Molecular Mechanisms Mediating the Induction of Apoptosis by Chemopreventive Selenium Compounds

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"There is only one serious philosophical problem. It is suicide. To judge whether life is or not worth living ....."

Camus, 1955

(who may have got it right for selenium and cancer prevention)

Abstract

Prospective studies and recent intervention trials suggest that the risk of some cancers may be inversely related to selenium intake and this is supported by strong experimental evidence from animal cancer models. How this cancer-protective effect is mediated is unclear but interference with the balance of growth/apoptosis during tumor outgrowth is a likely hypothesis. In general, there is a correlation between the effectiveness of selenium compounds as chemopreventive agents *in vivo* and their ability to inhibit cell growth and induce apoptosis *in vitro*. As processes of cellular growth regulation are intimately regulated by intracellular signalling, this study has investigated the signal transduction pathways affected by selenium compounds in several cell culture systems.

Using the cervical carcinoma cell line, HeLa, molecular mediators of selenium-induced apoptosis were investigated. These studies were later extended to biopsies of normal human oral mucosa cells (NOMCs) and human oral carcinoma cells (SCCs), using a primary culture system. Two selenium compounds were tested: selenodiglutathione (SDG), the primary metabolite of selenite, the most commonly used cancer-protective selenium compound in animal models, and the synthetic compound, 1,4-phenylenebis (methylene)selenocyanate (pXSC), one of the most potent chemopreventive selenium compounds. Both compounds induced apoptosis in the cells tested and this was associated with a strong induction of Fas ligand (Fasl). Blocking Fas activation resulted in inhibition of apoptosis. Activities of stress kinases JNK (c-jun N-terminal kinase) and p38 were also induced on selenium treatment and intervention studies using specific chemical inhibitors and/or dominant negative mutants suggested that JNK, but not p38, was functionally important for the induction of apoptosis and Fasl. The relative levels of induction of JNK activity and Fasl also correlated with the extent of apoptosis observed. On treatment with SDG, but not pXSC, SCCs were found to preferentially activate the JNK/Fasl pathway compared to NOMCs and this correlated with enhanced sensitivity of SCCs to SDG-induced apoptosis. Both SDG and pXSC also appeared to modulate activities of other signalling proteins like extracellular regulated kinases (ERKs) 1,2 & 5 and Akt. However, functional interference with these pathways using specific chemical inhibitors or constitutively activated mutants revealed either none or a small effect on apoptosis-induction. One exception was the activation of Akt by SDG in SCCs: inhibiting its activity sensitised the cells to apoptosis significantly. Finally, preliminary xenograft studies have confirmed the induction of apoptosis *in vivo*, using chemopreventive dietary levels of selenium.

Table of Contents

List of Tables

List of Figures

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Abbreviations

AIF	apoptosis inducing factor
ASK1	apoptosis signal-regulated kinase 1
bp	base pair
BrdU	5-Bromo-2'-deoxy-uridine
BSA	bovine serum albumin
BSC	benzylselenocyanate
Ca^{2+}	calcium
cdk	cyclin dependent kinase
cm	centimeter
CO_2	carbon dioxide
C-terminal	carboxy terminal
d	day/s
DAB	3,3'-diaminobenzidine
DMBA	7,12-dimethylbenz[a]anthracene
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid
ERK	extracellular signal regulated protein kinase
FACS	fluorescence activated cell sorting
FAD	flavin adenine dinucleotide
Fasl	Fas ligand
FCS	foetal calf serum
FGF	fibroblast growth factor
G0	quiescence phase
G1	gap/growth phase 1
G2	gap/growth phase 2
GADD	growth arrest and DNA damage inducible
GFP	green fluorescent protein
GGSMC	γ-Glutamyl-Se-methylselenocysteine
GPx	glutathione peroxidase
GST	glutathione-S-transferases

HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
IgG	immunoglobulin G
IL-2	interleukin-2
JNK	c-jun N-terminal kinase
kb	kilobases
kDa	kilodalton
m	month/s
M	molar
MAPK	mitogen activated protein kinase
μCi	microcurie
mg	milligram
μg	microgram
min	minute/s
ml	millilitre
μl	microlitre
mm	millimeter
mM	millimolar
μM	micromolar
MNU	methylnitrosourea
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NaF	sodium fluoride
NK cells	natural killer cells
NNK	4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone
NOMC	human normal oral mucosa-derived cell
N-terminal	amino terminal
O_2	oxygen
OD	optical density
o/n	overnight
PARP	poly(ADP-ribose)polymerase
p38	p38 kinase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PI	propidium iodide
PKA	protein kinase A

PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulphonyl fluoride
ppm	parts per million
pXSC	1,4-phenylene-*bis*-(methylene)selenocyanate
Rb	retinoblastoma protein
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription-polymerase chain reaction
S	synthesis phase
SAC	Se-allylselenocysteine
SCC	human squamous cell carcinoma cell
SDS	sodium dodecylsulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Sec	selenocysteine
SECIS	selenocysteine insertion sequence
SM	selenomethionine
SMC	Se-methylselenocysteine
SSB	single strand breaks
TEMED	N,N,N′,N′-Tetramethyl-ethylenediamine
TNFα	tumour necrosis factor-α
TPA	tetradecanoyl phorbol-13-acetate
TPS	triphenylselenonium chloride
tRNA	transfer RNA
TR	thioredoxin reductase
Trx	thioredoxin
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling
U	unit
UV	ultraviolet radiation
v/v	volume for volume
w/v	weight for volume
y	year/s

1 Selenium in Biology and Disease

1.1 Introduction

Selenium has been puzzling researchers ever since its discovery in 1817. In biology, it was long considered to be toxic (Franke, 1934) until it was identified as growth factor in certain bacteria (Pinsent, 1954) and an essential trace element in rats (Schwarz and Foltz, 1957); regarded as a potential carcinogen for years but now widely accepted as a potential anti-tumorigenic agent (Combs Jr et al., 2001); extensively used as an antioxidant, but has also been noted to undergo pro-oxidant redox cycling (Seko and Imura, 1997); an functional component of peroxidases but also shown to use peroxides to form cellular structures (Ursini et al., 1999).

Study of biological roles played by selenium started in the 1930's when it was demonstrated to be the dietary component responsible for certain diseases in animals grazing in specific areas of the American grasslands (reviewed in (Hatfield, 2000)). This prompted the United States Department of Agriculture (USDA) to map the selenium content of forage in the USA and it was shown that selenium content of the plants vary according to where they were grown. Thus effectively identifying selenium-deficient areas in the USA. Later, the identification of selenium as an essential nutrient prompted investigations into several veterinary diseases, like white muscle disease in sheep, endemic to the low-selenium areas. Supplementation of selenium not only had a positive response in these diseases but also on disease previously related to vitamin E deficiency, like mulberry heart disease of pigs, suggesting for the first time that selenium may function as an antioxidant (reviewed in (Hatfield, 2000)).

The evidence for selenium as a micronutrient in humans came from studies in China investigating the childhood cardiomyopathy, Keshan disease (Gu, 1983). The disease was endemic to certain regions and the veterinary history of these areas showed similar diseases in cattle as those caused by selenium deficiency. Supplementation of selenium to affected children protected them from the ravages of the disease, thus identifying selenium deficiency with increased susceptibility to cardiomyopathy in humans (Gu, 1983).

These studies proved to be a turning point in selenium research as the focus now shifted, from mechanisms of toxicity, to its role in normal biochemistry. This effort led to identification of selenoproteins, the major agents underlying the role of selenium in

promoting health. Research over the last 3 decades has unequivocally implicated selenium to be an essential nutrient of fundamental importance to human physiology and the role of selenoproteins in maintaining human health has become more apparent.

Selenium is a component of selenoproteins as the 21st amino acid, selenocysteine (Chambers et al., 1986; Zinoni et al., 1986). It is now clear that several of these selenoenzymes are selenium-dependent as selenocysteine forms a critical component of their catalytic sites (Chambers et al., 1986; Zinoni et al., 1986).

Additionally, selenium also influences other major human health issues like, cancer prevention and tumour cell surveillance but these are not likely to be mediated exclusively by selenoprotein activities (reviewed in (Rayman, 2000)).

1.2 Acute selenium deficiency and health conditions

Selenium enters the food chain through plants, which enrich it from the soil. Low soil selenium content, or soil low in bio-available selenium due to complex formation with metals, reduce the selenium content of plants growing in these conditions. The consequences are primarily observed in livestock grazing in such areas and also in humans predominantly reliant on local produce.

Animal-deficiency diseases have been extensively identified since the discovery that selenium was a trace nutrient necessary for anaerobic growth of E.coli on glucose and in the prevention of liver necrosis in rats (Pinsent, 1954; Schwarz and Foltz, 1957). Selenium deficiency-related pathologies, like reproductive defects and white-muscle disease, had such a severe impact on the economy that selenium supplementation of high-risk animals is now routine (Combs and Combs Jr, 1986). Recent studies have also implicated the incidence of transmissible spongiform encephalopathy (TSE) in cattle to be partially related to selenium deficiency (Purdey, 2000).

In humans, the major conditions recognised to stem from selenium-deficiency are the endemic cardiomyopathy, Keshan disease and Kashin-Beck disease – a debilitating osteoarthropathy. Both were first identified in an area of China with extremely low selenium content in the soil (Cheng et al., 1990; Gu, 1983; Li et al., 2000).

As discussed in detail later, low selenium status has also been associated with a significantly increased risk of cancer incidence. A 2-6 fold (depending on the study)

increased risk has been identified in the cohort with lowest serum selenium concentrations (Rayman, 2000).

1.3 Health effects of mild selenium deficiency

A large body of evidence has accumulated suggesting mild selenium deficiency can adversely affect health and enhance disease susceptibility. One of the confounding factors in such analyses is that while selenium may directly affect the disease process, it may also be an outcome of the condition itself, perhaps aiding disease progression. However, carefully controlled clinical and prospective trials can effectively discriminate between these alterations. Selenium status appears to influence a large number physiological activities with profound implications on human health, some of the major issues are discussed next.

1.3.1 Reproduction

Selenium deficiency has been recorded to be associated with incidence of miscarriage in livestock and selenium supplementation appears to prevent early pregnancy loss (Stuart and Oehme, 1982). In humans, an association between low serum selenium and first trimester and recurrent miscarriages have been reported (Barrington et al., 1996). This is thought to be associated with a reduced protection from oxidative damage resulting from diminished activity of glutathione peroxidases (GPx), the first selenoprotein to be characterised (Barrington et al., 1996). A later study, however, did not find differences in selenium levels between the control group and non-pregnant women with a history of recurrent miscarriage (Nicoll et al., 1999). However, as this study did not exclude women who had had a miscarriage from the control group, it is possible that this may have confounded the result.

Male fertility is affected by selenium as it is required for formation of testosterone and normal development of spermatozoa (Behne et al., 1996). A trial held in Scotland found supplementation of sub-fertile men with 100μg/day selenium for 3 months significantly increased sperm motility and reproductive success (Scott et al., 1998). However, a similar trial in Poland on sub-fertile men using 200μg/day for 3 months found no association with supplementation and sperm motility (Iwanier and Zachara, 1995).

A mechanistic explanation for these findings came from studies on rat spermatogenesis. Typically, the mid-piece of the sperm which embeds the helix of mitochondria in a keratin-

like material, is morphologically altered in selenium deficiency; not only is sperm motility decreased, but the head and the tail may become disconnected because of fractures of the midpiece material (Wu et al., 1973). In selenium-sufficient animals the element is associated with the mitochondrial capsule material, which becomes fragile on selenium deficiency (Ursini et al., 1999). It was demonstrated that an isoform of GPx, phospholipid hydroperoxide GPx (PHGPx or GPx4), is abundantly expressed in spermatids and protects them from oxidative damage. However, in fully mature spermatozoa it was found to have no peroxidase activity and was transformed to an insoluble structural protein accounting for almost 50% of all proteins in the capsule (Ursini et al., 1999). This polymerisation, conceivably, confers the structural stability required for normal sperm activity.

1.3.2 Immune function

Selenium deficiency has been strongly associated with loss of immunocompetence and is attributed to attenuated cell-mediated immunity and B-cell function (Spallholz et al., 1990). Supplementation with selenium, has a significant immunostimulatory effect, including clonal expansion of activated T cells (Kiremidjian-Schumacher et al., 1994). For example, supplementation with 200μg/day selenite resulted in enhanced response of leukocytes to antigen stimulation, development of cytotoxic T cells and destruction of tumour cells (Kiremidjian-Schumacher et al., 1994). The activity of natural-killer cells (NK-cells) was also increased on selenium supplementation (Kiremidjian-Schumacher et al., 1996).

The explanation for these effects may be as follows. Activated lymphocytes and NK-cells require interleukin-2 (IL-2) stimulation for clonal expansion and differentiation to cytotoxic T cells. Selenium is thought to promote IL-2 stimulation directly by transcriptionally upregulating the IL-2 receptors in these cells (Roy et al., 1992; Roy et al., 1994), thus providing a possible mechanistic explanation for immunostimulatory effects of selenium. There are indications that selenium may have more diverse roles in regulating the immune system. For example, several T-cell associated genes are putative selenoproteins and this is complemented by an increased selenophosphate synthetase (a crucial enzyme involved in selenocysteine synthesis) activity in activated T cells (Guimaraes et al., 1996; Taylor, 1995).

1.3.3 Viral Infection

A newly emerging area of selenium research is regarding its role in viral infection where it has been implicated in the occurrence, virulence and disease progression (Beck, 2000). The best evidence comes from studies on the coxsackie virus, which is thought to be a co-factor in the endemic cardiomyopathy, Keshan disease (Beck, 2000). Injection of selenium-deficient mice with a benign strain of the coxsackie virus resulted in mutations in the viral genome resulting in a cardiovirulent form that caused myocarditis (Beck et al., 1995). However, this was not seen in selenium-sufficient mice, but when the virus isolated from selenium-deficient mice showing cardiomyopathy was introduced to selenium-sufficient mice, it still induced heart damage (Beck et al., 1995). Further studies in GPx-1 knockout mice revealed that this enzyme was critical in avoiding mutations in the viral genome (Beck et al., 1998). Thus it appears that selenium-deficiency may affect viral genomes, presumably due to enhanced oxidative stress inducing mutations, resulting in emergence of virulent forms. In this context, it is intriguing to note the emergence of several new strains of influenza from the selenium-deficient regions in China and the evidence that the first putative crossing-over of HIV to humans occurred in a selenium-deficient population in Zaire (Beck et al., 1995; Rayman, 2000).

Plasma selenium has recently been demonstrated to be a strong predictor of the outcome of HIV infection. Individuals with plasma selenium concentrations below 85µg/l were found to be 20 times more likely to succumb to HIV-related causes than those with adequate levels (Baum et al., 1997). In fact, low plasma selenium was shown to be a greater risk factor (by a factor of 16) for mortality than low helper-T-cell count. In infected individuals, low plasma levels were significantly related to mortality and accelerated disease progression (Campa et al., 1999). This data, together with reports of concomitant reduction in plasma selenium with loss of helper-T-cells suggest that selenium-deficiency is a major risk factor for the outcome of HIV infection (Beck, 2000). Similar data for a protective role of selenium in the progression of hepatitis B and C infection to liver cancer (Yu et al., 1999; Yu et al., 1997) and for infection with the Ebola virus have been reported (Ramanathan and Taylor, 1997).

Thus, it appears that maintenance of proper redox processes and immunocompetence by selenium may be the critical mechanisms mediating its anti-viral effect. Selenium may be selectively incorporated by the viral proteins resulting in a decrease in available selenium in the host as described for HIV, coxackie virus, hepatitis and measles viruses (Zhang et al., 1999a; Zhang et al., 1999b; Zhao et al., 2000). It has been suggested by the Taylor

group (Zhao et al., 2000) that with adequate selenium the host immunity will be optimal and consequently the host cell is less likely to die as a result of the infection. In this situation the best viral strategy is to replicate at low levels and maintain a persistent disease. However, on selenium depletion, the resulting diminished immunocompetence, increased oxidative stress and enhanced host cell apoptosis and forces the virus to replicate faster leading to exacerbated disease progression.

1.3.4 Cardiovascular disease

Several studies have shown that selenium may have a protective effect against cardiovascular disease (Neve, 1996). A 2-3 fold risk of cardiovascular mortality was found to be associated with serum selenium concentrations below 45μg/l (Salonen et al., 1982). Another study found a significant inverse correlation between toenail selenium content and the risk of myocardial infarctions, but only in the population with the lowest level of selenium (Kardinaal et al., 1997). An inverse association between risk of ischaemic heart disease and serum selenium concentration below 79μg/l was reported from another study on Danish men (Suadicani et al., 1992). A few studies have failed to find a significant correlation, but most of these included either none or very few people with low plasma/toenail selenium concentrations (Neve, 1996; Salvini et al., 1995). It may well be that a significant effect is detectable only when low selenium status (and not moderate) is compared with higher levels. The levels of other antioxidants may also be a complicating factor.

As the transformation of macrophages to foam cells and the proliferation smooth muscle resulting from their uptake of oxidised low-density lipoprotein (oxLDL) are considered to initiate artherogenesis, and antioxidants may be able to inhibit this process by blocking LDL oxidation (Neve, 1996). This makes selenoproteins with antioxidant activities, like GPx's, attractive targets for mediating the protective effects of selenium. Selenium deficiency has been shown to modulate the eicosanoid metabolism and induce preferential production pro-coagulant thromboxanes over the anti-clotting prostacyclins (Neve, 1996). This may result in platelet aggregation and vasoconstriction and consequently lead to cardiovascular diseases: this is suggested by a study in Finland which reported an inverse association between platelet aggregability and selenium status in individuals suffering from ischaemic heart disease (Salonen et al., 1988).

1.3.5 Neurodegeneration and mood

Since the brain is deficient in catalase activity, selenoproteins with antioxidant functions are critical to the removal of damaging peroxides in this organ (Ramaekers et al., 1994). This crucial role is reflected in the observation that during selenium deficiency, the selenium supply to the brain receives priority (Hawkes and Hornbostel, 1996). Supplementation with selenium has been shown to reduce epileptic seizures in children (Weber et al., 1991) and neurotransmitter cycling is also affected in selenium deficiency (Castano et al., 1997). Alzheimer's patients have been reported to have significantly reduced selenium content in the brain (Hawkes and Hornbostel, 1996) and selenium deficiency is also associated with accelerated cognitive decline in the elderly (Berr et al., 2000; Hawkes and Hornbostel, 1996).

Several studies have revealed a significant association of selenium deficiency with negative mood states (Hawkes and Hornbostel, 1996). Individuals fed low selenium diets for 15 weeks showed significantly decreased positive mood indicators, like 'clearheaded/confused' and 'elated/depressed' ratios (Finley and Penland, 1998). Individuals on high selenium diets had more positive overall mood indicators, for example improved 'confident/unsure', 'composed/anxious' and 'clearheaded/confused' subscores (Finley and Penland, 1998). Selenium supplementation has also been shown to decrease anxiety, tiredness and depression, the effect being greatest in individuals on lowest selenium diets (Benton and Cook, 1990).

1.3.6 Other health issues

Glutathione peroxidase (GPx) activity in essential in the thyroid gland to prevent oxidation-induced toxicity (Coppinger and Diamond, 2000). Also, the production of thyroid hormones are dependent upon the catalytic activities of iodothyronine deiodinases, which are selenoproteins (Coppinger and Diamond, 2000). Selenium therefore regulates thyroid hormone metabolism and consequent endocrine effects. Supplementation with selenium increases deiodinase activity and enhanced production of the active hormone (Coppinger and Diamond, 2000). Symptoms of hypothyroidism are aggravated by selenium (and iodine) deficiency as reported from selenium-deficient areas (Vanderpas et al., 1990).

The ageing process is associated with accumulation of oxidative damage, reduced effectiveness of the immune system and endocrine dysfunction. As discussed earlier,

selenium can conceivably influence all these factors. In fact recent reports have noted longevity in areas with selenium-rich soils and has been hypothesised that areas of the world with higher lifespan than average are those where bioavailable soil selenium is high (McKenzie et al., 2000). Indicators of lipid peroxidation from elderly patients show a inverse correlation with plasma selenium content and lowered GPx activity is found in neutrophils from elderly individuals (McKenzie et al., 2000). In a study of individuals aged between 96-106 years, the percentage of NK-cells in circulation was related to the selenium status (Ravaglia et al., 2000). Finally, it has been suggested that telomere length, which progressively shortens with every cell division, may also be maintained by enhanced GPx activity (Serra et al., 2000).

Selenium also affects several human conditions associated with oxidative stress and inflammation. While oxidative stress is managed by antioxidant activities of several selenoproteins, inflammatory responses may be regulated by the influence of selenium on cycloxygenase and lipoxygenase pathways resulting in reduced production of inflammatory leukotrienes and prostaglandins. This may explain why selenium status has been inversely associated with relative risk and/or severity of diseases like asthma, pancreatitis and arthritis (Hasselmark et al., 1993; Knekt et al., 2000; McCloy, 1998).

1.4 The eukaryotic selenoproteome

Proteins containing the 21st amino acid, selenocysteine (Sec), which is cotranslationally incorporated and is coded by the UGA codon are termed selenoproteins (Chambers et al., 1986; Zinoni et al., 1986). Eukaryotic selenoproteins have no sequence homology to prokaryotic selenoproteins apart from selenophosphate synthetase, which itself participates in Sec biosynthesis (Gladyshev, 2000). This suggests independent origins of these two selenoproteomes (i.e. all selenoproteins in an organism). The eukaryotic selenoproteins of known functions participate in predominantly redox reactions which are crucial to normal physiology. The incorporation of selencysteine in the active sites of these proteins render them uniquely suited to participate in redox pathways.

1.4.1 Selenoproteins

The 22 known selenoproteins can be classified into groups based on the location of Sec on the polypeptide. The two major groups emerge from such analysis are the GPx group and the thioredoxin reductase (TR) group. The GPx group is by far the most abundant and includes proteins in which Sec is located in the N-terminal region. Several members

contain UXXC or CXXU motifs (U, Sec; C, cysteine and X, any amino acid) that resemble active centres of thioredoxins and glutaredoxins. Predicted structures in this group indicate presence of both α-helices and β-sheets and most appear to have the thioredoxin fold. During catalysis, the Sec in these proteins is oxidised to selenic acid or forms selenosulphide bonds. The TR group has Sec located in the C-terminal regions and this positioning in the conformationally flexible C-terminus ensures accessibility of this residue. All other selenoproteins are placed in group 3 and are often characterised by a CXU or UXC motif. In most selenoproteins, in all three groups, the Sec-flanking sequences are abundant in glycine, cysteine (Cys) and basic amino acids. The functional significance of this remains unclear.

Unlike the prokaryotic selenoproteins only a few physiological functions of selenoproteins in humans is well characterised. Broadly, these activities include prevention of oxidative damage, control of cellular redox processes, involvement in spermatogenesis and modulation of thyroid hormones. Since a recent review (Gladyshev, 2000) has exhaustively discussed the functions of selenoproteins, a short summary of the major mammalian selenoproteins is presented in Table 1.4.1.

1.4.2 Selenocysteine: function and evolution

Sec and Cys and Serine (Ser) differ by only one atom. However, their properties vary greatly, since at physiological pH, serine is completely protonated, the majority of cysteines are also protonated but Sec remains ionised (Stadtman, 1996). This property and the lower redox potential confers Sec its special properties as reflected in the inability of Cys to functionally replace Sec in known selenoproteins. Enzyme kinetic comparison between selenoproteins and their naturally occurring Cys-containing counterparts reveal the considerably higher k_{cat} for selenoproteins (Stadtman, 1996).

While selenoproteins are found in all three major groups of life forms (archaea, bacteria and eukaryotes) several species in each of these groups lack genes for selenoproteins. The evolutionary implications of this observation are unclear. Sec could have been encoded by UGA in primitive anerobic organisms, where it was found in abundance. However, with the advent of oxygen in the atmosphere the selection pressure may have turned against its use because of its lability in aerobic conditions. Some organisms, presumably with enhanced oxidant defences, could have retained Sec and its insertion machinery. The aerobic lability of Sec and the development of the specific Sec insertion system may have

Selenoproteins	Function
Group I (GPx group)	
1. Cytosolic glutathione peroxidase (GPx1) 2. Gastrointestinal glutathione peroxidase (GPx2) 3. Plasma glutathione peroxidase (GPx3) 4. Phospholipid hydroperoxide glutathione peroxidase (GPx4)	Antioxidant enzymes: removal of H_2O_2 and lipid and phospholipid hydroperoxides. Influencing eicosanoid synthesis, modifying inflammation. Gpx shield sperms from oxidative damage and later polymerises to a key structural protein.
5. Selenoprotein P	Protects endothelial cells from peroxynitrite-induced damage.
6. Selenoprotein W	Putative role in muscle function.
7. Selenoprotein T	Unknown function.
Group II (TR group)	
8. Cytosolic thioredoxin reductase (TR1) 9. Thioredoxin reductase in testis (TR2) 10. Mitochondrial thioredoxin reductase (TR3	Reduction of nucleotides, maintenance of cellular redox state, essential for cellular viability and proliferation, redox control of signalling molecules and transcription factors.
Group III (other selenoproteins)	
11. Thyroid hormone deiodinase 1 12. Thyroid hormone deiodinase 2 13. Thyroid hormone deiodinase 3	Production and regulation of levels of active thyroid hormone.
14. Selenoprotein R (Selenoprotein X)	Putative role in methionine sulphoxide reduction.
15. Selenoprotein N	Unknown function.
16. 15kDa selenoprotein	May contribute to cancers, particularly of the prostate, as it is differentially expressed in normals and tumours and the gene is located in a region commonly deleted or mutated in cancers.
17. Selenophosphate synthetase 2	Necessary for the the synthesis of selenophosphate, a precursor of selenocysteine, and consequently for selenoprotein synthesis.

Table 1.4.1 Known mammalian selenoproteins and their function.

All mammalian selenoproteins known to date, are listed along with their known functions. For a detailed discussion see Gladyshev, 2000.

then left UGA without an overt function, perhaps resulting in its evolution as a termination signal.

1.4.3 Selenocysteine incorporation

Unlike the other 20 naturally occurring amino acids, selenocysteine is synthesised on its tRNA and is coded for by the 'stop' codon UGA (Bock, 2000; Stadtman, 1996). This enigmatic dual function of the UGA codon has resulted in a lot of interest directed towards elucidating the molecular mechanisms involved in selenocysteine incorporation. The molecular mechanisms mediating selenocysteine incorporation in prokaryotes are extensively characterised and recently reviewed in detail (Bock, 2000). This section will therefore primarily deal with recent developments in the field of selenocysteine incorporation in eukaryotes.

Sec incorporation occurs co-translationally at the UGA codon. Specific secondary stem-loop structures in the selenoprotein mRNA, called the Sec insertion sequences (SECIS), have been defined by mutational analysis that signal the recoding of the UGA codon from translation termination to Sec insertion (Martin III and Berry, 2000). In E.coli, the selC gene codes for the $tRNA^{[Ser]Sec}$, which has an anticodon complementary to UGA and is initially charged with serine by seryl-tRNA synthetase. Sec synthase, encoded by the selA gene, later converts this seryl-$tRNA^{[Ser]Sec}$ to selenocysteyl-$tRNA^{[Ser]Sec}$. The activated selenium donor species is monoselenophosphate which is produced by the activity of the selD gene product, selenophosphate synthetase (Bock, 2000).

In order to prevent $tRNA^{[Ser]Sec}$ from functioning as an ubiquitous UGA suppressor, the presence of specific structural features make it unrecognisable by the standard elongation factor (EF). A specific Sec elongation factor, SELB binds selenocysteyl- $tRNA^{[Ser]Sec}$ and to the SECIS element but not the precursor seryl-$tRNA^{[Ser]Sec}$, thus tethering the selenocysteyl- $tRNA^{[Ser]Sec}$ and the mRNA in the ribosome (Bock, 2000).

Unlike prokaryotes, eukaryotes have the SECIS elements located in the 3'-untranslated region (3'-UTR) and these can recode any upstream, in frame UGAs in the entire mRNA, as long as a minimum distance criteria is met (Martin et al., 1996). Eukaryote SECIS elements contain, among other features, an A-G tandem purine pair at the base of the stem-loop structure which is required for Sec insertion (Mansell and Berry, 2000). Recently a mammalian protein, SECIS-binding protein 2 (SBP2), has been characterised which specifically binds to the purine pair and immunodepletion of SBP2 has been shown to

abolish Sec incorporation (Copeland and Driscoll, 1999; Copeland et al., 2000). Addition of recombinant SBP2 restores Sec incorporation demonstrating that, at least *in vitro*, SBP2 is required for the insertion of Sec (Copeland et al., 2000). However, SBP2 was found to have no detectable EF activity (Copeland et al., 2000).

Taking note of the identification of a Sec-specific EF in the archaeon *Methanococcus jannaschii*, which, while showing specificity for selenocysteyl- $tRNA^{[Ser]Sec}$, did not bind SECIS elements, researchers started to look for a putative mammalian Sec-specific EF that showed specificity towards selenocysteyl- $tRNA^{[Ser]Sec}$ but did not have a SECIS-binding domain. Finally by sequential database searches with the putative archaeal SELB sequence, the mammalian EF specific to Sec incorporation, eEFSec, was identified (Berry et al., 2001; Dennis, 1997).

Thus the current model in eukaryotes (Fig 1.4.1) suggest that the presence of SECIS elements in the 3'-UTRs of selenoproteins signals the binding of SBP2 to these specialised structural elements. This is followed by the recruitment of eEFSec-$tRNA^{[Ser]Sec}$ to the SECIS-SBP2 complex. Assembly of the complex at the SECIS element, followed by delivery of eEFSec-$tRNA^{[Ser]Sec}$ to a UGA codon occupying the ribosomal A site would allow translation of all in frame UGAs in the open reading frame as Sec (Fig 1.4.1).

What still remains obscure is the interaction of eEFSec-selenocysteyl-$tRNA^{[Ser]Sec}$ with translation release factors (eRF1 and 3) which bring about termination. The simple model of the UGA codon at the ribosomal A site being decoded by either the release factors or eEFSec-selenocysteyl-$tRNA^{[Ser]Sec}$ would have predicted some sort of competition where increase in eRF1 or eRF3 would favour termination. However, recent evidence suggests that overexpression of both eRF1 and eRF3 result in increased Sec incorporation (Grundner-Culemann et al., 2001). This may point towards non-canonical regulation of termination in ribosomes translating selenoprotein mRNAs; perhaps the presence or absence of SBP2 dictating whether UGA will be read as a Sec insertion codon or a termination signal. Other, as yet unidentified, proteins may also be involved in specifying the fate of the UGA codon.

1.4.4 Identification of new selenoproteins

With the sequencing of several genomes, the time is appropriate to use database screens to identify novel selenoproteins which can be later characterised. Since the characteristic of selenoprotein genes is the sequence specifying the SECIS elements in the 3'-UTR of the

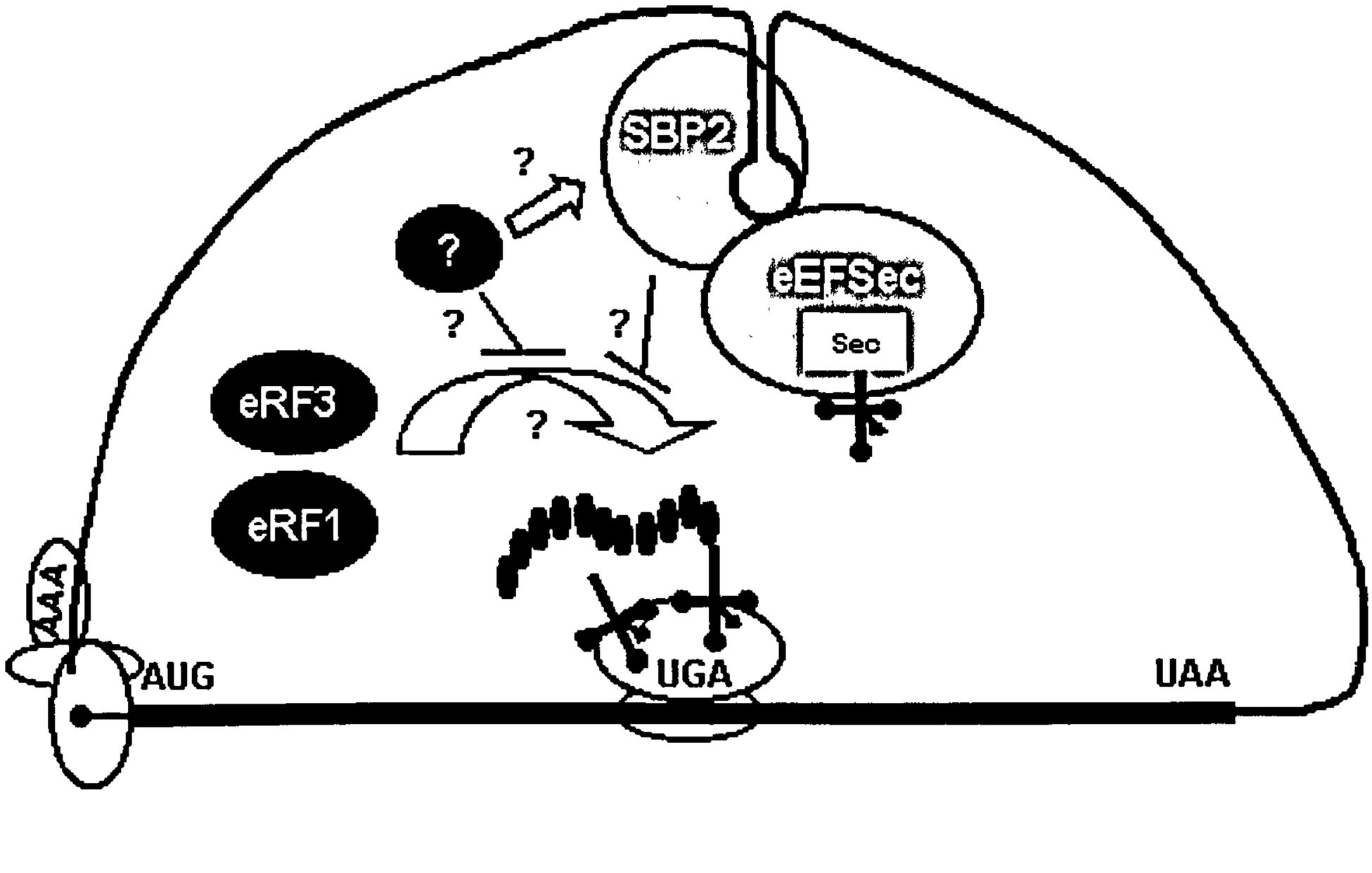


Fig 1.4.1 Model for eukaryotic selenocysteine insertion (Mansell and Berry, 2000).

A model of the current mechanisms believed to mediate Sec incorporation. The figure shows a mRNA molecule with the SECIS elements as a stem-loop structure. SBP2 and eEFSec interact with this structure and recruit selenocysteyl-tRNA which incorporates a Sec residue in the nascent polypeptide at the UGA 'termination' codon. Translation termination is brought about by either as yet unidentified factors or non-canonical regulation of eRF1 and eRF3. Additional factors required for the formation of this complex, association with ribosomes and regulation of release factor are as yet unidentified. Putative activities are marked with '?'.

corresponding mRNA, this has been successfully utilised to identify novel selenoproteins *in silico* (Gladyshev, 2000). An algorithm that scans database ribonucleotide sequences for SECIS elements, called SECISearch, has been recently developed based on primary sequence identification, secondary structure prediction and estimation of minimum free energy (Kryukov et al., 1999). Using this a number of hitherto unknown bona-fide selenoproteins have been identified in different eukaryotic organisms (Gladyshev, 2000; Martin-Romero et al., 2001).

1.5 Selenium metabolism

Selenium metabolism has been extensively reviewed recently (Ganther, 2000; Ganther, 2001; Harrison et al., 1997), therefore only a brief overview will be presented. Mammals have limited body stores of selenium, and require a regular supply of the element through their diet and/or water. The nutritional role of selenium is expressed not by the element *per se*, but through the biochemical functions of a number of selenium-containing proteins. Animals synthesise selenoproteins from hydrogen selenide after activation to selenophosphate. Reduction of inorganic salts like sodium selenite is a source of hydrogen selenide (Fig 1.4.2). In this process glutathione conjugation with selenite results in the formation of selenodiglutathione (SDG) which undergoes further reduction to form hydrogen selenide. Another source of hydrogen selenide is via the liberation of selenium from organoselenium compounds by scission of C-Se bonds. The metabolism of selenomethionine requires the conversion to selenocysteine, via the transsulphuration pathway, before the release of inorganic selenium by selenocysteine lyase (Fig 1.4.2). Hydrogen selenide is toxic, but only small quantities are needed for selenoprotein synthesis. Any excesses are rapidly methylated to form less toxic species which are eliminated from the body mainly in the form of trimethylselenonium in the urine and/or exhaled as dimethylselenide (Fig 1.4.2). Monomethylated methylselenol can also be formed directly from Se-methylselenocysteine (one of the major components in plants) and selenobetaine (a synthetic compound) (Andreadou et al., 1996b). In this case the inorganic pool is bypassed, however sufficient demethylation to inorganic selenium may occur to maintain selenoprotein biosynthesis.

Hydrogen selenide is methylated by S-adenosylmethionine in three successive reactions. The first two steps are catalysed by an arsenite-sensitive microsomal enzyme, thiol S-methyltransferase, forming dimethylselenide (Hsieh and Ganther, 1977; Mozier et al., 1988). The final step is catalysed by thioester S-methyltransferase and may become rate limiting as the rate of methylation decreases with the increase in the degree of methylation

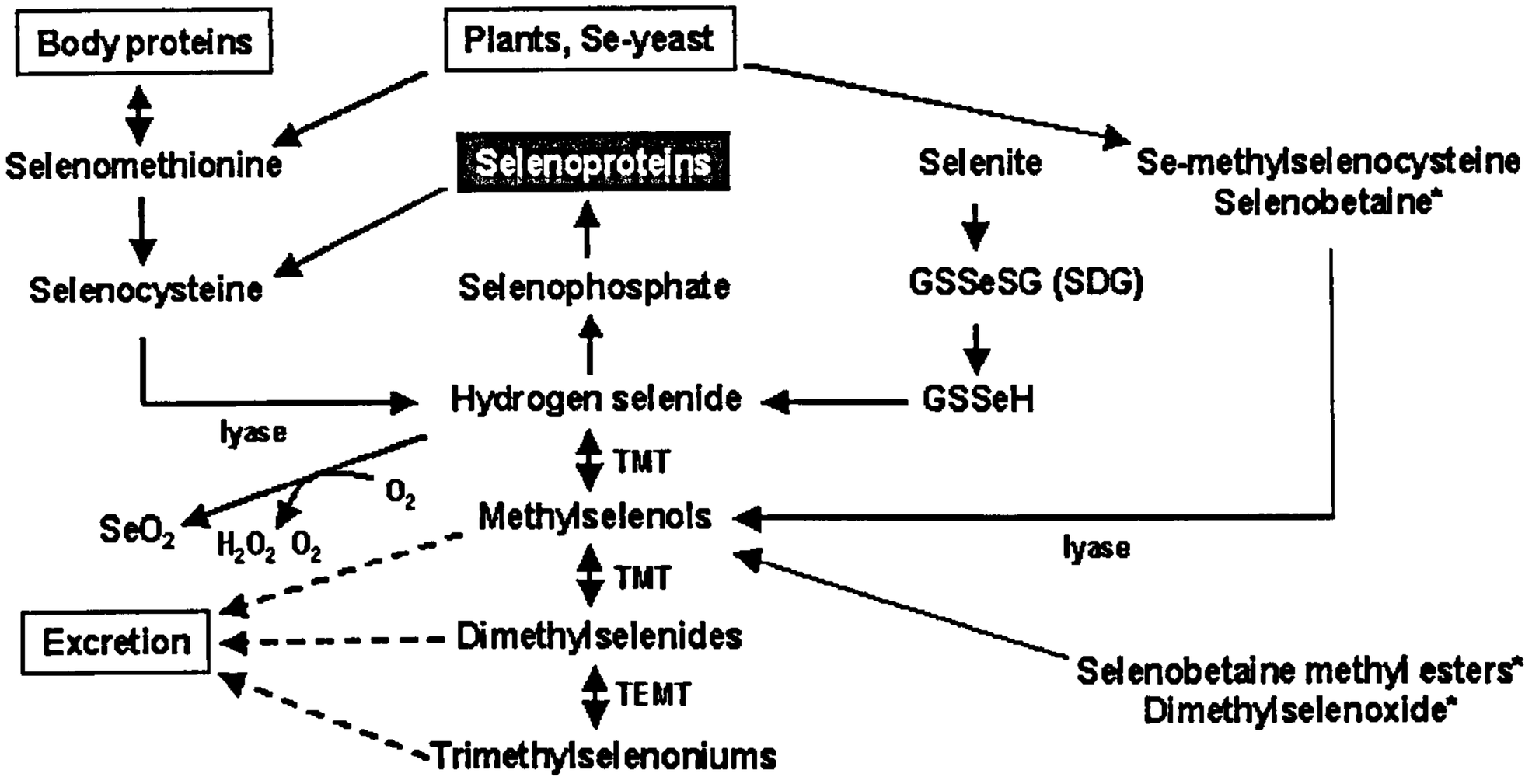


Fig 1.4.2 Schematic representation of selenium metabolism (adapted from Harrison et al, 1997 and Ganther, 2000)

Plants supply animals with methylated selenoaminoacids. Conversion to inorganic selenium is necessary for selenoprotein synthesis while excess of hydrogen selenide is detoxified by methylation and subsequently excreted. TMT, thiol S-methyltransferase; TEMT, thioester S-methyltransferase. Compounds marked with asterisk are synthetic.

(Mozier et al., 1988). At normal dietary levels of selenium, monomethylated forms are the major metabolites (Vadhanavikit et al., 1993). At chemopreventive levels monomethylated forms increase along with trimethylselenonium. At the highest levels of intake, dimethyl selenide (excreted in the breath) equals monomethyl selenium and surpasses trimethylselenonium levels.

1.6 Biochemical reactions of low molecular weight selenium compounds

As discussed in Section 1.2, 1.3 and the next chapter, low molecular weight selenium compounds have a profound influence on preserving normal health and protecting against disease incidence, like cancers. It is therefore pertinent to discuss the various biochemical reactions of these compounds that might modify proteins and have a major impact on disease prevention.

Research on biochemical reactions of selenium compounds have been mainly directed towards protein thiols, and four types of reactions identified (reviewed in (Ganther, 1999)):

Type 1, formation of selenenylsulphide bonds (S-Se);
Type 2, formation of selenotrisulphide bonds (S-Se-S);
Type 3, formation or disruption of disulphide bonds;
Type 4, formation of diselenide bonds (Se-Se)

Types 1 and 2 are the most commonly characterised and several examples are known. Recent work on proteins with cysteine clusters in their active sites, like protein kinase C (PKC) have underscored the importance of these reactions as selenite has been shown to inhibit the activities of such proteins but not those lacking such clusters (Gopalakrishna et al., 1997). With selenium metabolites that are further reduced, like SDG, inhibitory effects on thiol containing proteins are more pronounced (Spyrou et al., 1995). Selenium adduct formation on critical thiol containing residues can inactivate proteins, including transcription factors, and may thereby influence cellular processes (Kim and Stadtman, 1997; Spyrou et al., 1995).

Selenium may act as an catalyst in formation or breakage of disulphide bonds without actually getting incorporated (Type 3 reaction). Catalytic amounts of selenite have been demonstrated to initiate rapid disulphide formation leading to restoration of ribonuclease activity (Lundstrom et al., 1992). SDG, in turn, can efficiently oxidise reduced thioredoxin

by promoting disulphide bond formation (Ren et al., 1993). It has also been shown that selenium can catalyse intramolecular disulphide bond and thereby inactivate PKC (Gopalakrishna et al., 1997).

While there is no direct biological evidence of type 4 reactions, it remains an interesting possibility that may help identify the biochemical basis of selenoprotein action and other selenium mediated activities.

1.7 Conclusion

Over the last 50 years compelling evidence has accumulated for the beneficial role of selenium in human health. It appears that the activities of this trace nutrient impinge on several major health issues and its thought that selenium exerts its influence primarily through selenoprotein activity. While the molecular mechanism underlying Sec incorporation in mammals is gradually becoming clear, the functions of the majority of the selenoproteins remain obscure. For most of the health issues discussed in this chapter, data suggesting strong associations is rapidly accumulating, but the biological underpinnings of the role of selenium in animal health remain poorly defined. What has been achieved, however, is an appreciation of the fundamental role of selenium.

It has been realised that the biological activities of selenium are properties of its various compounds and not of the element *per se*. This means that additional research employing various metabolites and synthetic compounds of selenium needs to be carried out to identify the active metabolites. The next section discusses the role of selenium in prevention of cancer, where low molecular weight selenium compounds, rather than selenoproteins, appear to be the key mediators.

2 Selenium: an Anti-cancer, Chemopreventive Agent

2.1 Introduction

Evidence from several lines of investigation indicates that selenium may affect cancer incidence. Epidemiological evidence generally shows an inverse correlation between dietary selenium intake and cancer risk. The limited number of intervention trials conducted so far have also been supportive of this claim. A large number of studies on laboratory animals have repeatedly demonstrated supranutritional, but non-toxic, levels of selenium intake to be associated with reduced tumorigenesis. It should be noted that while, for reasons of convenience, the word 'selenium' is used the biological effects of selenium are due to particular chemical forms of selenium and not the element *per se*.

While nutritional functions of selenium are thought to be largely mediated by the activities of several selenoproteins, there is only limited evidence that these are important mediators of the anti-cancer activities of selenium compounds. There is convincing evidence, however, suggesting anti-cancer activities mediated by selenium compounds in individuals in whom the activities of selenoproteins are already maximal. It appears that several selenium-metabolites are the key players in anti-cancer activities though actual mechanisms are not known. It may well be that selenium deprivation may increase cancer-risk, though little evidence is forthcoming, but there is more widespread acceptance of the inverse association of supranutritional selenium exposure and cancer incidence. These hypotheses are perhaps not mutually exclusive and both nutritional and supranutritional modes influence the anti-cancer action of selenium.

Whereas evidence supporting selenium as an anti-cancer, chemopreventive agent is substantial, our understanding of molecular and cellular mechanisms mediating these activities remains sketchy. This chapter discusses the evidence implicating selenium as a potent modifier of cancer risk and the potential mechanisms that may be involved.

2.2 Evidence in humans

Evidence for selenium as a cancer risk modifier in humans has come from ecological, epidemiological and, most recently, intervention trials.

2.2.1 Epidemiological studies

The initial clue indicating a possible inverse correlation based on geographic association found the risk of mortality attributed to cancers of the lung, breast, gastrointestinal tract and peritoneum and lymphomas to be significantly lower for Americans living is areas of high to moderate forage selenium levels compared those low in forage selenium content (Shamberger and Willis, 1971). Later, a reanalysis of the same data extended the same conclusion to colorectal, ovarian and bladder cancers (Clark et al., 1991). Both total cancer mortality and age-corrected mortality due to leukaemia, lung, breast, ovarian and colorectal cancers were estimated to be inversely related to the per capita intake of selenium in a study comparing 27 different countries (Schrauzer et al., 1977). Similar data was also reported from China (Yu et al., 1985).

Several epidemiological studies have since noted that cancer patients generally have a low selenium status compared to healthy individuals, as assessed by plasma/serum concentrations (summarised in Table 2.2.1 and reviewed in (Combs and Gray, 1998; Vinceti et al., 2000b)). These studies are often complex to interpret due to methodological issues and variations in assessment techniques. The evaluation of selenium exposure is complicated by the fact that it is dependent on the type of food and where it was produced. Also, tissue selenium content can be affected by the disease stage and treatment. Therefore in the most valid assessments, selenium exposure is based on biological parameters from prediagnostic samples, commonly toenail clippings and blood plasma and serum. However, even these are not always accurate as, for example, blood selenium levels vary between fasting and morning blood collection, presumably because bolus doses of selenium causes transient increases in blood selenium (Patterson et al., 1989). Toe nail clippings, on the other hand, give an estimate of average selenium levels over a longer period of time (~6 months: the average time between initial nail formation and clipping). The situation may be further confounded if the individuals have access to multiple sources of selenium like high-selenium foods and other dietary supplements. Other problems often plaguing some of these studies are variation of selenium status within the population and low incidence of cancers in general, all of which affect the statistical power of the studies. Notwithstanding such complications, several studies have provided interesting evidence. A decade long prospective study in America found that initial plasma selenium concentration was inversely correlated to the later risk of colonic adenomatous polyps and non-melanoma skin cancer (Clark et al., 1993). Patients with plasma selenium level less than the population median were 4 times more likely to have one or more adenomatous polyps and had more than three times the number of polyps per person. Eight independent cohort

Study	Population (cases)	Follow-up	RR (95% CI)
Willett et al., 1983	USA, (111)	5	0.5 (0.3-0.9)
Salonen et al., 1984	Finland (128)	6	0.3 (0.1-0.7)
Peleg et al., 1985	Georgia (130)	12	1.0
Salonen et al., 1985	Finland (51)	4	0.2 (0.03-0.8)
Fex et al., 1987	Swedish men (35)	8	0.3, p trend=0.05
Kok et al., 1987	Holland		
	males (40)	9	0.4 (0.2-0.8)
	females (29)	9	0.7 (0.2-2.0)
Nomura et al., 1987	Japanese men in Hawaii (280)	11	0.8, p trend = 0.61
Virtamo et al., 1987	Finnish men (109)	9	0.9 (0.5-1.5)
Coates et al., 1988	USA (154)	12	1.0 (0.5-1.8)
Ringstad et al.,1988	Norway (60)	6	0.7 (0.3-1.7)
Knekt et al., 1990	Finland		
	males (597)	10	0.4, p trend = <0.001
	females (499)	10	0.9, p trend = 0.6
Garland et al., 1995	US females (934)	3.5	1.2 (0.9-1.7)

Table 2.2.1 Summary of major cohort studies on the relation between selenium and overall cancer risk (adapted from Vinceti et al., 2000 and unpublished work of Dr P. R. Harrison).

Major cohort studies investigating the relationship between selenium intake and overall cancer-risk are summarised in the table. The number of actual cases and the length of the follow up time (in years) is indicated. The results expressed in terms of relative risk (RR), usually for the highest to lowest quintiles of plasma selenium and are corrected for the common confounding factors like age and smoking. 95%confidence limits are indicated beside the RR in parentheses.

studies failed to detect any significant association with colorectal/gastrointestinal cancer risk and plasma selenium level but, interestingly, all of them found that individuals who did develop cancers had lower initial plasma levels than those remaining healthy (Knekt et al., 1988; Nomura et al., 1987; Peleg et al., 1985; Ringstad et al., 1988; Salonen et al., 1985; Schober et al., 1987; Virtamo et al., 1987; Willett et al., 1983). A meta-analysis of these data should be very illuminating. Several other prospective cohort studies have, however, shown prediagnostic serum selenium levels to be significantly lower in cases of cancer than in disease-free controls (Coates et al., 1988; Fex et al., 1987; Salonen et al., 1984) (summarised in Table 2.2.1).

Several studies have demonstrated protective effects of selenium against epithelial cancers including bladder (Helzlsouer et al., 1989), ovary (Helzlsouer et al., 1996), pancreas (Burney et al., 1989), thyroid (Glattre et al., 1989), lung (Knekt et al., 1990) and stomach cancers (Knekt et al., 1990). Protection from oesophagus (Jaskiewicz et al., 1988), lung (Gerhardsson et al., 1985), melanoma skin cancer (Reinhold et al., 1989), head and neck cancers (Westin et al., 1989), gastric (Caygill et al., 1989) and prostate cancers (Criqui et al., 1991) have been noted in other studies. A recent study has showed a strong inverse dose response association between toe-nail selenium level and prostate cancer incidence (Yoshizawa et al., 1998).

While the positive evidence from epidemiology and animal experiments is significant, several studies have failed to find any association of selenium with total cancer risk and cancers of the stomach, bladder, lung, cervix, oral cancer and colon-rectum (reviewed in (Vinceti et al., 2000b)). A recent study found a positive association of toenail selenium with cancer risk though not with an unadjusted analysis (Garland et al., 1995). However, this study was fraught with several methodological problems particularly changing levels of selenium within the cohort over time and possible overadjustment for smoking. Reports from a 'natural experiment' in Italy, on a population accidentally exposed to high doses of selenium from their drinking water have also argued the case for an elevated cancer risk (Vinceti et al., 2000a; Vinceti et al., 1998).

Gender specific effects have been note in several studies, generally in favour of men (Kok et al., 1987a; Kok et al., 1987b; Rogers et al., 1991). This, however, is not reflected in geographical studies and the biological basis remains obscure. There is extensive evidence in animals showing that selenium supplementation reduces the incidence of carcinomas of the breast, however, epidemiological data have almost consistently failed to find such an association (van den Brandt et al., 1994; van 't Veer et al., 1996; van 't Veer et al., 1990).

Taken together, the data from epidemiological studies indicate significant inverse correlation between selenium level and gastrointestinal, oesophageal, stomach, prostate, upper respiratory tract and lung cancers, and also possibly thyroid and pancreatic cancers. There is no suggestion of a link with breast cancer and evidence is also weak for colon cancer risk (though the length of follow up for the largest studies were quite short).

Supplementation trials of cancer patients with selenium have not been extensively reported. However, one study found selenium supplementation reduced bone marrow suppression and kidney damage induced by cisplatin (Hu et al., 1997). There is also animal data suggesting reduction of cardiac toxicity induced by adriamycin therapy, on selenium supplementation (Boucher et al., 1995). Thus, epidemiological evidence suggests that selenium is a likely cancer risk modifier, and appears to be protective against certain cancers, particularly those of epithelial origin.

2.2.2 Intervention trials

Intervention using selenium has been reasonably limited (summarised in Table 2.2.2). These trials have not only used different selenium sources (like selenite, selenized yeast, selenomethionine or selenate), sometimes in combination with other vitamins and minerals, the populations are also quite variable in terms of risk factors and geography.

The two studies conducted in China, in Quidong, Shandong Province, have only been reported in English very recently. One employed the use of a daily supplement of 200μg selenised yeast and concluded that almost complete prevention of liver cancer could be achieved among carriers of hepatitis surface antigen (otherwise with 1.58% incidence risk) (Yu et al., 1997). It also reduced the incidence of liver cancer among first-degree relatives by approximately 60%. The other study used table salt fortified with sodium selenite and reported attenuation of liver cancer incidence by 37% (Yu et al., 1991). It should be cautioned that these studies suffered from several methodological and quality control issues.

The third study, in Linxian county, China was technically sound employing a double-blind, placebo-controlled, randomised design in a region where oesophageal and stomach cancer causes a third of all deaths. Using a combination of 50 μg selenium (as selenised yeast), vitamin E and β-carotene (all administered at ~twice their Recommended Dietary Allowances – RDA) for 5 years, a protective effect was observed against mortality from total cancer (13%; RR=0.87 (0.75-1.00)) and stomach cancer (21%; RR=0.79 (0.64-0.99)),

No.	Dose and form of Se	Population	Cancer type (outcome)
1	200μg Se/day as selenised yeast	Hepatitis surface antigen carriers	Liver cancer (I)
2	Table salt fortified with 15mg/kg Se as selenite	Hepatitis surface antigen carriers	Liver cancer (I)
3	50μg Se/day as selenised yeast	General population	Stomach cancer (I)
4	50μg Se/day as selenate	Oesophageal dysplasia	Oesophageal cancer (NE)
5	100μg Se/day and 50μg Se/day as selenomethionine	Reverse smokers	Oral lesions (I)
6	200μg/day as selenised yeast	Patients with prior resected adenomatous polyps	New adenomatous polyps (I)
7	200μg Se/day as selenised yeast	Patients with prior skin cancer	Basal or squamous cell carcinoma (NE); lung (I); colorectal (I); prostate (I)

Table 2.2.2 Intervention trials employing selenium.

Summary of intervention trials employing selenium (Se) either alone or in combination with other minerals and vitamins. In study 3, selenium was used in combination with β-carotene and vitamin E. In study 4, selenium was used in combination with 25 other minerals and vitamins. In study 5, selenium was used in combination with zinc and vitamin A and riboflavin. In study 6, selenium was used in combination with vitamins A, C, E and zinc. I: statistically significant inhibition and NE: no effect.

and well as total mortality (9%) (Blot et al., 1993). However, no protection from oesophageal cancer was conferred when selenium (as 50μg sodium selenate/day) was given in combination with 25 other vitamins and minerals (Li et al., 1993).

A placebo-controlled trial in India, on reverse smokers diagnosed with premalignant oral lesions showed 57% remission in the treatment group (8% in placebo group) on supplementation with a cocktail vitamin A, riboflavin, zinc and selenomethionine (100 and 50μg/day) (Krishnaswamy et al., 1995; Krishnaswamy et al., 1993).

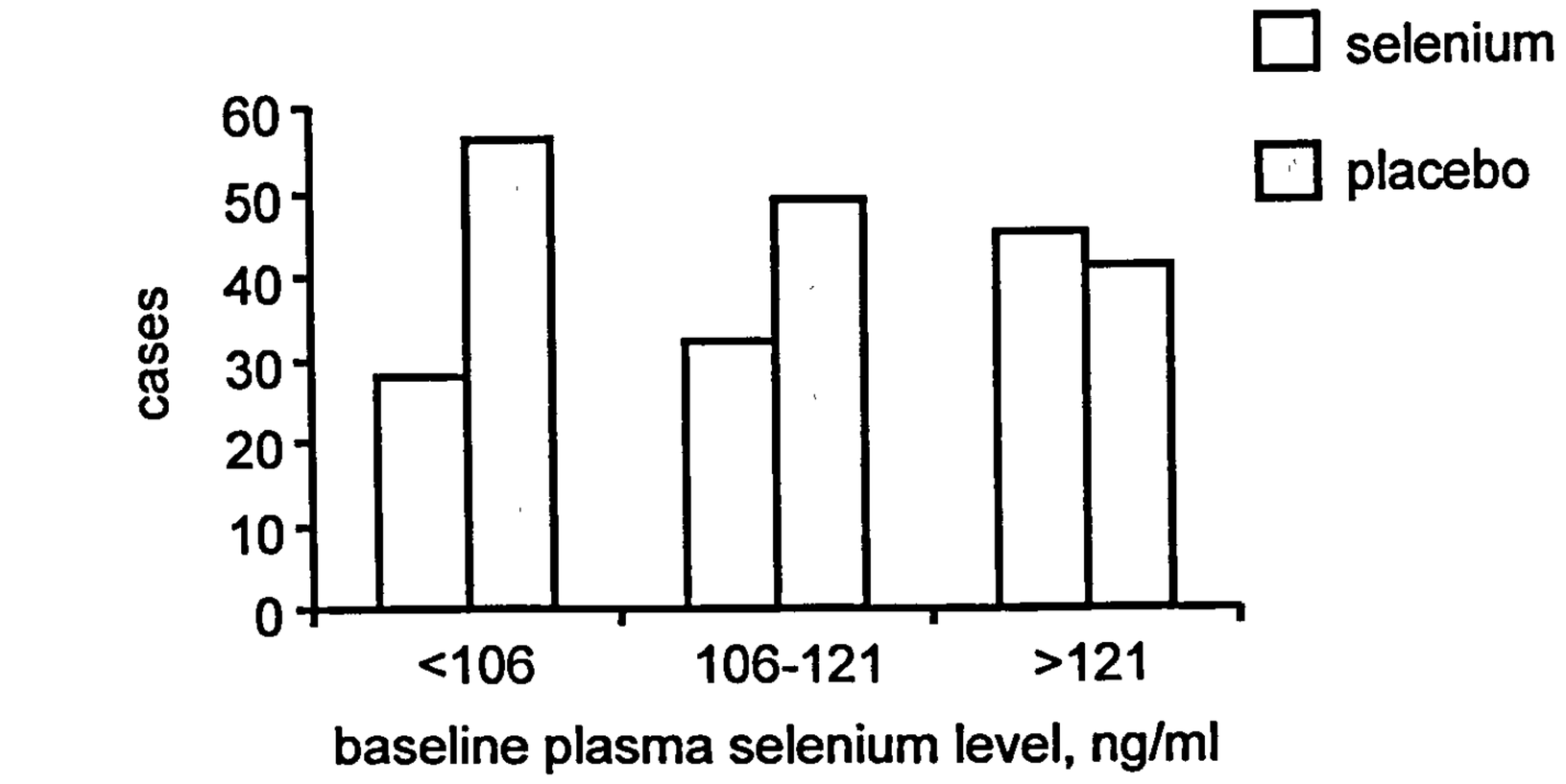
A recent study in Italy, using a randomised, double blind cancer prevention trial on patients with prior resected adenomatous polyps noted a 44% reduction in the incidence of new polyps using selenium (200μg/day as selenised yeast) along with zinc and vitamins A, C and E (Bonelli, 1998).

A pivotal prospective, double-blind, placebo-controlled, randomised selenium chemoprevention study, the Nutritional Prevention of Cancer (NPC) trial, was conducted in the US (Clark et al., 1996). 1312 subjects with a previous history of basal or squamous cell carcinoma of the skin were randomised and the treatment group supplemented with 200μg of selenium (as selenised yeast) per day for an average of 4.5 yrs and followed up for 6.4 years. While selenium supplementation did not affect the recurrence of skin cancer, it was associated with a statistically significant decrease in several secondary endpoints: total cancer incidence (RR=0.63 (0.47-0.85)) and incidence of lung, colorectal and prostate cancers (Table 2.2.3). The study also reported a decrease in the total cancer mortality in the supplementation arm (RR=0.50(0.31-0.80)). Recent updates on this trial have shown that men with the lowest baseline selenium blood levels prior to supplementation had the greatest reduction in cancer, especially so for prostate cancer (Clark et al., 1998; Combs Jr, 2000). It should be noted that none of the subjects had plasma serum levels below 60ng/ml and that very few had levels below 80ng/ml; thus this cohort was selenium-adequate and the cancer protection was shown by supranutritional levels of selenium. Yet, subjects with initial plasma selenium levels less than 106ng/ml showed not only the highest rates of cancer in the placebo group but also the strongest protective effects of supplementation with selenium (Fig 2.2.1A) (Clark et al., 1998; Combs Jr, 2000). Individuals with plasma selenium level above 121ng/ml showed no cancer-protective of selenium supplementation. Prostate cancer, in particular, demonstrated this trend most clearly (Fig 2.2.1B) (Clark et al., 1998; Combs Jr, 2000).

Cancer Site	Selenium	Placebo	RR	*P* value
Lung	17	31	0.54	0.04
Prostate	13	35	0.37	0.002
Colorectal	8	19	0.42	0.03
Total carcinomas	59	104	0.55	<0.001
Total cancer incidence	77	119	0.63	0.001
Total cancer mortality	29	57	0.50	0.002

Table 2.2.3 Cancer incidence and cancer-related mortality in the NPC study (adapted from Clark et al., 1996).

Summary of cancer incidence and cancer-related mortality found in the NPC trial. The numbers in the selenium and placebo columns indicate the number of cancer cases in the selenium supplemented and placebo administered cohorts, respectively. RR: relative risk and *P* value is calculated from log rank tests. Total cancers include both carcinomas and non-carcinomas like melanomas, leukaemia, lymphoma etc.



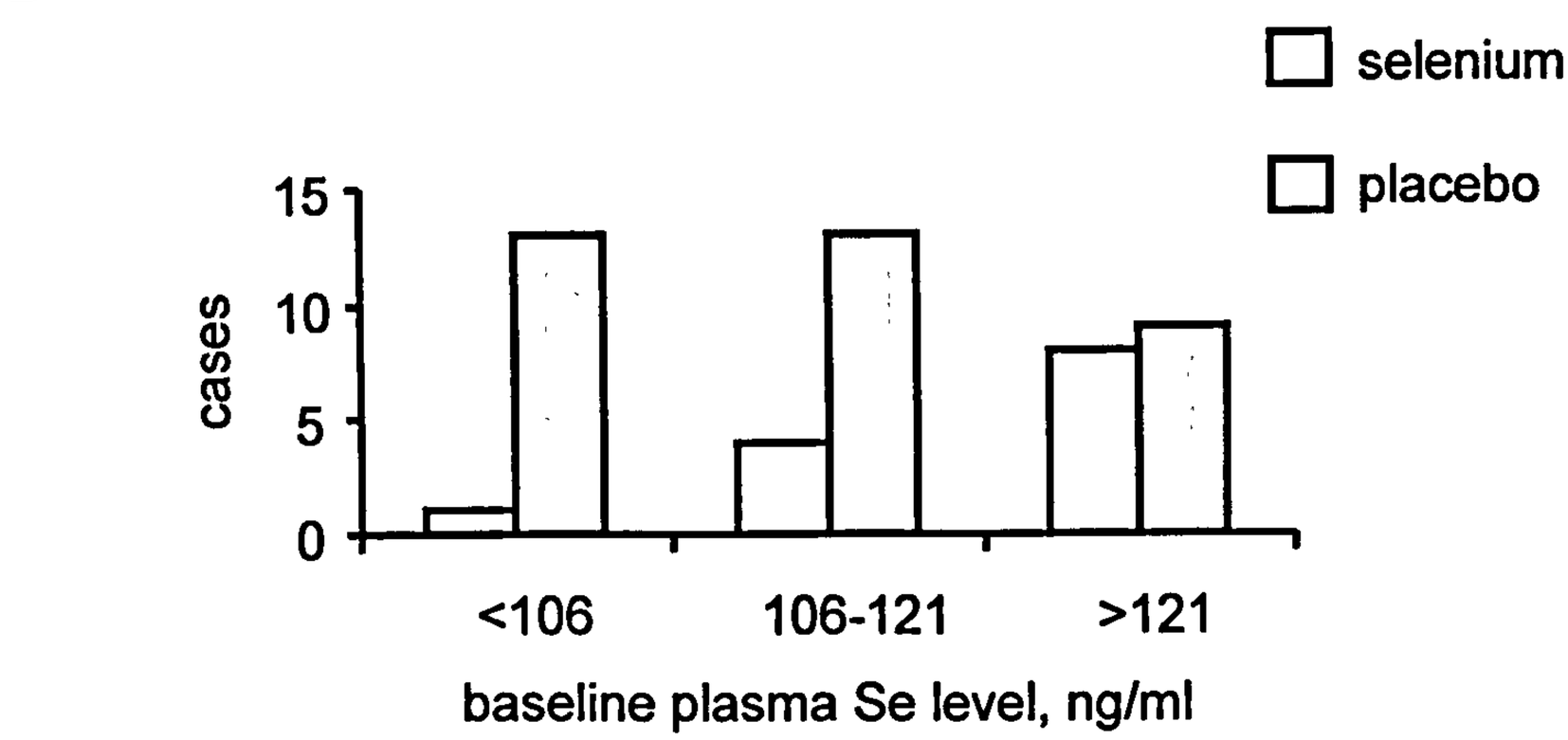


Fig 2.2.1 Effect of selenium on total cancer risk and prostate cancer risk by tertile of baseline plasma selenium (adapted from Clark et al., 1998 and Combs, 2000).

