyknosis could be induced in this cell type (Figure 3.15E).

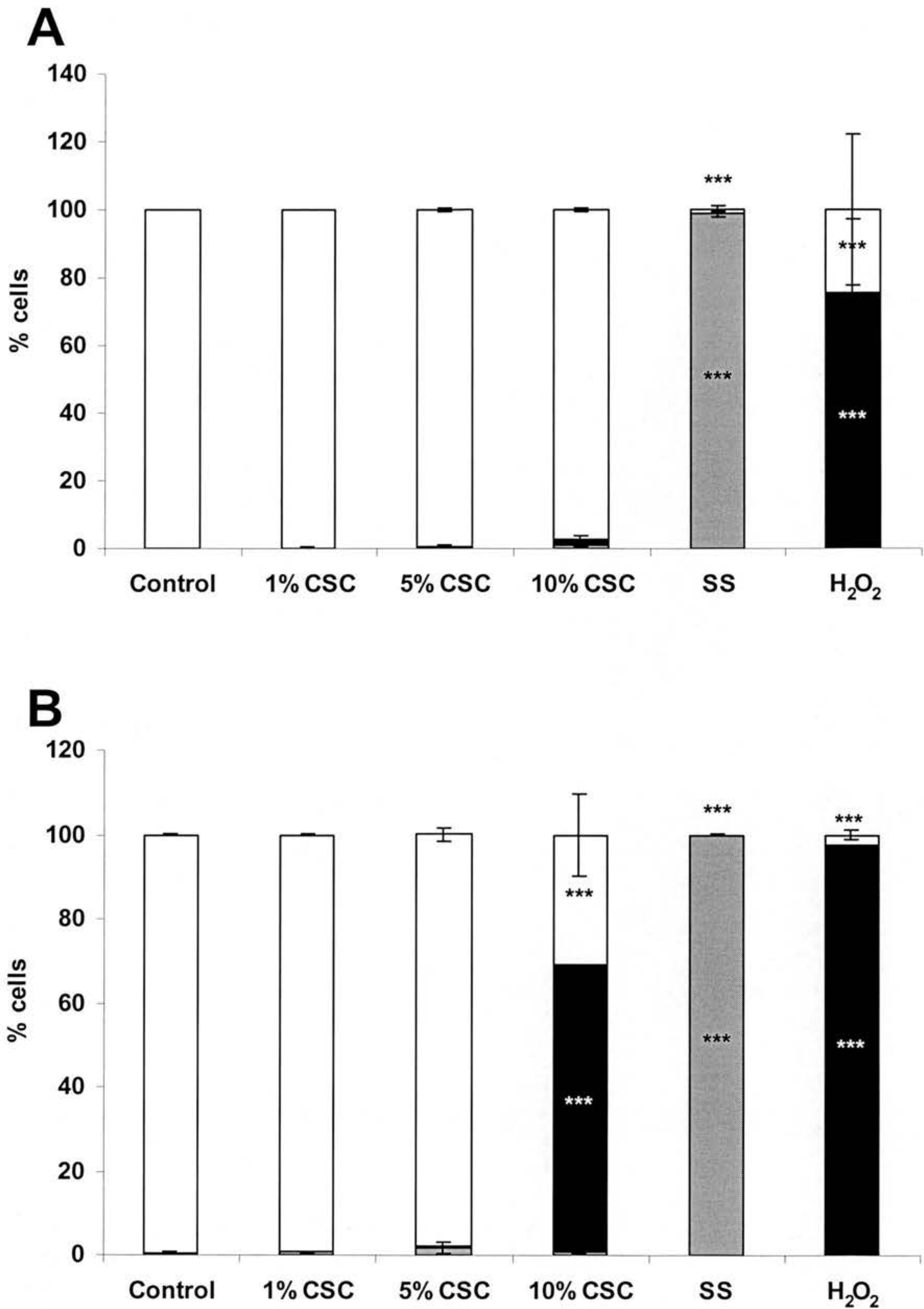


Figure 3.14. Cigarette smoke condensate induces necrosis in A549 alveolar epithelial cells. Cells were exposed to 1%, 5% or 10% CSC, 2μM SS, or 10mM H_2O_2 for 6 **(A)** or 24 **(B)** hours in serum-free media. Cells were stained with acridine orange and ethidium bromide and the percentage of viable (*open bars*), apoptotic (*grey bars*) and necrotic (*closed bars*) cells were counted. Results are mean of three experiments ± SEM. *** $p<0.001$

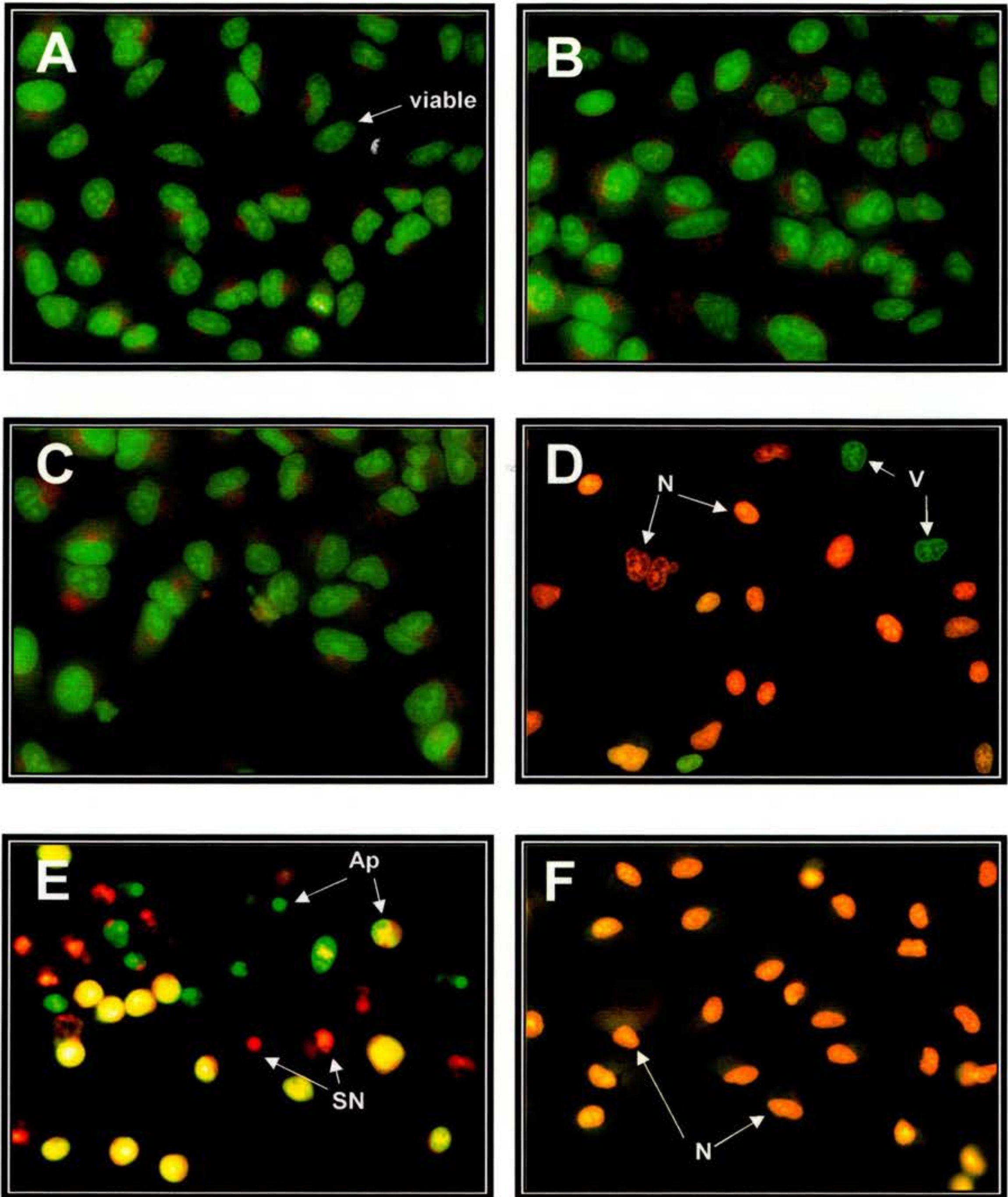


Figure 3.15. Cigarette smoke condensate induces necrosis not apoptosis in A549 cells. Cells were treated for 24 hours with either media alone **(A)**, 1% CSC **(B)**, 5% CSC **(C)**, 10% CSC **(D)**, 2μM SS **(E)** or 10mM H_2O_2 **(F)** and stained with acridine orange/ethidium bromide. Viable cells take up acridine orange and appear green and cells that have lost their cell membrane integrity take up ethidium bromide and appear orange. Note the nuclear pyknosis after SS treatment not present in cells treated with CSC or H_2O_2. Examples of viable cells (V), apoptotic cells (Ap), necrotic cells (N) and cells undergoing secondary necrosis (SN) are marked. Magnification x34

3.5.2. Development of LDH release assay

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme present in all cells, which upon loss of membrane integrity during necrosis, escapes from the cell and can be detected in the cell medium. This method was used to confirm the presence of necrosis after CSC exposure. The manufacturers instructions required that the optimum plating concentration be determined for this assay. Therefore a suspension of A549 cells at 2 x 10^6 cells per ml was used to prepare six replicates of doubling dilutions in a 96 well plate ranging from 2 x 10^5 to 100 cells per well. The cells were allowed to adhere overnight at 37°C before either fresh serum-free media or media containing 1% Triton X-100 was added in triplicate for each plating density. The cells were incubated at 37°C for 24 hours prior to harvesting as described in materials and methods. Subtracting the low control from the high control and determining the greatest difference determined the optimum plating density, which was greatest at 3000 cells per well (Figure 3.16). Use of over 3000 cells per well was impossible, as total lysis of the cells released LDH at levels that were above the range of the assay. In order that the plating density was not altered between experiments (usually 9000 cells per well in a 96-well plate) the harvested supernatants of future experiments were diluted threefold in serum-free media prior to the addition of LDH reagent. As it had been noted that CSC interfered with other assays, CSC was added to LDH reagent in the absence of cells in the optimisation experiment, no change in absorbance was evident in either the high or the low controls (data not shown)

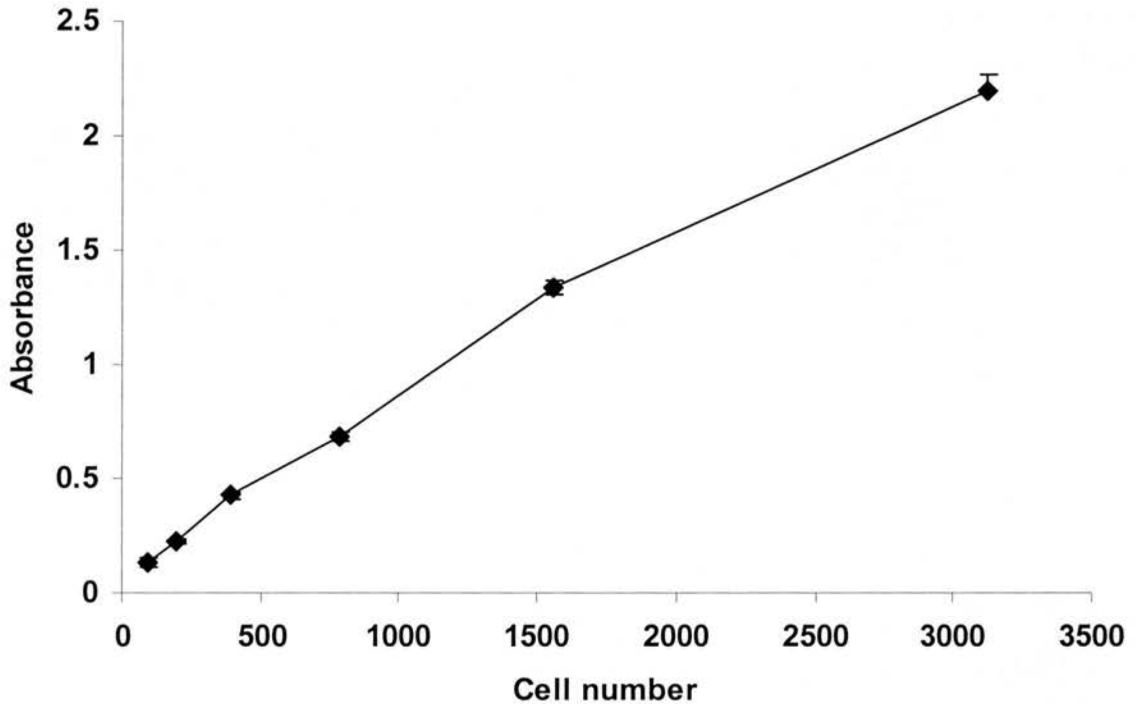


Figure 3.16. Effect of plating density on the LDH assay. Cells were plated at different densities and cultured O/N at 37°C. Fresh serum-free media (low control) or 1% Triton X-100 (high control) was added and incubated for 24 hours at 37°C. LDH reagent was added to the supernatants and incubated for 30 minutes at room temperature in the dark prior to measuring the absorbance at 500nm. The difference between the high and low control at each plating density was determined. Results expressed as the mean of one experiment performed in triplicate ± SEM.

3.5.2. A549 cells release LDH in response to CSC

The release of LDH from A549 cells was monitored after CSC treatment for 2, 4, 8, 24, 28 and 48 hours. Release of LDH from A549 cells treated with doses of 5% CSC and above was evident as early as 8 hours after exposure (Figure 3.17), which confirmed the presence of necrosis. LDH release was apparent with all doses of 5% CSC and above, and this occurred in a time-dependent but not dose-dependent manner.

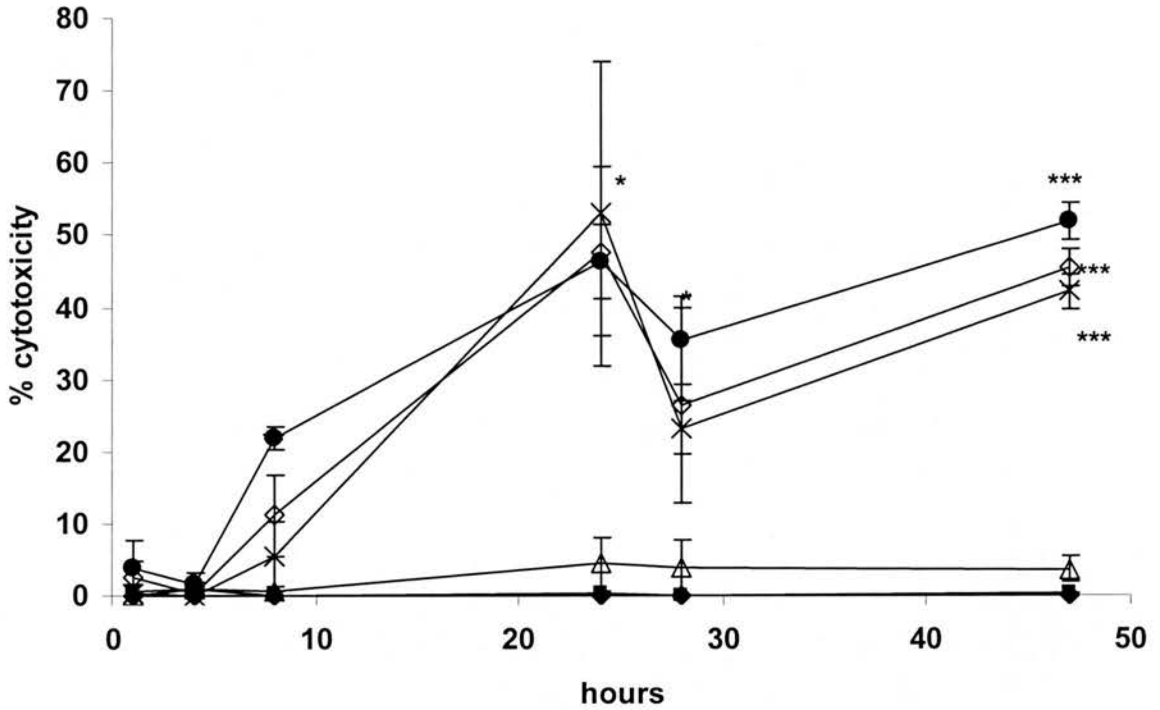


Figure 3.17. Cigarette smoke condensate induces necrosis in A549 alveolar epithelial cells, measured by LDH release. Cells were exposed to either media alone (*closed diamonds*) 1% (*closed squares*), 2.5% (*open triangles*), 5% (*crosses*), 7.5% (*open diamonds*) or 10% (*closed circles*) CSC for up to 48 hours in serum-free media. Supernatants were collected and amount of LDH released from the cells was determined and expressed as percentage of total lysis with 1% Triton X-100. Results are expressed as mean of three experiments performed in triplicate ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

3.6. CIGARETTE SMOKE CONDENSATE INDUCES NECROSIS IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC'S)

Apoptosis of both epithelial cells and endothelial cells has been implicated in the pathogenesis of emphysema (Kasahara *et al.,* 2000, 2001). Primary human umbilical vein endothelial cells (HUVEC's) were used to study the effect of CSC on endothelial cells. HUVECs underwent similar morphological changes to A549 cells (section 3.2.1) after exposure to 10% CSC (Figure 3.18.D). These morphological changes also occurred at the lower dose of 5% CSC (Figure 3.18.C), indicating that HUVECs are more susceptible to the deleterious effects of CSC. Moreover, acridine orange and ethidium bromide staining showed that, like A549 cells, HUVEC's underwent necrosis and not apoptosis in response to CSC exposure. Again, necrosis was detected at lower doses than in A549 cells, they underwent necrosis after 24 hour treatment with both 5% and 10% CSC (Figure 3.19), detected as uptake of ethidium bromide with the absence of any nuclear changes (Figure 3.20.C and D). Like treatment of A549 cells no nuclear swelling was observed, in fact the nuclei of permeable cells appeared smaller (Figure 4.20C, D) but still resembled the morphology of cells treated with H_2O_2, (Figure 3.20F; necrotic control). 2μM SS was used as an apoptotic control to demonstrate that classical morphological changes indicative of apoptosis could be obtained in this cell type (Figure 3.20E). To confirm CSC-induced necrosis in HUVECs, release of LDH was monitored. In accordance with preceding data, LDH was detected in the media from cells treated with doses greater than 5% CSC for 24 hours (Figure 3.21).

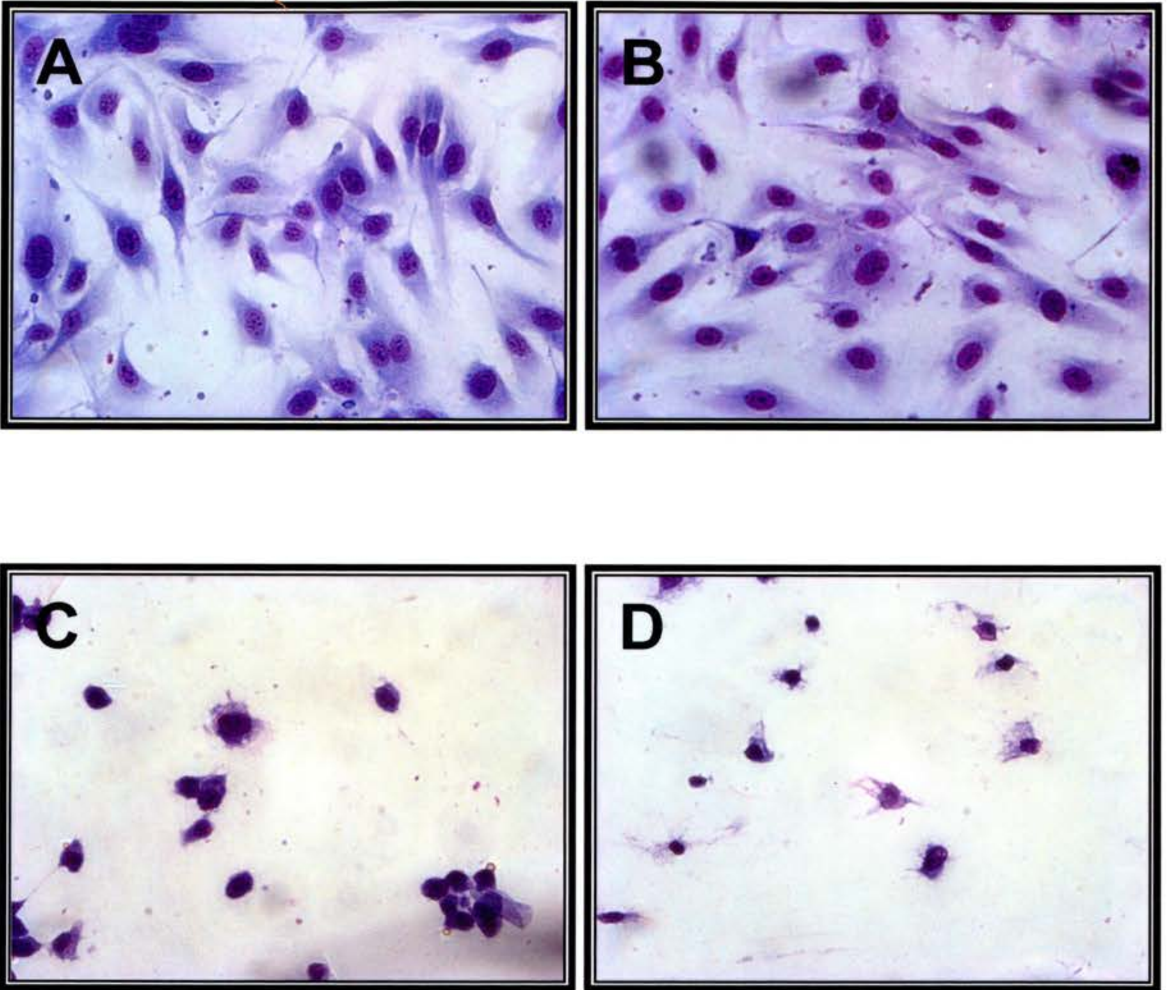


Figure 3.18. Cigarette smoke condensate induces necrosis not apoptosis in human umbilical vein endothelial cells (HUVEC's). Cells were grown on sterile glass coverslips, treated for 24 hours with either media alone **(A)**, 1% CSC **(B)**, 5% CSC **(C)**, or 10% CSC **(D)** and stained with DiffQuick. Magnification x34

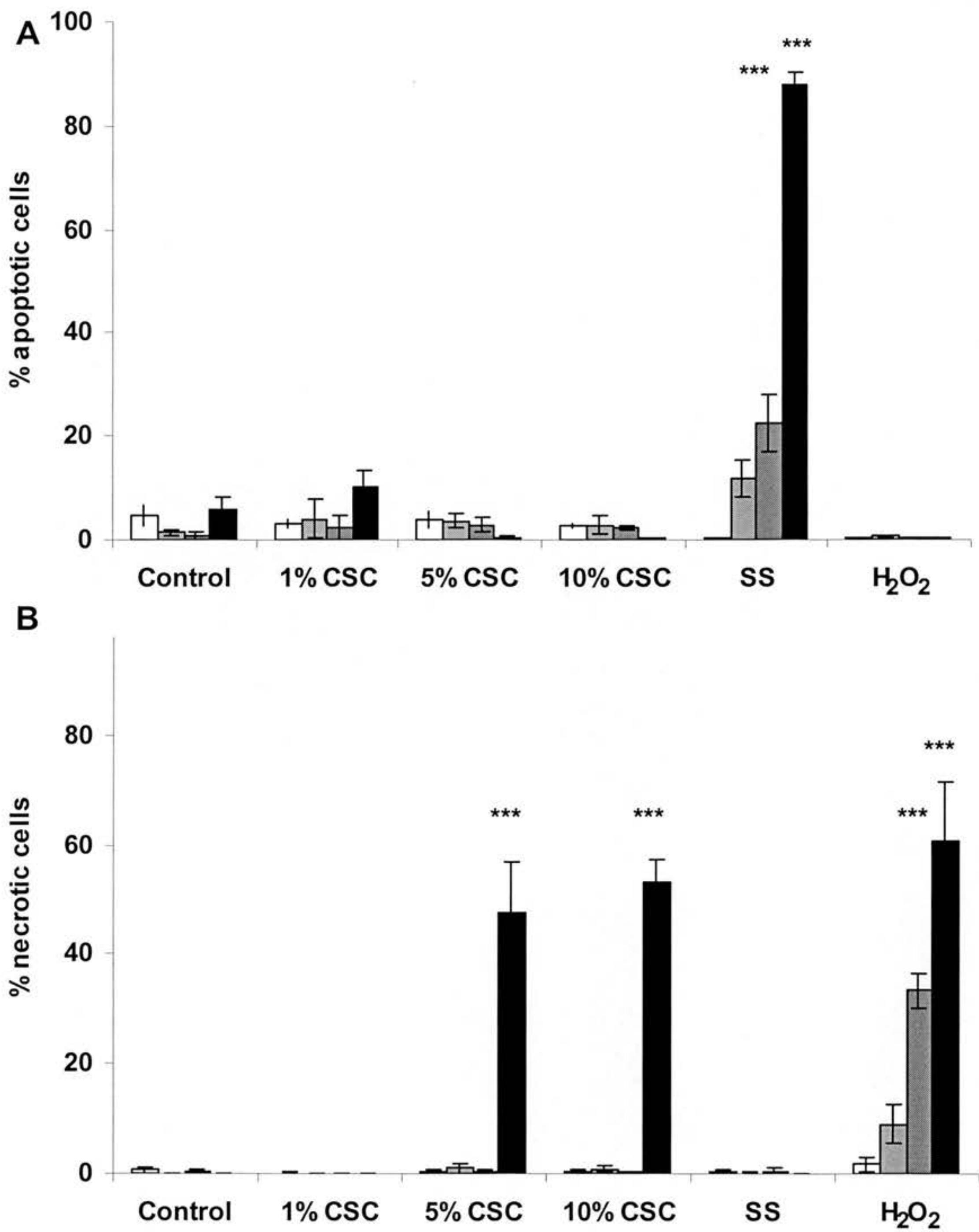


Figure 3.19. Cigarette smoke induces necrosis in human umbilical vein endothelial cells (HUVEC's). HUVEC's were treated in full media containing 1, 5 and 10% CSC, 2μM SS or 5mM H_2O_2 for 2 hours (*open bars*), 4 hours (*grey bars*), 8 hours (*hatched bars*) and 24 hours (*closed bars*). Cells were stained with acridine orange and ethidium bromide and the percentage of viable, apoptotic **(A)** and necrotic cells **(B)** were counted. Results are mean of three experiments performed in duplicate ± SEM. *** $p < 0.001$

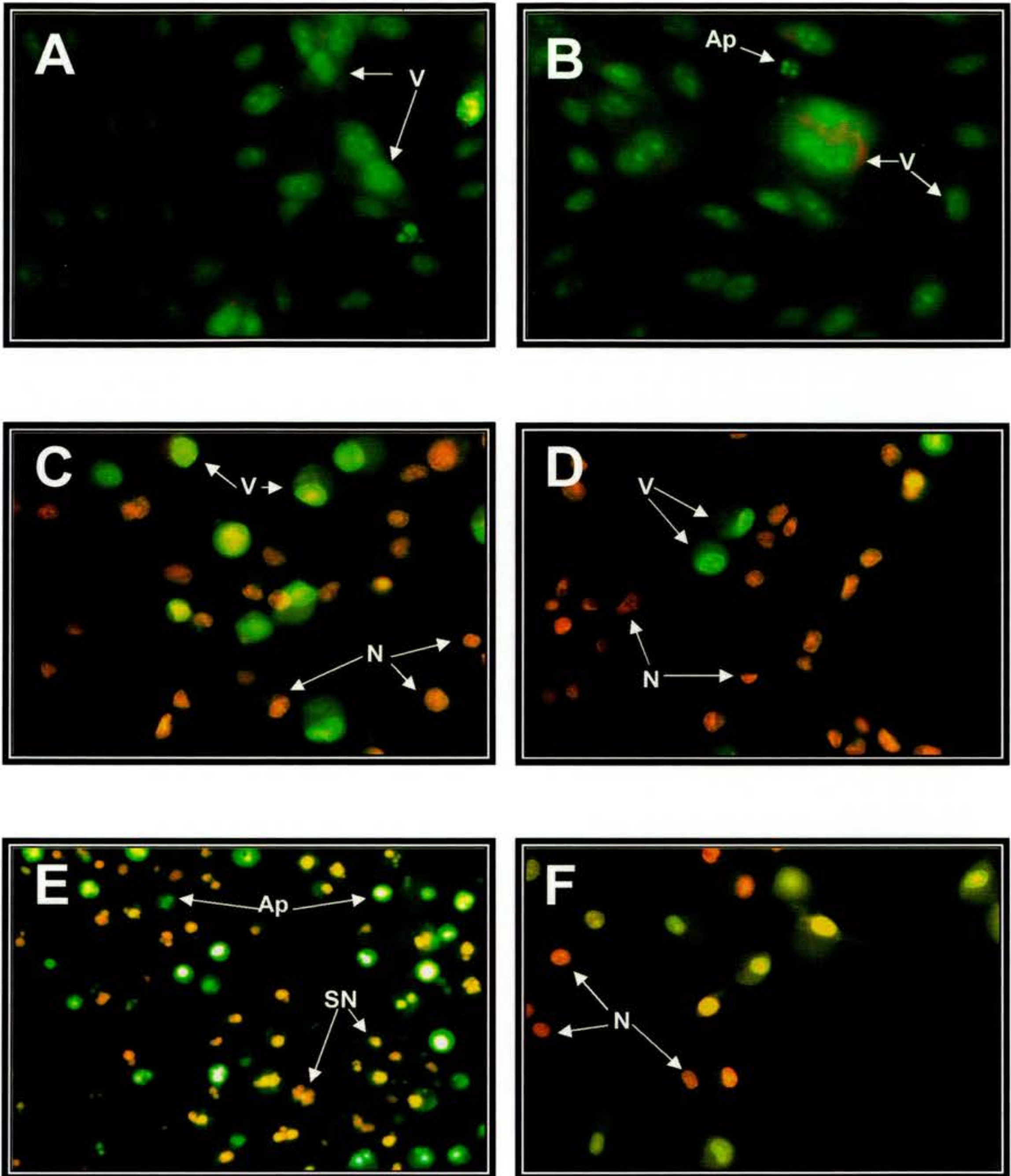


Figure 3.20. Cigarette smoke condensate induces necrosis not apoptosis in human umbilical vein endothelial cells (HUVEC's). Cells were treated for 24 hours with either media alone **(A)**, 1% CSC **(B)**, 5% CSC **(C)**, 10% CSC **(D)**, 2μM SS **(E)** or 10mM H_2O_2 **(F)** and stained with acridine orange/ethidium bromide. Viable cells take up acridine orange and appear green and cells that have lost their cell membrane integrity take up ethidium bromide and appear orange. Note the nuclear pyknosis after SS treatment not present in cells treated with CSC or H_2O_2. Examples of viable cells (V), apoptotic cells (Ap), necrotic cells (N) and cells undergoing secondary necrosis (SN) are marked. Magnification x34