Follow ups and reanalysis of data from the NPC trial showed that the strongest effect against total cancer **(A)** and prostate cancer **(B)** risk was seen in people in the lowest tertile of plasma selenium content. Cases indicated are the actual number of individuals diagnosed with cancer.

Thus selenium supplementation appears to protect against certain types of human cancers: prostate, lung, colorectal, oesophagal, gastrointestinal, oral and liver. However, no protection is apparent for breast and skin cancers. The encouraging evidence from the last few years has emphasised the need for detailed, better controlled, large-scale trials. One such trial, the Selenium and Vitamin E Chemoprevention Trial (SELECT), to be performed in North America has already started recruiting volunteers. It plans to enrol 32,400 healthy men without a prior history of prostate cancer. The individuals will be randomised into four arms and supplemented daily with 200μg selenium (as selenomethionine), 400mg vitamin E, both selenium and vitamin E, or placebo, for 7 years. The total follow up time is currently scheduled as 11yrs (Miller, 2001). A Canadian trial will be addressing similar questions randomising both male and female subjects having successful resection of stage IA non-small cell lung carcinoma to receive 200μg selenium as selenised yeast or placebo daily for 4 years. Another large trial is also planned in Europe in 4 countries including the UK. This Prevention of Cancer by Intervention by Selenium (PRECISE) trial is scheduled to recruit about 50,000 subjects of both sexes and selenium supplementation is planned for 6.5 years (M. Rayman, personal communication).

2.3 Evidence from experimental carcinogenesis models

Several laboratory models of carcinogenesis have demonstrated the ability of a variety of selenium compounds to inhibit/retard tumorigenesis (extensively reviewed in (Combs, 1989; Combs Jr, 1997; Ip, 1998)). It was estimated, in 1986, that among the 100+ studies performed until then, about two-thirds showed reductions in tumour production and/or preneoplastic endpoints and half showed reductions of 50% or more (Combs and Combs Jr, 1986). Since then, several further studies have found similar reductions tumour yield or experimental metastasis using supranutritional levels of selenium, and only a limited number (Appel et al., 1996) have found such treatments ineffective. It is generally accepted that exposure, at higher levels than those required to support maximal selenoenzyme expression, to some selenium compounds can be anti-tumorigenic.

In contrast to the vast body of experiments indicating otherwise, four studies indicate selenium supplementation to enhance tumorigenesis. Each of these used sodium selenite at supranutritional doses. Three of these found, upon selenium treatment, reduction of tumour yields at one site while enhancing them at a different site (Ankerst and Sjogren, 1982; Dorado et al., 1985; Nakadaira et al., 1996). The fourth reported enhanced incidence of papillomas only when the carcinogen was administered in small multiple doses but not

with a single large dose (Perchellet et al., 1987). These studies may reflect changes in tumour-site specificity rather than enhanced tumorigenesis *per se*.

Compared to the large number of studies that have examined the contribution of supranutritional levels of selenium, relatively few have addressed the complementary question of selenium deficiency potentially enhancing tumorigenesis. Literature here is inconsistent (reviewed in (Combs and Gray, 1998)); 12 studies have found no effect while 10 suggested selenium deficiency increased tumour yield. 5 studies have reported reduced tumorigenesis on selenium deficiency. It also appears that selenium deficiency may result in increased risk depending on the carcinogen used (Nelson et al., 1996b). Thus the present evidence is insufficient to determine unequivocally whether nutritionally suboptimal selenium status increases cancer risk in animal models.

2.4 Effects of selenium on various stages of carcinogenesis

As protective effects have been observed in different phases of carcinogenesis, in different models, it is not clear if anti-cancer activities of selenium are stage specific. It may well be that different mechanisms of action (see Section 2.6) are of varying influence during different stages of carcinogenesis.

For example, protection from oxidative damage by selenium-dependent enzymes like GPx's and TR or carcinogen detoxificatiom (discussed in Section 2.6.1) may play a role in inhibiting the initiation phases of cancer by removing potentially genotoxic free radicals. In support of this, selenite (only when administered during the initiation stage) have been shown to protect from dimethylhydrazine- (DMH) induced colon tumours in mice (Temple and Basu, 1987), 7,12-dimethylbenz[a]anthracene- (DMBA) induced mammary tumours (Thompson et al., 1982) and aflatoxin induced hepatic lesions in rats (Baldwin and Parker, 1987). Similar data has been obtained during the initiation of DMBA-induced mammary tumours in rats supplemented with a number of aliphatic selenocyanates (Ip et al., 1995) and 1,4-phenylene-*bis*-(methylene) selenocyanate (pXSC) (el-Bayoumy et al., 1992).

Other studies suggest that the effects of selenium may not be stage-specific. Selenium treatment after the initiation phase is also protective in several models of hepatic, colonic and mammary cancers (Curphey et al., 1988; Ip and Ganther, 1992b; Thompson et al., 1981). One study of azoxymethane-induced colon tumours has reported selenite to be effective only in the post-initiation phases (Reddy et al., 1988), while in DMBA-induced mammary cancers in rats, the optimal effect is obtained when selenium is present

continuously, starting during initiation (Ip and Daniel, 1985). Studies on experimental metastasis would suggest selenium to be capable of exerting protection against these later stages of carcinogenesis (Tanaka et al., 2000; Yan et al., 1999). The dose (perhaps also type) of carcinogen used may be a contributory factor, as higher doses of DMBA appear to shift the anti-cancer activities of selenium to the post-initiation stages (Ip and Daniel, 1985; Ip et al., 1981).

It is pertinent to note that the intervention trials in humans discussed earlier detected protective effects of selenium supplementation within relatively short time periods, suggesting that selenium prevents development of malignancy in already initiated preneoplastic cells. In fact, in the NPC trial, the authors reported that reduction in the incidence of prostate cancer among selenium-treated subjects was observed in each year, except the very first (Combs Jr, 2000). This also suggests selenium treatment to be effective in the later stages of carcinogenesis.

2.5 Chemical form, dose and chemopreventive efficacy

The biological effects, including anti-cancer activities, of selenium appear to be properties of its various compounds and not of the element *per se*. Therefore, study of form and dose of selenium compounds is essential to understand its chemopreventive activity.

2.5.1 Active selenium metabolites

As discussed in Section 1.5, selenium compounds undergo extensive metabolism. Evidence suggests that, while various chemical sources of selenium may vary in their efficiency of absorption and/or retention, the biochemical basis for the anti-tumorigenic effects may reside in certain selenium metabolites produced, following absorption, from those sources.

Most commonly, selenite and selenomethionine (abundant in foods and feedstuff, and in selenised yeast) have been used in animal studies because of their commercial availability, and both these have been shown to suppress carcinogenesis in many different models without affecting animal health adversely. The first indication that metabolism of primary sources of selenium is required for selenium-mediated chemopreventive effects came from the observations that selenomethionine was generally less effective as a anti-cancer agent than selenite, although tissue selenium concentrations were higher in animals given selenomethionine (Ganther and Lawrence, 1997). At the same time, it was noted that

chemopreventive activity of selenomethionine could be significantly reduced by a low methionine diet (Ip, 1988). This suggested that limiting methionine resulted in diverting the selenomethionine to be non-specifically incorporated into body proteins thereby sequestering it away from its metabolic pathway (Fig 1.4.2).

Employing a strategy of delivering selenium at specific points along the metabolic pathway, using specific precursor compounds, the active metabolites involved in cancer protection could be identified. Extensive use of this approach by the Ganther and Ip groups (extensively reviewed in (Ganther and Lawrence, 1997; Ip, 1998)) has led to the suggestion that the active ingredient is downstream of hydrogen selenide (H_2Se). Thus, while selenite is more active than selenomethionine (SM), Se-methylselenocysteine (SMC) is about two times more effective than selenite (Ip and Hayes, 1989). Other methylated forms of selenium and their precursors, like selenobetaine, that by-pass H_2Se formation also shows chemopreventive activity (Ip and Ganther, 1992a; Ip and Ganther, 1990; Ip et al., 1991). Thus delivery of selenium in a pre-methylated form, by-passing H_2Se, may avoid the putative non-specific toxicity associated with H_2Se presumably via superoxide generation (Garberg et al., 1988; Wilson et al., 1992; Yan and Spallholz, 1993). While ROS generation and subsequent non-specific toxicity of inorganic forms of selenium has been demonstrated *in vitro*, these compounds remain very effective chemopreventive agents, *in vivo*, and are not associated with any adverse side effects. Presumably, entry via the inorganic pool at *in vivo* supplementation levels, leads to the formation of the active metabolites reasonably efficiently without the associated superoxide mediated damage seen *in vitro*.

Three types of selenium metabolites (see Fig 1.4.2) merit special mention: a) selenodiglutathione (SDG), the reductive metabolite of inorganic salts (selenite and selenate); b) H_2Se, the common intermediate of the reductive pathway and the catabolic pathway for selenium-containing amino acids and, c) methylated metabolites of selenide ($[CH_3]_xSe$).

SDG has been shown to inhibit (more effectively than selenite) development of transplantable tumours *in vivo* (Milner, 1985; Milner, 1986; Poirier and Milner, 1983) and inhibit cell growth *in vitro* (Fleming et al., 2001; Ghose et al., 2001a; Lanfear et al., 1994). It is unlikely to be the active metabolite *per se* since it is rapidly metabolised to other metabolites like H_2Se. However SDG shows marked differences to its precursor compound, selenite, including considerably enhanced ability to mediate apoptosis and inhibit cell growth *in vitro* (Fleming et al., 2001). As tyrosine phosphorylation patterns of

SDG are dissimilar to those of oxidants (Fleming et al., 2001), this suggests that SDG may have substantially diminished non-specific toxicity.

H_2Se may also contribute to anti-carcinogenic activity by undergoing oxidative metabolism and producing superoxides and H_2O_2, which may stimulate apoptosis induction (Lu et al., 1995b; Spallholz, 1994; Zhu et al., 1996). Though demonstrated in tissue culture cells, ROS generation has not been shown *in vivo*. H_2Se mediated increase in selenoprotein activity may also have a chemoprventive function. Moreover, recent work has, indirectly, implicated H_2Se as being crucial for apoptosis generation by both inorganic selenium compounds and SMC (A-S. Chung, personal communication). This study has revealed that treatment with non-toxic doses of Hg^{2+}, which is a specific selenide trapper, inhibits apoptosis induction not only by selenite and SDG, but also SMC. Thus selenide appears to be a key metabolite involved in a critical step in apoptosis-induction by various selenium compounds. If this were indeed the case, then routeing of SMC for demethylation to selenide would appear to play a major role in chemoprevention by methylated selenium compounds.

Methylation products of H_2Se are also known to possess anti-cancer activities. The Ganther and Ip laboratories have shown methylselenols and its derivatives are potently anti-carcinogenic (reviewed in (Ip, 1998)). Using methylselenol precursors, selenobetaine and SMC, they have demonstrated that these compounds have enhanced anti-tumour activities than selenite (Ip and Ganther, 1992a; Ip and Ganther, 1990; Ip et al., 1991). However, dimethylselenoxide which gets metabolised rapidly to dimethylselenide, was very poorly chemopreventive possibly because of its prompt excretion (Ip et al., 1991). Similar lack of chemopreventive efficacy was noted for trimethylselenonium (Ip and Ganther, 1992a). This reduced activity can also be partially attributed to the diminished reactivity of the Se bound to two or three carbon atoms. At doses used in the experiment both the compounds, however, were able to support maximum GPx expression, presumably by feeding back into the H_2Se pool (Ip et al., 1991; Vadhanavikit et al., 1993). This suggests the reversibility of the methylation pathway to the H_2Se pool may be essential to maintain optimal selenoprotein expression, however, it may also be necessary for chemopreventive action but this possibility has not been investigated in detail.

At low doses, arsenic competitively inhibits H_2Se methylation and methylselenol (and analogous di- or tri-methylated species) demethylation. Selenite-mediated, but not methylated selenium compound- mediated, chemoprevention is inhibited when animals are exposed to low doses of arsenic (which have no chemopreventive activity itself) (Ip et al.,

1991). Thus methylselenol, its precursors and derivatives, appear to have anti-carcinogenic activity distinct from those associated with the inorganic selenium pool. This implies that the inorganic selenium pool needs to be metabolised to methylated derivatives to have any chemopreventive activity.

2.5.2 Other selenium compounds

Since methylselenol precursor, methylselenocyanate, demonstrated chemopreventive activity, an examination of a series of aliphatic selenocyanates with increasing lengths of the carbon side chain suggested the chemopreventive potencies as: heptyl=pentyl>propyl>methyl (Ip et al., 1995). Thus longer the alkyl chains better the efficacy of selenocyanates.

SMC (a common constituents of food and feedstuff and selenised yeast) is readily metabolised to mono-, di- and tri-methylated species of selenium in animals primarily by scission to methylselenol by cysteine conjugate β-lyase, which also shows considerable activity with other Se-alkylcysteine derivatives, therby releasing the active Se-alkyl moiety (Andreadou et al., 1996a). Accordingly, Se-methyl-, Se-propyl- and Se-allyl-derivatives have been tested for chemopreventive activity and while the first two gave about 50% reduction in tumour yield, Se-allyl derivatives inhibit tumour formation by almost 90% (Ip et al., 1999).

Sulphur analogues, such as S-allylcysteine and related allylsulphides, occur in abundance in garlic. Therefore, growth of garlic in high selenium medium results in selenium-enriched garlic by substitution of sulphur by selenium in these compounds. These selenium-derivatives of sulphur compounds from selenium-enriched garlic when tested on laboratory models of carcinogenesis showed strong chemopreventive activity that was about twice that shown with selenised yeast (Ip and Lisk, 1995; Ip et al., 1996). Recent analysis has revealed γ-Glutamyl-Se-methylselenocysteine (GGSMC) to be the major chemical form of selenium (~73%) in selenium-enriched garlic (Ip et al., 2000a). Experiments suggest that GGSMC may serve as a carrier/precursor for SMC since the biological activities of the two compounds (including, chemopreventive efficacies, tissue accumulation, excretion and gene expression patterns) appear to be similar (Dong et al., 2001b). It is interesting to note that both SMC and GGSMC when administered to animals for a short period (4 weeks) immediately after carcinogen-dosing, provides lasting protection from methylnitrosurea induced mammary lesions (Dong et al., 2001b). This

would suggest that these compounds cause irreversible damage to the transformed cells, possibly by inducing apoptosis although this is yet to be demonstrated.

Selenium-enriched garlic has potentially several advantages over other methods of selenium supplementation in the general population. As it is mainly used for flavouring, overconsumption is less likely. At nutritional levels, it supports optimal selenoprotein expression and at higher doses has a strong chemopreventive action. However, it has reduced toxicity as its active constituent, SMC, is prevented from accumulation by methylation to di- and tri-methylated excretion products. High-selenium broccoli also exhibits strong chemopreventive activity in rodent models (Finley et al., 2000).

Research into developing aromatic selenium compounds was pioneered by Dr Karam El Bayoumy at the American Health Foundation, New York, with a view to develop novel reagents with lower toxicity. *p*-Methoxybenzeneselenol was the first aromatic selenium compound synthesised which showed inhibiton of tumour yield at liver, kidney and colon on treatment with the carcinogen azoxymethane (Reddy et al., 1985; Tanaka et al., 1985). Benzylselenocyanate (BSC) was another successful compound which showed anti-tumorigenic activities in various different models of cancer utilising different carcinogens (el-Bayoumy, 1985; Nayini et al., 1989; Nayini et al., 1991). However, BSC has a very pungent odour which reduced the food intake of animals resulting in growth depression. Therefore, to reduce its volatility, a second methylselenocyanate group was added in the *para* position resulting in 1,4-phenylene-*bis*-(methylene)selenocyanate (pXSC; used in this study). This compound had significantly less toxicity than BSC and subsequently was shown to possess the highest chemopreventive index (defined as the highest cancer inhibitory activity in relationship to its toxicity *in vivo*) for selenium compounds to date (Conaway et al., 1992; el-Bayoumy et al., 1992).

pXSC was shown to inhibit DMBA-induced mammary carcinogenesis by interfering with DNA adduct formation (el-Bayoumy et al., 1992), to have an anti-initiation effect in the azoxymethane colon cancer model (Reddy et al., 1992) and also inhibit mammary and colon carcinogenesis at post-initiation phases (Ip et al., 1994a; Reddy et al., 1992). In contrast to selenite, which does not effect 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) induced lung cancer, pXSC strongly inhibited tumorigenesis in this cancer model (el-Bayoumy et al., 1993). This finding is particularly relevant as NNK is strongly implicated in pathogenesis of tobacco-related lung cancers in humans (Hecht and Hoffmann, 1988). Later studies have also revealed inhibition of lung metastasis of melanoma cells in mice treated with pXSC (Tanaka et al., 2000).

It is clear that pXSC and related compounds release some selenium to the inorganic selenide pool, leading to the nutritional bioavailability of selenium from these compounds (Ip et al., 1994a). However, the rate of selenium release cannot entirely explain the chemopreventive properties of these compounds: 10ppm of selenium as pXSC is equivalent to 3ppm of selenium as selenite in the efficacy of cancer protection; yet only 1ppm of selenium as pXSC can replete GPx in a selenium-deficient animal as opposed to 0.1ppm selenium as selenite (Ip et al., 1994a). Thus the relative selenium concentration required for anticancer activity compared to that required to replete GPx for pXSC is 10 while it is 30 for selenite, suggesting that the pXSC molecule has some inherent anti-cancer activity that is independent of the release of selenium from the parent molecule. Very recently, glutathione conjugates of compounds like pXSC (pXSSG) have been developed and strong cancer inhibitory activities demonstrated (Rao et al., 2001). It appears that, as with selenite, glutathione conjugation may be the first metabolic step undergone by pXSC (Rao et al., 2001). Interestingly, while the chemopreventive efficacies of pXSSG and pXSC are similar, the sub-chronic maximum tolerated dose of pXSSG is 4 times higher than that of pXSC indicating reduced toxicity (Rao et al., 2001). It would be interesting to see the efficacy of this class of aromatic selenium compounds in clinical trials.

Selenium bonded directly to a benzene ring has provided another class of low molecular weight selenium compounds. This bonding is very stable and less of selenium is released during metabolism from these compounds and subsequently these are not efficient in repleting GPx in deficient animals (Ip et al., 1998a). Of the different compounds in this group, triphenylselenonium chloride (TPS) shows the most promise as an anti-cancer agent (Ip et al., 1994b). TPS had potent chemopreventive activity at 5ppm but no detectable influence on selenoprotein expression at 30ppm, and even at 200ppm no toxic effects were observed (Ip et al., 1998a). This observation also underscores the suggestion that selenoproteins may not be involved in cancer preventive activities and in the case for TPS there appears to be a particularly distinct separation of bioavailability, anti-cancer activity and toxicity.

To put this discussion in perspective, one should note that the NPC trial used a complex selenium source, selenised yeast (Clark et al., 1996). Until recently, it has been assumed that the major component of selenised yeast was selenomethionine (SM). However, development of new analytical techniques have revealed that selenomethionine accounts for only ~20% of all selenium containing material (Bird et al., 1997). Another ~20% is in the form of SMC, selenocystine and selenoethionine, while the remaining 40-50%

comprises a combination of hitherto unidentified compounds (Bird et al., 1997). As there is no evidence how these unidentified compounds may have differing effects on the multistep process of carcinogenesis, our current knowledge cannot entirely explain the biological basis of the cancer-inhibitory effect observed by Clark et al. (1996). Curiously a later paper, using the same techniques and having some of the investigators in common, have again re-established the abundance of selenomethionine as the major constituent of selenised yeast (~85%), most of it immobilised in proteins (Ip et al., 2000b).

Again, until very recently, it was assumed that all selenium in the plasma is protein-bound. New techniques have now revealed that a small fraction (2-5%) of selenium in plasma is non-covalently bound to proteins (Combs Jr, 2000). Given the assumption that intermediates of selenium metabolism discharge the anti-cancer activities, this fraction is likely to contain such species: thus it would be valuable to resolve this fraction to individual components as they may have tremendous implications for developing effective biomarkers of the critical selenium metabolites responsible for selenium-mediated chemoprevention.

In conclusion, it appears that normal products of metabolism of both inorganic and organic forms of selenium have anti-carcinogenic potentials, as does several synthetic compounds. While inorganic selenium salts would yield SDG, further metabolism would produce selenide, which is also the metabolic fate SM, and SMC (Fig 1.4.2). Selenide itself may exert major anti-cancer activities, but when accumulated in sufficient quantities may produce apparently anti-carcinogenic metabolites like methylselenols (and its derivatives). ROS produced by selenide may also directly contribute to chemoprevention by inducing apoptosis in preneoplastic cells. Such a central role for selenide indicates that though dose efficiencies may vary, both inorganic and organic selenium compounds can be anti-tumorigenic. Synthetic compounds like pXSC, TPS and BSC, which appear to release their selenium very slowly for general metabolism, may employ more direct effects (perhaps as very effective analogs of the natural anticarcinogenic metabolities) to mediate their chemopreventive effects.

2.5.3 Selenium dose and anti-carcinogenic effect

Anti-tumorigenic effects observed for various selenium-compounds require exposure to supranutritional doses, typically, for laboratory animals, >1mg selenium/kg diet or 0.7mg selenium/l drinking water. These are 5-10 times the level required to prevent clinical signs of selenium deficiency and support maximal selenoprotein (known) expression (no greater

than 0.5mg selenium/kg of diet). On a unit body weight basis, this is also greater than those experienced by most people, which is rarely greater than 200μg/person/day.

It has been estimated that maximum expression of GPx and selenoprotein P in plasma would yield a plasma selenium concentration of 80ng/ml, a level that can be supported by a daily dietary selenium intake of 30-40μg/day (Combs Jr, 2000; Yang et al., 1989). The baseline plasma selenium concentration of patients entering the NPC trial was 114±23ng/ml, equivalent to a daily intake of >85μg from dietary sources (Clark et al., 1996; Clark et al., 1998). It is therefore interesting to note that use of 200μg/day was associated with reductions in cancer risk, although it did not increase plasma GPx activities or alter Thyroxine:3,5,3'-Triiodothyronine ratios (a measure of selenoprotein deiodinase activity) (Clark et al., 1996; Clark et al., 1998; Combs Jr, 2000).

This makes it very unlikely that anti-cancer activities of selenium compounds can be completely explained by the involvement of known selenoenzymes. Selenium-deficiency may, however, limit the activity/s of one or more selenoproteins and enhance cancer risk. Therefore, depending on dose, selenium may play different roles in cancer prevention, viz., deficient individuals may benefit from supplementation by optimal expression of selenoproteins and selenium-replete individuals may benefit from diverting selenium resources to other metabolic routes.

From the above discussion on dose and form of selenium (and with reference to particular biological responses mediating chemopreventive effects of selenium dicussed later – Section 2.6), it appears that selenium has two basic roles in cancer prevention: a) as a component of antioxidant enzymes, and b) as anticarcinogenic metabolites. This has led to the proposal of a 2-stage model (Fig 2.5.1) for role of selenium in cancer prevention (Combs and Gray, 1998). According to this model, different selenium-induced biological activities are induced at different doses. These activities are mediated through either the functions of specific selenium-dependent enzymes or of certain selenium metabolites produced in large amounts at relative high doses (supranutritional) of the elements. The cumulative effect of all these anti-cancer activities is proposed to account for the robust chemopreventive action seen at supranutritional doses of selenium. The model predicts that set of anti-cancer activities involved in protecting a nutritional deficient individual, on supplementation with nutritionally adequate doses, is likely to be different from those conferring a cancer-protective action at supranutritional levels.

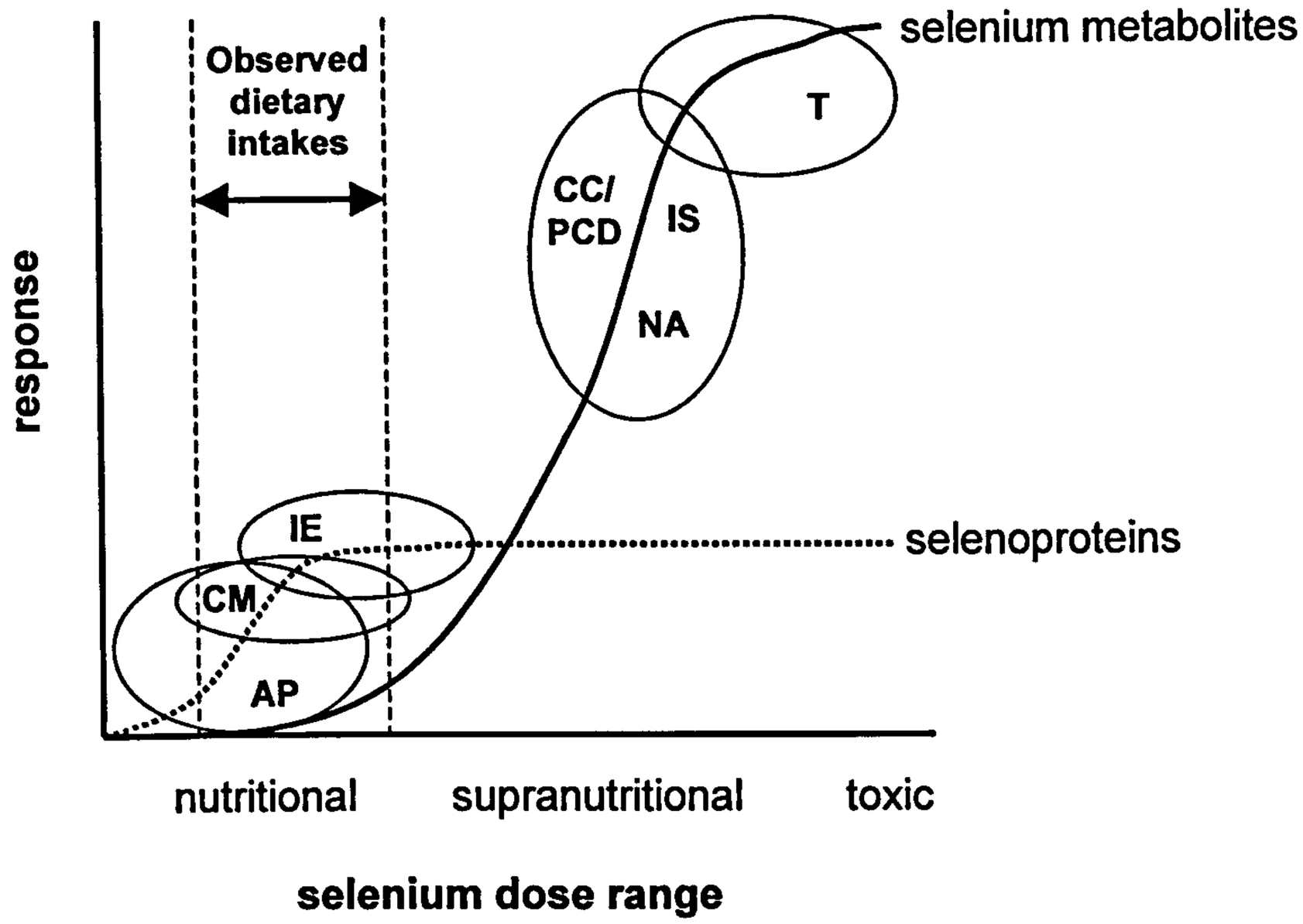


Fig 2.5.1 Two-stage model for roles of selenium in cancer prevention (adapted from Combs and Gray, 1998)

This model shows that selenium has two essential roles in chemoprevention, viz., regulation of antioxidant activity of selenoproteins and regulation of biological responses by selenium metabolites. Biological responses are: AP, antioxidant protection; CM, carcinogen metabolism; IE, immune enhancement; CC/PCD, cell cycle alterations and induction of programmed cell death (apoptosis); IS, enhanced immune surveillance and cell-mediated tumouricidal activity; NA, inhibition of neoangiogenesis; T, non-specific toxicity.

2.6 Candidate mechanisms mediating anti-cancer activities of selenium compounds

While a huge amount of data has accumulated over the years establishing selenium compounds as potent anti-cancer agents, little is known of the biological mechanisms that mediate this cancer-inhibitory activity. A particular problem has been the inability to address several of these issues in a clinical setting; therefore researchers are reliant on *in vitro* cell culture experiments and studies on laboratory animals.

2.6.1 Alteration of carcinogen action

Though no universal role has been delineated, selenium may exert some of its chemopreventive actions by altering carcinogen action. Chemopreventive doses of selenite and selenocyanates, but not selenomethionine, have been demonstrated to inhibit the formation of DNA-adducts by carcinogens like DMBA and bis(2-oxypropyl)nitrosamine (Ejadi et al., 1989; el-Bayoumy et al., 1992; Ip et al., 1995). While Phase I xenobiotic enzymes generally do not appear to be affected by selenium (reviewed in (Ip, 1986)), Phase II enzymes, like glutathione-S-transferases (GST) and uridine 5'-diphosphate-glucuronyltransferase, are induced in the liver and kidney upon selenium treatment at chemopreventive levels (Chidambaram and Baradarajan, 1996; Ip and Lisk, 1997). Consistent with this, some GSTs are upregulated in selenium-resistant variant cell lines (Wu et al., 1995b). However, as both chemopreventive and nutritionally repleting doses of selenium induce these enzymes to similar levels, their role in mediating the anti-cancer activities of selenium is debatable.

While selenite has been shown to suppress DNA adduct formation by aflatoxin in the chick (Chen et al., 1982b), the similar dietary levels do the opposite in rats (Chen et al., 1982a), suggesting a species-specific effect. Effect of azoxymethane is altered by selenium, possibly by increasing its hydroxylation and subsequent oxidation (Fiala et al., 1991). Promotion of ring hydroxylation of the carcinogen 2-acetylaminofluorene by selenium is thought to diminish the carcinogenic potential of this compound (Besbris et al., 1982). The production of mutagenic metabolites in the liver, by carcinogens such as DMBA, 2-acetylaminofluorene and benzopyrene, is also reduced by dietary selenium supplementation in rats (Gairola and Chow, 1982; Schillaci et al., 1982).

In summary, while considerable variation with respect to carcinogen type and host species is observed, chemopreventive levels of selenium can affect carcinogen action and may therefore influence the initiation phase of carcinogenesis.

2.6.2 Enhancement of immune surveillance

Selenium-deficiency has long been shown to impair T- and B-cell immune responses in animals. Antibody dependent cytotoxicity has been found not to be affected by selenium deprivation but some reports indicate that natural killer (NK) cell-dependent cytotoxicity is susceptible to selenium deficiency (Talcott et al., 1984). Selenium deprivation has also been reported to affect splenocyte differentiation and attenuated proliferative responses of T-cells on mitogenic stimulation (Chang et al., 1994; Finch and Turner, 1989).

Supranutritional levels of selenium have been found to stimulate cytotoxic activities of NK-cells (Kiremidjian-Schumacher et al., 1994; Kiremidjian-Schumacher et al., 1996; Petrie et al., 1989) and lymphokine activate killer cells in humans (Kiremidjian-Schumacher et al., 1996), though similar effects have not been reproduced in animals (Nair and Schwartz, 1990). Supplementation with 200μg of selenium/day, a dose equivalent to that used in the NPC trial, though the form of selenium was different, was found to promote destruction of tumour cells by cytotoxic lymphocytes and macrophages (Kiremidjian-Schumacher et al., 1994; Roy et al., 1994). Selenium exposure also leads to an increased production of high-affinity IL-2 receptor subunits (Roy et al., 1992; Roy et al., 1994) which may, conceivably, lead to accelerated clonal expansion of IL-2 stimulation-induced cytotoxic effector cells. Recently, investigations into several T-cell associated genes, like CD4, CD8 HLA-DR, have revealed the presence of open reading frames similar to selenoprotein P, potential SECIS elements and Sec insertion sequences in their mRNA (Guimaraes et al., 1996; Taylor, 1995). This raises the intriguing possibility that these may be functional selenoproteins that may confer an additional level of selenium-mediated regulation of the immune system.

2.6.3 Inhibition of angiogenesis and invasion

Growth of capillary blood vessels from existing ones, i.e., angiogenesis is essential for the growth, progression and metastasis of solid tumours (Folkman, 1971). Selenite, selenium-enriched garlic and SMC have all been shown to inhibit angiogenesis and tumour-induced neovascularisation in animal models (Jiang et al., 2000; Jiang et al., 1999; Lu, 2001; Schumacher et al., 2001). Whilst the molecular mechanisms involved in this process are

not clear, *in vitro* data suggests that reduction in the expression of a potent angiogenic growth factor, the vascular endothelial growth factor (VEGF), may be responsible (Jiang et al., 2000; Lu, 2001). pXSC has also been reported to inhibit VEGF and basic fibroblast growth factor (bFGF)-induced angiogenic sprouting in an *in vivo* model (Schumacher et al., 2001).

Selenium compounds have also been reported to modulate the activities of matrix metalloproteases (MMP), which mediate the degradation of the extracellular matrix, a prerequisite for cellular invasiveness and movement (McCawley and Matrisian, 2000). Inhibition of MMP-2 and MMP-9 gelatinolytic activities has been reported following selenium treatment (Jiang et al., 2000; Jiang et al., 1999; Yoon et al., 2001). Selenite has also been shown to increase the production of tissue inhibitor of matrix metalloprotease-1 (TIMP-1), an inhibitor of MMP activity (Yoon et al., 2001). These observations raise the possibility that modulation of cellular movement via alteration of MMP activities may contribute to angiogenic sprouting. Endothelial cells (lining capillaries) are sensitive to selenium-induced apoptosis; this may also be causally related to inhibition of neo-angiogenesis (Lu, 2001).

Reports of modulation of MMP activities suggest that these may contribute to reducing tumour invasiveness and metastasis. Several selenium compounds, including pXSC, inhibit experimental metastasis in animal models (Tanaka et al., 2000). *In vitro*, selenite has been shown to retard migration of tumour cells through a collagen matrix (Yoon et al., 2001). Cellular attachment and motility of HeLa cells is inhibited by non-growth inhibitory doses of selenite (Gong and Frenkel, 1994; Yan and Frenkel, 1992). Though this is a newly emerging aspect of selenium biology, evidence suggests selenium may contribute directly to inhibit neo-angiogenesis and tumour invasiveness.

2.6.4 Protection from oxidative damage

As the aetiology of several cancers is thought to involve mutagenic oxidative stress, the antioxidant activities of selenoproteins, such as GPxs, have been proposed to mediate chemopreventive effects of selenium. It is thought that these may operate by removing genotoxic H_2O_2 and lipid hydroperoxides, by blocking the production of ROS and malonyldialdehyde. Indeed, the production of UV-B and phorbol ester induced skin tumours in rat was inhibited by selenite and this correlated with increased GPx activity in the skin (Pence et al., 1994; Perchellet et al., 1987). Several other studies on rodents, using selenite, BSC, pXSC, selenomethionine and the synthetic selenium compound ebselen and

have shown inhibition of oxidative stress induced damage to DNA, lipids and proteins (El-Bayoumy et al., 2000; Fiala et al., 1997; Roussyn et al., 1996; Takada et al., 1992).

However, there is evidence that at least the cytosolic GPx (GPx1) is not involved since GPx1-null mice do not show any abnormal histopathologies up to 15 months age (Cheng et al., 1997; Ho et al., 1997) although they are more sensitive to exogenous oxidative stress (de Haan et al., 1998). In fact, GPx1-transgenic mice are actually more sensitive to DMBA/TPA-induced skin cancer, rather than the reverse, hypothetically because the generation of tumor-promoting lipoxygenase-derived peroxides is increased (Lu et al., 1997).

The anti-carcinogenic response seen in selenium-replete animals supplemented with supranutritional doses of selenium (>1ppm) cannot involve the known selenoproteins, as, animals show maximal GPx activities in most tissues at dietary levels of ~0.2ppm. Accordingly, the studies on selenium mediated inhibition of UV induced oxidative damage in rodents typically observed the said effect at dose ranges of 0.1-0.5ppm (Pence et al., 1994). Also, reports suggesting chemopreventive doses (1.5ppm) of selenium reduce lipid peroxidation only slightly (Lane and Medina, 1985) or not at all (Horvath and Ip, 1983) also go against this hypothesis.

2.6.5 Redox regulation and the thioredoxin system

Redox signalling is critical to the growth of several cancers and its modulation may be another underlying mechanism of cancer-inhibitory effects of selenium. Thiol residues on critical regulators and effectors, like transcription factors, are known to be involved in their regulation in response to changes in redox potential in the cell (Kamata and Hirata, 1999; Sun and Oberley, 1996). Selenium influences redox signalling in two ways; first, it may be a component of these active centres as selenocysteine and directly participate in redox reactions, secondly, selenium may modulate the activities of redox regulatory proteins by forming covalent adducts and catalysing thiol/disulphide reactions with crucial cysteine or Sec residues (as described in Section 1.6).

In vitro, low concentrations of selenite and SDG have been shown to inhibit PKC activity by covalently modifying catalytic cysteine clusters (Gopalakrishna et al., 1997). Thiol-dependent modification is also implicated in the inhibition of UV-induced stress-kinase activation by selenium (Park et al., 2000b). Cysteine modification is also the proposed mechanism for inhibition of the transcription factor NF-κB (Kim and Stadtman, 1997).

Thus, these activities could profoundly modulate redox signalling and subsequently affect cellular homeostasis. Such activities have been extended to a model (Ganther, 2000) where selenium may regulate redox signalling independently of its capacity to directly modulate changes in peroxide/ROS levels and the cellular redox potential. Redox-regulated transcription factors can have two states, ON and OFF, that differ by the oxidation state of regulatory cysteines that often involve presence or absence of intramolecular disulphide bonds, as in the OxyR transcription factor (Zheng et al., 1998). Selenium can reversibly catalyse redox-changes in these proteins by reversing or promoting the formation of disulphide bonds (type 3 mechanism, see Section 1.6), leading to resetting the protein activity to the basal state. Thus, by altering the redox state of important targets selenium may influence the activity of these molecules.

A recent report has shown that benzeneselenic acid can displace zinc from tightly bound zinc-sulphur clusters in metallothionein (Jacob et al., 1999). This mechanism can form the basis for removal of zinc by selenium from zinc-finger of transcription factor and other signalling proteins and could be a putative chemopreventive mechanism. Selenite has also been shown to inactivate the c-myc oncogene in hepatoma cells leading to the loss of its transforming potential (Yu et al., 1990). This appears to be mediated by the competition of selenium with zinc on the c-myc-activating zinc-finger (Maz) protein (Nelson et al., 1996a). Curiously, cysteine proteases like caspases have inhibitory zinc atoms blocking the active site cysteine. Thus direct displacement of this zinc molecule by selenium compounds can conceivably activate the caspases and induce apoptosis.

A prime candidate likely to have a significant influence on redox modulation by selenium is the selenoprotein thioredoxin reductase (TR). TR and thioredoxin (Trx) form a redox system and have diverse functions, like regulating transcription factor activities and providing reducing equivalents for DNA synthesis (recently reviewed in (Holmgren, 2000)). While TR expression is low in many normal tissues, it is overexpressed in transformed cells (Powis and Montfort, 2001). In fact, selenite and SDG are highly efficient oxidants of Trx and substrates for TR (Bjornstedt et al., 1992; Kumar et al., 1992) and so it has been proposed that the inhibitory effects of selenite and SDG on cell growth may involve competitive inhibition of the thioredoxin system and oxidation of structural cysteines in Trx to disulphides (Ganther, 1999).

In cancer cell lines and animals, supranutritonal doses of selenium as selenite, increases the activity of TR (Berggren et al., 1997). Interestingly such changes have not been observed *in vitro* with SMC and SM and in general appear to be specific to epithelial cells (Ganther,

1999). Animal studies have also noted that the increased TR activity is not permanent as prolonged exposure to high selenium levels result in a decline to basal levels (Berggren et al., 1999). Recent evidence suggests that TR activity is not affected in rats fed with chemopreventive doses of SMC and methylselenic acid (Ganther and Ip, 2001).

Trx is also overexpressed in many cancers, is secreted by tumour cells and stimulates proliferation while inhibiting apoptosis in cells in culture (Powis and Montfort, 2001). Cells transfected with Trx cDNA show increased tumour growth and decreased apoptosis *in vivo* and it appears that it is the reduced form that mediates these effects (Powis et al., 1998). A continuously high dietary level of selenium results in a reduction in TR activity, postulated to be due to formation of an irreversible diselenide 'trap' at the selenocysteine in its active site (Ganther, 1999): this would also result in a reduction in the level of reduced Trx . Of particular interest in this context is the fact that ASK1, a mediator of pro-apoptotic signalling, is inhibited by binding of reduced Trx to its N-terminal region (Ichijo et al., 1997; Saitoh et al., 1998): thus depletion of reduced Trx by selenium compounds via this mechanism or by direct oxidation of reduced Trx might be expected to activate ASK1 and induce apoptosis.

2.6.6 Activities of other selenoproteins and other new functions

In the NPC trial showing inhibition of human cancer incidence on selenium supplementation, very few, if any, patients had nutritionally limiting selenium intakes as judged by their baseline plasma selenium level (see Section 2.5.3). Thus it appears to be highly probable that anti-cancer activities of selenium at supranutritional doses involve mechanisms unrelated to the activities of known selenoproteins. However, a role for selenoenzymes in nutritionally deficient situations may be possible. If any as yet unidentified and/or uncharacterised selenoproteins are involved in mediating anti-cancer actions, selenium levels at which their activities are saturated is likely to be much higher than the known selenoenzymes.

Newly identified selenoprotein with unknown functions, like the 15kDa selenoprotein whose expression is reduced dramatically in some cancers is potentially interesting and may reveal clues to novel cancer-inhibitory mechanisms employed by selenium (Gladyshev et al., 2000).

Selenoenzymes like iodothyronine 5-deiodinases (DI) may also affect cancer cell growth by regulating thyroid hormone metabolism. Thyroid hormones have been shown to oppose

oestrogen-induced proliferation of breast cancer cells (Vorherr, 1987). This may be relevant as breast cancer patients have low selenium levels and lower plasma thyroxine levels (Combs Jr and Lu, 2000). Breast cancer incidence is also significantly higher in areas of endemic iodine deficiency compared to non-endemic areas (Combs Jr and Lu, 2000).

In several cancers, tumour suppressor genes may be inactivated by methylation of specific cytosine residues. Inhibition of methyltransferesae (MTase) activity has also been suggested as an important mechanism of chemoprevention by selenium compounds and recent work has demonstrated that selenite, BSC and pXSC can efficiently inhibit MTase activity (Fiala et al., 1998), presumably via reactions with the critical thiol residues on the MTase protein.

In contrast to increased methylation of key tumour suppressor genes, overall genomic hypomethylation of DNA has been recognised as a very consistent early event in human colorectal carcinogenesis (Feinberg and Vogelstein, 1983). Recently, *in vitro* and *in vivo* studies using selenite and selenomethionine have shown that selenium may prevent occurrence of hypomethylated DNA in the colon and liver, suggesting that this may also be a potential anti-cancer activity (Davis et al., 2000).

2.6.7 Growth inhibition by suppressing proliferation and/or apoptosis

From a cellular point of view, deregulation of tissue size homeostasis is the basis for cancer, thus the disease can occur in a tissue when the rate of proliferation exceeds the rate of cell death. Selenium compounds can have a negative influence on carcinogenesis by decreasing the rate of cellular proliferation and/or increasing the rate of apoptosis. A large corpus of data has accumulated indicating that selenium compounds can inhibit cellular proliferation and/or induce apoptosis. A strong correlation has been observed between the effectiveness of selenium compounds as chemopreventive agents *in vivo* and their ability to induce growth arrest and apoptosis *in vitro* (Lanfear et al., 1994; Ronai et al., 1995; Thompson et al., 1994; Wilson et al., 1992). Animal studies have also confirmed the growth inhibitory effects of selenium at chemopreventive conditions *in vivo* (Ip and Dong, 2001; Ip et al., 2000c; Ip et al., 2000d).

2.6.7.1 Cell cycle, apoptosis and their regulation

Although neoplasia involves many other processes, in almost all instances, the underlying platform for neoplastic growth is deregulated cell proliferation and suppressed cell death. It is understandable therefore, that evolution has forged very tight and complex regulatory mechanisms to maintain cellular homeostasis. Thus, conversely, intervention at the level of these fundamental biological underpinnings of tumour cells can form the basis of anti-cancer therapeutic strategies.

A cell normally undergoes a growth (or 'gap') phase (G1) prior to replicating its DNA in the synthesis phase (S). Following a further 'gap' phase (G2) the cell finally enters the mitotic phase (M) and divides (Murray and Hunt, 1993) (see Fig 2.6.1). The timing and order of cell cycle events are monitored during cell cycle checkpoints that occur at the G1/S phase boundary, in S phase, and during G2/M phases (reviewed in (Murray, 1994)). These checkpoints ensure that critical events in a particular phase are completed prior to the initiation of the next phase, thereby precluding the generation of genetically abnormal cells. Cell cycle progression can be blocked at these checkpoints in response to the status of both the intra- and extracellular environments. For example, growth arrest is induced on detection of incomplete DNA replication, DNA damage or chromosomal misalignment on the mitotic spindle (Murray, 1994). Also, the expression of some genes necessary for DNA replication occurs only in the presence of mitogens (Murray and Hunt, 1993). Upon rectification of the error, progression through the cell cycle resumes. However if the aberration is too severe or the cell is incapable of repairing the damage: apoptosis is invoked to eliminate damaged cells (Murray and Hunt, 1993). The choice of progression or delay while the error is rectified or apoptotic cell death is decided at the checkpoints.

The regulation of the cell cycle is achieved primarily by the activities of two protein families: the cyclins and the cyclin-dependent protein kinases (cdk's) (Hunter and Pines, 1994; Murray, 1994). The kinase activity of cdk's is dependent on their interaction with activating cyclins and subsequent phosphorylation of specific substrates allows progression through the cell cycle (Fig 2.6.1). The abundance of specific cyclins increases during the phase of the cell cycle where they are required and decline in phases when they are not needed (Murray and Hunt, 1993). For example, usually cyclin D associates with cdk4 and cdk6 during early G1, whereas cyclin E activates cdk2 during the G1 to S transition. Cyclin A binds to cdk2 or cdc2 during S phase and the G2 to M transition (Fig 2.6.1). Thus specific cyclin/cdk complexes are activated, and their phosphorylation of particular proteins permit the cell cycle to progress. The transcription of genes necessary for S phase,

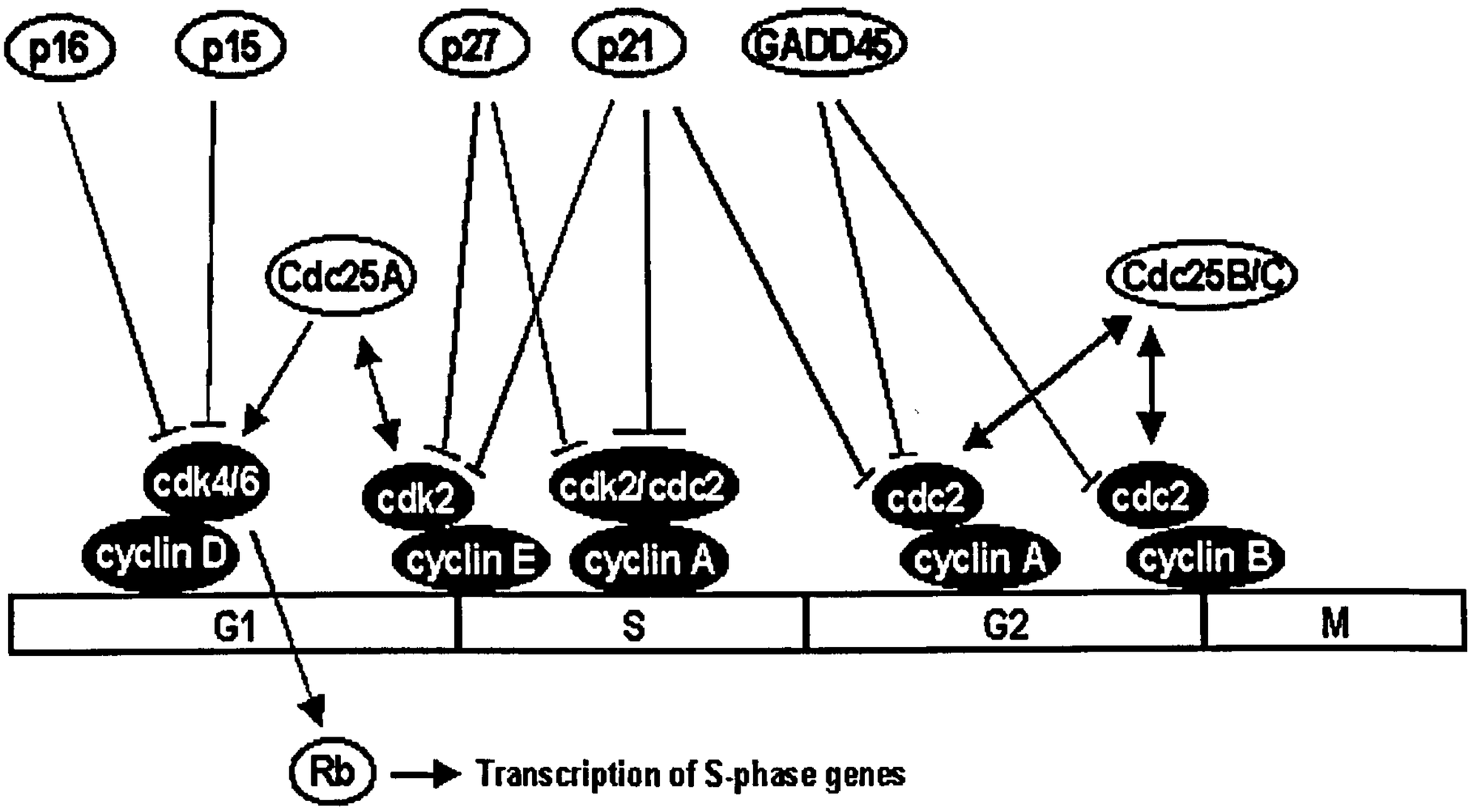


Fig 2.6.1 Regulation of cell cycle progression.

The principal regulatory components of the mammalian mitotic cell cycle are represented. The major cdk's (red) and cyclins (blue) and their cell cycle phase-specific regulation is shown (see text for discussion). Some other regulatory components are also indicated. G1, 'Gap' phase 1; S, synthesis (DNA) phase; G2, 'gap' phase 2; M, mitosis.

for example, is regulated by cyclin D/cdk4-dependent phosphorylation of the retinoblastoma (Rb) protein (Fisher, 1997; Morgan, 1995).

Cyclin/cdk complexes can also be bound by cdk inhibitor proteins which inhibit kinase activity and prevent cell cycle progression. Two major families of cdk inhibitor proteins have been identified: the CIP/KIP family, including p21, p27 and p57, predominantly inhibit G1 to S transition and the INK4 family, including p15, p16, p18 and p19, which are often mutated in certain cancers and specifically interact with cdk4 and cdk6 (Fig 2.6.1) (Harper and Elledge, 1996; Xiong, 1996).

Several other genes are known to modulate cell proliferation by affecting the formation or activity of cdk complexes. For example, the proto-oncogene c-myc is a transcription factor which with other factors, like Max, to promote mitogenesis (Amati et al., 1993). The expression of c-myc, which is modulated by growth factors, is required for quiescent cells to enter the cell cycle. This activity is dependent on c-myc induced transcription of Cdc25, a phosphatase that activates cdk's (Galaktionov et al., 1996).

Apoptosis is an evolutionary conserved, genetically regulated form of cell death necessary in many processes, ranging from embryonic development and tissue homeostasis. It is distinguished from necrosis by characteristic chromatin condensation, nuclear fragmentation, cell shrinkage and plasma membrane blebbing, whereby cells break into small, membrane-surrounded fragments (apoptotic bodies) that are phagocytosed without inciting an inflammatory response (Wyllie, 1997).

Survival of all somatic cells requires the continuous input of survival and trophic signals to suppress apoptosis (Evan and Vousden, 2001). The central engines of apoptosis are the caspases, cascades of cysteine aspartyl proteases that implement cell death by cleaving a variety of intracellular substrates triggering cell dissolution (Earnshaw et al., 1999; Wolf and Green, 1999). Caspases exist as latent zymogens and their activation comprises an activation cascade involving multiple caspases which, according to their sequence of activation, are organised into apoptotic initiator caspases (caspase 2, 8, 9 and 10) and apoptotic executor caspases (caspase 3, 6 and 7) (Earnshaw et al., 1999; Wolf and Green, 1999) (Fig 2.6.2). One such apical cascade pathway is mediated by the transmembrane death receptors of the Fas/TRAIL/tumour-necrosis factor (TNF) receptor 1 family, whose ligation triggers recruitment of adaptor proteins like Fas-associated death (FADD) domain protein and assembly of a multiprotein receptor-bound death-inducing signalling complex (DISC) that recruits and activates the initiator caspase 8 (Fig 2.6.2)

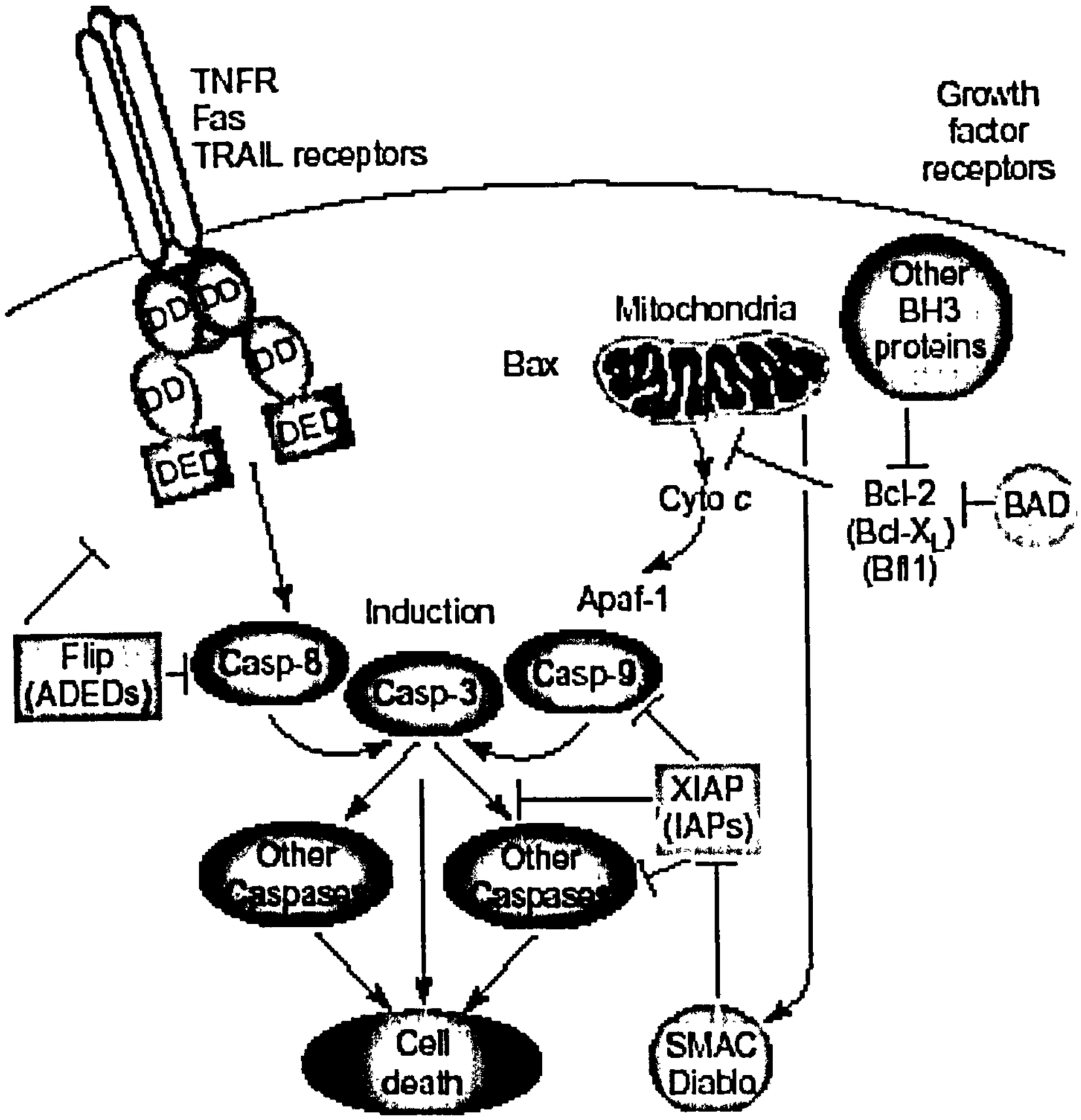


Fig 2.6.2 Molecular mechanisms of apoptosis induction (adapted from Reed, 2001).

The molecular machinery responsible for the induction of apoptosis is summarised in this figure. The mitochondria-dependent and the death receptor pathway and their major regulatory components are delineated. Functions of the components have been discussed in the text. DD indicates proteins with 'death domain' motifs; DED, proteins containing 'death effector domain' interaction motifs.

(Walczak and Krammer, 2000). The other major death-signalling pathway involves the mitochondrion, which acts as an integrating sensor of death insults by releasing cytochrome c into the cytosol where it triggers caspase activation (Hengartner, 2000). The mitochondrial pathway is thought to be the principal target of survival signalling, which acts by stabilising mitochondrial function and integrity and suppressing cytochrome c release. Once cytochrome c is released it directs the formation of an intracellular apoptosome complex that recruits the initiator caspase 9 via the adaptor protein Apaf-1 (Fig 2.6.2) (Hengartner, 2000). Activation of both these initiator caspase cascades lead to the activation of executor caspases like caspase 3 (Reed, 2001).

The inhibitor of apoptosis proteins (IAP's) are a family of evolutionary conserved apoptosis suppressors, which function mainly by inhibiting caspase activity (Reed, 2001). Consistent with their role, some IAP's like survivin are commonly overexpressed in cancers. Other members of this family include XIAP, cIAP1 and cIAP2. IAP's in turn, have been shown to be inhibited by the mitochondria-released protein SMAC(DIABLO) (Fig 2.6.2). Another family of proteins, the anti-apoptotic death effector domain (ADED), which include members like Flip, which inhibits FADD and caspase 8 activity (Fig 2.6.2) (Reed, 2001).

The mitochondria-dependent pathway is governed by the Bcl2 family of proteins which are conserved throughout metazoan evolution. Both pro-apoptotic (like Bax, Bad, Bak) and anti-apoptotic (like Bcl2, $Bclx_L$) Bcl2 family proteins exist, which often form complex networks of homo- and heterodimers (Reed, 2001). The relative amounts of active pro- and anti-apoptotic proteins determine the sensitivity or resistance of cells to a variety of impinging apoptotic stimuli (Hengartner, 2000). Alterations in the expression of Bcl2 family members have also been implicated in cancer (Reed, 2001). The primary function of these proteins appear to be the regulation of the release of cytochrome c and other apoptogenic proteins from mitochondria (Fig 2.6.2) (Reed, 2001).

The apoptotic programme is optimised to ensure that signals for phagocytosis are displayed well before the release of cellular constituents. A major phagocytosis signal is the translocation of phosphatidylserine to the outer leaflet of the plasma membrane (Henson et al., 2001).

In recent years, several instances of caspase-independent programmed cell death have identified (Leist and Jaattela, 2001). Non-caspase proteases like cathepsins and calpain have been implicated in triggering apoptosis by cleaving death substrates that lead to cell

death. Fas receptor has been also described to induce caspase-independent cell death, particularly in activated primary T-lymphocytes presumably via the induction of ROS (Leist and Jaattela, 2001). Mitochondria may also mediate a cell death, independent of caspase activation by the release of mitochondria-derived ROS or by the release of apoptosis-inducing factor (AIF) from the intermembrane space. This flavoprotein can directly induce nuclear changes and death induction (Leist and Jaattela, 2001).

Selenium treatment has been shown to modulate a number of key regulators of cell cycle progression and apoptosis (summarised in Table 2.6.1) and are discussed next.

2.6.7.2 Cell cycle progression and selenium

Post-initiation anti-cancer effects of selenium are likely to involve perturbation of tumour cell behaviour. Exposure of cells to selenium compounds like selenite has been shown to reduce DNA, RNA and protein synthesis and inhibit cellular proliferation and is accompanied by a block in the cell cycle at the S/G2-M phase ((Lu et al., 1995a; Sinha et al., 1996) and reviewed in (Combs Jr and Lu, 2000; Ip, 1998)). In contrast, SMC induces a much moderate growth inhibition that was accompanied by a block at the G1 phase of the cell cycle (Sinha et al., 1996). pXSC is known to inhibit the activity of thymidine kinase (TK) (Tillotson et al., 1994) which may directly affect the rate of DNA synthesis and subsequently cell cycle progression, though inhibition of cell cycle progression has not been reported for pXSC. As the first step in thymidine incorporation involves its phosphorylation by TK (Broet et al., 2001), the close correlation between TK inhibition and suppression of thymidine incorporation suggests a functional interaction.

SMC and its derivative, methylselenic acid, was found to inhibit cdk2 activity and reduce the cdk2-cyclin E content, which is compatible with the observed G1 arrest (Sinha et al., 1996; Sinha et al., 2001). However, selenite also decreased cdk2 activity but did not significantly affect its association with cyclins D1, A or E (Sinha et al., 1996). This observation is inconsistent with the observed S/G2-M arrest seen on treatment of cells with selenite (see Fig 2.6.1). A later study, using synchronised cells treated with SMC, revealed a significant delay in progression through the S phase rather than a block at G1 and this was associated with a reduction in cdk2 activity but only a transient inhibition of cdk2-cyclin E association (Sinha and Medina, 1997). While this would allow entry into the S phase, progression through S would be severely delayed. It should be noted that while these studies offer a plausible explanation for observed cell cycle perturbations upon SMC treatment, functional evidence (for example, whether constitutively active cdk2 could

Form	Parameter	Outcome
Selenite	ROS	E
	DNA SSBs	E
	eIF2	I
	p53	E
	AP-1	I
	NF-κB	I
	c-myc	I
	c-fos	E
	PKC	I
	PKA	I
	DNA cytosine methyltransferase	I
	cdk2	I
	cyclins A, D1, E	NE
	caspase 3	E/NE
	PARP cleavage	E/NE
	JNK	E
	p38	E
	GADD family	E
	Bcl2, Bax	NE
SDG	ROS	E
	p53	E
	AP-1	I
	PKC	I
	PKA	I
SMC	ROS	E
	DNA SSBs	NE
	PKC	I
	PARP cleavage	E
	Caspase 3, 8, 9	E
	JNK	NE
	p38	NE
	cdk2	I
	cyclin E	I (transient)
	GADD family	E
	PCNA, p21(in vivo)	NE
	cyclins A, D1 (in vivo)	I
	p27 (in vivo)	E
	Bcl2 (in vivo)	I
pXSC	PKC	I
	DGK	E
	PKA	I
	TK	I
	JNK	E
	DNA cytosine methyltransferase	I
	PKC (in vivo)	I

Table 2.6.1 Molecular targets of selenium compounds.

The current knowledge of candidate molecular targets of some major chemopreventive selenium compounds, that may mediate their proliferation-inhibitory and apoptosis-inducing abilities, is summarised in this table. I: inhibition, E: enhancement and NE: no effect. Other abbreviations have been discussed in the text.

override cell cycle defects) is not currently available. Selenomethionine, on the other hand, has been shown to induce a transient block in the G2/M stage accompanied by a concomitant decrease in cyclin B levels and cdc2 activity (Chigbrow and Nelson, 2001; Menter et al., 2000). Thus different, selenium compounds appear to block cell cycle progression at different stages, possibly involving modulation of different regulatory molecules.

Selenium compounds like pXSC, BSC, selenite, SDG and SMC have been shown to inhibit activity of PKCs (Foiles et al., 1995; Gopalakrishna et al., 1997; Rao et al., 2000; Sinha et al., 1999). This may explain the some of the cell cycle effects noted above, as cdk2 has been proposed to be a distal target for PKCs (Hamada et al., 1996). Several selenium compounds, including SDG and Se-allylselenocysteine (SAC) have been shown to induce accumulation of p53 which could also lead to cell cycle arrest (Jiang et al., 2001b; Lanfear et al., 1994). Consistent with this, SAC treatment can elevate p21 protein levels and inhibit Rb phosphorylation (Jiang et al., 2001b), which may influence cell cycle progression. Rb phosphorylation is initiated predominantly by cyclin D complex formation with cdk4 and is sustained by cyclin E-cdk2 complex formation (see Section 2.6.7.1). Interestingly, SAC treatment, *in vitro*, reduces the protein levels of cdks 2 and 4, and cyclins E and D (Jiang et al., 2001b; Zhu et al., 2000a). Inhibitors of cdk activities, p21 and p27 are also induced on exposure to SAC (Jiang et al., 2001b; Zhu et al., 2000a). Members of the growth arrest and DNA-damage inducible (GADD) family are induced by selenium compounds like selenite, methylselenocyanate and SMC (Kaeck et al., 1997; Sinha et al., 1999) and may represent functional pathways leading to cell cycle modulation, though this is not reproducible *in vivo* (Lu, 2001).

Selenite and SDG have been shown to inhibit overall protein synthesis, possibly via phosphorylation (and subsequent inactivation) of eukaryotic initiation factor 2 (eIF-2) (Safer et al., 1980; Vernie, 1987). Such an inhibition has been shown to associate with inhibition of tumour growth (Combs Jr and Lu, 2000).

Whether cell cycle arrest and apoptosis induction are mutually exclusive events (perhaps dependent on form of selenium and cell type) or whether these events are interrelated is not known. However, evidence from other contexts suggests that these cellular responses are intimately related, often sharing major regulatory components. For example, SAC induces both cell cycle arrest and apoptosis and both appear to be associated with p53 expression (Jiang et al., 2001b). There is precedent, for example on UV exposure, that a delay in cell cycle progression may initiate apoptosis-induction (Bulavin et al., 2001), and a similar

observation has been made in colon cancer cells treated with selenomethionine (Chigbrow and Nelson, 2001).

In terms of chemopreventive effects, growth inhibition not accompanied by apoptosis will only be effective as long as the agent is present continuously, because elimination of transformed cells is not possible. This is the response observed in studies with TPS, which inhibits DNA synthesis but does not induce apoptosis (Lu et al., 1995a). When administered continuously during the entire phase of tumour promotion and progression it very efficiently suppresses tumour growth, presumably by cytostasis. However, when the treatment period is shortened, its efficacy decreases dramatically (Ip et al., 1998b). Selenium compounds that are potent inducers of apoptosis, a shortening of selenium treatment does not affect chemopreventive efficacy (Baldwin and Parker, 1987; el-Bayoumy et al., 1992; Ip et al., 1995).