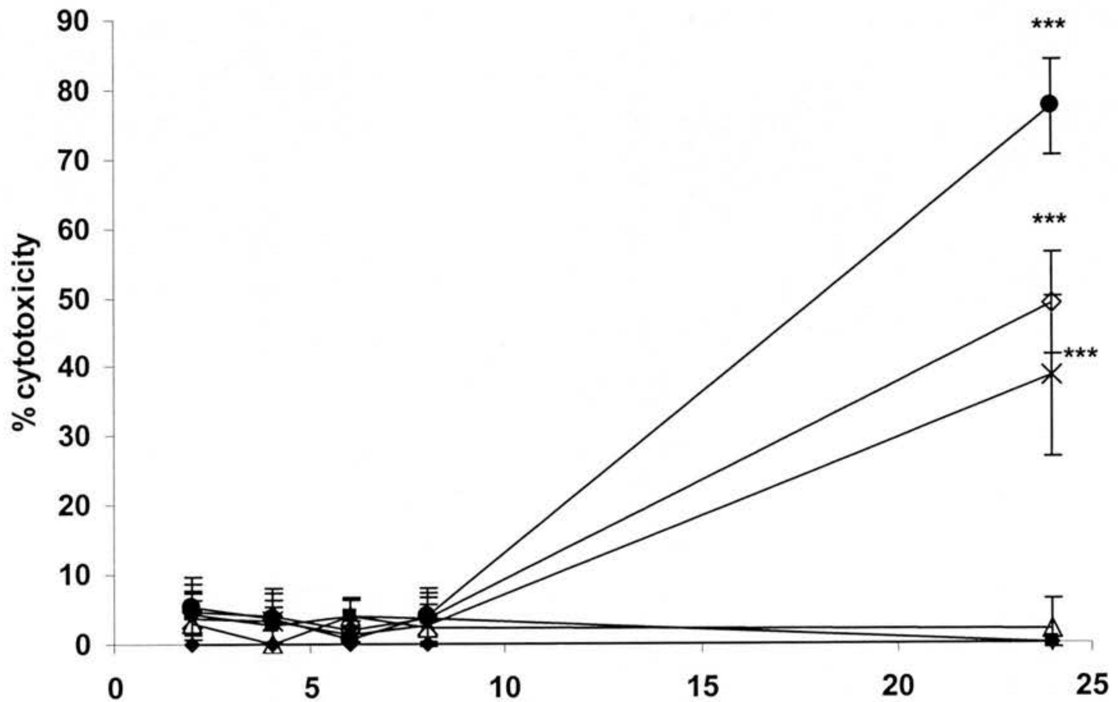


Figure 3.21. Cigarette smoke condensate induces necrosis in HUVEC's, measured by LDH release. HUVEC's were treated in full media alone (*closed diamonds)* or containing 1% (*closed squares*), 2.5% (*open triangles*), 5% (*crosses*), 7.5% (*open diamonds*) and 10% CSC (*closed circles*) for 2, 4, 6, 8 and 24 hours. Supernatants were harvested and the amount of LDH released from the cells was determined and expressed as percentage of total lysis with 1% Triton X-100. Results are mean of three experiments performed in triplicate ± SEM. *** $p < 0.001$

3.7. Discussion

Although the predominant hypothesis is that emphysema results from loss of alveolar epithelial and endothelial cells by apoptotic cell death, the data obtained in this chapter did not support this hypothesis. This chapter demonstrated that apoptotic changes did not occur in either epithelial or endothelial cells after CSC treatment, in fact CSC induced necrotic cell death in both cell types. Studies by Kasahara *et al.* (2000, 2001), provided the foundations for this thesis, however the authors concentrated on the role of VEGF and VEGF-R in the pathogenesis of emphysema, not on cigarette smoking itself. Study of the lungs of emphysema patients showed that increased numbers of terminal deoxytransferase-mediated dUTP nick end labelling (TUNEL) positive cells were evident as compared with healthy non-smokers. Moreover, increased levels of oligonucleosomal DNA fragmentation were evident when compared with normal subjects. No significant increase in TUNEL positive cells or DNA fragmentation was evident in the lungs of healthy smokers (Kasahara *et al.,* 2001). Thus, while these studies propose a role for apoptosis in the development of emphysema, they do not provide evidence that cigarette smoking induces apoptotic cell death. However, a number of previous studies have demonstrated that cigarette smoke induces apoptosis both *in vivo* and *in vitro* (Aoshiba *et al.,* 2001; Carnevali *et al.*, 2003; D'Agostini *et al.,* 2000, 2001; Hoshino *et al.,* 2001; Ishii *et al.,* 2001; Ma *et al.,* 1999; Rajpurkar *et al.,* 2002; Vayssier *et al.,* 1998; Wang *et al.,* 2000, 2001)

Direct comparisons between the majority of these studies with the data presented in this chapter is not appropriate as different cell types and/or smoke exposure methods were used both *in vitro* and *in vivo*. However, two of these studies observed cigarette smoke-

induced apoptosis in the cell lines used in this chapter, A549 cells (Hoshino *et al.*, 2001) and HUVECs (Wang *et al.*, 2001). Both studies treated cells with cigarette smoke condensate, however different preparation methodologies were used compared to this thesis. Although the use of condensate is a common method for cigarette smoke exposure *in vitro*, no standardised protocol exists for its production. Each procedure can isolate a slightly different spectrum of components, which may account for the inconsistencies in results between laboratories (discussed further in chapter 5).

CSC-induced apoptosis has been documented in A549 cells using DNA laddering and a variation of the acridine orange/ethidium bromide assay (Hoshino *et al.*, 2001), however use of these methods in this chapter provided no evidence of apoptosis in response to CSC. However, like many carcinoma cell lines, A549 cells are a non-homogeneous cell population that consist of multiple sub-populations (Croce *et al.*, 1999; Watanabe *et al.*, 2002). In such cultures, subpopulations can become prevalent with relative ease. For example, in both studies a population with a significantly lower doubling time was isolated, thus frequent sub-culturing of the heterogeneous population may result in slower growing subpopulations being eliminated from the culture. A549 subpopulations have demonstrated variability in response to toxicological agents (Watanabe *et al.*, 2002). Thus, laboratory-specific subpopulations, as a result of different culture methodologies, may explain the inconsistency in results when using A549 cells.

Although cell cycle analysis was primarily undertaken to investigate the occurrence of apoptotic cells by the presence of a Sub G_1 peak, these experiments revealed interesting

information about the effect of cigarette smoke on the cell cycle. Treatment with 10% CSC for 8 hours after removal of mimosine resulted in profiles where the cells remained in G_1/S. This indicated that cigarette smoke had induced an arrest at this stage of the cell cycle. A number of studies have demonstrated that oxidative stress induces cell cycle arrest at G_1/S (Chen and Ames, 1994; Chen *et al.*, 1998, 2000), with a phenotype resembling senescence (Chen and Ames, 1994; Chen *et al.*, 1998, 2000). Moreover, increased expression of p53, p21 and under-phosphorylated retinoblastoma (Rb) protein was observed (Chen *et al.*, 1998). DNA strand breaks occur in response to oxidative stress (Leanderson, 1993; Schraufstatter *et al.,* 1986), and such damage can induce p53. Expression of p53 results in induction of p21, which acts to inhibit cyclin-dependent kinases (CDKs). CDKs act to phosphorylate Rb resulting in the release of the transcription factor E2F, which then activates transcription of early S-phase genes, inadequate phosphorylation of Rb results in cell cycle arrest in G_1. Thus, oxidative stress-induced cell cycle arrest may be a direct consequence of DNA damage, activation of p53 and under-phosphorylation of Rb.

When investigating a hypothesis it is important to apply appropriate techniques and to understand the limitations of the assay of choice. Moreover, improper interpretation of data can produce misleading results. Thus, care must be taken to ensure the technique is applied appropriately and data are analysed properly. Many of the investigations that documented apoptosis in response to cigarette smoke identified apoptotic cells by TUNEL or, *in situ* end labelling (ISEL) (Aoshiba *et al.,* 2001; D'Agostini *et al.,* 2000, 2001; Ma *et al.,* 1999; Rajpurkar *et al.,* 2002; Wang *et al.,* 2001). During apoptosis caspase-activated DNase (CAD) cleaves DNA into 200bp fragments, or multiples

thereof (Enari *et al.,* 1998; section 1.4.2.4), producing classical "DNA ladders" upon electrophoresis. TUNEL and ISEL are widely used, simple and sensitive techniques that enable monitoring of this phenomenon quantitatively, at the single-cell level. These techniques use the enzyme terminal deoxytransferase (TdT), or DNA polymerase I in the case of ISEL, to incorporate labelled nucleotides into the 3' OH-terminal of DNA strand breaks (Gavrieli *et al.*, 1992). However, these methods are not specific for DNA strand breaks obtained through apoptosis, and may detect damage induced by other mechanisms. DNA fragmentation also occurs during necrosis, although a spectrum of fragments of low molecular mass are produced, often resulting in a smear when analysed by electrophoresis (Bicknell and Cohen, 1995). Moreover, DNA strand breaks that arise during necrosis reveal a 3'-OH terminus and consequently are detected by TUNEL (Hayashi *et al.*, 1998). DNA strand breaks occur during situations of oxidative stress (Leanderson, 1993; Schraufstatter *et al.,* 1986), which can also be detected by TUNEL. For example, exposure of lung fibroblasts to cigarette smoke results in TUNEL positive cells that display no additional markers of apoptosis (Kim *et al.,* 2003). This was the result of reversible oxidative damage not cell death, the cells continued to proliferate and were TUNEL negative following removal of smoke and application of fresh media. Thus, false positives can be obtained when oxidative stress is the stimulus or necrosis the outcome. Additionally, TUNEL is unreliable as the preservation methods used to prepare cells can have a dramatic effect on detection of strand breaks (Labat-Moleur *et al.,* 1998; Negoescu *et al.,* 1998). Thus, care must be taken when interpreting the results of TUNEL and additional identification of apoptosis is required. D'Agostini *et al.* (2000) observed increased TUNEL positive cells in the skin of mice after whole-body exposure to cigarette smoke, which was associated with

areas of alopecia, however no further analysis into the cause of DNA damage was undertaken.

Ma *et al.* (1999) also relied solely on TUNEL to detect apoptotic cells in the gastric mucosa after whole body exposure to smoke. It cannot be assumed that TUNEL positive cells in the gastric mucosa are apoptotic in this study without additional investigation. However, a more recent study from the same laboratory, confirmed apoptosis in the gastric mucosa after cigarette smoke exposure, detected by both TUNEL and western blotting for caspase-3 cleavage (Wang *et al.,* 2000). In this study the gastric mucosa is not directly exposed to cigarette smoke and thus different mechanisms may be involved in cell death than presented in this chapter.

CSC exposure has been documented to induce apoptosis in fibroblasts (Ishii *et al.*, 2001) detected by annexin-V-PE/7-amino-actinomycin D (7-AAD) staining. However, careful examination of the data shows that, in fact, necrosis was the predominant form of cell death not apoptosis. Moreover, apoptosis was overestimated in these studies. The percentage of apoptotic cells was determined as a percentage of 7-AAD negative cells, discounting necrotic cells, which in some cases was over half the population. Therefore, this study in reality supports the data presented in this chapter. However, as cell death was evaluated by flow cytometry alone in this study the occurrence of autofluorescence must be considered. In this chapter autofluorescence was observed when annexin-V binding was analysed by FL-1 (emission wavelength 507nm). This study employed annexin-V detected by FL-2 (emission wavelength 570nm) and 7-AAD detected by FL-3 (emission wavelength 660nm), autofluorescence may not interfere

with the signal at these wavelengths. However, it appears that no investigations were undertaken to determine whether autofluorescence occurred in this study, and therefore care must be taken when interpreting this paper.

Cellular autofluorescence has been documented in macrophages isolated from the lungs of smokers (Skold *et al.,* 1989; Streck *et al.,* 1994) and from rats exposed to smoke (Skold *et al.,* 1993). *In vitro* studies demonstrated that macrophages endocytose CSC-derived fluorescent material (Skold *et al.,* 1992). Endocytosis is a universal mechanism by which cells can non-specifically internalise material dissolved in the extracellular fluid (Lodish *et al.*, 1995). Thus, all cells have the capability to internalise fluorescent material from cigarette smoke. Quenching of cigarette smoke-mediated autofluorescence with crystal violet prior to staining has been successful in some studies (Hallden *et al.,* 1991; Skold *et al.,* 1996; Umino *et al.,* 1999). However, this method was developed to study intracellular staining and preservation of the cell membrane integrity was not a concern. Annexin-V/PI staining depends on the preservation of cell membrane integrity, use of crystal violet resulted in membrane permeabilisation, and all cells taking up both stains. Thus it was not possible to use this method to study apoptosis in cells exposed to CSC in this chapter.

As discussed in section 1.4.4, the basic description of necrosis is defined as osmotic swelling of the cell and organelles, cytoplasmic vacuolisation and eventually cell lysis (Wyllie *et al.*, 1980). Early and late characteristics can be detected morphologically; early abnormalities include marginal clumping of chromatin, nuclear swelling, dilatation of the endoplasmic reticulum, and gross swelling of the mitochondria. The

later stages of necrosis can be detected by rupture of nuclear, organelle and plasma membranes (Wyllie *et al.*, 1980). In this chapter necrosis was determined by loss of plasma membrane integrity, detected by uptake of ethidium bromide and release of LDH with no morphological changes of the nucleus reminiscent of apoptosis. However, these methods only monitor the end point of necrotic cell death, loss of plasma membrane integrity. Other forms of cell death may result in loss of plasma membrane integrity, either directly or due to lack of phagocytic clearance in an *in vitro* system. Therefore it should not be assumed that loss of plasma membrane integrity indicates the cells have died in a necrotic manner. Interestingly, other markers of necrosis were not evident after cigarette smoke exposure, no nuclear swelling was evident either by TEM or light microscopy, however TEM analysis of cells treated with doses of 5%CSC appear to possess swollen mitochondria. In fact, TEM showed that 10% CSC treatment may induce an autophagic-like cell death of A549 cells. The absence of oligonucleosomal DNA fragmentation confirmed that cigarette smoke was not inducing apoptosis however, as discussed earlier, DNA fragmentation also occurs in necrotic cell death. The absence of necrotic smears after electrophoresis indicates that "classical" necrotic cell death may not be occurring and further studies are required to characterise the form of cell death induced by cigarette smoke in more detail.

Studies exist in the literature, however, which support the observation that necrosis occurs in response to cigarette smoke (Ambalavanan *et al.,* 2001; Hopkin and Steel, 1980; Pouli *et al.,* 2003). Exposure of neonatal porcine vascular smooth muscle cells to CSC resulted in necrotic cell death with no evidence of apoptosis, using a modified TUNEL method (Ambalavanan *et al.,* 2001). In addition, exposure of mouse lung

epithelial cells to vapour phase CSC resulted in LDH release within 6 hours of exposure to 2.5 (35ml) puffs of smoke (Pouli *et al.,* 2003). Blood derived lymphocytes were unable to exclude a vital dye in a dose-dependent manner after CSC exposure (Hopkin and Steel, 1980). Exposure of rats to cigarette smoke resulted in a significant increase in TUNEL positive cells in the bronchial epithelium, however TEM analysis revealed cytoplasmic vacuolisation and organelle swelling, indicating necrotic, not apoptotic, cell death (Jung *et al.,* 2000).

This chapter does not support previous hypotheses that emphysema results from cigarette smoke-induced apoptosis of alveolar epithelial and endothelial cells, however it provides evidence that destruction of alveolar septa may result from cigarette smoke-induced necrosis. Loss of these cells by any means would result in loss of alveolar epithelium and endothelium, which may not be repaired due to the inhibitory effect of cigarette smoke on repair mechanisms (section 1.2.5). Of great importance however, is the finding that type II lung epithelial cell death occurs (Kasahara *et al.,* 2001; Hoshino *et al.,* 2001). As discussed previously, among many other important functions these cells are considered the "stem-cell" of the alveoli, as the gas-exchange type I cells are replenished by division and differentiation of the type II cells. Thus loss of these cells results in a further inability of the alveolar epithelium to regenerate itself and would therefore result in loss of alveolar walls.

Apoptosis enables removal of dying cells with little damage to the surrounding tissue if effectively cleared by phagocytes. However, necrosis ultimately results in cell lysis and the release of intracellular hydrolytic enzymes and lysosomal contents. This may

amplify local tissue damage by causing additional cells to undergo necrosis and also by recruiting inflammatory cells to the site of injury. It is well documented that inflammation occurs in smokers and individuals with emphysema/COPD. Retamales *et al* (2001) recruited patients undergoing lung volume reduction surgery (LVRS) and using computed tomography (CT) determined severity of emphysema. The numbers of inflammatory cells present in tissues and airspaces were determined, increased inflammation was associated with increased severity of emphysema. Thus, although much interest has been generated on the involvement of apoptosis, necrosis and subsequent inflammatory responses are more likely candidates in the pathogenesis of emphysema.

Chapter 4: Cigarette Smoke Condensate inhibits caspase activation and switches apoptosis to necrosis

4.1. Introduction

In the previous chapter it was shown that treatment of A549 cells and HUVECs with CSC resulted in necrotic cell death. As discussed in section 1.2.4.2, the cigarette smoking habit places a high oxidative burden on the smoker both systemically and directly to the lung, which is thought to cause many of the toxic effects of cigarette smoke. Studies of the effect of oxidants on cell death *in vitro* have demonstrated that oxidative stress inhibits apoptosis and induces necrosis, in Jurkat, U937, Burkitts lymphoma, and HepG2 cells (Hampton and Orrenius, 1997; Lee and Shacter, 1999, 2000; Palomba *et al.,* 1996; Samali *et al.,* 1999). It was hypothesised that cigarette smoke may be inhibiting apoptosis in addition to inducing necrosis, therefore the effect of CSC on apoptosis was studied.

A549 epithelial cells and HUVEC endothelial cells were not used to study the cell death pathway for a number of reasons. It was exceedingly difficult to induce A549 cells to undergo classical apoptosis; staurosporine (SS) induced phosphatidylserine (PS) externalisation with a morphology that vaguely resembled apoptosis, however many other markers of apoptosis were absent (Chapter 3). It was also not possible to use HUVEC cells to study the cell death pathway in great detail, due to a difficulty in obtaining sufficient numbers of cells on a regular basis. Jurkat T-cells undergo apoptosis readily and display classical apoptotic markers such as oligonucleosomal DNA fragmentation, chromatin condensation, caspase activation, and cell shrinkage, and therefore are often used as a model to elucidate the mechanism of cell death in response to various stimuli (Hampton *et al.*, 2002; Hampton and Orrenius, 1997; Leist *et al.,* 1997; Malhotra *et al.*, 2001; Saleh *et al.*, 2000; Tepper *et al.,* 1999). It has also

been shown that the cellular mechanisms that execute the apoptotic process are neither cell-type, nor species, specific (Zamzami *et al.,* 1996). Therefore Jurkat cells were used in this chapter as a model system to investigate the effect of CSC exposure on apoptosis.

4.2. CSC inhibits apoptosis and induces necrosis in Jurkat T-cells.

Jurkat cells treated with 10% CSC underwent necrosis as detected morphologically (Figure 4.1C and 4.2.A) and confirmed by LDH release (Figure 4.3). Cells treated with SS displayed the classical features of apoptosis, shrunken cells with pyknotic nuclei (Figure 4.1B), and oligonucleosomal DNA fragmentation (Figure 4.2.B SS). A small increase in LDH release was observed after longer treatments with SS (Figure 4.3), however this was attributed to secondary necrosis in the absence of phagocytic clearance. Interestingly, cells cultured with a combination of SS and CSC displayed a necrotic-like morphology (Figure 4.1D), swollen nuclei with no definite edge to the cytoplasm, indicating that plasma membrane integrity had been lost. SS/CSC treated cells also displayed no evidence of apoptosis, either by morphology (Figure 4.1D), or DNA laddering (Figure 4.2.B SS/CSC). Like A549 cells, the absence of necrotic smears after electrophoresis indicated that these cells were not undergoing a classical necrotic death.

It has been shown that apoptosis and necrosis can be induced by the same stimulus dependent on its intensity (Lennon *et al.*, 1991). However, cells treated for shorter incubations with CSC showed no evidence of apoptosis prior to undergoing necrosis (Figure 4.4). In addition, apoptosis was not observed when cells were treated with lower doses of CSC, cells treated with 1% CSC remained viable and those treated with 5% CSC underwent necrosis (Figure 4.5). Moreover, there was no significant

difference between the levels of necrosis induced by 5% and 10% CSC. Treatment with 1% CSC in combination with SS did not prevent apoptosis, however co-culture with 5% CSC prevented apoptosis and induced necrosis to a similar extent as with 10% CSC (Figure 4.5).

SS is a broad-ranging inhibitor of protein kinases that induces apoptosis of numerous cell types by activation of the intrinsic apoptotic pathway (section 1.4.2.3). Using anti-Fas activating antibody (CH-11) as a stimulus, the effect of CSC on the extrinsic pathway was studied. CH-11 treatment of Jurkat cells resulted in classical apoptotic morphological changes and induced oligonucleosomal DNA fragmentation (Figure 4.6), which was prevented by co-culture with CSC resulting in necrosis (Figure 4.6). Thus, CSC affects both the intrinsic and extrinsic apoptotic pathways.

To further clarify the stage of the apoptotic process CSC was affecting, cells were induced to undergo apoptosis with SS, exposed to 10% CSC at hourly intervals and co-cultured for the remainder of the experiment. Whereas, the predominant form of cell death was necrosis when CSC was added at early time points, this decreased when CSC was added more than 1 hour after SS and increasing levels of apoptosis occurred with each, later addition, of CSC (Figure 4.7A). This was coupled with increased oligonucleosomal DNA fragmentation as apoptosis became more predominant (Figure 4.7B).

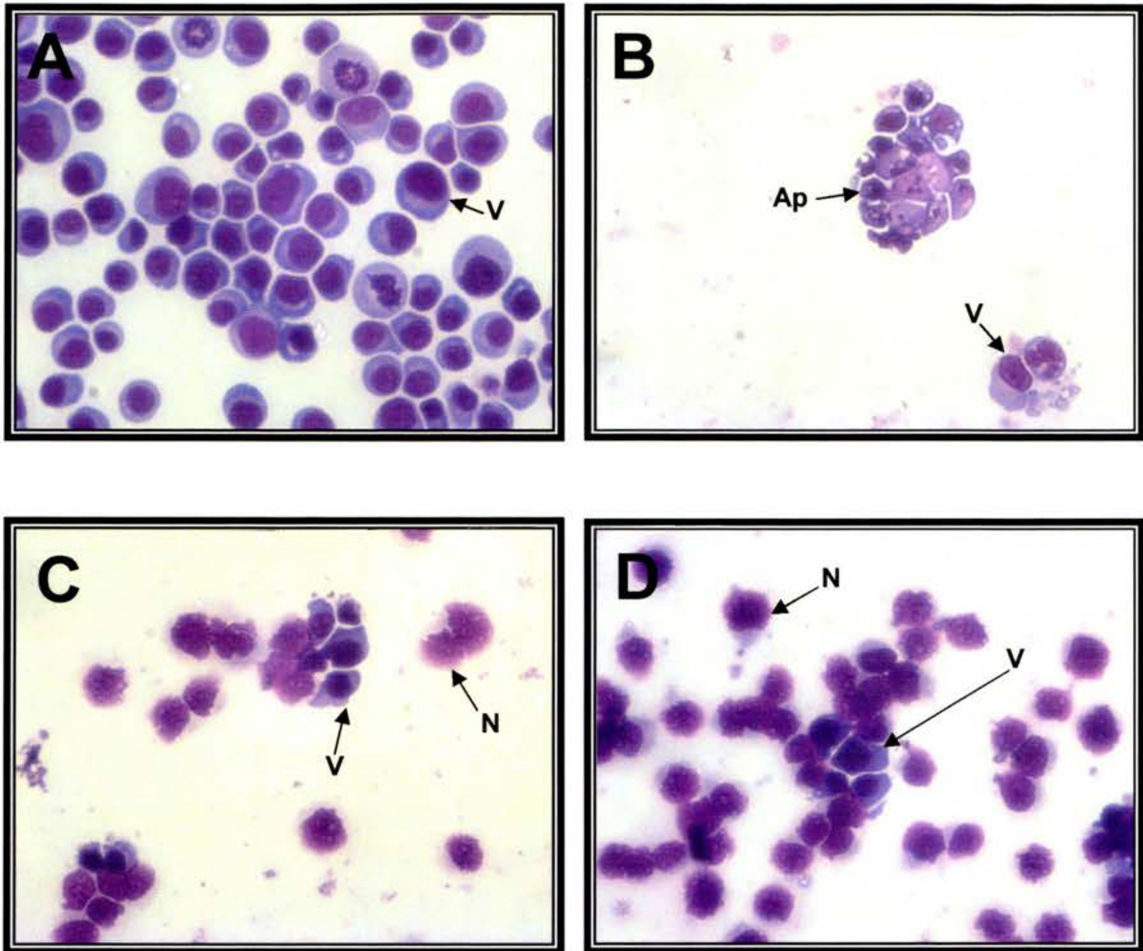
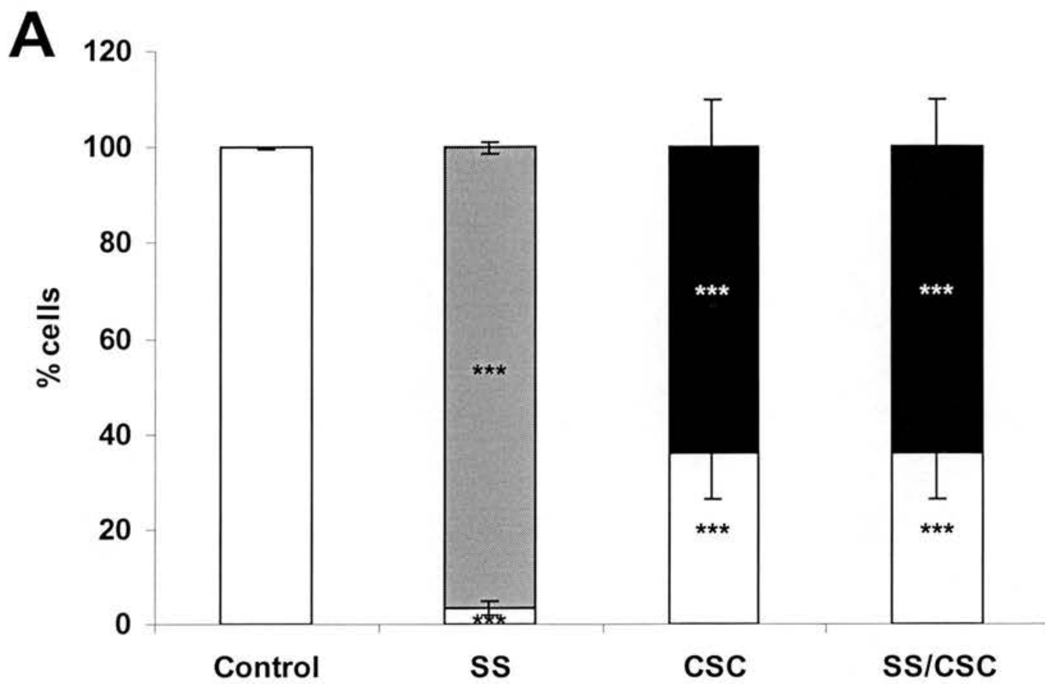


Figure 4.1: Cigarette smoke condensate inhibits apoptosis and induces necrosis in Jurkat cells. Cells were treated for 6 hours with either media alone **(A)**, 2μM SS **(B)**, 10% CSC **(C)** or a combination of 2μM SS and 10% CSC **(D)**. Cytospins were prepared and stained with DiffQuick and the percentage of viable **(V)**, apoptotic **(Ap)** and necrotic **(N)** cells were counted. Magnification x34



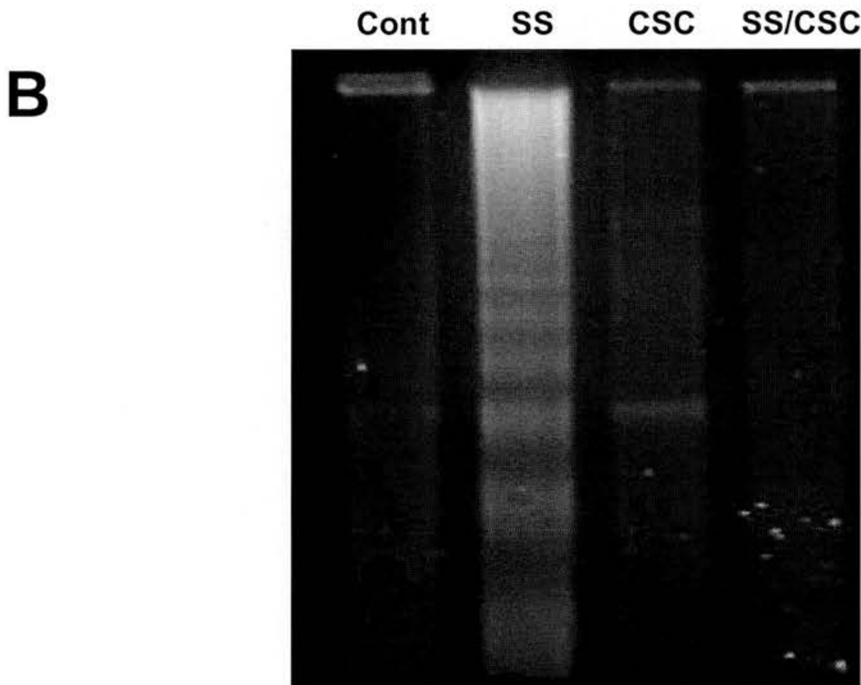


Figure 4.2. Cigarette smoke condensate prevents apoptosis and induces necrosis in Jurkat cells. Jurkat cells were incubated for 6 hours at 37°C with either normal media, 2μM SS, 10% CSC or a combination of SS and CSC. Cytospins were prepared and the percentage of viable (*open bars*), apoptotic (*grey bars*) and necrotic (*closed bars*) cells were determined **(A)**. Results expressed as the mean of three experiments where at least 200 cells were counted per slide ± SEM. *** $p<0.001$ compared to control. Apoptosis was confirmed by the presence of oligonucleosomal DNA fragmentation **(B)**. Representative gel of three experiments is shown.

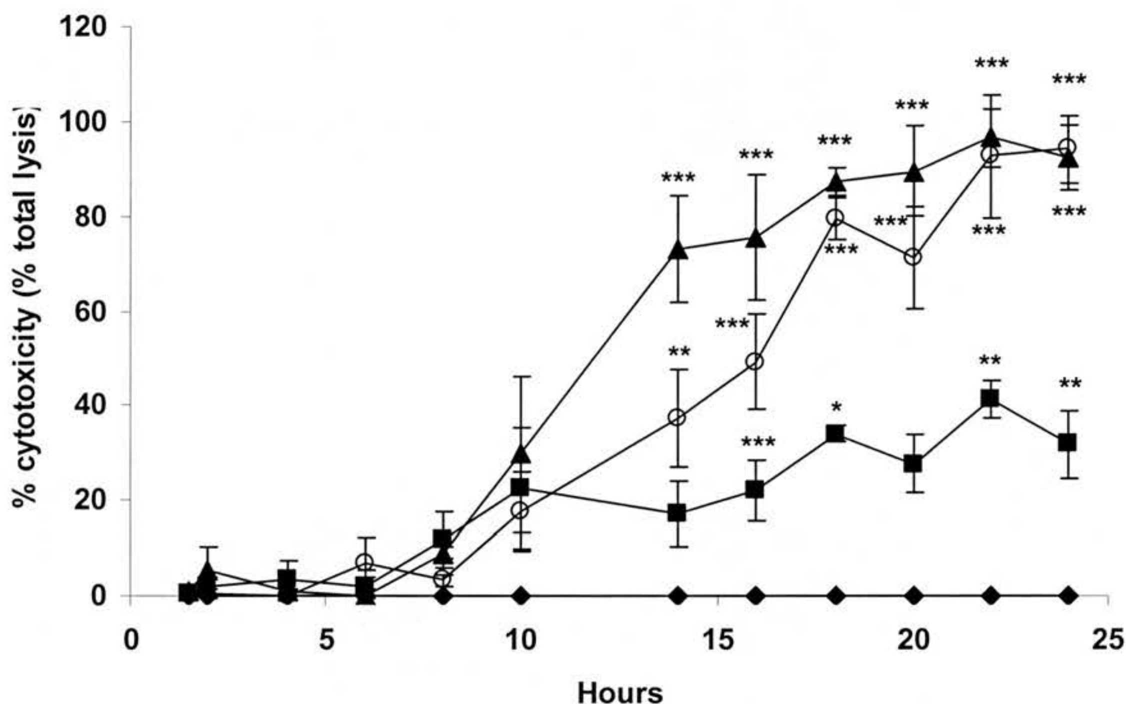


Figure 4.3. Cigarette smoke condensate induces necrosis in Jurkat cells, measured by LDH release. Jurkat cells were incubated for up to 24 hours at 37°C with either normal media (*closed diamonds*), 2μM SS (*closed squares*), 10% CSC (*closed triangles*) or a combination of SS and CSC (*open circles*). Cells were centrifuged at 250*g*, the media was collected and analysed for LDH content. Results expressed as the mean of three experiments ± SEM. *** $p<0.001$ compared to control.

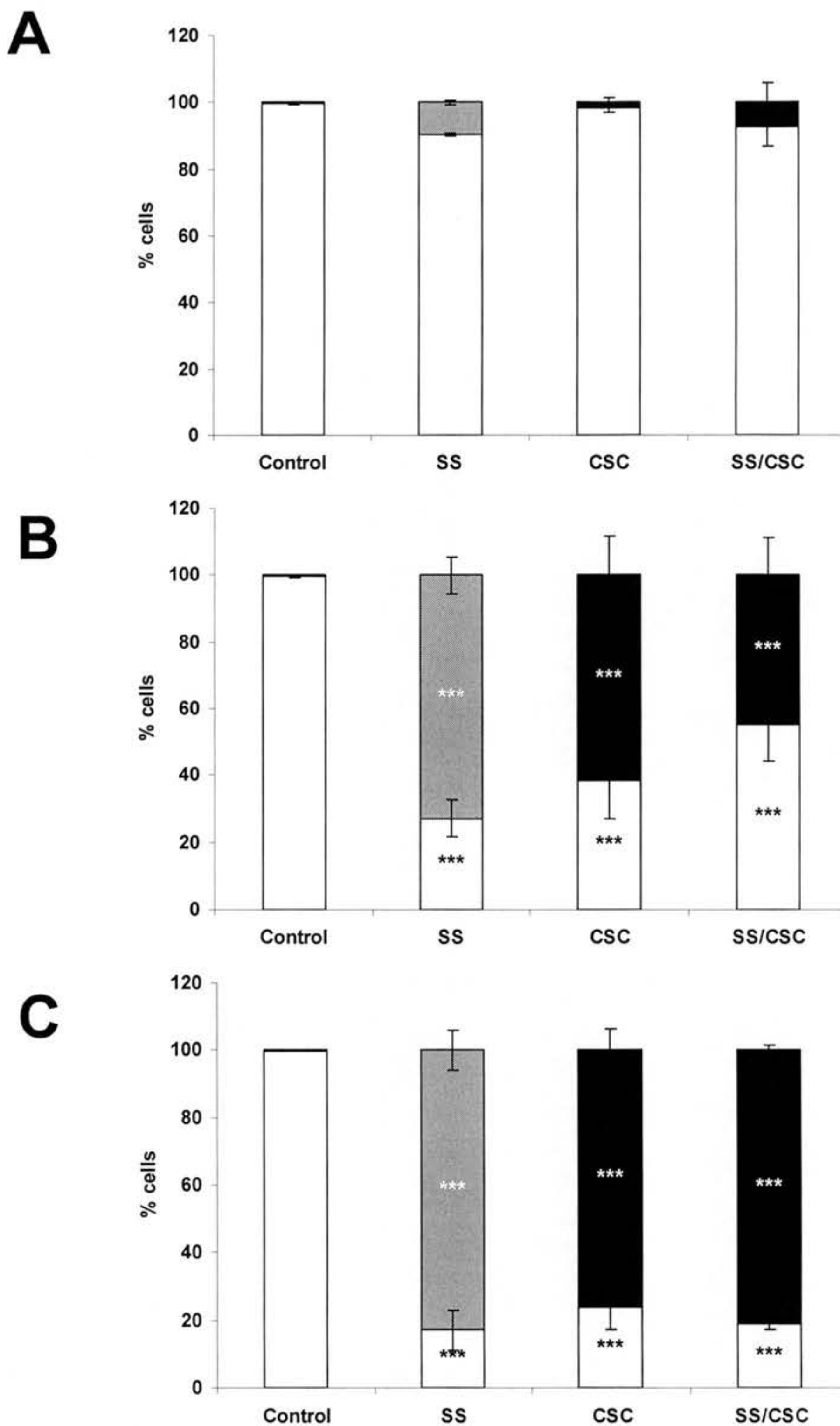


Figure 4.4. Time course of apoptosis and necrosis induced by SS and CSC. Jurkat cells were incubated for 2 hours **(A)**, 4 hours **(B)** or 6 hours **(C)** at 37°C with either normal media, 2μM SS, 10% CSC or a combination of SS and CSC. Cytospins were prepared and the percentage of viable (*open bars*), apoptotic (*grey bars*) and necrotic (*closed bars*) cells were determined. Results expressed as the mean of three experiments where at least 200 cells were counted per slide ± SEM. *** $p<0.001$ compared to control.

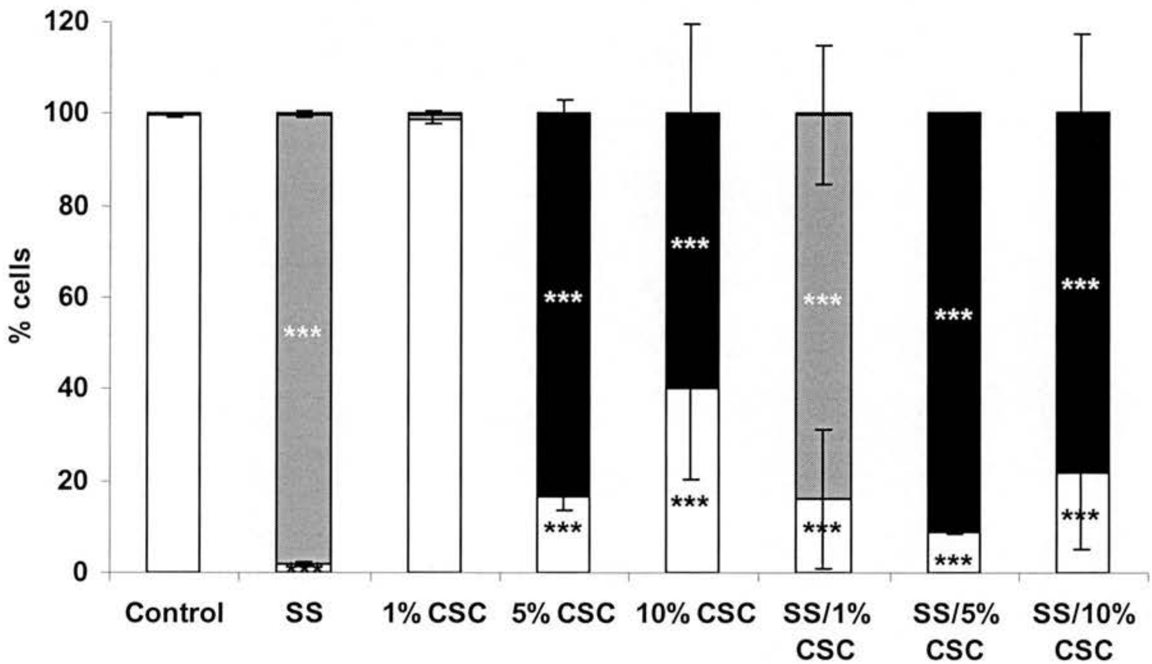
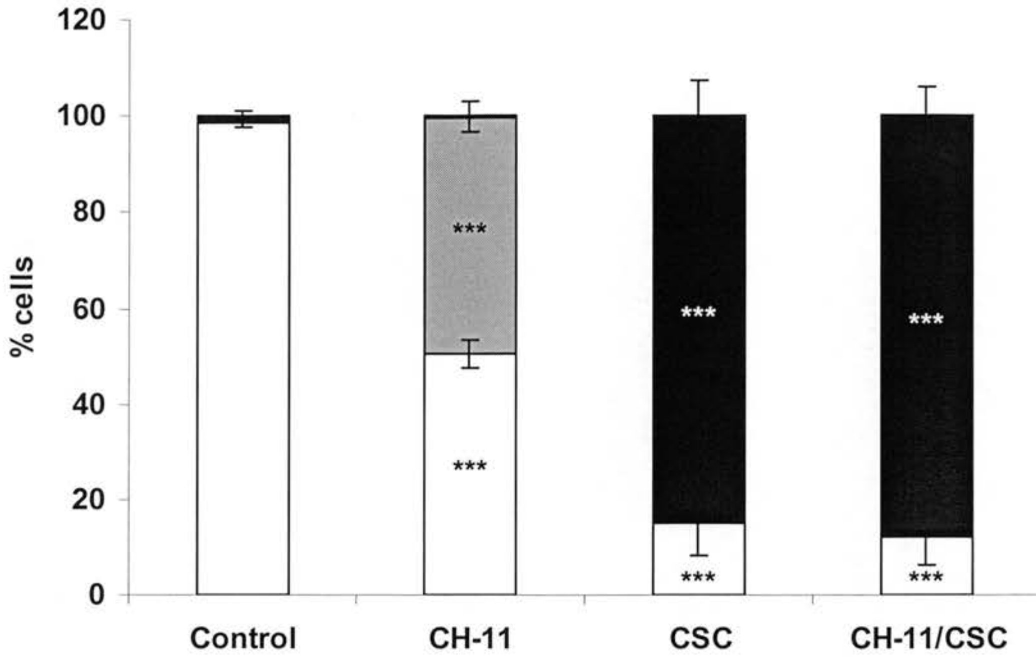


Figure 4.5. Dose response to CSC. Jurkat cells were incubated for 6 hours at 37°C with either normal media, 2μM SS, 1% CSC, 5% CSC 10% CSC or a combination of SS and 1%, 5% or 10% CSC. Cytospins were prepared and the percentage of viable (*open bars*), apoptotic (*grey bars*) and necrotic (*closed bars*) cells were determined. Results expressed as the mean of three experiments where at least 200 cells were counted per slide ± SEM. *** $p<0.001$ compared to control.

A



B

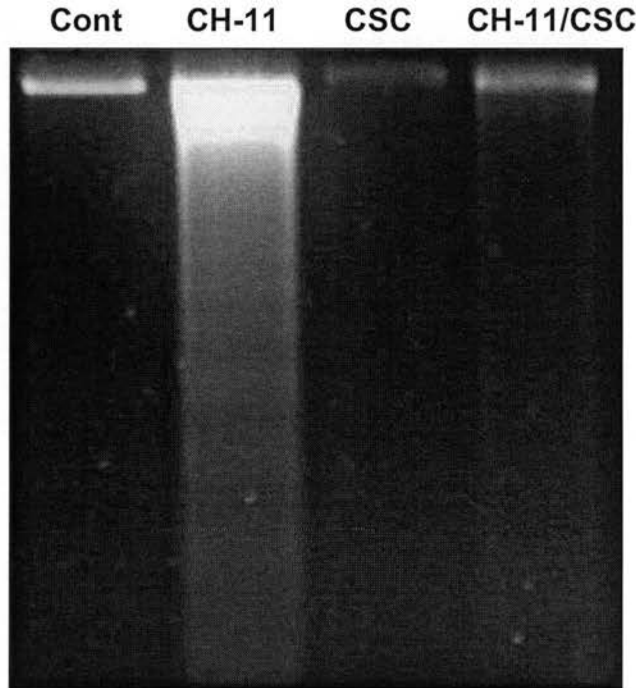


Figure 4.6. Cigarette smoke condensate inhibits apoptosis induced by Fas ligation. Jurkat cells were incubated with either normal media, 50ng/ml CH-11, 10% CSC or a combination of CH-11 and CSC for 10 hours at 37°C. Cytospins were prepared and the percentage of viable (*open bars*), apoptotic (*grey bars*) and necrotic (*closed bars*) cells were determined **(A)**. Results expressed as the mean of three experiments where at least 200 cells were counted per slide ± SEM. *** $p<0.001$ compared to control. Apoptosis was confirmed by the presence of oligonucleosomal DNA fragmentation **(B)**. Representative gel of three experiments is shown.

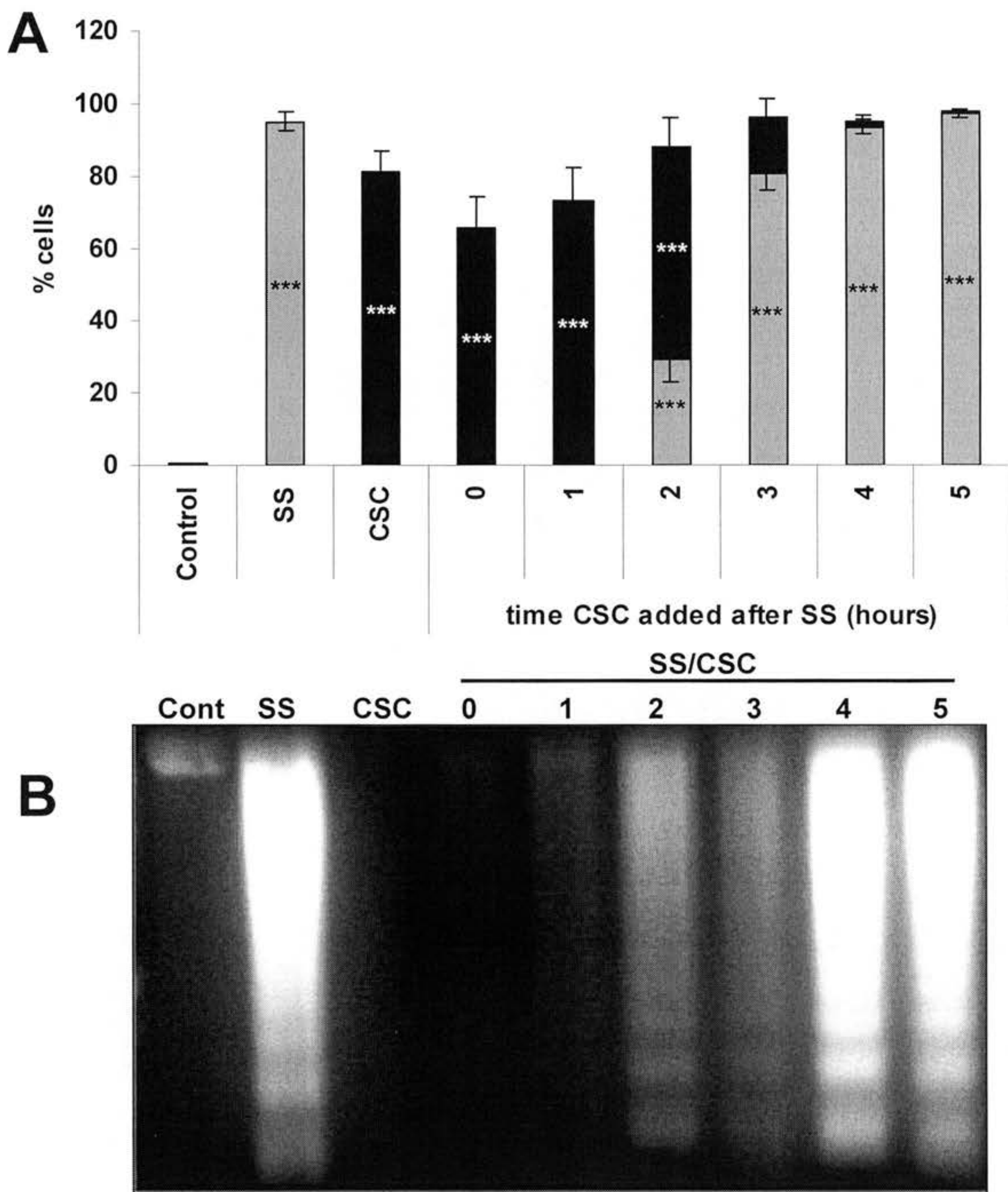
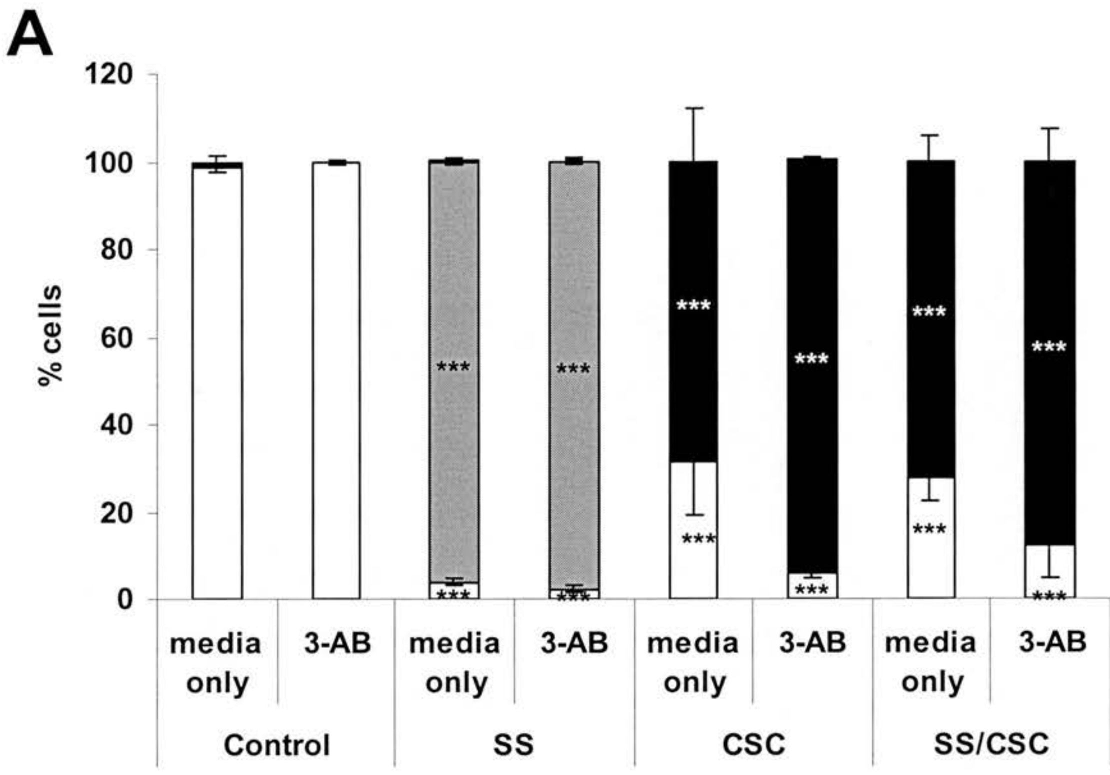


Figure 4.7. Cigarette smoke condensate inhibits an early stage in the apoptotic pathway. Jurkat cells were incubated with either normal media, 2μM SS or 10% CSC and incubated at 37°C for 6 hours. Apoptosis was initiated in 5 separate flasks with 2μM SS and 10% CSC was added at hourly intervals. Cells were incubated at 37°C for a total of 6 hours from the addition of SS. Cytospins were prepared and the percentage of viable, apoptotic (*grey bars*) and necrotic (*closed bars*) cells were counted **(A)** Results expressed as mean of three experiments ± SEM. *** $p<0.001$ compared to control. Apoptosis was confirmed by the presence of DNA ladders **(B).** Representative gel of three experiments shown..

4.3. Inhibition of apoptosis and induction of necrosis by CSC does not involve poly (ADP-ribose) polymerase (PARP) activation.

Inhibition of apoptosis by oxidants has been shown to involve the activation of the DNA repair enzyme poly (ADP-ribose) polymerase (PARP) in response to oxidant-induced DNA strand breaks (Lee and Shacter, 1999; Palomba et al., 1996). Moreover, PARP activation was higher in lymphocytes from patients with COPD compared to healthy controls (Hageman *et al.*, 2003). As outlined in section 1.4.4, activation of PARP in situations of large scale DNA damage may result in ATP depletion, and an inability to execute the apoptotic program. In Chapter 3 it was suggested that CSC may in fact induce significant DNA damage as evidenced by the G1/S cell cycle block. Inhibition of PARP activity prevented oxidant-mediated necrosis and resulted in apoptotic cell death (Lee and Shacter, 1999; Palomba *et al.,* 1996).

Therefore, the involvement of PARP in CSC-induced necrosis and inhibition of apoptosis was determined. Jurkat cells were pre-treated with the PARP inhibitor, 3-aminobenzinamide (3-AB) for 30 minutes prior to addition of SS and/or CSC. There was no significant difference in the apoptotic and necrotic profiles of the cells after treatment in the presence or absence of 3-AB (Figure 4.8). Therefore it was deduced that CSC does not prevent apoptosis by depletion of ATP as a result of induction of PARP.



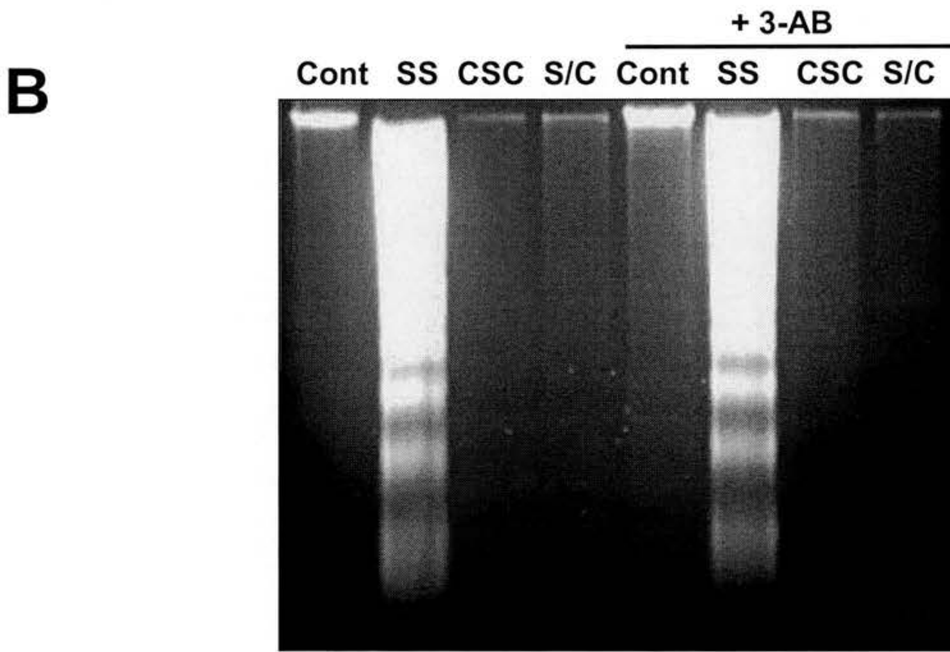


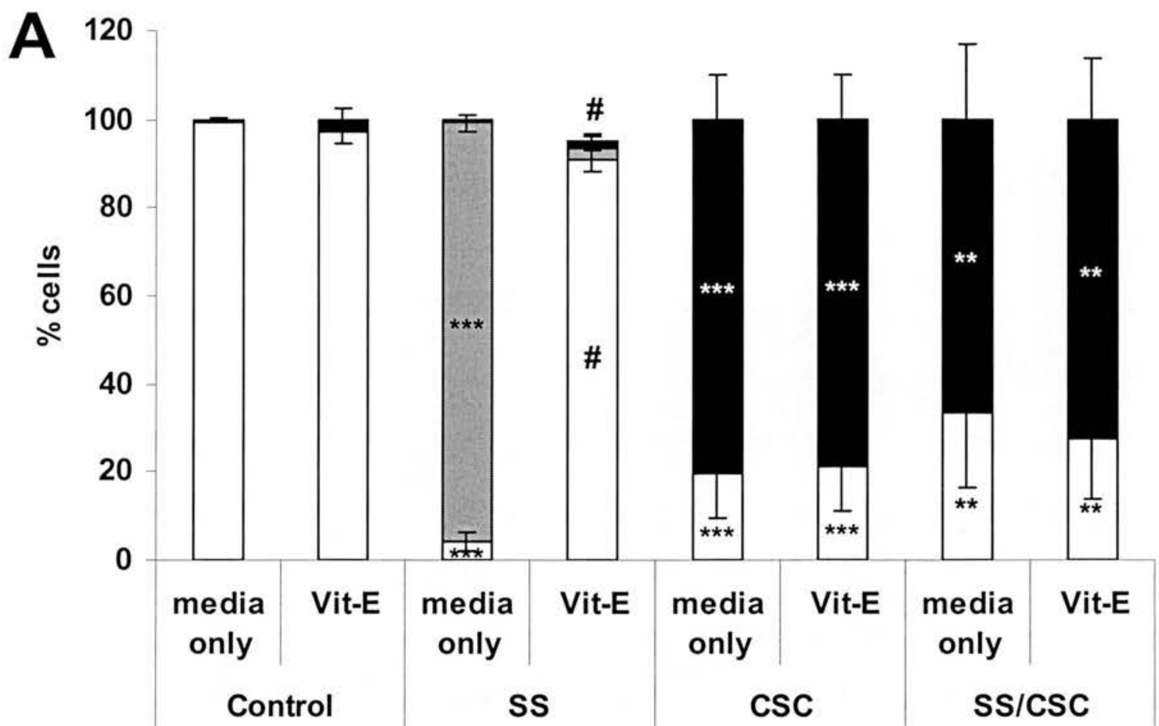
Figure 4.8. 3-aminobenzinamide pre-treatment does not prevent CSC-induced necrosis or apoptosis inhibition. Jurkat cells were incubated with 1mM 3-AB for 30 minutes at 37°C prior to treatment with 2μM SS, 10% CSC or a combination of SS and CSC (S/C) at 37°C for 6 hours. Cytospins were prepared and the percentage of viable (*open bars*), apoptotic (*grey bars*) and necrotic (*closed bars*) cells were counted **(A)** Results expressed as mean of three experiments ± SEM. *** $p<0.001$ compared to appropriate control. Apoptosis was confirmed by the presence of DNA ladders **(B)** representative gel of three experiments shown..

4.4. Inhibition of apoptosis and induction of necrosis can be prevented by some antioxidants but not others.

In order to investigate the involvement of oxidants in CSC-mediated necrotic cell death and apoptosis inhibition, antioxidants were added to the media prior to the addition of SS and/or CSC, in order to quench the reactive oxygen species (ROS) from the smoke.

4.4.1. Vitamin-E and mannitol do not protect against CSC-induced necrosis

Vitamin-E (α-tocopherol) is a chain breaking antioxidant, which scavenges peroxyl radicals and prevents lipid peroxidation (Wang and Quinn, 1999). Lipid peroxidation is induced after cigarette smoking (section 1.3.3.1 and 1.2.4.2) and therefore the role of the peroxyl radical in CSC-mediated necrosis was investigated. Pre-treatment with 1mM vitamin-E for 30 minutes prior to the addition of stimuli did not protect against CSC-induced necrosis. Cells exposed to SS in the presence of vitamin-E did not undergo apoptosis (Figure 4.9), and as a result it was difficult to evaluate the role of oxidants in inhibition of apoptosis caused by CSC using vitamin-E as an antioxidant. However, the presence of vitamin-E did not prevent necrosis induced by CSC and thus it was assumed that peroxyl radicals were not involved in CSC-induced necrosis.



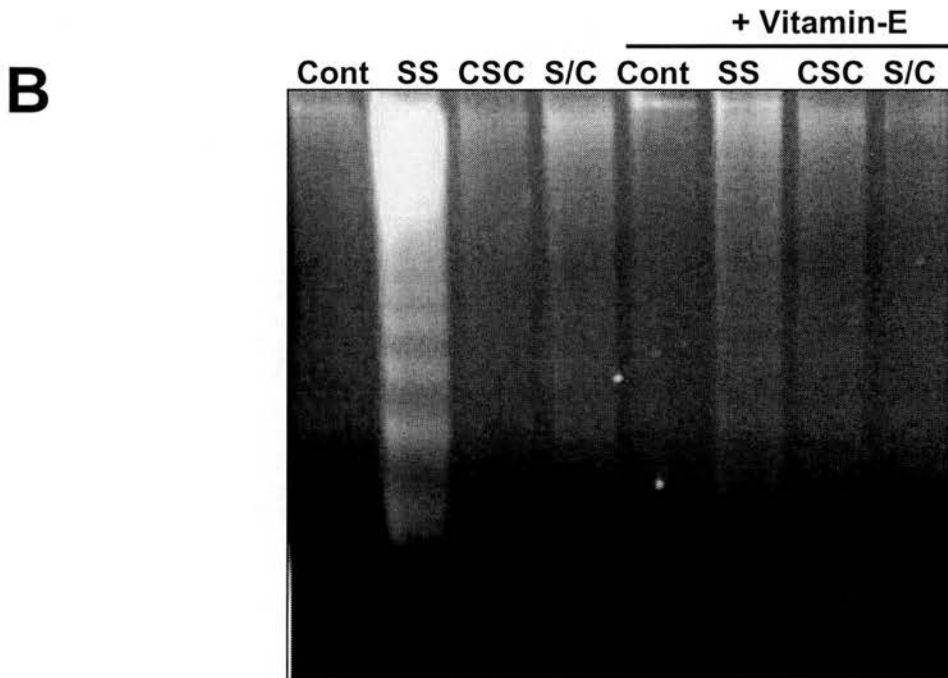


Figure 4.9. Vitamin-E pre-treatment does not prevent CSC-induced necrosis or apoptosis inhibition. Jurkat cells were incubated with 1mM vitamin-E for 1 hour at 37°C prior to treatment with 2μM SS, 10% CSC or a combination of SS and CSC (S/C) at 37°C for 6 hours. Cytospins were prepared and the percentage of viable (*open bars*), apoptotic (*grey bars*) and necrotic (*closed bars*) cells were counted **(A)** Results expressed as mean of three experiments ± SEM. *** $p<0.001$, ** $p<0.01$ compared to appropriate control; # $p<0.001$ compared to media only. Apoptosis was confirmed by the presence of DNA ladders **(B)** representative gel of three experiments shown..