A recent report, using SMC, in a much used model for selenium-mediated chemoprevention of mammary gland lesions, found no alteration in BrDU labelling or the expression of cell cycle biomarkers PCNA and cyclin D1 in either normal epithelia or in premalignant lesions – while the progression of the latter to carcinoma was inhibited (Ip et al., 2000c). This suggests that inhibition of cell growth is not a primary response to SMC. In contrast, TPS, consistent with its activity described in the last paragraph, decreased BrDU incorporation and expression of PCNA and cyclin D1, and while the total number of lesion were not reduced, there were significantly less larger lesions (Ip et al., 2000c).

Further interesting observations were reported in a follow-up of the study discussed above. Detailed analysis revealed that SMC significantly inhibited BrDU incorporation and levels of cyclins A and D1, and elevated p27 expression only in physically smaller lesions (Ip and Dong, 2001). In larger lesions no effect on cellular proliferation (assessed by the same criteria) was detectable and considering all lesions together (prior to size-dependent grouping) no overall inhibition of proliferation rates was observed (Ip and Dong, 2001). Apoptosis induction, however, was evident irrespective of size. These observations imply that selenium-mediated inhibition of cellular proliferation may be sensitive to the pathological stage of the lesions. A 'rheostat' mechanism could be invoked to explain this phenomenon where either early lesions are more responsive to SMC-induced inhibition of proliferation than pathologically advanced ones or transformed cells become more resistant to SMC-induced antiproliferative effects as they acquire a certain number of mutations. As apoptosis is induced, irrespective of lesion size (equivalent to pathological stage?), this might be the primary mechanism of chemoprevention. This is borne out by the fact that

short-term treatment of SMC offers comparable chemopreventive efficacies as continuous treatment (Ip et al., 1991). Since the number of the premalignant lesions in the mammary gland far exceeds the number eventually progressing to carcinomas, further studies need to be performed to address which subset of lesions ultimately progress. Perhaps only those pre-disposed to progress (due to accumulation of a certain number of mutational events?) are targeted by the primary response, i.e. apoptosis, induced by selenium. Others, likely to be smaller if pathological stage correlates with physical size, may be subject to secondary effects like inhibition of proliferation.

2.6.7.3 Apoptosis and selenium

Induction of apoptosis is a plausible mechanism for selenium-mediated chemoprevention, as it can delete carcinogen-initiated cells and suppress clonal expansion of a transformed cell population. Our laboratory, and others described apoptosis-induction by selenium compounds *in vitro* in the early 1990s (Lanfear et al., 1994; Thompson et al., 1994; Wilson et al., 1992). Since then a substantial proportion of the literature has focused on this phenomenon and strong evidence for apoptosis at chemopreventive levels have been shown to occur both *in vitro* and *in vivo* (el-Bayoumy et al., 1995; Ip et al., 2000c; Ip et al., 2000d; Ronai et al., 1995). While p53 appears to be induced by several selenium compounds, our group, using a mouse erythroleukemia cell line, was able to show that induction of apoptosis by SDG did not necessarily require functional p53 activity (Lanfear et al., 1994). This has since been confirmed for methylselenocyanate in a mouse mammary cell line with a p53 null phenotype (Kaeck et al., 1997) and for selenomethionine in a colon cell lines expressing a mutated p53 (Redman et al., 1997). As p53 mutations are present in a majority of human cancers, the fact that selenium-induced apoptosis does not require functional p53 is of potential usefulness.

A major issue in the field has been the involvement of non-specific genotoxicity. Selenite has been shown to induce DNA single strand breaks (SSB) in a murine leukaemia cell line possibly induced by the generation of ROS (Lu et al., 1994). DNA SSBs have also been shown to precede cell death and apoptotic DNA fragmentation induced by selenite treatment (Lu, 2001). Specific inhibition of endonucleases responsible for apoptotic DNA fragmentations prevented loss of viability but not the generation of SSBs (Lu, 2001). Similar genotoxicity via the induction of ROS has been reported for selenite and SDG in other *in vitro* systems, though this remains to be verified *in vivo*. In appears that the generation of H_2Se by the inorganic pool of selenium is critical for induction of ROS and subsequent genotoxicity, since inhibition of H_2Se formation attenuates selenium-induced

loss of cellular viability (Garberg et al., 1988). Moreover, methylated compounds that bypass the H_2Se stage induce apoptosis without accompanied genotoxicity (i.e. SSBs) (Wilson et al., 1992). Our group has recently demonstrated that free radical scavengers like mannitol and pyrollidine dithiocarbamate (PDTC) do not inhibit SDG-induced loss of cellular viability while they attenuate pXSC-induced loss of viability suggesting different molecular targets of these two compounds (Fleming et al., 2001). N-acetylcysteine (NAC), another antioxidant, however, inhibits anti-proliferatory effects of pXSC, selenite and SDG (Fleming et al., 2001). Similar results have been reported elsewhere for selenite-induced apoptosis, where mannitol also failed to inhibit DNA SSBs (Lu, 2001). These results would suggest that free radicals do not mediate the generation of SSBs and loss of viability. Inhibition of cellular viability by inorganic selenium compounds but not methylated compounds has been assumed to involve ROS as established by ROS generation assays and protective effects of NAC supplementation. However, the situation is not entirely clear since a recent report has shown that apoptosis induced by SMC, which is believed to enter the metabolic pathway by-passing selenide formation, also involves generation of ROS and is inhibited on NAC treatment (Jung et al., 2001).

The vast majority of animal chemoprevention studies have used selenite, which is effective in the absence of any evidence for non-specific toxicity *in vivo* or adverse effects on animal health; thus, it appears that at physiological doses, non-specific genotoxicity may not be relevant. *In vitro* results could be an artefact of cell culture settings or even a result of excessive selenium dosage. One potential problem is that, for practical reasons, detection of a significant amount of apoptosis and associated biochemical changes *in vitro* requires much higher doses than those tolerated *in vivo*, whereas a difference of as small as 1% in apoptotic rates may have a significant contribution in attenuating growth of pre-malignant lesions. A further technical issue is that a large proportion of the *in vitro* studies have used leukaemia cell lines which are exceptionally sensitive to genotoxic damage. As the evidence for selenium-mediated chemoprevention has been obtained primarily using epithelial systems, choice of leukaemia cell lines for *in vitro* studies may have been unfortunate and misleading. In human context, the NPC study also failed to detect any protective effect of selenium supplementation on incidence of leukaemias and lymphomas.

Molecular targets of selenium, functionally associated with induction of apoptosis, have not been clearly defined. There are conflicting reports as to whether selenite induces caspase 3 activation and it appears that this may be cell type specific. Induction of caspase 3 activity (assayed by a fluoregenic peptide cleavage) by selenite is reported in $HepG_2$ hepatoma cells (Shen et al., 2001) but not in HL-60 leukaemic cells (Kim et al., 2001) and

DU-145 prostate cancer cells (Jiang et al., 2001a). However, cleavage of poly(ADP-ribose)polymerase, PARP (a physiological substrate of caspase 3), is observed in HepG2 (Shen et al., 2001), DU-145 (Menter et al., 2000), two other prostate cancer cell lines (Menter et al., 2000), suggesting activation of caspase 3 by selenite. However, functional intervention using caspase 3 inhibitors were not performed in the studies reporting activation. In contrast, SMC and methylselenic acid have been shown to induce caspase 3 activity in HL-60 (Kim et al., 2001) and DU-145 cell lines (Jiang et al., 2001a), respectively. In these cases, inhibitors of caspase 3 activation attenuated the induction of apoptosis suggesting caspase 3 to be a functional component of the apoptotic pathway.

Activation of caspase 9 and release of cytochrome c from the mitochondria has been documented for SMC treatment (Jiang et al., 2001a) but intervention experiments on these have not been performed. Cytochrome c release has also been reported for selenite in hepatic cells (Shen et al., 2001) but not in DU-145 prostate cells (Jiang et al., 2001a). Activation of caspase 8 (comparable to the extent of caspase 9 activation) by methylselenic acid but not sodium selenite has also been reported recently (Jiang et al., 2001a). Thus in the case of SMC, mitochondria dependent apoptosis could be involved; however, the observation that caspase 8 is activated may suggest a role for death receptors.

Apart from the evidence for involvement of caspases described above, little is known about other components of the signalling pathway mediating selenium-induced apoptosis. While components involved in selenium-mediated cell cycle regulation may contribute to the induction of apoptosis (as described in Section 2.6.7.2), it is very likely that apoptosis specific signalling would also be involved. Inhibition of polyamines has been suggested as one mechanism for selenomethionine-induced apoptosis and exogenous administration of spermine has been shown to prevent apoptosis on selenomethionine exposure (Redman et al., 1997).

p53 is a known mediator of apoptosis and while it has shown that p53 may not be necessary for apoptosis induction by selenium in cancers (discussed earlier in this section), it may still be an important player in p53 wild type cells. It has been demonstrated that p53 levels are increased on treatment with apoptosis-inducing doses of SDG, selenide and SAC (Jiang et al., 2001b; Lanfear et al., 1994). Comparing apoptosis induction by SAC and methylselenic acid in a cell line with non-functional p53 and its parental line with wild type p53 revealed that the presence of functional p53 considerably enhanced apoptosis (Ip et al., 2000d; Zhu et al., 2000b). Inhibition of Rb phosphorylation and induction of the

GADD family proteins may also contribute to apoptosis induction (discussed in Section 2.6.7.2).

In vitro, selenide has been shown to downregulate the expression of the anti-apoptotic protein Bcl2 (Wei et al., 2001), while selenite and methylselenocyanate had no effect (Kaeck et al., 1997). Expression of the pro-apoptotic protein, Bax, was also unaffected by selenite and methylselenocyanate treatment (Kaeck et al., 1997). Recent *in vivo* studies using SMC have revealed a downregulation of Bcl2 expression (Ip and Dong, 2001). It remains to be seen whether in situations where Bcl2 is downregulated, overexpression of exogenous Bcl2 confers protection from apoptosis. Previous studies in our laboratory have indicated this may not be the case, at least for SDG (Blower, 1998).

There is also strong evidence that selenium can modulate the activities of key transcription factors, thereby altering gene expression patterns which may subsequently influence induction of apoptosis and cellular proliferation rates. Covalent modifications of thiol groups on critical residues have been postulated to mediate the inhibition of NF-κB seen on treatment with selenite and ebselen (Kim and Stadtman, 1997; Makropoulos et al., 1996; Shimohashi et al., 2000). NF-κB has been implicated to be intimately involved in regulation of apoptosis and cellular proliferation (Abraham, 2000; Bours et al., 2000; Gilmore, 1999) and this inhibition may form the basis for growth inhibition by selenium. Similar inhibition has been reported for other transcription factors like AP-1 (Handel et al., 1995; Spyrou et al., 1995) and c-myc (Nelson et al., 1996a; Yu et al., 1990) with known functions influencing cellular growth. A caveat to these studies is that the responses were measured at non-apoptosis-inducing conditions and may not necessarily be the same at higher doses of selenium. Such studies need to be performed at chemopreventive doses of selenium and further supplemented by functional intervention approaches in order to establish their contribution to apoptosis.

2.6.7.4 Signal transduction and cellular growth: a target for selenium?

The survival of an organism depends on its ability to respond to environmental changes. In metazoans, this dynamic relationship extends to every individual cell, which may proliferate, differentiate, exhibit motility or undergo apoptosis in response to physical or chemical cues. For a cell to elicit a response, appropriate to the environmental cues, three critical criteria need to be fulfilled. A cell should have a) sensory mechanisms to detect environmental alterations; b) molecular systems to interpret and integrate this information, and finally c) 'effector' machinery to carry out appropriate cellular responses. In the case

of selenium mediated growth inhibition some clues exist as what the first and third criteria may comprise of. It is likely that accumulation of selenium compounds and/or selenoprotein activities above a threshold acts as a sensory mechanism. The inhibition of proliferation and induction of apoptosis, perhaps mediated by the candidate molecular targets discussed in Sections 2.6.7.2 and 2.6.7.3 (summarised in Table 2.6.1), comprise the cellular response and the immediate 'effector' machinery. What remains obscure and until very recently completely uninvestigated, is the machinery that interprets and integrates the sensory information following selenium exposure to ensure an appropriate response, in this case of tumour growth inhibition.

Many signal transduction pathways co-operate and participate in this decision-making process (reviewed in (Chang and Karin, 2001; Dhanasekaran, 1998)). Regulation of cell growth is mediated by a complex array of signalling pathways precisely co-ordinated by different families of cell surface receptors. The vast majority of signal transduction occurs via phosphorylation cascades mediated by the activities of kinases and phosphatases (Dhanasekaran, 1998). In the last decade, several signalling paradigms modulating cellular proliferation and apoptosis have emerged and selenium biologists are poised to extend this knowledge to their subject of study (reviewed in (Ghose et al., 2001b)). Also, we have recently reported that selenium compounds like pXSC and SDG induce widespread tyrosine phosphorylation (Fleming et al., 2001).

In the literature, there are indications that some signalling components that may have a role in growth inhibition are influenced by selenium (summarised in Table 2.6.1). The best described is the inhibition of several PKC isoforms by selenium compounds like selenite, SDG, pXSC (also *in vivo*), BSC and SMC, in manner dependent on the number of conserved cysteine residues at the catalytic domain (Foiles et al., 1995; Gopalakrishna et al., 1997; Rao et al., 2000; Sinha et al., 1999). PKC is a major signal transduction molecule regulating several key cellular processes including mitogenesis, apoptosis, cell migration, angiogenesis, cell adhesion and invasion (Gopalakrishna and Jaken, 2000). Also PKC mediates cellular transformation by phorbol esters, an event selenium can inhibit both *in vitro* and *in vivo* (Jaken, 1990; Perchellet et al., 1987). However, while selenite was shown to decrease phorbol ester binding to PKC, selenecysteine and SDG did not affect binding but were able to inactivate the kinase activity (Gopalakrishna et al., 1997). In principle, inhibition of PKC may also lead to inhibition of AP-1 and NF-κB activity as observed on selenium treatment (Gopalakrishna and Jaken, 2000). However such a signalling event is unlikely as mutagenesis studies have shown that a direct modification of cysteine residues in these proteins is involved, suggesting a direct interaction (Kim and Stadtman, 1997;

Spyrou et al., 1995). cdk2, whose activity is inhibited by SMC and is correlated to SMC induced cell cycle deregulation has also been suggested to be a distal target for PKCs (Hamada et al., 1996): this therefore warrants investigation. Finally, while inhibition of PKC may play a critical role in chemopreventive activities of selenium compounds, it is unlikely that its inhibition is sufficient. Evidence for this comes from studies on the synthetic selenium compound, ebselen, which can very efficiently inhibit PKC activity but has been shown to lack anti-cancer activity (Ip and Ganther, 1990; Wakamura et al., 1990).

pXSC has been reported to increase the activity of diacylglycerol kinase (DGK) which suggests an enhanced conversion of diacylglycerol (DAG) to phosphatidic acid (Rao et al., 2000). DAG is a well characterised allosteric activator of PKC: suggesting the enhanced DGK activity could be functionally upstream of PKC inhibition (Gopalakrishna and Jaken, 2000). DGK may have other signalling functions, apart from inhibiting PKC: it has been shown to influence ras activity which may subsequently alter mitogen activated protein kinase (MAPK) pathways (Topham and Prescott, 2001).

Studies have also revealed SDG and selenocysteine, but not selenite, can inhibit protein kinase A (PKA) but at higher concentration than those required to inhibit PKC activity (Gopalakrishna et al., 1997). The aromatic selenium compounds, pXSC and BSC, have also been shown to efficiently inhibit PKA activity (Foiles et al., 1995). PKA may influence both proliferation and apoptosis (Cross et al., 2000). It has been shown to have enhanced expression in many cancers and its inhibition results in a suppression of proliferation (Alper et al., 1999). Activation of PKA has been reported to inhibit apoptosis induction by several apoptotic stimuli, perhaps via modulation of activities of the Bcl2 family of proteins (Cross et al., 2000).

The mitogen activated protein kinases (MAPK) are an evolutionary conserved family of enzymes that regulate several critical cellular processes including proliferation and apoptosis (Chang and Karin, 2001; Ichijo, 1999). MAPK activity is regulated through a three-tiered kinase cascade composed of a MAPK, MAPK kinase (MAPKK or MEK) and a MAPKK kinase (MAPKKK or MEKK). MAPK's affect a number of cellular targets including transcription factors and other effector kinases (Fig 2.6.3). In mammals, four distinctly regulated groups of MAPK's have been identified: the extracellular signal-regulated kinases (ERK1/2), c-jun-N-terminal kinases (JNK1/2/3), p38 kinases (p38$\alpha/\beta/\gamma/\delta$) and ERK5 (Fig 2.6.3) (Chang and Karin, 2001).

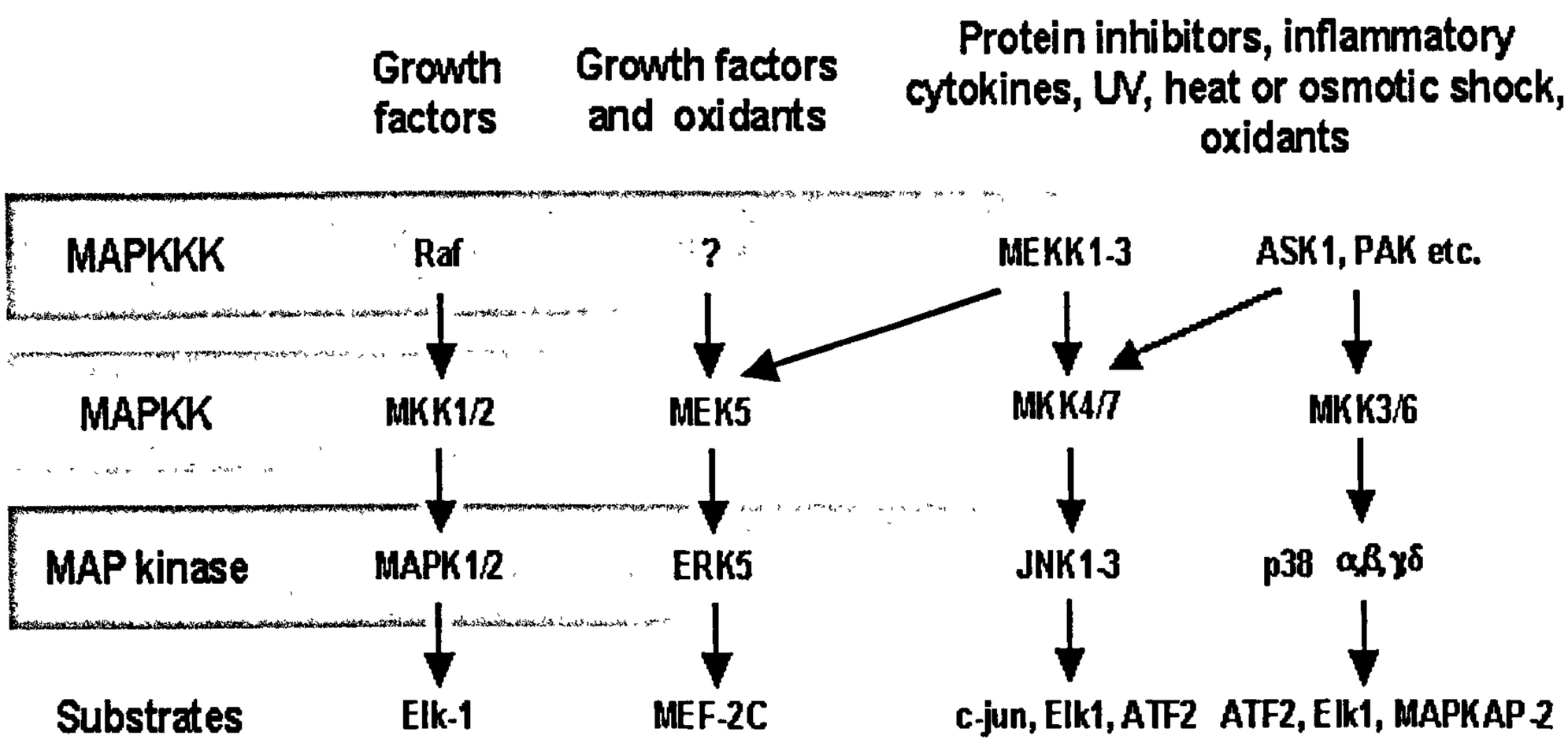


Fig 2.6.3 Mammalian mitogen activated protein kinase (MAPK) pathways.

The three-tiered cascade modules of the mammalian MAPK pathways are schematically represented. MAPK's have a diverse range of substrates including transcription factors, other kinases and a variety of cellular regulators, some of which are indicated. Principal inductive stimuli for the four major groups of MAPK's are shown above each group.

At the time of commencement of this project, there was only one study addressing the role of these signalling proteins in cancer chemoprevention by selenium. This study demonstrated that pXSC at chemopreventive doses was capable of inducing JNK activation and also potentiating JNK activation by UV (Adler et al., 1996). JNK is activated by several cellular stresses, like oxidative stress, DNA damage, chemotherapeutic drugs, and has been shown to functionally mediate apoptosis (Davis, 2000). The paper however provided no functional evidence nor was any correlation made with inhibition of proliferation and induction of apoptosis by selenium.

Another set of studies has, somewhat serendipitously, indicated modulation of MAPK pathways by selenium. These authors, while investigating the insulin-mimetic properties of selenium, have observed that selenate induces phosphorylation of the epidermal growth factor receptor (EGF receptor) and the insulin receptor (Stapleton, 2000; Stapleton et al., 1997). They also showed, in accordance with the activation of EGF receptor, activation of the ERK 1 and 2 kinase activities which are strongly implicated in growth regulation and apoptosis (Stapleton, 2000; Stapleton et al., 1997). Selenate has also been reported to activate p70 S6 kinase via a pathway that is sensitive to rapamycin (Hei et al., 1998), this may be of some relevance as p70 S6 kinase is known to regulate cell size, growth and proliferation (Proud, 1996).

Finally, an interesting body of information has been generated from studies on the fruit fly, *Drosophila*. Mutation of the *Drosophila* homologue of the selenophosphate synthetase gene completely inhibits selenoprotein synthesis and cells harbouring this mutation exhibited lower levels of proliferation and accumulation in the G2 phase, coincident with enhanced levels of cyclin B (Alsina et al., 1999). This suggests an important role for selenoproteins in regulating proliferation. The same authors have noted that the effects observed in these mutants were similar to those described for mutants in the EGF receptor pathway and have subsequently detected genetic interactions with components of the ras/MAPK pathway (Morey et al., 2001). This would suggest that selenoprotein function may modulate activity of some components of the EGF signalling pathway, presumably via altering the redox state of the cell.

2.7 Project rationale and aims

Evidence for selenium compounds as cancer-risk modifiers is considerable; therefore it is pertinent to investigate candidate mechanisms that may be mediating this effect. As discussed earlier (Section 2.6) selenium-mediated anti-cancer activity may be plausibly

mediated by a number of candidate mechanisms. The aim of chemoprevention by dietary supplementation, ideally, is to eliminate the incidence and progression of pre-neoplastic lesions: therefore mechanisms that affect initiation stages of cancer are of great importance. Rodent studies indicate selenium-mediated chemoprevention is evident at the initiation stages of cancer inhibiting incidence of pre-neoplastic lesions, thus candidate mechanisms like inhibition of neoangiogenesis and invasion and perhaps even enhanced immune surveillance are irrelevant in these situations. However, these mechanisms may significantly affect established cancers and are well worth investigating as they may provide novel opportunities for intervention. As suggested by the two-stage model (Fig 2.5.1), supranutritional doses of selenium are likely to induce anti-cancer activities that are different from those induced by nutritional repletion of selenium in deficient individuals. In the latter situation selenoprotein mediated activities like protection from oxidative stress are likely to be key mediators. Whilst, in the case of selenium supplementation at supranutritional levels, where selenoprotein activity is saturated, the action of selenium metabolites is likely to mediate anti-cancer activities predominantly. It appears from animal experiments that at chemopreventive levels of intake, several selenium compounds confer a lasting protection from cancer incidence even when provided for a short period during the early promotion stages of carcinogenesis (Ip, 1998). This suggests that selenium treatment is capable of eliminating transformed cells *in vivo*. As tissue homeostasis is maintained by a balance of proliferation and cell death, it is conceivable that selenium exerts its anti-tumour activity by modifying the rates of these events. While inhibition of cellular proliferation at chemopreventive doses is a possibility, recent studies have failed to demonstrate this for some very potently anti-carcinogenic selenium compounds (see Section 2.6.7.1). Apoptosis-induction however, is readily detectable at these conditions, suggesting cell death to be a key player in selenium-mediated cancer chemoprevention.

While apoptosis-inducing abilities of selenium compounds are well documented, the molecular/cellular mechanisms that mediate this activity is unknown. Elucidation of these molecular mediators is absolutely critical in the evaluation of selenium compounds as potential anti-cancer agents in humans. Delineating apoptosis-inducing pathways would be invaluable in assessing the efficacy and toxic consequences of selenium compounds and could serve as candidate surrogate biomarkers of selenium-mediated chemopreventive activity. Detailed knowledge of apoptosis mediators also presents the opportunity to enhance the efficacy and/or reduce toxicity of existing compounds via chemical manipulations. This is particularly relevant to the synthetic organoselenium compounds discussed in Section 2.5.2 because of their amenability to structural manipulations.

The explosion of interest in apoptosis in the last two decades has led to the identification of several apoptotic signalling pathways, particularly those mediated by the MAPKs. As discussed in Section 2.6.8, there is some evidence that such phosphorylation dependent signalling cascades may be significantly altered upon exposure to selenium. The primary aim of this study was therefore to identify the molecular mediators of selenium-induced apoptosis, to investigate systematically the role of cellular signalling in initiating such a response, and finally, using molecular genetic and pharmacologic tools, to address the functional significance of the putative mediators.

As apparent from the literature, different selenium compounds have disparate abilities to induce apoptosis, possibly utilising different signalling pathways and mediators. This study investigated apoptosis-induction by a natural, inorganic selenium compound, selenodiglutathione (SDG), and a synthetic organoselenium compound, 1,4-phenylene-*bis*-(methylene)selenocyanate (pXSC). SDG is the primary metabolite of selenite, the most commonly used selenium compound in animal chemoprevention studies and pXSC is a synthetic, aromatic compound which has the highest chemopreventive index (the ratio of the maximum tolerated dose to the dose required to give a 50% inhibition of total tumour yield) of all currently tested selenium compounds. The rationale for choosing these compounds is based on the fact that these have been demonstrated to potently inhibit cancerous lesions in several different animal models of cancer, although their significance to human cancers remains unaddressed. Also, the fact that one is a natural, inorganic compound and the other an organic synthetic compound may reveal potential difference in the mechanisms underlying apoptosis-induction by these compounds. This argument is borne out by the fact that some cancer types are sensitive to one and not the other (see Section 2.5.2). SDG was preferred over selenite as it has been shown to be a more potent inducer of apoptosis than selenite and there are indications that growth inhibitory effects of selenium are dependent on its conversion to SDG (Fleming et al., 2001).

3 Materials and Methods

3.1 Materials

3.1.1 Selenium compounds

Selenodiglutathione (SDG) was prepared by J. Fleming (Beatson Institute, UK) as described previously (Ganther, 1971). 1,4-phenylene-*bis*-(methylene)selenocyanate (pXSC) was provided as a part of an ongoing collaboration with Dr K. El-Bayoumy (American Health Foundation, USA).

3.1.2 Cell lines and primary cultures

Human cervical carcinoma cell line, HeLa was obtained from S. Boldt, Beatson Institute, UK. A431 cells, a human vulvar epidermoid cancer cell line, stably transfected with TAM67 or the corresponding 'empty' vector was obtained from Dr G. Stapleton. Primary cultures of oral keratinocytes, derived from biopsy material was obtained from Dr F. McGregor, Beatson Institute, UK.

3.1.3 Tissue culture

3.1.3.1 Beatson Institute Central Services

Sterile distilled water; sterile glycerol; sterile phosphate buffered saline (PBS); phosphate buffered saline + EDTA (PE); sterile glass pipettes; sterile Pasteur pipettes.

3.1.3.2 Becton Dickinson, UK

60mm, 90mm tissue culture dishes; 10ml, 25ml, 50ml plastic pipettes; Falcon 1059 polypropylene tubes; Falcon 2059 polypropylene tubes; Falcon 2098 polypropylene tubes; sterile Plastic pack syringes; 18, 25 gauge sterile syringe needles; 40μm nylon cell strainers.

3.1.3.3 Bibby Sterilin Ltd, UK

Sterile plastic 7ml bijoux and 20ml universal containers.

3.1.3.4 Costar Corporation, UK

6, 12 and 96 well tissue culture plates; disposable Cell scrapers.

3.1.3.5 Gibco BRL Europe Life Technologies Ltd., UK

Dulbecco's medium (Beatson formulation); 10x Dulbecco's Modified Eagles medium; Ham's F12 medium; 2.5% trypsin; 200mM L-glutamine; 7.5% sodium bicarbonate; 100mM sodium pyruvate; Geneticin (G418); EGF.

3.1.3.6 Gelman Sciences, UK

Sterile 0.2 μm acrodisc filters.

3.1.3.7 Harlan Sera-Lab Ltd

Foetal calf serum.

3.1.3.8 Nunc, Denmark

T25, 80, and 175 cm^2 tissue culture flasks; cryotubes.

3.1.3.9 Qiagen, UK

Effectene™ Transfection Reagent

3.1.3.10 Sigma, UK

Bufferall; transferrin; adenine; cholera toxin; hydrcotisone.

3.1.4 Antibodies

3.1.4.1 Becton Dickinson, UK

Fas ligand, anti-human Fas ligand monoclonal antibody; Bcl2 monoclonal antibody; Bax monoclonal antibody; $Bclx_L$ monoclonal antibody.

3.1.4.2 Calbiochem, USA

c-jun (Ab-3) monoclonal antibody – to detect TAM67 expression.

3.1.4.3 New England Biolabs, UK

Phosphospecific (Ser63/73) c-Jun polyclonal antibody kit; PhosphoPlus™ p38 kinase (tyr182) polyclonal antibody kit; PhosphoPlus™ Akt (ser 473) polyclonal antibody kit; cleaved caspase 3 ploYclonal antibody; cleaved PARP monoclonal antibody.

3.1.4.4 Promega, UK

Activated ERKs1/2 polyclonal antibody.

3.1.4.5 Roche, Germany

Anti-HA High Affinity rat monoclonal antibody (clone 3F10).

3.1.4.6 Santa Cruz Biotechnology, USA

Activated JNK monoclonal antibody p-JNK(G-7); total ERK1(C-16)-G polyclonal antibody; ERK5(C-20) antibody.

3.1.4.7 Sigma, UK

FLAG tag monoclonal antibody.

3.1.4.8 Upstate Biotechnology, USA

MAPKAP-2 kinase monoclonal antibody.

3.1.5 Plasmids

3.1.5.1 dnFADD-GFP

Dominant negative Fas associated death domain (FADD) protein fused to green fluorescent protein (GFP) was a gift from Dr H. Wajant, University of Stuttgart, Germany.

The construct was prepared by replacing the amino terminal death effector domain of FADD by full length GFP.

3.1.5.2 pEGFP-C1

pEGFP-C1 is a commercially available eukaryotic expression plasmid that encodes the enhanced green fluorescence protein (EGFP) (a red-shifted variant of GFP which contains the double amino-acid substitution of Phe64 to Leu and Ser65 to Thr). In the following text, this plasmid is referred to as 'GFP'.

3.1.5.3 TAM67

TAM67 is a dominant negative c-jun construct lacking amino acids 3-122. The plasmid was a kind gift from Dr D. Gillespie (Beatson Institute, UK).

3.1.5.4 c-jun S63/73A

c-jun S63/73A is a c-jun construct with the two JNK phosphorylation sites, Ser63 and Ser73 mutated to Ala. This was a kind gift from Dr M. Karin (University of California, San Diego, USA).

3.1.5.5 MEK5(D)

MEK5(D) is a HA-tagged constitutively activated mutant of the MAPKKK MEK5 where Ser313 and Thr317 has been replaced aspartic acid residues. This construct was a kind gift from Dr J-D. Lee (Scripps Research Institute, USA).

3.1.5.6 ERK5 and ERK(AEF)

Both construct are gifts from Dr J-D. Lee (Scripps Research Institute, USA). ERK5 is the wild type MAPK ERK5 in a mammalian expression vector and is FLAG tagged. ERK5(AEF) is a FLAG tagged ERK5 mutant with Thr218 and Tyr220 replaced with Ala and Phe, respectively.

3.1.6 Bacteriology

3.1.6.1 Beatson Institute Central Services

L-Broth (Luria Bertani) liquid medium.

3.1.6.2 Becton Dickinson, UK

Falcon 1059, 2059, 2501 polypropylene tubes.

3.1.6.3 Bibby Sterilin Ltd., UK

90mm bacteriological petri dishes.

3.1.6.4 Difco Laboratories, UK

Bacto-Agar

3.1.6.5 Gibco BRL, UK

E.coli DH5α competent cells.

3.1.6.6 Sigma, UK

Ampicillin

3.1.7 Chemicals, radiochemicals, reagents and kits

3.1.7.1 Sigma, UK

All chemicals unless otherwise mentioned.

3.1.7.2 Amersham, UK

[γ^{32}] dATP; [^{3}H]-thymidine.

3.1.7.3 Calbiochem, UK

Ac-DEVD-CHO; SB203580; PD98059; LY294002; SB202190.

3.1.7.4 Clontech, UK

Annexin V-FITC apoptosis detection kit.

3.1.7.5 Fischer Chemicals, UK

Histoclear

3.1.7.6 Fisons Scientific Equipment, Uk

Ammonium acetate; Butan-2-ol; Chloroform; Dimethyl sulfoxide (DMSO); Ethylene diamine tetra acetate (EDTA) disodium salt; Ethylene Glycol-bis(b-aminoethyl Ether)N,N,N',N'-tetraacetic acid (EGTA); Methanol; Propan-2-ol; Sodium acetate; Sodium dodecyl sulphate (SDS).

3.1.7.7 Gibco BRL, UK

Agarose (ultrapure electrophoresis grade); Tris base.

3.1.7.8 Hughes and Hughes, UK

HistoMount™

3.1.7.9 Intergen, USA

ApoTag® fluorescein direct *in situ* apoptosis detection kit.

3.1.7.10 James Burrough Ltd., UK.

Ethanol

3.1.7.11 Premier Brands, UK

Marvel dried skimmed milk.

3.1.7.12 Qiagen Ltd, UK

QIAGEN Plasmid Maxi Kit.

3.1.7.13 R & D Systems, UK

Fas/Fc chimera

3.1.7.14 Severn Biotech Ltd.

30% (w/v) acrylamide: 0.8% (w/v) bis-acrylamide; 40% (w/v) acrylamide: 2.1% (w/v) bis-acrylamide.

3.1.7.15 Surgipath, UK

Eosin; haemotoxilin.

3.1.7.16 Transgenomic, UK

RNase A

3.1.7.17 Upstate Biotechnology, USA

Human soluble FasL and accompanying potentiator antibody.

3.1.7.18 Vector Laboratories, USA

Vectastain ABC kit; Peroxidase substrate DAB kit; Vectashield mounting medium.

3.1.8 Miscellaneous

3.1.8.1 Amersham, UK

Hybond ECL™ nitrocellulose membrane; ECL ™ enhanced chemoluminiscence reagent

3.1.8.2 Elkay laboratory products Ltd., UK

1.5ml microcentrifuge tubes; 1.5ml screw-cap microcentrifuge tubes.

3.1.8.3 Fuji Ltd., Japan

Fujifilm $Super_{RX}$ X-ray film.

3.1.8.4 GibcoBRL, UK

BenchMark™ pre-stained protein ladder

3.1.8.5 Kodak Ltd, UK

X-OMAT AR X-Ray film; X-OMAT RA X-ray film processor.

3.1.8.6 Whatman international Ltd

Whatman 3mm filter paper

3.1.9 Water

Distilled water for the preparation of buffer stocks was obtained from a MilliRO 15 system (Millipore, UK), and for protein, enzyme, RNA or recombinant DNA procedures was further purified on a Millipore MilliQ System (Millipore, UK), to 18 MΩ/cm. Sterile distilled water for making up tissue culture media was supplied by the Beatson Institute Central services.

3.1.10 Xenograft studies

3.1.10.1 Charles River Ltd., UK

CD-1 nu/nu mice

3.1.10.2 Harlan Tekland, UK

Selenium supplemented and depleted mouse diets.

3.1.10.3 Stratech, UK

Matrigel

3.2 Methods

3.2.1 Cell culture

All cell culture work was performed using strict aseptic techniques inside a laminar flow hood (Class II Microbiological Safety Cabinets, Medical Air technology Ltd., Manchester, UK). Cells were incubated at 37°C in a dry atmosphere containing 5% (v/v) CO_2 (Heraeus, Essex, UK) and were routinely screened for mycoplasma infection using a fluorescent dye technique. All media used contained Bufferall to maintain the pH at 7.0.

HeLa cells were maintained in modified Dulbecco's medium (Beatson formulation) supplemented with 10% foetal calf serum and 1mM glutamine. Cells were maintained in culture for a maximum of 6-8 w before being replaced with cells from frozen stocks.

A431 cells stably transfected with TAM67 or the corresponding empty vector were obtained from Dr G. Stapleton (Beatson Institute, UK): their generation has been described previously. Both lines were maintained in Dulbecco's MEM (Beatson formulation) supplemented with 10% foetal calf serum, 1mM glutamine and under G418 selection.

The derivation and characterisation of the primary cultures of biopsies of normal human oral mucosa or carcinomas has been described previously (Edington et al., 1995; McGregor et al., 1997). All cells were maintained on irradiated (60Gy from a Co^{60} radiation source) Swiss 3T3 feeders, either in FAD+ medium (1:3 Ham's F12/Dulbecco's MEM with 10% foetal calf serum and insulin, EGF, transferrin, cholera toxin, hydrocortisone and adenine) in the case of normal cells or 10H medium (Dulbecco's MEM plus 10% foetal calf serum without added growth factors except hydrocortisone) in the case of carcinomas (Edington et al., 1995; McGregor et al., 1997). Normal cultures were used within the first 2-3 passages from frozen stocks, before their growth rate deteriorated significantly. Oral cultures were used for experimentation whilst rapidly growing at not more than 70% confluence after which the irradiated 3T3 feeders were carefully removed by treatment with phosphate buffered saline (PBS)/0.02%EDTA and the cultures re-fed with normal medium. Swiss 3T3 cells were maintained in 10C medium (Dulbecco's MEM plus 10% donor calf serum). Immediately prior to all experimentation both carcinomas and normals were grown on 10H medium to avoid complications arising from growth in different media.

To freeze cell stocks for storage, semi-confluent cultures were trypsinised, pelleted and the pellet resuspended at a concentration of approximately 10^6 cells/ml in chilled medium containing 50% serum and 10%(v/v) DMSO. Suspensions were aliquoted into 1ml Nunc cryotubes and placed in a polystyrene box and frozen, well insulated, at -70°C overnight to ensure a slow rate of cooling. The ampoules were then transferred to a liquid nitrogen tank until required.

Frozen stocks were recovered by removing the ampoules from liquid nitrogen and placed into a small, covered bucket of water at 37°C. Once thawed, the cells were added to 10 ml of the appropriate pre-warmed growth medium, centrifuged, resuspended in fresh growth medium and transferred to T125 flasks.

3.2.1.1 Transient transfection of HeLa cells

10^6 cells/100mm plate were seeded the night before transfection and incubated o/n. The next day 2μg of DNA were transfected into cells using Effectene™ transfection reagent following manufacturer's instructions. All plasmids used were verified by diagnostic restriction digest analysis and sequencing. 18 hours later cells were washed once in PBS, re-fed with complete medium, and incubated o/n. Cells were treated and/or harvested 16h later.

GFP-transfected cells were sorted in sterile conditions for further analysis (like immunoblotting) using the CellQuest software (Becton Dickinson, UK) and FACSVantage SE (Becton Dickinson, UK) flow cytometry.

3.2.2 DNA synthesis/thymidine incorporation assay

10^4 HeLa cells were plated in 200μl of medium per microtitre well. After 18h, the medium was replaced with medium containing SDG or pXSC and incubated overnight. Each well was then given 0.5μCi of tritiated thymidine for 6h, the medium removed and the cells trypsinised and transferred onto a filter paper mat (printed filtermat A, Pharmacia) using a microtitre plate harvester (Skatron Combi Harvester, model 11900 (Skatron, Norway, distributed by LKB). After adding scintillator, the mat was scanned and counted using a plate counter (model 1205 Betaplate™, Pharmacia). Four replicate wells were used for each condition. The radioactivity incorporated into DNA was calculated as a percentage of untreated cultures.

3.2.3 Trypan blue assessment of cellular viability

1.5 x 10^6 HeLa cells/100mm plate were seeded 18h prior to treatment with selenium compounds. After treatment cells were trypsinised and harvested. Trypan Blue was added for 2min before cells were counted using an haemocytometer. Cells not staining with trypan blue were considered viable.

3.2.4 Apoptosis assays

3.2.4.1 TUNEL assay

5 x 10^5 cells/60mm plate (or 5 x 10^3 cells on mm glass cover slips) were seeded 18h prior to treatment with selenium. Apoptosis in was measured by the Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assay using the ApoTag® kit according to the manufacturer's protocol. For oral cells, after plating the cells for 2d, irradiated 3T3 feeders were carefully removed from the cultures prior to experimentation by washing the cultures vigorously three times with PBS/0.02% EDTA. Selenium was added 18h after the removal of feeders. Assays were performed with 3 replicate plates of cells and at least 5 randomly selected fields totalling a minimum of 300 cells per plate were counted for TUNEL-positive cells.

3.2.4.2 Accumulation of hypodiploid DNA of transfected cells

Assessment of apoptosis among the HeLa cells transiently co-transfected with a green fluorescent protein (GFP)-encoding vector was performed by measuring the sub-G0/G1 DNA content of the GFP-positive population. Cells were harvested after treatment, washed in cold PBS, fixed in 1% paraformaldehyde for 1 h on ice and permeabilized with 70% ethanol for 1h on ice. Cells were then resuspended in 1 ml of PBS to which 0.5 ml of Phosphate-citric acid buffer (0.2M NaH_2PO_4, 4mM citric acid) was added and incubated at room temperature for 5 min. After RNase treatment (250μg/ml), the DNA was stained with propidium iodide (PI; 10 μg/ml) and the cell cycle distribution determined by analysing 10^4 GFP positive cells with the CellQuest software using a FACScan flow cytometer (Becton Dickinson, UK). Similar protocol was used to assess sub-G0/G1 DNA content of untransfected cells excluding the selecting of GFP positive cells. All treatments were in triplicate.

3.2.4.3 Annexin assay

For the annexin V assays the cells were plated on 60mm plates or coverslips as described earlier (Section 3.2.4.1). Annexin V staining was carried out using the Annexin V-FITC apoptosis detection kit according to the manufacturer's protocol. The percentage of Annexin V-staining cells was then determined microscopically. Cells positive for Annexin V and negative for PI staining were scored as apoptotic.

3.2.5 Detection of proteins by SDS-PAGE and Western blotting

To prepare whole cell protein extracts for immunoblotting, the cells were washed twice with ice-cold PBS and then scraped off in 0.2ml of lysis buffer (20mM Hepes, pH 6.8, 5mM EDTA, 10mM EGTA, 5mM NaF, 0.1μg/ml okadaic acid, 1mM DTT, 0.4M KCl, 0.4% Triton X-100, 10% glycerol, 5μg/ml leupeptin, 50μg/ml PMSF, 1mM benzamidine, 5μg/ml aprotinin, 1mM sodium orthovanadate), incubated on ice for 20 min, followed by centrifugation at 13,000 rpm in a microfuge for 20 min and the lysate (supernatant) recovered. The supernatant was stored at -70^{0}C. Up to 50μg of protein sample (quantitated using the bicinchonic acid–copper(II)sulphate method) was mixed with an equal volume of 2x loading buffer (187.5 mM Tris-HCl, pH 6.8, 30% glycerol, 6.9% SDS, 2.1M β-mercaptoethanol, 0.1% bromophenol blue), boiled for 10 min before electrophoresis on a SDS-polyacrylamide gel electorphoresis (SDS-PAGE) gel.

Gels containing different concentration of polyacrylamide (i.e. 6%, 8%, 10%) were used to resolve proteins according to their molecular weight. The resolving gel was made by adding the appropriate volume of 30% (w/v) acrylamide: 0.8% (w/v) bis-acrylamide to a solution of 0.45M Tris, 0.1% SDS, 0.08% TEMED and 0.1% (w/v) APS. The stacking gel comprised of 0.125M Tris, pH6.8, 0.1% SDS, 1.7ml 30% (w/v) acrylamide: 0.8% (w/v) bys-acrylamide, 0.2% APS and 0.3% TEMED. Protein were loaded along with BenchMark^{TM} pre-stained protein ladder and electrophoresis was performed o/n prior to transfer onto nitrocellulose using a Camlab semi-dry blotter (Camlab, UK), following the manufacturer's protocol..

Western blots were blocked in the presence of TBST (50mM Tris pH 7.5, 50mM NaCl, 1mM EDTA, 0.1% Tween-20) containing 5% non-fat dried milk for 1h at room temperature; washed 3x 10min with TBST and then incubated with antibodies diluted in TBST containing 0.1-1% bovine serum albumin (according to antibody manufacturer's instruction) and 0.1% sodium azide. The antibody dilutions and the length of incubation

were according to manufacturer's instructions. Following incubation with the primary antibody the membrane was washed 3x 10 min with TBST. This was followed by incubation in 20ml of a 1: 3000 dilution of the appropriate IgG HRP-linked antibody in blocking buffer for 1.5h. The secondary antibody solution was removed and the membrane was washed 3x 10 minutes with TBST. After removal of excess surface liquid, bound primary antibody was detected using ECL chemiluminescent methodology according to manufacturer's instructions.

For loading controls, blots were stripped by incubation for 30min with 1x Stripping buffer (100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH6.7) at 50^{0}C. The membrane was washed with 2x TBST prior to proceeding to blocking and subsequent manipulations as described in the last paragraph.

3.2.5.1 Detection of ERK5 mobility shift

Phosphorylation of ERK5 results in a shift in its electrophoretic mobility detectable by western blotting, and a consistent correlation between the shifted fraction of phosphorylated ERK5 and the activity of ERK5 as measured by protein kinase assay has been observed (Kato et al., 1998). This electrophoretic mobility shift was thus used as an assay for ERK5 activity. Essentially a 6%SDS-PAGE was used as described in Section 3.2.5, with exception of 30% (w/v) acrylamide: *0.2%* (w/v) bis-acrylamide stock solution to resolve the band shift.

3.2.6 Immunecomplex kinase assays

Cells were washed in ice cold PBS and then scraped off in 0.15ml of lysis buffer (25mM Hepes pH 6.8, 75mM NaCl, 2.5mM $MgCl_2$, 0.1mM EDTA, 25mM NaF, 0.5mM DTT, 1% NP-40, 10μg/ml leupeptin, 1mM PMSF, 3mM benzamidine, 20mM β-glycerophosphate and 0.1mM sodium orthovanadate), incubated on ice for 20min, followed by centrifugation at 13,000 rpm in a microfuge for 20min and the lysate (supernatant) recovered. After protein quantitation, 100-200μg of protein in 100μl was incubated o/n on a roller at 4^{0}C with 2μg of either MAPKAP-2, JNK or ERK5 antibody and 50μl of ~50% protein A sepharose slurry. Following immunecomplex formation the beads were washed 4x in 1ml of wash buffer (20mM Hepes pH 6.8, 50mM NaCl, 2.5mM $MgCl_2$, 0.1mM EDTA and 0.05% Triton X-100) and incubated for 30min with 30μl of kinase reaction mix at 30^{0}C. 30μl kinase reaction mix comprised of 15μl 2x kinase buffer (20mM HEPES, 20mM $MgCl_2$, 0.1μg/ml okadaic acid, 20mM β-glycerophosphate, 0.1mM sodium orthovanadate

and 2mM DTT), 10mM ATP, 5μCi γ^{32} ATP and 5μg of appropriate substrate (hsp25 for MAPKAP-2; GST-c-jun for JNK; MBP for ERK5). The reaction was stopped by adding 30μl of 2x loading buffer (Section 3.2.5), boiled for 10min and electrophoresed on a 10%SDS-PAGE prior to Western Blotting (Section 3.2.5). Radioactivity was detected by exposing membranes to X-ray films.

3.2.7 Transformation of Competent Bacterial Cells

E.coli DH5α competent cells were used for the propagation of plasmid DNA. A 20μl aliquot of the competent cells were thawed on ice and then transferred to a chilled polypropylene tube (Falcon 2059). The appropriate amount of DNA was added to each tube and left to incubate on ice for 30 minutes. Cells were then heat shocked for 45 seconds in a water bath at 42°C. 80μl of L-broth was added to each sample and each tube was incubated at 37°C for 1 hour with vigorous shaking. The whole of the transformation reaction was then plated on to L-agar plates containing 50μg/ml of ampicillin. Plates were inverted and incubated o/n at 37°C to allow colony formation.

Single bacterial colonies were picked and used to inoculate a sterile universal tube containing 5 ml of L-broth medium containing ampicillin. After incubation for 6 hr at 37°C accompanied by vigorous shaking, the culture was added to 500 ml of L-broth with ampicillin and incubated o/n under the same conditions.

Plasmid preparation of was carried using the Qiagen maxiprep kit following the manufacturer's recommendations. The concentration of nucleic acid was determined spectrophotometrically using a Beckman DU 650 spectrophotometer. Optical density (OD) readings were obtained at 260 nm and 280 nm; concentration was calculated by assuming an OD reading of 1 at 260 nm ($A_{260} = 1$) to approximately correspond to a concentration of 50 μg/ml of double stranded DNA. The ratio between readings at 260 nm and 280 nm (A_{260}:A_{280}) provided an estimate of the sample purity; a ratio of ~1.8 indicated that preparations were sufficiently pure. The DNA concentration was determined as described below and the plasmid DNA was aliquoted and stored at minus 20°C.

3.2.8 Production and purification of GST-c-jun protein

GST-c-jun (1-79) fusion vector was used to transform *E.coli* DH5α as described in Section 3.2.7. The follow day individual colonies were picked and grown in culture o/n. 2ml of o/n culture was diluted to 100ml of L-broth containing ampicillin and incubated at culture

growth conditions till the OD at 600nm reached 0.3-0.5. At this stage isopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 1mM and the culture incubated for 4h in order to induce fusion protein expression. Bacterial cell were centrifuged at 3,000rpm for 5min and resuspended pellet added to the NETN buffer (20mM Tris pH8.0, 100mM NaCl, 1mM EDTA and 0.5% NP-40) prior to sonication on ice for 3x 15sec. The suspension was pelleted by a microfuge and the supernatant (crude bacterial extract) recovered. 3ml of crude extract was mixed with 150µl of ~50% glutathione sepharose slurry (prepared in NETN buffer) and allowed to mix for 30min at room temperature on a roller. The beads were pelleted by a microfuge and supernatant discarded, washed 3x with NETN buffer and finally resuspended in an equal volume of NETN buffer. GST-fusion protein bound sepharose was either prepared fresh or used within a week of preparation. For kinase assays (described in Section 3.2.6), 20µl of the GST-fusion protein bound to sepharose was washed thrice in wash buffer (see Section 3.2.6), resuspended in 30µl of kinase buffer (see Section 3.2.6) and kinase assay performed as described in Section 3.2.6.

3.2.9 Pharmacological inhibitors and Fas-Fc chimera experiments

Pharmacological inhibitors Ac-DEVD-CHO (50µM), SB203580 (10µM), PD98059 (50µM), LY294002 (50µM) and SB202190 (10µM and 30µM) were used at the indicated concentrations and added 30min prior to adding SDG and pXSC.

For the Fas/Fc chimeric protein experiments cells were grown on 3T3 feeders on glass coverslips. Cells were grown to the appropriate density, the feeder layer removed and then incubated for a further 16h. Cells were then treated with the Fas/Fc chimeric protein (250ng/ml) for 1h prior to treatment with either selenium compound or recombinant soluble human Fas ligand (150ng/ml) and the potentiator antibody (2µg/ml). After 16h incubation, the TUNEL assay was performed as described in Section 3.2.4.1.

3.2.10 Xenograft studies

Immunosuppressed 'nude' mice of CD-1 nu/nu background were bred and supplied by Charles River Ltd., UK. The animals were maintained in a protected and controlled environment. Animal handling and procedures were carried out in a laminar air flow hood and in accordance with the permit and guidelines issued by the Home Office, UK.

18 mice were divided into 2 groups, on high (2ppm) and low selenium (<0.1ppm) diets. 3d after switching to the experimental diets the mice were injected with human squamous cell

carcinoma (SCC) cells. SCC primary culture BICR31 was harvested and 10^7 cells in 200µl was mixed with an equal volume of matrigel. All treatments were performed on ice using chilled reagents and plasticware. Nude mice were injected subcutaneously with 0.4ml of the cell suspension/Matrigel mixture using a chilled 25 gauge needle.

Engraftment was determined by direct visualisation of tumour growth and caliper aided measurement. Day to day maintenance was carried out by Technological (Animal House) Services, Beatson Institute, UK and tumour-size measured every week.

3.2.11 Paraffin-embedded sections and immunohistochemistry

The xenograft tissue was fixed in neutral buffered formalin o/n and processed in a Shandon Citadel^{2000} processor. The tumours were dehydrated through a series of graded alcohols, embedded in paraffin wax, sectioned at 5µm, and stained with haematoxylin and eosin for pathological analysis (processed by Technological Services, Beatson Institute, UK).

For immunohistochemistry, the sections were deparaffinized for 30min using Histoclear followed by xylene for 10min. Rehydration was carried out sequentially in 100% and 20% ethyl alcohols (5min in each step) and finally water. Sections were incubated in 0.3%H_2O_2 for 15min followed by a 5min wash in distilled water. Following treatment with 0.05% Saponin for 15min and washing with PBS, immunohistochemistry was carried out using the Vectastain ABC (avidin-biotin-peroxidase) kit for rabbit IgG according to manufacturer's instructions. Caspase 3 antibody was used at 1:100 dilution (or Rabbit IgG at 0.5µg/ml as negative control) for 1.5h at room temperature in a moist chamber. Peroxidase substrate DAB (3,3'-diaminobenzidine) kit was used to visualise the primary antibody according to manufacturer's instructions. Counterstaining was carried out using eosin and the section dehydrated with sequential treatment with water, 70% and 100% ethanol, and xylene. Finally the section was mounted in Histomount and covered with a cover slip.

3.2.12 Microscopy

Immunohistochemistry, Annexin V staining and TUNEL staining were visualised using a Zeiss Axioskop 20 microscope (Zeiss, Germany) with an appropriate light source and photomicrographs recorded using the AxioVision ver. 3.0.6 software (Zeiss, Germany). For some high resolution images/scoring the Leica TCS SP2 spectral confocal multiphoton system (Leica, Germany) using a Leica DMI RBE microscope (Leica, Germany) and Leica

Confocal Software (Leica, Germany) was used. For visualising growing cultures an inverted Zeiss Axiovert 25 microscope (Zeiss, Germany) was used.

3.2.13 Statistical analysis

Differences between experimental groups were compared using the equal variance, two-sided Student *t*-test. A $p<0.05$ was considered significant.

4 Induction of Apoptosis by Chemopreventive Selenium Compounds

4.1 Background

Ever since the Clark et al., 1996 study there has been a rejuvenated interest in elucidating the molecular mechanisms underlying selenium-mediated chemoprevention. A survey of published data reveals that the chemopreventive activities of selenium compounds *in vivo* closely parallel their growth inhibitory properties exhibited *in vitro*. This is an attractive hypothesis as it could potentially check the expansion of transformed cells to limit tumour development.

Several laboratories, including ours, have demonstrated the induction of apoptosis by a variety of chemopreventive selenium compounds (see Section 2.6.7.3). Perturbations in cycle progression have also been shown in some systems; however, the significance of this to the mode of action to the most potent selenium compounds remains unclear. However the evidence for apoptosis induction is overwhelming but it remains to be seen if cell cycle defects and apoptosis-induction are functionally interlinked.

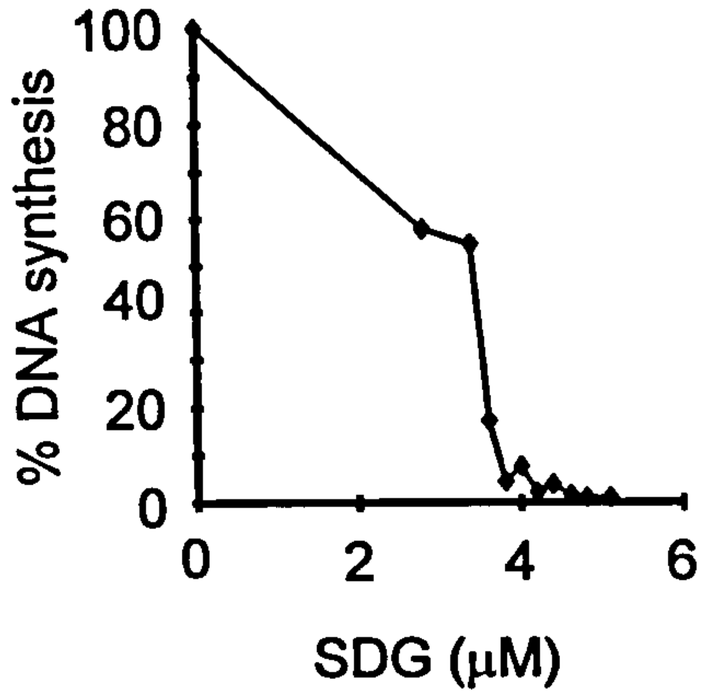
Though the induction of apoptosis is generally accepted to have a major contribution to the anti-cancer activities of selenium compounds, at the commencement of this study little was known about the molecular mechanisms involved in this process.

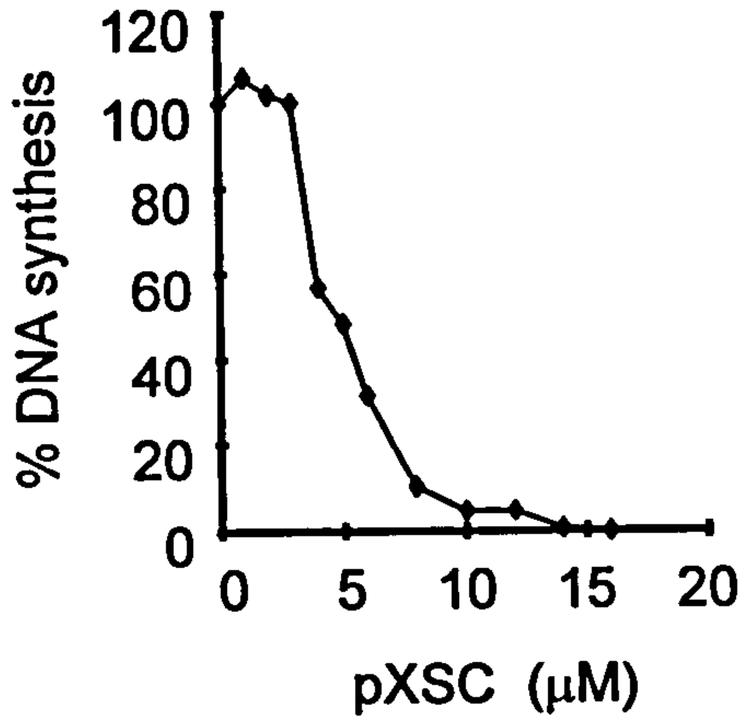
The objective of this part of the study was therefore primarily to characterize the nature of the growth inhibitory mechanisms employed by two compounds showing very strong anti-tumour activity *in vivo*; SDG and pXSC. The experiments were carried out in a human cervical carcinoma cell line, HeLa, which has dysfunctional p53.

4.2 Inhibition of cellular proliferation by selenium

In order to test whether SDG and/or pXSC resulted in some form of growth inhibition in HeLa cells, incorporation of tritiated thymidine into DNA was measured. HeLa cells treated overnight with either compound showed a dose dependent decrease in DNA synthesis (Fig 4.2.1A). This is in keeping with previous assays for cellular viability (assessed by tetrazolium salt reduction) which had shown a similar response in different cell lines (data not shown). Higher concentrations of pXSC were required to achieve

A





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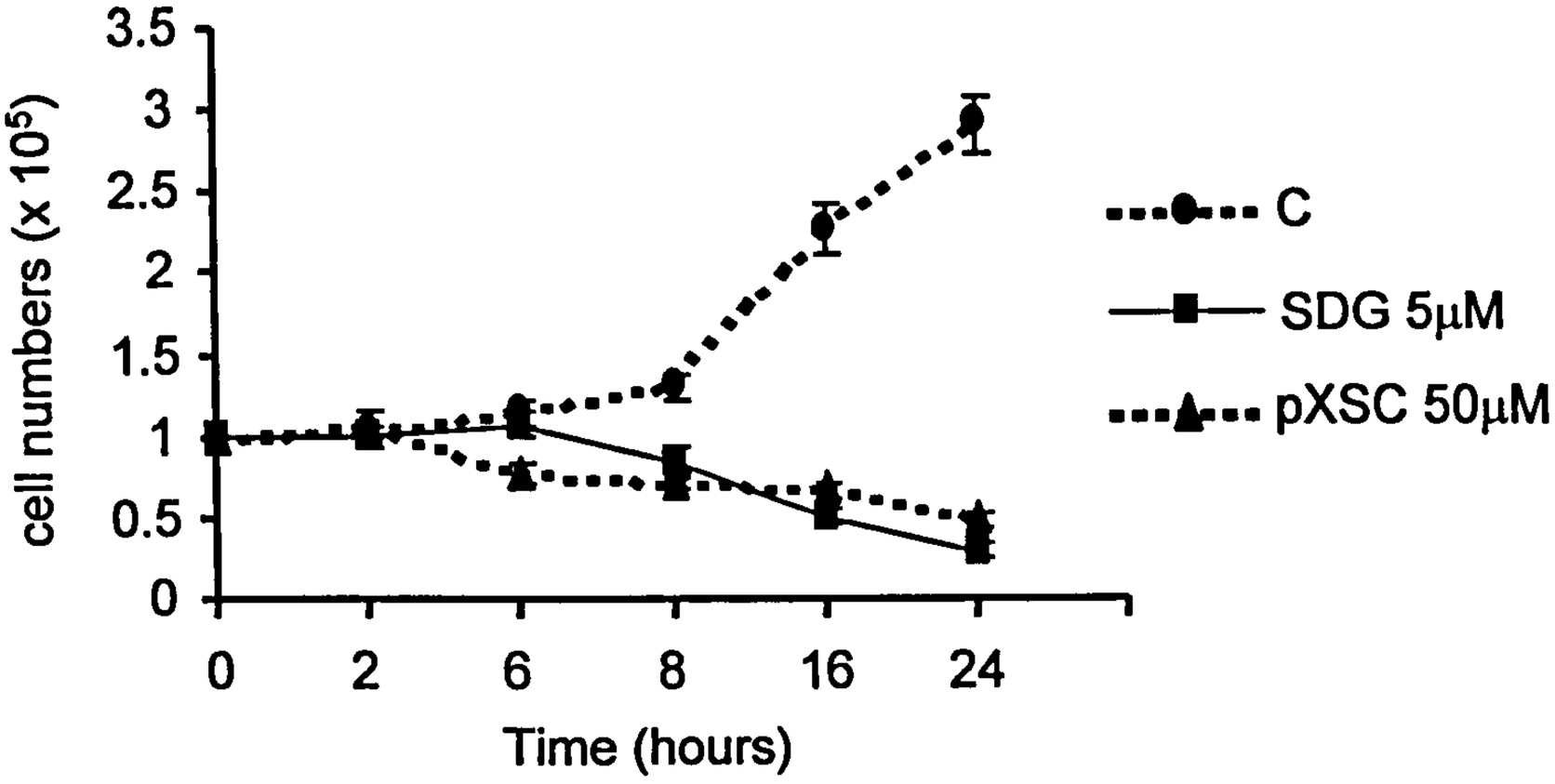


Fig 4.2.1 Growth inhibition of HeLa cells by treatment with SDG or pXSC.

(A) HeLa cells were treated overnight with the indicated concentrations of SDG or pXSC and incorporation of tritiated thymidine measured to assess inhibition of DNA synthesis. **(B)** SDG (5μM) and pXSC (50μM) were added to HeLa cells for the indicated times and cellular viability assessed by the Trypan blue dye exclusion method.

inhibition of DNA synthesis than SDG, which reflects the previously published observation that a higher dose of pXSC, compared to selenite, is required to show chemopreventive activity *in vivo* (see Section 2.5.2).

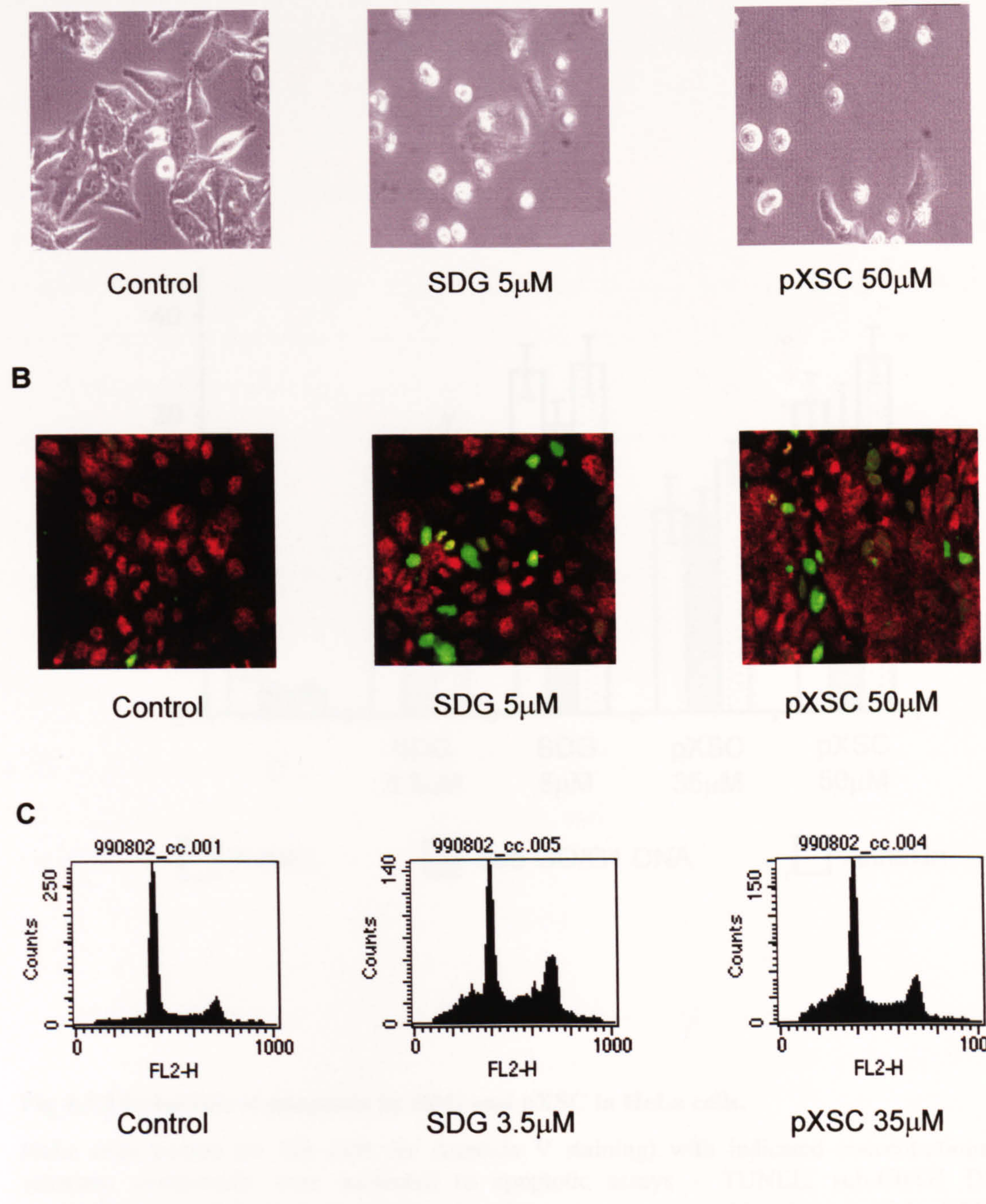
Exposure of HeLa cells to higher doses of selenium showed a decrease in the actual number of viable cells, over time, as assessed by Trypan blue exclusion (Fig 4.2.1B). This suggested that a mechanism involving loss of cellular viability and not a cell cycle arrest was the key event mediating the observed inhibition of growth.

4.3 Apoptosis induction by SDG and pXSC

Morphological analysis of HeLa cells exposed to selenium displayed apoptotic characteristics like increased refractile nature, cell shrinkage and rounding up, chromatin condensation and suggestions of membrane blebbing (Fig 4.3.1A).

HeLa cells treated with SDG or pXSC, at doses that had a marked effect on cellular viability, were analysed by several different apoptosis assays. One of the reasons for performing a variety of assays was to prove conclusively that the cell death observed was not due to non-specific necrosis. In retrospect, a major indirect benefit of standardising several different assays was the flexibility of adapting one or the other to suit specific needs of later experiments.

Apart from establishing concordance by 3 different assays, cell death evaluation was by two independent criterion. TUNEL (Fig 4.3.1B) and the sub-G0/G1 DNA content quantification (Fig 4.3.1C) addressed the characteristic DNA fragmentation in apoptosis whereas AnnexinV staining measured specific alteration in membrane composition. Use of AnnexinV allowed a robust discrimination between apoptotic and necrotic cells as the assay is dependent on the exposure of phosphotidylserine to the outer surface of the plasma membrane while maintaining membrane integrity – a characteristic of early apoptosis. Necrotic cell are also AnnexinV positive as membrane disruption allows staining. Therefore propidium iodide (PI) is used to discriminate between early apoptosis and necrosis: it enters and stains the DNA of necrotic cells but not *early* apoptotic cells (since the plasma membrane is still intact). Only AnnexinV positive and PI negative cells were scored as apoptotic. The extent of apoptosis measured by this assay was comparable to those evaluated by measuring the accumulation of hypodiploid DNA or TUNEL (Fig 4.3.2) suggesting limited non-specific toxicity by SDG and pXSC, at least at the doses used.



Control SDG 3.5μM pXSC 35μM

Fig 4.3.1 Analysis of apoptosis induction in HeLa cells by SDG and pXSC.

HeLa cells were treated with the indicated concentrations of selenium compounds for 16h before assessing apoptosis induction by **(A)** morphology, **(B)** TUNEL assay and **(C)** accumulation of hypodiploid DNA content. **(A)** light microscope photomicrographs of morphological changes associated with SDG and pXSC treatment. **(B)** represents merged photomicrographs of TUNEL assay showing FITC (green) and PI (red) fluorescence. FITC fluorescence indicates apoptotic cells while PI is a general DNA stain. **(C)** FACS generated profiles of cellular DNA content in all stages of the cell cycle showing accumulation of sub-G0/G1 DNA framents on SDG and pXSC treatment. Control in all cases is DMSO.

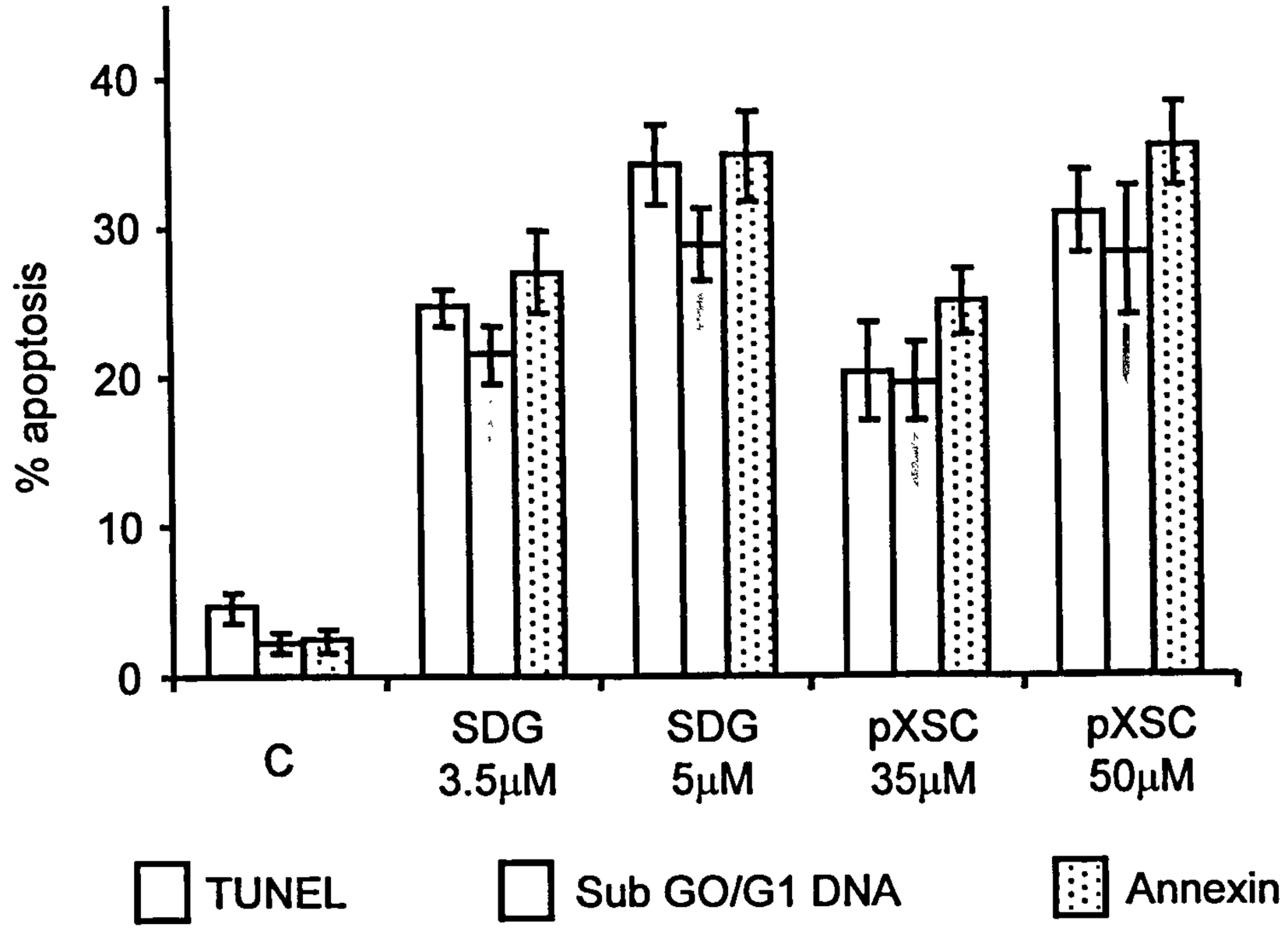


Fig 4.3.2 Induction of apoptosis by SDG and pXSC in HeLa cells.

HeLa cells treated for 16h (10h for Annexin V staining) with indicated concentrations of selenium compounds were subjected to apoptotic assays – TUNEL, sub-G0/G1 DNA accumulation and Annexin V staining. C, represents treatment with solvent vehicle, DMSO. Error bars indicate standard errors. Each treatment was performed in triplicate. Data presented are representative of 3 independent experiments.

TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) assay (Fig 4.3.1B), AnnexinV staining and appearance of hypo-diploid DNA content (Fig 4.3.1C) conclusively proved the induction of apoptosis in HeLa cells by SDG and pXSC. Cell death was dose-dependent (Fig 4.3.2) and corroborated well with loss viability seen in Section 4.2.

4.4 Involvement of Caspase 3

The major downstream effectors of the apoptotic cascade are the caspases. Caspase activation cascades converge on caspase 3 which, on activation, proteolytically cleaves (thereby activating) several key proteins including PARP (poly(ADP-ribose) polymerase) which bring about DNA fragmentation and cell death (see Section 2.6.7.1).

Treatment of HeLa cells with SDG or pXSC resulted in the activation of caspase 3 assessed by using an antibody specifically recognising the cleaved fragment of caspase 3 (Fig 4.4.1A). At concentrations that induce significant apoptosis, SDG induced caspase 3 only slightly whereas this induction was greater with pXSC.

Treatment of cells with a specific pharmacologic inhibitor of caspase 3, Ac-DEVD-CHO, resulted in a decrease in apoptosis in response to both SDG and pXSC (Fig 4.4.1A). This inhibition was more marked (55%, p=0.04) with pXSC than SDG (27%, p=0.032) perhaps reflecting the disparate abilities of the two compounds to induce caspase 3. The efficacy of the inhibitor was tested by assessing cleavage of PARP – a downstream proteolytic target of caspase 3. Use of a cleaved PARP fragment specific antibody revealed that the inhibitor did reduce cleavage of PARP, again more efficiently for pXSC (53% compared to 27%) than for SDG.

It is clear from the extent of inhibition of PARP cleavage that 50μM of the caspase 3 inhibitor has a partial effect, perhaps explaining the partial protection from apoptosis induction. Higher concentrations of the inhibitor proved to be toxic to HeLa cells precluding the possibility of inhibiting caspase 3 activity completely. It is possible that there may be a caspase independent apoptotic mechanism, as has been previously documented in other contexts (see Section 2.6.7.1), but this investigation does not provide any conclusive evidence for such a route.

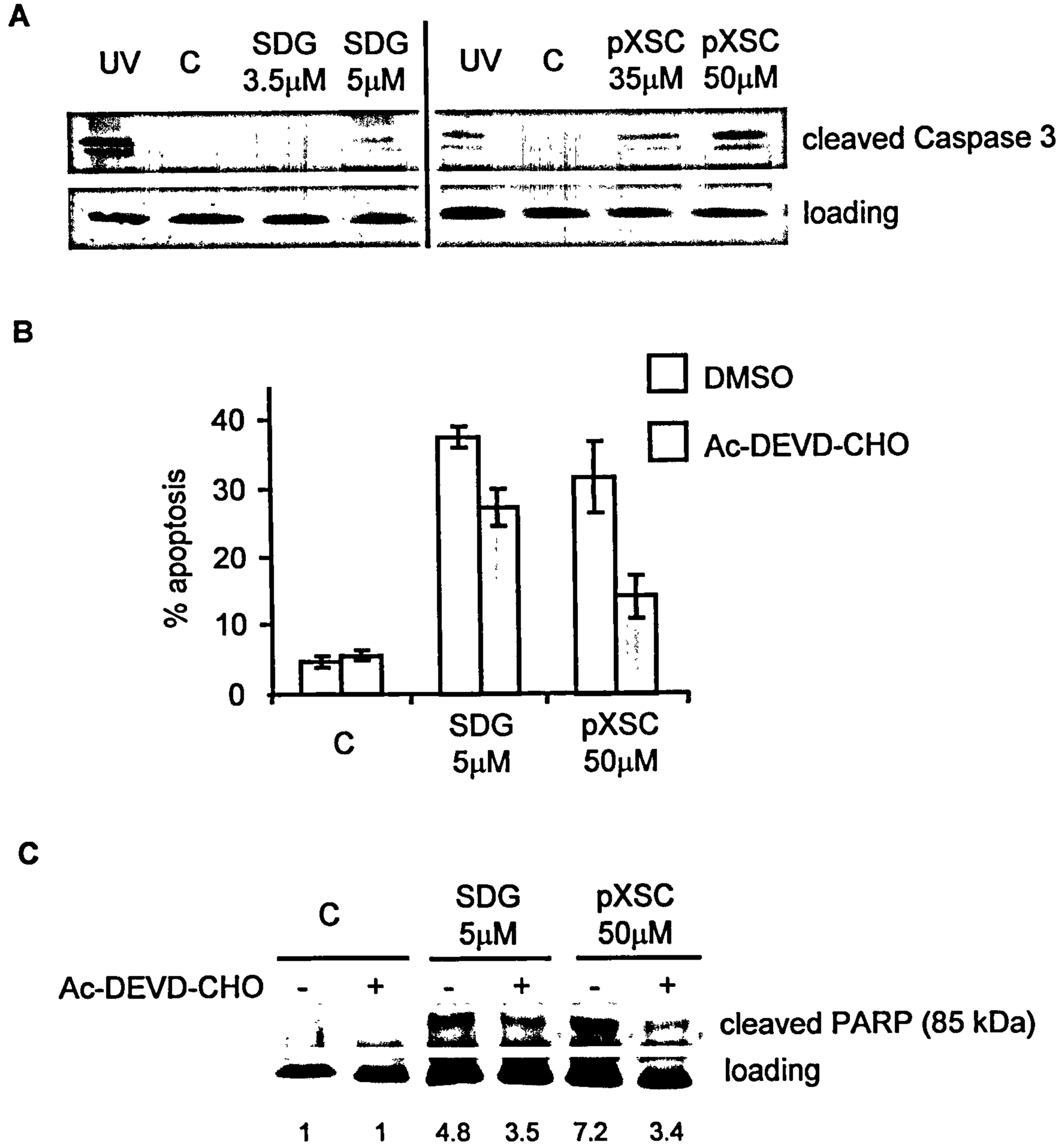


Fig 4.4.1 Caspase 3 activity in SDG- and pXSC-induced apoptosis.

HeLa cells treated with indicated concentrations of selenium compounds for 16h were assayed for the involvement of caspase 3. **(A)** Cell lysates were prepared and Western blotted with either an antibody against the cleaved fragment of caspase 3 or a total p38 kinase antibody as a loading control. UV, HeLa cells exposed to 200Jm^{-2} UV irradiation 6h prior to the preparation of cell lysates. **(B)** TUNEL assay measuring the extent of apoptosis induction by selenium compounds in the presence or absence of specific caspase 3 inhibitor, Ac-DEVD-CHO (at 50μM, added 30min prior to selenium treatment). Error bars indicate standard errors. Each treatment was performed in triplicate. Data presented is representative of 2 independent experiments. **(C)** HeLa cells were treated with the indicated concentrations of SDG and pXSC for 16h in the presence or absence of Ac-DEVD-CHO. Cell lysates were western blotted using antibodies against the cleaved fragment of PARP or total p38 kinase as loading control. The figures below the blots indicate the fold increase in cleaved PARP above untreated level, normalized to the loading control. C, in all cases, represents treatment with solvent vehicle, DMSO.

4.5 Molecular mediators of SDG and pXSC induced apoptosis

Programmed cell death can be broadly classified into two major groups; receptor mediated and mitochondria dependent (See Section 2.6.7.1). In recent years, however, the boundary between these two classes is getting increasingly blurred with evidence mounting for cross-talk and synergistic activation of both modes leading to amplification of the response. These mechanisms are not mutually exclusive and, while they may function separately in some situations, they may co-operate in other scenarios.

4.5.1 Role of the Bcl2 family members

The Bcl2 family members (see Section 2.6.7.1) critically regulate mitochondrial cytochrome c release (and therefore apoptosis) and have been implicated in several apoptotic responses induced by oxidants, chemotherapeutic drugs etc (Reed, 2001). These proteins are often regulated at the transcriptional level and fall into pro- and anti- apoptotic classes. Bcl2 and $Bclx_L$ are anti-apoptotic and are often downregulated in response to several apoptotic stimuli while their overexpression alleviates the apoptotic response. Bax , on the other hand, is acutely pro-apoptotic and recent evidence suggests that it is perhaps indispensable in mediating most forms of mitochondria mediated cell death (Zhang et al., 2000b).

To test whether Bcl2 family members were involved in the apoptosis induction by selenium compounds, SDG and pXSC were added at apoptosis-inducing concentrations for various times to assess any alterations in the levels of Bcl2, $Bclx_L$ and Bax proteins. However, Western blotting revealed that none of the protein levels were altered on treatment with pXSC or SDG (Fig. 4.5.1).

This corroborates with an earlier study that found no induction of Bcl2 family members on treatment with selenite (Kaeck et al., 1997). However, other selenium compounds like SMC have been shown to decrease levels of Bcl2 *in vivo* (Ip and Dong, 2001). There is no information regarding pXSC in other systems. Overexpression of Bcl2 protein in mouse C57 cells have been shown to have no added protection from SDG treatment (Blower, 1998). However, the relevance of this data is confounded by the fact that in this system no protection from H_2O_2 treatment was observed.

However, the lack of change in protein levels of the Bcl2 family members does not necessarily exclude the possibility of mitochondrial involvement in SDG/pXSC mediated

Fig 4.5.1 Protein levels of Bcl2 family members on exposure to selenium.

HeLa cells were treated as indicated with SDG and pXSC and the cell lysates western blotted using antibodies against Bcl2, Bclx$_L$, Bax and total p38 kinase as loading control. C, represents treatment with solvent vehicle, DMSO.

cell death. Bcl2 family members not included in our study, like Bad and Bak, may be involved. There is also the possibility of post-translational modification of Bcl2 family members. For example, phosphorylation-mediated inactivation of Bcl2 and $Bclx_L$ by JNK (Deng et al., 2001; Fan et al., 2000) and p38 (Torcia et al., 2001) has been documented, as well as the phosphorylation of BAD by the Akt kinase (del Peso et al., 1997). These remain very real possibilities particularly because other experiments reported elsewhere in the thesis have shown that both the selenium compounds under investigation can induce JNK, p38 and Akt activities. Identification of such post-translational modifications of Bcl2 family members is difficult since phosphorylation site-specific antibodies for these modifications are not generally available. Alternative strategies, for example assaying alterations in migration patterns due to phosphorylation and overexpressing of putative phosphorylation site/s mutants need to be considered.

The involvement of the mitochondrial pathway could also perhaps be addressed by examining cytochrome c release or changes in mitochondrial membrane potential. Caspase 8 is activated by the receptor mediated pathway while the mitochondrial route utilises caspase 9 (see Section 2.6.7.1). Therefore assays for individual activities of these caspases followed by assessment of functional contribution using of specific inhibitors could indicate the relative contibution of the two apoptotic modes. However, the emerging evidence for overlap between the pathways might make such simplistic experiments difficult to interpret.

4.5.2 Involvement of Fas ligand (FasI) in selenium mediated cell death

Apoptosis-related receptors are activated by cognate apoptosis-inducing ligands and thereby trigger programmed cell death by several diverse stimuli. This often involves transcriptional upregulation of the ligand which is secreted into the extracellular space thereby inducing autocrine or paracrine apoptosis in cells bearing the complementary receptors. Fasl is one such cell death mediating ligand which has been shown to be upregulated, both *in vitro* and *in vivo*, in response to various cellular stresses and chemotherapeutic agents (Debatin, 1999).

Treatment of HeLa cells with SDG and pXSC led to the rapid induction of Fasl protein in a dose dependent manner (Fig. 4.5.2A, B). Only the soluble form of Fasl was detectable, which fits well with the idea of autocrine/paracrine apoptosis. The expression of the Fas receptor in HeLa cells has been previously described (Wajant et al., 1998) making the

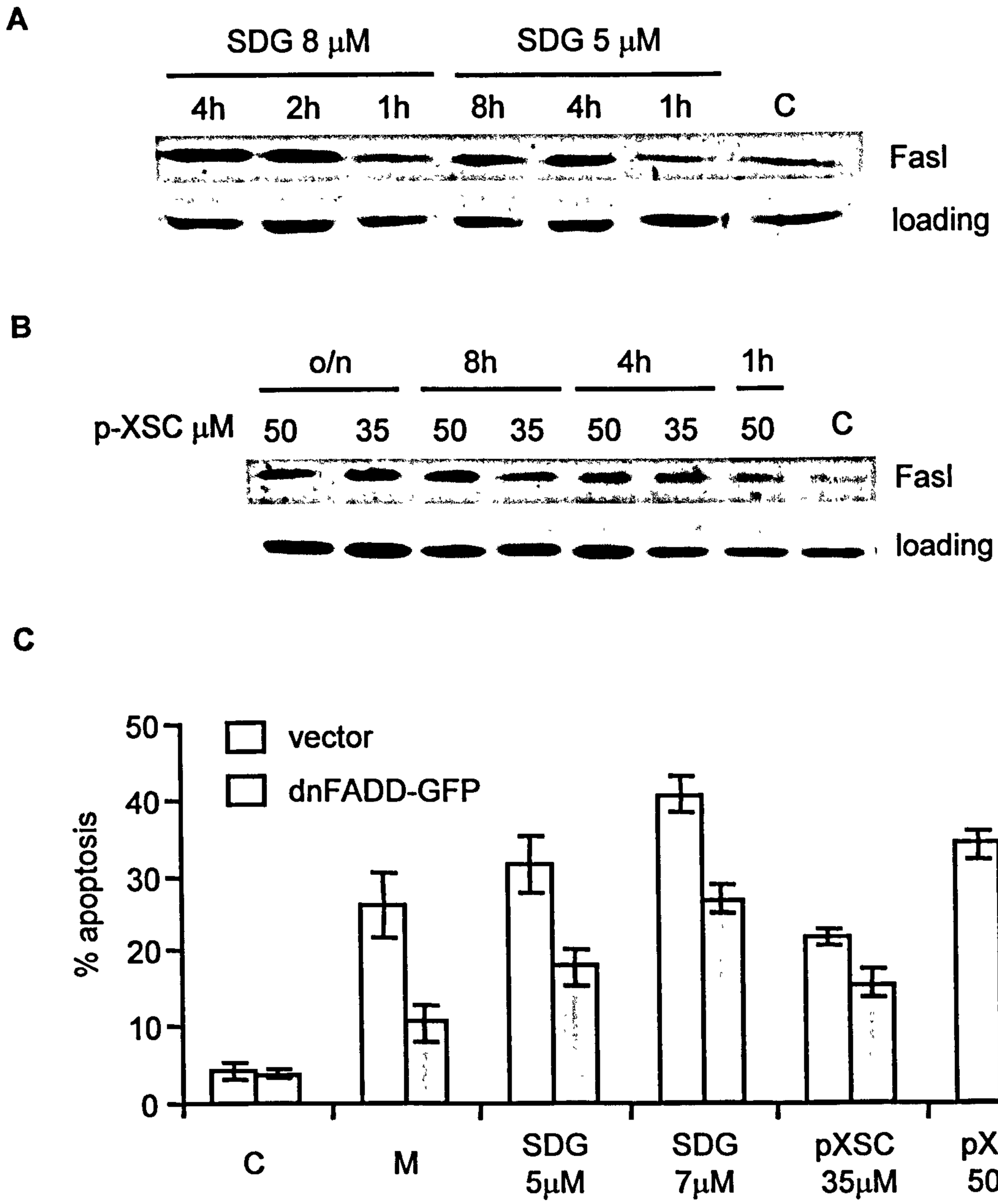


Fig 4.5.2 Induction of Fas ligand mediates SDG- and pXSC-induced apoptosis.

HeLa cells were treated as indicated with SDG **(A)** and pXSC **(B)**. Cell lysates were Western blotted using antibodies against Fas ligand or total p38 kinase as loading control. **(C)** Levels of apoptosis of HeLa cells transiently transfected with dnFADD-GFP or an empty vector (GFP co-transfected) were assessed after selenium treatment for 16h by measuring the accumulation of hypodiploid DNA within the GFP positive fraction of cells. M represents a positive control treatment with 400μM MMS for 8h. Error bars indicate standard errors. Each treatment was performed in triplicate. Data presented is representative of 3 independent experiments. C, in all cases, represents treatment with solvent vehicle, DMSO.

involvement of the Fas mediated cell death pathway a putative candidate mechanism in selenium-induced apoptosis in these cells.

The levels of Fasl induction correlated well with the cell death observed, therefore we tested if this was functionally required. Stimulation of Fas leads to the formation of a receptor-bound death-inducing signaling complex (DISC), consisting of FADD and two different forms of caspase-8 (see Section 2.6.7.1). It has been shown that disruption of FADD protects cells from Fas mediated apoptosis (Wajant et al., 1998): providing us with a tool to assess functional requirement of the Fas pathway in SDG/pXSC-induced programmed cell death.

Dr H Wajant (University of Stuttgart, Germany) very kindly provided us with a dominant negative FADD (dnFADD) expression construct where the amino-terminal death effector domain of FADD is replaced by GFP. HeLa cells were transiently transfected with the dnFADD or empty vector for 24h followed by treatment with selenium compounds. Using fluorescence activated cell sorting (FACS) the sub-G0/G1 DNA content of the cells expressing GFP was assessed (Fig 4.5.2A). Exogenous expression of dnFADD resulted in a reduction in of SDG-induced (43% and 34% for 5 and 7 μM, respectively; p=0.014 and 0.002) and to a lesser extent for pXSC-induced apoptosis (28% and 29% for 35 and 50 μM, respectively; p=0.02 and 0.034).

These studies have therefore shown for the first time induction of Fasl by selenium compounds and have also confirmed its functional involvement apoptosis induction. One consideration, prior to the transfection studies, was as HeLa cells expressed relatively low levels of Fas receptor (Wajant et al., 1998), the induction of the ligand observed could be non-specific and may not contribute to cell death. However, use of dnFADD successfully demonstrated the functional relevance of this pathway suggesting that the low levels of Fas receptor expression was sufficient. No evidence for the induction of the Fas receptor itself was found (work of J.Fleming).

Expression of exogenous dnFADD results in partial protection from apoptosis suggesting either only partial protection is attained by transfecting dnFADD or Fas-independent pathways also contribute to the induction of apoptosis. It is a possibility that other cytotoxic ligands (like TRAIL – TNF-related apoptosis inducing factor) may be involved or perhaps there may be some contribution of mitochondrial dysfunction. Experiments suggested in Section 4.5.1 may be of some importance in order to dissect the relative importance of the mitochondria-mediated pathway.

Finally, given that both pXSC and SDG induce JNK activation (Section 4.3.1) the involvement of Fasl described here opens up the possibility of an apoptosis signalling pathway from JNK activation to Fasl induction via transcriptional activation of c-jun as has been shown in other systems (Kolbus et al., 2000; Sharma et al., 2000).

4.6 Conclusions

This chapter describes apoptosis induction by chemopreventive selenium compounds, SDG and pXSC, in HeLa cells. Several different assays have been used to assess cell death which appears to be, at least partially, dependent on caspase 3 induction as judged by the caspase inhibitor experiments. Bcl2, $Bclx_L$ and Bax protein levels do not change when cells are treated with cytotoxic doses of selenium compounds. This however does not exclude the possibility of post-translational regulation of these key mediators of cell death. Selenium compounds were also shown to induce Fasl which appears to have a functional contribution to the induction of cell death. This is of particular importance, since it is the first functional mediator of selenium-induced apoptosis to be identified.

4.7 Chapter summary

- Chemopreventive selenium compounds, pXSC and SDG, reduce proliferation of HeLa cells accompanied by a loss of cellular viability.

- The loss of cellular viability is primarily due to the induction of apoptosis.

- Caspase 3 is activated by selenium and inhibition of its activity partially protects from selenium-induced apoptosis.

- The levels of Bcl2, $Bclx_L$ and Bax proteins do not change during the course of selenium exposure.

- FasL is induced by pXSC and SDG and disruption of Fas signalling by overexpressing a dominant negative FADD protects from apoptosis induction.

5 Signalling Mechanisms Mediating Selenium-induced Cell Death

5.1 Background

As established in the previous chapter and described extensively in the literature, several chemopreventive selenium compounds induce programmed cell death (see Section 2.6.7.3). A major issue remaining unresolved is what integrates the cellular sensing of chemopreventive levels of selenium to an appropriate response – in this case that of cell death (including an involvement of the Fas mediated pathway in the context of this thesis).

The evolutionary conserved, Mitogen activated protein kinase (MAPK) signal transduction pathways have been shown to critically regulate cell growth, survival and apoptosis by accurately interpreting the sensory cues (see Section 2.6.7.4). The MAPK pathways are extremely context dependent and it is believed that a balance of pro- and anti-apoptotic signals regulate the ultimate cellular response. The context-specificity and the intimacy of the cross-talk between different pathways result in a highly sophisticated signal transduction system sensitive to even small alterations of its environment. Given the central role of MAPK signalling and the fact that several known apoptotic stimuli involve functional alterations in MAPK signal transduction, this study investigated whether any of the major MAPKs are involved in selenium induced cell death. Precedent for the involvement of MAPKs has been discussed in Section 2.6.7.4. Our lab has also found evidence for the induction of haemoxygenase (HO-1) by selenium compounds (Fleming et al., 2001) which is a typical marker for a cellular stress response, often involving the activation of the stress induced MAPKs like JNK and p38, thus strengthening the case for investigation into these pathways.

5.2 Involvement of p38 kinase

The induction of p38 stress kinases have been documented for a number of apoptotic stimuli including those mediated by the activation of the Fas pathway (Zhang et al., 2000a). Depending on the context, p38 can be both pro- or anti- apoptotic (Martin-Blanco, 2000). It is believed that p38 kinase activation is involved in DNA damage-induced apoptosis, but recent evidence suggests that its primary role may be in initiating a block in the G2/M phase of the cell cycle which may, in turn, potentiate apoptosis (Bulavin et al., 2001). The 'pro-proliferatory' function of p38 kinase is less well elucidated, but the current

evidence suggests it may be involved in fibroblast growth factor 2-induced proliferation of Swiss 3T3 and granulocyte colony-stimulating factor-induced proliferation of haematopoetic cells (Nebreda and Porras, 2000). p38 has also been shown to mediate survival in cardiomyocytes from anisomycin-induced apoptosis and in neuronal survival, probably via the activation of the MEF2 transcription factor (Nebreda and Porras, 2000).

5.2.1 Activation of p38 kinase by SDG and pXSC

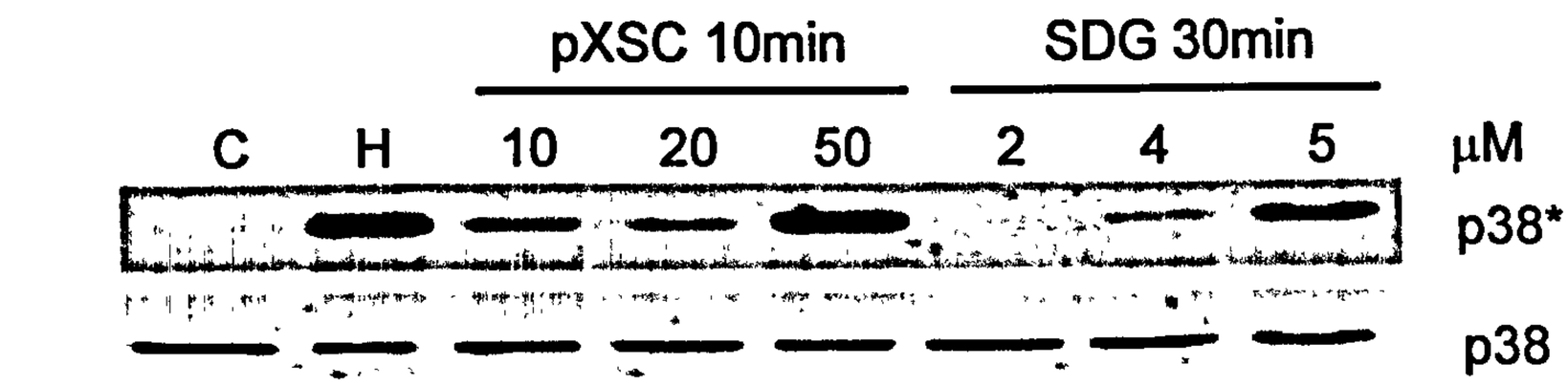
Fig 5.2.1A shows the dose dependent activation of p38 kinase on treatment with both SDG and p-XSC using phosphorylation state specific antibodies against the Thr180/Tyr182 residues. While the phosphorylated fraction (activated) of p38 gets induced, there is no change in the levels of the total protein. The activation is fairly rapid – within 10 min for pXSC and 30 min for SDG.

As the duration of p38 activity may influence its downstream response, HeLa cells were treated with chemopreventive doses of selenium for longer time periods (Fig 5.2.1B). This revealed that p38 activation was sustained throughout the period of the treatment and when substantial apoptosis was detectable.

The antibodies used recognise the phosphorylated fraction of the p38 isoforms α and β. p38 has two other major isoforms, δ and γ, which have been implicated in certain physiological responses. However, the unavailability of phospho-specific or total antibodies against these isoforms precludes the analysis of the endogenous activities of these forms.

5.2.2 Specific inhibition of p38 activity does not reveal a functional role in apoptotic signalling

A very highly specific pharmacological inhibitor for p38α and β activities have been developed and extensively characterised. This inhibitor, SB203580, was used to assess the contribution of p38 activation to cell death induction by SDG and pXSC. HeLa cells were pretreated with 10μM SB203580 prior to addition of selenium. At these conditions the inhibitor completely abolished p38 activity, determined by assaying the kinase activity of MAPKAP-2 (MAPK activated protein kinase 2; see Fig 2.6.3) – an immediate downstream p38 target (Fig 5.2.2A). The specificity of the inhibition was reflected in its failure to affect JNK activity, assessed by the specific phosphorylation of c-jun by JNK at serine 63, at conditions that completely attenuated p38 activity (Fig 5.2.2B).



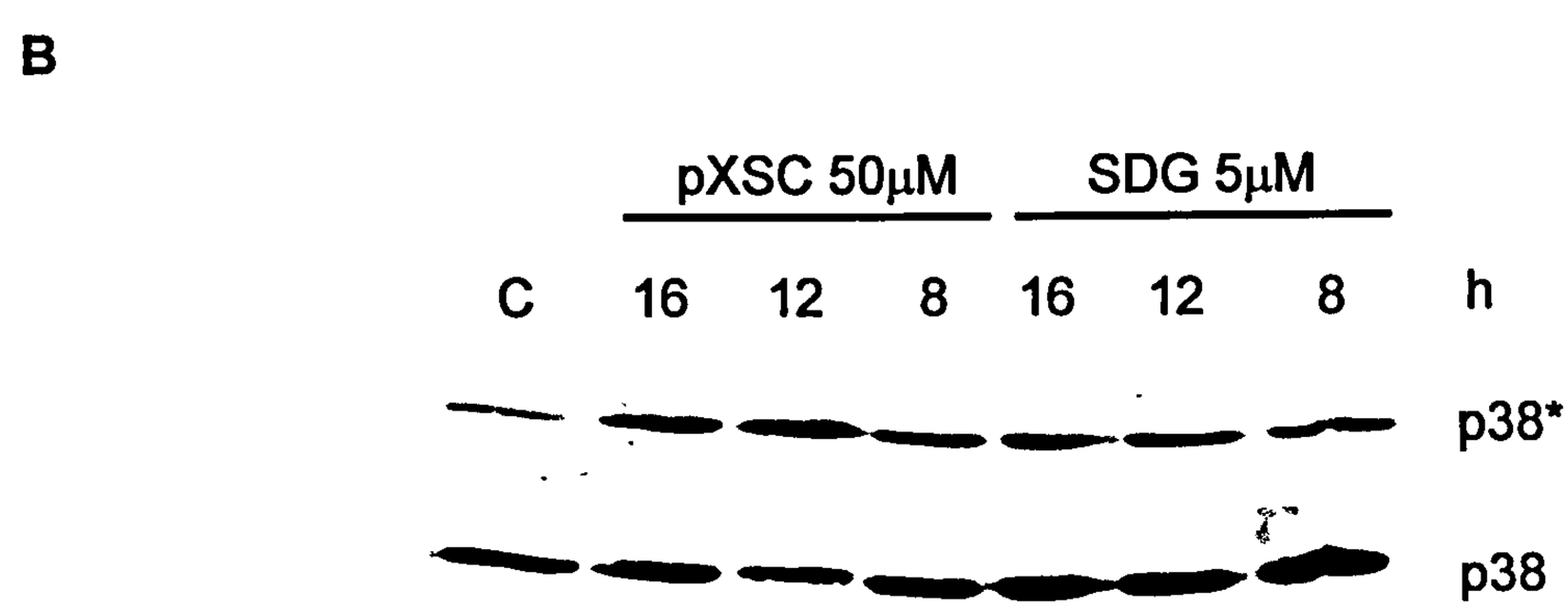


Fig 5.2.1 Activation of p38 kinase by SDG and pXSC.

HeLa cells were treated as indicated with SDG or pXSC, cell lysates prepared, and analysed by SDS/PAGE and western blotting with antibodies against the activated form of p38 (p38*). As loading controls, the blots were then stripped and western blotted with antibodies against total p38. **(A)** Shows activation of p38 kinase after a short exposure to selenium compounds, while **(B)** shows the same after longer treatments. H, represents a positive of 2mM H_2O_2 treatment for 30min. C, in all cases represents treatment with solvent vehicle, DMSO.

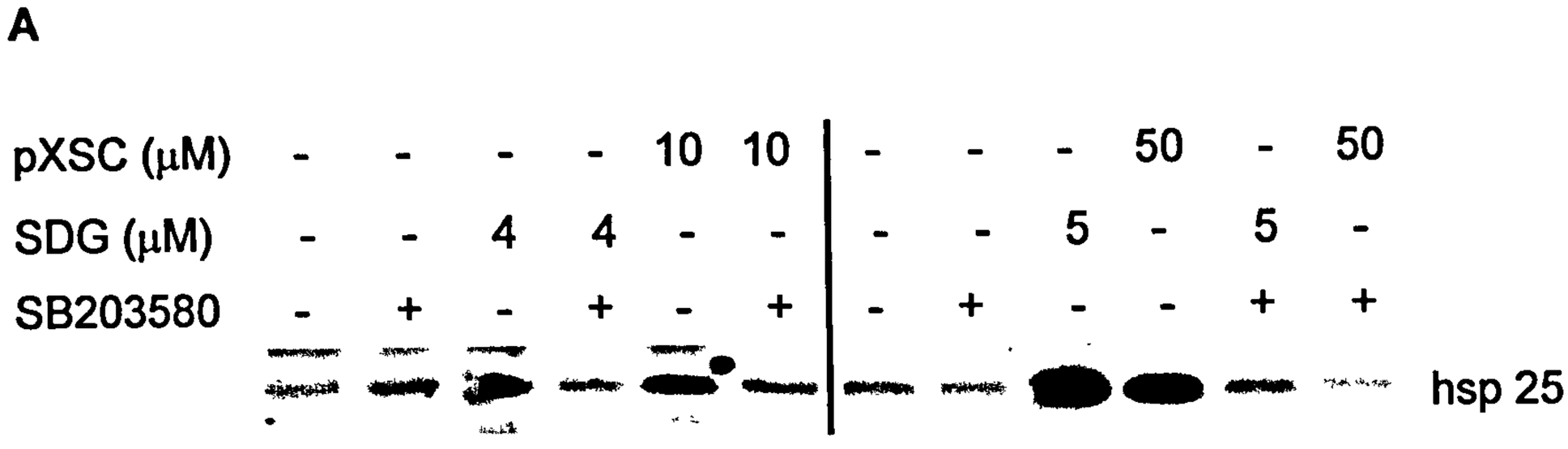


Fig 5.2.2 Effect of inhibition of p38 activity on apoptosis induction by SDG and pXSC.

(A) HeLa cells were treated with indicated concentrations of selenium compounds for 16h in the presence or absence of specific p38 inhibitor, SB203580 (at 10μM, added 30min prior to selenium treatment). Cell lysates were analysed for p38 kinase activity by an immunecomplex kinase assay for endogenous MAPKAP-2 (direct substrate of p38 kinase) activity using hsp25 as a substrate. **(B)** JNK activity in cell lysates prepared after 30min of selenium treatment in the presence or absence of SB203580 (at 10μM, added 30min prior to selenium treatment) were measured by western blot analysis using antibodies against phosphorylated Serine 63 of c-jun (c-jun*(Ser 63)). **(C)** Levels of apoptosis induction by selenium compounds, with or without pre-treatment with SB203580 (at 10μM, added 30min prior to selenium treatment), was measured by TUNEL assay. UV, HeLa cells exposed to 200Jm^{-2} UV irradiation 12h prior to the preparation of cell lysates. Error bars indicate standard errors. Each treatment was performed in triplicate. Data presented is representative of 4 independent experiments. C, in all cases, represents treatment with solvent vehicle, DMSO.

On measuring the extent of cell death induced by either selenium compound in HeLa cells treated with or without SB203580, no difference in the number of cells undergoing apoptosis was found (Fig 5.2.2C). A control experiment exposing cells to UV showed marked reduction of apoptotic cells when p38 activity was inhibited (Fig 5.2.2C). These experiments suggest that apoptosis induction by selenium does not require p38 activation.

Though no obvious role for p38 could be demonstrated by these experiments, it is quite possible that p38 contributes to selenium-mediated cyto-toxicity by more subtle means. For example, p38 may promote some deregulation of cell cycle progression that may potentiate apoptosis. This view is supported by the evidence that treatment with some selenium compounds induces several members of the GADD (Growth arrest and DNA damage) family of proteins that regulate cell cycle progression and are known to be influenced by p38 activity (Sheikh et al., 2000). Selenomethionine induces a cell cycle block associated with the inhibition of cdc2 activity. Evidence from other studies suggest that cdc2 activity may be inhibited by p38, possibly via inactivation of phosphatases (Pearce and Humphrey, 2001). Our experiments perhaps employed an apoptotic impetus that was too acute to detect these differences. A detailed study of cell cycle patterns in synchronous cultures treated with or without SB203580 would perhaps be more appropriate to investigate whether p38 induced by SDG and pXSC has a role in altering the cell cycle. One cannot, however, expect a dominant effect arising from possibilities mentioned above as no effect on apoptosis induction was revealed in the inhibitor studies.

p38 activity may also be downstream of the induction of apoptosis, perhaps in some form of a postive feedback loop to strengthen an acute cytotoxic response. There is evidence that casapase 3 may activate MEKK1 which, in turn, may activate p38 (Cardone et al., 1997; Nakagami et al., 2001). Having cells treated with or without a caspase 3 inhibitor prior to selenium treatment and following the kinetics of p38 activation could verify this particular phenomenon. Again such a response enhancement mechanism appears to be unlikely given the very rapid activation of p38, low caspase 3 activity (particularly for SDG) and the fact that if it had a major contribution then it would have been revealed in the inhibitor experiment.

Due to the fact that available reagents (phospho-specific antibodies and inhibitors) only recognise/affect the p38α and β isoforms, the role of p38δ and γ could not be investigated. It is possible that one or both of these are activated concomitantly with α and β and share a redundant (or even perhaps independent) role in apoptosis induction with the latter. Whether SDG/pXSC activates the δ and γ isoforms could be assessed by activation of an

overexpressed tagged construct by 'pull-down' kinase assays. If found activated, specific dominant negative constructs could then be utilised, individually or in combination with SB203580, to investigate their possible functional involvement.

A final relevant fact is that in HeLa cells, in response to certain stimuli, p38α appears to promote apoptosis whereas p38β enhances survival (Nemoto et al., 1998). A detailed investigation into this isoform specificity would again entail overexpression studies with wild type and dominant negative constructs.

Thus, though SDG and pXSC rapidly induced sustained p38 activity, its association with cell death in this particular context remains unclear.

5.3 Involvement of c-jun N-terminal kinase (JNK)

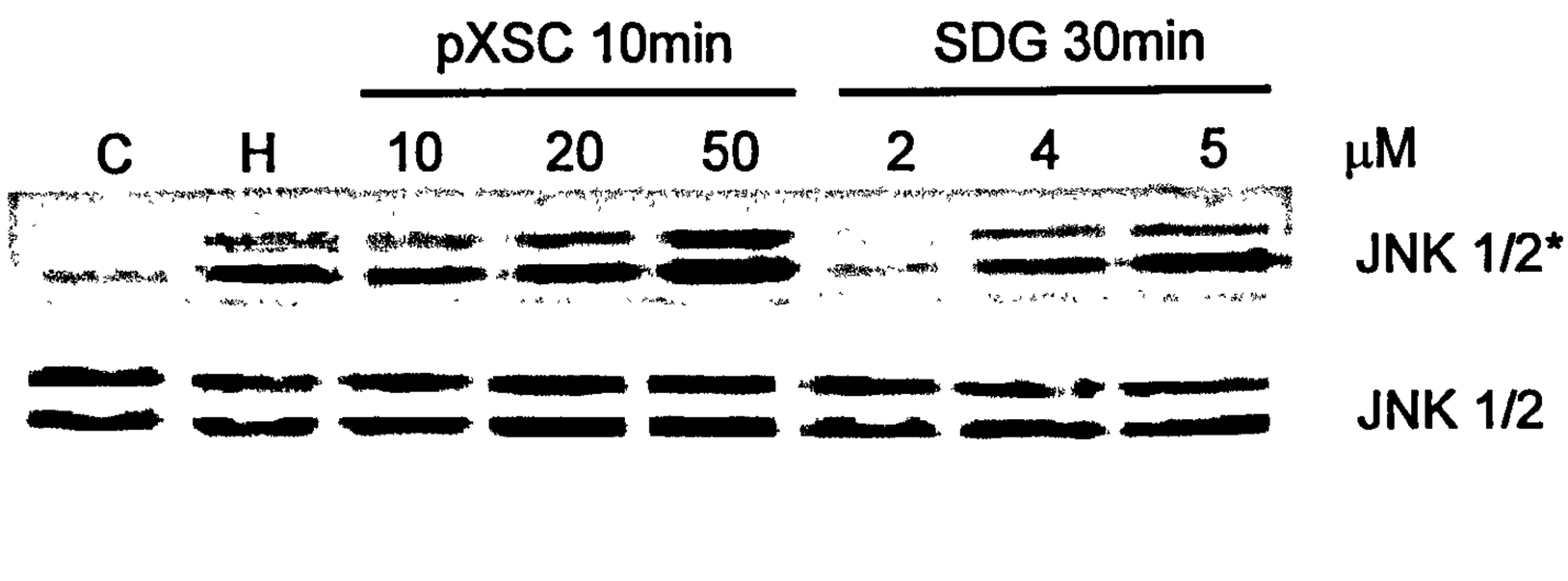
The JNK pathway is activated by the exposure of cells to several types of stress, though its role in the stress response is unclear (Davis, 2000; Dong et al., 2001a). It is possible that it may mediate some of the effects of stress on the cells, like induce apoptosis, or, alternatively be a survival response. Thus the specific role depends on the cellular context and both apoptotic and survival signaling by JNK has been documented (Leppa and Bohmann, 1999). Phosphorylation of c-jun on the sites that are phosphorylated by JNK (Ser-36 and Ser-73) causes increased transcriptional activity (Derijard et al., 1994; Kyriakis et al., 1994). Apoptosis mediated by c-jun-regulated effector proteins like Fasl are often preceded by enhanced JNK activity and this altered gene expression mediated cell death is well documented (Kolbus et al., 2000; Sharma et al., 2000).

5.3.1 SDG and pXSC activate JNK

Phospho-specific antibodies against Thr183/Tyr185 of JNK1 and 2, the major isoforms in non-neuronal cells, are excellent markers of JNK activity (Derijard et al., 1994; Kyriakis et al., 1994). Treatment with SDG and pXSC revealed a rapid, dose dependent increase in JNK activity within 10-30 min(Fig 5.3.1A).

As with p38, the duration of the JNK induction appears to determine the specific response, for example proliferation or apoptosis (Davis, 2000). HeLa cells were therefore treated with the selenium compounds over longer time periods: this revealed that JNK activity was sustained over the course of the treatment (Fig 5.3.1B). This fits well with the current

A



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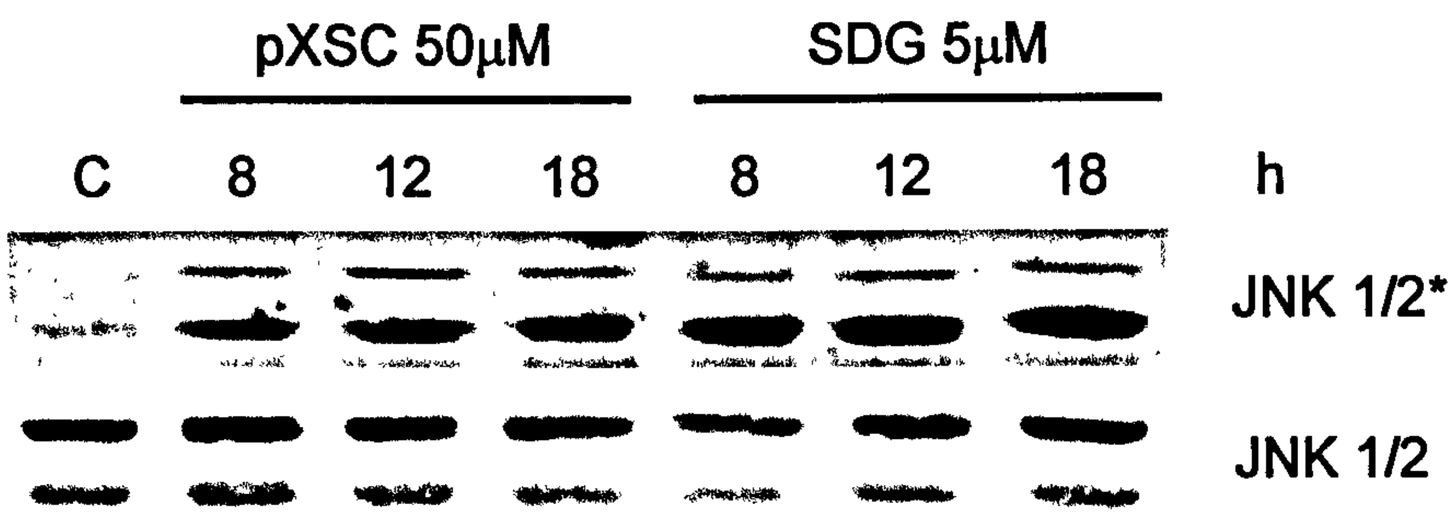


Fig 5.3.1 Activation of the stress kinase, JNK by SDG and pXSC.

HeLa cells were treated as indicated with SDG or pXSC, cell lysates prepared, and analysed by SDS/PAGE and western blotting with antibodies against the activated forms of JNK1/2 (JNK 1/2*). As loading controls, the blots were then stripped and western blotted with antibodies against total JNK1/2. **(A)** Shows activation of JNK kinase after a short exposure to selenium compounds, while **(B)** shows the same after longer treatments. H, represents a positive of 2mM H_2O_2 treatment for 30min. C, in all cases, represents treatment with solvent vehicle, DMSO.

model for pro-apoptotic JNK signalling where sustained JNK activation is thought to induce apoptosis (Davis, 2000; Dong et al., 2001a).

5.3.2 JNK activation leads to the phosphorylation of c-jun

To ensure the JNK activity observed resulted in the phosphorylation of its major downstream target, c-jun, a GST-fusion of the N-terminal half of c-jun was used as a JNK substrate in a 'pull-down' kinase assay (Fig 5.3.2A). An increased in GST-c-jun phosphorylation was observed confirming the results from the phospho-specific antibody experiments.

To confirm that JNK was indeed phosphorylating c-jun at the appropriate sites (Ser-63 and Ser-73), phospho-specific antibodies recognizing these particular sites were used. Fig 5.3.2B shows the increased phosphorlyation of c-jun at residues Ser-63 and Ser-73 on treatment with both SDG and pXSC.

5.3.3 JNK mediates selenium-induced apoptotic signalling via c-jun activity

As specific pharmacologic inhibitors of the JNK pathway are not very well characterised and there is considerable signalling redundancy amongst the known upstream JNK activators, c-jun was chosen as an appropriate level for disruption of the JNK pathway. JNK1 and 2 kinase dead constructs have been previously described but were not used as they are not very efficient (Dr S. Gutkind, personal communication).

A dominant negative c-jun construct, TAM67 (a kind gift from Dr D. Gillespie, Beatson Institute, UK), has been used extensively in the literature (Brown et al., 1994; Fan et al., 2001; Freemerman et al., 1996). This construct was used to transiently transfect HeLa cells prior to selenium treatment. To monitor changes in the population of transfected cells, the plasmid of interest was co-transfected with a GFP construct and FACSCAN used to evaluate accumulation of hypodiploid DNA content only in the fraction positive for GFP fluorescence. TAM67 expression in the GFP sorted population was at least as high as endogenous c-jun (Fig 5.3.3A). The level of induction of apoptosis SDG in TAM67-expressing cells was reduced by about 40% ($p=0.003$ and 0.006 for 5μM and 7μM SDG, respectively) compared to cells transfected with vector alone (Fig 5.3.3B). The protection from pXSC, however, was only about 20% though this was statistically significant ($p=0.003$ for both 35μM and 50μM).

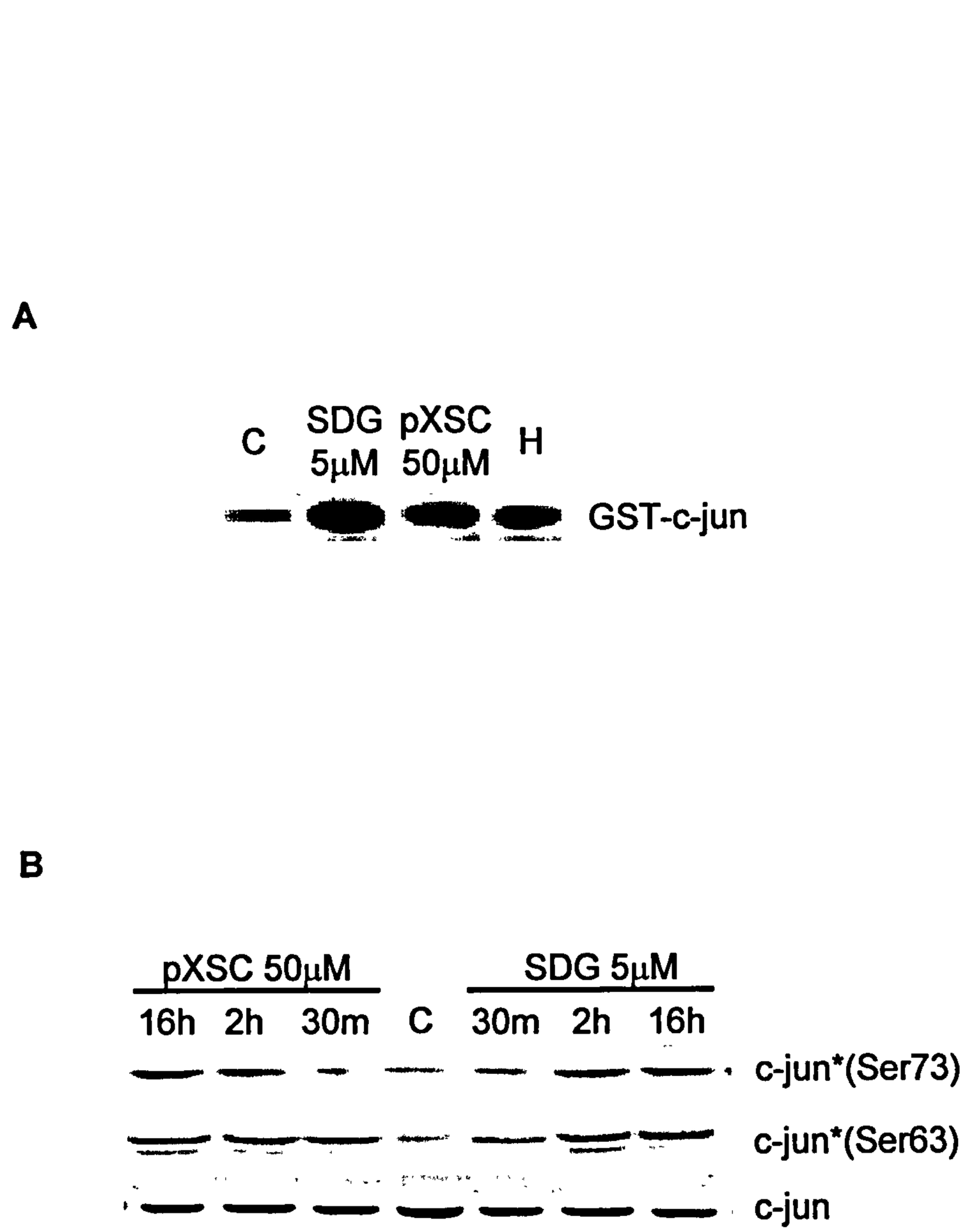
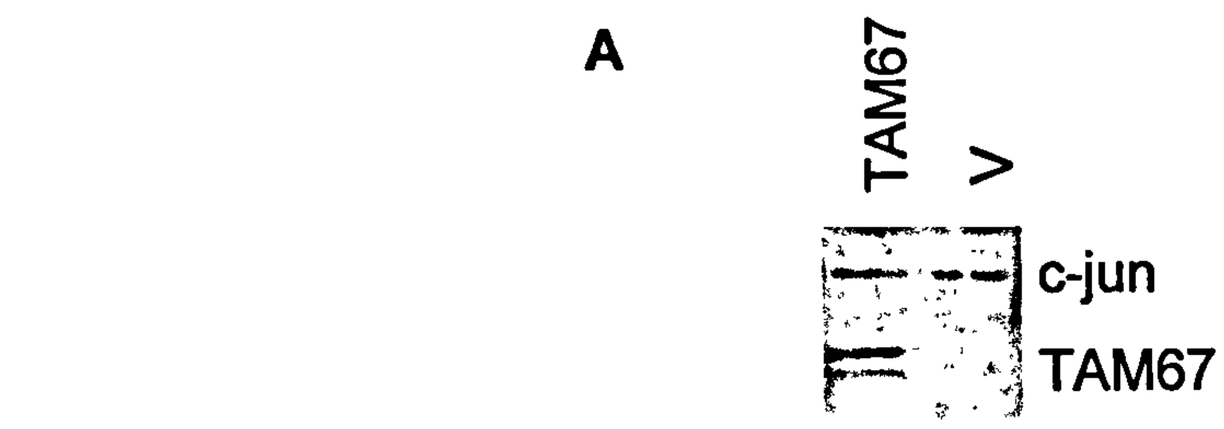
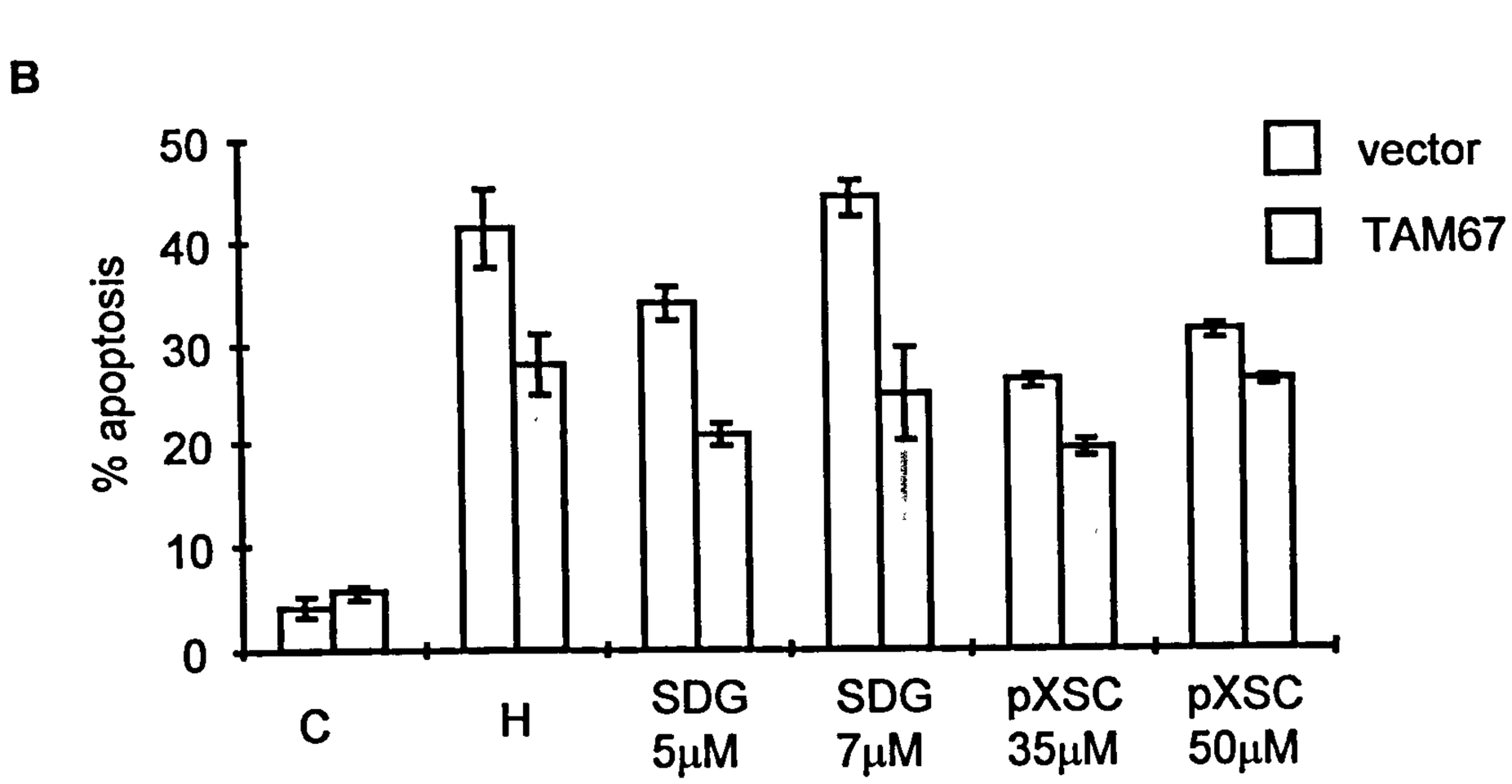


Fig 5.3.2 Activation of c-jun by SDG and pXSC.

(A) Immnunecomplex kinase assay of JNK activity was performed on HeLa cell lysates that were treated for 30min with indicated concentrations of selenium compounds, using a GST fused N-terminal fragment of c-jun as substrate. H, represents a positive control of 2mM H_2O_2 treatment for 30min **(B)** Activation of c-jun by selenium treatment was assessed by western blot analysis of cell lysates derived from HeLa cells treated with SDG and pXSC as indicated. Antibodies against phosphorylated forms of c-jun at serines 63 (c-jun*(Ser63)) and 73 (c-jun*(Ser73)) were used to measure c-jun activation. As loading controls, the blots were then stripped and western blotted with antibodies against total c-jun. C, in all cases, represents treatment with solvent vehicle, DMSO.





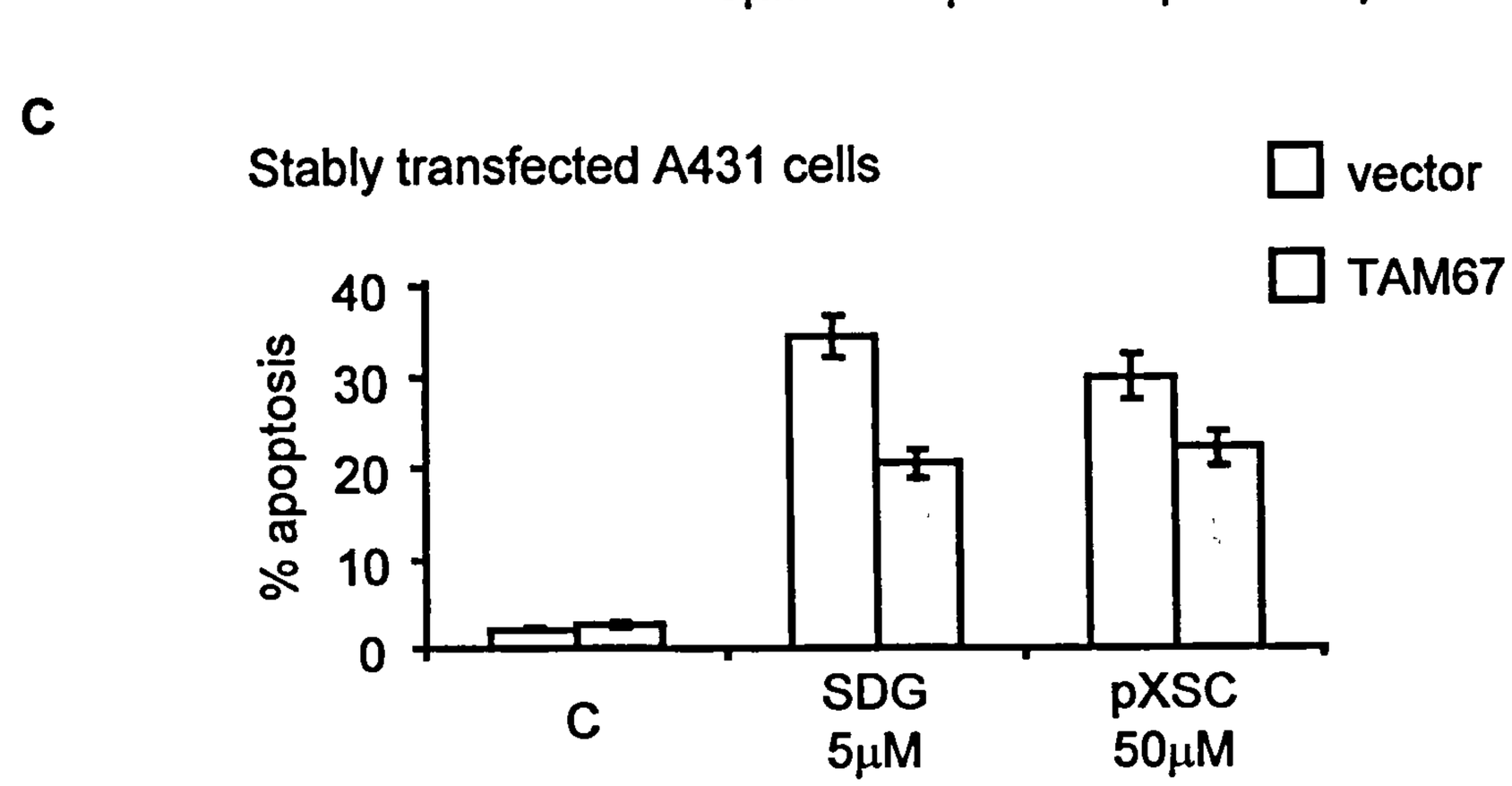


Fig 5.3.3 Effect of overexpression of a c-jun dominant negative, TAM67, on SDG- and pXSC-induced apoptosis.