Mannitol is a hydroxyl radical (·OH) scavenger, but in doing so is itself converted to a free radical. Hence it acts as a protective agent only if its radical is less damaging than the radical it quenched (Gillbe *et al.,* 1996). The presence of 5mM mannitol in the media had no effect on SS-, or CH-11-induced apoptosis or CSC-induced necrosis (Table 4.1 and 4.2). A small number of apoptotic cells were detected after exposure to the combination of SS and CSC in the presence of mannitol but the level of necrosis was not affected (Table 4.1). Similarly, mannitol did not protect against CSC-induced necrosis when added in combination with CH-11, however unlike in response to SS/CSC no apoptotic cells were detected (Table 4.2). Morphological analysis of apoptosis was supported by analysis of oligonucleosomal DNA fragmentation, which was only seen after treatment with the apoptosis inducers, SS and CH-11 in the presence of mannitol (Figure 4.10 C and F).

Table 4.1. Inhibition of apoptosis and induction of necrosis mediated by CSC is prevented by DTT and GSH. Jurkat cells were incubated with either normal media, 5mM mannitol, 1mM DTT or 1mM GSH containing 2μM SS, 10% CSC or a combination of 2μM SS and 10% CSC for 6 hours at 37°C. Cytospins were prepared and the percentage of viable, apoptotic and necrotic cells were determined. Results are mean of three experiments ± SEM. *** $p<0.01$ compared to appropriate control; # $p<0.001$ compared to no-antioxidant control.

		% Apoptosis	% Necrosis
Control	No-antioxidants	0.3 ± 0.1	0.1 ± 0.1
	Mannitol	0.3 ± 0.2	0.2 ± 0.1
	DTT	2.4 ± 1.3	0.7 ± 0.4
	GSH	0.4 ± 0.2	0.2 ± 0.2
SS	No-antioxidants	94.9 ± 2.6 ***	0.4 ± 0.2
	Mannitol	97.4 ± 1.2 ***	0.9 ± 0.7
	DTT	90.3 ± 4.2 ***	0.1 ± 0.1
	GSH	93.4 ± 4.5 ***	0.2 ± 0.1
CSC	No-antioxidants	0.0 ± 0.0	74.6 ± 5.0 ***
	Mannitol	0.0 ± 0.0	75.4 ± 11.8 ***
	DTT	0.4 ± 0.3	16.3 ± 1.7 #
	GSH	12.9 ± 4.7	0.3 ± 0.3 #
SS/CSC	No-antioxidants	0.0 ± 0.0	69.5 ± 4.9 ***
	Mannitol	23.2 ± 18.4 #	61.4 ± 18.5 ***
	DTT	79.5 ± 11.4 *** #	0.6 ± 0.4 #
	GSH	98.5 ± 0.5 *** #	0.3 ± 0.2 #

4.4.2. GSH and DTT protect against CSC-induced necrosis

GSH is a thiol antioxidant, which scavenges free radicals by sacrificing its sulfydryl group resulting in the formation of glutathione disulphide (GSSG) (Meister and Anderson, 1983; section 1.1.1.3.). The level of apoptosis in cells incubated with SS, or CH-11 was unaffected by the presence of GSH, however necrosis was abolished in response to CSC with a small percentage of cells undergoing apoptosis. Co-culture with the apoptosis inducers and CSC in the presence of GSH again prevented necrosis with 98 percent of cells undergoing SS-induced apoptosis and 81 percent undergoing CH-11-induced apoptosis (Table 4.1 and 4.2 respectively). Moreover, oligonucleosomal DNA fragmentation, absent after co-culture with CSC and SS/CH-11 alone (Figures 4.2B and 4.6B respectively), occurred when treated in the presence of GSH (Figure 4.10B and E). Therefore, the presence of GSH prevented both the induction of necrosis and the inhibition of the apoptotic pathway as a result of CSC exposure.

DTT, also a thiol antioxidant, was used to study the role of oxidants in CSC-induced necrosis and apoptosis inhibition. DTT did not affect control cells or affect apoptosis induced by SS, but did significantly reduce the level of necrosis seen after CSC exposure. SS-induced apoptosis occurred with no evidence of necrosis in cells co-cultured with SS and CSC in the presence of DTT (Table 4.1). However, DTT itself prevented apoptosis induced by CH-11, and cells treated with CSC in the presence of DTT underwent equivalent levels of necrosis as when no-antioxidants were used (Table 4.2). These results were confirmed by electrophoresis of DNA, figure 4.10A shows that DNA fragmentation occurred after treatment with SS but not CSC alone, and unlike after treatment with SS and CSC without antioxidants (Figure 4.2B) cells treated with

SS and CSC in the presence of DTT demonstrated DNA fragmentation (Figure 4.10A). No DNA fragmentation was seen with CH-11, CSC or CH-11/CSC in the presence of DTT (Figure 4.10D), mimicking the absence of morphological apoptosis observed. This indicated that both DTT and GSH may be quenching a component(s) of CSC responsible for inducing necrosis while preventing apoptosis induced by SS, but only GSH was effective at attenuating the effects when CH-11 was the stimulus.

Table 4.2. Inhibition of apoptosis and induction of necrosis mediated by CSC is prevented by DTT and GSH. Jurkat cells were incubated with either normal media, 5mM mannitol, 1mM DTT or 1mM GSH containing 50ng/ml CH-11, 10% CSC or a combination of 50ng/ml CH-11 and 10% CSC for 9 hours at 37°C. Cytospins were prepared and the percentage of viable, apoptotic and necrotic cells were determined. Results are expressed as the mean of two experiments ± SEM. *** $p<0.01$ compared to appropriate control; # $p<0.001$ compared to no-antioxidant control.

		% Apoptosis	% Necrosis
Control	No-antioxidants	0.6 ± 0.2	0 ± 0
	Mannitol	0.6 ± 0.6	0 ± 0
	DTT	4.2 ± 0.4	6.0 ± 0.3
	GSH	0.7 ± 0.4	0 ± 0
CH-11	No-antioxidants	49 ± 5.1 ***	0.1 ± 0.1
	Mannitol	49.1 ± 0.9 ***	0 ± 0
	DTT	8.4 ±1.2 #	5.9 ± 3.4
	GSH	56.9 ± 2.6 ***	0 ± 0
CSC	No-antioxidants	0 ± 0	84.2 ±12.5 ***
	Mannitol	0 ± 0	94 ± 1.1 ***
	DTT	0 ± 0	72.1 ± 18.1 ***
	GSH	8.9 ± 6.1	0 ± 0 #
CH-11 /CSC	No-antioxidants	0 ± 0	87.2 ± 10.5 ***
	Mannitol	0 ± 0	91.3 ± 0.3 ***
	DTT	2.1 ± 2.1	92.9 ± 5.5 ***
	GSH	81.7 ± 0.4 *** #	0 ± 0 #

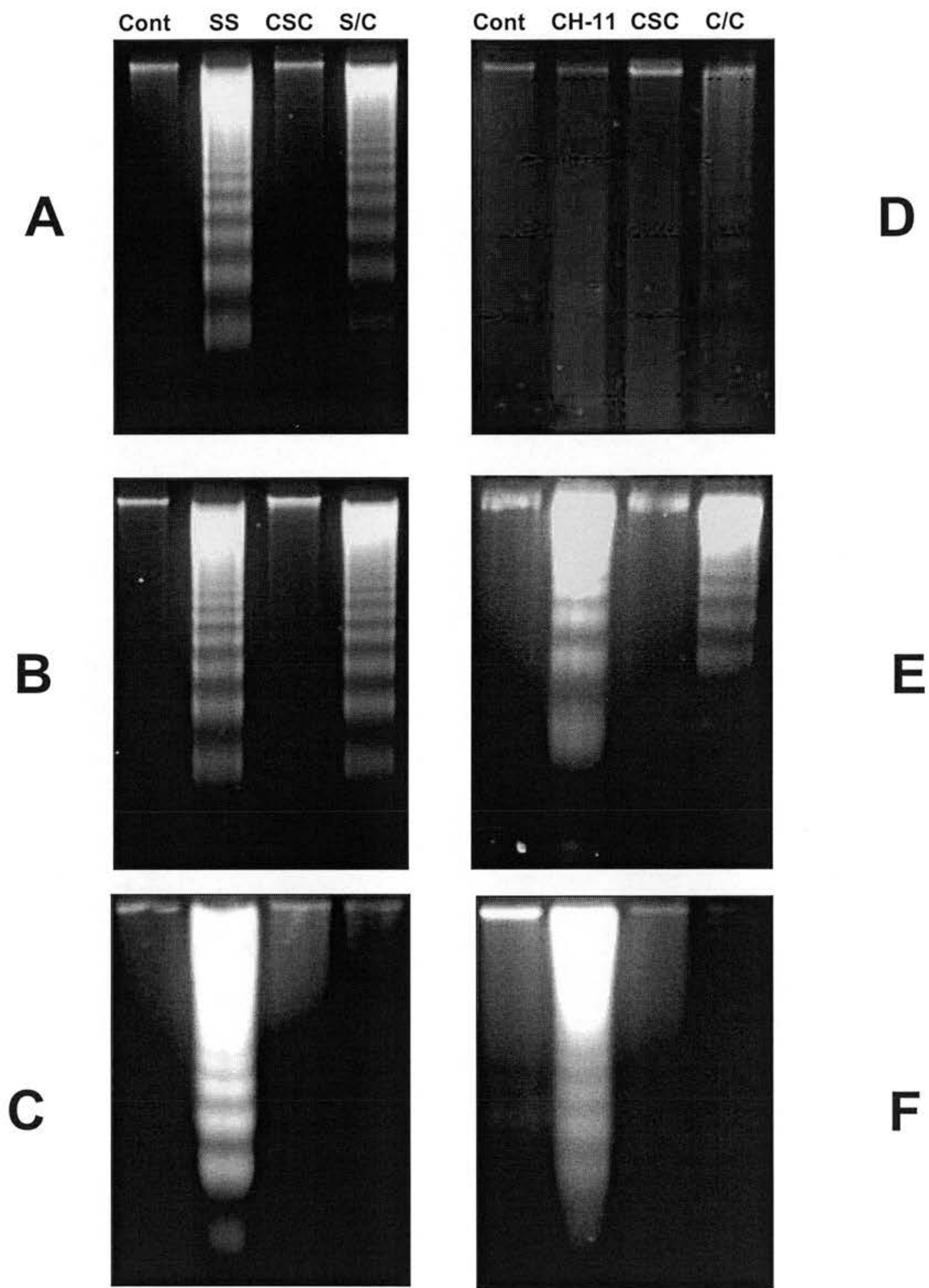


Figure 4.10. Oligonucleosomal DNA fragmentation in response to SS and/or CSC in the presence of antioxidants. Jurkat cells were incubated with media containing 1mM DTT **(A, D),** 1mM GSH **(B, E)** or 5mM mannitol **(C, F)** with 2μM SS, 10% CSC or a combination of 2μM SS and 10% CSC for 6 hours **(A-C)** or 50ng/ml CH-11, 10% CSC or a combination of 50ng/ml CH-11 and 10% CSC **(D-F)**. Representative gels of three experiments **(A, B, C)** and two experiments **(D, E, F)** data are shown.

4.4.3. CSC conjugates directly with GSH

In vivo, in addition to its antioxidant properties, GSH acts as a detoxifying agent conjugating to electrophilic compounds via its thiol group both spontaneously and with the aid of GSH transferases (Meister and Anderson, 1983; Section 1.1.1.3.). In order to determine whether GSH-CSC conjugates occur *in vitro*, in the absence of the enzyme, various concentrations of CSC were incubated with GSH at 37°C (Figure 4.11). A dose dependent decrease in GSH was observed, indicating that GSH may be forming conjugates with the many electrophilic compounds present in cigarette smoke and would thus not be detected by the GSH assay.

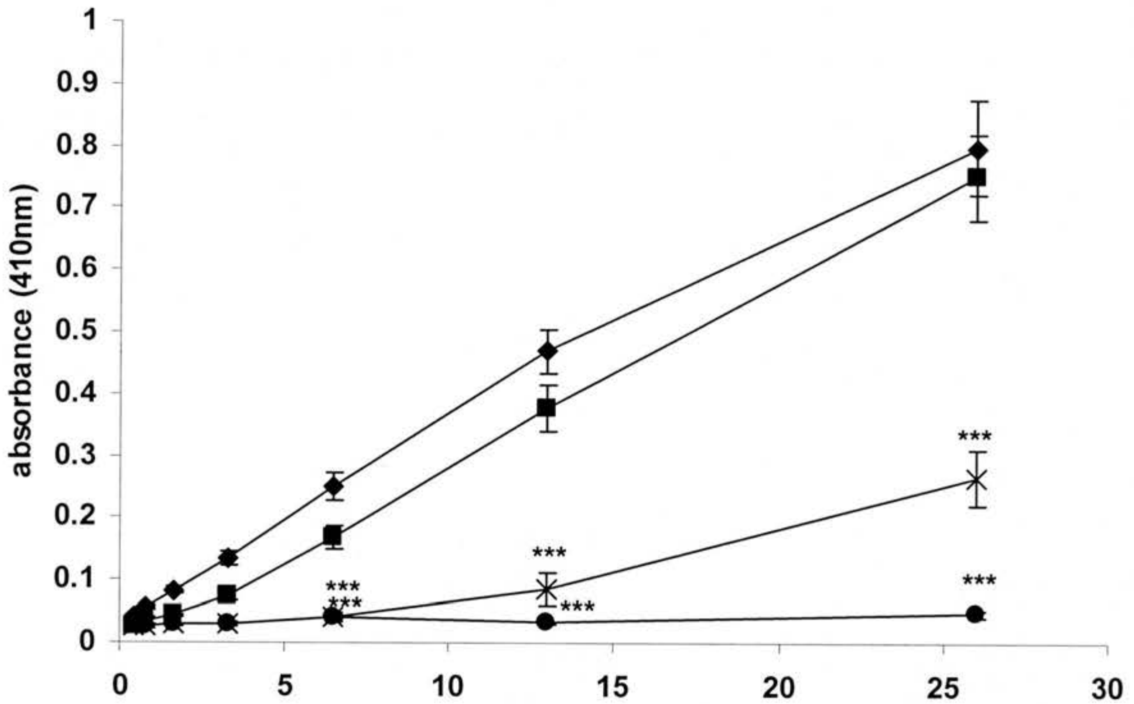


Figure 4.11. Cigarette smoke condensate conjugates with GSH in a non-enzymatic manner. Glutathione was incubated either alone (*closed diamonds*) or with 1% CSC (*closed squares*), 5% CSC (*crosses*) or 10% CSC (*closed circles*) for 1 hour at 37°C. The level of detectable GSH was determined using the "Tietze" GSH assay. Results expressed as mean of three experiments performed in triplicate ± SEM. ***$p<0.001$.

4.5. Inhibition of apoptosis is coupled with an inhibition of caspase activation

4.5.1. Cigarette smoke condensate prevents caspase activation in Jurkat cells

Caspases are the main effectors of the apoptotic pathway thus, as cigarette smoke was shown to affect early stages of apoptosis, the caspase pathway was studied in more detail. Procaspase-3 cleavage was monitored by western blot in cytoplasmic lysates from Jurkat cells treated with SS and/or CSC (Figure 4.12A), although the antibody detects both the proform and the active subunits of caspase-3 it was difficult to detect the subunits under the conditions of this assay, possibly as a result of ubiquitination and proteolytic degradation. As a result, processing of procaspase-3 was detected by a decrease in intensity or total loss of the 37kDa band, representative of procaspase-3, by Western blot. In support of the previous data, cleavage of caspase-3 proform was only observed in cells exposed to SS, not in cells exposed to either CSC or a combination of SS and CSC (Figure 4.12A). This indicated that in addition to preventing the morphological features of apoptosis, CSC prevented the activation of caspases.

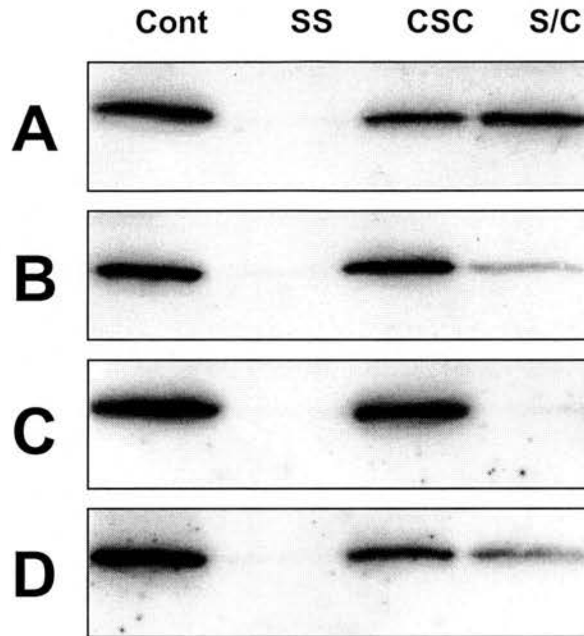


Figure 4.12. Cigarette smoke condensate prevents caspase-3 cleavage in Jurkat cells. Jurkat cells were treated for 6 hours with either normal media **(A)** or media containing, 1mM DTT **(B)**, 1mM GSH **(C)**, 5mM mannitol **(D)** in the presence of media alone (cont), 2μM SS, 10% CSC or with a combination of 2μM SS and 10% CSC (S/C). Whole cell lysates were prepared and caspase-3 cleavage was determined by Western blot. Representative blots of three experiments are shown.

4.5.2. GSH and DTT protect against CSC-induced caspase inhibition

The antioxidants GSH and DTT prevented the inhibition of apoptosis induced by CSC in addition to preventing CSC-induced necrosis, and therefore their role in preventing caspase inhibition was investigated. Jurkat cells were incubated with SS and/or CSC in the presence of 1mM GSH, 1mM DTT or 5mM mannitol and the effect on procaspase-3 cleavage was determined (Figure 4.12). In the presence of mannitol (Figure 4.12D) activation of caspase-3 was observed in cells treated with SS but not in cells treated with CSC. However, a small proportion of procaspase-3 was activated after treatment with SS and CSC in the presence of mannitol, which correlates with the small percentage of apoptotic cells observed morphologically (Table 4.1). However, in contrast to cells treated without antioxidants, procaspase-3 cleavage occurred in cells treated with SS and CSC in the presence of both DTT (Figure 4.12B) and GSH (Figure 4.12C), reflecting the morphological apoptosis seen in cells treated with SS and CSC in the presence of DTT or GSH (Table 4.1).

4.6. Cigarette smoke does not affect caspase activity directly

4.6.1. Cigarette smoke condensate does not affect recombinant caspase-3 activity

Caspases contain a thiol group in the active site, which is essential for function and is sensitive to inactivation by oxidation, nitrosylation or alkylation (Melino *et al.,* 1999). Previous studies have demonstrated that oxidative stress decreases the activity of caspases (Borutaite and Brown, 2001; Hampton *et al.,* 2002; Lee and Shacter, 1999, 2000; Samali *et al.,* 1999). In addition, in a similar manner to that observed with GSH (Figure 4.10), CSC may conjugate to the thiol group in the caspase active site resulting in inactivation of the enzyme. To determine whether this was occurring, recombinant caspase-3 was incubated with various concentrations of CSC and the activity of the enzyme was determined by its ability to cleave a colorimetric caspase substrate. CSC did not directly affect caspase-3 activity as no decrease in absorbance was observed (Figure 4.13). This indicated that the inhibition of apoptosis observed in the presence of smoke was not due to a direct effect on caspase activity. A positive control of 1mM H_2O_2 significantly decreased recombinant caspase-3 activity, in accordance with the literature (Bourtaite and Brown, 2001).

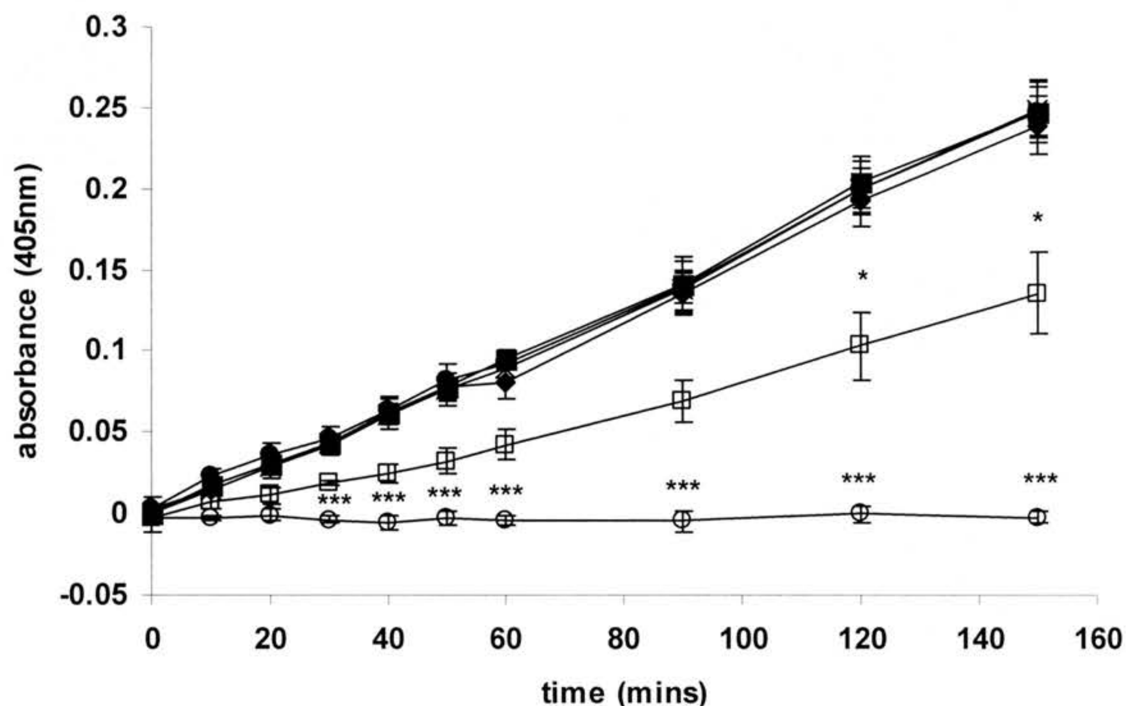


Figure 4.13. Cigarette smoke condensate does not inhibit recombinant caspase-3 activity directly. Recombinant caspase-3 was incubated either alone (*closed diamonds*) or with 1% CSC (*closed squares*), 5% CSC (*crosses*), 10% CSC (*closed circles*) 1mM H_2O_2 (*open squares*) or a caspase inhibitor (*open circles*) in the presence of a colorimetric caspase-3 substrate. Absorbance at 405nm was measured at determined time intervals. Results expressed as mean of three experiments ± SEM. * $p<0.05$ ***$p<0.001$.

4.6.2. Cigarette smoke condensate does not affect caspase activation in a cell-free system

Although CSC was not directly affecting caspase activity, caspase-3 cleavage did not occur in the presence of CSC; therefore an investigation into the direct effect of CSC on activation of the caspase pathway was performed. The caspase pathway was activated artificially in lysates from Jurkat cells by initiating formation of the apoptosome with 100μM cytochrome-c and 1mM dATP (Slee *et al.,* 1999). Subsequent caspase activation was detected by monitoring processing of procaspases-3 and -9 by Western blot. CMF-PBS was added to the lysates to a final concentration of 10%, as a negative control, and after addition of cytochrome-c and dATP, caspase activation was observed (Figure 4.14A and C). However, addition of 10% CSC to cytoplasmic lysates did not prevent processing of caspases-3 or –9 (Figure 4.14B and D), which taken alongside the effect of CSC on recombinant caspase-3, it was evident that CSC did not directly prevent caspase activation.

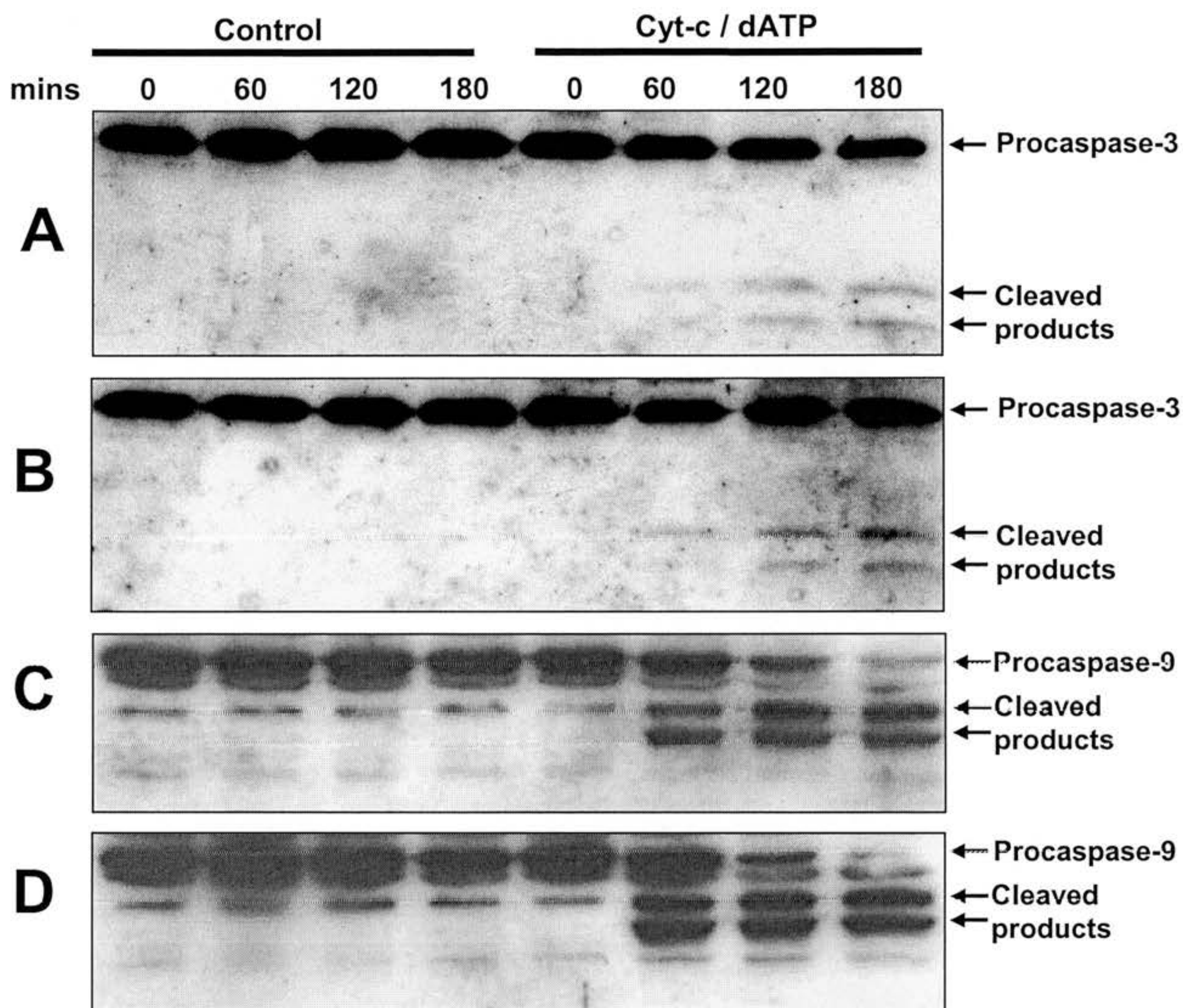


Figure 4.14. Cigarette smoke condensate does not directly inhibit caspase activation. Cytoplasmic extracts were prepared from Jurkat cells and either 10% CMF-PBS **(A and C)** or 10% CSC **(B and D)** was added. Apoptosome formation was initiated by addition of cytochrome-c and dATP at 37°C and samples were taken at hourly intervals and boiled for 5 minutes in laemmli buffer. Cleavage of caspase-3 **(A and B)** or caspase-9 **(C and D)** was monitored by Western blotting. Result is representative of three experiments.

4.7. Cigarette smoke condensate indirectly affects caspase activation

4.7.1. Pre-incubation with cigarette smoke condensate prevents caspase-3 activation in a cell-free system

Given that CSC treatment prevented activation of the caspase cascade, but did not affect caspase activity or activation directly, the indirect effect of CSC on caspase activation was investigated. Jurkat cells were incubated with either media alone or media containing 10% CSC for 2 hours prior to preparation of cytoplasmic lysates and formation of the apoptosome. Caspase-3 processing was evident in lysates from cells incubated with media alone (Figure 4.15A). However, in lysates from cells treated with 10% CSC no caspase-3 processing was observed after addition of dATP and cytochrome-c (Figure 4.15B). This indicates that CSC prevents caspase activation at a point upstream of caspase-3 cleavage.

4.7.2. Pre-incubation with cigarette smoke condensate prevents caspase-9 activation in a cell-free system

As outlined in section 1.4.2.2 and 1.4.2.3, the extrinsic apoptotic pathway can activate the intrinsic apoptotic pathway via Bid. In this situation both pathways have a common effector caspase upstream of caspase-3 activation, caspase-9. As CSC prevents apoptosis induced by both of these pathways (section 4.2), caspase-9 activation was studied. Western blot analysis of cell-free extracts revealed that caspase-9 activation was prevented following a 2 hour incubation with 10% CSC (Figure 4.15D). Therefore, it was evident that CSC was preventing the activation of the caspase cascade at the level of caspase-9 activation.

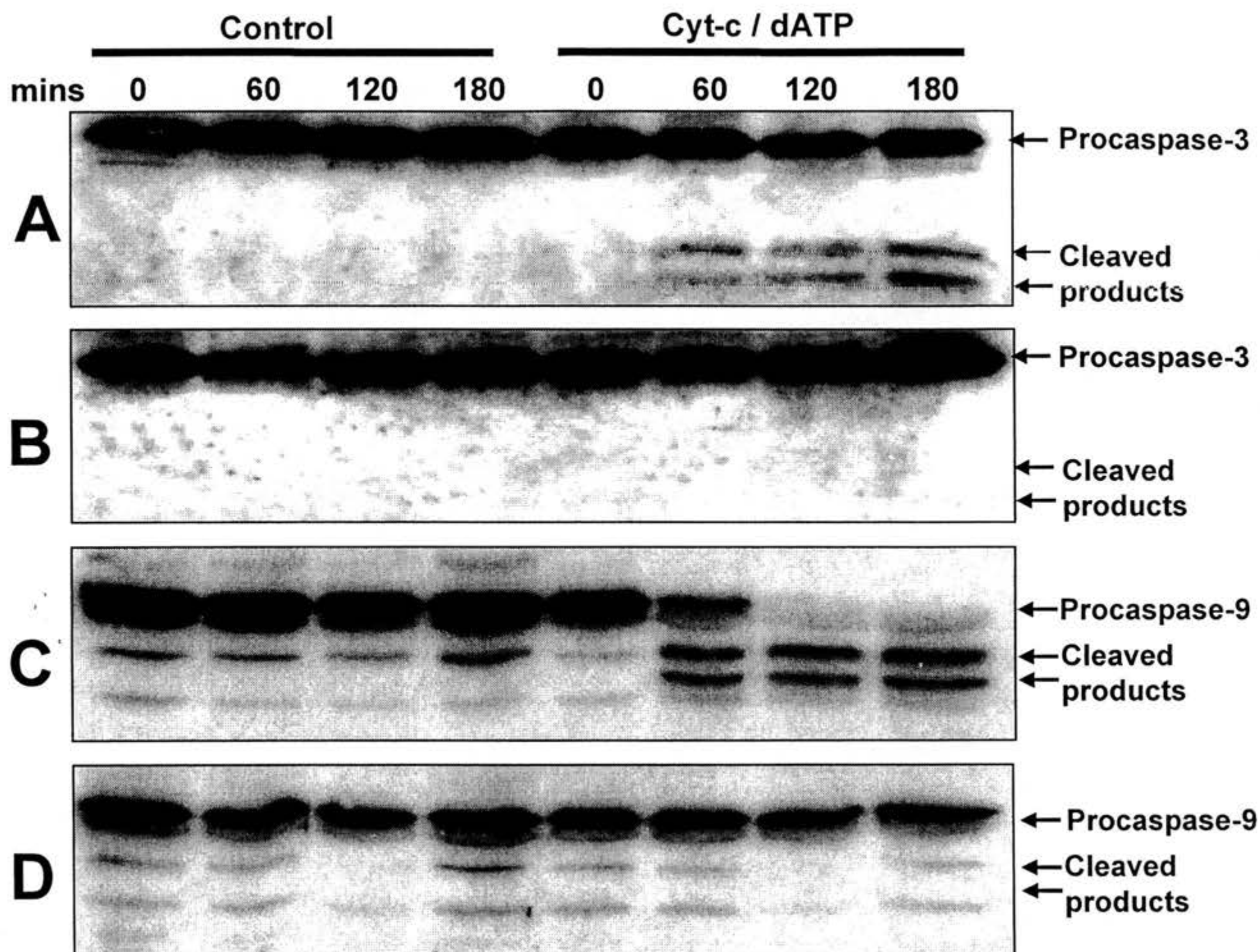


Figure 4.15. Cigarette smoke condensate inhibits caspase activation. Jurkat cells were treated for 2 hours with normal media **(A and C)** or 10% CSC **(B and D)** and cytoplasmic extracts were prepared. Apoptosome formation was initiated by addition of cytochrome-c and dATP at 37°C and samples were taken at hourly intervals and boiled for 5 minutes in laemmli buffer. Cleavage of caspase-3 **(A and B)** or caspase-9 **(C and D)** was monitored by Western blotting. Result is representative of three experiments.

4.8. Inhibition of caspase activation by cigarette smoke is not the result of a redox imbalance.

Caspases are redox sensitive enzymes that require reducing conditions to function (Melino *et al.*, 1997). It is possible that CSC treatment prevents caspase activation by inducing an oxidative intracellular environment. Therefore an antioxidant was added to the lysates prior to the addition of cytochrome-c and dATP. GSH is an important cytosolic antioxidant, which was effective at preventing the effects of CSC in earlier experiments. Either 1mM GSH, or an equivalent volume of cell extract buffer (CEB), was added to the lysates of Jurkat cells treated with either media alone or 10% CSC for 2 hours. However, the presence of GSH did not reverse CSC-induced inhibition of caspase-3 activation (Figure 4.16).

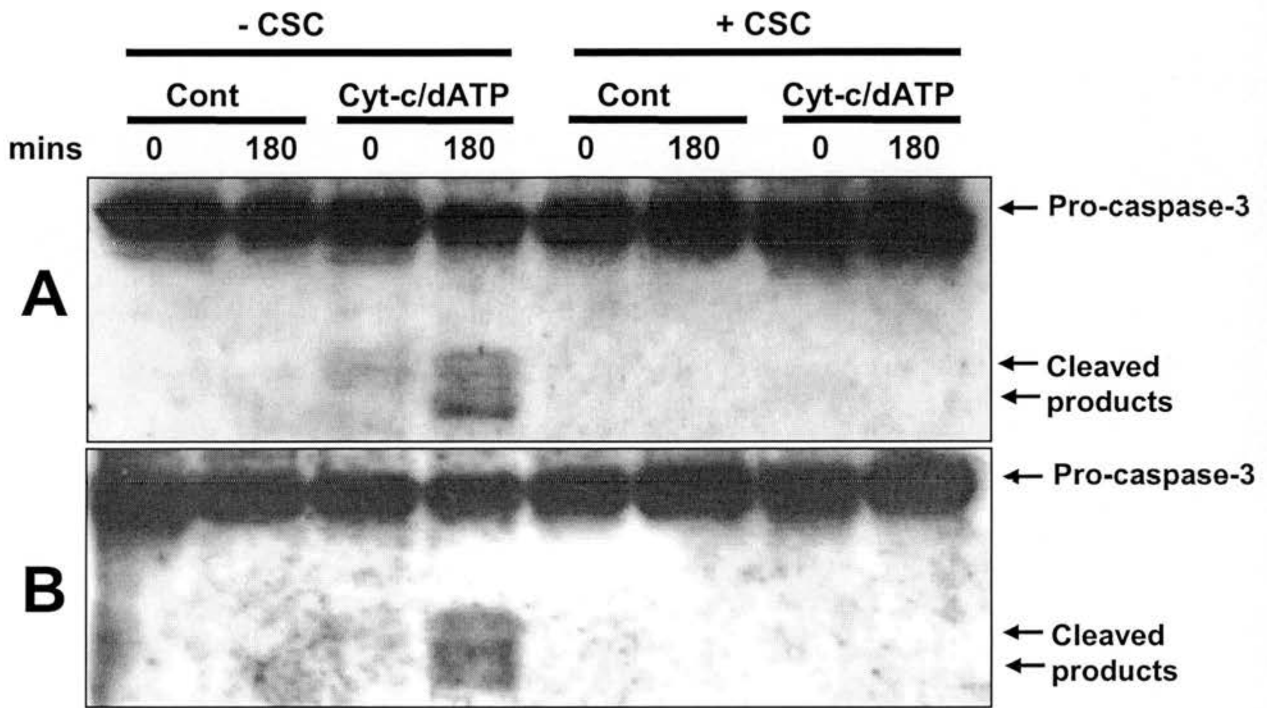


Figure 4.16: Addition of GSH does not prevent CSC induced caspase inhibition. Jurkat cells were treated for 2 hours with normal media or 10% CSC and cytoplasmic extracts were prepared. Either an equivalent volume of CEB **(A)** or, 1mM GSH **(B)** was added to the lysates, apoptosome formation was initiated by addition of cytochrome-c and dATP at 37°C and samples were taken at hourly intervals and boiled for 5 minutes in laemmli buffer. Cleavage of caspase-3 was monitored by Western blotting. Result is representative of two experiments

4.9. Inhibition of caspase activation by cigarette smoke is not the result of MAP kinase activation.

As discussed in section 1.4.2.5, phosphorylation of caspase-9 can prevent its activation. Cardone *et al.* (1998) has demonstrated that this can be mediated by PI3-kinase therefore this pathway was investigated initially. Jurkat cells were pre-treated with the PI3-kinase inhibitor, LY294002 (50μM) for 1 hour at 37°C. CSC was added to a final concentration of 10% and cells were incubated for a further 2 hours at 37°C. Analysis of apoptosome formation in lysates from these cells revealed that inhibition of PI3-kinase did not prevent caspase inhibition induced by CSC (Figure 4.17). More recently the ERK1/2 pathway has been implicated in caspase-9 phosphorylation (Allan *et al.*, 2003) and phosphorylation of ERK was observed in A549 cells in response to CSC (Figure 4.18). The inhibitor PD98059, inhibits the ERK pathway by inhibiting the kinase responsible for phosphorylation of ERK1/2, MEK1/2. However, CSC-mediated inhibition of caspase activation was not prevented in the presence of this inhibitor (Figure 4.19).

These experiments indicated that CSC-mediated caspase inhibition did not result from phosphorylation of caspase-9 by currently known mechanisms. Numerous phosphorylation sites may be present in caspase-9, therefore phosphorylation by other kinase pathways was considered. Phosphorylation of p38 was observed when A549 cells were treated with CSC (Figure 4.20), therefore this experiment was repeated using the p38 inhibitor SB203580. Caspase-3 cleavage was not detected after addition of cytochrome-c and dATP to lysates from cells treated with SB203580 and CSC (Figure 4.21), indicating that p38 was not involved in inhibition of caspase activation by CSC.

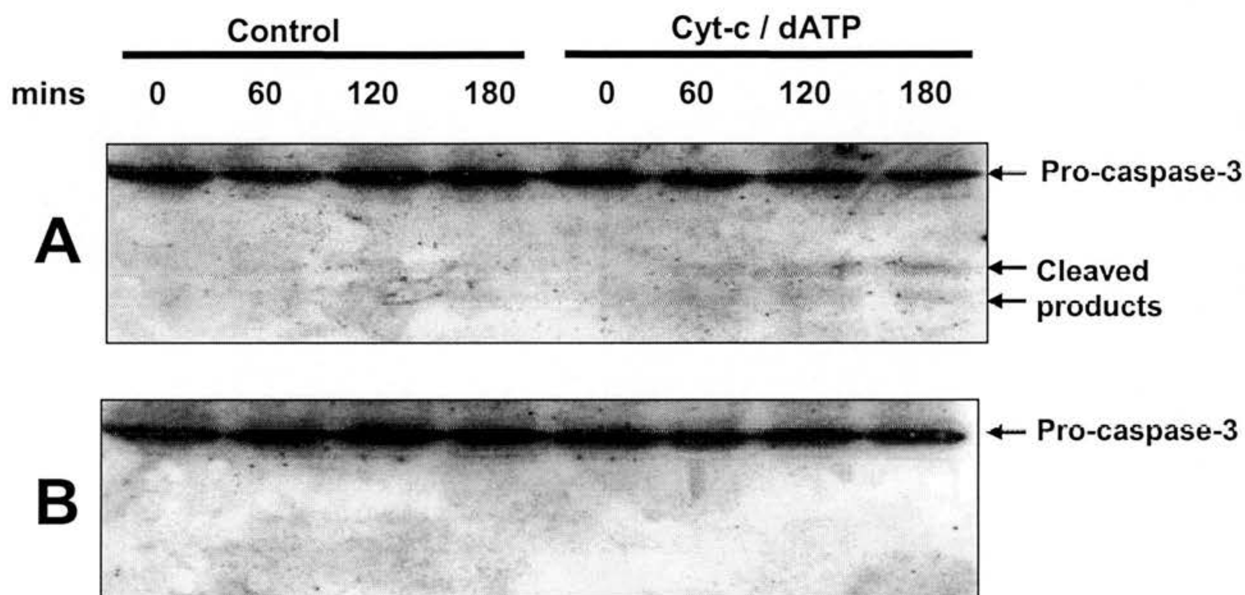


Figure 4.17: Inhibition of the P-I-3 kinase pathway does not prevent CSC induced caspase inhibition. Jurkat cells were pre-treated with 50μM LY294002 for 1 hour at 37°C prior to treatment for 2 hours with normal media **(A)** or 10% CSC **(B)** and cytoplasmic extracts were prepared. Apoptosome formation was initiated by addition of cytochrome-c and dATP at 37°C and samples were taken at hourly intervals and boiled for 5 minutes in laemmli buffer. Cleavage of caspase-3 was monitored by Western blotting. Result is representative of two experiments

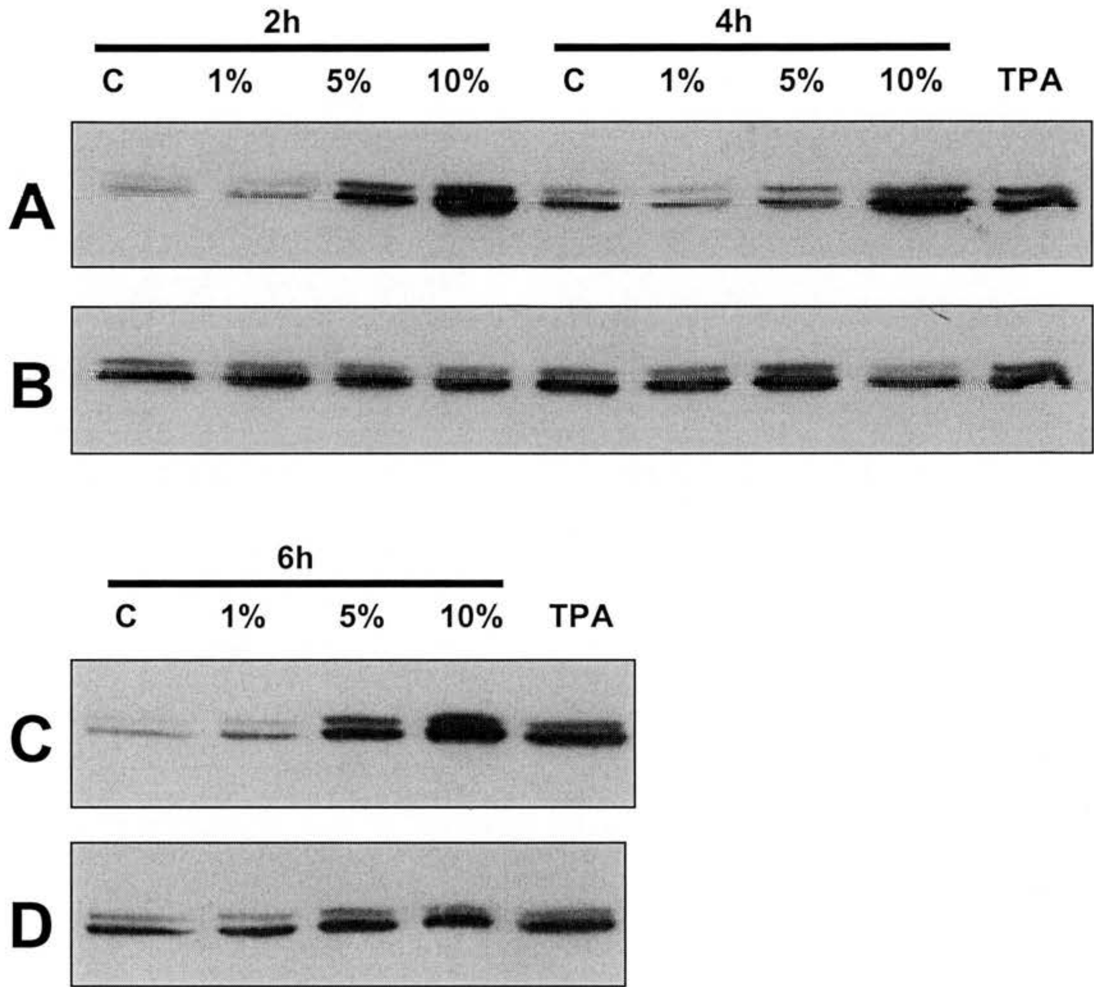


Figure 4.18: Cigarette smoke condensate induces ERK1/2 phosphorylation in A549 cells. A549 cells were treated for 2, 4 and 6 hours with either media alone, 1% CSC, 5% CSC or 10% CSC. Whole cell lysates were prepared and the levels of phosphorylated ERK was determined by Western blot **(A, C)**. To ensure increases were not due to increased expression of ERK, blots were stripped and reprobed with an antibody to native ERK **(B, D)**. Treatment with 100ng/ml 12-O-Tetradecanoylphorbol 13-acetate was used as a positive control for phosphorylation (TPA). Result representative of two experiments.

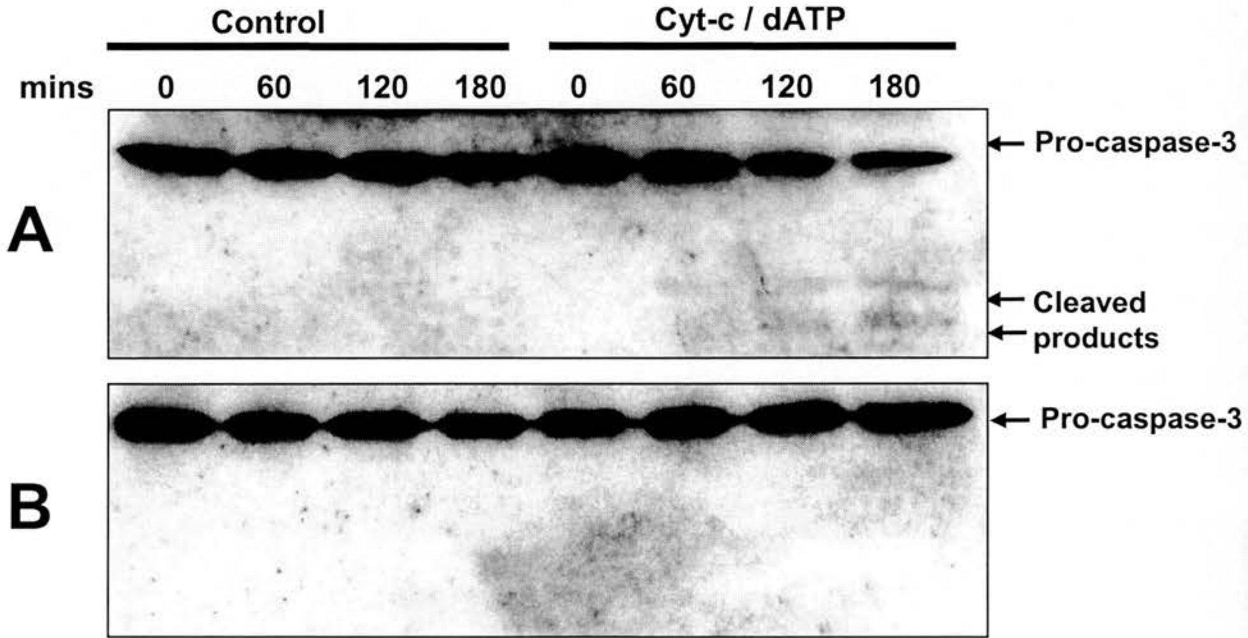


Figure 4.19: Inhibition of the ERK pathway does not prevent CSC induced caspase inhibition. Jurkat cells were pre-treated with 10μM PD98059 for 30 minutes at 37°C prior to treatment for 2 hours with normal media **(A)** or 10% CSC **(B)** and cytoplasmic extracts were prepared. Apoptosome formation was initiated by addition of cytochrome-c and dATP at 37°C and samples were taken at hourly intervals and boiled for 5 minutes in laemmli buffer. Cleavage of caspase-3 was monitored by Western blotting. Result is representative of two experiments

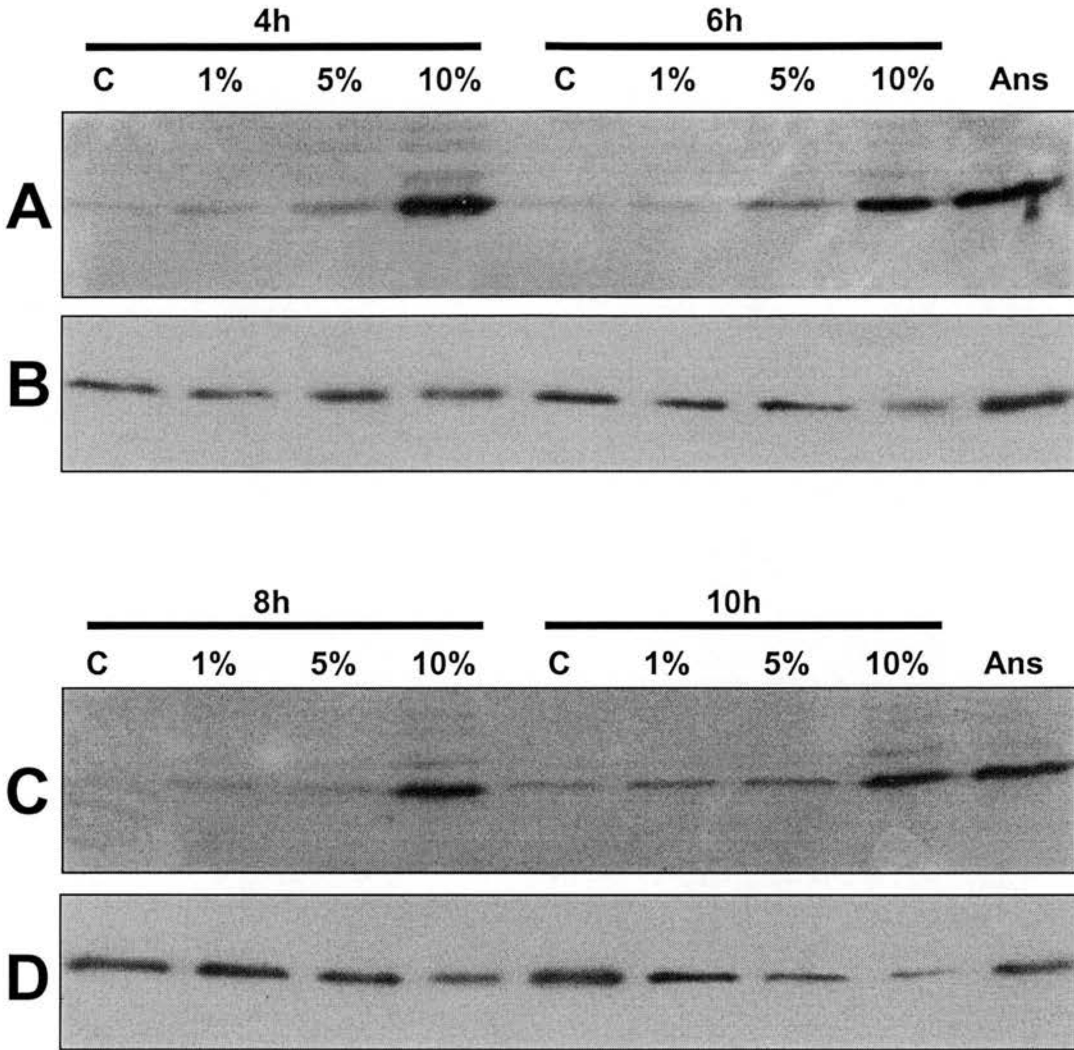


Figure 4.20: Cigarette smoke condensate induces p38 phosphorylation in A549 cells. A549 cells were treated for 2, 4 and 6 hours with either media alone, 1% CSC, 5% CSC or 10% CSC. Whole cell lysates were prepared and the levels of phosphorylated p38 was determined by Western blot **(A, C)**. To ensure increases were not due to increased expression of p38, blots were stripped and reprobed with an antibody to native p38 **(B, D)**. Treatment with 1mM Anisomycin was used as a positive control for phosphorylation (Ans). Result representative of two experiments.

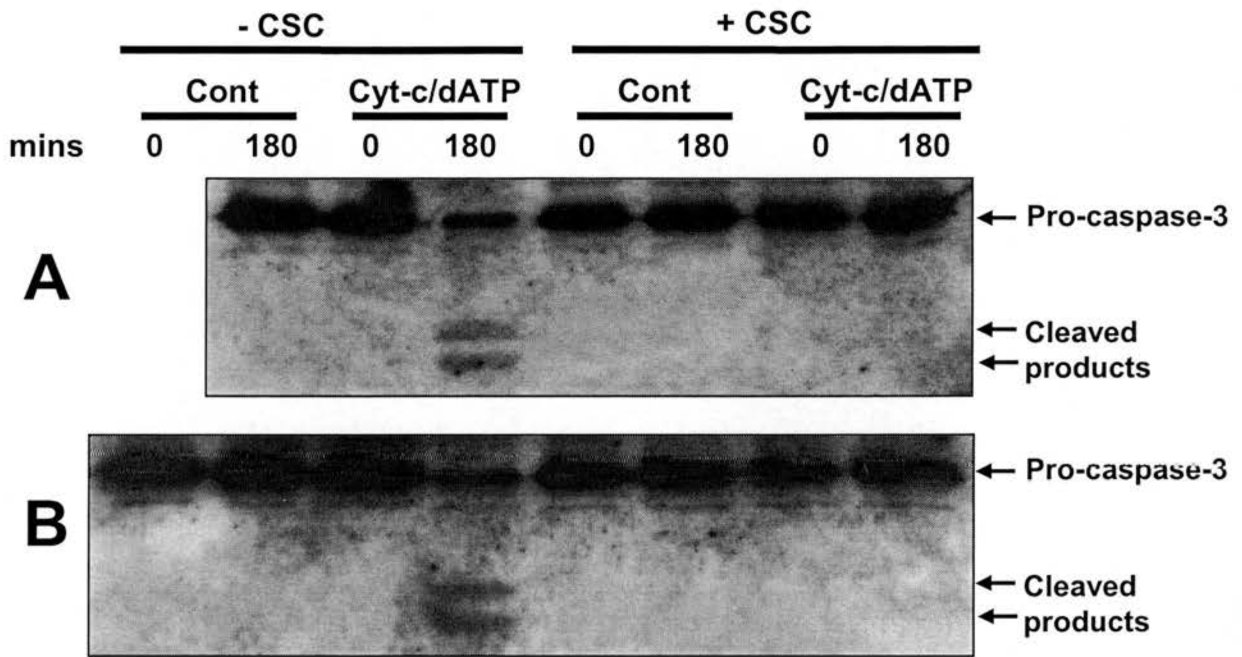


Figure 4.21: Inhibition of the p38 pathway does not prevent CSC induced caspase inhibition. Jurkat cells were pre-treated with 10μM SB203580 for 30 minutes at 37°C prior to treatment for 2 hours with normal media **(A)** or 10% CSC **(B)** and cytoplasmic extracts were prepared. Apoptosome formation was initiated by addition of cytochrome-c and dATP at 37°C and samples were taken at hourly intervals and boiled for 5 minutes in laemmli buffer. Cleavage of caspase-3 was monitored by Western blotting. Result is representative of two experiments

4.10. Cigarette smoke condensate does not affect expression of HSP-27, -70 or –90

As outlined in section 1.4.2.5, the molecular chaperones heat-shock proteins-27, -70 and -90 can regulate caspase activation by binding and sequestering components of the apoptosome. Western blot analysis of HSP-27, -70 and -90 levels in lysates from Jurkat cells treated for 1, 2, 4 and 6 hours with either normal media, SS, CSC or the combination of SS and CSC showed that the expression of these proteins was not affected by any of these treatments (Figure 4.22).

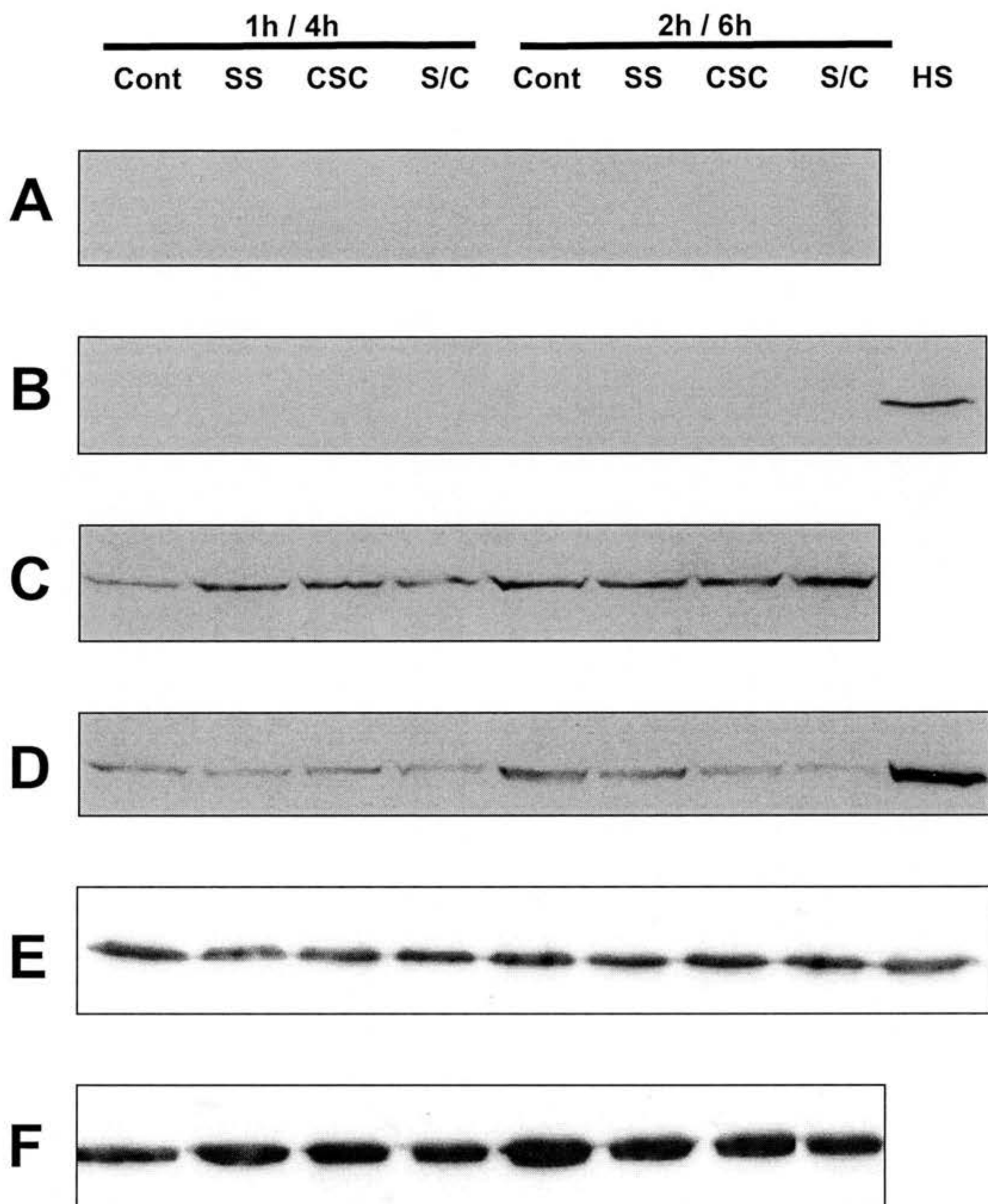


Figure 4.22: HSP-27 -70 or –90 are not increased by CSC treatment. Jurkat cells were treated for 1, 2 **(A, C. E)**, 4 and 6 hours **(B, D, F)** with normal media, 2μM SS, 10% CSC or a combination of SS and CSC prior to preparation of cytoplasmic extracts. Levels of HSP-27 **(A, B),** HSP-70 **(C, D)** and HSP-90 **(E, F)** were monitored by Western blotting. Extracts from Jurkat cells heated at 44°C for 30mins were used as a positive control (HS).