(A) HeLa cells transiently co-transfected with GFP and TAM67 or an empty vector were sorted using FACS to enrich the GFP positive cells. Western blotting using a total c-jun antibody was carried out to analyse TAM67 expression between empty vector transfected (V) and TAM67 transfected cells. **(B)** Levels of apoptosis of HeLa cells transiently co-transfected with GFP and TAM67 or an empty vector were assessed after selenium treatment for 16h by measuring the accumulation of hypodiploid DNA within the GFP positive fraction of cells. H, represents a positive control treatment with 2mM H_2O_2. Each treatment was performed in triplicate. Data presented is representative of 3 independent experiments. **(C)** A431 cells stably transfected with TAM67 or the 'empty' expression vector were treated with SDG and pXSC as indicated, for 16h, and levels of apoptosis measured by TUNEL assay. Error bars, in all cases, indicate standard errors. C, in all cases, represents treatment with solvent vehicle, DMSO.

To confirm these findings from transient transfection studies described above, we tested cell death induction in A431 cells stably expressing TAM67 or an empty vector (kindly provided by Dr G. Stapleton, Beatson Institute, UK). The expression of TAM67 and its functional attenuation of c-jun activity in these cells have been previously described (Malliri et al., 1998) and was confirmed by Dr G. Stapleton (Beatson Institute, UK) for the particular batch of cells used in our study. Studies with A431 cells stably expressing TAM67 confirmed the conclusions from the transient transfection studies: compared to empty vector transfected cells, stable expression of TAM67 showed an attenuated response to SDG and pXSC induced apoptosis (Fig 5.3.3C). Again the protection from pXSC (25% reduction, p=0.04) was less than that from SDG-induced cell death (41% reduction, p=0.001).

Disruption of c-jun function results in cells cycling considerably more slowly (Schreiber et al., 1999). It is conceivable that the reduction in apoptosis observed could be due to this non-specific effect in TAM67 expressing cells. However, other experiments showed that this was not the case for transient overexpression of TAM67 in HeLa cells, since the incorporation of tritiated thymidine, prior to selenium treatment, was comparable for both TAM67 and empty vector transfected cells (data not shown).

As TAM67 completely disrupts c-jun function, it is possible that use of this strategy might disrupt other non-JNK mediated activities of c-jun and therefore the protection against selenium-induced apoptosis observed might not be specific to the disruption of JNK activity. To address this issue similar transient overexpression experiments were performed using a HA-tagged c-jun construct whose serine residues at positions 63 and 73 (sites on c-jun specifically phosphorylated by JNK) had been altered to alanine, c-jun S63/73A (a kind gift from Dr M. Karin, University of California, San Diego, USA). The expression of the mutant protein was confirmed using antibodies against the HA epitope tag (Fig 5.3.4A). Comparing levels of apoptosis, between cells expressing c-jun S63/73A and empty vector, attenuation of cell death mediated by SDG (45% reduction, p=0.0001) and pXSC (18% reduction, p=0.04) was found due to c-jun S63/73A overexpression (Fig 5.3.4B).

To test if there was any co-operative effect between JNK and p38 activation, HeLa cells transiently transfected with TAM67 were treated with the p38 inhibitor SB203580 (Fig 5.3.5). This study revealed no enhanced protection by p38 and JNK inhibition from cell death induction compared to TAM67 expression on its own.

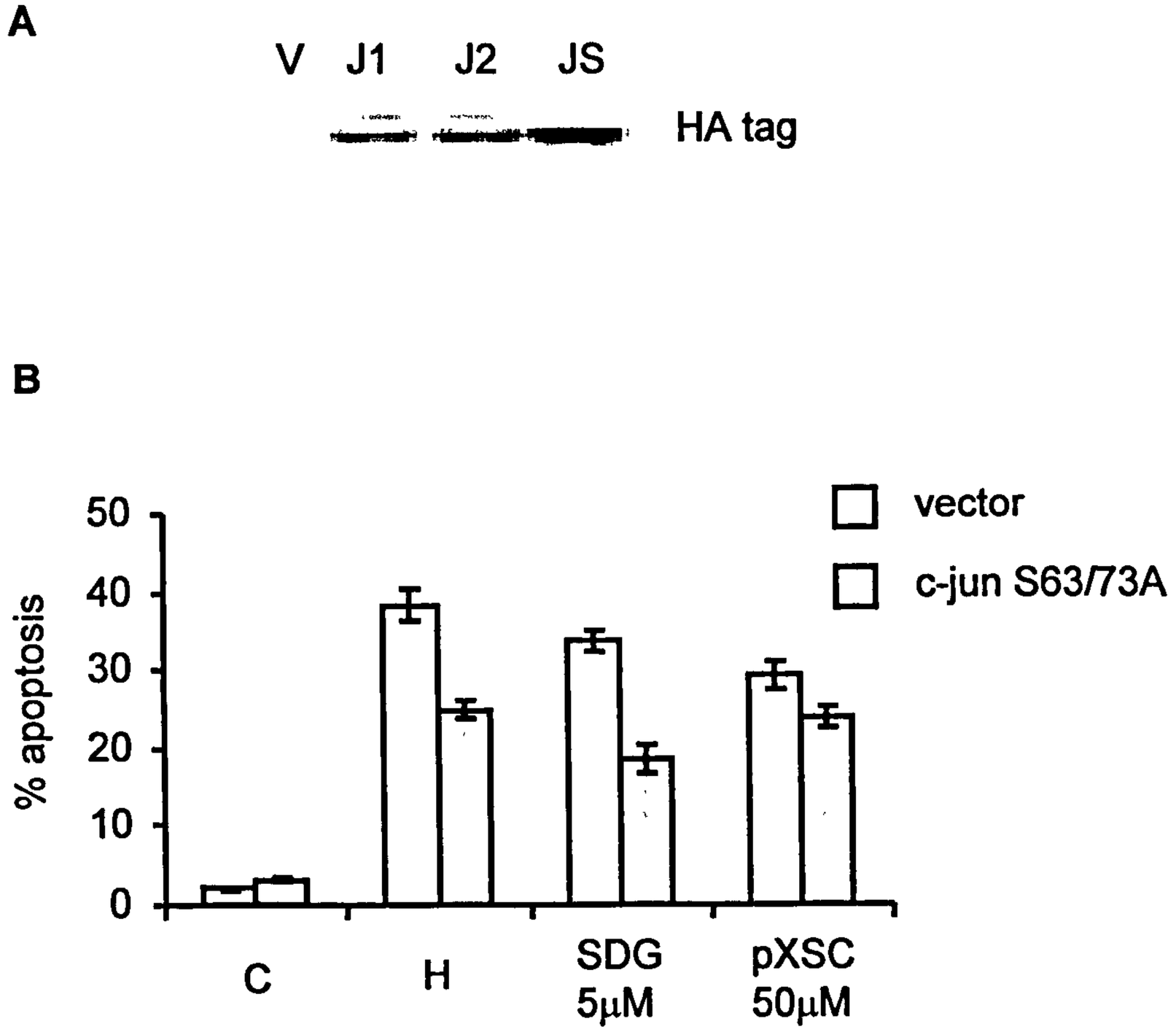


Fig 5.3.4 Effect of overexpressing a phosphorylation defective mutant, c-jun S63/73A, on SDG- and pXSC- induced apoptosis.

(A) HeLa cells transiently co-transfected with GFP and HA-tagged c-jun S63/73A or an empty vector were sorted using FACS to enrich the GFP positive cells. Western blotting using an anti-HA tag antibody was carried out to compare c-jun S63/73A expression between empty vector transfected (V) and c-jun S63/73A transfected cells. J1 and J2 are two pools of c-jun S63/73A transfected cells prior to FACS sorting. JS, is c-jun S63/73A transfected cells after FACS enrichment. **(B)** Levels of apoptosis of HeLa cells transiently co-transfected with GFP and c-jun S63/73A or an empty vector were assessed after selenium treatment for 16h by measuring the accumulation of hypodiploid DNA within the GFP positive fraction of cells. H, represents a positive control treatment with 2mM H_2O_2. Each treatment was performed in triplicate. Data presented is representative of 2 independent experiments. Error bars indicate standard errors. C, represents treatment with solvent vehicle, DMSO.

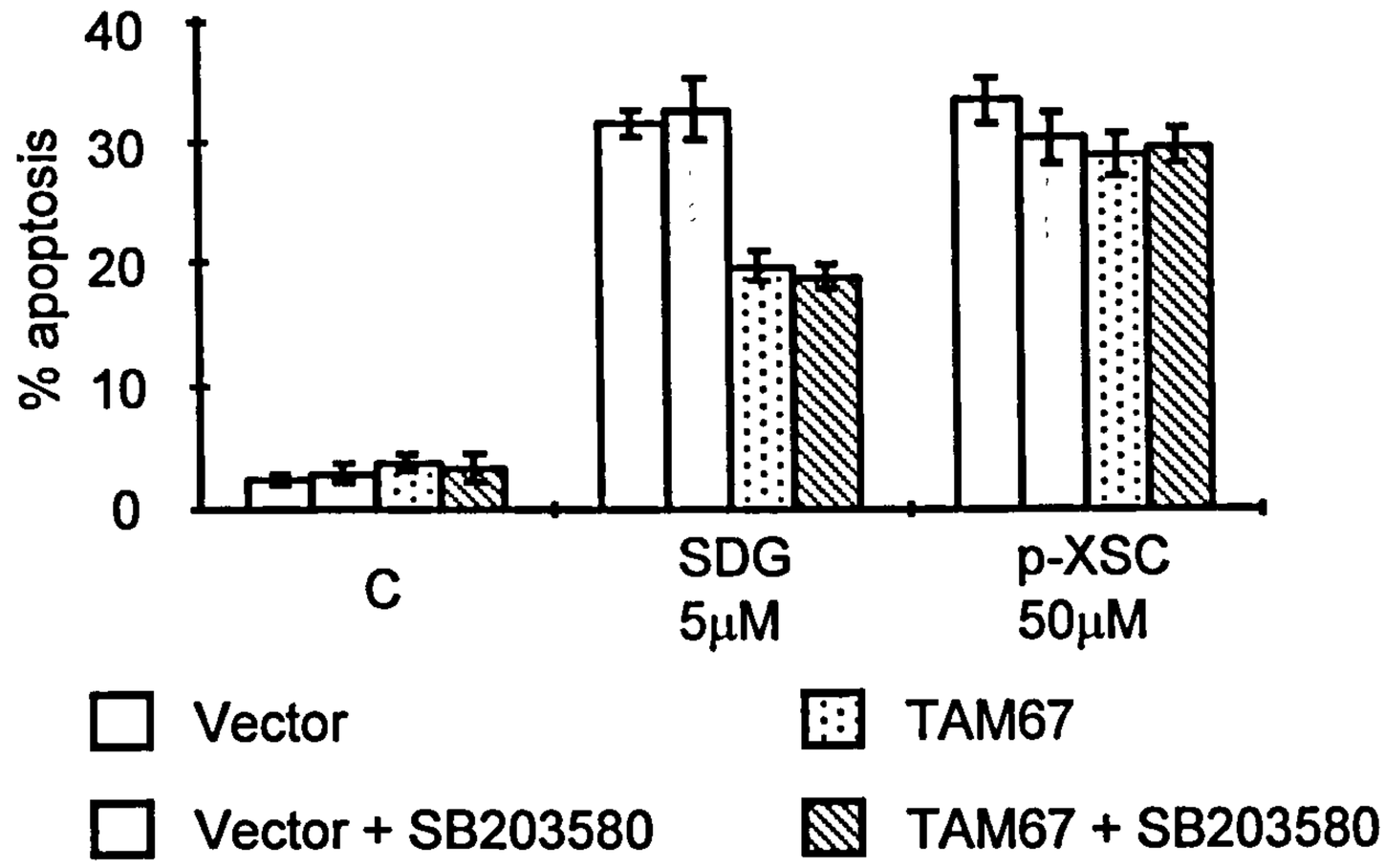


Fig 5.3.5 Effect of simultaneous abrogation of p38 kinase and c-jun activity on cell death induced by selenium compounds.

HeLa cells transiently co-transfected with GFP and TAM67 or an empty vector were treated with SDG and pXSC in the presence or absence of SB203580 (at 10μM, added 30min prior to selenium treatment – see Fig 5.2.2A). Extent of apoptosis of all combinations of transfections and inhibitor was assessed by measuring the accumulation of hypodiploid DNA within the GFP positive fraction of cells. Each treatment was performed in triplicate. Data presented is representative of 2 independent experiments. Error bars indicate standard errors. C, represents treatment with solvent vehicle, DMSO.

This data indicates that activation of JNK by the selenium compounds is a functionally-required pro-apoptotic signalling pathway acting through c-jun. This in turn implies that, at least partially, selenium induced apoptosis requires new gene expression. One candidate is clearly Fasl in view of the data already noted in Section 4.5.2: this hypothesis is explored in the next section. However, the relative contribution of the JNK pathway to SDG and pXSC induced apoptosis appears to be different. It is clear from these experiments that mechanisms apart from the JNK/c-jun pathway are involved particularly in the case of pXSC. Whether these are entirely JNK independent mechanisms or they involve other JNK functions remain unclear. The use of the recently developed JNK1-/-, JNK2 -/- double knockout cells might reveal the complete spectrum of JNK functions in selenium-induced apoptosis.

The most plausible role of JNK in mediating apoptosis, apart from the c-jun mediated pathway, is to influence mitochondrial cytochrome c release. Evidence for JNK phosphorylating and inactivating antiapoptotic functions of Bcl2 and $Bclx_L$ in certain contexts have already been described (Deng et al., 2001; Fan et al., 2000). It would be very illuminating to study JNK mediated post-translational modifications of the Bcl2 family of proteins in response to selenium – but with currently available reagents the endogenous activities of these proteins cannot be studied.

5.4 JNK regulates the induction of Fas ligand

The induction of Fasl in response to various stresses and alkylating agents has been shown to be dependent on c-jun transcriptional activity. Given that phosphorylation of c-jun at serines 63 and 73 by JNK increases its transcriptional activity, it is possible that JNK activity is required for Fasl induction. As Fasl is strongly induced by SDG and pXSC and attenuation of Fas signalling resulted in partial protection from apoptosis (about the same extent as that obtained by disrupting JNK mediated c-jun activation; Fig 4.5.2), this hypothesis was tested directly by evaluating whether disruption of c-jun activity affected selenium induced FasL expression.

Cells transfected with either TAM67 or an empty vector were treated with selenium and the levels of Fasl induction examined (Fig 5.4.1A). The transfection efficiency of vector only and TAM67 expressing cells were comparable (85% and 83%, respectively) as judged by GFP co-transfection. TAM67 expression was found to reduce the induction of FasL by SDG (by ~5 fold), but not that by pXSC. This perhaps reflects the lower extent of

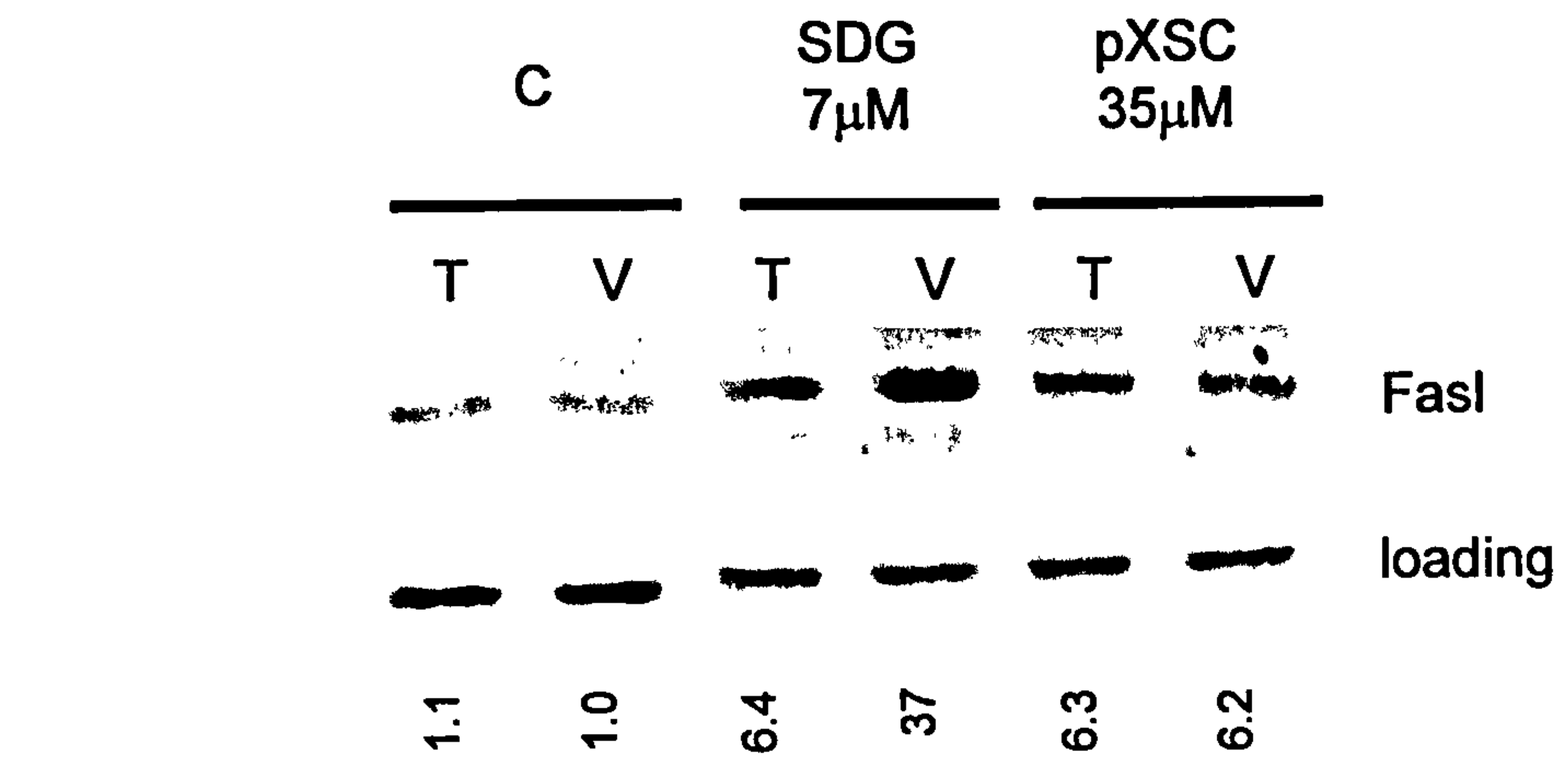


Fig 5.4.1 Effect of c-jun and p38 activation on FasI induction by SDG and pXSC.

(A) Vector (V)- or TAM67 (T)-transfected HeLa cells, co-transfected with a GFP-expression vector, were treated with the indicated concentrations of SDG or pXSC for 18h and then analysed for FasI expression using western blotting. The loading control is total p38 kinase. The percentages of transfected cells, determined from the percentage of GFP-expressing cells, were 85% and 83% for vector and TAM67 transfections, respectively. **(B)** HeLa cells were treated with the indicated concentrations of SDG and pXSC in the presence or absence of SB203580 (at 10μM, added 30min prior to selenium treatment), cell lysates prepared and subjected to western blotting using anti-FasI and total p38 kinase (for loading) antibodies. C, represents treatment with solvent vehicle, DMSO. The figures below the lanes indicate the fold increase in FasI expression above control level after selenium treatment, normalised to the loading.

protection from pXSC-induced apoptosis by TAM67 and dnFADD (see Sections 5.3.3 and 4.5.2) overexpression compared to SDG-induced apoptosis.

The role of p38 kinase activation in the induction of Fasl was tested by pre-treating cells with SB203580 prior to addition of the selenium compounds. No differences in the levels of Fasl induction resulted from inhibiting p38 kinase activity (Fig 5.4.1B).

This set of data therefore implicate the JNK pathway to be responsible for the induction of Fasl (via c-jun transcriptional activity) which, at least partially, contributes to the induction of apoptosis by chemopreventive selenium compounds.

5.5 ERKs 1/2 in selenium-induced programmed cell death

Historically, ERKs 1 and 2 have been considered to be the major mediators of cellular proliferation (Cross et al., 2000; English et al., 1999). However, in the recent years ERKs have also been found to be actively involved in apoptosis inhibition (Cross et al., 2000; Dhanasekaran, 1998). This view is rapidly gaining momentum particularly with the recent Raf (the upstream activator of ERKs) knockout studies where greatly enhanced apoptotic sensitivity has been noted (Huser et al., 2001; Mikula et al., 2001; Wojnowski et al., 1997). ERK activity has often been found to be inhibited in cells undergoing apoptosis on exposure to a range of cytotoxic stimuli (Cross et al., 2000). Conversely, forced activation of ERK inhibits apoptosis in response to a wide range of stimuli including oxidative stress and chemotherapeutic drugs (Cross et al., 2000).

5.5.1 ERKs 1/2 activities are altered on selenium exposure

In order to determine whether ERK 1/2 activities were altered on selenium treatment, HeLa cells were serum starved overnight (to reduce basal levels of ERK activity) and then treated with SDG and pXSC at concentrations that induce apoptosis. Both the compounds resulted in the activation ERKs 1 and 2 after treatment for 2h as measured by Western blotting using phospho-specific antibodies (Fig 5.5.1A). The activation by SDG was stronger than that by pXSC under these conditions.

In order to see if these compounds prevented the activation of ERKs 1 and 2 by mitogenic stimuli, as would be the case in the tumour environment, serum-starved cells were pre-treated with selenium prior to the addition of epidermal growth factor (EGF). Compared to

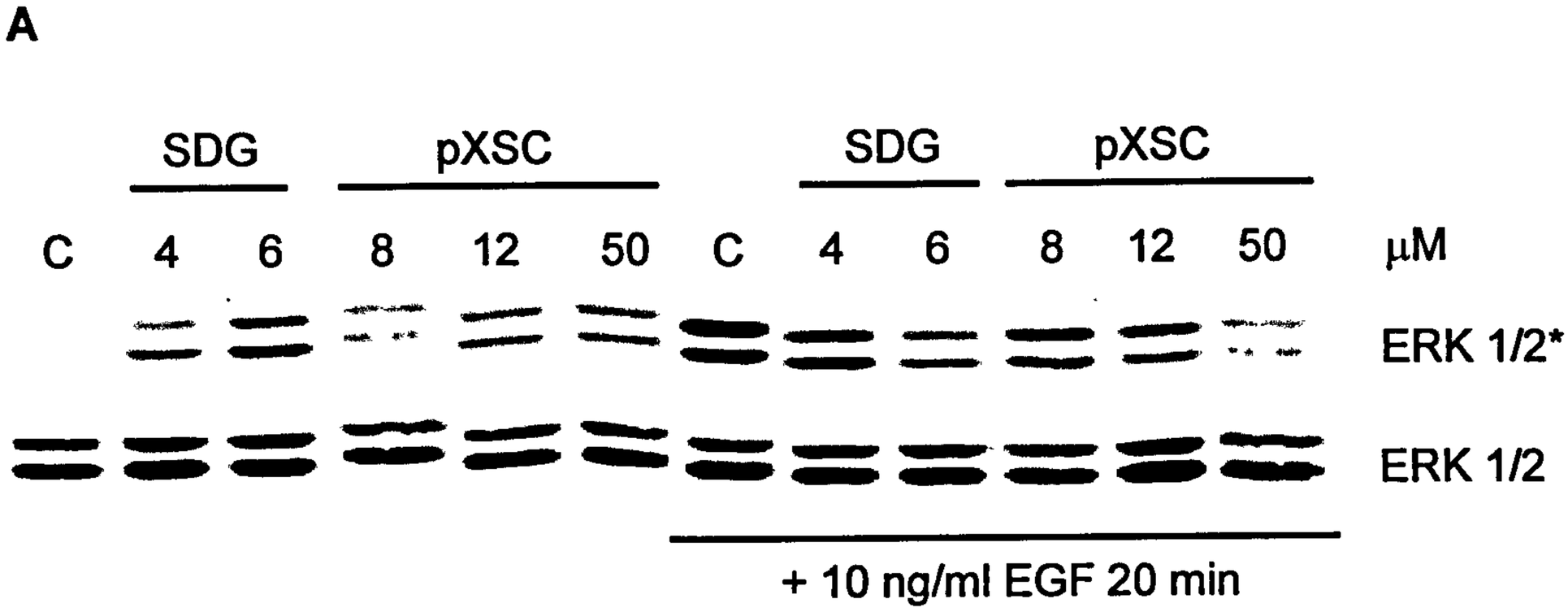


Fig 5.5.1 Effect of selenium compounds on ERK1/2 activity.

(A) HeLa cells were serum-starved for 16h and then treated with the indicated concentrations of SDG or pXSC for 2h, in some cases followed by 10ng/ml EGF treatment for 20min. Cell lysates were prepared and run on SDS/PAGE gels and Western blotted using antibodies that recognise total ERK1/2 or only the activated form (ERK1/2*). **(B)** HeLa cells were serum-starved for 16h and then treated as indicated. ERK1/2 activities were measured as in (A).

EGF only control, treatment with SDG and pXSC showed a dose dependent inhibition of ERK activation by EGF (Fig 5.5.1A).

Treatment of HeLa cells by SDG and pXSC for longer time periods showed a substantial degree of ERK 1/2 activity which is maintained over the entire period of the treatment (Fig 5.5.1B). Again SDG seemed to be a more potent inducer of ERK activity than pXSC though not to the extent as seen during treatments for shorter times.

Thus a complex scenario of ERKs 1/2 activity modulation by selenium compounds is emerging. On one hand apoptosis-inducing doses induce activation but, on the other, selenium compounds retard activation by mitogens like EGF. Changes in ERK activity in the continuous presence of mitogens, over a time period that induces substantial apoptosis, could not be resolved as prolonged exposure to mitogens (like measuring ERK activity in cells growing in the presence of serum) led to an overwhelming activation that did not allow quantitative discrimination between the activity levels to be made.

5.5.2 Complete inhibition of ERKs 1/2 has only a small effect on selenium-induced apoptosis

In order to investigate the functional relevance of modulation of ERK 1/2 activity to selenium-induced apoptosis the effect of complete inhibition of ERK activity on apoptotic signalling was investigated. If complete inhibition resulted in decreased apoptosis then this would indicate that ERK activation was pro-apoptotic, whilst increased apoptosis would implicate a protective function.

The well characterised MEK inhibitor, PD98059, when added prior to selenium treament completely abolished ERK 1/2 activation by both SDG and pXSC (Fig 5.5.2A). This was associated with slight sensitisation (~ 20%) of HeLa cells to selenium-induced apoptosis (Fig 5.5.2B). This difference was only statistically significant for some of the concentrations of the selenium compounds used ($p=0.039$ and 0.041 for SDG 5μM and pXSC 35μM, respectively). The other doses, though showing the same trend, were not significant ($p=0.054$ and 0.05 for SDG 3.5μM and pXSC 50μM, respectively). This suggests the ERK activation may be a survival response, albeit an ineffective one. Given the small difference in sensitivity, further complicated by statistical variation over concentration ranges, the biological significance of the phenomenon remains unclear.

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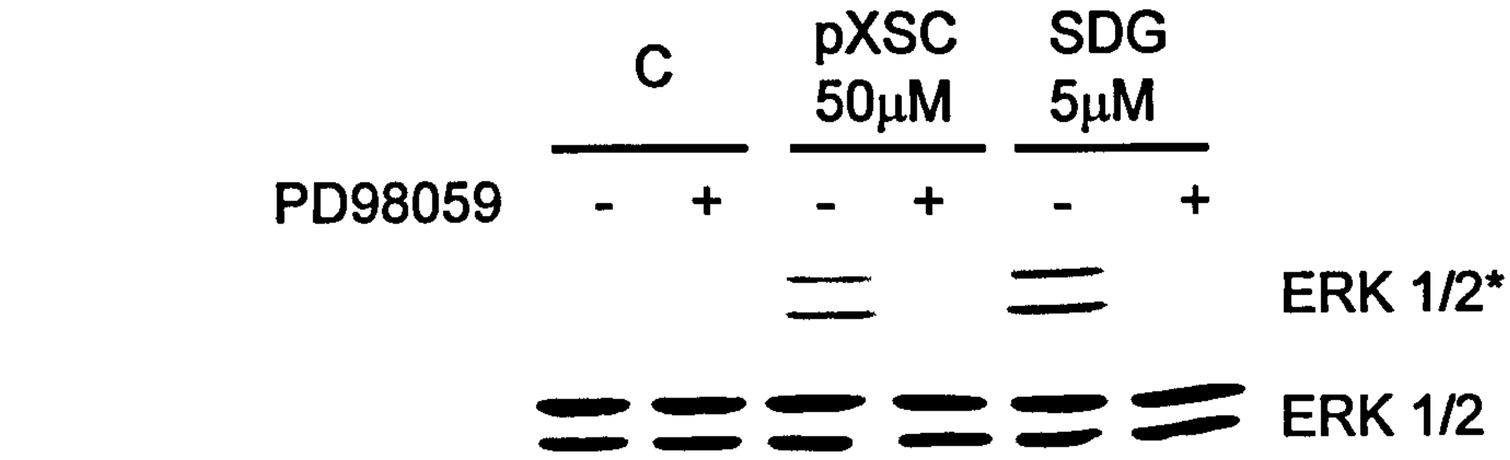

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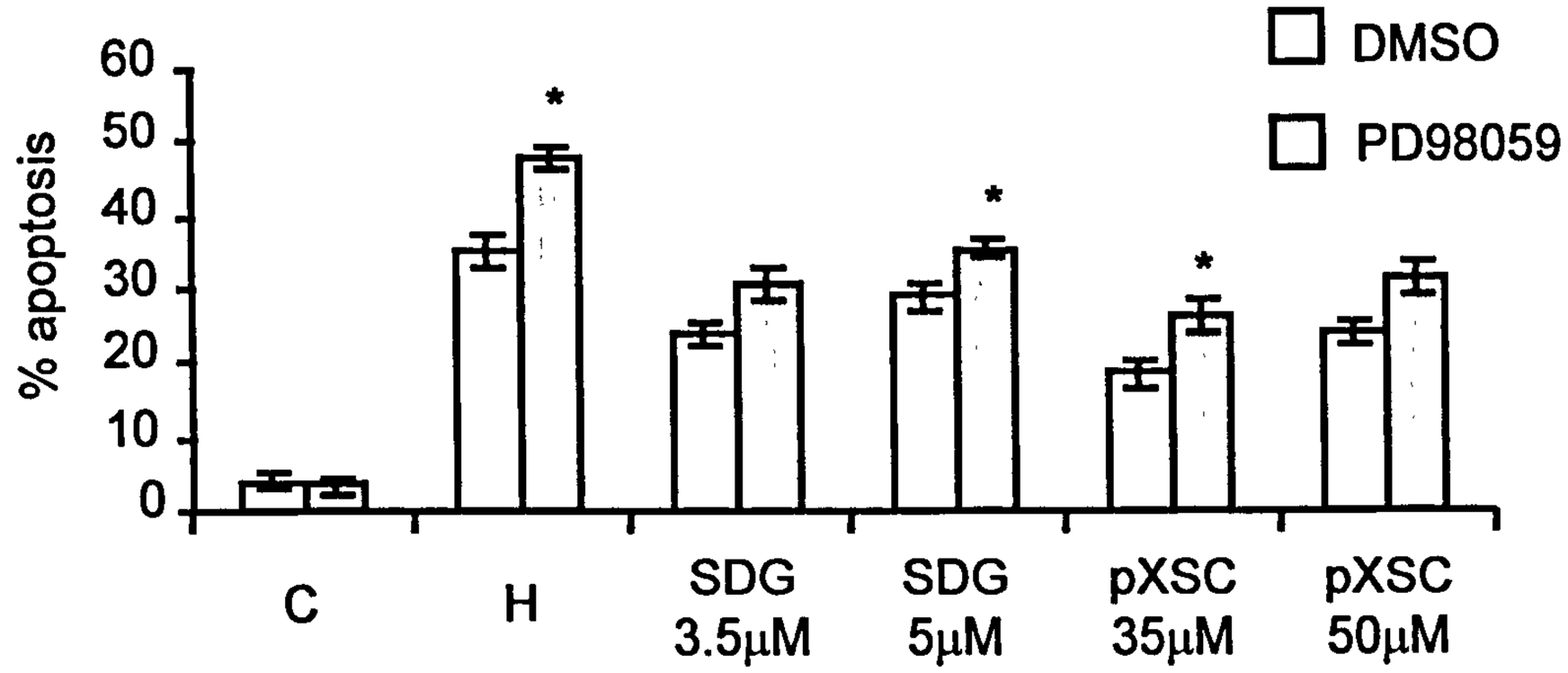

Fig 5.5.2 Effect of inhibition of ERK1/2 activity on apoptosis induction by SDG and pXSC.

(A) Serum-starved HeLa cells were pre-treated with 50μM PD98059 for 30min and then treated with indicated concentrations of SDG and pXSC for 16h. The effect on activation of ERK1/2 was determined by western blotting using antibodies that recognise phospho-specific (ERK1/2*) or total ERK. **(B)** Induction of apoptosis following treatment with indicated concentrations of selenium compounds for 16h, in the presence or absence of the ERK inhibitor, was measured by TUNEL assay. Each treatment was performed in triplicate. Data presented is representative of 3 independent experiments. Error bars indicate standard errors. H, represents a positive control treatment with 200mM H_2O_2 for 16h. C, in all cases, represents treatment with solvent vehicle, DMSO.

Thus, while ERK 1/2 activation may be involved in anti-apoptotic survival signalling in response to selenium compounds – its contribution is relatively small. It is possible that the overall effect of selenium is inhibition of ERK activity which could not be directly assessed in our earlier experiments. Therefore, forcible activation of the ERK pathway, using constitutively activated constructs of upstream activators, may reveal a functional role. However, the overall inhibition, if any, can only be partial as no differences in ERK activity can be seen on treating cells growing in serum with selenium. If inhibition of ERK activity by selenium compounds were functionally significant, the complete abolition of ERK activity by PD98059 would have been expected to reveal a strong sensitisation, to apoptosis unlike the small trend observed. Thus it is likely that the overall effect of selenium treatment, in the presence of continued mitogenic stimulation, is not a functionally relevant inhibition of ERK activity.

The mechanisms mediating the anti-apoptotic function of ERK activity is unclear. There is evidence that caspase 3 activation may cleave and inactivate Raf-1, and hence block ERK activation (Widmann et al., 1998). ERK has also been shown to inhibit caspase activation following cytochrome c release possibly by a phosphorylation dependent mechanism (Erhardt et al., 1999). All these possibilities could be addressed by standard caspase assays used in conjunction with specific caspase inhibitors. The activities of the Bcl2 family members may also be regulated post-translationally by the ERK pathway (Scheid and Duronio, 1998). However, in view of the small functional contribution of ERK 1/2 activation on apoptosis induction by chemopreventive selenium compounds such studies were considered to be of lower priority.

5.6 Big Mitogen Kinase 1 (or ERK5) in SDG and pXSC mediated cell death

Recently, a new member of the MAPK family, named the Big Mitogen Kinase 1 (BMK1) or ERK5, has been described (Han et al., 1996; Lee et al., 1995). Apart from being activated by mitogens, like EGF (Kato et al., 1997; Kato et al., 1998), it is also redox sensitive and induced by oxidants (Abe et al., 1996). The role of ERK5 in cellular proliferation has been particularly well characterised in HeLa cells where it appears to be the major pathway mediating EGF induced proliferation (Kato et al., 1998). Though no direct role of ERK5 in anti-apoptotic signalling has been elucidated and its downstream targets remain elusive, it could potentially be an important regulator of apoptosis.

5.6.1 Selenium compounds inhibit BMK1 (or ERK5) activity

Phosphorylation (and thereby activation) of ERK5 results in a shift in electrophoretic mobility detectable by western blotting. This shifted fraction of phosphorylated ERK5 is consistently correlated to its activity as measured by kinase assays (Kato et al., 1998). However, by this assay, serum-starved cells treated with SDG and pXSC showed no increase in ERK5 (Fig 5.6.1A). An EGF treated control showed a reproducible shift in ERK5 mobility indicating activation (Fig 5.6.1A).

To investigate if selenium compounds affected mitogen mediated activation of ERK5, serum-starved cells were pre-treated with SDG and pXSC prior to stimulation with EGF. Compared to the EGF control, selenium-treatment caused an acute dose-dependent inhibition of ERK5 activation by EGF (Fig 5.6.1A). This inhibition of EGF stimulation was confirmed by an endogenous ERK5 'pull down' kinase assay using myelin basic protein (MBP) as a substrate (Fig 5.6.1B).

Similar experiments also tested whether the selenium compounds could inhibit ERK5 activity in the continued presence of mitogens. HeLa cells grown in 10% foetal bovine serum (FBS) were treated with apoptosis-inducing doses of SDG and pXSC. This revealed that, even with persistent mitogenic stimulation, selenium compounds still attenuated ERK5 activity (Fig 5.6.1C).

These studies therefore demonstrated that both pXSC and SDG potently inhibit ERK5 activity at doses that induce substantial apoptosis. This raises the intriguing possibility of ERK5 inhibition being a functional component of selenium initiated pro-apoptotic signalling.

5.6.2 Constitutive activation of ERK5 protects from selenium-induced apoptosis

The MAPK kinase MEK5 is the immediately upstream activator of ERK5. A constitutively activated mutant of MEK5, MEK5(D), has been generated by substituting the conserved phosphorylation sites, serine 313 and threonine 317, with aspartate residues (Kato et al., 1997). A mutant ERK5, ERK5(AEF), with its tyrosine and threonine phosphorylation sites mutated to alanine and phenylalanine has also been developed. These were made available to us by a kind gift from Dr J-D. Lee (Scripps Research Institute, USA). We used the ERK5(AEF) expression construct along with wild type ERK5 expression constructs, in

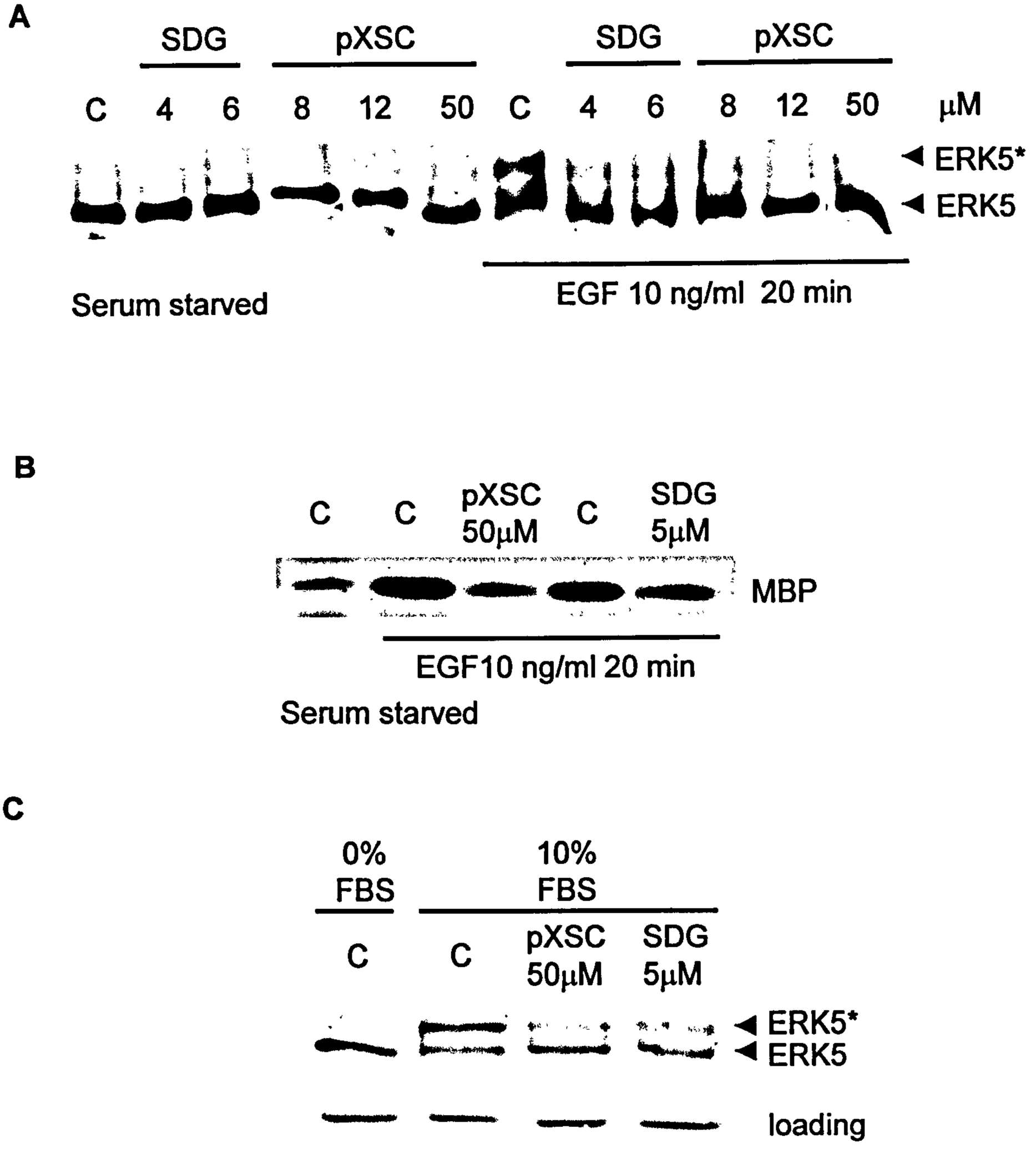

Fig 5.6.1 Inhibition of ERK5 activity by chemopreventive selenium compounds.

(A) HeLa cells were serum starved for 16h and then treated with the indicated concentrations of SDG or pXSC for 2h, in some cases followed by 10ng/ml EGF treatment for 20min. Cell lysates were prepared and run on a 6% SDS/PAGE gel (see Section 3.2.5.1). Activation of ERK5 was measured by its change in migration detected by Western blotting with anti ERK5 antibody. **(B)** Serum starved cells were treated as in (A) and an immunecomplex kinase assay using MBP as a substrate performed to measure ERK5 kinase activity. **(C)** HeLa cells growing in 10% FBS were treated with indicated concentrations of SDG and pXSC for 16h and western blot analysis performed as before to compare ERK5 activities. Serum-starved cells were also analysed as a control. The level of total p38 was used as the loading control. C, in all cases, represents treatment with solvent vehicle, DMSO.

transient transfection studies where ERK5 activity was persistently activated by overexpressing MEK5(D). Co-expression with GFP constructs, as described earlier, was used to identify transfected cells.

The expression of these proteins after transfection was confirmed by western blotting using antibodies against their specific tags (HA-tag for MEK5(D) and Flag-tag for the two ERK5 constructs) (Fig 5.6.2A). The specificity of persistent ERK5 activation by the constitutively activated MEK5 construct was addressed by immunecomplex kinase assays for ERK5 activity (Fig 5.6.2B). Only the combination of MEK5(D) and wild type ERK5 showed substantial activity.

FACS analysis of accumulation of subG0/G1 DNA content within the GFP positive population revealed that constitutive activation of ERK5 resulted in a small (~ 20%) but reproducible protection from SDG and pXSC induced apoptosis (Fig 5.6.2C). This protection was consistent over different doses and was statistically significant ($p=0.03$ and 0.04 for SDG 5μM and 7μM, respectively and $p=0.04$ for both 35μM and 50μM pXSC). The levels of apoptosis in the ERK5(AEF) mutant expressing cells showed no consistent or statistically significant differences.

This very specific, albeit small, protection of constitutive activation of ERK5 suggests that inactivation of ERK5 by chemopreventive selenium compounds may be of biological relevance.

It is possible the protection observed is an artefact of alterations in proliferation potential as thymidine incorporation levels are enhanced on constitutive ERK5 activation. However, the *rate* of inhibition of DNA synthesis did not vary between empty vector and MEK5(D) + ERK5(AEF) transfected cells (data not shown). Furthermore, in general, rapidly cycling cells show enhanced sensitivity to apoptosis compared to slowly cycling cells, whereas, in these experiments the converse is the case.

Given the dearth of knowledge of downstream ERK5 targets, how ERK5 may mediate anti-apoptotic signalling is unclear. However, transcription factors, MEF2C and myc (English et al., 1998; Kato et al., 2000), are known downstream targets of ERK5 and have been implicated in mediating cell survival/apoptosis. Suppression of activities of these proteins by ERK5 inhibition may therefore constitute a survival signal of some biological significance. These would perhaps therefore be reasonable starting points to further dissect ERK5's role in anti-apoptotic signal transduction.

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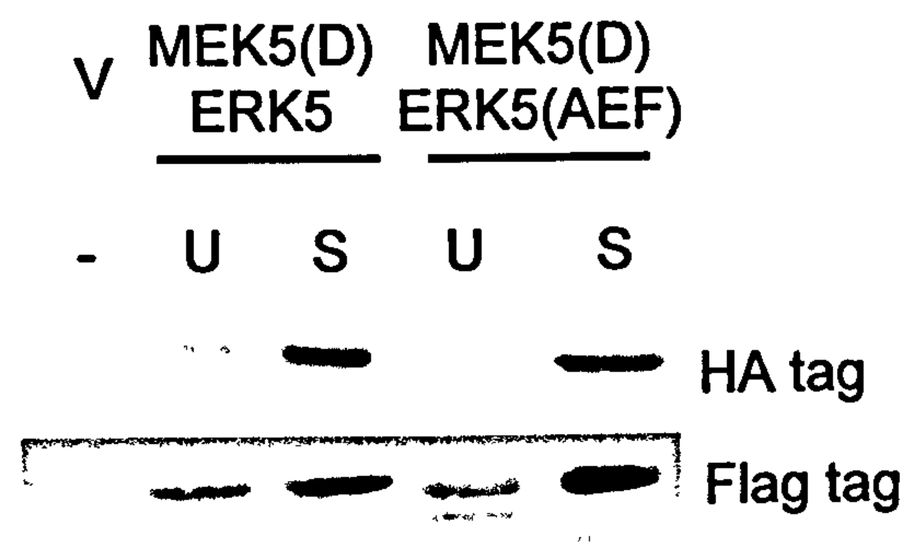

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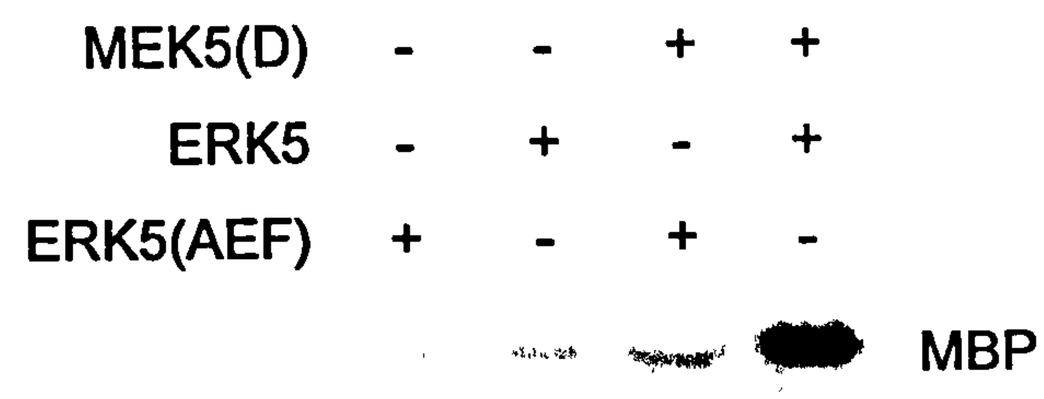

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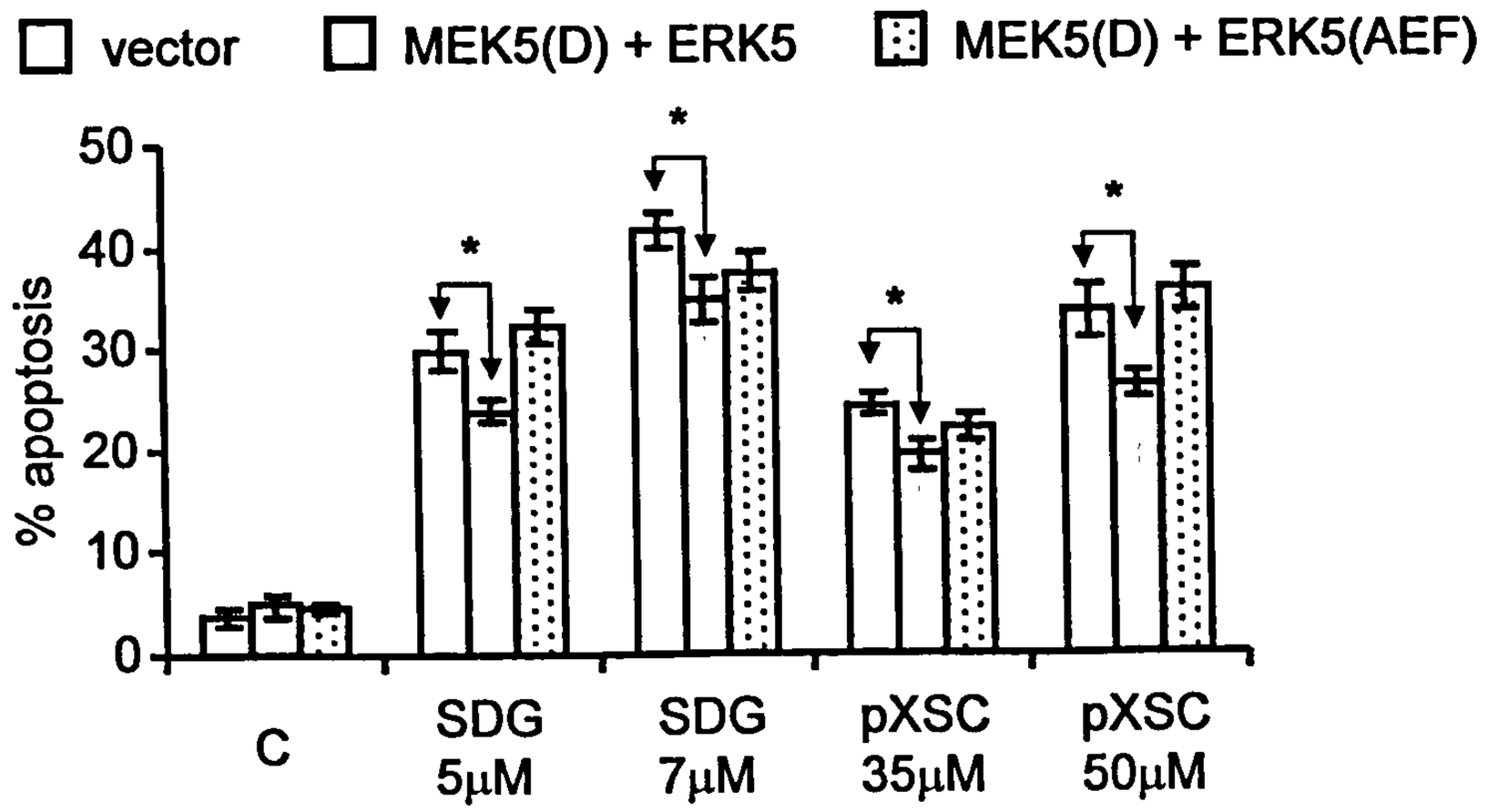

Fig 5.6.2 Effect of constitutive activation of ERK5 on selenium-induced apoptosis.

(A) HeLa cells were transiently co-transfected with GFP and HA-tagged MEK5(D) or an empty vector together with either Flag-tagged ERK5 or Flag-tagged ERK5(AEF). Transfected cells were sorted using FACS to enrich the GFP positive cells. Western blotting using anti-HA tag or anti-Flag tag antibody was carried out to analyse MEK5(D), ERK5 and ERK5(AEF) expression between empty vector transfected (V) and cells transfected with the various constructs. U is transfected cells prior to FACS sorting. S, transfected cells after FACS enrichment. MEK5(D) is a constitutively activated mutant, ERK5 is wild type and ERK5(AEF) is a phosphorylation defective mutant. **(B)** ERK5 kinase assay using MBP as a substrate was carried out on cells expressing combinations of the various constructs. **(C)** Measurement of apoptosis induction was performed by analysing the subG0/G1 DNA content in the GFP positive fraction of cells. Each treatment was performed in triplicate. Data presented is representative of 2 independent experiments. Error bars indicate standard errors. *, indicate significance, $p<0.05$. C, represents treatment with solvent vehicle, DMSO.

5.7 Role of Akt in selenium mediated programmed cell death

Protein kinase B (PKB)/Akt is a serine threonine kinase (not of the MAPK family) whose activity is very closely linked to enhanced cell survival (Downward, 1998). Akt is activated downstream of phosphoinositide 3-kinase (PI3K) in signalling cascades effected by a wide range of receptors, including those for growth and survival factors (Alessi and Cohen, 1998). The downstream actions of PKB appear to include phosphorylation of proteins involved in the apoptotic cascade (like Bad) (del Peso et al., 1997) and regulation of expression of apoptotic proteins (by inhibiting transcription factors like forkhead (Brunet et al., 1999) and/or activating others like NF-κB (Romashkova and Makarov, 1999)). Thus Akt is key mediator of cell survival and it comes as no surprise that it can be cleaved and inactivated by caspase 3 during apoptosis (Widmann et al., 1998).

Several members of the PI3K-Akt signalling pathway has been shown to upregulated in a variety of human cancers, and this is correlated to enhanced cellular survival (Cantley and Neel, 1999; Shayesteh et al., 1999; Staal, 1987). Several apoptotic stimuli inhibit Akt activity, whose overexpression leads to protection (Cross et al., 2000). Other apoptotic insults, like H_2O_2, activate Akt as a survival response and its inhibition leads to enhanced sensitivity (Shaw et al., 1998).

5.7.1 Enhanced Akt activity on selenium exposure

To investigate the role of Akt in selenium-induced apoptosis HeLa cells were serum-starved overnight to reduce Akt activity to basal levels, followed by treatment with chemopreventive selenium compounds. Treatment with both SDG and pXSC increased the phosphorylated fraction of Akt whereas total Akt remained constant (Fig 5.7.1A, B). This implicated a dose-dependent activation of Akt, particularly on pXSC exposure. SDG only weakly activated Akt.

To assess if Akt activation by mitogens was reduced by the selenium compounds, serum-starved cells were pre-treated with SDG and pXSC prior to stimulation with 10% FBS. Phospho-specific antibodies revealed no change in the levels of Akt activations between EGF only control and those pre-treated with selenium (Fig 5.7.1A, B).

These experiments concluded that selenium compounds, especially pXSC, rapidly activated Akt but do not inhibit Akt activation by mitogenic stimulation.

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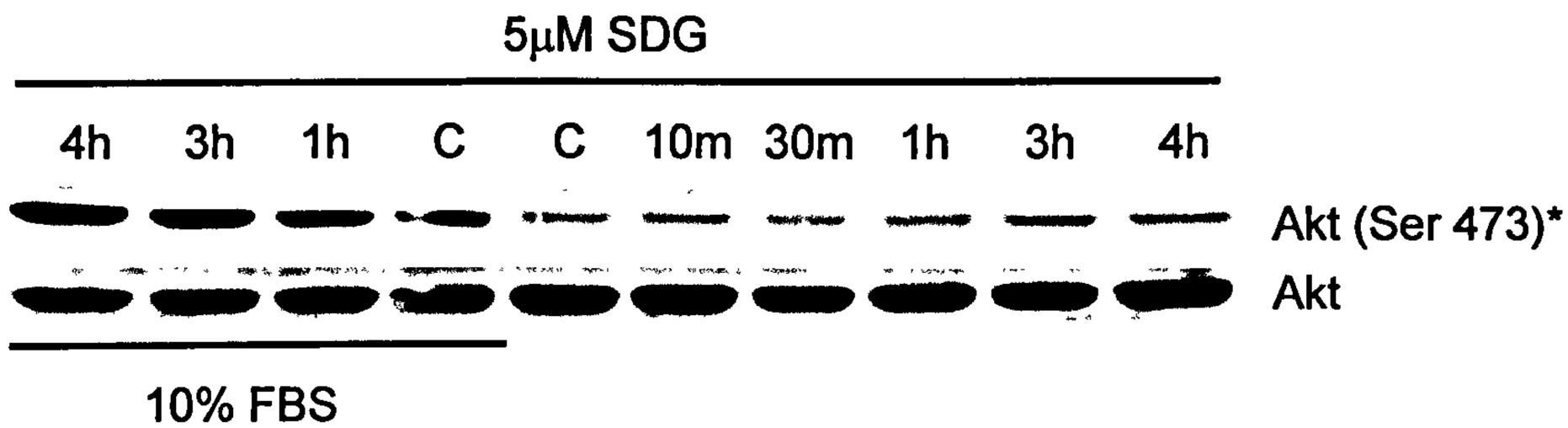

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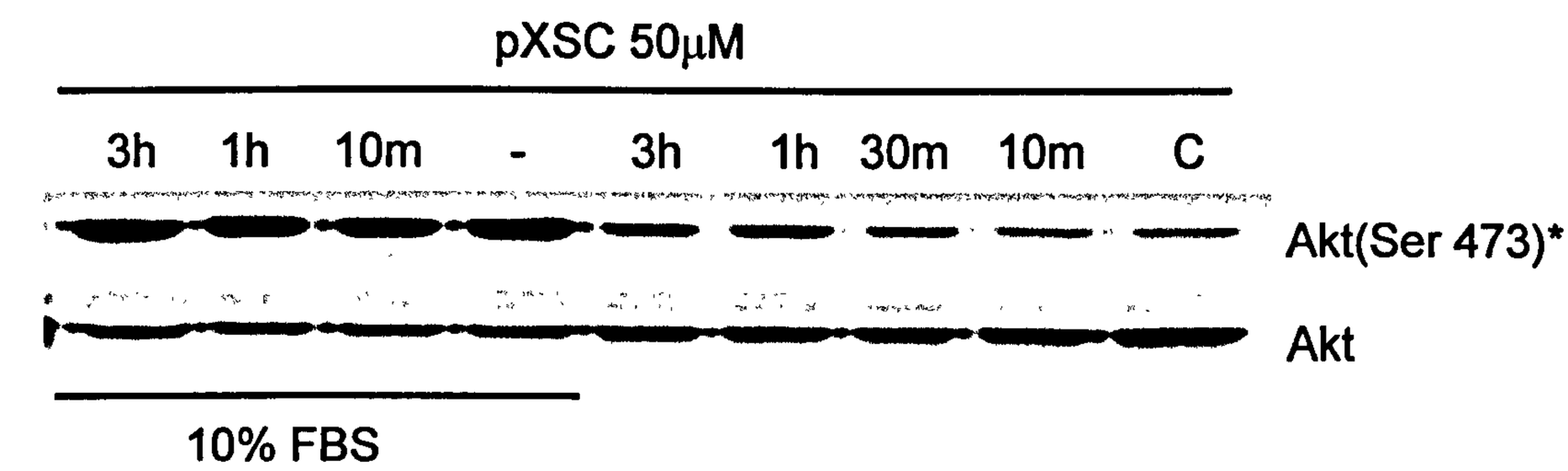

Fig 5.7.1 Activation of Akt by SDG and pXSC treatment.

HeLa cells were serum starved for 16h and then treated as indicated with SDG **(A)** or pXSC **(B)**, in some cases followed by 10% FBS treatment for 30min. Cell lysates were prepared and run on SDS/PAGE gels and Western blotted using antibodies that recognise total Akt or only the activated form (Akt*).

5.7.2 Pharmacologic inhibition of Akt activation

LY294002 is a well characterised, specific inhibitor of PI3K activity, which is necessary for Akt activation (Vlahos et al., 1994). Pre-treating HeLa cells with 50μM of LY294002 completely abolished Akt activation by either selenium compound (Fig 5.7.2A).

Quantifying apoptosis in HeLa cells treated with cytotoxic doses of SDG and pXSC, the inhibition of Akt activity was found to have no significant effect on the levels of cell death observed (Fig 5.7.2B). In contrast, a H_2O_2 positive control in the same experiment showed a 40% increase in cell death by LY294002 treatment (Fig 5.7.2B).

This suggests that, although Akt activity is associated with selenium exposure, it is unlikely that this has any substantial contribution to apoptosis induction. Perhaps activation of Akt, is a failed survival response that is overwhelmed by the acute apoptotic stimuli.

It is possible that Akt activation is an artefact of general PI3K activity which regulates other aspects of cellular physiology not detected in the cell death assays used in these experiments. Akt, acting through GSK3, has been implicated in inhibiting DNA binding activity of c-jun (Nikolakaki et al., 1993). This attenuation of AP-1 transactivation, taken together with the increased, pro-apoptotic c-jun activity seen on exposure to selenium compounds, underscores the suggestion that the Akt activation observed in our study may be a failed survival response.

5.8 Conclusion

This chapter describes an investigation of the major signalling pathways that may mediate selenium-induced apoptosis. Chemopreventive selenium compounds, SDG and pXSC, induced global changes in several of major signalling pathways and functional evidence suggests that at least some of these modulations directly mediate selenium-induced programmed cell death.

The stress activated protein kinase (SAPK), JNK, was shown to be a major player acting and through Fasl induction via c-jun transactivation. However, compared to SDG, the importance of this pathway for pXSC-induced cell death is considerably less. Although p38 activity was induced by both the compounds, experiments using a p38 specific inhibitor did not reveal any functional role for this kinase. Inhibition of ERK 1/2 activity showed a possible sensitisation whereas the forced activation of ERK5 resulted in a small,

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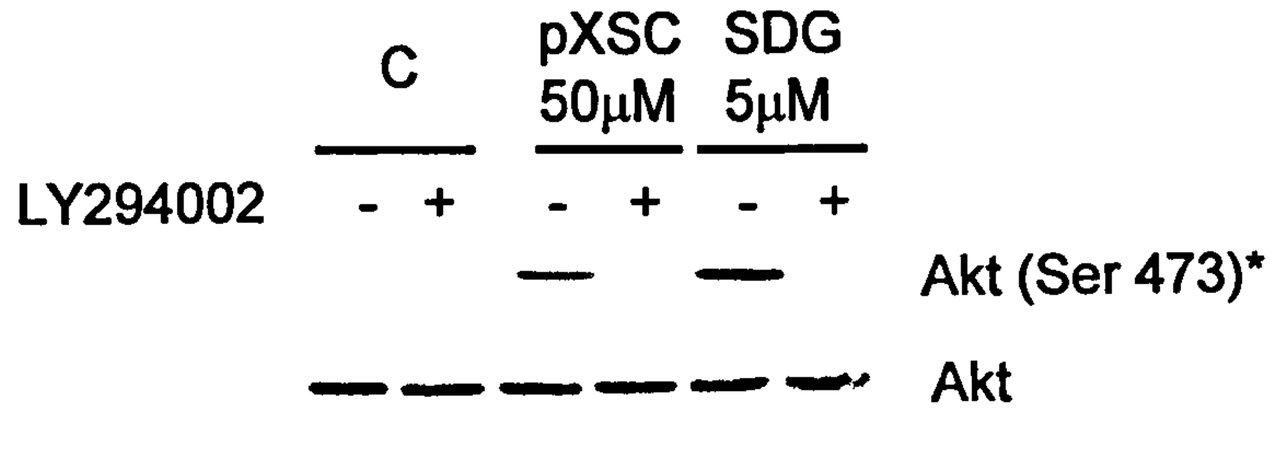

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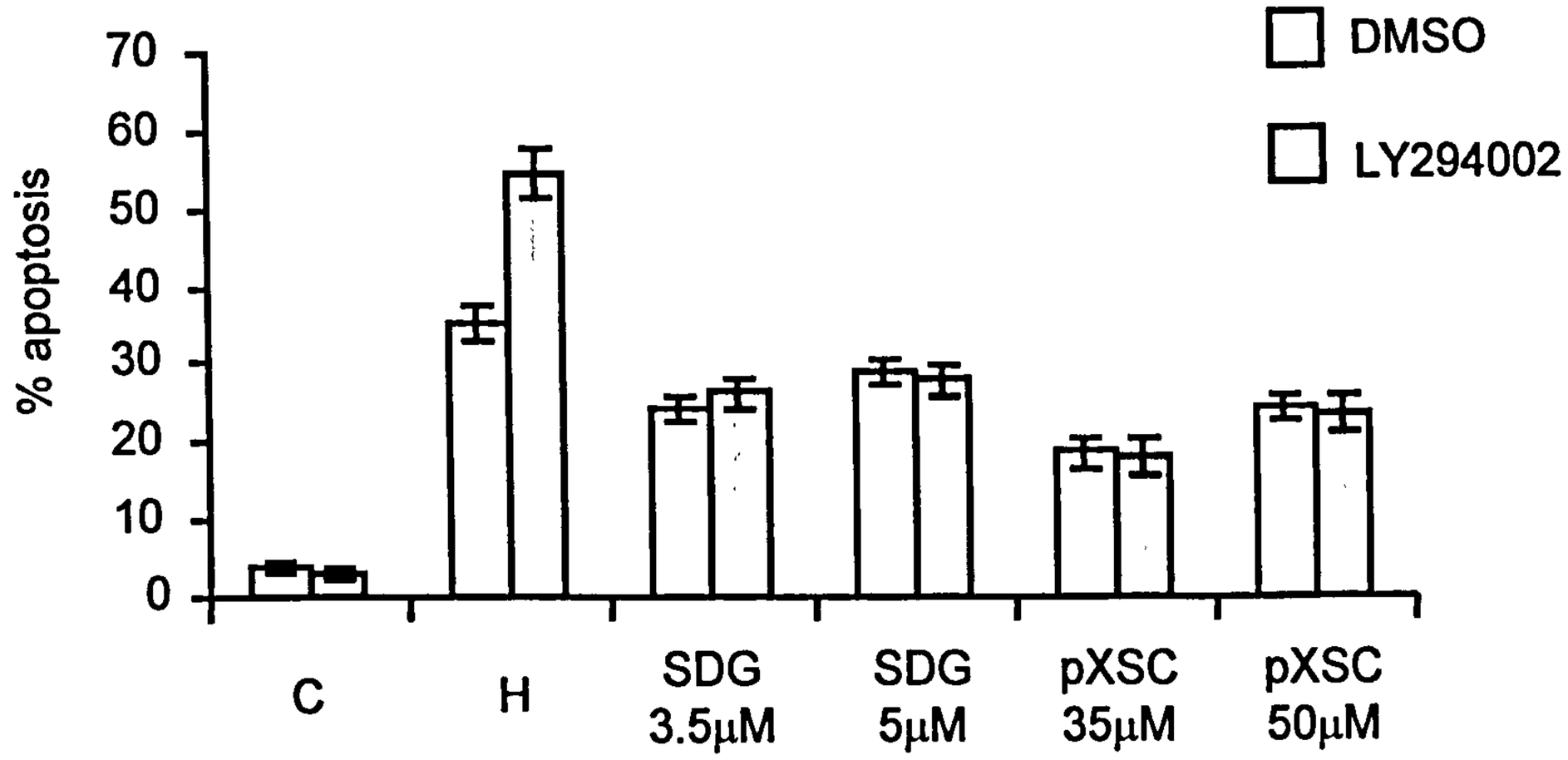

Fig 5.7.2 Effect of inhibition of Akt activity on apoptosis induction by SDG and pXSC.