4.11. Cigarette smoke condensate inhibits caspase activation and apoptosis in A549 cells.

The studies in this chapter demonstrate that CSC inhibits caspase activation and apoptosis in a Jurkat model system. However this thesis was aimed at elucidating the effects of CSC on cell death in the lung with the intention of providing insight into the pathogenesis of emphysema. Therefore these experiments were repeated using lung epithelial cells. A549 cells were treated with 2μM SS and 1, 5, or 10% CSC for 24 hours, prior to staining with acridine orange/ethidium bromide to monitor levels of apoptosis and necrosis. In accordance with data obtained in the Jurkat model, CSC prevented SS-induced apoptosis, promoting necrosis (Figure 4.23). Moreover, treatment of A549 cells with 10% CSC prior to preparation of cytoplasmic extracts, prevented caspase activation induced by addition of cytochrome-c and dATP (Figure 4.24).

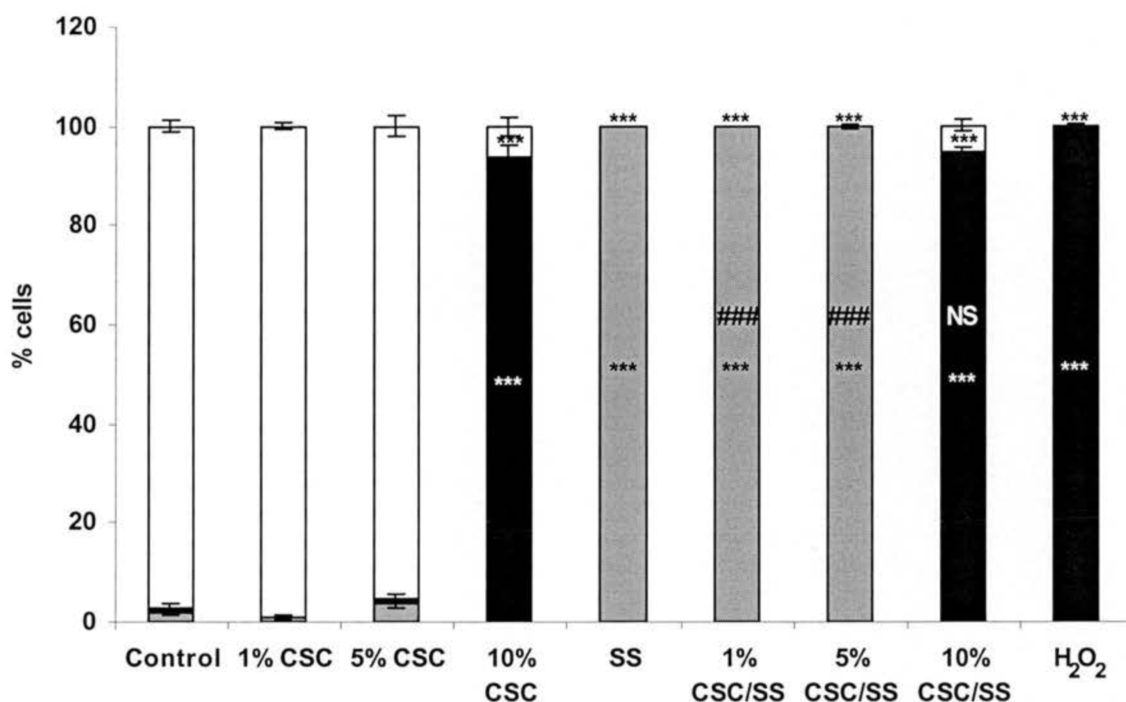


Figure 4.23. Cigarette smoke condensate prevents apoptosis and induces necrosis in A549 cells. A549 cells were incubated with either media alone, or media containing CSC, SS, H_2O_2 or a combination of SS and CSC for 24 hours. Acridine Orange and ethidium bromide were added and the percentage of viable (*open bars*), apoptotic (*grey bars*) and necrotic (*closed bars*) cells were counted. Results expressed as the mean of 3 experiments ± SEM performed in duplicate where at least 300 cells were counted per well. *** $p<0.001$ compared to control, NS non-significant; ### $p<0.001$, compared to relevant smoke alone treatment (1% and 5% CSC both apoptotic and viable values).

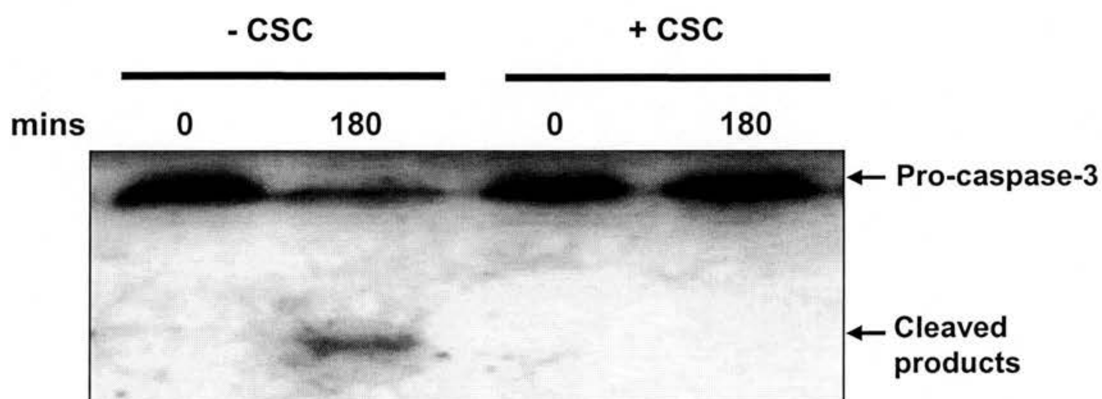


Figure 4.24: Cigarette smoke condensate prevents caspase-3 activation in A549 cells. A549 cells were exposed to CSC for 2 hours prior to preparation of cytoplasmic lysates. Apoptosome formation was initiated by addition of cytochrome-c and dATP at 37°C. Samples were taken at 0 and 180 minutes and boiled for 5 minutes in laemmli buffer. Cleavage of caspase-3 was monitored by Western blotting. Result is representative of 2 experiments

4.12. DISCUSSION

Chapter 3 showed that that CSC induced necrosis in the alveolar epithelial type II cell line (A549) and primary endothelial cells (HUVEC's) with no evidence of apoptosis. Cigarette smoking results in cellular oxidative stress and previous studies have shown that oxidative stress induces necrosis (Hampton and Orrenius, 1997; Lee and Shacter, 1999, 2000; Palomba *et al.,* 1996; Samali *et al.,* 1999). Moreover, in these studies oxidative stress prevented caspase activation and apoptosis induced by classical stimuli. Therefore it was hypothesised that CSC may act via a similar mechanism. Thus this chapter aimed to elucidate the effects of CSC on the apoptotic machinery in Jurkat cells.

That Jurkat cells also underwent necrosis in response to CSC, associated with an inhibition of apoptosis induced by SS or CH-11, was similar to results obtained by previous researchers investigating the effect of oxidative stress on cell death (Lee and Shacter, 1999, 2000; Palomba *et al.,* 1996; Samali *et al.,* 1999). Many cellular mechanisms are redox sensitive and it is assumed that the oxidants in cigarette smoke are responsible for many of the cellular responses to smoke (MacNee, 2000). However, in contrast to the previous studies (Lee and Shacter, 1999; Palomba *et al.*, 1996), CSC-induced necrosis was not a result of oxidant-induced PARP activation. In order to elucidate further the possible role of oxidative stress in CSC-mediated inhibition of apoptosis and induction of necrosis, the antioxidants mannitol, vitamin-E, GSH and DTT were used.

That CSC-mediated necrosis was not prevented by mannitol, and thus not driven by the hydroxyl radical, was a unexpected outcome given that numerous studies of the effects

of hydrogen peroxide had demonstrated almost exactly the same results as seen with CSC (Lee and Shacter, 1999, 2000; Palomba *et al.,* 1996). Hydrogen peroxide can easily break down, catalysed by the presence of reduced iron (appendix 1), to produce the most reactive oxygen radical, the hydroxyl radical (Cheesman and Slater, 1993; Nordberg and Arnér, 2001). As outlined in section 1.3.3.1, hydroxyl radicals are generated in the tar phase of cigarette smoke. Therefore, the hydroxyl radical was expected to be responsible for the effects of CSC. Mannitol had little effect on CSC-mediated necrosis. However a significant increase in apoptotic cells was observed in the co-culture experiments when SS, but not CH-11, was the apoptotic stimulus. This may indicate that removal of ·OH radicals partly restores the ability of the cell to undergo apoptosis in the presence of CSC. However this appears not to be sufficient for longer incubations. This chapter concentrated on the effects of CSC on the intrinsic apoptotic pathway, the contrasting effects of ·OH removal on apoptosis inhibition executed by the intrinsic (SS) and extrinsic (CH-11) pathway may indicate that different components of CSC prevents activation of each apoptotic pathway.

One of the consequences of hydroxyl radical production is lipid peroxidation resulting in the production of peroxyl radicals (Cheesman and Slater, 1993). Therefore peroxyl radicals could be responsible for hydrogen peroxide-mediated apoptosis inhibition and necrotic cell death (Lee and Shacter, 1999, 2000; Palomba *et al.,* 1996; Samali *et al.,* 1999). Cigarette smoke contains peroxyl radicals (section 1.3.3.1), and markers of lipid peroxidation are increased in response to cigarette smoking (Koul *et al.,* 2001; section 1.2.4.2), which can be reduced by treatment with vitamin-E (Koul *et al.,* 2001). However, the use of vitamin-E revealed that production of radicals by lipid peroxidation

was not responsible for cigarette smoke-mediated necrosis. Apoptosis induced by SS was prevented by addition of vitamin-E, which made determining the role of peroxyl radicals in CSC-mediated apoptosis inhibition difficult. This phenomenon has been observed previously (Ahlemeyer and Krieglstein, 2000; Krohn *et al.,* 1998), however no mechanistic explanation has been provided.

The thiol antioxidants GSH and DTT prevented the inhibition of caspases and induction of necrosis by CSC when SS was the stimulus. However, DTT was ineffective at preventing necrosis in the CH-11 studies. This may be due to the nature of the CH-11 molecule, an anti-Fas activating antibody of the IgM class. The antibody molecule consists of two heavy and light chains held together by interchain disulphide bonds. IgM molecules are polymers of five antibody molecules held together by a cysteine-rich J-chain. Binding of the pentameric IgM molecule to multiple Fas receptors on the cell surface mimics trimerisation, resulting in activation of caspase-8. The presence of DTT may separate the heavy and light chains of the antibody by reduction of the disulphide bonds (Roitt, 1994), disrupting the pentamer and preventing Fas ligation.

Although GSH is a scavenger for most ROS, it also functions as a detoxification agent, conjugating to electrophilic compounds via its thiol group (section 1.1.1.3.). This conjugation function was demonstrated by the ability of CSC to cause a dose-dependent decrease in total GSH, which was not purely due to oxidation, as glutathione reductase was present to convert any GSSG formed to GSH. Conjugation of GSH with components of cigarette smoke has been observed previously, the electrophilic α,β-unsaturated aldehydes, acrolein and crotonaldehyde, were identified as the predominant

compounds conjugating with GSH (Reddy *et al.,* 2002). Inhibition of the caspase pathway by electrophilic compounds has also previously been reported (Lawson *et al.,* 1999; Nobel *et al.,* 1997). Therefore, that GSH prevented CSC-mediated apoptosis inhibition and necrosis, implicated electrophilic compounds in the effects of CSC. Acrolein, which as stated earlier, is an α,β-unsaturated aldehyde that conjugates readily to thiol-containing compounds such as GSH (Chasseaud, 1979; Reddy *et al.,* 2002), has been shown to inhibit caspase activation and apoptosis (Finkelstein et al., 2001; Kern and Kehrer, 2002). However, acrolein inhibited caspase activation by direct interaction with the enzyme (Kern and Kehrer, 2002), which was not seen in response to CSC. Therefore, it is not likely that the inhibitory effects of CSC are mediated via acrolein.

As discussed earlier, cigarette smoke posseses major oxidative potential, which can be illustrated by the ability of cigarette smoke to induce oxidative modification of biological molecules (Asami *et al.*, 1999; Cross *et al.*, 1993, Reznick *et al.*, 1992). Comprehensive studies by Panda *et al.* (1999, 2001) showed that whole-phase and tar-phase cigarette smoke induces oxidation of guinea pig microsomal proteins and bovine serum albumin as detected by the formation of protein carbonyls. Such, oxidative modification induced subsequent proteolytic degradation of these proteins. Oxidative modification by cigarette smoke was completely prevented by the addition of ascorbate, partially prevented by GSH but not affected by mannitol or vitamin-E. The authors also determined that the oxidative potential resided in the tar fraction, which possesses the long-lived radicals present in cigarette smoke (section 1.3.3.1). These studies have a very similar protection profile as observed in this chapter for cigarette smoke-induced inhibition of apoptosis. Thus it may be hypothesised that the apoptosis-inhibitory

potential observed may also reside in the tar fraction. However, in order to confirm this, fractionated smoke extracts would need to be utilised.

Although oxidative stress is a major mechanism for many of the effects of cigarette smoke, nicotine, a major constituent of cigarette smoke, has also been shown to inhibit apoptosis. Nicotine can prevent apoptosis induced by UV-light (Sugano *et al.,* 2001; West *et al.,* 2003; Wright *et al.,* 1993), hydrogen peroxide (Sugano and Ito, 2000; West *et al.,* 2003), dexamethasone (Hakki *et al.,* 2002), etoposide (West *et al.,* 2003) and chemotherapy agents (Heusch and Manakjee, 1998). Moreover, incubation with nicotine has been shown to prevent caspase activation (Sugano and Ito, 2000; Sugano *et al.,* 2001; Hakki *et al.,* 2001, 2002). Treatment of cells with nicotine results in increased oxidative stress and depletion of intracellular GSH (Chang *et al.*, 2003). However none of the above studies investigated the role of oxidative stress in nicotine mediated inhibition of apoptosis. Sugano *et al.* (2001) demonstrated that nicotine prevents apoptosis at the level of cytochrome-c release. However, it is unlikely that nicotine is responsible for caspase inhibition seen in response to CSC, although cytochrome-c release was not monitored in response to CSC, inhibition of the apoptotic machinery was observed downstream of mitochondrial involvement.

In addition to preventing the morphological aspects of apoptosis, CSC treatment prevented activation of the caspase pathway. Caspases contain an active site thiol which, when modified by alkylation, oxidation or s-nitrosylation, affects their activity (Melino *et al.,* 1997). In addition to being a potent oxidising agent, cigarette smoke contains nitric oxide (Pryor and Stone, 1993) and therefore has the potential to

nitrosylate proteins. Thus, it was hypothesised that CSC may directly inactivate caspases by direct conjugation, oxidation, or nitrosylation. However, no direct effect of CSC was evident on both recombinant caspase-3 or, apoptosome formation and subsequent caspase activation. Incubation of Jurkats with CSC prior to preparation of lysates prevented activation of caspases-9 and -3, which indicated that additional factors were required to prevent caspase activation.

The caspase pathway is regulated at many stages, however few known inhibitors act at the level of caspase-9 activation. The members of the Bcl-2 family act to prevent release of cytochrome-c from the mitochondria (section 1.4.2.5). In studies in this chapter, involvement of the mitochondria was bypassed as exogenous cytochrome-c was added to the lysates, therefore the possibility that inhibition was as a result of the actions of Bcl-2 proteins was unlikely. The Inhibitor of Apoptosis Proteins (IAP's) act upon active caspase-9 and prevent activation of downstream caspases (section 1.4.2.5). As CSC treatment prevented the activation of caspase-9 it was assumed that IAP's were not involved. It was hypothesised that CSC treatment prevented formation of a functional apoptosome complex. The molecular chaperones, heat-shock proteins (HSPs) –27, -70 and –90 sequester components of the apoptosome and prevent its formation (section 1.4.2.5). Although named for their induction during the heat-shock response, these proteins are also expressed during many other situations of cellular stress, such as oxidative stress. Western blot analysis demonstrated that the expression of these proteins was not altered after CSC exposure and thus may not be involved in the prevention of caspase activation by CSC. However, while most studies that document the role of HSPs in apoptosis inhibition have monitored only upregulation or

have used overexpression studies, the ATPase function of these proteins may be necessary (Jäattella, 1999). In addition, it is also thought that co-chaperones from the DNA-J, TPR and BAG families may be required for HSP function (reviewed in Takayama *et al.*, 2003). Thus in order to exclude HSPs from CSC-mediated caspase inhibition, further investigation into the activity of the protein or presence of co-chaperones would be necessary. It has been shown that phosphorylation of procaspase-9 can prevent its activation (Allan *et al.*, 2003; Cardone *et al.*, 1998). In the experiments by Cardone *et al.* (1998) the phosphatidylinositol 3-kinase (PI3-K) pathway was acting via Akt to phosphorylate caspase-9. Whereas in the studies by Allan *et al.* (2003) procaspase-9 was phosphorylated by ERK. However the use of inhibitors revealed that neither pathway was responsible for the prevention of caspase activation by CSC. Few other endogenous inhibitors of caspase activation are known, thus CSC-mediated caspase inhibition may involve a yet unknown inhibitor, or mechanism.

Prevention of caspase activation does not always result in necrosis, in some cases inhibition of caspases enables cell survival (Chen *et al.*, 2002; Detjen *et al.*, 2001; Ko *et al.*, 2000; Lawson *et al.*, 1999; Marzo *et al.*, 2001). However, in many other situations inhibition of caspases is not sufficient to prevent death, it simply occurs via another mechanism (Coelho *et al.*, 2000; Kim *et al.*, 2000; Lemaire *et al.*, 1998; Sané and Bertrand, 1999; Uzzo *et al.*, 2001). Cigarette smoke contains many compounds and radicals, of which individual components have cytotoxic actions, thus although cigarette smoke prevents caspase activation, survival may not be possible as a result of additional insult by other components and necrosis occurs. It may also be possible that while one

component of cigarette smoke is responsible for caspase inhibition, another may induce another pathway that also leads to cell death. Cell death in response to cigarette smoke appears to be occurring via necrosis, however many forms of necrosis-like, caspase-independent, cell death occur (Leist and Jäättelä, 2001) and further investigation is needed to establish the exact mode of cell death.

An inhibition of caspase activation and apoptosis by cigarette smoke is an interesting finding however, as discussed in section 3.7, previous studies have shown an apoptotic form of cell death in response to smoke (Aoshiba *et al.,* 2001; D'Agostini *et al.,* 2000, 2001; Hoshino *et al.,* 2001; Ishii *et al.,* 2001; Ma *et al.,* 1999; Vayssier *et al.,* 2001; Wang *et al.,* 2000, 2001). Remarkably, although these studies claim that cell death occurs via apoptosis, which by definition involves the activation of caspases, only four of these studies investigated caspase activation (Aoshiba *et al.*, 2001; Hoshino *et al.*, 2001; Wang *et al.,* 2000, 2001). The study by Wang *et al.* (2000) monitored the level of apoptosis in the gastric mucosa of the rat after a whole body exposure of smoke for up to nine days. They demonstrated increased apoptosis in a time- and dose- dependent manner as detected by terminal deoxytransferase-mediated dUTP nick end labelling (TUNEL) and Western blotting for caspase-3 processing. In this investigation the tissues involved were not directly exposed to cigarette smoke, but were affected systemically after smoke inhalation by the rat. Caspase activation and apoptosis may be occurring in sites distant from the lung, either as a result of systemic oxidative stress, or the presence of elevated pro-apoptotic factors. For example, as discussed in section 1.2.2.2, increased plasma TNF-α is observed in smokers and COPD patients, which in addition to its pro-inflammatory effects, induces apoptotic cell death (reviewed in

Aggorwal, 2003). Cigarette smoke-mediated caspase inhibition is not observed in this study. This may be due to the responsible component(s) not being absorbed into the body, or not in sufficient quantities to affect other tissues. Otherwise they may have a short half-life and consequently are not present in the body for a sufficient length of time to be effective systemically. This is supported by the study by Hoshino *et al.* (2001) where CSC aged in air was no longer cytotoxic.

In the study by Wang *et al* (2001) HUVEC's were exposed to cigarette smoke extract (CSE), prepared by bubbling the smoke from 1 filter-less medium tar cigarette (8mg/cigarette) through 10mls of media (DMEM). Apoptosis was determined by oligonucleosomal DNA fragmentation, TUNEL and a caspase-3 activity assay. DNA fragmentation was observed after cigarette smoke exposure, however the data were not presented in the paper. The method used to monitor caspase activity in this study is susceptible to false interpretation; these compounds are based chemically on caspase inhibitors, which vary in their specificity (Chang and Yang, 2000). Ideally caspase activation should have been confirmed by additional methods such as Western blotting.

Although the form of cell death is classified as apoptotic, two studies support the data presented in this chapter. Alveolar macrophages treated with CSC underwent morphological changes associated with apoptosis and stained positive by TUNEL, however cell death was independent of caspase activation (Aoshiba *et al.*, 2001). Moreover, although chromatin condensation at the nuclear periphery occurred, nuclear fragmentation was rare. As outlined in section 1.4.3, caspase-independent cell death displays similar morphological changes to classical apoptosis, but with subtle

differences. In particular, AIF induces such nuclear changes however caspase activation is required for further chromatin condensation and formation of nuclear bodies (Daugas *et al.*, 2000b). Therefore, CSC treatment may have resulted in caspase-independent cell death, possibly via AIF translocation in this study.

CSC-induced apoptosis of A549 cells, detected by morphological changes, could not be prevented by treatment with the broad-spectrum caspase-inhibitor, ZVAD-fmk (Hoshino *et al.*, 2001). Therefore the authors proposed that caspase-independent cell death was occurring. However, they report oligonucleosomal DNA fragmentation in response to smoke, a marker that is deemed to be caspase-dependent. Additional similarities between the findings of this chapter and the data presented by Hoshino *et al.* (2001) are on the effects of quenching the cytotoxic effect of smoke. The GSH precursor, N-acetyl-L-cysteine (NAC) and an aldehyde scavenger, aldehyde dehydrogenase, completely abolished the cytotoxic effects of CSC. Thus this corroborates the hypothesis presented by this chapter that the electrophilic compounds in cigarette smoke may be responsible for its effects on cell death.

In conclusion, this chapter demonstrated that in addition to inducing necrotic cell death, CSC treatment prevented caspase activation and apoptosis. However, although many possible explanations were investigated, no insight into the mechanism of CSC-mediated inhibition was elucidated and further study into this area is needed. The results in this chapter were primarily obtained in a model system using Jurkat T-cells, yet emphysema is a disease of the alveoli. Although execution of the apoptotic machinery is neither cell type, nor species, specific (Zamzami *et al.,* 1996), data

obtained in a T-cell model system may not be relevant to disease pathogenesis in the lung epithelium. Therefore it was confirmed that inhibition of caspase activation and apoptosis also occurred in lung epithelial cells (A549). Consequently the effects of CSC on the Jurkat cell death machinery may also be occurring in lung epithelium, and contributing to the development of emphysema.

Chapter 5: General Conclusions and Future Work

This thesis initially aimed to elucidate the mechanisms of CSC-induced apoptosis *in vitro*. However, contrary to expectation, CSC did not induce apoptosis in A549, HUVEC or Jurkat cells, but instead promoted a necrotic form of cell death. Moreover, treatment of cells with CSC prevented caspase activation and apoptosis induced by classical stimuli, resulting in necrosis.

The initial hypothesis arose from previous studies demonstrating increased apoptotic cells in the lungs of emphysema patients (Kasahara *et al.*, 2001; Segura-Valdez *et al.*, 2000), which was supported by observed increases in apoptotic cells both *in vitro* and *in vivo* after smoke exposure (Aoshiba *et al.*, 2001; D'Agostini *et al.*, 2000, 2001; Hoshino *et al.*, 2001; Ishii *et al.*, 2001; Ma *et al.*, 1999; Vayssier *et al.*, 2001; Wang *et al.*, 2000, 2001). Discussion of the data within this thesis in the context of these previous studies is complicated, as the study of cigarette smoking both *in vivo* and *in vitro* has its limitations. Among these is the fact that human smokers only develop clinical symptoms after many years of smoking, yet animals are only exposed to smoke for a matter of weeks, and cell cultures for a matter of hours. Additionally, as discussed in section 1.2.7, only a proportion of smokers develop COPD suggesting there is a susceptibility to acquire the disease, which is difficult to incorporate into *in vivo* and *in vitro* model systems.

The use of *in vitro* cigarette smoke exposure models is further complicated by the various delivery systems. Condensate is a common surrogate for cigarette smoke exposure *in vitro*, however its production is not standardised (also discussed in section 3.7). Many different protocols are used, with each one isolating a slightly different

spectrum of components. This is dependent on a number of factors; the type of fluid the smoke is dissolved in, the volume of fluid used per cigarette, and the preparation method. The system used by many researchers involves bubbling cigarette smoke through fluid. The method of exposing cells to CSC in this thesis was used to replicate the situation in a smoker's lung, whereby passing the smoke over the buffer in the tonometer system more accurately mimics smoke filling the airspace and exposing the lung lining fluid. The complicated nature of *in vitro* cigarette smoke exposure can be evidenced by the fact that in many of the *in vitro* studies that observe cigarette smoke-induced apoptosis (Aoshiba *et al.*, 2001; Carnevali *et al.*, 2003; Hoshino *et al.*, 2001; Vassier *et al.*, 1998; Wang *et al.*, 2001), it appears that more dilute CSC was prepared and thus cells were exposed to lower doses of cigarette smoke than used in this thesis. This may explain the inconsistency in results as induction of cell death by a stimulus has been shown to be dose dependent, whereby lower doses induce apoptosis and higher doses induce necrosis (Lennon *et al.*, 1991). However in this thesis lower doses of CSC did not induce apoptosis in A549, HUVEC, or Jurkat cells. In the case of cigarette smoke exposure this dose-dependent explanation is too simplistic, due to the diverse composition of CSC obtained by different preparation methods.

An alternative explanation can be proposed for the increased numbers of apoptotic cells evident in the lungs of emphysema patients (Kasahara *et al.* 2000, 2001), and in the bronchial epithelium of rats after smoke exposure (D'Agostini *et al.*, 2001). The study of apoptosis *in vivo* is more complex than *in vitro* systems as evidence of cell death does not persist. Apoptotic cells are rarely detected *in vivo* due to their rapid and efficient removal by phagocytes (Savill *et al.*, 1993). Therefore the detection of increased

number of apoptotic cells in diseases, like emphysema, may reflect defects in clearance mechanisms rather than increases in cell death. Decreased phagocytic capability has been observed after smoke exposure (Braun *et al.,* 1998; Ortega *et al.,* 1992, 1994; Zappacosta *et al.,* 2001). Moreover, phagocytosis has been demonstrated to be depressed in individuals with COPD (Ferrara *et al.,* 1996; Muns *et al.,* 1995; Prieto *et al.,* 2000). Therefore, the observation of increased apoptotic cell death after smoke exposure may actually result from decreased phagocytosis of apoptotic cells resulting from normal tissue turnover, rather than increased apoptosis.

An interesting contrast between the data presented here and previous studies is that not only did CSC not induce apoptotic cell death, it actually prevented execution of the caspase pathway. Initial investigations provided little insight into the mechanistic aspects of CSC-mediated caspase inhibition. Therefore there is substantial scope for extension of these findings into this area. One of the hypothetical mechanisms for CSC-mediated caspase inhibition was that phosphorylation of procaspase-9 prevents its activation. However, specific kinase inhibitors had no effect on the inhibition of caspase activation induced by CSC. Therefore it would be interesting to determine whether phosphorylation of procaspase-9, or other components of the apoptosome, occurs in response to CSC. This could be determined by using alkaline phosphatase to remove any phosphate groups, which may have been added as a result of incubation with CSC, before inducing formation of the apoptosome. Further study to elucidate the phosphorylation target could be performed by P^{32} radiolabelling and immunoprecipitation for components of the apoptosome.

Nitric oxide (NO) acts at many stages to inhibit execution of the caspase pathway. Caspases can be inactivated by nitrosylation of the active site thiol (Melino *et al.*, 1999). CSC contains high levels of NO (section 1.3.3.1), and has the potential to nitrosylate proteins directly. However caspase activation was only prevented by incubation of cells with CSC prior to preparation of cytoplasmic lysates. This indicates that protein expression may be required for caspase blockade, which could be determined by treatment of cells with CSC and the protein synthesis inhibitor, cyclohexamide. The inducible form of the enzyme nitric oxide synthase (iNOS), which generates NO from arginine, is elevated after smoke exposure *in vivo* (Chang *et al.*, 2001). Thus CSC treatment may prevent activation of the caspase pathway in this system by inducing expression of iNOS. To determine whether increased iNOS expression was causative of caspase inhibition, iNOS protein levels could be monitored. In addition, CSC treatment in the presence of iNOS inhibitors, prior to apoptosome formation would demonstrate whether caspase inhibition was associated with iNOS expression.

Nitric oxide can also prevent formation of a functional apoptosome complex (Zech *et al.*, 2003). Cigarette smoke-mediated caspase inhibition was occurring at the level of caspase-9 (Chapter 4), activation of which requires the formation of a functional apoptosome. Therefore it may be that CSC prevents caspase activation by preventing apoptosome formation. Immunoprecipitation, and subsequent Western blotting for components of the apoptosome, would determine whether these proteins still associate after smoke exposure.

The involvement of hydroxyl and peroxyl radicals in CSC-mediated caspase inhibition was excluded. However, although previous studies that observed inhibition of apoptosis have used hydrogen peroxide to induce oxidative stress, other reactive oxygen and nitrogen radicals are released by cigarette smoke and their role in apoptosis inhibition should be investigated. In this thesis the deleterious effects of CSC could be prevented by the antioxidant GSH, a potent antioxidant able to quench most free radical species. Inhibition of caspase activation and apoptosis has also been prevented by the quinone compound menadione (2-methyl-1,4-naphthoquinone) (Samali *et al.*, 1999), which produces superoxide by redox cycling (Thor *et al.*, 1982). The quinone radical system in the tar phase of cigarette smoke also releases superoxide radicals and therefore the possibility that superoxide is involved in CSC-mediated caspase inhibition and necrosis should be considered. Superoxide dismutates to form hydrogen peroxide and oxygen, a reaction that can occur spontaneously or can be catalysed by superoxide dismutase (Cheesman and Slater, 1993). However, as discussed in section 4.12, the hydrogen peroxide produced can further break down to form hydroxyl radicals. In the study by Samali *et al.* (1999) the inhibitory effect of menadione on caspases could be prevented by the addition of the hydrogen peroxide scavanger, catalase, but not by superoxide dismutase. Therefore it is likely that caspase inhibition induced by menadione occurs via hydrogen peroxide and not by superoxide itself. This would involve the hydroxyl radical, which has been eliminated as being responsible for CSC-mediated caspase inhibition.

The antioxidant GSH is native to the lung and forms one of the most important lung antioxidant defences described to date (Kelly, 1999). Therefore it could be deduced that

the effects of CSC observed in this thesis would not occur *in vivo*. However, previous studies have demonstrated that in situations of acute smoking, GSH is decreased in lung lining fluid with a subsequent rebound to levels higher than that of non-smokers (Cantin *et al.,* 1987; Li *et al.,* 1994, 1996; Rahman *et al.,* 1996). It is during this window of antioxidant depletion that the cells of the lung may be susceptible to caspase inhibition and necrotic cell death as a consequence of additional cigarette smoke exposure.

As with all studies into disease pathogenesis *in vitro* findings should be further investigated in an *in vivo* model. However, there would be many problems in trying to replicate the data obtained in this thesis in *in vivo* experiments. Initially it would be important to determine the form of cell death that occurred after smoke exposure. However, while many morphological and biochemical markers of apoptotic cell death are detectable *in vivo*, it is more difficult to detect necrosis *in vivo* as many of the markers used to identify necrotic cells are not suited to the study of fixed histological sections. Theoretically, necrosis could be detected after acute smoke exposure by monitoring levels of lactate dehydrogenase (LDH) in BAL fluid after acute smoke exposure. However, determining whether cigarette smoke inhibits caspase activation and apoptosis *in vivo* would be more complex. Whereas the presence of apoptotic cells and activation of caspases in tissues can be easily detected, it is virtually impossible to detect an absence of apoptosis or lack of caspase activation *in vivo*. In order to adequately investigate the inhibitory effect of cigarette smoke, lung cell apoptosis would need to be induced by instillation of an apoptotic stimulus. Therefore, this effect may be best investigated *ex vivo*. Animals could be exposed to cigarette smoke prior to the lungs being excised and relevant cell types isolated. The ability to undergo

apoptosis in response to classical stimuli could then be determined. As discussed earlier, damage to the type I pneumocyte layer can be repaired by proliferation and differentiation of type II pneumocytes, therefore it would be important to determine whether caspase activation is prevented in the type II cell.

In addition to COPD, cigarette smoking results in the development of numerous other diseases (section 1.3.2). The data obtained in this thesis may also contribute to elucidating the mechanism by which cigarette smoking results in lung cancer. In order for an organism to remain healthy, a balance must exist between cellular proliferation and death. Cancer develops when mutated cells survive and proliferate inappropriately. Many genes have been identified that are involved in tumour development, dysregulation of these genes often confers resistance to apoptosis resulting in tumourigenesis. Cigarette smoke contains numerous carcinogenic compounds and thus has the potential to transform cells and induce tumour formation. Normally, intracellular controls "sense" the integrity of the cell and induce apoptosis if irreparable defects are present, thereby preventing proliferation of mutated cells. The combination of caspase inhibition, as observed in this thesis, and the tumourigenic potential of cigarette smoke may have catastrophic consequences for the smoker. The data obtained in this thesis demonstrates that although apoptosis is prevented, cell death still ensues; yet this was observed after continual exposure of the cells to cigarette smoke. The lungs of smokers are not relentlessly exposed to cigarette smoke, and in this thesis CSC-mediated caspase inhibition occurred within 2 hours of exposure, at which time cells remained viable. It would be interesting to investigate whether cells remain viable after

removal of cigarette smoke; and importantly, whether caspase inhibition is maintained after cigarette smoke is removed.

This thesis aimed to elucidate the role of cell death in the development of emphysema, with the hypothesis that excessive cell death would result in destruction of alveolar walls. However, the data obtained did not conform to the popular opinion that cigarette smoking results in apoptotic cell death within the lung. Instead, cigarette smoke exposure resulted in necrotic cell death. These two forms of cell death have different downstream implications. Apoptosis is thought to be an anti-inflammatory process, due to rapid phagocytic clearance before cell membrane integrity is lost, thereby preventing release of intracellular contents which would provoke inflammation and tissue damage (Savill *et al.*, 1993). However it has been demonstrated that phagocytic clearance of apoptotic bodies actively suppresses inflammation. Macrophages that ingest apoptotic cells suppress the inflammatory response in a paracrine/autocrine fashion by releasing soluble inhibitors, resulting in decreased expression of pro-inflammatory cytokines, such as IL-12, IL-8 and TNF-α (Fadok *et al.*, 1998). In contrast, exposure to necrotic cells results in increased expression of IL-8 and TNF-α by macrophages (Fadok *et al.*, 2001). Interestingly, COPD is an inflammatory lung disease with increased levels of IL-8 and TNF-α observed in the lungs and systemic circulation of COPD patients (section 1.2.2.2). Thus these inflammatory effects may be a direct consequence of cigarette smoke-induced necrosis.

Contrary to expectation, this thesis demonstrated that cigarette smoke did not induce apoptotic cell death. Moreover, cigarette smoke prevented activation of the apoptotic

machinery, and instead resulted in cell death by an alternative mechanism, necrosis. The cell death program is important for the removal of unwanted or potentially dangerous cells. As discussed in section 1.4 many active cell death pathways, often with a necrotic phenotype, have been identified indicating a degree of redundancy in the process leading to elimination of these cells. Yet, with respect to the role of cigarette smoke in the development of emphysema, necrotic cell death would not change the overall outcome of tissue destruction.

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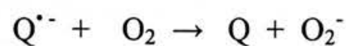
Appendices

APPENDIX 1. FREE RADICAL CHEMISTRY

A. Quinone Cycling

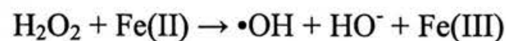
$$Q + QH_2 \rightleftharpoons 2H^+ + Q^{\bullet -}$$



$$Q^{\bullet -} + O_2 \rightarrow Q + O_2^-$$

$$2O_2^- + 2H_2 \rightarrow O_2 + H_2O_2$$

B. Fenton Chemistry

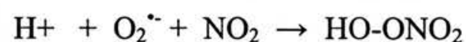


$$H_2O_2 + Fe(II) \rightarrow \bullet OH + HO^- + Fe(III)$$

$$Fe(III) + O_2^{\bullet -} \rightarrow Fe(II) + O_2$$

C. Peroxynitrite/peroxynitrate

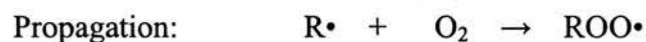
$$H+ + O_2^{\bullet -} + NO \rightarrow HO\text{-}ON{=}O$$



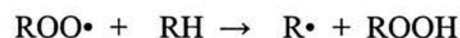
$$H+ + O_2^{\bullet -} + NO_2 \rightarrow HO\text{-}ONO_2$$

D. Lipid Peroxidation

Initiation: $In\bullet + RH \rightarrow InH + R\bullet$



Propagation: $R\bullet + O_2 \rightarrow ROO\bullet$



$$ROO\bullet + RH \rightarrow R\bullet + ROOH$$

Termination: $2ROO\bullet \rightarrow [ROOOOR] \rightarrow$ non radical products $+ O_2$

APPENDIX 2. CHEMICALS THAT HAVE BEEN REPORTED TO BE PRESENT IN CIGARETTE SMOKE

Compound	Compound
TOTAL PARTICULATE MATTER	
carbon monoxide	2-nitropropane [2B]
nicotine	hydrogen cyanide
nitrogen oxides	
ALCOHOLS	
butan-1-ol	methanol
butan-2-ol	2-methylpropanol-1
ethanol	propan-1-ol
POLYAROMATIC COMPOUNDS	
anthanthrene	7-H dibenzo-[c,g]carbazole [2B]
anthracene	dibenzo[a,e]pyrene
benzo[a]anthracene [2A]	dibenzo[a,h]pyrene
benzo[b]fluoranthene [2B]	dibenzo[a,i]pyrene [2B]
benzo[j]fluoranthene [2B]	dibenzo[a,l]pyrene [2B]
benzo[k]fluoranthene [2B]	dimethylphenanthrene
benzo[ghi]fluorene	fluoranthene
benzo[b]fluorine	fluorene
benzo[ghi]perylene	indeno[1,2,3-sd]pyrene
benzo[c]phenanthrene	1-, 2-, 3-, 4-, 5-, 6-metylchrysenes
benzo[e]pyrene	2-, 3-methylfluoranthenes
carbazole	1-methylphenanthrene
chrysene [3]	perylene
coronene	phenanthrene
dibenz[a,h]acridine	pyrene
dibenz[a,j]acridine	triphenylene
CARBOXYLIC ACIDS	
acetic acid	lactic acid
benzoic acid	phenylacetic acid
n-butyric acid	propionic acid
formic acid	succinic acid
AMMONIA AND VOLATILE AMINES	
ammonia	2-, 3-, 4-methylpyridines
dimethylamine	1-methylpyrrolidine
2,5-dimethylpyrazine	pyridine
ethylamine	pyrrolidine
methylamine	trimethylamine
methylpyrazines	hydrazine
PHENOLIC COMPOUNDS AND QUINONES	
catechol [2B]	1-napthol
4-ethylcatechol	2-napthol
ethylphenols	phenol
eugenol	resorcinol
guaiacol	4-vinylcaechol
hydroquinone	4-vinylguaiacol
3'-hydroxyisoeugenol	2-, 3-, 4-vinylphenols
isoeugenol	xylenols
3-, 4-methylcatechol	ortho-, meta-, para-cresol

AROMATIC AMINES

2-, 3-, 4-aminophenyls
aniline
2-, 3-, 4-ethylanilines
2-methyl-1-napthylamines
1-, 2-napthylamines
ortho-, meta-, para-toludines
2,3-, 2,4-, 2,5-, 2,6-dimethylanilines

LACTONES

coumarin
γ-butyrolactone

AGRICULTURAL CHEMICALS

captan
carbaryl
para-, para'-, ortho-DDD
para-, para'-, ortho-DDT
endrin
malathioin
maelic hydrazide
thiodan

VOLATILE ALDEHYDES AND KETONES

acetaldehyde [2B]
acetone
acrolein [3]
crotonaldehyde [3]
formaldehyde [2A]
furfural

OTHER VOLATILE COMPOUNDS

benzene [1]
urethane [2B]
vinyl chloride [1]

PYRIDINES AND PYRAZINES

2,3-dimethylpyrazine
2,4-, 2,5-, 2,6-lutidines
2-methylpyrazine
3-methylpyridine
2-; 3-, 4-picolines
pyridine
2-vinylpyridine
N-nitrosamines
4-[methylnitrosamino]-1-[3-pyridyl]-1-butanone
N'-nitrosoanabasine
N'-nitrosoanatabine
N-nitrosodi-*n*-butylamine
N-nitrosodiethanolamine
N-nitrosodiethylamine
N-nitrosidimethylamine
N-nitrosomethylethylamine
N'-noricotine
N-nitrosopiperdine
N-nitrosopyrrolidine

METALS

aluminium
antimony
arsenic [1]
bismuth
cadmium [1]
caesium
chromium [1]
cobalt
copper
gold
iron
lanthanum
lead [2B]
magnesium
manganese
mercury
nickel [1]
potassium
scandium
selenium
silver
sodium
telluriam
zinc
polonium-210

IARC classifications
1: known human carcinogen
2A: probable human carcinogen
2B: possible human carcinogen
3: unclassifiable as human carcinogen

APPENDIX 3: REAGENTS AND SOLUTIONS

Acridine Orange Ethidium Bromide staining

Acridine Orange/Ethidium bromide staining solution

100µl 1mg/ml AcO in PBS
200µl EtBr (500µg/ml, Sigma)
700µl PBS

Agarose gel electrophoresis

0.5M EDTA pH 8

18.61g Na_2 EDTA.$2H_2O$ (Sigma) in 70ml dH_2O
Adjust pH to 8 with 10M NaOH.
Add dH_2O to 100ml
Store at room temperature

10x TBE buffer

108g (890mM) Trizma Base (Sigma)
55g (890mM) Boric Acid (Sigma)
40ml 0.5M EDTA pH 8
Make up to 1L with dH_2O
Store at room temperature

Annexin-V/propidium Iodide staining

Annexin-V binding buffer

2mM $CaCl_2$ in Hanks balanced salt solution (HBSS, Sigma)
Store at room temperature

Propidium Iodide Solution
1mg Propidium Iodide (Molecular Probes) in 1ml dH_2O
Store at 4°C

DNA Laddering reagents

"CURRENT PROTOCOLS IN MOLECULAR BIOLOGY" PROTOCOL

5M NaCl

14.6g Sodium Chloride (Sigma) in 50ml ddH_2O
Store at room temperature

1M Tris.Cl pH 7.6

24.2g Trizma Base (Sigma) in 70ml ddH_2O,
pH to 7.6 with HCl
make up to 100ml with ddH_2O.
Store at room temperature

TE buffer (10mM Tris Cl pH 7.6, 1mM EDTA)
5ml 1M Tris.Cl pH 7.6 and 495ml dH_2O.
Add 0.185g EDTA
Store at room temperature

TTE solution (TE buffer containing 0.2% Triton X-100)
50 ml TE with 100μl Triton X-100 (Sigma)
(store at 4°C)

10x DNA loading buffer
1g Ficoll 400 (20%) Sigma, F-4375
1ml 0.5M Na_2EDTA pH 8 (0.1M)
0.5ml 10% SDS (1%)
1.25ml 1%(0.01g/ml) Bromophenol blue (0.25%)

Make to 5ml with dH_2O
Store at room temperature

<u>GSH Assay reagents</u>
0.1M Phosphate Buffer with 5mM EDTA, pH 7.5 (KPE)
Make solution A by adding 6.8g KH_2PO_4 to 500mls dH_2O – store at 4°C
Make solution B by adding 8.5g K_2HPO_4 or 11.4g $K_2HPO_4.3H_2O$ to 500mls dH_2O – store at 4°C
Make 0.1M phosphate buffer by adding 16mls of solution A to 84mls of solution B – make up fresh each time
Adjust pH to 7.5, then add 0.327g of EDTA

Extraction buffer (0.1% Triton / 0.6% SSA in KPE)
Add 20μl of Triton X-100 and 120mg of SSA to 20mls of 0.1M Phosphate buffer with 5mM EDTA, pH 7.5 – make up immediately prior to use and keep on ice.

<u>Sub G_1</u>
DNA extraction buffer
192ml 0.2M Na_2HPO_4
8ml Citric Acid
Store for several months at 4°C

PI staining solution
To 10ml 0.1% Triton X-100 in PBS add
2mg DNase free RNase A
200μl 1mg/ml Propidium Iodide
Prepare freshly

Western blotting Reagents

RIPA Lysis Buffer (whole cell lysate)

90ml CMF-PBS
1ml NP-40
0.5g deoxycholic acid
1ml 10% SDS
make up to 100ml with CMF-PBS
Store at 4°C

Anti-protease cocktail
1 protease inhibitor tablet (Roche 1836153)
dissolve in 1ml RIPA buffer
10x solution
Add 100μl to 900μl RIPA lysis buffer before use

Cell Extract Buffer (CEB, for caspase westerns)

0.075g KCl
0.03g $MgCl_2$
0.037g EDTA
0.038g EGTA
in 80ml dH_2O

Add 200μl leupeptin at 5mg/ml and 200μl aproteinin at 1mg/ml
Make up to 100ml with dH_2O.
Store at 4°C

Immediately prior to use
Prepare 100mM stock DTT in CEB (15.42mg/ml)
And 0.5M stock PMSF in DMSO (87.1mg/ml)
Use both solutions at a working concentration of 1mM

SDS PAGE Gel

	Final acrylamide concentration	
	10%	**12%**
Acrylamide-Bisacrylamide (37.5:1)	5ml	6ml
4x Tris.Cl/SDS pH 8.8	3.75ml	3.75ml
dH_2O	6.25ml	5.25ml
10% (w/v) ammonium persulphate	50μl	50μl
TEMED	10μl	10μl

4% Stacking gel	
Acrylamide-Bisacrylamide (37.5:1)	0.65ml
4x Tris.Cl/SDS pH 6.8	1.25ml
dH_2O	3.05ml
10% (w/v) ammonium persulphate	25μl
TEMED	5μl

4x Tris.Cl pH 8.8 (1.5M Tris.Cl containing 0.4% SDS)
Dissolve 91g Trizma base in 300ml H_2O.
Adjust to pH 8.8 with 1M HCl.
Add H_2O to 500ml total volume.
Filter through 0.5micron syringe filter, add 2g SDS.
Store 4°C for up to 1 month.

4x Tris.Cl pH 6.8 (0.5M Tris.Cl containing 0.4% SDS)
Dissolve 6.05g Trizma base in 40ml H_2O.
Adjust to pH 6.8 with 1M HCl.
Add H_2O to 100ml total volume.
Filter through 0.5micron syringe filter, add 0.4g SDS.
Store 4°C for up to 1 month.

1x SDS Reducing Agent
1.0ml glycerol
0.5ml β-mercaptoethanol
2.0ml 10% SDS
1.25ml 1M Tris.Cl pH 6.7
3ml 0.05% bromophenol blue
Store at 4°C

5x Running Buffer
15.1g Trizma base
72g Glycine
5g SDS
Dissolve in 400ml dH_2O
Make up to 600ml with dH_2O
Store at 4°C

Transfer Buffer (PVDF)
14.4g Glycine
3g Trizma base

Dissolve in 1L dH_2O
Store at 4°C

Transfer Buffer (nitrocellulose)
2.9g Glycine
5.8g Trizma base
0.37g SDS
Dissolve in 500ml dH_2O
Add 200ml methanol

Make up to 1L with dH_2O
Store at 4°C

10x Tris Buffered Saline (TBS)
24.2g Trizma base
87.7g NaCl
Dissolve in 800ml dH_2O
Adjust pH to 7.4 with HCl and make up to 1L with dH_2O
Store at room temperature

Strip Buffer
100ml 10% SDS (10g/100ml)
400ml dH_20
3.78g Trizma base

pH to 6.7 with conc HCl
Make up to 1L with dH_2O
Store at room temperature
Immediately prior to use add 400µl β-mercaptoethanol to 50ml strip buffer

Cigarette Smoke Prevents Apoptosis through Inhibition of Caspase Activation and Induces Necrosis

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Emphysema is characterized by enlargement of the distal airspaces in the lungs due to destruction of alveolar walls. Alveolar endothelial and epithelial cell apoptosis induced by cigarette smoke is thought to be a possible mechanism for this cell loss. In contrast, our studies show that cigarette smoke condensate (CSC) induces necrosis in alveolar epithelial cells and human umbilical vein endothelial cells. Furthermore, study of the cell death pathway in a model system using Jurkat cells revealed that in addition to inducing necrosis, CSC inhibited apoptosis induced by staurosporine or Fas ligation, with both effects prevented by the antioxidants glutathione and dithiothreitol. Time course experiments revealed that CSC inhibited an early step in the caspase cascade, whereby caspase-3 was not activated. Moreover, cell-free reconstitution of the apoptosome in cytoplasmic extracts from CSC-treated cells, by addition of cytochrome-c and dATP, did not result in activation of caspases-3 or -9. Thus, smoke treatment may alter the levels of pro- and antiapoptogenic factors downstream of the mitochondria to inhibit active apoptosome formation. Therefore, unlike previous studies, cell death in response to cigarette smoke by necrosis and not apoptosis may be responsible for the loss of alveolar walls and inflammation observed in emphysema.

Cigarette smoke is a complex mixture of chemicals containing high levels of oxidants and is the major etiologic factor in the development of chronic obstructive pulmonary disease (COPD), of which emphysema is a major component. Emphysema is characterized by enlargement of distal airspaces due to destruction of alveolar wall endothelial cells, epithelial cells, and connective tissue resulting from both protease/antiprotease and oxidant/antioxidant imbalances (1, 2). Recently, it has been proposed that apoptosis of alveolar wall cells occurs in response to cigarette smoking, resulting in progressive cell loss and emphysema (3–7).

The study of cell death, including apoptosis, has attracted intense interest as the physiologic program for deletion of harmful or unwanted cells *in vivo*. A variety of newly characterized forms of cell death have highlighted the complexity, diversity, and redundancy that exist in a cell's ability to die (8). In general, there are two main forms of cell death: apoptosis and necrosis. Apoptosis is a well defined programmed response that results in characteristic morphologic and biochemical changes, such as cell shrinkage and the condensation and fragmentation of nuclear material (9). Inappropriate apoptosis has been implicated in many pathologic conditions, such as neurodegenerative disorders and cancer (10). Necrosis, however, is regarded as a passive response to extremes of environmental stimuli, such as heat and ultraviolet light, and is characterized by cytoplasmic swelling, the rapid loss of plasma membrane integrity, and eventually cell lysis (11). It has been documented that the mode of cell death may be dependent on the cell type, the concentration of stimulus employed, and its environmental setting (12).

The most characterized effectors of apoptotic cell death are the caspases, a family of cysteine proteases that interact with each other in a hierarchical manner (13). One pathway involves induction of apoptosis by ligation of surface death receptors such as Fas and tumor necrosis factor. This so-called "extrinsic" pathway results in auto-activation of caspase 8, and the subsequent cleavage of procaspase-3 into its active subunits (14). The "intrinsic" mitochondrial pathway can be activated in response to stimuli such as ultraviolet light and oxidative stress, and results in the release of mitochondrial cytochrome c, initiating formation of the apoptosome complex (15, 16). Consisting of APAF-1, cytochrome-c, and caspase 9, in the presence of dATP, formation of the apoptosome results in the autoactivation of caspase 9 and again, activation of the effector caspase 3 (17). Through cleavage of a distinct subset of cellular substrates, caspase-3 initiates many of the key changes witnessed during apoptosis, thus explaining how a diverse range of stimuli manifest identical phenotypic outcomes during cell death (13). It has been shown that interference with one or more of these stages may result in inhibition of the entire apoptotic process (18).

Reactive oxygen species (ROS) are molecules that have been implicated in mediating apoptotic processes. Depending on the concentration, ROS have been shown to both promote and inhibit apoptosis (12, 19). Studies have also shown that oxidants, including hydrogen peroxide (H_2O_2), inhibit the apoptotic process initiated by other stimuli (19, 20–22). Inhibition of the caspase cascade (19–23) and activation of poly (ADP-ribose) polymerase (PARP) (21, 22, 24) have been proposed as mechanisms for the oxidant-mediated inhibition of apoptosis.

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Abbreviations: calcium magnesium-free phosphate-buffered saline, CMF-PBS; chronic obstructive pulmonary disease, COPD; cigarette smoke condensate, CSC, dithiothreitol, DTT; glutathione, GSH; glutathione disulphide, GSSG; lactate dehydrogenase, LDH; human umbilical vein endothelial cells, HUVECs; poly (ADP-ribose) polymerase, PARP; reactive oxygen species, ROS; staurosporine, SS.

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The aim of the present study was to characterize cigarette smoke–mediated induction of cell death in epithelial and endothelial cells. An alveolar epithelial type II cell line (A549) and primary human umbilical vein endothelial cells (HUVECs) were chosen as surrogate cells to represent alveolar epithelial type II cells and pulmonary microvascular endothelial cells. Here we demonstrate that, in contrast to previous studies (3, 4, 25–28), cigarette smoke induced necrosis with no evidence of apoptosis, and in addition was able to inhibit apoptosis induced by staurosporine (SS). The cell death machinery is ubiquitous and highly conserved; thus, Jurkat T cells are commonly used to study the mechanisms of cell death, given that they undergo apoptosis readily and display classical apoptotic markers. Therefore, Jurkat cells were used as a model system to determine the effect of cigarette smoke condensate (CSC) on their well-characterized apoptotic pathway. Here we report that cigarette smoke exposure prevents caspase activation, thus inhibiting apoptosis, and instead promoting necrosis.

Materials and Methods

Materials

All chemicals were of analytical reagent grade and purchased from Sigma Chemical Co. (Poole, UK) unless stated otherwise.

Cell Culture

A549 cells (ECACC; Porton Down, UK) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (LabTech International, Ringmer, UK), 100 U/ml penicillin, 100 μg/ml streptomycin (P/S; Invitrogen, Paisley, UK) and 2 mM L-Glutamine (L-Glut; Invitrogen). A549 cells were seeded into 6-well plates at a density of 0.3×10^6 cells per well or 96 well plates at 9×10^4 cells per well for treatment. Cells were quiesced overnight in serum-free media and subsequently treated under serum-free conditions. HUVECs were grown in EBM-2 media (BioWhittaker, Verviers, Belgium) with supplements supplied by the manufacturer. HUVECs, between passages 4 and 6, were seeded into 24-well plates at a density of 5×10^4 cells per well or 96-well plates at 1×10^4 cells per well, and cultured overnight before treatment. Cells were washed once in calcium magnesium-free phosphate-buffered saline (CMF-PBS) before being exposed to varying concentrations of CSC in full media. Jurkat cells (ECACC) were grown in RPMI supplemented with 10% fetal bovine serum, P/S, and L-Glut and treated at a cell density of 1×10^6 cells/ml with either normal media, 2 μM SS (Calbiochem, Nottingham, UK), 100 ng/ml anti-Fas activating antibody (CH-11; Upstate Biotechnology, Lake Placid, NY), 10% CSC or a combination of 2 μM SS and 10% CSC or 100 ng/ml CH-11 and 10% CSC.

Preparation of CSC

CSC was prepared fresh at a concentration of 1 cigarette/ml in CMF-PBS. Whole smoke from a king-size medium tar filter-tipped cigarette was drawn into a glass syringe and passed over CMF-PBS in a tonometer with agitation (29, 30). The condensate was sterile filtered through a 0.22-mm syringe filter before use.

Determination of Apoptosis and Necrosis

Acridine orange/ethidium bromide staining was performed as previously described (31). Briefly, treated cells were stained with 4 μg/ml acridine orange and 4 μg/ml ethidium bromide and visualized by epifluoresence microscopy. Viable (normal, green nuclei), early apoptotic (condensed, green nuclei), late apoptotic (condensed, red nuclei), and necrotic (normal, red nuclei) cells were counted. For assessment of DNA fragmentation, 2×10^6 cells were lysed in 500 ml 7 M guanidine hydrochloride and applied to Wizard SV miniprep columns (Promega, Madison, WI). Columns were centrifuged at 10,000 × *g* for 2 min, column wash solution (9 mM Tris.Cl pH 7.4, 90 mM NaCl, 2.25 mM EDTA, 55% ethanol) was applied and the columns were re-centrifuged. The wash was repeated before eluting DNA with 50 ml TE/RNase and performing electrophoresis on a 1.8% agarose gel. The lactate dehydrogenase (LDH) assay (Roche, Lewes, UK) was performed on cells grown in 96-well plates. Treated cells were incubated at 37°C for times as indicated, plates centrifuged at 250 × *g* for 10 min and 100 μl of supernatant transferred to a fresh 96-well plate. The LDH assay was performed as per manufacturer's instructions. Jurkat cells were prepared by Cytospin (Shandon, Pittsburgh, PA), stained with DiffQuick (Dade Behring, Marburg, Germany), and viable, apoptotic and necrotic cells counted by brightfield microscopy (Olympus, London, UK). Two Cytospins were prepared for each treatment and at least 300 cells counted per slide.

Cell-Free Apoptosis Assay and Western Blotting

The assay was performed as previously described (32). Briefly, Jurkat or A549 cells, were incubated in cell extract buffer (CEB, 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 100 μM PMSF, 10 μg/ml leupeptin, 2 μg/ml aprotinin) on ice for 10 min, before passing through a 25-G needle ten times and centrifuged at 10,000 × *g* for 15 min at 4°C. Supernatants were removed and stored at −70°C until used. Cell-free apoptosis was initiated by the addition of 10 μM cytochrome-c and 1 mM dATP to the extracts, followed by incubation at 37°C. Aliquots were removed at the time points indicated, 4× Laemmli buffer added, and the sample heated to 96°C for 5 min. Samples equivalent to 1×10^6 cells were analyzed by Western blot using a polyclonal anti–caspase-3 antibody, or a polyclonal anti–caspase-9 antibody (Pharmingen, Oxford, UK) recognizing both pro- and active forms. The signal was detected using a horseradish peroxidase–conjugated secondary antibody (Santa Cruz, Wembley, UK) and enhanced chemiluminesence (ECL; Amersham, Little Chalfont, UK).

Assessment of Recombinant Caspase Activity

Caspase-3 activity was determined using the caspase-3 assay kit (Calbiochem), as per manufacturer's instructions. Briefly, active caspase-3 (30 U) was placed into a half-volume 96-well plate before addition of inhibitor, 1, 5, 10% CSC, or 1 mM H_2O_2. The plate was incubated at 37°C for 1 h before addition of colorimetric caspase-3 substrate, DEVD-pNA. Caspase-3 activity was determined by measuring the change in absorbance at 405 nm after 2.5 h at 37°C.

Measurement of Glutathione Levels

Four sets of glutathione (GSH) standards were prepared in KPE buffer (0.1 M phosphate buffer, 5 mM EDTA, pH 7.4), CSC added to a final concentration of 1, 5, or 10%, and solutions incubated with agitation for 1 h at 37°C. GSH levels were measured using a microplate assay adapted from the enzymatic method developed by Tietze and coworkers (33) and Vandeputte and colleagues (34).

In brief, 1.67 U/ml GSH reductase and 0.2 mg/ml dithiobisnitrobenzoic acid were added to the GSH standards for 30 s to enable the conversion of GSSG to GSH before 0.2 mg/ml reduced β-nicotinamide adenoside diphosphate (β-NADPH) was added. The change in absorbance was measured over 2 min at 405 nm in a microplate reader (MRX).

Statistical Analysis

ANOVA was performed using the MiniTab package with Tukey's post-testing to determine significance between treatments. A P value < 0.05 was deemed significant.

Results

CSC Induces Necrosis in Epithelial and Endothelial Cells

A significant increase in LDH release from A549 cells and HUVECs was seen in a dose-dependent manner after a 24-h exposure to CSC (Figure 1A). LDH release from A549 cells was also time-dependent (Figure 1B). In addition, acridine orange and ethidium bromide staining revealed that CSC induced necrosis, with no evidence of apoptosis in either A549 cells or HUVECs (Figures 2 and 3). The absence of apoptosis in response to CSC was further confirmed by a number of methods, including electron microscopy, oligonucleosomal DNA fragmentation, and chromatin condensation assessed by Hoechst 33342, over a range of doses and time points (data not shown). In contrast, however, apoptosis was induced by staurosporine in both cell types (Figures 2E and 3E). Interestingly, A549 cells co-cultured with SS and CSC together did not die by apoptosis, but instead underwent necrosis (Figure 4), thereby implicating a potential for a direct inhibitory effect of CSC on the apoptotic machinery. These results clearly demonstrate that epithelial and endothelial cells undergo necrosis, not apoptosis, in response to CSC.