(A) Serum-starved HeLa cells were pre-treated with 50μM LY294002 for 30min and then treated with indicated concentrations of SDG and pXSC for 16h. The effect on activation of Akt was determined by Western blotting using antibodies that recognise phospho-specific (Akt*) or total Akt. **(B)** Induction of apoptosis following treatment with indicated concentrations of selenium compounds for 16h, in the presence or absence of the Akt inhibitor, was measured by TUNEL assay. Each treatment was performed in triplicate. Data presented is representative of 2 independent experiments. Error bars indicate standard errors. C, represents treatment with solvent vehicle, DMSO.

but consistent, protection. Akt, though activated by both compounds, does not seem to be adequate to affect apoptosis as revealed by inhibiting its activation.

It is believed that an intimate interaction between pro- and anti- apoptotic signals determines the cell fate and probably reflects the importance of the evolutionary conserved genetic programme leading to apoptosis. Thus, it is only to be expected that several signalling modules will orchestrate signal transduction in a concerted manner, thereby increasing the complexity. The complexity itself enforces a tight control over the entire process which, if disregulated, would be of disastrous consequence to the organism. When seen from this perspective, it is quite possible that the small functional trends described for some of the kinases may have a physiological role which a reductionist approach of studying individual kinases in isolation may fail to resolve.

Several other pathways have also been implicated in apoptotic signalling and it will be interesting to look at the relative contributions of these pathways. For example, as described in Sections 2.6.7.2, 2.6.7.3 and 2.6.7.4, other studies have shown selenium mediated inhibition of protein kinase A, Ca^{2+} dependent and independent protein kinase C's (PKC), cyclin-dependent kinase2 (cdk2) and thymidine kinase (TK). However, the significance of these to apoptosis remains to be clarified. Another major modulator of apoptotic signalling in response to various stimuli, like oxidative stress, is the NF-κB pathway. Selenium compounds (at sub-micromolar doses) have been shown to inhibit NF-κB activity (Kim and Stadtman, 1997), but the implications of this to apoptosis is yet to be addressed. Tyrosine kinases like c-Abl may also be involved as certain selenium compounds have been shown to induce DNA damage.

5.9 Chapter Summary

- Chemopreventive selenium compounds induce sustained p38 kinase activity but pharmacologic inhibition did not affect cell death induction.

- The stress kinase, JNK, is rapidly activated by SDG and pXSC and leads to the phosphorylation of c-jun. Dominant negative and phosphorylation defective mutants of c-jun suppress SDG induced apoptosis; the inhibition is much smaller for pXSC treatment.

- The induction of Fasl by SDG is attenuated by overexpression of a dominant negative c-jun construct. Fasl induction by pXSC remains unaffected.

- Pro-proliferation signalling kinases ERKs 1 and 2 are induced by both compounds but their activation by exogenous mitogenic stimulation is reduced. Complete inhibition of ERK1/2 activities slightly sensitises cells to apoptosis.

- ERK5 activity is strongly inhibited by SDG and pXSC, and forced activation of ERK5 results in a small, but consistent, protection from apoptosis.

- Anti-apoptotic signalling mediated by Akt is induced by selenium compounds (particularly pXSC) but functional significance of this is unclear since inhibition of PI3K activity does not influence selenium mediated cell death.

6 Mechanisms of Selenium-induced Apoptosis in Primary Cultures of Oral Keratinocytes

6.1 Background

In Chapters 4 and 5, molecular mechanisms mediating apoptosis induction by chemopreventive selenium compounds were investigated in the established human cervical carcinoma cell line, HeLa, primarily due to the exploratory nature of the work and the ease of manipulation of these cells. These investigations identified several functional mechanisms underlying the induction of apoptosis by these compounds. However, the significance of these studies to the *in vivo* situation remains unclear. This is because firstly, epidemiological evidence suggests that selenium mediated anti-cancer activity is restricted to particular cancer types (see Section 2.2) and secondly, since established cell lines accumulate genetic changes as a result of prolonged culture, their relevance to 'real' cancer cells *in vivo* is questionable.

In this study, the premises adopted was that induction of apoptosis may be a major mechanism underlying the chemopreventive actions of selenium. The data presented in previous chapters has established that apoptosis-induction by selenium is functionally regulated by signal transduction pathways. Thus, one may argue that a detailed investigation into the regulatory signalling pathways mediating programmed cell death may reveal how selenium compounds discriminate between normal and tumours. It is to these ends that our group has attempted to develop an *in vitro* primary culture system relevant to human cancers where differences in sensitivity between normal and tumour cells may be investigated.

In view of the fact that lung, upper respiratory tract and oesophageal cancers are three of the cancer types in which the epidemiological evidence for a cancer protective effect of selenium is strongest (see Section 2.2), we have focused on human oral cancers as they share similar risk factors to lung and other head and neck cancers. It is also relevant to note that one of the handful of intervention trial using selenium found a significant inverse correlation between selenium supplementation and incidence of oral lesions (see Section 2.2.2). To obtain data as relevant as possible to human squamous cell carcinomas (SCC), a panel of primary cultures of biopsies of oral squamous cancers and normal oral mucosa cells (NOMC) which have been characterised previously at the Beatson Institute were used (Edington et al., 1995; McGregor et al., 1997). Putative functional pathways defined earlier

in HeLa cells (Chapters 4 and 5) were tested in this oral cell model to establish their relevance to chemoprevention in humans.

The panel of primary cultures of biopsies of NOMCs and SCCs were maintained using the 3T3 feeder layer system (see Section 3.2.1). The irradiated 3T3 feeder layer was carefully removed by EDTA treatment prior to experiments commencing with the remaining oral cells, typically at 60-70% confluence. The NOMCs were used within 2-3 passages from frozen stocks, while they were still proliferating rapidly, prior to senescence occurring.

6.2 Enhanced sensitivity of human oral carcinomas to induction of apoptosis by selenium compounds

Two independently-derived primary cultures of SCCs (BICR31 and BICR56) and NOMCs (FNB5 and FNB6) were treated with SDG and pXSC and the extent of apoptosis measured by AnnexinV staining. While both the compounds induced cell death in oral keratinocytes, SCCs were markedly more sensitive to SDG than NOMCs ($p<<0.001$, for pooled SCC and NOMC data) whereas any differences in sensitivity to pXSC were marginal and not statistically significant ($p=0.51$, for pooled SCC and NOMC data)(Fig 6.2.1).

This result has since been confirmed by TUNEL assay using these and other independently-derived SCCs and NOMCs (Ghose et al., 2001a). We have also observed that increased sensitivity of SCCs to SDG treatment can be reproduced at the level of growth inhibition, as measured by tritiated thymidine uptake. Again, as with apoptosis, no consistent differences in sensitivity was seen between SCCs and NOMCs on pXSC treatment.

It might be argued that the NOMCs may be progressing through the cell cycle more slowly and were thereby more resistant to apoptosis. However, this is unlikely as pXSC did not show any tumour selectivity and, more importantly, thymidine uptake levels of the SCCs and NOMCs are comparable at the conditions they were used to measure apoptosis (data not shown).

Thus we have developed an *in vitro* system, relevant to selenium mediated chemoprevention in humans which can be used to dissect molecular mechanisms responsible for selective apoptosis induction. Recently, since this work was completed, an independent study has shown similar tumour selectivity of apoptosis induction by selenite and selenomethionine comparing primary cultures of normal prostate cells to prostate

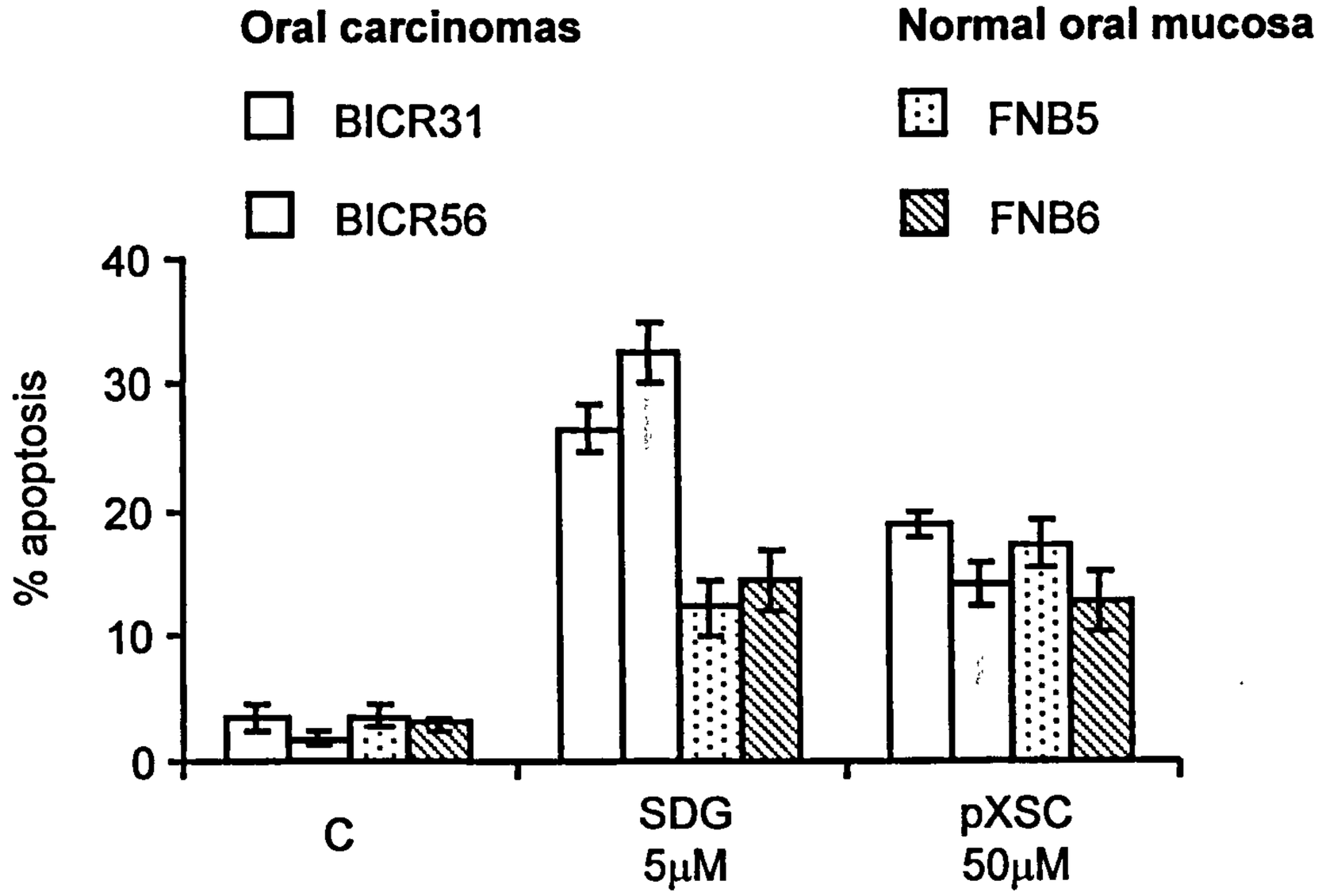

Fig 6.2.1 Sensitivity of NOMCs and SCCs to selenium induced apoptosis.

The degree of apoptosis after treatment of NOMCs or SCCs with solvent control (C), SDG or pXSC for 16h was quantitated by the AnnexinV staining protocol. Error bars indicate standard errors. Each treatment was performed in triplicate. Data presented is representative of 2 independent experiments. C, represents treatment with solvent vehicle, DMSO.

cancer cell lines (Menter et al., 2000). As SDG is the primary physiological metabolite of selenite this evidence corroborates our conclusions. It remains unclear why pXSC, though potently chemopreventive in animal studies, does not show such a selectivity. This could be a reflection of differing tumour-type specificity between SDG and pXSC.

6.3 Fasl mediates selenium-induced programmed cell death in oral keratinocytes

Fasl was induced readily on selenium treatment and was shown to be a functional mediator of cell death in HeLa cells, therefore, this was tested in the primary cultures. Both SDG and pXSC induced Fasl in SCCs and NOMCs (Fig 6.3.1A, B) at apoptosis inducing doses. Strikingly, the induction of Fasl by SDG in SCCs was considerably greater than that in NOMCs (3 and 6.3 times more at SDG concentrations 5μM and 8μM, respectively)(Fig 6.3.1A). No such differences between SCCs and NOMCs were seen with pXSC treatment (Fig 6.3.1B). Thus the degree of Fasl induction appears to correlate closely with the extent of apoptosis induced by selenium compounds.

As transfection efficiencies of these primary keratinocytes is extremely poor, a Fas/Fc chimeric peptide was used to block the engagement of Fas receptor by Fasl (Itoh et al., 1991). Apotosis mediated by addition of recombinant soluble Fasl, as a control, was attenuated on pretreating oral cells with Fas/Fc. The extent of apoptosis induced by SDG (43% inhibition; p=0.021) and pXSC (39% inhibition; p=0.044) was significantly reduced by blocking receptor engagement by Fasl (Fig 6.3.1C). This confirmed that selenium-induced apoptosis was mediated by Fasl in oral cells. However, the protection confered by blocking Fas activation on selenium-induced apoptosis was less than that achieved for exogenous sFasl treatment (Fig 6.3.1C): this suggests that Fas-independent pathways may also contribute to selenium-mediated apoptosis.

In keeping with the mechanistic role of Fasl in inducing apoptosis in oral cells it appears that the relative levels of Fasl induction determines the extent of apoptosis. This may well explain why SDG, and not pXSC, exhibits tumour selectivity. A point of interest is that in the oral cells the importance of the Fas pathway is comparable between SDG and pXSC, whereas in HeLa cells it appeared to be more important to SDG-induced apoptosis than pXSC (Fig 4.5.2C).

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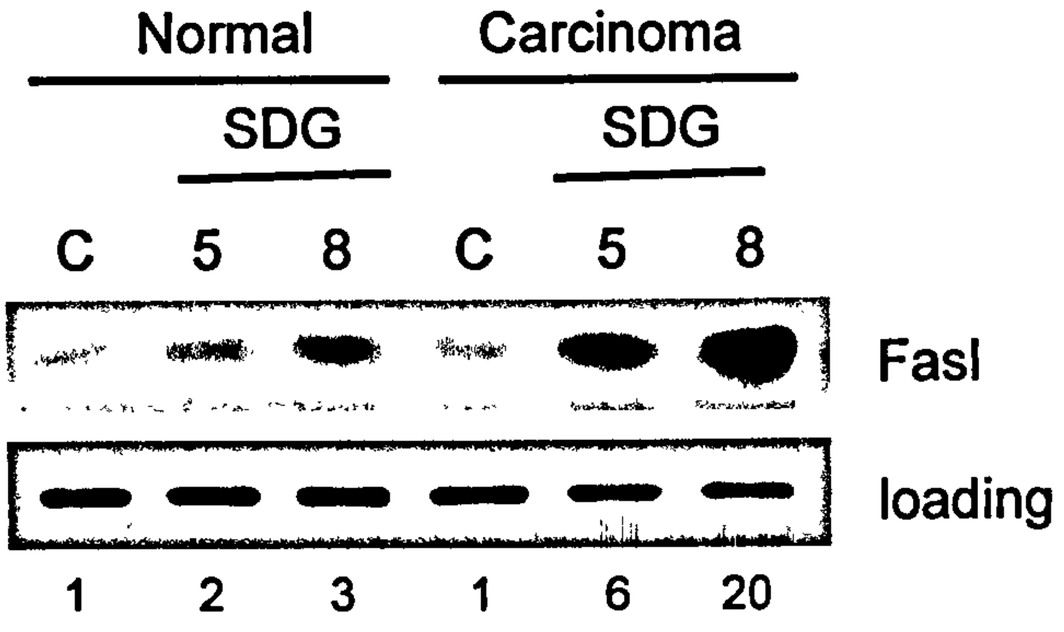

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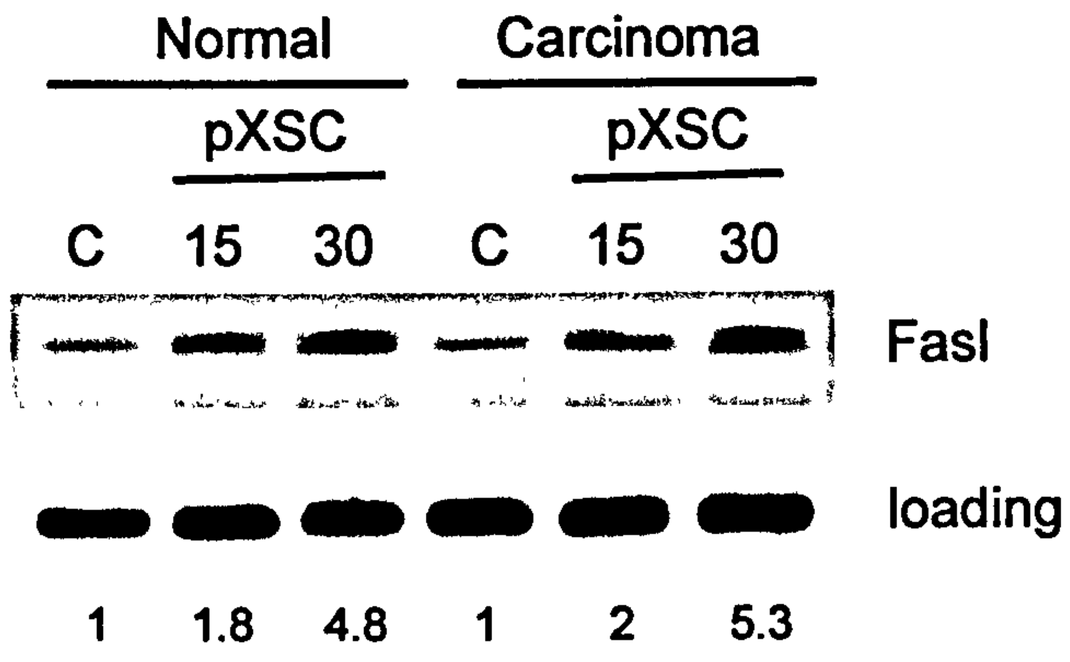

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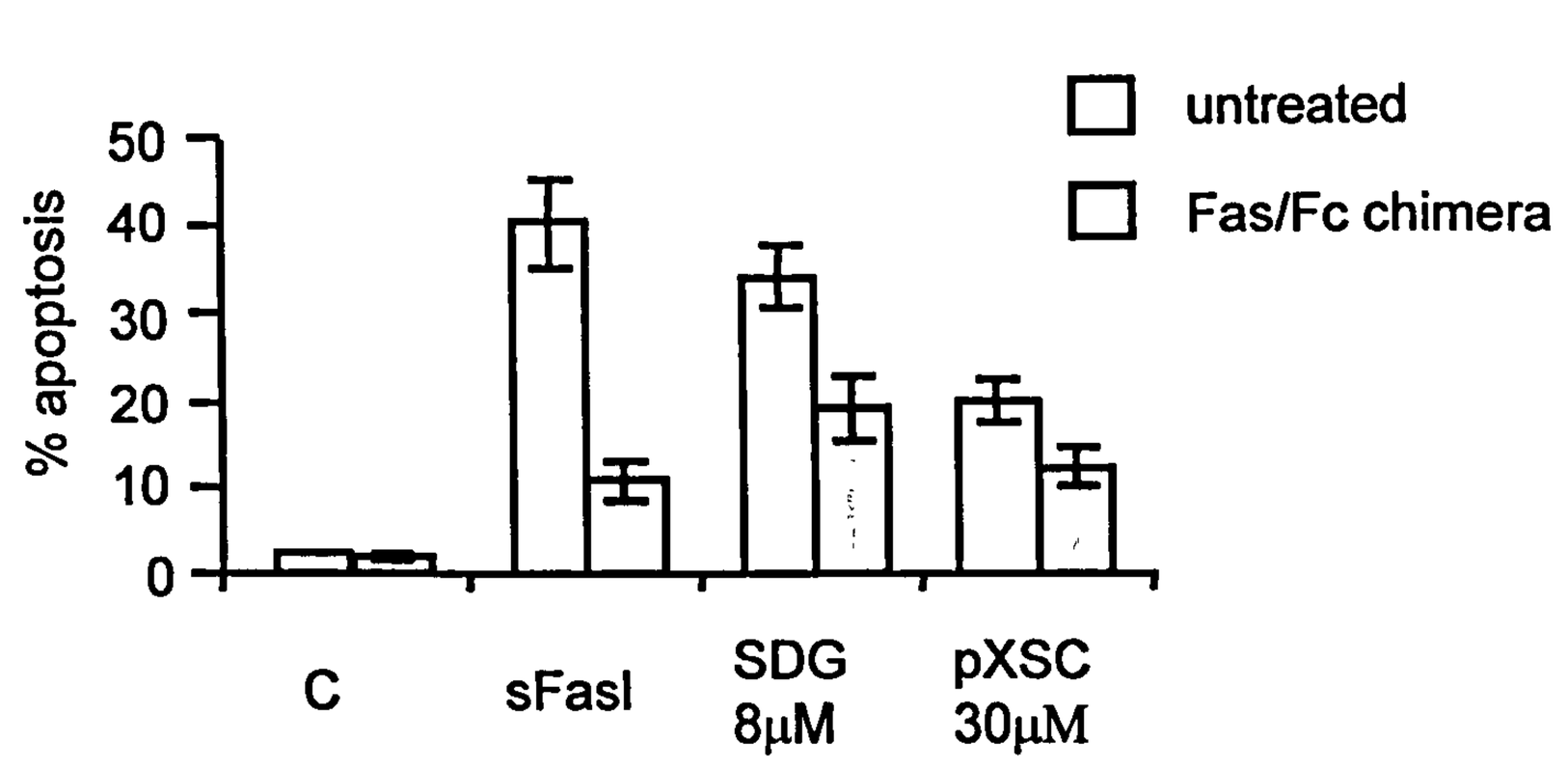


Fig 6.3.1 Induction of Fas ligand by SDG and pXSC in oral keratinocytes.

NOMCs and SCCs were treated with the stated concentrations of SDG **(A)** or pXSC **(B)** for 16h, cell lysates prepared and Western blotted with either an anti-Fasl antibody or antibody against total p38 kinase as a loading control. The figures below the blots indicate the fold increase in Fasl expression above untreated level, after selenium treatment, normalised to the loading control. **(C)** Protection against SDG- and pXSC-induced apoptosis (measured by the TUNEL assay) after pre-treatment of SCCs with a Fas/Fc chimera (250ng/ml) for 1h followed by the indicated concentrations of SDG or pXSC for 16h. As a positive control cells were also treated with soluble Fasl (sFasL). Each treatment was performed in triplicate. Data presented is representative of 2 independent experiments. Error bars indicate standard errors. C, represents treatment with solvent vehicle, DMSO.

6.4 Stress kinases mediate selenium-induced programmed cell death in oral keratinocytes

In HeLa cells, the activities of stress kinases, JNK and p38, were induced on treatment with selenium compounds. While no functional role for p38 activation could be determined, inhibition of the JNK pathway at the level of c-jun led to an attenuated apoptotic response involving inhibition of Fasl induction.

6.4.1 Induction of JNK and p38 activity by SDG and pXSC

Using phosphospecific antibodies, the activation of p38 and JNK after selenium exposure was tested in the primary cultures of the oral cells. Both SDG and pXSC caused activation of JNK and p38 in both normal and carcinoma cells under conditions in which apoptosis was induced (Fig 6.4.1A,B). There was no significant differences between NOMCs and SCCs in the extent of induction of JNK and p38 kinase by pXSC (Fig 6.4.1A, B). However, SDG was reproducibly more efficient at activating JNK and p38 in SCCs than NOMCs (Fig 6.4.1B), thus mirroring the greater sensitivity of SCCs to induction of apoptosis by SDG (Fig. 6.2.1).

6.4.2 JNK, but not p38, mediates selenium-induced cell death in oral keratinocytes

All further functional experiments were mainly performed using SCCs in view of their increased sensitivity to apoptosis induction, particularly by SDG. As transfection strategies, such as those used in the HeLa cell experiments described in Section 5.3.2, could not be utilised due to low transfection efficiencies a pharmacologic approach was adopted. The well documented differential sensitivities of p38 kinase, JNK and ERKs 1/2 to the SB202190 chemical inhibitor of the MAPK family was exploited: low concentrations (up to about 10μM) have been shown to selectively inhibit p38 kinase, at 30μM both p38 and JNK are inhibited, whereas ERKs 1&2 are not affected until even higher concentrations (Jacinto et al., 1998; Le-Niculescu et al., 1999; Whitmarsh et al., 1997).

Control experiments confirmed that 10μM SB202190 prevented SDG or p-XSC induced activation of p38 kinase (measured by activation of its downstream target MAPKAPK-2 in an immunecomplex kinase assay), but not activation of JNK (measured by phosphorylation of c-Jun at Ser63) or ERKs 1&2 (measured using phosphospecific antibodies); whereas

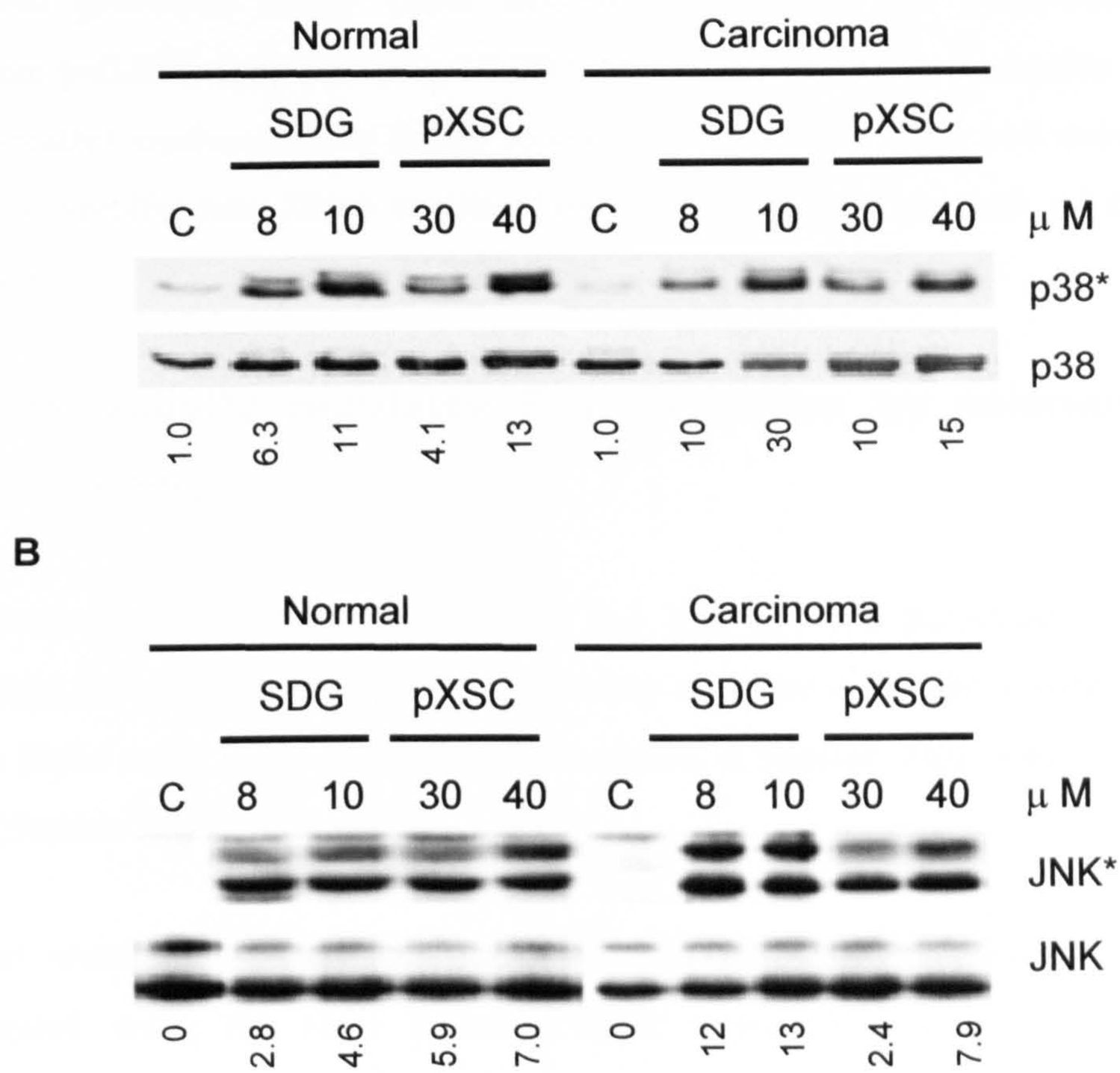


Fig 6.4.1 Activation of JNK and p38 kinase by SDG and pXSC in oral keratinocytes.

NOMCs or SCCs were left untreated (C) or treated with the indicated concentrations of SDG or p-XSC for 1h or 30min, respectively, cell lysates prepared, and analysed by SDS/PAGE and western blotting with antibodies against the activated forms of **(A)** p38 (p38*) or **(B)** JNK (JNK*). As loading controls, the blots were then stripped and western blotted with antibodies against total p38 or JNK. The figures below the blots indicate the relative levels of p38, JNK activation after normalisation to the loading control levels. C, represents treatment with solvent vehicle, DMSO.

30μM SB202190 prevented activation of both p38 kinase and JNK, but did not affect activation of ERKs 1&2 (Fig 6.4.2A).

Measurement of apoptosis indicated while 10μM SB202190 had no effect, 30μM SB202190 prevented SDG- (36% inhibition; p=0.033) and p-XSC-induced (33% inhibition; p=0.041) apoptosis (Fig 6.4.2B). This suggests that the activation of JNK is (at least partially) mechanistically linked to apoptosis induction while p38 activation has no functional significance. These results closely mirror the earlier work on HeLa cells in Chapter 5.

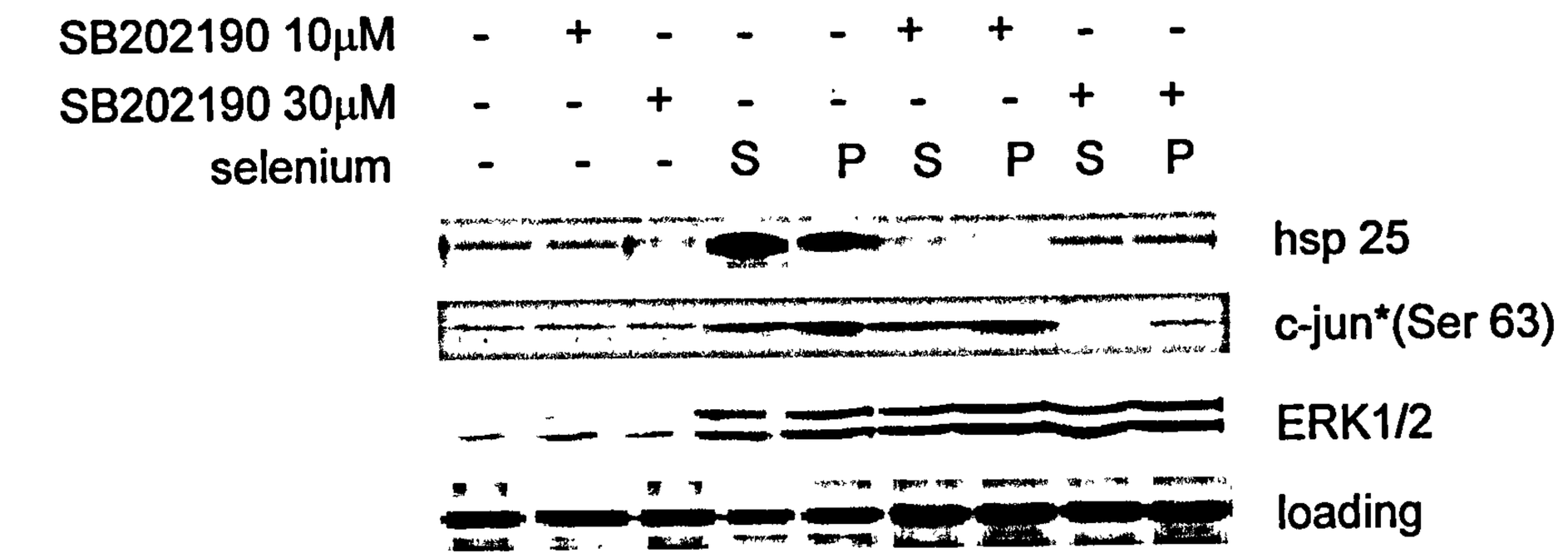
6.5 JNK activity regulates FasI induction by selenium in oral carcinomas

The observations described in Sections 6.3 and 6.4 are particularly interesting as differential induction of FasI appears to be a key regulator of enhanced sensitivity of SCCs and, in HeLa cells, JNK activity has been shown to regulate FasI induction (at least for SDG) (Section 5.4).

Whether stress kinase activities influenced the induction of FasI in oral cells was investigated using the same pharamacologic approach described in Section 6.4.2. Inhibition of p38 only did not affect FasI induction, while inhibition of both JNK and p38 resulted in reduced FasI production in response to both pXSC and SDG by about 50% (Fig 6.5.1). This strongly suggests that activation of the JNK pathway is necessary for the induction of FasI and apoptosis by SDG and pXSC, whereas activation of the p38 pathway is not.

A point of interest is that the pharmacologic approach in the oral cells revealed the role of JNK in inducing FasI in response to both pXSC and SDG. In HeLa cells however, transfection of TAM67 reduced FasI induction by SDG only. This may suggest that pXSC can induce FasI via JNK activation but independent of c-jun activation. The pharmacologic strategy applied to HeLa cells could resolve this issue but the increased toxicity to HeLa cells of high doses of inhibitor precludes this analysis.

A



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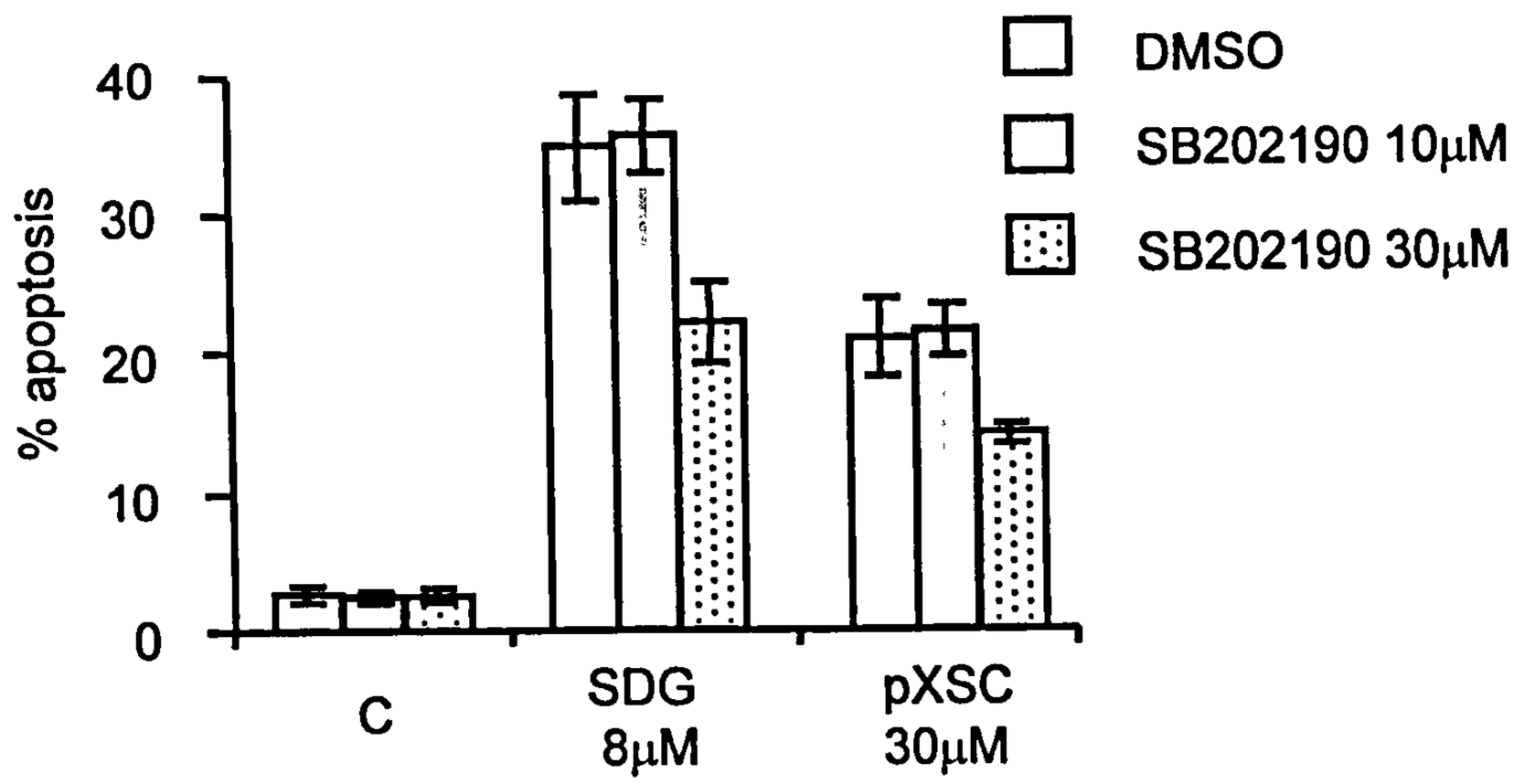


Fig 6.4.2 Effect of inhibition of p38 and JNK activation on induction of apoptosis by SDG and pXSC in SCCs.

(A) Effect of varying concentrations of SB202190 on the activation of p38 kinase, JNK and ERKs1/2 by 8μM SDG (S) or 30μM pXSC (P) was analysed. p38 kinase activity was measured by activation of its direct substrate MAPKAPK-2 in an immunoprecipitation kinase assay using hsp25 as MAPKAPK-2 substrate. Activation of JNK and ERK1/2 was assayed by phosphorylation using a phospho-specific anti-c-jun serine 63 (c-jun*(Ser63)) or ERK1/2 (ERK1/2*) antibodies. **(B)** SCCs were pre-treated for 30 min with the indicated concentrations of SB202190 followed by 8μM SDG or 30μM pXSC for 16h. Apoptosis was measured by TUNEL staining. Each treatment was performed in triplicate. Data presented is representative of 2 independent experiments. Error bars indicate standard errors. C, represents treatment with solvent vehicle, DMSO.

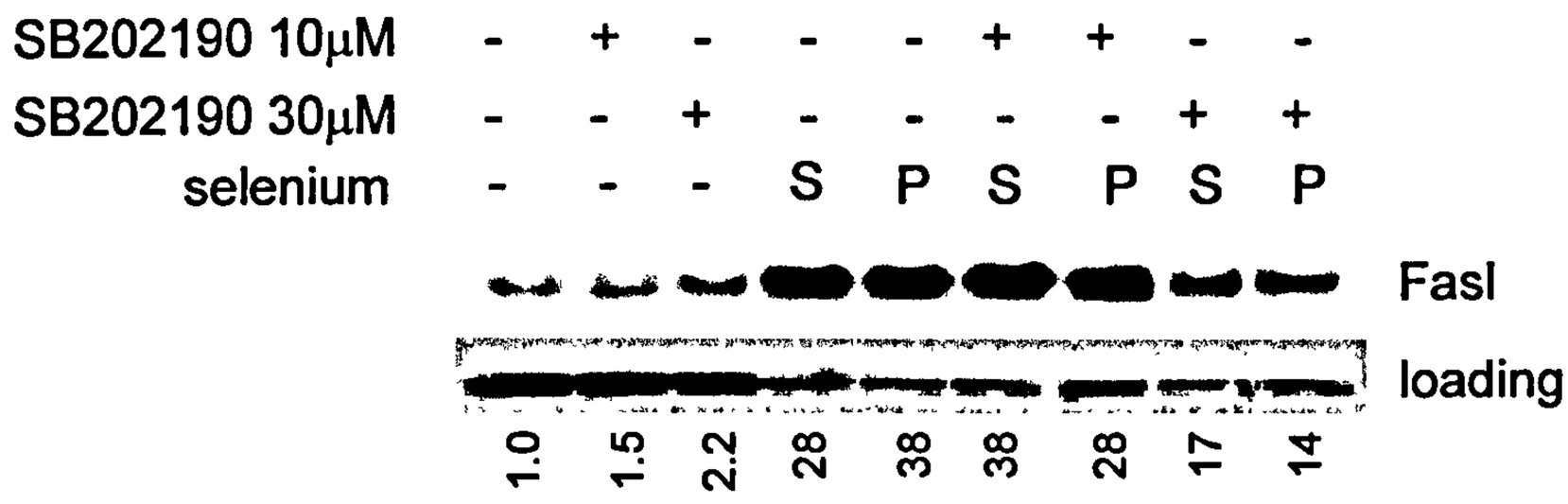


Fig 6.5.1 Effect of inhibition of p38 and JNK activation on induction of FasI by SDG and pXSC in SCCs.

SCCs were pre-treated for 30 min with the indicated concentrations of SB202190 followed by 8μM SDG or 30μM pXSC for 16h. FasI levels were measured by Western blotting using an anti-FasI antibody. Total p38 levels were used as loading controls. The figures below the blots indicate the fold increase in FasI expression above control level, after selenium treatment, normalised to the loading control level.

6.6 Inhibition of ERK 1/2 and Akt activation by chemopreventive selenium compounds

As with HeLa cells (Sections 5.5.1 and 5.7.1), both ERKs 1/2 and Akt were activated by both selenium compounds (work of J. Fleming). However, in contrast to HeLa cells, there was no reduction of mitogen-induced ERK 1/2 stimulation on selenium pre-treatment. ERK5 does not appear to be a prominent signalling pathway in these cells as it cannot be activated by known stimulants like EGF (work of J. O'Prey).

To test the functional role of ERK1/2 and Akt activation, specific inhibitors were employed as described in Sections 5.5.2 and 5.7.2. Control experiments in oral cells showed that under conditions of use PD98059 and LY294002 completely inhibited selenium induced activation of ERK 1/2 and Akt, respectively (Ghose et al., 2001a).

Parallel experiments measuring the extent of apoptosis revealed no difference in pXSC-induced cell death on inhibition of ERK1/2 or Akt activity (Fig 6.6.1). However, while inhibition of ERK1/2 activity had no effect on SDG-induced apoptosis, treatment with LY294002 resulted in a significant increase (59%) in apoptosis (Fig 6.6.1). This would suggest that the activation of Akt by SDG is a potent survival response whose inhibition sensitises the cells. ERK 1/2 activation by both selenium compounds and activation of Akt by pXSC appear to have no functional role and may be unsuccessful survival responses.

Thus we have for the first time identified a definite survival response which acts to check the extent of apoptosis (in HeLa there was a small trend with ERK1/2 and a small effect via ERK5 – Sections 5.5.2 and 5.6.2). Once this inhibition is removed by the LY294002 inhibitor, the extent of apoptosis increase considerably. Further experiments aimed towards identifying downstream targets of Akt activation may reveal its underlying function. Intriguingly, Akt can inhibit the forkhead transcription factor which may regulate Fasl transcription (Brunet et al., 1999). Inhibition of Akt could presumably lift this repression leading to increased induction of Fasl and thereby enhanced rate of apoptosis. Obviously other downstream survival related activities of Akt, including phosphorylation and inactivation of caspase 9 (Cardone et al., 1998) and Bad phosphorylation (del Peso et al., 1997), may complicate such a simplistic analysis. However, given the importance of Fas in SDG mediated apoptosis, analysing levels of Fasl induced by SDG, with or without inhibition of Akt activity should address this issue. Very recently a report has described that inhibition of PI3K activity sensitises of HL60 leukaemia cells to both chemotherapeutic drug- and Fas-induced apoptosis (O'Gorman et al., 2001). It also showed

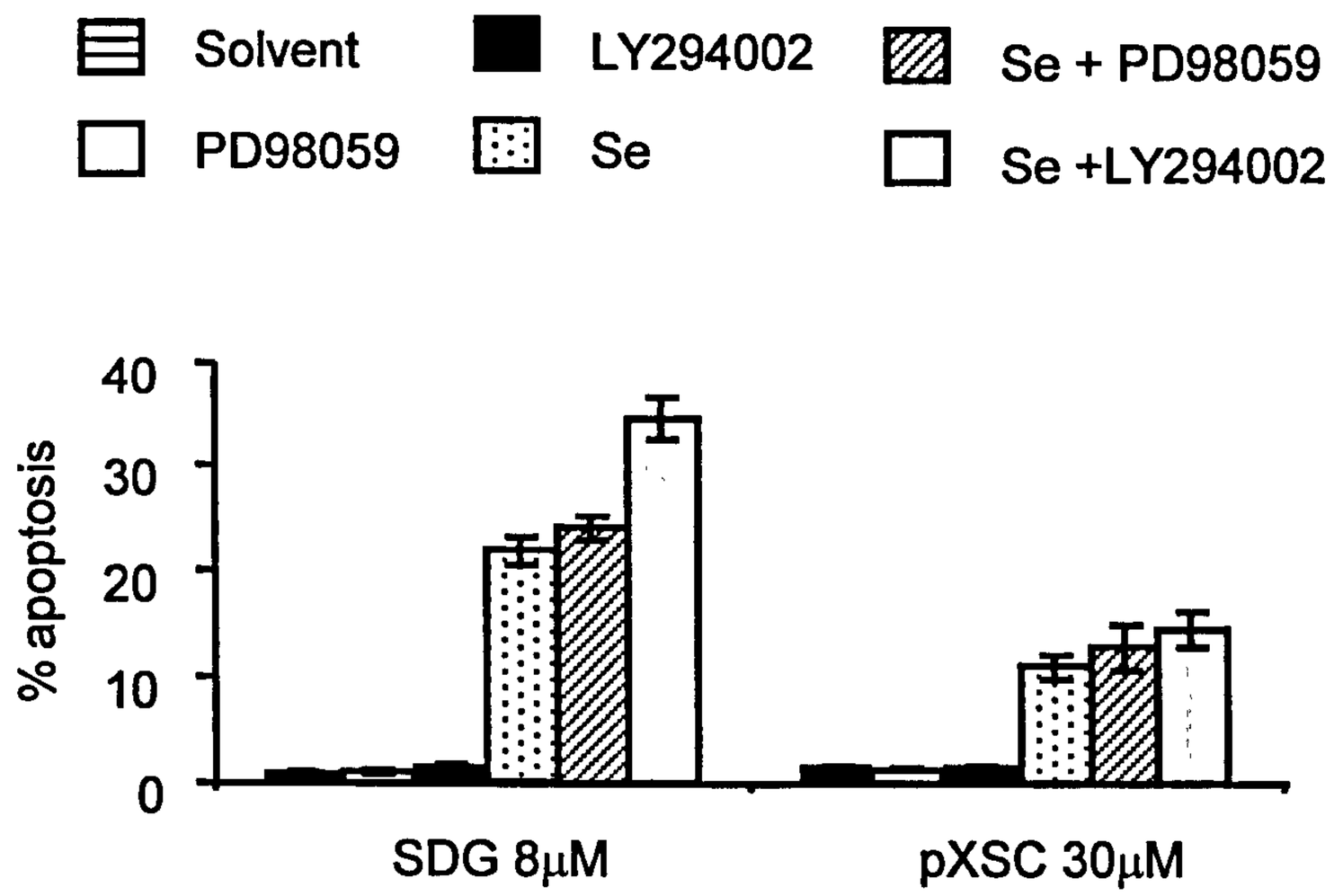


Fig 6.6.1 Inhibition of selenium-induced ERK1/2 and Akt activities in SCCs.

SCCs were pre-treated with 50μM LY294002 or 50μM PD98059 and then treated with 8μM SDG or 30μM pXSC for 18h. Induction of apoptosis was measured by TUNEL staining. Each treatment was performed in triplicate. Data presented is representative of 2 independent experiments. Error bars indicate standard errors. C, represents treatment with solvent vehicle, DMSO.

that PI3K inhibition significantly increases chemotherapeutic drug-induced JNK activation but whether this was of functional relevance remains unclear.

6.7 Conclusions

The main purpose of this work was to identify the signal transduction mechanisms responsible for apoptosis induced by cancer-protective selenium compounds using a relevant human model and to test whether selenium-induced apoptosis had a tumour selective component. The major novel finding is that human SCCs are more sensitive to induction of apoptosis by the SDG than NOMCs. This is clearly of considerable relevance to understanding the cancer-protective effect of selenium compounds, particularly selenite. It is not clear why pXSC does not show the tumour-selectivity exhibited by SDG but it may reflect the relative importance of the JNK/Fasl pathway in mediating the effects of the two compounds which is strongly correlated to the extent of apoptosis induced.

For the most part, molecular mechanisms of selenium-induced apoptosis identified in HeLa cells are quite similar to those seen in the primary cultures. Thus, we have successfully extended our work on established cell lines to a model more akin to the *in vivo* conditions. Taken together, the two sets of studies in different cellular models, very strongly implicates the critical involvement of the JNK/c-jun/Fasl pathway. Selenium is known to influence a myriad of biological processes and it is possible that other major mediators are involved which the current set of experiments have not addressed. This is particularly relevant as most of our functional studies have always demonstrated partial inhibition. An outstanding issue, given that the JNK/c-jun/Fasl pathway may form the basis of tumour selectivity, is the differential induction of upstream components of this pathway. To this end, there is an urgent need to analyse the relative activities of the known upstream JNK activators in SCCs and NOMCs.

Apart from defining this particular mechanistic pathway, these experiments may also have broader implications. The differential induction of JNK and Fasl, if reproduced *in vivo*, can be used as surrogate biomarkers of selenium activity as an anti-cancer agent. It is true that primary cultures, at best, provide only a close approximation of the *in vivo* situation. This and the fact that we have already identified possible major regulators of selenium-mediated apoptosis makes the case for venturing into *in vivo* experimentation very strong.

6.8 Chapter Summary

- A novel primary culture model, relevant to selenium mediated chemoprevention in humans developed from biopsies of SCCs and NOMCs, has been used to analyse the mechanism of action of SDG and pXSC.

- SCC's showed enhanced sensitivity to SDG-, but not pXSC-, induced apoptosis. This correlated strongly with the level of Fasl induction which (at least partially) mediates apoptosis induction by both compounds.

- SCCs were more efficient in activating JNK than NOMCs and this correlated well with their enhanced sensitivity and levels of Fasl induction.

- JNK, but not p38, activation mediated cell death and Fasl induction by SDG and pXSC.

- Inhibition of Akt activation by SDG, and not pXSC, increased the apoptotic rate. No difference in apoptosis levels was seen on inhibition of ERK 1/2 activation.

7 Molecular Mechanisms Mediating Anti-cancer Activities of Selenite *in vivo*

7.1 Background

The previous chapter has identified that treatment of oral keratinocytes with chemopreventive selenium compounds results in the induction of apoptosis, *in vitro*. Some of the dominant pathways that are likely to regulate selenium-induced programmed cell death in these cells have been identified and it appears that a signalling route leading from the induction of JNK activity to the induction of Fasl, via activation of c-jun, is of particular significance.

Active discrimination between tumour and normal cells form the basis of any anti-cancer agent and identification of its underlying mechanisms are of great importance. Chapter 6 also implicated the differential utilisation of this JNK-mediated signalling module to be the putative mechanism underlying tumour selectivity, at least for SDG.

These findings are generally similar in established cell lines and primary cultures relevant to humans, but if any real biological relevance is to be attached to these findings, it is necessary to reproduce them *in vivo*. To this end, in collaboration with J. Fleming (Beatson Institute, UK), the chemopreventive activities of selenium have been investigated in mouse models.

Human SCCs growing in primary cultures were used in xenograft studies in immuno-supressed 'nude' mice. The fact that SDG, the primary metabolite of selenite, showed significant tumour selectivity and activation of the JNK/c-jun/Fasl pathway, together with the historically strong chemopreventive activity of selenite in several mouse models of cancer, prompted us to choose selenite as the compound of choice in these animal experiments.

It needs to be stressed that the study described here is still ongoing and is designed as an pilot for a possible larger study. The results described here are preliminary and considerable caution is therefore necessary in interpreting the data.

7.2 Human SCC xenografts in nude mice

A human oral SCC (BICR31) was chosen for xenograft studies in nude mice because of its previous characterisation in xenograft studies by Dr J. Brown (Beatson Institue, UK; unpublished data) which demonstrated its aggressive growth upon transplantation. Immuno-suppressed 'nude' mice were divided into two groups of nine and placed on either high (2ppm) or depleted selenium (<< 0.1ppm) diets, in the form of sodium selenite. The concentration of selenium in a standard mouse diet is ~ 0.16 ppm, we chose to compare a higher and a depleted dose on the simplistic assumption that possible biological differences may be enhanced.

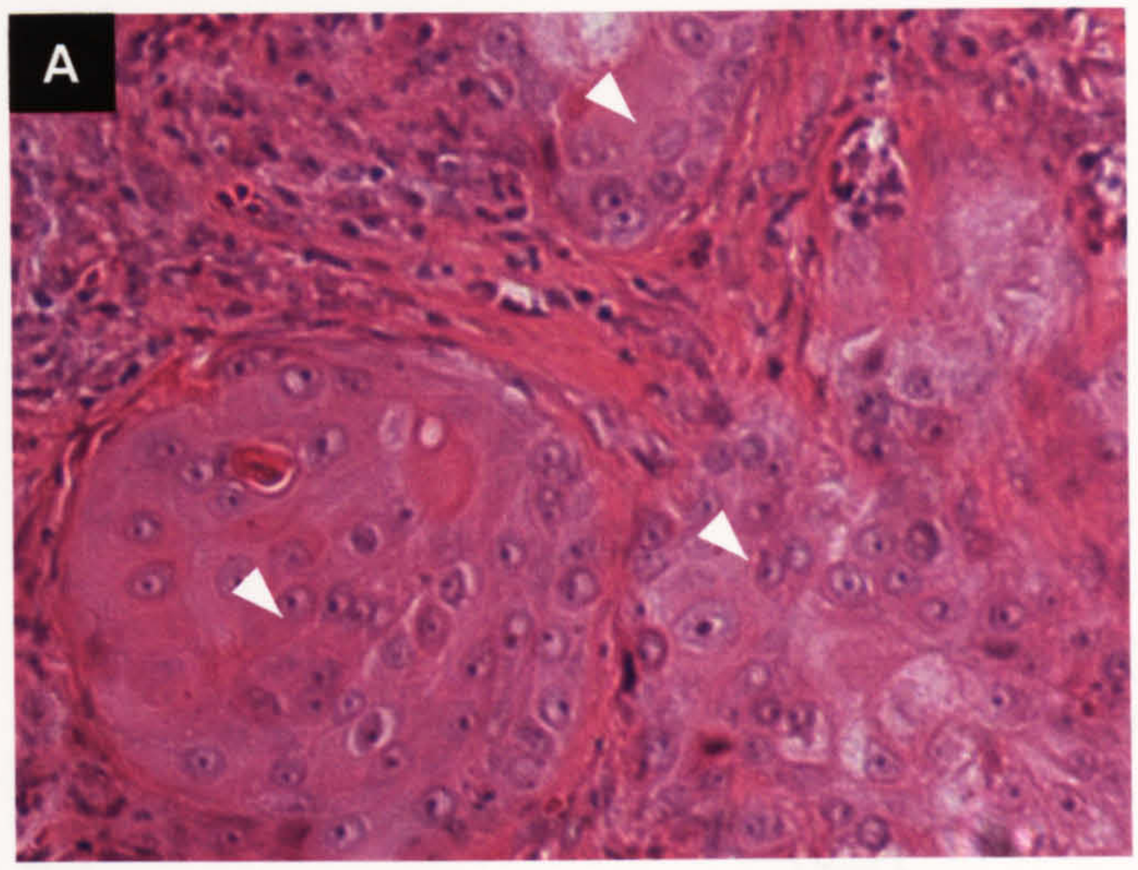
BICR31 cells in a matrigel matrix were subcutaneously injected into nude mice and tumour growth followed over time. As per the relevant Home Office project grant regulations, mice bearing a tumour load of $1cm^2$, as measured in any two dimensions, were killed and the tumour removed for analysis. At the time of writing this report the mice were on the 15^{th} week after the injection.

Preliminary analysis of week 12 tumours revealed the growth of the SCC's in tumour nests surrounded by mouse tissue (Fig 7.2.1A, B). The invading mass of tumour cells were obvious and other indicators of malignancy like vacuolar and clear cells were sometimes present. No immediate differences were observed at this level between tumours from mice on high or low selenium diets (Fig 7.2.1A, B). Detailed pathological analysis of these sections will be carried out when the experimental endpoint is reached.

7.3 Effect of high selenium diet on xenograft growth

Weekly measurement of tumour size was carried out to establish whether this dose of selenite in the diet affected the growth of xenografts. As the study is currently ongoing it is difficult to statistically represent differences in growth characteristic.

There is however, an indication that high selenium in the diet inhibits or at least delays xenograft outgrowth. Fig 7.3.1 plots the number of live mice, an inverse indicator of tumour burden (in view of the requirement that mice are killed when a tumour size of $1cm^2$ is reached), as a function of time. It is clear that at the later stages of the experiment, a lower number of mice in the low selenium cohort are alive. This suggests that high selenium in the diet may delay the growth of xenografts.



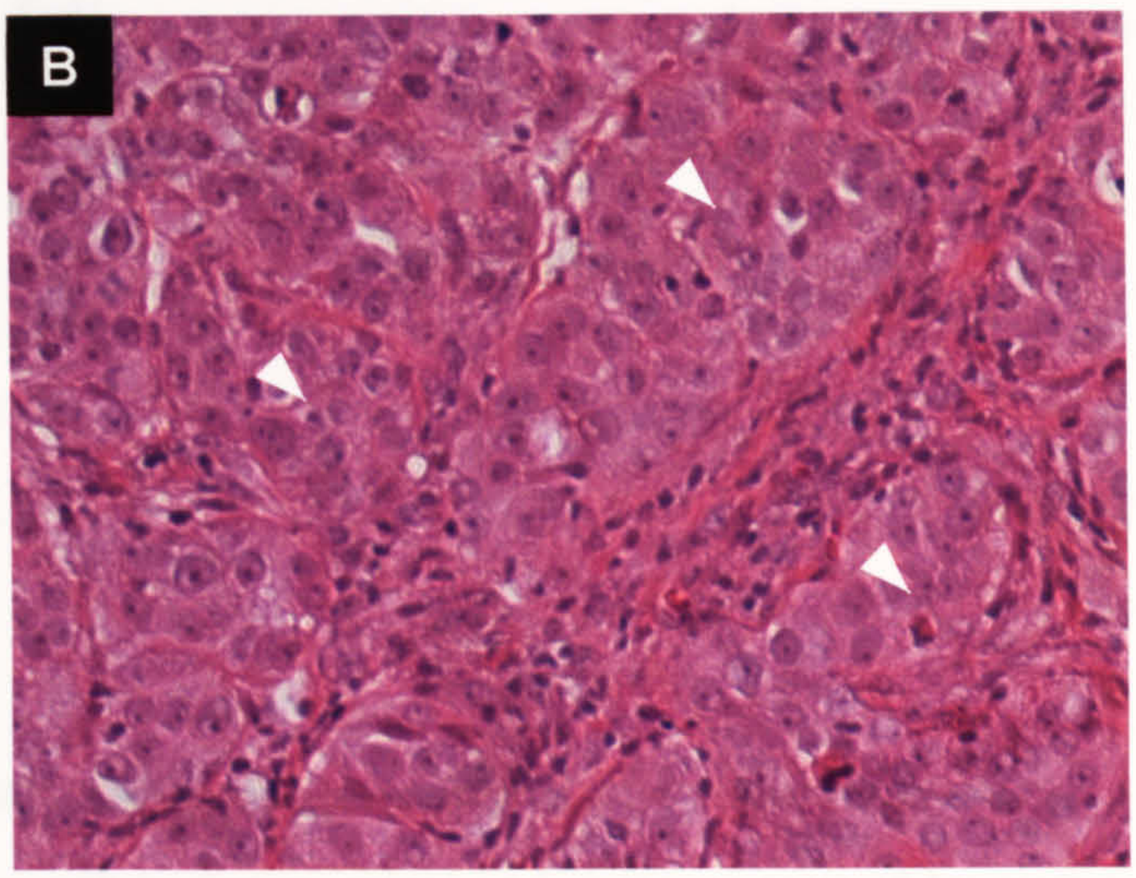


Fig 7.2.1 Histology sections of human SCC xenografts in nude mice.

5μM paraffin embedded histology sections from human SCC xenografts growing on nude mice were stained with haematoxylin/eosin to visualise histological characteristics. **(A)** is a histology section of a tumour growing on a mouse fed with a depleted selenium diet while **(B)** is from a mouse on high selenium diet. White arrowheads show 'tumour nests' of human SCC surrounded by mouse tissue.

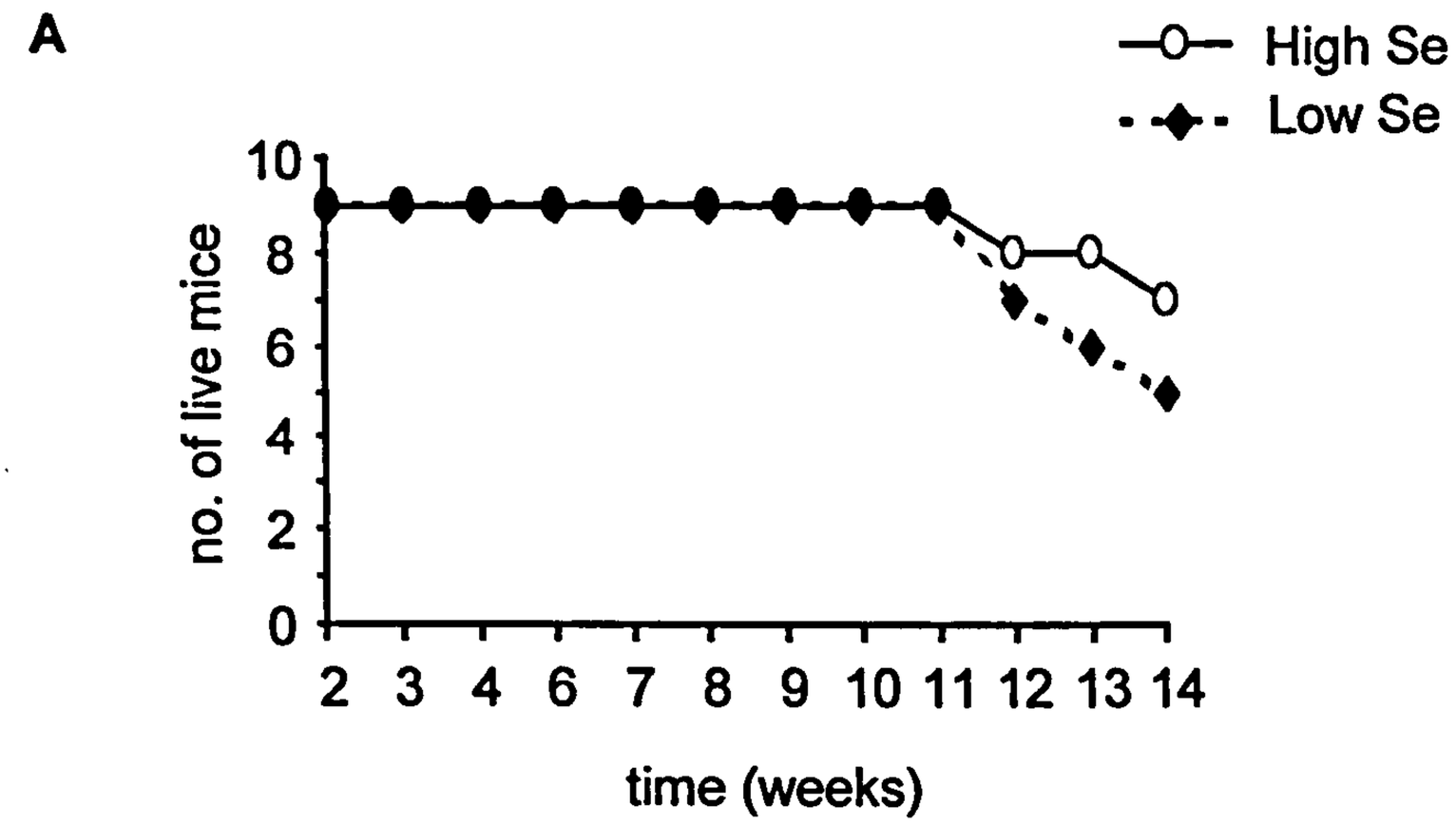


Fig 7.3.1 Sensitivity of SCC xenograft growth to dietary selenium levels.

Sensitivity of xenograft growth to dietary selenium levels was assayed by plotting the number of mice with tumours less than 1cm^2 (after which they are killed) as a function of time after xenograft initiation.

Comparison of the actual tumour sizes also reveal a trend towards smaller tumours in the high selenium cohort (data not shown). It is clear from this that the tumour sizes in both the groups remain fairly constant till the 11th week after which they start to proliferate and establish themselves. It is after this stage that the trend towards smaller tumour sizes in mice on high selenium diet begin to emerge. Our study has so far reached a few weeks after the xenografts have started to expand, therefore later timepoints may yield biologically meaningful results. However, going by the observed trend it is a possibility that we may be looking at early stages of selenite mediated inhibition of human SCC xenograft outgrowth.

7.4 High dietary levels of selenium correlates with apoptosis induction *in vivo*

The major objective of this study was to validate apoptotic mechanisms elucidated in cell lines in an *in vivo* situation. In view of the slight inhibition of tumour outgrowth by high selenium diet observed at these early stages of analysis, the contribution of apoptosis induction to this phenomenon was investigated.

Tissue sections from tumours were immunostained with an antibody against the cleaved fragment of caspase 3, accumulation of which is a strong marker for apoptosis induction (Riss, 2001). Three, week 12 tumours, 1 from high and 2 from low selenium cohorts (one showing signs of ulceration) were assessed. A significantly large induction of apoptosis was seen in the high selenium tumour compared to the two others on low selenium (Fig 7.4.1). The extent of apoptosis was more widespread and there was a dramatic increase in the intensity of staining. The apoptosis appeared to be mainly restricted to the tumour nests suggesting specificity.

Its is clear from this particular experiment that increased apoptosis appears to occur in tumours in mice on the high selenium diet. However, quantification of the extent of apoptosis and analysis of more tumours (from later timepoints) is necessary to confirm that this conclusion is statistically valid. There is also the need to confirm the same using a different assay, like TUNEL. To judge if high selenium in diet induces targeted cell death in tumours or enhances the rates of apoptosis generally, unrelated mouse tissue matched with the tumours needs to be analysed.