CSC Prevents SS- and CH-11–Induced Apoptosis, Resulting in Necrosis

The Jurkat cell is ubiquitously used to study the "core" molecular machinery of cell death, thus this model was employed to elucidate the effect of CSC on the caspase pathway in particular. Jurkat cells treated simultaneously with SS or CH-11 underwent the classical morphologic and biochemical changes indicative of apoptosis, such as chromosomal condensation and oligonucleosomal DNA fragmentation, or laddering (Figures 5A–5C). However, as ob-

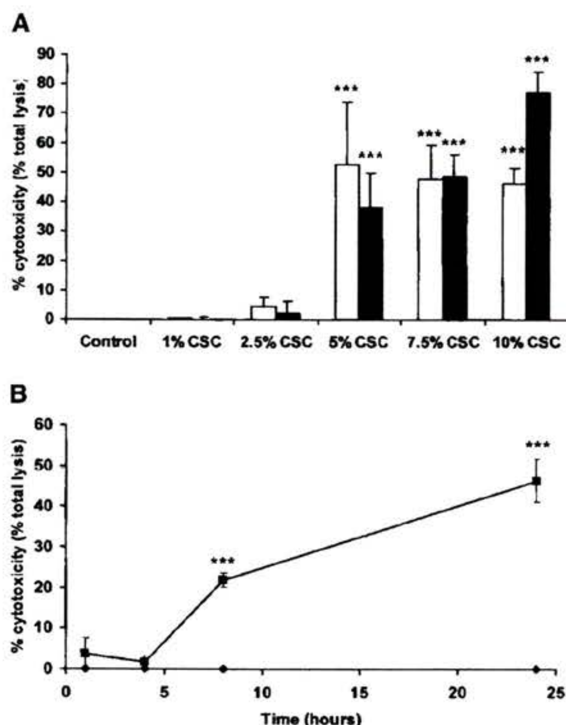

Figure 1. CSC induces necrosis in A549 alveolar epithelial cells and HUVECs. (*A*) A549 cells (*open bars*) and HUVECs (*closed bars*) were exposed to 1–10% CSC for 24 h and the percentage of LDH released into the culture media was determined, compared with a total lysis control (1% Triton X-100). (*B*) Cytotoxicity to A549 cells in response to 10% CSC (*closed squares*) was time-dependent; control media (*closed diamonds*). Results are mean of three experiments ± SEM. $^{***}P < 0.001$ compared with control.

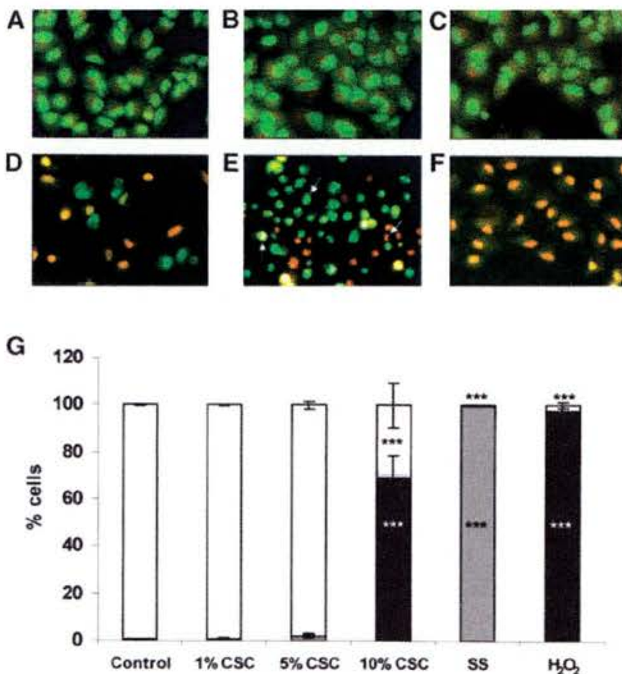

Figure 2. CSC induces necrosis in A549 alveolar epithelial cells with no evidence of apoptosis. A549 cells were exposed to media alone (*A*), 1% CSC (*B*), 5% CSC (*C*), 10% CSC (*D*), 2 μM SS (*E*), or 5 mM H_2O_2 (*F*) for up to 24 h. Acridine orange and ethidium bromide staining was performed, and the percentage of viable (*white bars*), apoptotic (*gray bars*), and necrotic (*black bars*) cells was determined (*G*, 24 h shown). Note classical apoptotic nuclei in *E* (*arrows*) and absence of this morphology in *B*, *C*, and *D*. Results are mean of three experiments ± SEM. $^{***}P < 0.001$ compared with control. *Magnification:* ×36.

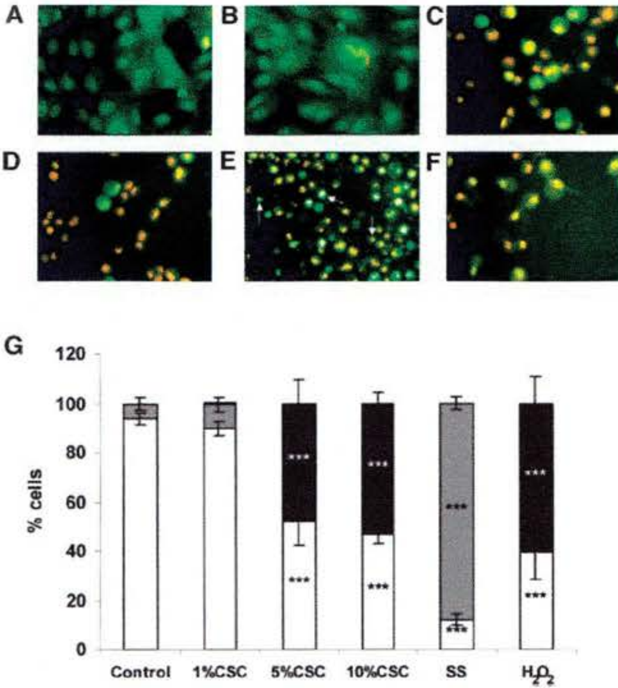

Figure 3. CSC induces necrosis in HUVECs with no evidence of apoptosis. HUVECs were exposed to media alone (*A*), 1% CSC (*B*), 5% CSC (*C*), 10% CSC (*D*), 2 μM SS (*E*), or 5 mM H_2O_2 (*F*) for up to 24 h. Acridine orange and ethidium bromide staining was performed and the percentage of viable (*white bars*), apoptotic (*gray bars*), and necrotic (*black bars*) cells was determined (*G*, 24 h shown). Note classical apoptotic nuclei in *E* (*arrows*) and absence of this morphology in *B*, *C*, and *D*. Results are mean of three experiments ± SEM. $***P < 0.001$ compared with control. *Magnification:* ×36.

served with A549 cells, apoptosis did not occur when CSC was added to the SS- or CH-11–treated cells or after CSC treatment alone (Figures 5D–5G). Morphologically, the predominant form of cell death in the presence of CSC was necrosis. This necrotic mode of cell death was underscored by the concurrent release of LDH from the cells treated with CSC or a combination of SS and CSC over 24 h (Figure 6). A low level of LDH release was observed from cells incubated with SS alone and this was attributed to secondary necrosis given the absence of phagocytic clearance. These results indicate that cigarette smoke prevents apoptosis induced by SS or CH-11, and instead promotes necrosis.

To ascertain more accurately the stage of apoptosis that was inhibited, Jurkat cells were treated with SS and 10% CSC was added at hourly intervals, after which the cells were coincubated for the remainder of the experiment. A higher ratio of apoptotic cells to necrotic cells was seen as the time between SS and CSC treatment increased (Figure 7). This suggests that CSC may impede an early phase in the apoptotic pathway, as CSC only affects apoptosis when present in the early stages of the process.

CSC-Induced Necrosis Is Not Mediated by Oxidative Stress

Inhibition of apoptosis and induction of necrosis has been demonstrated in response to oxidants such as H_2O_2 (20–22), which is present in cigarette smoke (35). Therefore, to determine whether the oxidant component of cigarette smoke was mediating inhibition of apoptosis, SS-exposed Jurkat cells were treated with CSC in the presence of various antioxidants and evaluated by morphology and DNA laddering. No effect was seen on the necrosis-inducing ability of CSC in the presence of the antioxidant mannitol. However, GSH and dithiothreitol (DTT) prevented necrosis induced by CSC, with cells remaining viable, whereas cells cocultured with SS and CSC underwent apoptosis rather than necrosis (Table 1 and Figure 8).

An alternative role for GSH *in vivo* is in the detoxification of electrophilic compounds by direct conjugation via the thiol group. A dose-dependent decrease in measurable GSH was observed after incubation with CSC (results expressed as percentage of control: 1% CSC, 80.66 ± 4.4; 5% CSC, 17.97 ± 5.15, $P < 0.001$; 10% CSC, 7.11 ± 0.44, $P < 0.001$). The assay employed measures both reduced (GSH) and oxidized (GSSG) glutathione. If oxidation had taken place, then no decrease in total GSH would have occurred.

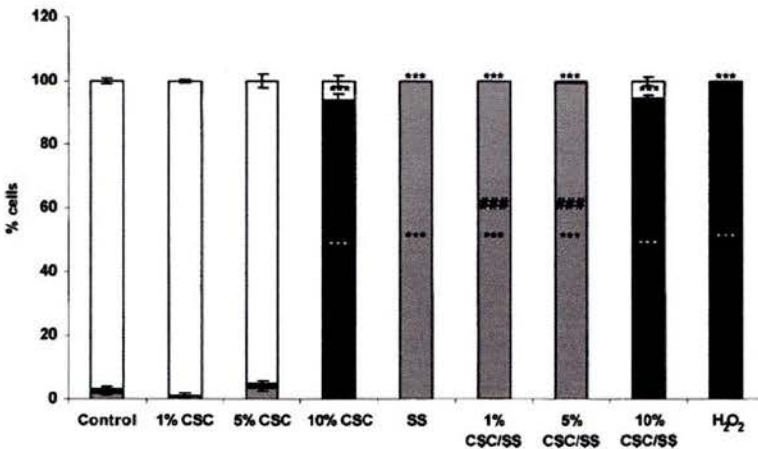

Figure 4. CSC prevents apoptosis and induces necrosis in A549 cells. A549 cells were incubated with either media alone, or media containing CSC, 2 μM SS, 5 mM H_2O_2, or a combination of SS and CSC for 24 h. The percentage of viable (*white bars*), apoptotic (*gray bars*), and necrotic (*black bars*) cells was determined following acridine orange and ethidium bromide staining. Results expressed as the mean of three experiments ± SEM. $***P < 0.001$ compared with control. 1% CSC/SS and 5% CSC/SS were significantly different from smoke alone treatments ($P < 0.001$).

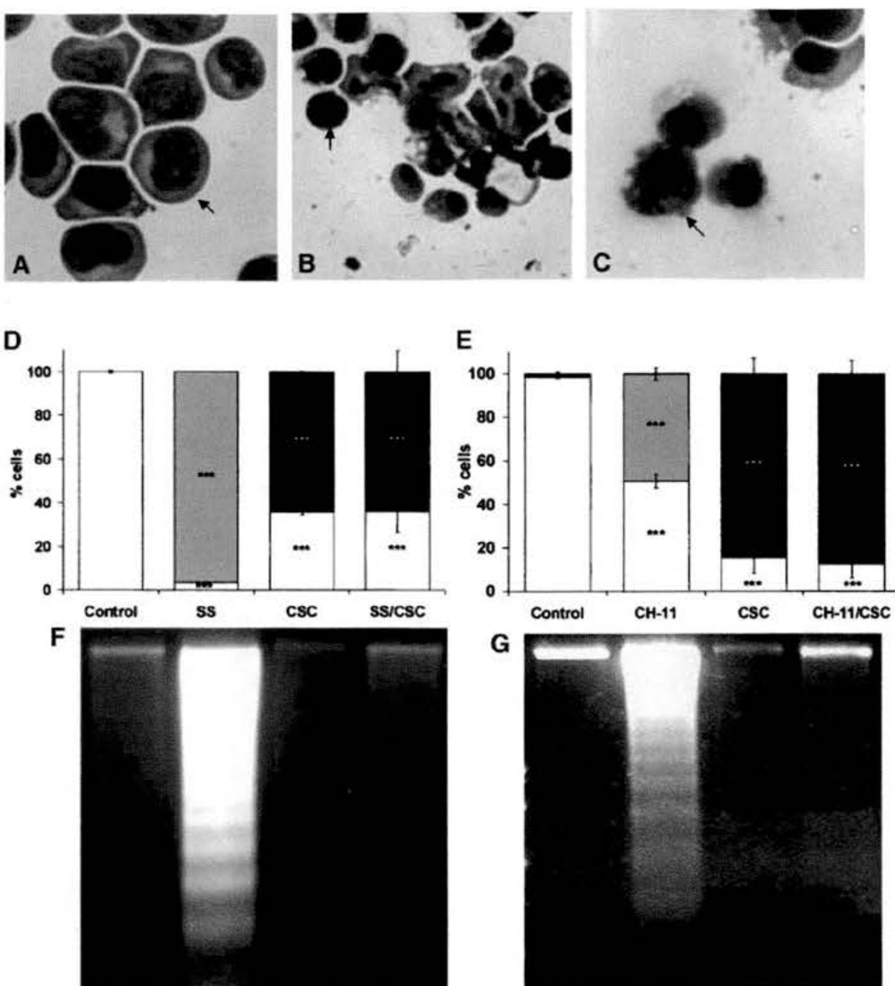

Figure 5. CSC switches apoptosis induced by SS or CH-11 to necrosis. Jurkat cells were treated with SS, CH.11, 10% CSC, or a combination as indicated. After a 6-h exposure (*D*) or 9-h exposure (*E*), death was assessed by morphology and the percentage of normal (*A*, *white bars*), apoptotic (*B*, *gray bars*), and necrotic (*C*, *black bars*) cells was determined. Results represent mean of three experiments ± SEM, where at least 300 cells were counted per slide. $^{***}P < 0.001$ compared with control. Apoptosis was confirmed by the presence of oligonucleosomal DNA fragments, or so-called "ladders" (*F*, *G*).

Thus, this data implies that cigarette smoke components may conjugate with GSH.

In addition, unlike in response to oxidants (21, 22, 24), the presence of a PARP inhibitor, 3-aminobenzinamide, did not alter the relative levels of apoptosis and necrosis in response to SS and CSC (data not shown), implying that depletion of ATP as a result of PARP activation is not involved in CSC-mediated cell death. These data suggest that oxidative stress induced by CSC is not responsible for inhibition of apoptosis and induction of necrosis.

CSC Inhibits Caspase-3 Cleavage but Not Activity

Because it was established that CSC affected an early stage of apoptosis, caspase activation was investigated. Caspases can be inhibited by direct modification; however, CSC had no direct effect on recombinant caspase-3 activity (data not shown). Caspases exist as an inactive proform that is cleaved to yield active subunits; thus, activation can be monitored by Western blot. Assessment of caspase-3 activation in Jurkat cells revealed cleavage of the proform after SS treatment, but not after CSC or coculture with SS and CSC (Figure 8), indicating that the caspase pathway may be halted in the presence of CSC. Furthermore, when cells were treated with SS and CSC in the presence of DTT or GSH, caspase-3 cleavage occurred, whereas mannitol was again not effective (Figure 8). Thus, GSH and DTT are able to "quench" or antagonize components in CSC that prevent caspase-3 activation.

Cell-Free Reconstitution of the Apoptosome Reveals CSC-Mediated Inhibition of Apoptosome Formation

To ascertain how CSC affected the caspase pathway, an active apoptosome was reconstituted in a cell-free system with cytochrome-c and dATP, in either the presence of CSC, or in extracts prepared from cells treated with CSC. No effect on activation of caspase-3 was observed when CSC was added to the lysates directly (data not shown). However, in extracts from Jurkats exposed to CSC for 2 h before preparation, neither caspase-3 nor caspase-9 activation occurred, in contrast to the progressive processing to the active form seen in lysates from untreated cells (Figures 9A–9D). This caspase inhibition was not dependent on PI3-kinase activation, due

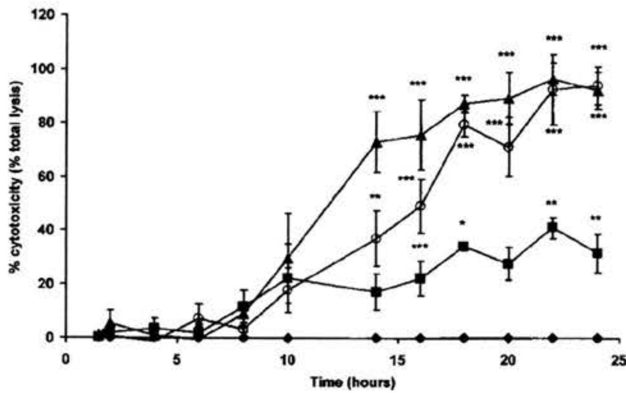

Figure 6. CSC induces LDH release from Jurkat cells in a time-dependent manner. Cells were treated with control media (*diamonds*), 2 μM SS (*squares*), 10% CSC (*triangles*), or a combination of SS and CSC (*open circles*), and the level of released LDH measured over 24 h was determined. Results are expressed as percentage LDH release compared with a total lysis control. Mean of three experiments performed in triplicate ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with untreated cells.

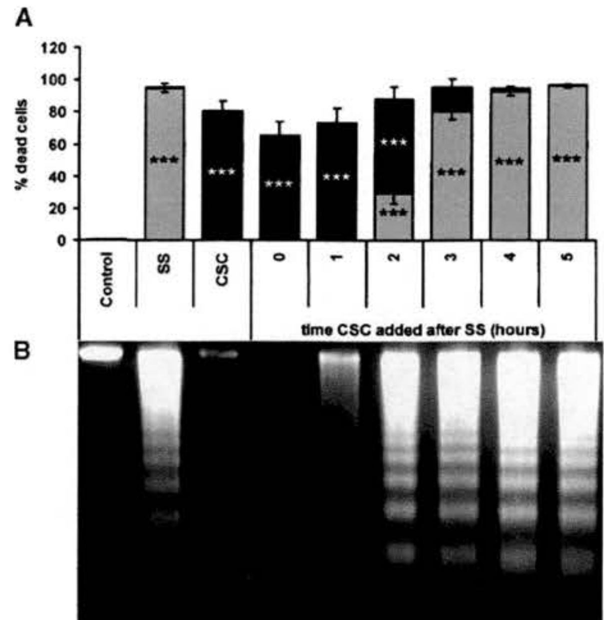

Figure 7. Cigarette smoke can switch SS-induced apoptosis to necrosis when added after SS treatment. Jurkat cells were treated with 2 μM SS, followed by addition of 10% CSC at hourly intervals. Six hours after initial SS treatment cytospins were prepared and the percentage of normal (*white bars*), apoptotic (*gray bars*), and necrotic (*black bars*) cells was determined (*A*). Apoptosis was confirmed by the presence of oligonucleosomal DNA "ladders" (*B*). Results are mean of three experiments ± SEM, where at least 300 cells were counted per slide. $***P < 0.001$ compared with control.

to the lack of effect of the inhibitor LY294002 (data not shown). Moreover, caspase-3 was not cleaved after reconstitution of the apoptosome in lysates from A549 cells treated with CSC (Figure 9E). Thus, cigarette smoke prevents apoptosis by inhibiting the formation of an active apoptosome complex and the subsequent activation of caspase-9 and -3.

Discussion

Cigarette smoking is the major etiologic factor in the pathogenesis of emphysema. The gas phase of cigarette smoke contains many free radicals and oxidant molecules, with the potential to generate additional oxidant molecules via redox cycling, leading to an increased oxidative burden in the lungs of smokers (35). Therefore, cigarette smoke inhalation leads to a depletion of antioxidants, release of inflammatory mediators, and an increase in epithelial permeability (36–39). Recent evidence has suggested that apoptosis of lung cells may be a factor in cigarette smoke–induced emphysema (3–5, 7, 40). In contrast, our studies show that CSC does not induce apoptosis and in fact induces necrosis in the alveolar epithelial type II cell line (A549) and primary endothelial cells (HUVECs). Interestingly, CSC also prevented apoptosis induced by SS in A549 cells.

Oxidative stress is responsible for many of the effects of cigarette smoking. Given that oxidants, such as hydrogen peroxide, have been shown to inhibit apoptosis and induce necrosis (19–22) we hypothesized that CSC may function in a similar manner. Jurkat cells, which undergo apoptosis readily and display easily identifiable markers, treated with CSC underwent necrosis. Moreover, Jurkat cells cocultured with a combination of SS or CH-11 and CSC also underwent necrosis, comparable to A549 cells, with no evidence of apoptosis. Therefore it appeared that CSC was inhibiting apoptosis and promoting necrosis.

An investigation into the role of oxidants demonstrated that GSH and DTT protected against CSC-induced necrosis, and prevented the inhibition of apoptosis usually seen during SS and CSC coculture. However, the antioxidant mannitol was ineffective. GSH is an important antioxidant *in vivo*; however, it also fulfils other vital roles such as regulation of immune function, signal transduction, metabolism, and the detoxification of electrophilic compounds (41–45). Detoxification occurs via the thiol group under the control of GSH–S-transferases, although conjugation is also observed in the absence of the enzyme (43, 45). A dose-dependent decrease in measurable GSH, in the absence of GSSG formation, after incubation with CSC was observed, indicating that GSH may form conjugates with the many electrophilic compounds present in cigarette smoke. Considering that the thiol compounds GSH and DTT were both able to prevent CSC-induced necrosis and CSC-mediated inhibition of apoptosis, whereas mannitol was not, indicates that electrophilic compounds, not oxidants, may be responsible for these effects.

To elucidate the mechanism of apoptosis inhibition, the caspase pathway was studied in more detail. Western blot analysis revealed that caspase-3 activation did not occur in cells treated with CSC. Moreover, treatment with GSH and DTT, and not mannitol, prevented the inhibitory effect of CSC on caspase activation. Caspases contain a central thiol group that is essential for function and prone to oxidation,

TABLE 1

The antioxidants DTT and GSH prevent CSC-induced necrosis and inhibition of apoptosis

		% Apoptosis	% Necrosis
Control	No antioxidants	0.3 ± 0.1	0.1 ± 0.1
	Mannitol	0.3 ± 0.2	0.2 ± 0.1
	DTT	2.4 ± 1.3	0.7 ± 0.4
	GSH	0.4 ± 0.2	0.2 ± 0.2
SS	No antioxidants	94.9 ± 2.6*	0.4 ± 0.2
	Mannitol	97.4 ± 1.2*	0.9 ± 0.7
	DTT	90.3 ± 4.2*	0.1 ± 0.1
	GSH	93.4 ± 4.5*	0.2 ± 0.1
CSC	No antioxidants	0.0 ± 0.0	74.6 ± 5.0*
	Mannitol	0.0 ± 0.0	75.4 ± 11.8*
	DTT	0.4 ± 0.3	16.3 ± 1.7†
	GSH	12.9 ± 4.7	0.3 ± 0.3†
SS/CSC	No antioxidants	0.0 ± 0.0	69.5 ± 4.9*
	Mannitol	23.2 ± 18.4†	61.4 ± 18.5*
	DTT	79.5 ± 11.4*†	0.6 ± 0.4†
	GSH	98.5 ± 0.5*†	0.3 ± 0.2†

$^*P < 0.001$ compared with antioxidant control.

$^†P < 0.001$ compared with no antioxidant treatment.

Jurkat cells were treated as indicated with 5 mM mannitol, 1 mM dithiothreitol, or 1 mM glutathione for 6 h. Cytospins were prepared and the percentage of normal, apoptotic and necrotic cells were determined. Results are mean of three experiments ± SEM where at least 300 cells were counted per slide.

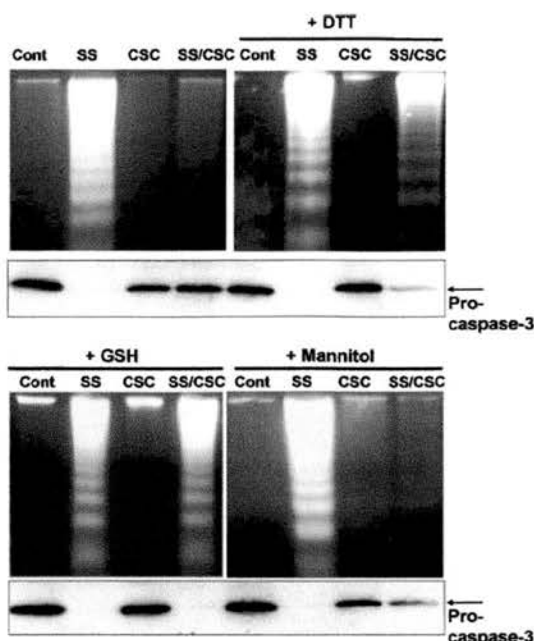

Figure 8. Activation of caspase-3 and subsequent DNA "laddering" does not occur in Jurkats treated with CSC or SS/CSC. Cells were treated with 2 μM SS and/or 10% CSC in the presence of the thiol antioxidants DTT or GSH, or mannitol. Activation of caspase-3 was determined by Western blot for loss of the pro form and the resultant DNA "laddering" was determined by agarose gel electrophoresis. Representative of three experiments.

alkylation, and s-nitrosylation (46). However, direct addition of CSC to recombinant caspase-3 had no effect on the ability of the enzyme to cleave its substrate.

The effect of CSC on caspase activation was studied further by reconstitution of the apoptosome, in cytoplasmic extracts of Jurkat cells, on addition of cytochrome-c and dATP. The occurrence of caspase-3 cleavage, as determined by Western blot, was used as a positive indicator of apoptosome formation and caspase-9 activation. Initially, CSC was incubated with the extracts before addition of cytochrome-c and dATP, whereby caspase-3 cleavage was observed, indicating that CSC had no direct effect on formation of an active apoptosome. Interestingly, in extracts from Jurkat cells treated with CSC for 2 h before preparation, caspase-3 activation was prevented. Moreover, caspase-9 activation did not occur, indicating CSC treatment prevented the formation of a functional apoptosome. Importantly, apoptosome formation and caspase-3 cleavage was also prevented by CSC treatment of A549 lysates, thus underscoring this effect on the cell death machinery to be more general and not Jurkat-specific. Numerous inducible regulators of apoptosis exist, presenting the possibility that CSC exposure may mediate an alteration of the intracellular balance between pro- and antiapoptogenic factors.

Although Jurkat cells were used as a model here, the involvement of T cells in the development of emphysema must not be overlooked. Increased numbers of T cells are observed in the lungs of emphysema sufferers, and their presence is correlated with increased lung destruction (47). Necrosis of T cells present in the lung may also contribute to the progression of emphysema by increasing local tissue damage by release of intracellular contents.

The data presented here are in contradiction to some previous studies (3, 4, 25–28). However, a number of these have used TUNEL nick end labeling to identify apoptotic cells (26, 28), a method that merely detects DNA strand breaks. Cigarette smoke exposure results in oxidant-induced DNA strand breaks (48), and so these studies may have inadvertently identified cells with oxidant-induced DNA damage as apoptotic. Moreover, caspase activation was either not involved (4), or not studied (27), in "cigarette smoke–induced apoptosis". Characterization of cell death is becoming increasingly complex. A number of alternative forms of cell death have been identified with many of the "classical" markers of apoptosis evident. However, in some cases cell death is independent of caspase activation (8). For this reason, it is becoming increasingly necessary to characterize cell death by a number of methods. This presents a plausible explanation as to why previous studies have purported to observe apoptosis in response to smoke exposure. Additionally, no standardized protocol for the production of CSC exists; each procedure can isolate a slightly different spectrum of components. Nevertheless, we believe that our method of exposing cells to CSC replicates the situation in a smoker's lung, whereby passing the smoke over the buffer in the tonometer system more accurately mimics smoke filling the airspace and exposing the lung lining fluid.

The key finding of this study is that cigarette smoke induces necrosis in alveolar type II cells, endothelial cells, and Jurkat cells. Moreover, CSC inhibited caspase activation and apoptosis in A549 and Jurkat cells. From these

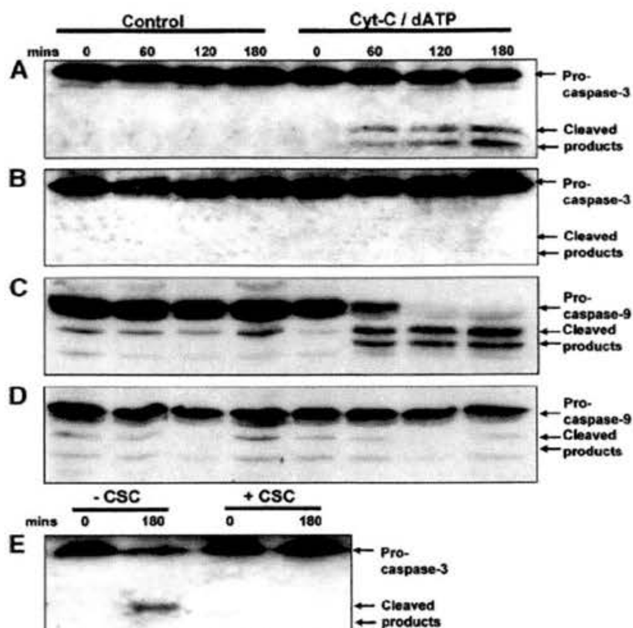

Figure 9. CSC treatment inhibits apoptosome formation and subsequent activation of the caspase pathway. Jurkat cells were incubated for 2 h with either control media (*A*, *C*) or 10% CSC (*B*, *D*). Cytoplasmic extracts were prepared and reconstitution of the apoptosome was initiated by addition of 10 mM cytochrome-c and 1 mM dATP. Western blotting for caspase-3 (*A*, *B*), or caspase-9 (*C*, *D*) indicated that apoptosome formation and activation of the caspase cascade did not occur in CSC-treated lysates. Similarly, CSC treatment inhibited apoptosome formation and caspase-3 activation in identically prepared A549 cell lysates (*E*). Result is representative of three experiments.

findings, we suggest a mechanism whereby cigarette smoke may induce emphysema. Although initially thought to be due to apoptosis (3–5, 7, 40), emphysema may in fact result from loss of alveolar tissue by necrosis of lung epithelial and endothelial cells. In these experiments, the effects of CSC could be prevented by the presence of extracellular thiol compounds such as GSH, which is native to the lung and forms one of the most important lung antioxidant defenses (49). Therefore, it could be deduced that the risk of necrosis in response to cigarette smoking is insignificant. However, in situations of acute smoking, GSH has been shown to be decreased in lung lining fluid with a subsequent rebound to levels higher than those of nonsmokers (36, 38, 50, 51). During this window of antioxidant depletion, the cells of the lung are likely to be susceptible to necrotic cell death as a consequence of additional cigarette smoke exposure. Moreover, local tissue damage may be amplified by the subsequent release of intracellular enzymes and lysosomal contents, resulting in recruitment of inflammatory cells to the site of injury and further necrosis of surrounding tissue. This scenario is supported by Retamales and coworkers (52), who observed increased inflammation in increased severity of emphysema. Thus, although much interest has been generated on the involvement of apoptosis, our studies emphasize the role of necrosis and the subsequent proinflammatory responses as more likely candidates in the pathogenesis of emphysema.

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