The induction of caspase 3 indicates a difference from *in vitro* cell culture data where, according to the available literature, its induction by selenite is contentious (see Section

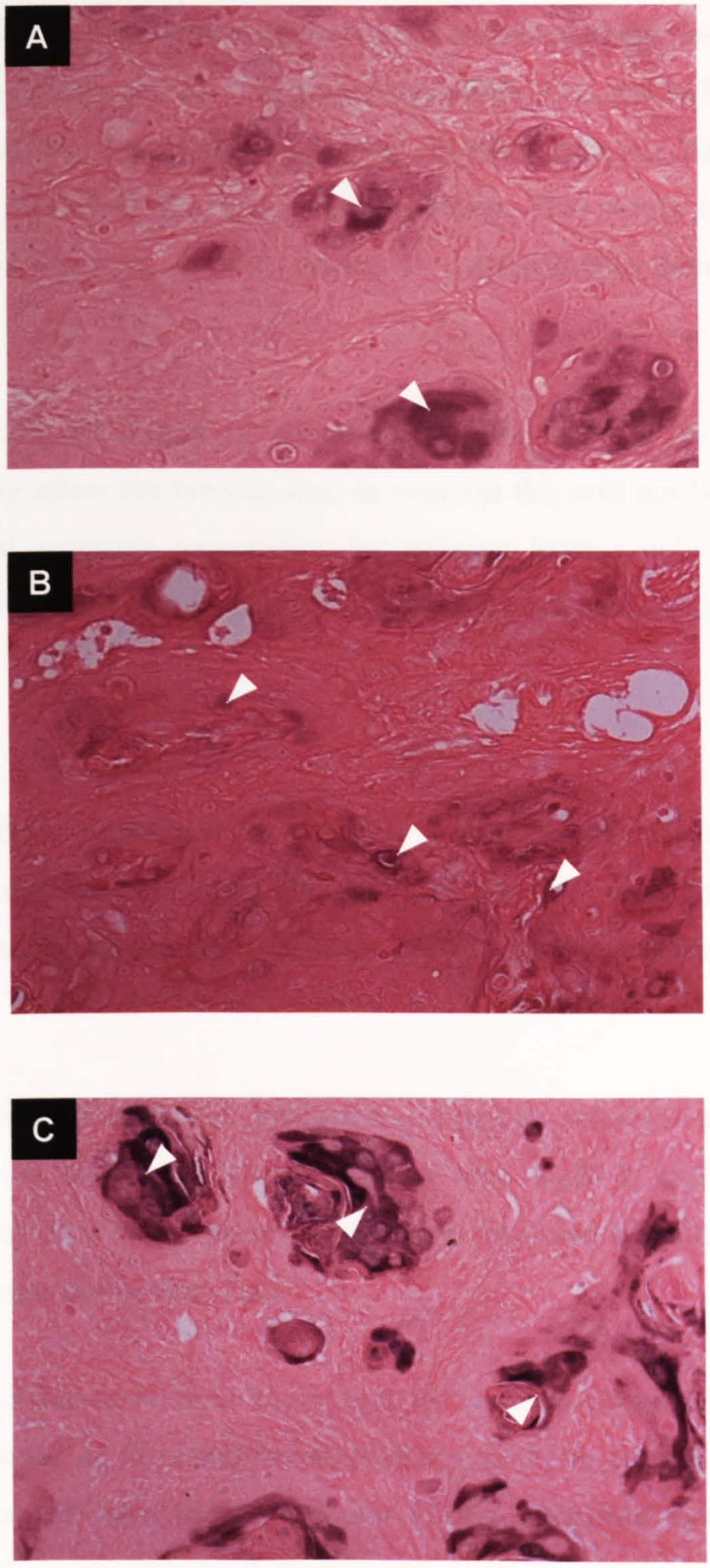


Fig 7.4.1 Enhanced induction of *in vivo* apoptosis in SCC xenografts in response to a supplemented selenium diet.

Paraffin embedded xenograft sections from 3 mice killed 12 weeks after xenograft initiation were subjected to immunohistochemistry using a antibody against cleaved caspase 3 (activated). Background staining was with eosin. 2 mice were from the depleted selenium cohort **(A,B)** while the third was from the supplemented selenium cohort **(C)**. Caspase 3 staining is indicated with white arrowheads. Tissue section in **(A)** was from a tumour sample that showed signs of ulceration.

2.6.7.3). Our *in vitro* studies have, however, found induction of caspase 3 by both pXSC and SDG (Section 4.4). Also, as its induction appears to be restricted to the tumour nests, it suggests some form of specificity and not a general toxic effect. One has to bear in mind that, even in tissue culture, the low doses of selenite exposure does not have the non-specific genotoxic effects described for higher concentrations. This would imply, that a small increase in selenium exposure, *in vivo*, results in a considerable tumour-selective effect.

One final issue is regarding the extent of apoptosis, *in vivo*. Even a 1% increase in apoptosis will considerably effect the tumour size, *in vivo* but this will not be relevant *in vitro*. Thus a very careful apoptotic quantitation for tumours from a individual mouse needs to be performed and correlated to its particular tumour growth characteristics.

7.5 Conclusions

This ongoing study is addressing the need to elucidate molecular mechanisms mediating the anti-cancer activities of chemopreventive selenium compounds *in vivo*. As our *in vitro* work has already identified several putative candidates, we now have defined targets to investigate *in vivo*.

The data discussed in this section, though preliminary, suggests possibilities that fit our current model for selenium mediated chemoprevention very well. It appears that the induction of apoptosis may be a major player in selenium mediated chemoprevention *in vivo* and significantly bolsters our *in vitro* data.

However, a lot of work remains to be done to confirm the tentative conclusions presented in this chapter. Apart from taking the study to completion (ideally to a stage where all the mice in the low selenium diet have been killed due to their increased tumour burden) several other aspects need to be addressed. Firstly, the level of selenium in the blood plasma of individual animals has to determined. Secondly, activation of JNK (and possibly c-jun) and Fasl needs to be investigated using immunostaining. Thirdly, detailed pathological analysis should be performed to identify histological features of selenium-mediated inhibition of xenograft outgrowth. Immunohistochemical studies directed at markers of cellular proliferation will also be carried out to assess if cytostatic activity correlate with the inhibitory effect on xenograft growth.

7.6 Chapter Summary

- An *in vivo* model of human SCC xenograft in nude mice has been developed and tumour outgrowth in response to high or low selenium containing diet is under investigation

- Preliminary data suggests that inhibition of xenograft growth is correlated with selenium enhanced diet.

- This inhibition is reflected in the increased levels of apoptosis observed in tumours growing on mice fed with selenium supplemented diet.

8 General Discussion

8.1 Summary of results

The large corpus of data from epidemiological, ecological and clinical prospective trials suggesting a strong inverse correlation between dietary selenium intake and incidence of several human cancers has led to an explosion of interest in elucidating cellular and molecular mechanisms that mediate anti-cancer activities of selenium compounds. This study investigated the molecular mechanisms mediating anti-cancer activities of two chemopreventive selenium compounds. Both pXSC and SDG (and its metabolic precursor selenite) have been extensively characterised in different models of animal cancers and found to have strong chemopreventive activity against the incidence of neoplastic growth. The approach undertaken in this study was to define molecular pathways responsible for chemopreventive actions of these compounds, *in vitro*, in established cell lines. The knowledge generated here was then translated into a primary culture system more relevant to human cancers. Finally, *in vivo* validation of *in vitro* data is being undertaken using xenograft analysis of mice on selenium depleted or supplemented diets.

The study found that both compounds, pXSC and SDG, induce apoptosis in cell lines which is, at least partially, dependent on caspase 3 activation. While protein levels of the major Bcl2 family members do not change on selenium treatment, induction of Fasl appears to be necessary. Activities of several members of the MAPK family were modulated by both the compounds, and activation of JNK appear to be particularly important. JNK activation was required for induction of Fasl (particularly with SDG) and appeared to be working via the activation of c-jun. Thus a signalling pathway from JNK activity leading c-jun activation and finally Fasl induction appeared to be functionally mediating induction of apoptosis by SDG and pXSC.

Use of a panel of primary cultures of SCCs and NOMCs underlined the importance of this pathway as its relative usage strongly correlated to the extent of apoptosis induction. SCCs showed enhanced sensitivity to SDG induced apoptosis compared to NOMCs and this was reflected in the levels of induction of Fasl and JNK activity. Activation of Akt by SDG was shown to have a putative role in cell survival by inhibiting the level of apoptosis.

Immunosuppressed mice harbouring xenografts derived from human SCCs are being currently used as an *in vivo* model. Preliminary data suggests that a high dietary

concentration of selenium retards tumour growth, possibly due to tumour-selective induction of apoptosis.

8.2 Selenium concentration ranges *in vivo* and its relevance to *in vitro* studies

The relative efficacies of selenium derivatives as chemopreventive agents *in vivo* parallel their growth inhibitory effects *in vitro* and their ability to induce apoptosis (Lanfear et al., 1994; Ronai et al., 1995; Thompson et al., 1994; Wilson et al., 1992). Though evidence for growth inhibitory activities is forthcoming from several *in vivo* studies, the vast majority of the current knowledge (including all the evidence in human systems) is from *in vitro* studies in tissue culture settings. Thus a logical concern regarding translation of *in vitro* knowledge to human conditions is the dose of selenium used. While it is true that *in vivo*, factors like selective tissue selenium uptake, immune reactions and detoxification may complicate analysis, it is of paramount importance to maintain some sort of relationship with physiologically relevant doses and *in vitro* experimentation.

It appears from human studies that the plasma selenium levels have the strongest correlation with the effectiveness of selenium to inhibit cancer incidence. An analysis of data from people living in seleniferous areas reveal plasma selenium concentrations of upto 5μM, although higher than the average level, can be found in humans (Fig 8.2.1) with no apparent adverse effects (Fleming et al., 2001; Longnecker et al., 1991). *In vitro*, induction of apoptosis and modulation of signalling pathways by SDG, as described in this study, is readily detectable in this concentration range underscoring the potential relevance of this data to *in vivo* conditions. The concentrations of the synthetic selenium derivative, pXSC, required for apoptosis induction are higher than for SDG but it is well established in animal models that pXSC has a higher chemopreventive index than selenite, exerting its maximum cancer-protective effect at high dietary levels (about 30ppm compared to 1-2ppm for selenite) presumably due to its lower toxicity (Tanaka et al., 1985).

8.3 Apoptosis induction by selenium compounds

8.3.1 Non-specific toxicity and selenium

As discussed in Sections 2.5.1 and 2.6.7.3, selenium compounds can induce the generation of ROS, which may contribute to non-specific genotoxicity leading to necrotic cell death. However, at least at doses used in this study, this does not seem to be the case for both the

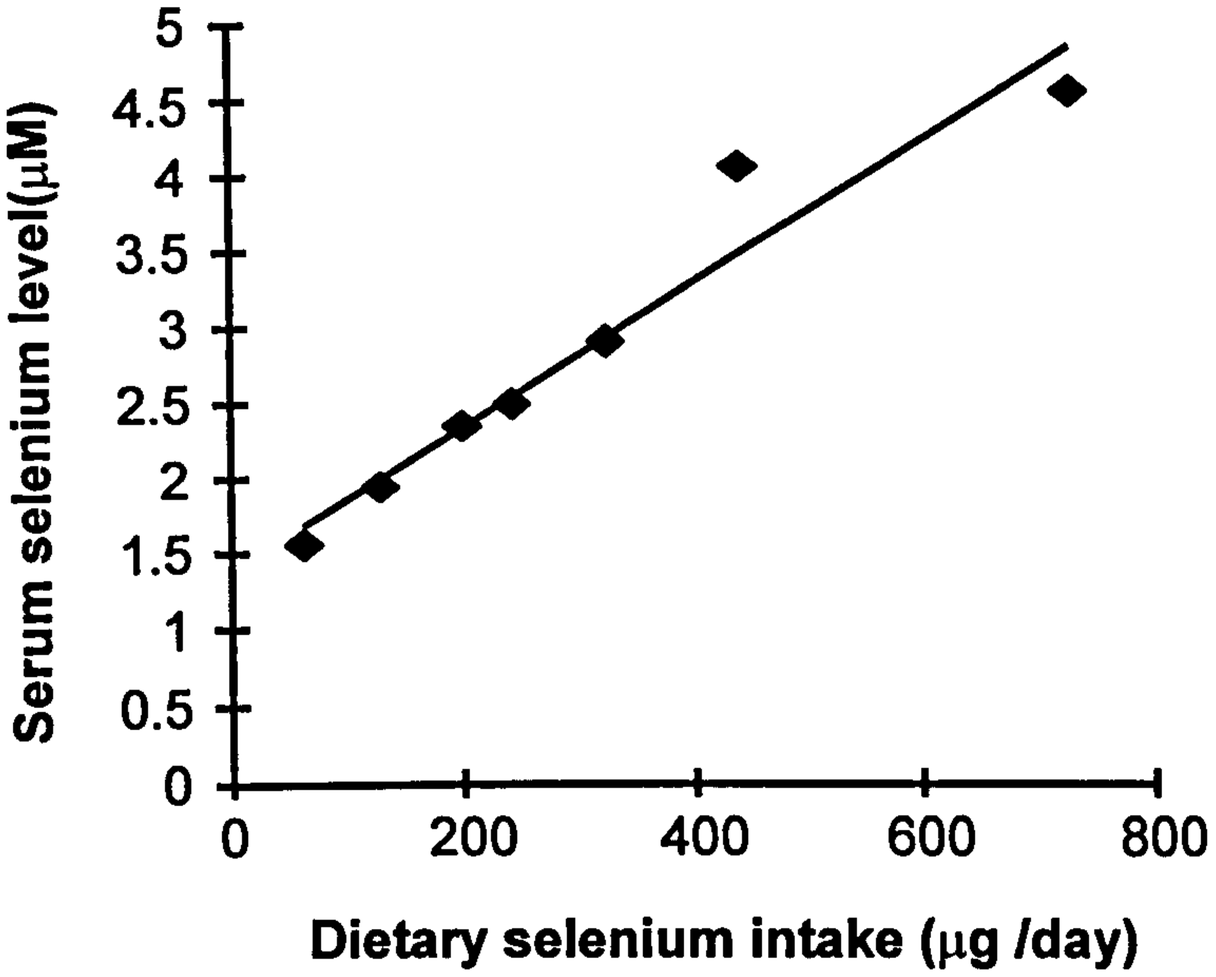


Fig 8.2.1 Range of selenium levels *in vivo* (compiled from data in Longnecker et al., 1991).

Plotting the selenium intake of people (μg/day) living in seleniferous areas against the measured serum selenium levels (μM) revealed that serum concentrations of 3-5 μM selenium can be achieved, *in vivo*, by people taking high doses of selenium without any apparent adverse effects.

SDG and pXSC as evident from AnnexinV experiments discussed in Section 4.3. Furthermore, most of the functional intervention experiments described in Chapter 5 were also reproducible using AnnexinV staining as an assay (data not shown), suggesting that the involvement of signalling pathways investigated pertain to apoptosis and not non-specific necrosis.

Stoichiometry of the biochemical reactions leading to generation of ROS by selenium indicates an equimolar ratio between selenium and H_2O_2 generated (Fig 8.3.1). However, concentration of H_2O_2 required for similar levels of growth inhibition is about 100 times higher than the selenium level required (Wu et al., 1995a). It is also pertinent to note that despite the interest in pXSC as a chemopreventive agent, it has not been shown to induce any form of non-specific toxicity. Yet, interestingly, our earlier work has revealed that free radical scavengers like mannitol can prevent pXSC-, but not SDG-, induced loss of viability and that tyrosine phosphorylation patterns of pXSC (but not of SDG) were somewhat comparable to those induced by H_2O_2 (Fleming et al., 2001). Curiously, a recent study has reported that overexpression of the same phosphorylation defective c-jun mutant that protects against selenium-induced apoptosis in our study (Section 5.3.3), *sensitises* apoptosis induced by a range of DNA damaging agents but not by other apoptosis inducers which do not cause DNA damage (Potapova et al., 2001).

ROS, for years, have been regarded as unwanted toxic by-products – a price paid for living in an aerobic environment. The large body of knowledge about cellular defences against ROS and their damaging nature has bolstered this prejudice. However, recent research suggests that ROS, in appropriate amounts, may serve as specific intracellular second messengers in signal transduction and are indispensable to normal physiology (reviewed in (Finkel, 1998; Finkel, 2000)). In cultured cells, stimulation by EGF, PDGF, angiostensin II and a host of cytokines trigger rapid production of ROS that may mediate tyrosine phosphorylation cascades. While it is true that accumulation of large quantities of ROS cause severe cellular damage, a careful investigation into the physiological role of ROS is also warranted.

Most studies of ROS induction by selenium have used chemical scavengers or overexpression of antioxidant proteins to investigate functional significance. The doses used in most studies are greatly higher than antioxidant levels achieved *in vivo* and are thus difficult to interpret. It might may well be that some selenium compounds, at certain doses (say in *in vitro* conditions), generate excessive amounts of ROS that cause non-specific toxicity, however in other situations (such as *in vivo* or in low concentrations) may be

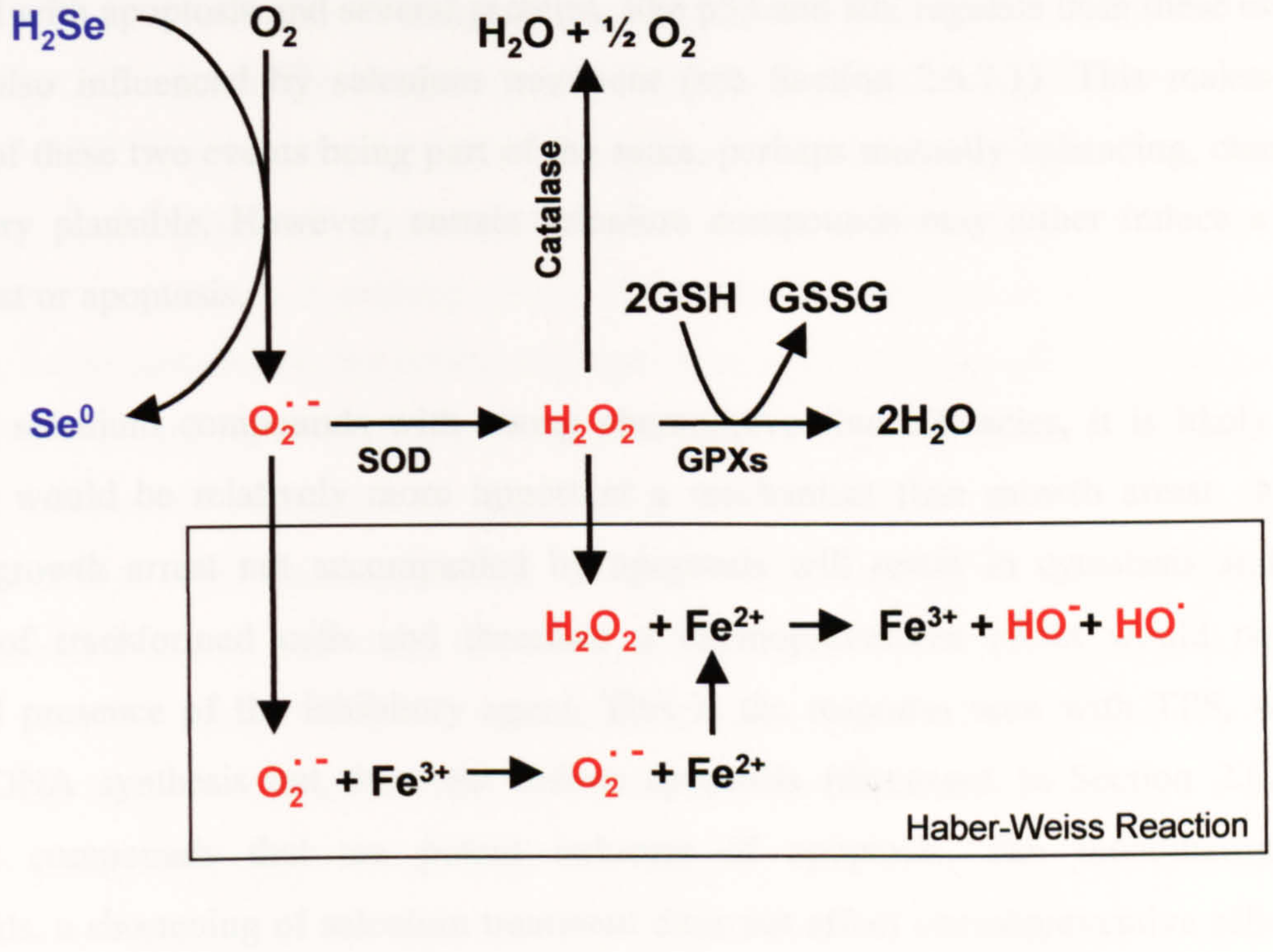


Fig 8.3.1 Generation of ROS and hydrogen peroxide from oxidation of hydrogen selenide (adapted from Harrison et al., 1997).

Schematic representation of generation of hydrogen peroxide, superoxide and free radicals from oxidation of selenium compounds. Selenide can release reactive oxygen species (ROS) which is converted to H_2O_2 by the action of SOD. H_2O_2 can either by removed by the action of GPXs or lead to the further formation superoxides via catalase activity or ferrous/ferric oxido-reductive reactions (Haber-Weiss reaction). ROS and H_2O_2 are shown in red. GSH/GSSH, reduced/oxidised glutathione; GPXs, glutathione peroxidases; SOD, superoxide dismutase.

generating just enough to mediate intracellular signalling and perhaps a regulated enhancement of ROS, may directly contribute to the anti-cancer activities of selenium.

8.3.2 Growth arrest or programmed cell death?

A large body of data implicate growth arrest and apoptosis to be important mediators of selenium-induced anti-cancer activity (Section 2.6.7.3). The cell cycle is very closely associated with apoptosis and several proteins, like p53 and Rb, regulate both these events and are also influenced by selenium treatment (see Section 2.6.7.1). This makes the scenario of these two events being part of the same, perhaps mutually enhancing, chain of events very plausible. However, certain selenium compounds may either induce a cell cycle arrest or apoptosis.

For most selenium compounds with strong chemopreventive efficacies, it is likely that apoptosis would be relatively more important a mechanism than growth arrest: this is because growth arrest not accompanied by apoptosis will result in cytostasis and not deletion of transformed cells and therefore a chemopreventive effect would require continued presence of the inhibitory agent. This is the response seen with TPS, which inhibits DNA synthesis but does not induce apoptosis (discussed in Section 2.6.7.2). Selenium compounds that are potent inducers of apoptosis, like monomethylated compounds, a shortening of selenium treatment does not affect chemopreventive efficacy. The compounds used in this particular study are of the latter class so while deregulation of the cell cycle may be concomitant and may even potentiate apoptosis, it is unlikely to be the dominant component of their chemopreventive actions. It is relevant to note in this context that previous attempts at measuring cell cycle alterations induced by pXSC have been uninformative (Ronai et al., 1995), presumably because of an overwhelming apoptotic response. Our work with SDG also indicates a similar observation (data not shown), suggesting that the selenium compounds used in our study predominantly induce apoptosis.

Also, a recent study using one of the animal models for breast cancer (methylnitrosourea-induced lesions) in which a large amount of chemoprevention work has been conducted, has reported that SMC does not alter the cellular proliferation rates in premalignant lesions, thus disputing the fact that selenium inhibits progression to malignancy via inhibition of proliferation (discussed in Section 2.6.7.2). Apoptosis, on the other hand, was easily detectable in this study. This, together with the issues discussed above, points to the need

to investigate detailed mechanisms of apoptosis-induction by chemopreventive selenium compounds, particularly *in vivo*.

8.3.3 SDG- and pXSC-induced programmed cell death

The only functional evidence in the literature describing mechanisms involved in selenium-induced apoptosis is for activation of caspase 3. Our data also implicates, particularly for pXSC, the involvement of caspase activity in apoptosis induction (Section 4.4). Caspase 3 is a distal effector cysteine protease that executes the apoptotic process (see Section 2.6.7.1). Once the caspase activation cascade is initiated leading to the activation of caspase 3, the apoptotic process is essentially irreversible. Thus, while detailed investigation into caspase 3 activity reveal the execution patterns of selenium-induced apoptosis, it does not address the critical issue of what initiates this process or what confers the specificity towards tumour cells as evidenced from the absence of non-specific toxicity in animal studies and restriction of apoptosis to tumour nests as shown in Section 7.4. Instead, studies of upstream caspases like caspases 8 and 9 may be valuable to dissect the relative contributions of the death receptor and the mitochondria mediated cell death pathways (see Fig 2.6.2). Induction of caspase 3, particularly by SDG, was very weak (Section 4.4). Also, the specific caspase 3 inhibitor reduced apoptosis only partially: this may be indicative of caspase independent mechanisms. Interestingly, it has been reported that in certain contexts the Fas pathway may induce caspase-independent cell death via RIP protein dependent induction of ROS (Vercammen et al., 1998): this may explain the relatively small induction of caspase 3, particularly by SDG which may predominantly use this alternative route. Caspase-independent cell death by SDG is also supported by the reported formation of 50kb DNA fragments with SDG (Lanfear et al., 1994) which is typical of AIF-induced apoptosis that does not require caspase activity (see Section 2.6.7.1).

A novel finding is that human SCCs are more sensitive to induction of apoptosis by the SDG than NOMCs. This is clearly of considerable relevance to understanding the cancer-protective effect of selenium compounds, particularly selenite. It is not clear why pXSC does not show the tumor-selectivity exhibited by SDG but it may reflect the relative importance of JNK/c-Jun pathway in mediating the effects of the two selenium compounds (see Sections 6.3 and 6.4.1). Selenite (SDG)- and pXSC-mediated chemoprevention studies on animal models have shown that their efficacies against various cancer types are different (for example, pXSC, but not selenite, protects from NNK-induced lung cancers). Thus, it may be that pXSC will not protect from the incidence of oral cancers in humans

while SDG (or selenite) will. However, in murine model, pXSC does show chemopreventive activity against the incidence of certain oral cancers (Tanaka et al., 1997).

As evident from selenium-mediated chemoprevention studies on animal models of cancer, while the inhibition of neoplastic growth is considerable there is no evidence of general animal health to be affected adversely. This very strongly suggests some form of tumour selectivity to be operational. This study and another reported recently (Menter et al., 2000) have for the first time demonstrated this *in vitro* and, significantly, in cancer types for which the evidence for selenium-mediated chemoprevention is the strongest (i.e. in oral and prostate cancers). The major advantage of our system over the prostate model is that it is a direct comparison between corresponding primary cultures of SCCs and NOMCs and not primary normal cells and cancer cell lines. We also provide functional evidence in our study which is lacking in the other.

Given the widespread occurrence of p53 mutations in several human cancers, it is interesting to note that selenium-induced apoptosis does not necessarily require functional p53 (discussed in Section 2.6.7.3). Both the SCCs used in our study have been shown to have dysfunctional p53. The p53 status of the NOMCs is wild type as evident from ongoing sequencing by Dr A. Muntoni and J. Fleming.

In vitro models, like the one described in this study, are invaluable tools to assess chemopreventive activity of selenium compounds. They provide an opportunity to evaluate the molecular mechanisms underlying tumour-selectivity, the very basis of the mode of action, of any promising chemopreventive agent. Apart from testing currently available selenium compounds, such a system could form the basis for a primary screen to select for novel compounds that may have potential chemopreventive activity. Again, due consideration has to be given to the cancer type-specificity of particular selenium compounds.

8.3.4 FasI and selenium-induced apoptosis

A novel finding from this study is the induction and functional requirement of FasI in SDG- and pXSC-induced apoptosis (Sections 4.5.2 and 6.3). The size of the FasI produced corresponded to the soluble form of the protein which could therefore be secreted and act intercellularly. The option of a secreted death-inducing protein is particulary relevant as it

may contribute to enhance apoptosis induction by initiating cycles of autocrine and paracrine apoptosis.

Fasl expression in biopsies of squamous carcinomas of the head and neck has also been demonstrated in a recent report and shown to be biologically active in inducing apoptosis in co-cultivated activated T lymphocytes (Gastman et al., 1999). This is of considerable interest since the Fasl/Fas receptor interaction is one of the major pathways initiating apoptosis by a variety of agents, including alkylating agents (Kolbus et al., 2000) and drugs used in chemotherapy (Debatin, 1999). Since both NOMCs and SCCs express the Fas receptor constitutively (work of J. Fleming), induction of Fasl may explain why selenium compounds induce apoptosis in human oral cells.

Fasl was induced considerably more strongly by SDG in SCCs than in NOMCs, whereas pXSC seemed to be equally effective in both normal cells and carcinomas. Thus, the extent of Fasl induction by the selenium compounds correlated closely with the level of apoptosis induced in normal mucosa or carcinomas and inhibition of the Fas pathway in SCCs by a Fas/Fc chimera attenuated the induction of apoptosis by SDG and p-XSC. Activation of the Fas pathway results in the activation of caspase 8 (see Section 2.6.7.1). Interestingly, a recent paper has reported the induction of caspase 8 by methylselenic acid but not selenite, the parent compound of SDG (Jiang et al., 2001a).

A recent study has reported the cleavage of G1/S checkpoint regulator proteins Rb and murine-double-minute-2 (mdm2) by caspase activity induced by Fas activation (Bi et al., 2001). This results in the accumulation of p53 and the E2F-1 transcription factor. As E2F-1 overexpression induces apoptosis and E2F-1-deficient cells are resistant to Fas induced apoptosis, it is possible that E2F-1 may be a mediator of selenium-induced cell death.

In vivo, the augmented levels of soluble Fasl produced by carcinoma cells could also enhance immunological responses that could target the carcinoma cells (Krammer, 2000). Tumours expressing Fasl are rapidly destroyed, with these sites regressing into abscesses (Restifo, 2000b) and this activity is thought to be mediated by the activation of caspase 1 (interleukin(IL)-1β-converting enzyme – ICE). In mouse models Fas activation has been linked to the production of IL-1β and IL-18 (Miwa et al., 1998; Puren et al., 1998) which in turn leads to the induction of the inflammatory cytokine IL-8. IL-1β is known to trigger a variety of immune activating chemokines and cytokines possibly via NF-κB transactivation (reviewed in (Restifo, 2000a)). Activation of the Fas pathway by selenium

compounds could therefore be a factor explaining their effects in enhancing anti-tumor immune responsiveness.

IL-2 is known to actively induce tumour regression and is widely touted as a major anti-tumour, immunotherapeutic agent (reviewed in (Rosenberg, 2001)). Selenium treatment can induce IL-2 mediated activities by upregulating IL-2 receptor subunits (Roy et al., 1992; Roy et al., 1994). It is therefore interesting to note that IL-2 treatment has been shown to induce Fasl expression and apoptosis resulting in the complete regression of mammary carcinomas and inhibition of neoplastic transition in mice (Wigginton et al., 2001).

In this context it is particularly interesting to note that a recent study has reported that supplementation of 200μg/day of sodium selenite in head and neck SCC patients resulted in a significantly enhanced cell-mediated immune-responsiveness, as reflected in the ability of the patients lymphocytes to respond to mitogenic stimulation, to generate cytotoxic lymphocytes, and to destroy tumour cells (Kiremidjian-Schumacher et al., 1994; Kiremidjian-Schumacher et al., 1996).

Our data show that Fasl induction by the selenium metabolite, SDG, is readily detectable at a selenium concentration within the range of plasma concentrations found in humans, although higher than the average level (Section 8.2). The concentration necessary for Fasl induction by pXSC is higher and correlates with the dose required to induce apoptosis by this compound.

8.4 Signalling pathways in selenium-mediated apoptosis

This study has demonstrated for the first time global modification of signal transduction pathways upon selenium treatment (summarised in Fig 8.4.1). Some of the modulations in MAPK activities have been shown to be functionally required for apoptosis induction. While alterations in ERK5 and possibly ERK1/2 may have a small effect on selenium-induced apoptosis, the dominant functional effect by far (and reproduced in a variety of tissue culture models) is the induction of JNK activity in response to SDG and pXSC (see Chapters 5 and 6).

JNK activity leads to the phosphorylation of c-jun at serines 63 and 73 and its subsequent transactivation (Derijard et al., 1994; Kyriakis et al., 1994) (Fig 8.4.1). Unlike other stimuli, like UV (Shaulian et al., 2000), that induce persistent JNK activation selenium

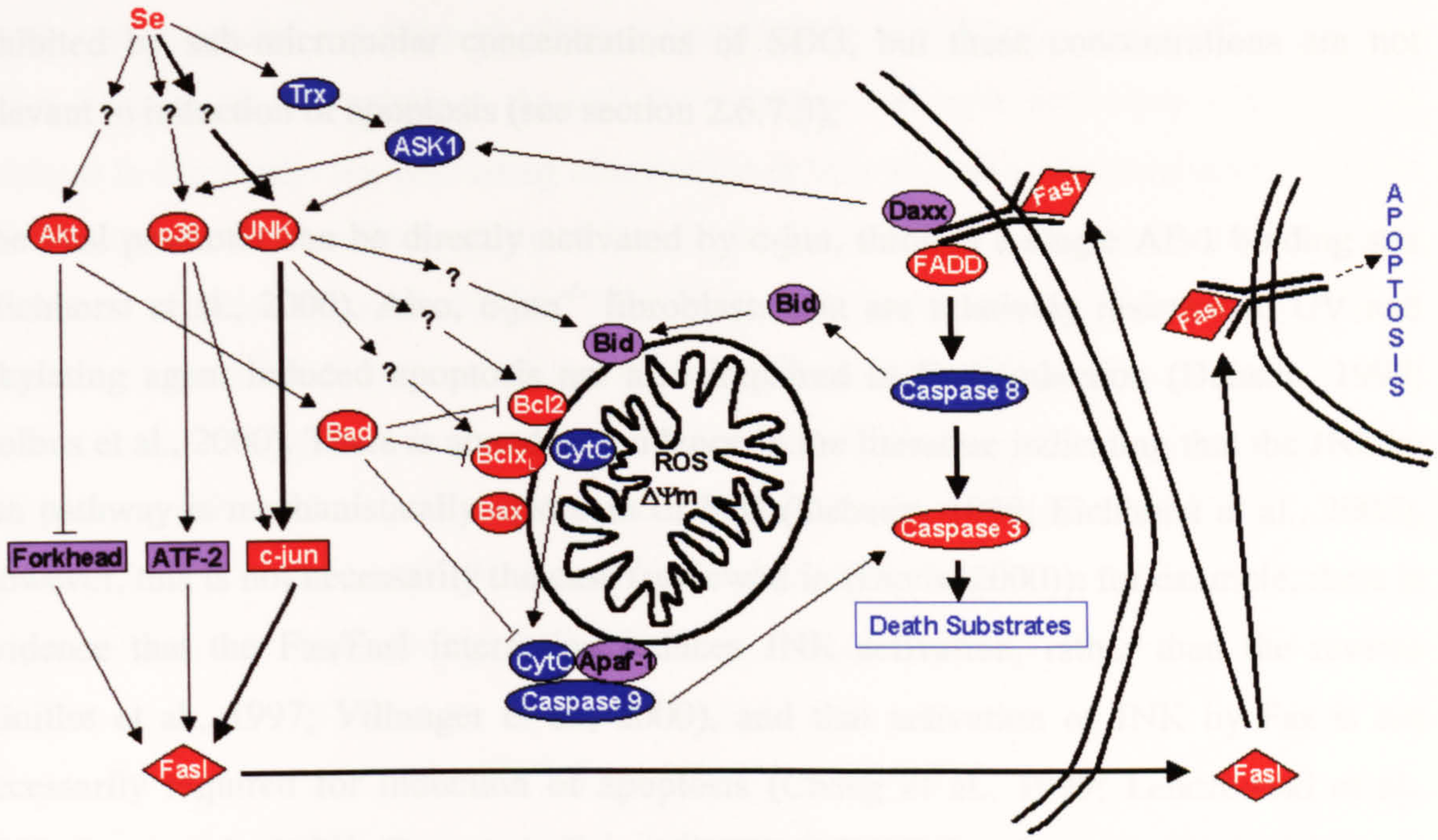


Fig 8.4.1 Schematic representation of major signalling pathways mediating selenium-induced apoptosis.

The major signalling pathways investigated in this study and found likely to be functionally involved in selenium-induced apoptosis are represented in this diagram. The molecules directly investigated in this study are in red. The thick arrows mark the signal transduction route as revealed in this study. Candidates of outstanding importance that are likely to contribute directly to the regulation of the identified apoptotic signalling route are represented in blue. Molecules whose function may also influence our model are in purple. Several other major signalling components which might influence apoptosis induction profoundly are not shown as these were not investigated, thus this scheme is, at best, a partial representation.

compounds tested did not induce c-jun protein expression (Section 5.3.2). However, c-jun transactivation is enhanced as reflected in the induction of Fasl (Section 4.5.2 and 6.3) which is attenuated by inhibition of JNK activity (Section 6.4) or expression of a dominant negative c-jun mutant (Section 5.4). c-jun homodimers and heterodimers (with other members of the jun and fos family of proteins) form the AP-1 transcription complex (Shaulian and Karin, 2001). AP-1 DNA-binding activity has been previously shown to inhibited by sub-micromolar concentrations of SDG, but these concentrations are not relevant to induction of apoptosis (see section 2.6.7.3).

The Fasl promoter can be directly activated by c-jun, through a single AP-1 binding site (Eichhorst et al., 2000). Also, c-jun$^{-/-}$ fibroblasts that are relatively resistant to UV and alkylating agent induced apoptosis are also impaired in Fasl induction (Debatin, 1999; Kolbus et al., 2000). There is abundant evidence in the literature indicating that the JNK/c-Jun pathway is mechanistically upstream of Fasl (Debatin, 1999; Eichhorst et al., 2000). However, this is not necessarily the case (reviewed in (Davis, 2000)): for example, there is evidence that the Fas/Fasl interaction induces JNK activation, rather than the reverse (Goillot et al., 1997; Villunger et al., 2000), and that activation of JNK by Fas is not necessarily required for induction of apoptosis (Chang et al., 1999; Lenczowski et al., 1997; Low et al., 1999). Recent studies on JNK1 $^{-/-}$ JNK2 $^{-/-}$ cells have shown that these cells are not resistant to Fas induced apoptosis (but resistant to UV and methyl methanesulphonate) suggesting that the JNK activation is not downstream of Fas activation (Tournier et al., 2000). Fas activation has also been shown to activate both JNK and p38 activation in Jurkat cells: however, in this case, activation of the MKK3/p38 pathway appeared to be important as a downstream target for Fas induced caspases (Juo et al., 1997). Moreover, targeted disruption of FADD, the adaptor protein required for Fas-induced apoptosis, does not eliminate adriamycin-induced apoptosis (Yeh et al., 1998). This may be because the Fas death domain of Fas binds independently to two effector molecules: FADD, which couples Fas to pro-caspase-8; and Daxx, which couples Fas to the JNK pathway, possibly activating an independent apoptotic programme (Goillot et al., 1997) (Fig 8.4.1).

One possibility is that the complexity in JNK/p38 signalling and its role in Fas mediated apoptosis is the formation of self-enhancing feedback signals. This would suggest dual functions for JNK (and possibly p38) function; first, in the activation of c-jun and Fasl induction; and second, following Fas receptor activation, to amplify the apoptotic response by activating further cell death cascades by augmenting Fasl transcription and perhaps other pathways. If this were the case, it may explain the partial protection obtained in the

intervention experiments on the JNK/c-jun/Fasl pathway. The role of p38 may be post-Fas activation, though the rapid induction of p38 activity and the lack of effect of p38 inhibitors on apoptosis induction suggests otherwise.

As far as other signalling pathways are concerned, activation of p38 and ERK 1/2 and inhibiton of ERK5 activity (though only in HeLa cells reflecting its particular importance in this cell type) did not seem to affect apoptosis induction greatly. However, these may either have a role in 'priming' the cell for apoptosis or have secondary functions not resolved in this study. An interesting observation in this context is that inhibition of ERKs 1/2 activities in HeLa cells by the specific inhibitor PD98059 enhanced selenium-mediated apoptosis slightly (Section 5.5.2). This may reflect the observation that selenium compounds inhibit activation of ERKs 1/2 by EGF (Sections 5.5.1). Forced activation of ERK5 conferred a small but consistent inhibition of selenium-mediated apoptosis (Section 5.6.2) suggesting a functional role for the observed downregulation of ERK5 activity (Section 5.6.1). In the oral cells activation of Akt by SDG, but not pXSC, appeared to constitute a potent survival signal (Section 6.6) though molecular targets of this remain obscure.

Modulation of a MAPK in isolation is not very meaningful, as its qualitative value is absolutely dependent on its context, duration of activity and to the activation status of other signalling pathways. Extensive crosstalk and redundancy underscore the tight control of the specificity of these pathways. From this perspective, it is likely that the other alterations observed in this study with apparently non-functional roles could be components of a specificity-generating process for apoptosis.

Exactly how selenium compounds induce the stress kinase pathways is not yet clear. One possibility is that selenium inhibits phosphatase action that leads to activation of kinases (usually mediated by phosphorylation). However, this is unlikely to be the case for the activation of JNK as the expression of MAPK phosphatase 1 (MKP-1), which specifically dephosphorylates and inactivates JNK (Liu et al., 1995), did not protect from growth inhibition by SDG and pXSC (data not shown). Another hypothesis to explain induction of apoptosis by SDG is that it may alter the redox status of the cells by manipulating level of a cellular reducing agent, such as Trx, which has been implicated in growth control in various contexts and is over-expressed in many tumors. This may be a plausible idea since SDG can inhibit Trx reduction by TR (see Section 2.6.5).

Of particular interest in this context is the fact that ASK1, one of the upstream activators of both JNK and p38 (Ichijo et al., 1997) (see Fig 8.4.1), is inhibited by binding of reduced thioredoxin to its N-terminal region (Saitoh et al., 1998): thus depletion of reduced thioredoxin by selenium compounds by one of the mechanisms described above might be expected to activate ASK1 and induce apoptosis. Studies on Ask1 $^{-/-}$ cells have suggested that it is critically required for persistent JNK and p38 activation (Tobiume et al., 2001), as seen with pXSC and SDG. Thus, studies of ASK1 activation by selenium compounds are clearly of some importance.

While this project was ongoing three papers appeared that had investigated the role of MAPKs in the context of selenium. The first two used non-growth inhibitory doses of selenite (nM range), therefore their relevance to cancer-chemopreventive mechanisms is debatable. One showed that selenite inhibited peroxynitrite-induced activation of p38 but not JNK (Schieke et al., 1999). The second study reported selenite-mediated inhibition of UV-induced JNK and p38 activity (Park et al., 2000b). Similar data was reported for selenite mediated inhibition of caspase 3 activity (Park et al., 2000a). However, the use of non-growth inhibitory doses make these studies irrelevant to the present discussion and presumably reflect the ability of low doses of selenite to stimulate antioxidant activities in the cells. The third report addressed the issue of apoptosis induction by selenium compounds directly and reported the activation of p38 and JNK kinase activities by selenite (the metabolic precursor of SDG) but not methylselenic acid (Jiang et al., 2001a), however no functional evidence was forthcoming.

8.4.1 A two-stage cellular response to selenium treatment

The activation of p38, JNK, c-jun (and thereby, indirectly, AP-1) and caspase 3 found in our study is in apparent contrast to data obtained by non-apoptosis inducing, sub-micromolar doses of selenium (selenite and SDG) discussed above and in Section 2.6.7.3. Cellular responses to selenium treatment appear to reflect the two-step cancer prevention model described in Section 2.5.3. At low concentrations, selenoprotein activities are induced resulting in alterations in redox potential which *quench* the redox-dependent activation of the mentioned entities (JNK, AP-1, caspase 3 etc.) (Fig 8.4.2). At higher concentrations, more akin to those showing chemopreventive activities, selenoprotein activity is already saturated and selenium metabolites induce growth inhibitory pathways reflected in the *activation* of these molecules (Fig 8.4.2). As discussed in Section 2.6.7.3 inhibition of NF-κB DNA binding activity has also been shown for selenite treatment, again the doses used were far lower than those inducing apoptosis.

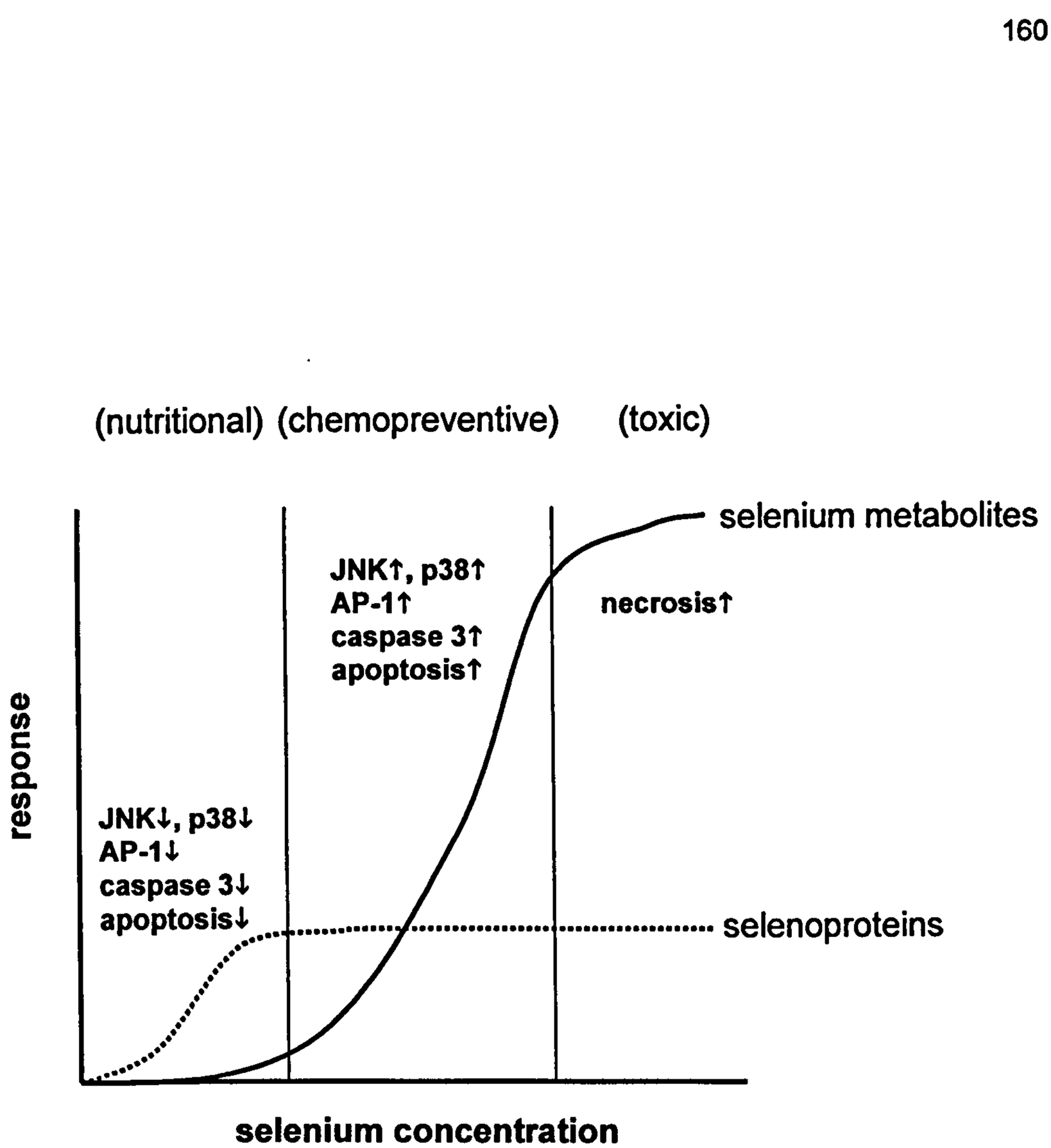


Fig 8.4.2 Two-stage model for selenium-induced *in vitro* cellular responses.

This model shows that selenoprotein activities increase in response to low doses of selenium treatment which can explain the inhibition of apoptosis, stress kinases, AP-1 and caspase 3. At higher concentrations, selenoprotein activities are saturated and apoptosis/cell cycle arrest is induced with concomitant increase in JNK/p38, AP-1 and caspase 3 activity. Even higher concentration result in non-specific toxicity. The role of signalling components in the toxic dose ranges have not been investigated. The words in parentheses refer to equivalent response *in vivo*.

In terms of cancer prevention, both these responses could result in a positive synergy. Selenium supplementation and the resultant saturation of selenoprotein activities could conceivably protect against damaging oxidative stress in selenium deficient individuals while the activities of the selenium metabolites may actively inhibit neoplastic growth in selenium replete individuals.

8.5 Tumour selectivity of selenium compounds: a molecular hypothesis

Chemopreventive selenium compounds appear to specifically target transformed cells, as normal cells and tissues appear to be generally unaffected. This study and another on prostate cells (Menter et al., 2000) referred to earlier have recapitulated this *in vitro*. It remains a critical issue to elucidate the underlying mechanisms of the selectivity. Our study implicates the relative use of the JNK/c-jun/Fasl signalling pathway to be critical, however the molecular basis of this differential activation remains unresolved.

Neoplastic transformation occurs as a result of mutations which lead to the changes in the structure and function of the proteins they encode. Cellular transformation, clonal expansion and progression result from accumulation of abnormal protein/s in cell. This genotypic, and the resultant phenotypic, alteration distinguishes normal cells from mutated cells in the same tissue. As selenium compounds cannot, conceivably, detect alterations at the genetic level, abnormal proteins are its most likely targets.

As discussed in Section 1.6, low molecular weight selenium compounds can undergo a number of characteristic biochemical reactions. It can therefore be hypothesised that chemopreventive selenium compounds may selectively counteract the activities of oncoproteins by direct interactions while leaving the wild-type protein unaffected. Such an alteration could not only abrogate oncoprotein activity, it may potentially confer novel chemical functionality, perhaps with respect to redox regulation. Such a model could therefore form the basis for tumour-selective activity of selenium compounds.

Given the versatility of selenium mediated biochemical reactions, there are a number of conceivable ways such an activity could be achieved. There is considerable evidence that activating mutations in proto-oncogenes often manifest in the substitution of glycine, arginine or serine by cysteine (see the following paragraphs for evidence). It is feasible that selenium compounds can bind to the sulphur of the thiol group (of the substituted cysteine) as an adduct and alter the oncogenic property/s of the protein in question (e.g. type 2

reaction in Section 1.6). Other selenium mediated biochemical modifications like formation of selenotrisulphide bond (S-Se-S) and formation/disruption of disulphide bonds are candidate mechanisms that could potentially alter the acquired functionality of oncoproteins which have gained thiol containing aminoacids (like cysteine) by mutational events. In this way selenium could selectively alter the properties of transformed cells but not of untransformed, normal cells.

Signal transduction proteins with altered activities are often oncogenic and frequently acquire cysteine substitutions. This, according to our hypothesis, could allow selenium compounds to differentially influence signalling pathways between transformed and untransformed cells. For example, cysteine substitutions are quite common in K-ras which lead to its acquisition of transforming activity. Glycine to cysteine activating mutations in K-ras have been seen in sporadic hepatic angiosarcomas (Przygodzki et al., 1997), oral squamous cell carcinoma (Kuo et al., 1994) and in non-small cell lung carcinomas (where it is an indicator of poor prognosis) (Noda et al., 2001; Slebos and Rodenhuis, 1992).

Constitutively-activating mutations of signalling molecules are frequently oncogenic. Often activation of signalling proteins are dependent on dimerisation and cysteine substitutions could aid this process by forming covalent links via disulphide bonds. Stat-3, when constitutively activated, is a putative oncogene and is frequently mutated in several tumours, particularly in head and neck cancers (Garcia and Jove, 1998; Grandis et al., 1998). It is thought cysteine substitutions in Stat-3 results in dimerisation (via disulphide bridges) leading to constitutive activity (Bromberg et al., 1999). Mutation in GTPases constitutively activate α subunits of signal-transducing guanine nucleotide-binding proteins (G-proteins) and arginine to cysteine mutations have been documented in the activation of several G proteins, like the oncogene gip2 (Pace et al., 1991).

Apart from direct modifications of signal transduction components, similar alterations in growth factor receptors could be affected by selenium and thereby influence cellular signalling. Activating cysteine substitutions in growth factor receptors, like fibroblast growth factor receptor 3 (FGFR3), have been have been reported to be associated with a number of cancers (Wu et al., 2000).

Alternatively, mutations in genes whose products protect against cancer initiation may act as oncogenes. Increased risk of oesophagal SCC incidence has been related to serine to cysteine mutations in the hOGG1 gene (Xing et al., 2001). The hOGG1 gene product is necessary for the repair of oxidative DNA lesions, the activity of which is abrogated by

this mutation (Kohno et al., 1998). Curiously, the study which found this association was carried out on the same population which showed decreased mortality of oesophagal and gastric cancer in an intervention trial with supplementation with antioxidants, including selenium (Blot et al., 1993). A recent follow-up study has shown that participants in this trial who had enhanced plasma selenium levels, as a result of supplementation, had significantly reduced risk of developing these cancers (Mark et al., 2000).

The suggested hypothesis would predict that the efficacy of selenium as a chemopreventive agent will be depend on the relative amount of the activated tumour-promoting proteins (levels of one particular protein or different ones) and available selenium for biochemical activity (eg. adduct formation). This fits in rather well with the evidence from animal studies where selenium appears to have a more profound effect at initiation stages (presumably lower number of mutational events) than on later ones (el-Bayoumy et al., 1992; Ip et al., 1995; Thompson et al., 1982). Also chemopreventive action of selenium compounds is generally dose-dependent (Ip et al., 1991; Ip et al., 1995; Reddy et al., 1997). Several studies have shown that higher doses of the initiating carcinogen tend to shift the anti-tumorigenic effects of selenium to the post-initiation phase (Ip and Daniel, 1985; Ip et al., 1981). This again could be explained by a temporal delay before selenium compounds have altered the activities of the oncoproteins in sufficient levels to display an effect at the organismal level.

It should be cautioned that early effects of selenium in chemical-induced cancer models can be attributed, at least partially, to carcinogen detoxification, reduction of oxidative stress and/or inhibition of carcinogen-DNA adduct formation (see Section 2.6). These phenomena suggest a possible role for selenoprotein induction and the hypothesis presented above need not be invoked.

To test this hypothesis, the following preliminary information/experiments would be useful: a literature trawl should reveal the key gene products with known oncogenic potential for those cancer types most effectively protected against by selenium supplementation. Elucidation of differentially activated pathways between tumours and corresponding normals, resulting from *in vitro* studies such as those described in Chapter 6, could also suggest potential candidates meriting such investigations. Data from pre-existing polymorphism studies could be screened to identify cysteine substitutions with putative contribution to activity. Overexpression of wild-type and cysteine substituted tagged candidate proteins in cells treated with or without radiolabelled selenium followed by immunoprecipitation, measurement of incorporated radioactivity and electrophoretic

mobility shift should indicate selenium-adduct formation. This approach is obviously not applicable to selenoproteins and other proteins that may physically bind selenium. More precise characterisation of candidate proteins is possible by exploiting recent advances in mass spectroscopy. Potential candidates would then have to be investigated further to see if their normal and oncogenic activities are altered in response to adduct formation.

Finally, selective uptake of selenium by tumour cells could also be a component of selectivity. However, no evidence for a mechanistic pathway that may facilitate such an activity is currently available. Also, the relatively broad spectrum of cancers affected by selenium treatment makes this possibility unlikely as one would expect either a global or very tissue specific enrichment of selenium.

8.6 Translational considerations and chemoprevention strategies

A candidate chemopreventive agent ideally has to satisfy several criterion before it can be can recommended for general use. These would include supporting evidence from epidemiological and animal studies; detailed knowledge of metabolism and identification of active metabolites; knowledge of most likely mechanisms at the molecular and cellular level; low incidence of adverse effects that are easily reversible; knowledge of endpoint biomarkers and the ease of administration. Finally, the possibility of developing 'fine-tuned', second generation chemopreventive agents based on the features of the original should also be an important criteria as this may allow greater flexibility, increased accessibility and also enhanced specificity.

While evidence in support of anti-tumorigenic activities of selenium has been accumulating from both epidemiological studies and those on animal models, much less is known about the mechanisms involved. Similarly, selenium metabolism is reasonably well characterised but there is a vigorous debate regarding the identity of the active metabolites. Surrogate end-point biomarkers are usually derived from the underlying mechanism/s of action. As these molecular and cellular dynamics have remained elusive, direct end point markers for selenium mediated anti-tumour activity have been largely unavailable.

This study has, for the first time, identified signalling pathways that form the underlying molecular/cellular basis for selenium-induced apoptosis - considered to be a major candidate mechanism of anti-tumorigenic activity. Also, by demonstrating the induction of Fasl that can be secreted into the extracellular space, a putative candidate that may influence the anti-tumour immune functions of selenium has been recognised. These

findings have implications on addressing some of the major issues relevant to the use of selenium compounds as chemopreventive agents.

Assessment of effects of selenium on cancer incidence without using cancer as an endpoint is an almost insurmountable problem. Surrogate endpoint biomarkers offer the potential of evaluating the efficacy of chemopreventive agents without the high costs, and time commitments of large-scale intervention trials. Therefore it is crucial to identify and develop candidate surrogate markers of selenium efficacy and safety. Apoptosis appears to be a plausible component of anti-tumourigenic activities of selenium. Therefore it can be considered as a putative surrogate biomarker to directly assess efficacy of selenium compounds. Selenium has been shown to induce apoptosis in xenografts under conditions where xenograft growth is reduced (Chapter 7). Correlating chemopreventive activities with the ability to induce apoptosis, *in vivo*, could therefore provide a measure of the potential of apoptosis as a biomarker assessing efficacy of selenium compounds. Similar approaches could also be used to assess JNK activity (using phospho-specific antibodies) and Fasl induction as candidate biomarkers.

In humans, procedures to detect apoptosis and JNK activation are likely to be complicated and of an invasive nature. It is unlikely enough biomaterial could be obtained from non-invasive procedures, such as sputum or urine collection, to evaluate these markers convincingly. Instead, the use sensitive ELISA based assays that can detect small increases of circulating Fasl in plasma may be of more practical use since induction of Fasl appears to correlate with the extent of apoptosis induction (Sections 4.5.2 and 6.3). It should be cautioned that some cancers result in higher levels of circulating Fasl which may mask any selenium-induced increase (Anastassiou et al., 2001; Bennett et al., 2001): confounding issues such as this needs to be investigated thoroughly. The issue of circulating Fasl will be addressed, *in vivo*, in the course of our ongoing xenograft analysis.

The Clark, 1996 study reported a significant reduction in the incidence of mortality due to cancer of the lung, prostate and colon. This was achieved by supplementation of the diet with 200μg of selenium per day. While this report has generated a lot of interest in using selenium as a chemopreventive agent, little has been achieved in the form of comparative analysis of these clinical findings with what is known from *in vitro* and animal studies. In this trial selenium was administered in the form of selenised yeast, which is a complex source of selenium compounds like SM, SMC, selenocysteine and selenothionine (Bird et al., 1997; Ip et al., 2000b) – thus the form of selenium responsible for cancer prevention remains undefined. It is thus of paramount interest to identify the active form. Detailed

knowledge of molecular/cellular mechanisms mediating the induction of cell death by selenium compounds could also be a tool in the identification of active metabolites of selenium. This would particularly be the case for pathways, like the JNK/c-jun/Fasl, relative use of which is tightly linked to the extent of apoptosis. If such a pathway was critical to the anti-cancer activities of selenium, then one might expect that the active metabolites would induce this pathway more efficiently than other compounds. Obviously such an approach could be confounded by stability and specific biochemical transformations of the particular metabolite, but when interpreted together with data from *in vivo* chemoprevention studies may yield valuable insights.

Molecular mechanisms of action could also provide ample evidence for specificity, efficacy and for possible adverse side-effects. The other aspect where such knowledge will prove invaluable is in the field of novel drug development. Based on identified molecular/cellular mechanisms new drugs can be specifically tailored for enhancement of efficacy and minimisation of toxicity. This is particularly a possibility for organic selenium compounds. However, such an approach primarily needs the establishment of detailed structure-function relationships. Therefore, how alteration in the structure may affect induction of JNK, say, might provide useful data for design of novel anti-tumourigenic selenium based compounds. On the other hand if activities of selenoproteins are involved in cancer-protective effects, then gene therapy approaches ectopically modifying expression/activity of critical selenoproteins need to be considered.

Laboratory animals and *in vitro* studies have greatly aided the understanding of mechanisms responsible for the protective role of selenium against cancer. How far this knowledge is applicable to humans remains unclear. Therefore there is a need to initiate pilot studies to determine the role of various forms of selenium (particularly those approved for human use) on cellular and molecular targets that are critical to the process of carcinogenesis. These studies are necessary to learn whether data obtained in laboratory animals and *in vitro* are applicable, if so how far, to humans. Again it is in the selection of these candidate molecular/cellular targets, whose investigation is proposed, that knowledge obtained from this and similar studies will be invaluable.

8.7 Future work

This section discusses some of the issues that need to be addressed in order to obtain a clearer picture of selenium-induced programmed cell death. Some deal with mechanistic

considerations resulting directly from this particular study (see Fig 8.4.1) while others are peripheral issues of considerable importance.

8.7.1 Mechanistic considerations of SDG- and pXSC-induced apoptosis.

In order to characterise the molecular mechanisms underlying apoptosis induction by SDG and pXSC, the expertise developed during the course of this study needs to be extended and appropriately exploited. Work presented here, taken together with a survey of the current literature, suggests that there are several mechanistic pathways, working at different levels that may mediate cancer protective effects of selenium; thus the picture is far from complete. It is obvious from the intervention studies that though the JNK/c-jun/Fasl pathway is important, it does not completely account for the level of apoptosis induction. All interventions produced only partial abrogation of apoptosis suggesting other mechanisms are also involved. A simplistic, linear route has been characterised (see Fig 8.4.1), whereas, current knowledge of other signalling mechanisms suggests that pathways rarely represent a simple, linear chain of events. Thus, functions of other contributing factors need to be addressed.

Within our specific model of proapoptotic signalling several issues need to be addressed (see Fig 8.4.1 and discussion in Section 2.6.7.1). Mitochondria mediated cell death plays a pivotal role in apoptosis and is regulated by the Bcl2 family of proteins. However, at the protein level, no alterations in the Bcl2 family members were detectable. Given their critical role in regulating apoptosis and evidence in the literature about post-translational modifications, particularly inhibitory modifications mediated by JNK, investigation into such phenomenon is warranted. Release of cytochorome c from the mitochondria is an early event in apoptosis induction via these organelles. Therefore, an initial investigation assessing cytochrome c release should indicate the involvement of mitochondria. If this were so, detailed analysis of whether post-translational modifications alter Bcl2 activities could be considered. Relative contributions of receptor mediated and mitochondria-dependent apoptosis can be assessed by the relative activations of caspases 8 and 9, respectively. However, there is indication that selenium compounds activate both capsase 8 and 9, suggesting both these pathways may be acting concomitantly.

The role of p38 induction remains intriguingly unresolved. Its activation kinetics suggests a putative role but this was not confirmed in the functional intervention studies in the context of apoptosis induction by SDG and pXSC. However, p38 can induce cell cycle arrest and potentiate apoptosis indirectly in other contexts (Bulavin et al., 2001). If this

were the case, initiating an acute apoptotic response could obscure its early activity. To investigate this possibility, synchronously growing cells will need to be treated with a broad dose range of selenium compounds and their progression through the cell cycle observed.

The functional role of Akt as a survival signal (in SDG treated SCCs) is also very intriguing. Akt can directly block transcription of Fasl by inhibiting transactivation of the Forkhead transcription factor (see Fig 8.4.1) (Brunet et al., 1999). As overexpression of c-jun dominant negative and JNK inhibition only reduces Fasl induction partially, it remains a possibility that other transcription factor regulate its transcription. Initially, the effect of inhibition of Akt activation on the induction of Fasl could be measured. Akt can also directly inhibit capspase 9 activity (Bulavin et al., 2001), as selenite has been shown to process pro-caspase 9 but not enhance its activity (Jiang et al., 2001a), it is possible that Akt activation functions to inhibit casapase 9 activation. Initially this could be tested by simply assessing caspase 9 activity in response to selenium in the presence or absence of a specific PI3K inhibitor. Similar inhibitor-mediated experiments could be performed to assess whether Akt activation retards c-jun activity as has been previously reported (Nikolakaki et al., 1993).

As discussed in Section 8.4, ASK1 is a potential upstream target for induction of apoptosis and stress kinase activation. It is suggested that its redox regulation via thioredoxin may make it a suitable target for modulation by selenium compounds. This hypothesis can be tested by primarily looking for activation of ASK1 by selenium compounds at apoptosis-inducing doses. Our initial studies failed to detect any substantial activation, however these are inconclusive as known inducers of ASK1 also failed to show any effect on its activation. Optimisation of the technique is currently ongoing. If ASK1 activation is implicated, dominant negative mutants of ASK1 will be used to resolve its contribution to apoptosis induction. Modulation of ASK1 activity by thioredoxin can be initially tested by immunoprecipitation to analyse changes in ASK1-thioredoxin binding in response to selenium treatment. If necessary, further characterisation including assays for alterations in subcellular localisation can be performed using immunocytochemistry.

Three major signalling entities known to influence apoptosis significantly and whose modulation by selenium is already recorded are PKC, PKA (see Section 2.6.7.4). Investigation of the activities of these molecular entities at apoptosis-inducing doses followed by functional intervention using overexpression and pharmacologic strategies are necessary to dissect their role/s. It is interesting to note that Fas mediated apoptosis may be

inhibited by PKC activation (Gomez-Angelats et al., 2000; Gomez-Angelats and Cidlowski, 2001), this may suggest that the inhibition of PKC observed on selenium treatment may have a functional role. The transcription factor NF-κB induces the expression of several apoptosis suppressing proteins of the Bcl2 and IAP families (Reed, 2001). Therefore it is intriguing to note that selenite can inhibit NF-κB activity (Kim and Stadtman, 1997), though at non-apoptosis inducing doses. This therefore warrants investigation of NF-κB status at growth inhibitory condition and whether any such modulation is functionally significant to apoptosis-induction.

The relevance of signalling pathways needs to be investigated *in vivo*. With the advent of phosphorylation state specific antibodies, descriptive studies should be relatively less complicated. However, intervention approaches are still technically limiting and require complicated genetic procedures. A sensible approach to *in vivo* signalling studies, given the limited amount of biological material and complicated procedures involved, is a translational approach of tissue culture knowledge to look for targets that are likely to be involved. It is from this consideration that the xenograft approach described in Chapter 7 was initiated and should be instrumental in validating several mechanistic issues *in vivo*.

8.7.2 Broader perspectives on selenium mediated chemoprevention

As discussed in the Chapter 2, the biological activities of selenium are properties of its various covalent compounds rather than intrinsic properties of the element *per se*. Keeping this in mind and the vigorous debate on the identity/s of active compounds, there is a need to investigate whether a molecular mechanism identified for a particular compound is relevant to other compounds. *In vitro* assays, as described in this study, can be used to test different selenium compounds for candidate mechanisms. While this study shows the natural metabolite, SDG and the synthetic organoselenium compound, pXSC to share similar mechanisms, it may not necessarily be so for other compounds. Monomethylated selenium compounds are reported to mediate enhanced chemopreventive activities accompanied with reduced non-specific DNA damage in comparison to inorganic compounds (see Section 2.5.1). It has been recently reported that methylselenic acid did not activate JNK at apoptosis inducing doses, however p38 activity is induced (Jiang et al., 2001a). In contrast, recent work by J. Fleming has shown that SMC (a methylselenic acid precursor) induces JNK activation at apoptosis-inducing doses. While there is some evidence that the modulation of the MAPKs may be a common feature of several chemopreventive selenium compounds, no overarching conclusions can be made until all the major compounds are uniformly tested and relative contributions assessed.

A major step in understanding tumour-specific effects of selenium treatment would be the qualitative and quantitative analysis molecular mechanisms underlying anti-cancer activities of selenium compounds. The use of sensitive cDNA microarray technology can provide a global perspective of selenium-induced differential gene expression profiles in normal and tumour tissues. Apart from implicating differential gene expression (for e.g., Fasl), our study also suggests that differential modulation of activities of signalling entities (for e.g., JNK) may form the basis of tumour-selectivity. To resolve such issues proteomic approaches need to be considered.

All mechanistic phenomenon underlying anti-tumour activities, particularly programmed cell death, of selenium compounds in tissue culture settings need to be validated *in vivo*. This is also the route for identification of novel biomarkers of selenium activity as a chemopreventive agent. As seen with Fasl and immune enhancement by selenium, a study of underlying mechanisms of a particular process may often reveal interesting clues to possible molecular/cellular basis of other, perhaps not directly overlapping, phenomenon. Validations of these processes *in vivo* will not only address the primary activity under scrutiny but may also reveal mechanistic foundations of other related activities.

Chemoprevention of cancer is likely to have a significant impact on the health of the world's population. One of the candidates likely to be considered (perhaps in combination with other agents) is selenium. While evidence from laboratory animals and clinical intervention trials consistently report a reduction in cancer incidence on selenium supplementation, the molecular mechanisms underlying this anti-cancer activity remains unresolved. Advances in the recent years have implicated several candidate mechanisms, but translation of this body of data (mainly *in vitro*) to clinical situations has remained agonisingly slow. Its is recommended that a network of clinicians, chemists, biologists, public health practitioners and epidemiologist be established, as in the chemotherapeutic field, to aid the translation of laboratory findings to human application. In the same context, a comprehensive database of pharmacologic properties of selenium compounds along with their candidate mechanisms of action needs to be developed to help the process of translation.

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10 Appendix

Publications arising from this work are included in this section:

1) Ghose, A., Fleming, J. and Harrison, P.R. (2001). Selenium and signal transduction: Roads to cell death and anti-tumour activity. Biofactors *14*, 117-123.

2) Ghose, A., Fleming, J., El-Bayoumy, K. and Harrison, P.R. (2001). Enhanced sensitivity of human oral carcinomas to induction of apoptosis by selenium compounds: involvement of Mitogen activated protein kinase and Fas pathways. Cancer Res. *61*, 7479-7487.

3) Fleming, J., Ghose, A. and Harrison, P.R. (2001). Molecular mechanisms of cancer prevention by selenium compounds. Nutr Cancer *In Press*.

Selenium and signal transduction: roads to cell death and anti-tumour activity

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Abstract

Accumulated evidence from prospective studies, intervention trials and studies on animal models of cancer have suggested a strong inverse correlation between selenium intake and cancer incidence. Several putative mechanisms have been suggested to mediate the chemopreventive activities of selenium: of these, the inhibition of cellular proliferation and the induction of apoptosis are particularly attractive.

The mitogen activated protein kinase (MAPK) pathways are known to be important regulators of cell death and our recent work has focused on the involvement of these pathways in selenium-induced apoptosis in primary cultures of oral cancers and corresponding normal mucosa derived from biopsy material.

Using this system, the oral carcinoma cells were found to have enhanced sensitivity to apoptosis when treated with certain selenium compounds compared to normal oral mucosa. Induction of Fas ligand was associated with selenium-induced apoptosis.

Signal transduction studies suggests that selenium induces several changes in the MAPK signalling pathways but functional intervention/inhibitor studies indicate that activation of the JNK pathway seems to be most important.

1. Introduction

Selenium is an essential trace nutrient of fundamental biological importance. Apart from its normal physiological role in maintaining the activities of a number of selenoproteins with important antioxidant or detoxification functions, selenium has also been implicated in several disease conditions. Recent research suggests that selenium-deprived populations are at risk of several pathological disorders such as exacerbated viral disease progression (e.g. HIV infection), loss of

immunocompetence, defects in reproduction, cardiovascular disease, negative influence on mood and several inflammatory conditions (for a recent review see [24]).

Several prospective studies and recent intervention trials suggest that the risk of several cancers are inversely related to selenium intake at plasma levels in the range of 0.5 - 2μM [4] (reviewed in [10]). Work with animal cancer models also strongly supports this evidence: the maximum chemopreventive effect occurs at supra-nutritional, but non-toxic, dietary levels (for review see [6,11]), but there is also less consistent evidence that selenium deprivation increases cancer incidence/growth rates [5]. This has therefore led to a considerable interest in the mechanisms that may be involved in the cancer-protective effect of selenium.

Several putative mechanism have been suggested to explain the cancer protective role of selenium, but none appear to account for the observed dose response relationship. As selenium is known to be essential for the activities of several antioxidant and redox regulatory enzymes in the form of selenocysteine in the catalytic site, the resulting increased ROS scavenging activity at higher selenium levels is a plausible mechanism [27]. This increased antioxidant activity probably accounts for the inhibition of UV-induced apoptosis after pre-treatment with low concentrations (nM range) of selenium [21,22]. Selenium also induces Phase II detoxification enzymes and influences the carcinogen detoxification process [3]. However, the activities of these selenoenzymes are saturated at selenium concentrations considerably less than those shown to have maximum chemopreventive effect in animal models [35]. This implies that even though the antioxidant and carcinogen detoxification processes may contribute significantly to the chemopreventive effect, other mechanisms must also be involved.

Selenium appears to have a protective effect at various stages of carcinogenesis. It is thought that it is most effective in the early stages but there is also evidence of it influencing later stages of cancer progression [14]. It is therefore very likely that selenium works through several different mechanisms which vary in importance at different stages of cancer progression. Thus, the carcinogen detoxification and ROS scavenging activity induced by selenium may be more relevant at the early stages of carcinogenesis, whereas others, like growth inhibition and apoptosis induction, may be critical in later advanced stages.

A strong correlation has been observed between the effectiveness of selenium compounds as chemopreventive agents *in vivo* and their ability to induce growth arrest and apoptosis *in vitro* [18,26]. Extensive animal studies have also confirmed the inhibition of cellular proliferation and apoptosis induction at chemopreventive conditions *in vivo* [7]. As these mechanisms can potenially prevent clonal expansion of transformed cells and remove carcinogen initiated cells from the body, their induction by selenium could be of some importance.

In this article we review the accumulating evidence for selenium-induced apoptosis as a major chemopreventive mechanism and discuss the potential molecular mechanisms which may be involved. In the past decade, several molecular switches regulating apoptosis have been discovered and there is considerable interest in exploiting this knowledge to design novel anti-tumour drugs. As chemopreventive concentrations of selenium compounds induce apoptosis, it is worthwhile to study the mechanisms involved and to identify the actual enzymes/proteins that are the targets of selenium action. Since known selenoproteins may not be the enzymes mediating the cancer

protective effects of selenium, such studies may identify new biomarkers suitable for evaluating functionally relevant levels of selenium *in vivo*.

Signal transduction processes mediated by the MAPK's (Mitogen activated protein kinase) regulate cellular responses, like proliferation and apoptosis, in response to extracellular stimuli. We have therefore systematically invesigated whether selenium-mediated changes in the MAPK pathways are responsible for the induction of apoptosis by selenium compounds.

2. Selenium and apoptosis

The first evidence for selenium induced apoptosis came in the early 1990's from *in vitro* studies on several different cell lines of both human and rodent origin [18,31]. This was soon followed by *in vivo* experimental evidence in animal cancer models clearly establishing the involvement of apoptosis in selenium mediated anti-cancer activities [7,13]. There was an initial controversy that selenium compounds, selenite in particular, induced necrotic cell death associated with single strand DNA damage; however, further reports using methylated forms of selenium have successfully dissociated DNA damage induced necrosis from the apoptotic response observed [33].

It has always been evident that the chemopreventive abilities of selenium compounds vary depending on their chemical structure. Monomethylated compounds are especially effective as chemopreventive agents, with much reduced non-specific toxicity compared to other forms, like selenite [33]. The fact that these monomethylated derivatives induce apoptosis rather than a necrotic cell death underlines the importance of apoptosis as a major chemopreventive mechanism. Another illumminating case is that of triphenylselenonium which inhibits DNA synthesis and consequently induces cytostasis but not apoptosis [12]. As this does not

destroy transformed cells in the body, this compound is only effective when present continuously and loses its efficacy as a chemopreventive agent when the treatment is withdrawn [12].

Our group, using a mouse erythroleukemia cell line, was able to show in 1994 that induction of apoptosis by selenodiglutathione (SDG), the primary metabolite of selenite, was independent of wild-type p53 activity [18]. This has since been confirmed for methylselenocyanate in a mouse mammary cell line with a p53 null phenotype [15] and for selenomethionine in a colon cell lines expressing a mutated p53 [25]. As p53 mutations are present in a majority of human cancers, the fact that selenium-induced apoptosis is p53 independent is of potential usefulness.

Alterations in cell cycle proteins and inhibition of DNA synthesis have also been observed with selenium treatment. A detailed study using synchronised cells revealed that Se-methylselenocysteine treatment resulted in a prolonged delay in the S-phase, rather than a classical cell cycle block [30]. This was coincident with a significant loss of cdk2 activity and was preceded by an early transient decrease in cyclin E levels allowing the cells to enters S-phase but then impeding further cell cycle progress [30]. Consistent with this, inhibition of DNA synthesis activity has also been reported and has been attributed to the loss of thymidine kinase activity [32]. Whether this delay sensitises cells to apoptosis or is an independent effect remains to be investigated.

Thus, to summarise, the current consensus is firstly that selenium induces apoptosis both *in vivo* and *in vitro* at physiologically relevant doses, and this may be a major mechanism mediating the anti-tumour activity of selenium. Secondly, this phenomenon, at least for the selenium compounds tested, is independent of p53

function and this is of enormous significance in terms of designing effective therapeutic regimens.

3. Selenium and apoptotic signal transduction

A major new finding to arise from our recent studies is that induction of apoptosis by selenium compounds appears to be due to the induction of Fas ligand (data submitted for publication). We have also systematically studied the involvement of the MAPK pathways in apoptosis induction by selenium compounds, such as selenodiglutathione (SDG) and 1,4-phenylenebis(methylene)selenocyanate (p-XSC). SDG is the primary metabolite of selenite, the most commonly used form of selenium used in animal studies, while p-XSC is a synthetic, organoselenium compound with one of the highest chempreventive indices known for any form of selenium [7].

We have performed these studies on human oral cancers and their normal mucosa counterparts, using a panel of primary cultures derived from biopsy material. The rationale for this being the fact that respiratory tract cancer is one of the cancer types for which the epidemiological evidence for a cancer protective effect of selenium is strongest. Our major findings are also reproducible in established cell lines in which signalling pathways have been well characterised and appear to be conserved in different cell biology systems.

A potentially important observation is that in this oral system is that carcinomas are significantly more sensitive to induction of apoptosis SDG than normal oral mucosa (data submitted for publication). Detailed investigation into the differences between the selenium-mediated apoptosis-inducing mechanisms in normal and tumour cells in this novel *in vitro* culture system, may provide crucial insight into the tumour specificity observed.

Our work shows that the activities of the so called 'pro-proliferative' pathways like ERKs 1,2 are very slightly enhanced whereas the so called 'stress activated kinases', c-jun N-terminal kinase (JNK) and p38 kinase are strongly induced (see Fig. 1 for summary of the signalling pathways involved)(data submitted for publication). This considerably extends the earlier evidence for induction of JNK by p-XSC [1]. Taken together it appears that these alterations in the MAPK pathways tip the balance in favour of an apoptotic response.

We have also demonstrated by functional studies that the JNK pathway is of particular importance since blocking this pathway by cotransfection of a dominant negative c-jun mutant significantly decreased the induction of apoptosis, whereas, inhibiting the p38 kinase pathway with a specific chemical inhibitor (SB203580) had no effect (data submitted for publication). Activation of JNK by selenium is probably responsible for the induction of Fas ligand since the JNK pathway has been shown to be involved in Fas ligand regulation (via c-jun transcriptional activity) in other contexts [17].

In Hela cells the situation is more complex since SDG and p-XSC also inhibit the activities of the mitogenic signalling pathways mediated by ERK's 1,2 and 5 and this inhibition is of some functional significance.

Thus, it appears that, depending on the tumour/cell type, the modulation of the activities of the various MAP kinases is functionally significant in selenium-induced apoptosis. Further research is required to elucidate both the upstream modulators and the downstream targets of these pathways and their contribution to the induction of apoptosis.

4. Upstream changes and downstream regulators

Since selenium has been shown to influence the redox state of the cells, a redox-sensitive mechanism may be, at least partially, involved in influencing the stress kinase pathways or even apoptosis directly. For example, selenium has been shown to directly affect the thioredoxin system which may drastically influence the cellular redox state [2,8]. Redox changes may then act as potential upstream modulator of various signalling pathways or even as regulators of trancriptional activity [16,20]. Interestingly, there is evidence for thioredoxin directly inhibiting ASK1, a MAP kinase kinase kinase which can induce JNK and p38 activity (see Fig.1) and also apoptosis, in a redox-dependent manner [28]. Whether selenium compounds affect ASK1 activity is currently being investigated.

Selenium compounds are also known to inhibit several other signalling pathways such as protein kinase A, Ca^{2+} dependent and independent protein kinase C's (PKC), Diacylglycerol kinase and thymidine kinase [9,23,32]. Whether these pathways directly influence apoptosis is yet to be established. The inhibition of the PKC's, especially, may have some significance as there are precedents of such inhibition influencing the induction of apoptosis [19].

ROS generation by selenium compounds at concentrations that induce apoptosis have been documented and inhibition of selenium-induced loss of cellular viability on addition of ROS scavengers and antioxidants have also been observed [29]. However, ROS generation does not account for the apoptotic response completely as there are several differences observed between the selenium response and that by H_2O_2 [34].

The Bcl-2 family of proteins are key regulators of mitochondria mediated apoptosis and selenium is known to influence mitochondrial activity directly [31], however, in a previous study no changes is expression of Bcl-2 family members was found [15].

Consitent with this, we have found that overexpression of bcl-2 in a mouse mammary cell line showed no effect on selenium induced loss of cellular viability (unpublished data).

Caspases are thought to be the downstream executors of the apoptotic programme and though there are some documented evidence of caspase-independent apoptosis, this appears to be quite infrequent. It has been suggested that selenium compounds like methylselenol may directly activate caspases, thereby inducing apoptosis, by removing the inhibitory zinc atom from the catalytic site of these cysteine proteases [8]. Our data would suggest that selenium induced apoptosis is, at least partially, caspase-dependent as a pan-caspase inhibitor reduces the number of cells undergoing apoptosis (unpublished data). Cleavage of the PARP protein, a downstream target of caspase 3, is also observed in some situations (unpublished data).

5. Conclusion

From the evidence reviewed here, it is clear that the apoptosis induction is a major mechanism involved in the anti-cancer activities of selenium compounds. It is also evident that selenium brings about global alterations in the MAPK signalling pathways and the induction of apoptosis is a direct consequence of these changes.

The case for selenium compounds as a potentially useful class of chemopreventive agents is becoming compelling. However, extensive basic research into the molecular mechanisms involved is necessary before selenium compounds can be tested effectively in chemopreventive and therapeutic programmes.

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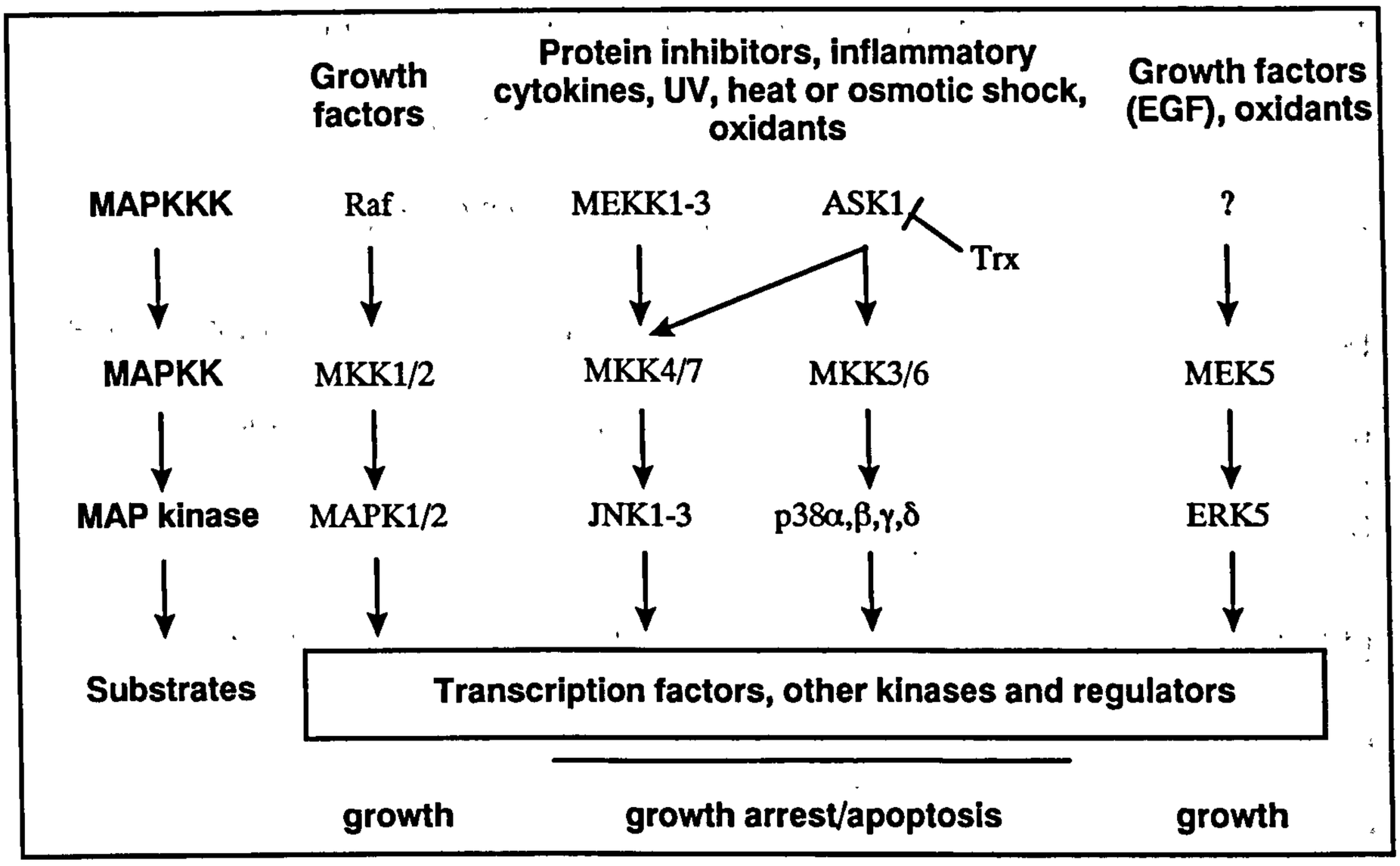

Fig 1. A. Ghose, J. Fleming and P.R.Harrison

Fig 1. Schematic representation of MAPK cascades in mammalian cells depicting the various inducers, substrates and activators. Trx, Thioredoxin

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Enhanced Sensitivity of Human Oral Carcinomas to Induction of Apoptosis by Selenium Compounds: Involvement of Mitogen-activated Protein Kinase and Fas Pathways[1]

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ABSTRACT

Prospective studies and recent intervention trials suggest that the risk of some cancers, including respiratory tract cancers, may be inversely related to selenium (SE) intake, and this is supported by strong experimental evidence with chemical-induced animal cancer models. How this cancer-protective effect is mediated is unclear, but interference with the balance of growth/apoptosis during tumor outgrowth is one plausible hypothesis. In general, there is a correlation between the effectiveness of SE compounds as chemopreventive agents *in vivo* and their ability to inhibit cell growth and induce apoptosis *in vitro*. This study has investigated the signal transduction pathways affected by SE compounds in biopsies of normal human oral mucosa cells and human oral squamous carcinoma cells (SCCs), using a primary culture system. Two SE compounds were tested: selenodiglutathione (SDG), the primary metabolite of selenite and the most commonly used cancer-protective SE compound in animal models, and the synthetic SE compound, 1,4-phenylenebis(methylene)selenocyanate (p-XSC), one of the most potent chemopreventive pharmacological SE compounds. Three novel findings are reported: (*a*) SCCs were found to be significantly more sensitive to induction of apoptosis by SDG than normal human oral mucosa cells, though the differences were marginal with p-XSC; (*b*) both SE compounds induced the expression of Fas ligand (Fas-L) in oral cells to a degree that correlated with the extent of apoptosis induction; and (*c*) both SDG and p-XSC induced the stress pathway kinases, Jun NH_2-terminal kinase (JNK) and p38 kinase, at concentrations causing apoptosis; p-XSC, and to a lesser extent SDG, also activated extracellular regulated kinases 1&2 (ERKs 1&2) and protein kinase-B or Akt. To test their functional involvement, the effect of inhibiting each of these pathways on induction of apoptosis by SDG and p-XSC was determined in SCCs. Inhibiting the ERKs 1&2 or Akt pathways with specific chemical inhibitors (PD98059 or LY294002, respectively) did not affect the extent of apoptosis induced by SDG or p-XSC (with the exception of LY294002, which actually enhanced the level of induction of apoptosis by SDG). The JNK pathway appeared to be most important for induction of Fas-L and apoptosis because concentrations of SB202190 that inhibited activation of both the JNK and p38 kinase (but not ERKs 1&2) in SCC reduced the extent of induction of Fas-L and apoptosis by SDG and p-XSC, whereas lower concentrations that inhibited activation only of p38 kinase did not. This was confirmed by the fact that exogenous expression of a dominant negative deletion mutant of c-Jun (TAM67) reduced the induction of both apoptosis and Fas-L by SDG.

INTRODUCTION

There is currently much interest in the potential cancer-protective effects of SE.[4] Human epidemiological evidence indicates a statistically significant inverse relationship between SE intake (which is primarily in the form of selenomethione) and risk of cancer overall, particularly in men (1, 2). In terms of individual cancer types, the evidence from the largest studies is strongest for lung, esophageal, prostate, and gastric cancers, but there is little evidence of any link with breast or colon cancer, although the length of follow-up in most of these studies was quite short. Three intervention trials also suggest that SE supplementation in the form of selenized yeast, either alone or with other antioxidant vitamins, reduces the risk of cancer (1). The most recent randomized placebo-controlled intervention trial with men with a prior history of skin basal or squamous cell carcinoma found no evidence for a reduction in risk of recurrence of skin cancer with 200 μg/d SE, but total cancer rates in the treatment arm were reduced significantly (by 37%), as were the rates of lung, colorectal, and prostate cancers (3, 4). The strongest treatment effect was observed in subjects with the lowest plasma SE levels before supplementation.

This evidence for a cancer-protective effect of dietary SE in humans is supported by very convincing animal evidence indicating that a high dietary level of SE, usually in the form of sodium selenite, substantially reduces the incidence of a wide variety of animal cancers under conditions where animal growth and health are not affected (1, 5, 6). Most studies have observed the maximum cancer-protective effect at nontoxic levels considerably higher than normal nutritional levels, but there is also more limited and less consistent evidence that sub-nutritional SE deprivation increases cancer risk. SDG, the primary metabolite of selenite, is potently anticarcinogenic, but selenomethionine, the most abundant form of SE in natural foods, has generally been found to be less effective than selenite (reviewed in Ref. 1). However, plant foods also contain other SE derivatives, such as Se-methylselenocysteine, which is an effective cancer-protective agent in animal models (5). There is therefore considerable interest in developing novel synthetic SE compounds that may have greater chemopreventive properties than naturally occurring forms. In animal models, the most effective chemopreventive agents seem to be those that are metabolized directly to monomethylated SE metabolites (1, 5, 7), including aliphatic or benzyl selenocyanates, such as p-XSC (8–10), used in this study. In particular, SE derivatives like p-XSC are effective in animal carcinogenesis models in which selenite is not so effective, *e.g.*, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone-induced lung cancers (10).

Thus, overall, the available evidence indicates a definite cancer protective effect of SE in animal models and probably also in humans. However, as noted above, the human intervention trials have used a complex SE source (selenized yeast) rather than specific SE compounds, and so it cannot be assumed that the mechanisms responsible for the cancer-protective effect in animals is necessarily relevant to the human studies (reviewed in Refs. 1, 2, 11, and 12). Arguably, the animal studies with selenite or synthetic SE derivatives may be more

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[4] The abbreviations used are: SE, selenium; ERK, extracellular regulated kinase; NOMC, normal oral mucosa cell; SDG, selenodiglutathione; SCC, oral squamous carcinoma cell; P-I-3 kinase, phosphatidyl inositol-3-phosphate kinase; p-XSC, 1,4-phenylenebis(methylene)selenocyanate; GPX, glutathione peroxidase; JNK, Jun NH_2-terminal kinase; EGF, epidermal growth factor; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; GSH, glutathione; MAPKAPK, mitogen activated protein kinase activated protein kinase; FADD, Fas-associated death domain; Fas-L, Fas ligand.

relevant to devising potential future chemoprevention strategies in high-risk humans than to the putative cancer protective effect of SE from natural human diets.

Several possible mechanisms have been proposed to explain the cancer-protective effects of SE compounds. They can modulate immune responses (reviewed in Ref. 1), but the detailed mechanisms involved require to be elucidated, and it remains to be established that this effect could generate a sufficient antitumor response at the levels of SE that are cancer-protective *in vivo*. A second hypothesis proposed that the chemopreventive effect of SE might be mediated by selenoproteins, such as the GPXs, eliminating tumor-promoting reactive oxygen species; however, this now seems less plausible because the activities of known selenoproteins seem to be saturated at a much lower dietary SE level than that required for the maximum chemopreventive effect of SE (13, 14). There is additional evidence that at least the cytosolic GPX1 is not involved because GPX1-null mice do not show any abnormal histopathologies up to 15 months of age (15, 16), although they are more sensitive to exogenous oxidative stress (17), and GPX1-transgenic mice are actually more sensitive to 7,12-dimethylbenz[*a*]anthracene/12-*O*-tetradecanoylphorbol-13-acetate-induced skin cancer, rather than the reverse, hypothetically, because the generation of tumor-promoting, lipoxygenase-derived peroxides is increased (18). However, thioredoxin reductase remains a possible candidate because it has been shown to be inhibited by protracted high SE levels, possibly attributable to diselenide bond formation at the selenocysteine in the active site (19). Additionally, new selenoproteins have been discovered recently (reviewed in Ref. 6), but it is not yet known what their functions are or the SE levels at which their activities are saturated. A third hypothesis is that SE compounds act as antipromotion agents, possibly by inducing apoptosis in initiated premalignant cells. This is suggested by animal evidence that selenite and p-XSC are effective if given after carcinogen in the early phase of tumor progression, although they may also reduce DNA damage by carcinogens (recently reviewed in Refs. 1 and 5). It is also consistent with the fact that, in general, the relative efficacies of SE derivatives as chemopreventive agents *in vivo* parallel their growth inhibitory effects *in vitro* and their ability to induce apoptosis (20–24); moreover, this could operate in premalignant cells because it does not necessarily require a functional p53 pathway (10, 21, 25). Recent work showing that both inorganic and organic forms of SE induce gadd34, gadd45, and gadd153 (25, 26), and inhibit cdk2 activity (26) suggests that interference with cell cycle checkpoint controls is associated with growth arrest/apoptosis induced by SE compounds.

The work reported in this paper, therefore, tested directly whether SE compounds with the most pronounced cancer protective properties affect any of the signal transduction pathways that control cell growth, survival, and apoptosis. In view of the fact that lung and esophageal cancers are two of the cancer types in which the epidemiological evidence for a cancer-protective effect of SE is strongest, we have focused on human oral cancers because they share similar risk factors to lung and other head and neck cancers. To obtain data as relevant as possible to human SCCs, we have used a panel of primary cultures of biopsies of oral squamous cancers and NOMC, which we have characterized previously (27, 28). We have investigated primarily two of the most potent cancer-protective SE compounds in animal studies (the primary metabolite of selenite, SDG, and p-XSC) and shown that they act by a similar mechanism (induction of Fas-L/JNK-dependent apoptosis).

MATERIALS AND METHODS

Cells and Culture Conditions. The derivation and characterization of the primary cultures of biopsies of normal human oral mucosa or carcinomas has been described previously (27, 28). All cells were maintained on irradiated 3T3 feeders, either in FAD+ medium (1:3 Ham's F-12/DMEM with 10% FCS and insulin, EGF, transferrin, cholera toxin, hydrocortisone, and adenine) in the case of normal cells or 10H medium (DMEM plus 10% FCS without added growth factors, except hydrocortisone) in the case of carcinomas (27, 28). Normal cultures were used within the first two to three passages from frozen stocks, before their growth rate deteriorated significantly. Oral cultures were used for experimentation while rapidly growing at not >70% confluence, after which the irradiated 3T3 feeders were carefully removed by treatment with PBS/0.02% EDTA, and the cultures were refed with normal medium. 3T3 cells were maintained in 10C medium (DMEM plus 10% donor calf serum). HeLa cells were maintained in modified DMEM (Beatson formulation; Life Technologies, Inc., Inchinnan, United Kingdom) supplemented with 10% FCS and 1 mM glutamine. Cells were maintained in culture for a maximum of 6–8 weeks before being replaced with cells from frozen stocks. All media used contained Bufferall (Sigma Chemical Co., Gillingham, United Kingdom) to maintain the pH at 7.0.

Chemicals. SDG was prepared as described previously (29). p-XSC was purified to 99.9% homogeneity as described by El-Bayoumy *et al.* (30). SB202190, SB203580, PD98059, and LY294002 were obtained from Calbiochem, Nottingham, United Kingdom.

DNA Synthesis/Proliferation Assay. 10^4 irradiated 3T3 cells were plated in 200 μl of 10C medium per microtiter well. After 4d, the medium was removed, and oral epithelial cells were added (10^4 in the case of normal cells or 5×10^3 carcinoma cells) in 100 μl of 10H medium. After 48 h, the medium was replaced with medium containing SDG or p-XSC and incubated overnight. Each well was then given 0.5 μCi of tritiated thymidine for 6 h, the medium was removed, and the cells were trypsinized and transferred onto a filter paper mat (printed filtermat A; Pharmacia) using a microtiter plate harvester (Skatron Combi Harvester, model 11900; LKB, Skatron, Norway). After adding scintillator, the mat was scanned and counted using a plate counter (model 1205 Betaplate; Pharmacia). Four replicate wells were used for each condition. Control experiments showed that the irradiated feeders supported growth of the oral cells for the duration of the experiment but contributed an insignificant background thymidine incorporation (<5% of that of the oral cells). The radioactivity incorporated into DNA was calculated as a percentage of untreated cultures.

Apoptosis Assays. Apoptosis in the oral cells was measured by TUNEL staining using the Apotag kit (Intergen, Purchase, NY), according to the manufacturer's protocol. The irradiated 3T3 feeders were carefully removed from the cultures before experimentation by washing the cultures vigorously three times with PBS/0.02% EDTA. Assays were performed with two to three replicate plates of cells, and ≥10 randomly selected fields per plate were counted for TUNEL-positive cells. Assessment of apoptosis among the HeLa cells transiently cotransfected with a GFP-encoding vector was performed by measuring the sub-G_0/G_1 DNA content of the GFP-positive population. Cells were harvested after treatment, washed in cold PBS, fixed in 1% paraformaldehyde for 1 h on ice, and permeabilized with 70% ethanol for 1 h on ice. Cells were then resuspended in 1 ml of PBS to which 0.5 ml of Phosphate-citric acid buffer was added and incubated at room temperature for 5 min. After RNase treatment (250 μg/ml), the DNA was stained with propidium iodide (10 μg/ml), and the cell cycle distribution was determined by analyzing 10^4 GFP-positive cells with the CellQuest software using a FACScan flow cytometer (Becton Dickinson, Cowley, United Kingdom). For the annexin V assays, the cells were plated on coverslips for 2 days, and then the 3T3 feeder cells were removed before SE treatment. Annexin V-FITC staining was carried out according to the manufacturer's protocol (CLONTECH, Basingstoke, United Kingdom). The percentage of annexin V-staining cells was then determined microscopically.

In some experiments (*e.g.*, using the Fas/Fc chimeric protein), cells were grown on 3T3 feeders on glass coverslips. Cells were grown to the appropriate density, and the feeder layer was removed and then incubated for an additional 16 h. Cells were then treated with the Fas/Fc chimeric protein (250 ng/ml) for 1 h before treatment with either SE compound or recombinant soluble human Fas-L (150 ng/ml) and the potentiator antibody (2 μg/ml). After 16-h incubation, the TUNEL assay was performed. Soluble recombinant human Fas-L and mouse monoclonal IgG1 potentiator antibody were obtained from Upstate Biotechnology, Lake Placid, NY; recombinant human Fas/Fc chimera protein was obtained from R & D Systems, Abingdon, United Kingdom.

Immunoblotting and MAPK Assays. To prepare whole cell protein extracts for immunoblotting, the cells were washed twice with ice-cold PBS and

then scraped off in 0.2 ml of buffer [20 mM HEPES (pH 6.8), 5 mM EDTA, 10 mM EGTA, 5 mM NaF, 0.1 μg/ml okadaic acid, 1 mM DTT, 0.4 M KCl, 0.4% Triton X-100, 10% glycerol, 5 μg/ml leupeptin, 50 μg/ml phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μg/ml aprotinin, and 1 mM sodium orthovanadate] and incubated on ice for 20 min, followed by centrifugation at 13,000 rpm in a microfuge for 10 min. The supernatant was stored at −70°C. Up to 50 μg of protein sample in 40 μl of buffer was mixed with 20 μl of loading buffer [187.5 mM Tris-HCl (pH 6.8), 30% glycerol, 6.9% SDS, 2.1 M β-mercaptoethanol, and 0.1% bromphenol blue] before electrophoresis on a SDS/8%PAGE gel. The proteins were then blotted onto nitrocellulose (Amersham, Little Chalfont, United Kingdom) using a Camlab semidry blotter, following the manufacturer's protocol. Western blots were preincubated in the presence of Tris-buffered saline-T [50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, and 0.1% Tween-20 containing 5% dried milk] and then incubated with antibodies diluted in Tris-buffered saline-T containing 5% BSA. Activated or total amounts of specific kinases were measured using the following antibodies: activated JNK monoclonal antibody p-JNK (G-7; Santa Cruz Biotechnology, Santa Cruz, CA); phospho-specific (Ser63/73) c-Jun antibody kit (New England Biolabs, Hitchin, United Kingdom); activated and total p38 kinase, PhosphoPlus p38 MAPK (tyr182) polyclonal antibody kit (New England Biolabs); activated MAPKs (ERKs 1&2), polyclonal antibody (Promega, Southampton, United Kingdom), and total MAPK, ERK 1(C-16)-G polyclonal antibody (Santa Cruz Biotechnology); ERK5 kinase, ERK5(C-20) polyclonal antibody (Santa Cruz Biotechnology); activated Akt and total Akt, PhosphoPlus Akt (ser 473) polyclonal antibody kit (New England Biolabs); and Fas-L, antihuman Fas-L monoclonal antibody (PharMingen/Becton Dickinson, Cowley, United Kingdom). In all cases, bound primary antibody was detected using enhanced chemiluminescence methodology (Amersham). Phosphorylation of ERK5 results in a shift in its electrophoretic mobility detectable by Western blotting and a consistent correlation between the shifted fraction of phosphorylated ERK5 and the activity of ERK5 as measured by protein kinase assay has been observed (data not shown and Ref. 31). This electrophoretic mobility shift was thus used as an assay for ERK5 activity. p38 kinase activity was measured indirectly by a protein kinase assay for the p38 substrate, MAPKAP kinase-2 (32).

Transient Transfection of Cells. HeLa cells were plated at a density of 1.2×10^6 cells in 90-mm dishes. The next day, cells were cotransfected with 1 μg each of a GFP construct, pEGFP (CLONETECH), the c-Jun dominant negative construct (TAM67; provided by Prof. B. Ozanne, Beatson Institute, Glasgow, United Kingdom), or a dominant negative FADD-GFP construct (donated by Dr. H. Wajant, University of Stuttgart) using the Effectene (Qiagen, Crawley, United Kingdom) transfection reagent, according to the manufacturer's protocol. After overnight incubation, fresh medium was added and incubated for another 6 h, before the addition of SE compounds or chemical inhibitors for the appropriate time. Cells were then harvested, and apoptosis assays were performed to assess apoptosis in the GFP-positive population (see above for details).

RESULTS

Sensitivity of NOMCs and SCCs to growth inhibition by SE compounds. Our panel of primary cultures of biopsies of NOMCs and SCCs were derived and maintained using the well-established 3T3 feeder layer system (27, 28), which was designed to permit growth of keratinocytes at all stages of cancer progression. The 3T3 feeder layer was then removed before experiments commencing with the remaining oral epithelial cells. The NOMCs were used within two to three passages from frozen stocks, while they were still proliferating rapidly, before senescence occurring.

We have tested a variety of SE compounds. Our previous studies found that the most effective cancer-protective SE compounds in animal studies, such as selenite, Se-methylselenocysteine, and p-XSC, all induced growth arrest in various cell types, unlike selenomethione, which only affected cell growth at very high concentrations (21). However, whereas most of these SE compounds only inhibit cell growth after a lag period (21), p-XSC induces growth arrest/apoptosis much more quickly, presumably because it is metabolized more rapidly to the directly acting SE derivative. The fact that the lag period required for selenite was reduced by the addition of GSH (33) suggested that the active inhibitor might be SDG, which would be formed by reaction of selenite with GSH either intra or extracellularly, and this was consistent with the fact that purified SDG inhibited cell growth without a significant lag period (33). In the present studies, we therefore focused on elucidating the signal transduction pathways affected by SDG and p-XSC, because their effects were rapid and arguably most likely to be relevant to the chemopreventive effects of the most effective SE compounds, at least in animal models.

Fig. 1 shows the dose-response curves for growth inhibition of NOMCs or SCCs by SDG or p-XSC; to assess whether there were any consistent differences in sensitivity between NOMCs and SCCs, the results for three independently derived NOMCs, or cultures of four carcinoma biopsies, have been pooled. This shows that NOMCs may be less sensitive to SDG than carcinoma cultures in the range 4–5 μM ($P < 0.001$, using Student's t test), whereas with p-XSC, any differences are small and not consistent throughout the dose range.

Induction of Apoptosis and Fas-L by SE Compounds. We (21, 34) *et al.* (20, 22–24) reported previously that SE compounds induce apoptosis in rodent mammary, ovarian, and leukemia established cell lines. We found that both SDG and p-XSC induced apoptosis in oral cell cultures as judged by TUNEL staining, annexin V staining, or measurement of sub-G_0 cells by fluorescence-activated cell sorter analysis. Care was taken to ensure that all cultures were subconfluent and growing at their maximum growth rates. Using either TUNEL staining (Fig. 2, *A* and *C*) or annexin V staining (Fig. 2, *B* and *D*) as assays of apoptosis, SCCs were significantly more sensitive to induction of apoptosis by SDG than NOMCs [Fig. 2, *C* and *D*; $P = 0.0003$ (TUNEL data), $P = 0.003$ (Annexin data), using the Student's t test], whereas any differences in sensitivity to p-XSC were marginal and not statistically significant (Fig. 2, *C* and *D*; $P = 0.39$, $P = 0.5$, respectively). The concentrations of SE compounds required to obtain a significant level of apoptosis were somewhat higher than those required to detect growth arrest by inhibition of DNA synthesis (compare Figs. 1 and 2). All additional molecular studies were performed at SE concentrations found to induce a significant level of apoptosis (usually in the range 20–40%).

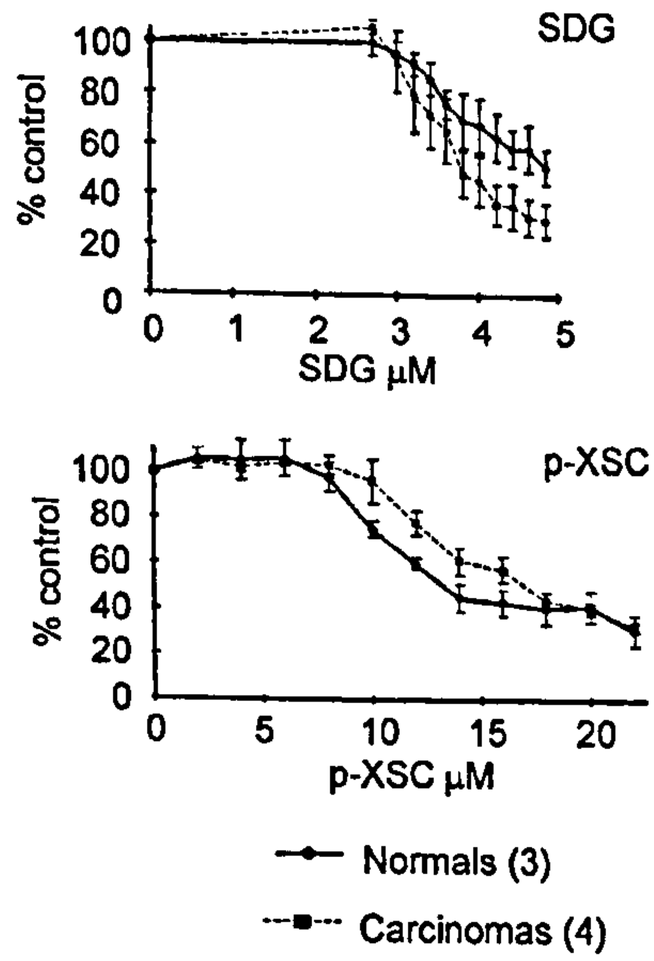

Fig. 1. Sensitivity of NOMCs and carcinomas to growth arrest/inhibition of DNA synthesis by SDG and p-XSC. Cells were grown in the indicated concentration of SE compound for 20 h and analyzed using the DNA synthesis/proliferation assay. ———, average of three normal cultures (±SE); —, average of four carcinoma cultures (±SE).

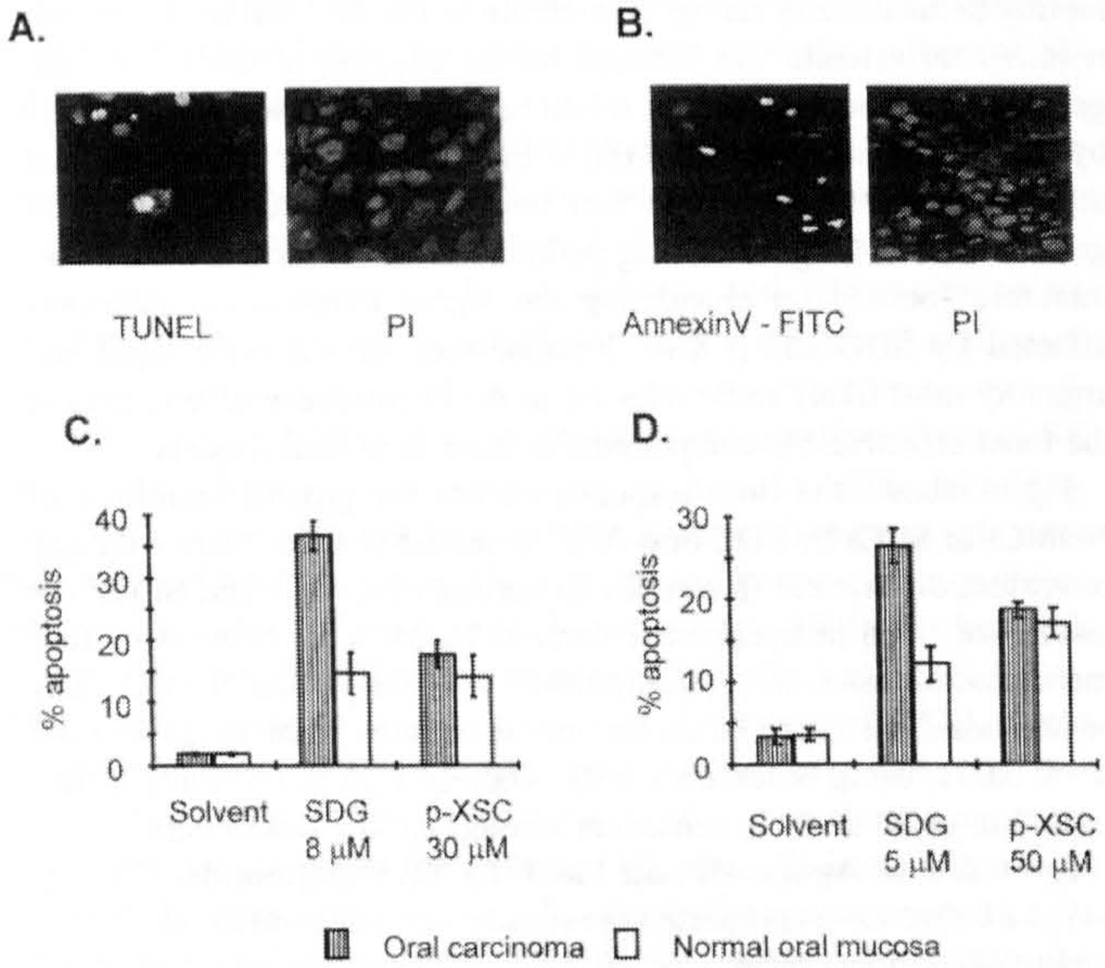

Fig. 2. Sensitivity of NOMCs and SCCs to SE compounds. The degree of apoptosis after treatment of NOMCs or SCCs with solvent control, SDG, or p-XSC for 16 h was quantitated by counting the percentage of TUNEL-positive cells (*A* and *C*) or annexin V-positive cells (*B* and *D*). The results are the averages of two independent NOMCs or carcinoma cultures (±SEs).

In the course of testing whether any of the known apoptotic pathways were induced by SE compounds, we discovered that both SDG and p-XSC induced Fas-L in NOMCs and SCCs under conditions where significant apoptosis was induced (Fig. 3*A*). The size of the Fas-L produced corresponded to the secreted form of the protein (35). Fas-L was induced considerably more strongly by SDG in SCCs than in NOMCs, whereas p-XSC seemed to be equally effective in both normal cells and carcinomas (Fig. 3*A*). Thus, the degree of Fas-L induction correlated closely with the extent of apoptosis (compare Fig. 2). Additional experiments showed that inhibition of the Fas pathway with a Fas/Fc chimera (36) significantly reduced the extent of induction of apoptosis by both SDG and p-XSC (Fig. 3*B*; $P = 0.021$, $P = 0.044$, respectively). Thus, induction of Fas-L is functionally required for induction of apoptosis by these SE compounds.

Effects of SE Compounds on ERKs 1/2, ERK5, and Akt Kinase Signal Transduction Pathways. We next investigated whether SDG or p-XSC interfered with kinase signal transduction pathways known to control growth or cell survival. ERKs 1&2 (37) and ERK5 (31) have both been implicated in control of cell proliferation by serum mitogens or EGF, whereas the P-I-3 kinase/Akt pathway plays an important role in cell survival in a wide variety of cell types (38). ERK5 has also been reported to be redox sensitive and induced by oxidants (39). In view of their increased sensitivity to induction of apoptosis, particularly by SDG, these experiments were performed mainly using SCCs, rather than NOMCs. One way in which SE compounds might induce apoptosis is by interfering with the activation of mitogenic or survival pathways by growth factors. To test whether SDG or p-XSC interfered with activation of the ERKs 1&2 or Akt pathways, SCC cultures were serum starved for 16 h, then pretreated for 2 h with SDG or p-XSC, and then given EGF or serum. Cell extracts were prepared and then analyzed for the extent of activation of ERKs 1&2 or Akt by Western blotting with antibodies that recognize either the total amount of each kinase or only the activated (phosphorylated) forms. These experiments revealed no evidence for a reduction in activation of ERKs 1&2 or Akt by SDG or p-XSC after treatment with EGF or serum (Fig. 4, *A* and *B*). Control experiments showed that p-XSC itself induced significant activation of ERKs 1&2, though this was much less marked with SDG (Fig. 4*A*). Although it appears paradoxical that an agent causing growth arrest should activate a mitogenic pathway, this has been shown to occur also with other stress factors, such as oxidants (40). However, neither p-XSC nor SDG had any very significant effect on Akt activation (Fig. 4*B*).

To investigate more thoroughly by functional experiments whether the ERKs 1&2 and Akt pathways were involved in mediating the induction of apoptosis by SE compounds, we tested whether the apoptotic effects of SDG or p-XSC were altered by inhibiting the ERKs 1&2 or Akt pathways with the specific chemical inhibitors, PD98059 (which inhibits activation of ERKs 1&2 by inhibiting MAP extracellular signal-regulated kinase activity) and LY294002 (which inhibits P-I-3 kinase and therefore activation of Akt; Ref. 41). Control experiments showed that under the conditions used, PD98059 prevented activation of ERKs 1&2, and LY294002 prevented activation of Akt by SDG and p-XSC (Fig. 5*A*). However, neither inhibitor protected SCCs from induction of apoptosis by SDG or p-XSC (Fig. 5*B*); in fact, inhibition of Akt activation by treatment with LY294002 significantly increased the level of SDG-induced apoptosis by ~55% ($P < 0.001$; Fig. 5*B*). This might imply that the induction of Akt activation by SDG may be an (inadequate) attempt to protect the cells from apoptosis. These experiments therefore confirm that the ERKs 1&2 and Akt pathways are not of major importance in mediating the induction of apoptosis by either SDG or p-XSC in this oral cell system.

ERK5 is another member of the MAPK family that has also been implicated in control of cell proliferation by EGF in certain cell types (31). We therefore determined whether activation of ERK5 could be detected in our oral cultures by the characteristic change in its migra-

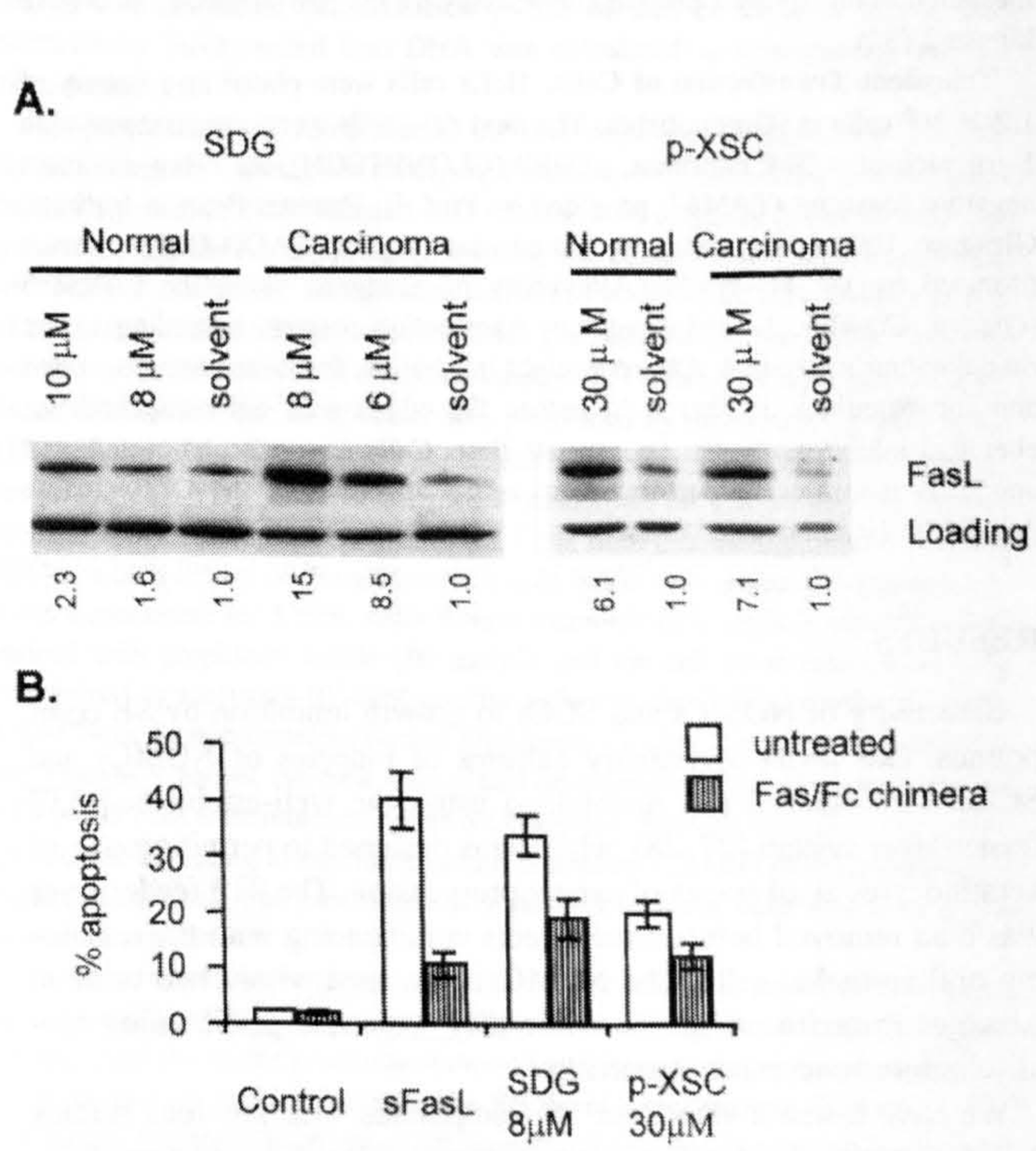

Fig. 3. Induction of Fas-L by SE compounds. In *A*, NOMCs and SCCs were treated with the stated concentrations of SDG or p-XSC for 16 h, and cell lysates were prepared and Western blotted with either an anti-Fas-L antibody or antibody against total p38 kinase as a loading control. The figures below the blots indicate the fold increase in Fas-L expression above untreated level after SE treatment, normalized to the loading control. *B*, protection against SDG- and p-XSC-induced apoptosis (measured by the TUNEL assay) after pretreatment of SCCs with a Fas/Fc chimera (250 ng/ml) for 1 h, followed by the indicated concentrations of SDG or p-XSC for 16 h. As a positive control, cells were also treated with soluble Fas-L (*sFasL*).

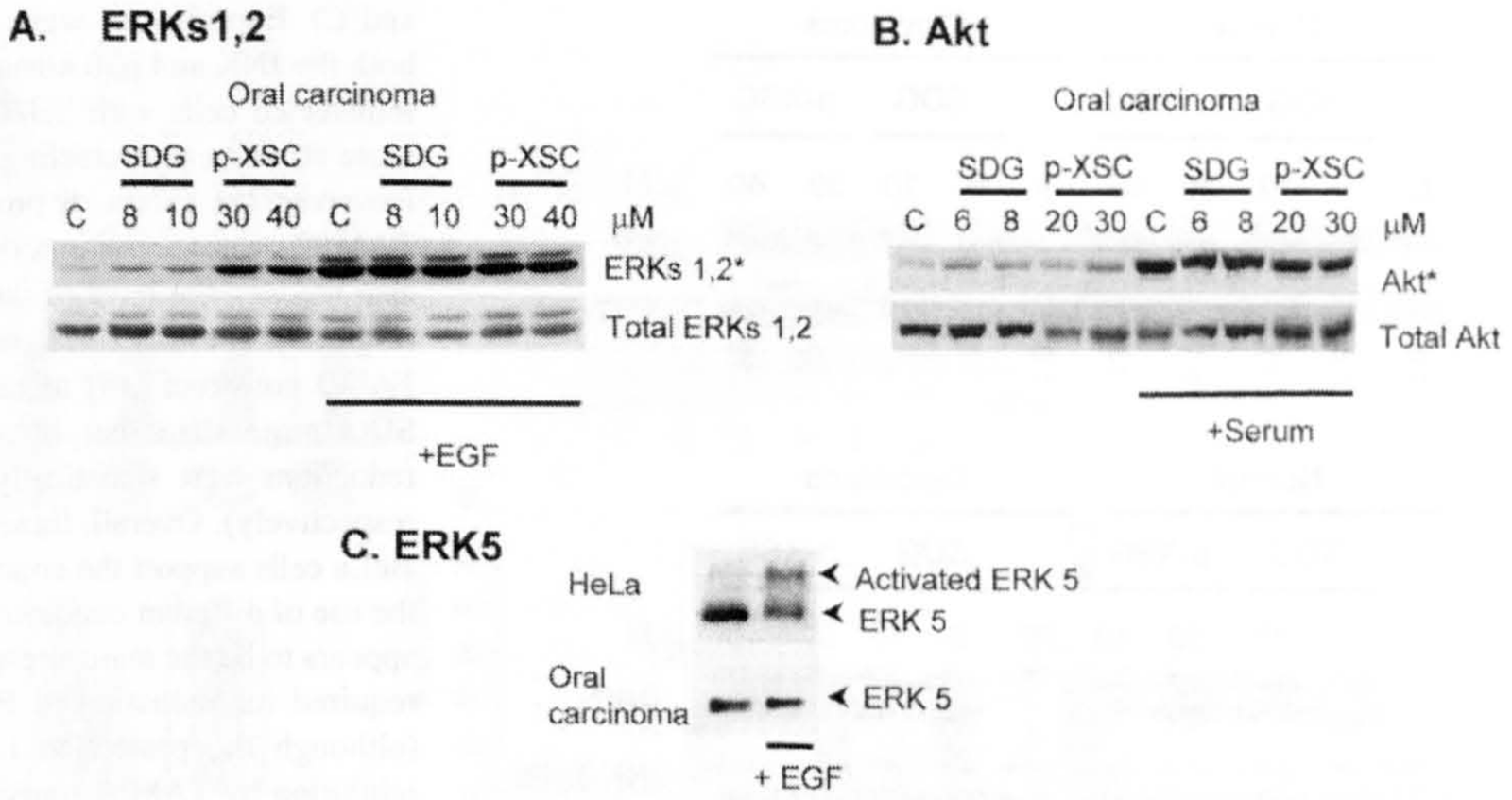

Fig. 4. Effect of SE compounds on activation of ERKs 1&2 (*A*), Akt (*B*), or ERK5 (*C*). SCCs (*A* and *B*), or HeLa cells (*C*), were starved for 16 h and then treated with the indicated concentrations of SDG or p-XSC for 2 h, in some cases followed by 10 ng/ml EGF for 20 min for ERKs 1&2 and ERK5 assays or 10% serum for 30 min for Akt assays. Cell lysates were then prepared and run on SDS-PAGE gels and then Western blotted using antibodies that recognize the total amount of ERKs 1&2 (*A*) or Akt (*B*) or only their activated forms (*ERKs 1,2** and *Akt**). Activation of ERK5 (*C*) was measured by its change in migration detected by Western blotting with anti-ERK5 antibody (36).

tion in SDS/polyacrylamide gels after Western blotting (31). However, no activation of ERK5 by EGF was found with either NOMCs or SCCs, whereas ERK5 activation was readily observed in parallel experiments with HeLa cells (Fig. 4*C*), as others have reported (31).

Induction of Stress Kinases by SE Compounds. We next investigated whether the SE compounds induced the JNK and p38 stress kinase pathways because they have been strongly implicated in induction of growth arrest/apoptosis by a wide variety of signals (40). Both SDG and p-XSC caused activation of JNK and p38 in both normal and carcinoma cells under conditions in which apoptosis was induced (Fig. 6). There was no significant differences between NOMCs and SCCs in the extent of induction of JNK and p38 kinase by p-XSC (Fig. 6). However, SDG was reproducibly more efficient at activating JNK and p38 in SCCs than NOMCs (Fig. 6), thus mirroring the greater sensitivity of SCCs to induction of apoptosis by SDG (Fig. 2).

To test whether the JNK or p38 pathways were functionally important, we performed experiments to determine whether preventing the activation of JNK and/or p38 reduced the extent of apoptosis induced in SCCs by SDG or p-XSC. In the first approach, we exploited the known differential sensitivities of p38 kinase, JNK, and ERKs 1&2 to two chemical inhibitors of the MAPK family, SB202190 and SB203580. Low concentrations (up to ~10 μM) selectively inhibit p38 kinase; at 30 μM, both p38 and JNK are inhibited, whereas ERKs 1&2 are not affected until even higher concentrations (42–44). We therefore tested whether concentrations of SB202190 affected induction of Fas-L and apoptosis by SDG or p-XSC in SCCs. Control experiments confirmed that 10 μM SB202190 prevented SDG- or p-XSC-induced activation of p38 kinase (measured by activation of its downstream kinase MAPKAPK-2 in an immunoprecipitation/kinase assay) but not activation of JNK (measured by phosphorylation of c-Jun at Ser63) or ERKs 1&2 (measured using phospho-specific antibodies), whereas 30 μM SB202190 prevented activation of both p38 kinase and JNK but did not affect activation of ERKs 1&2 (Fig. 7*A*). Parallel experiments indicated that 30 μM SB202190 prevented SDG and p-XSC induction of Fas-L (Fig. 7*B*) and apoptosis (for SDG, $P = 0.033$; for p-XSC, $P = 0.041$; Fig. 7*C*), whereas 10 μM SB202190 had no effect (Fig. 7, *B* and *C*). This strongly suggests that activation of the JNK pathway is primarily responsible for the induction of Fas-L and apoptosis by SDG and p-XSC.

Using phospho-specific c-Jun antibodies, we found that the SE compounds induced phosphorylation of c-Jun at Ser 63/73, residues known to be phosphorylated by JNK (Fig. 7*A* and data not shown). Phosphorylation of c-Jun at Ser63/73 is known to be associated with increased transcriptional activity (45), and induction of Fas-L is known to be dependent on c-Jun transcriptional activity (46, 47). Thus, our data provide a plausible explanation as to how induction of JNK by SDG and p-XSC leads to induction of Fas-L and apoptosis. To confirm the functional importance of the JNK/c-Jun pathway directly, we also performed experiments interfering with c-Jun function using a dominant negative mutant of c-Jun, TAM67 (48). Because the primary oral cultures are extremely difficult to transfect in

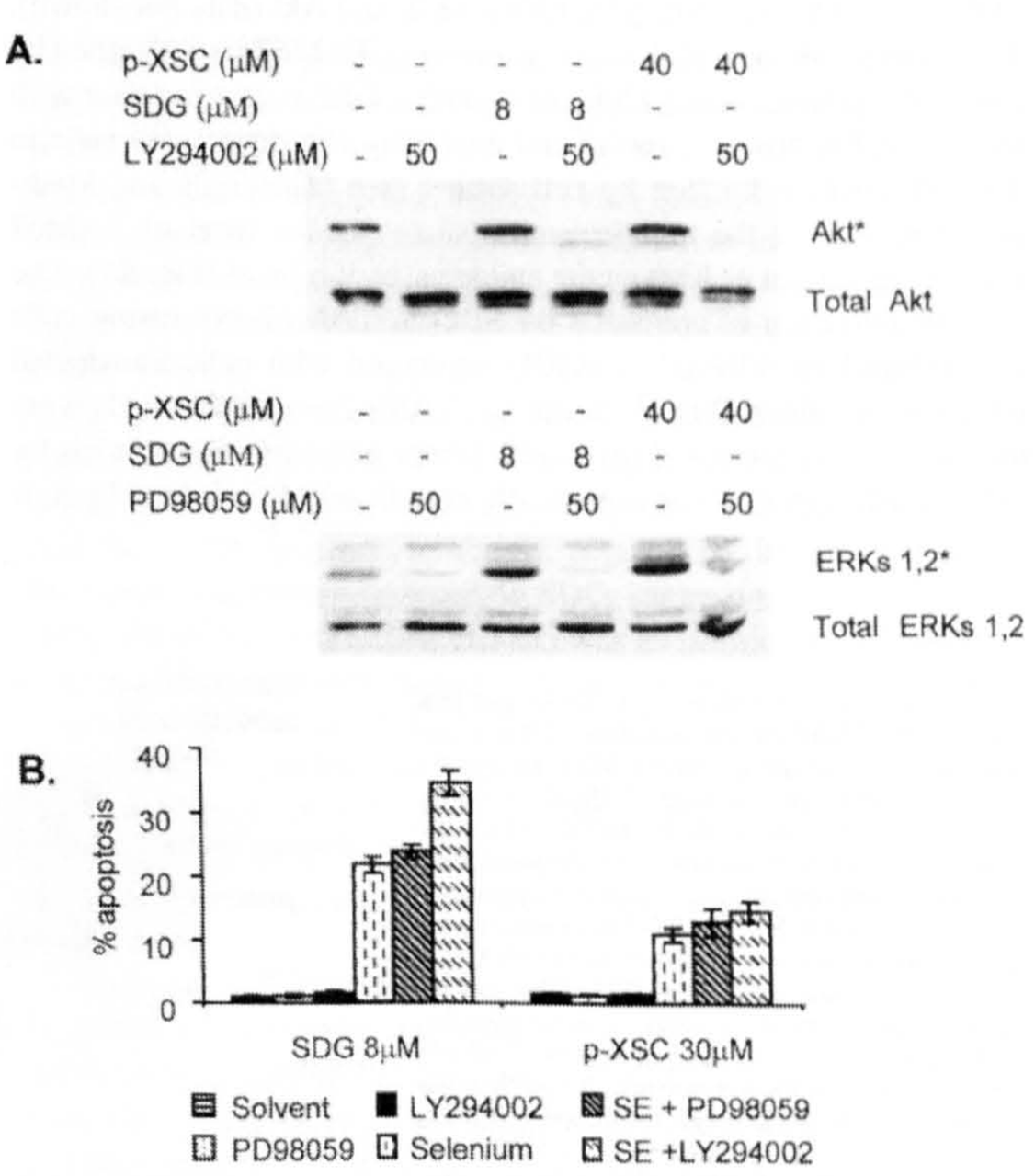

Fig. 5. Effect of treatment of SCCs with the P-I-3 kinase/Akt pathway inhibitor (*LY294002*) or the ERKs 1&2 pathway inhibitor (*PD98059*) on induction of apoptosis by selenium compounds. *A*, SCCs were pretreated with 50 μM LY294002 or 50 μM PD98059 and then treated with 8 μM SDG or 30 μM p-XSC for 18 h, and the effect on activation of ERKs 1&2 or Akt was determined by Western blotting using antibodies that recognize phospho-specific or total Akt or ERK. *B*, induction of apoptosis under the same conditions measured by TUNEL staining as described in Fig. 2.

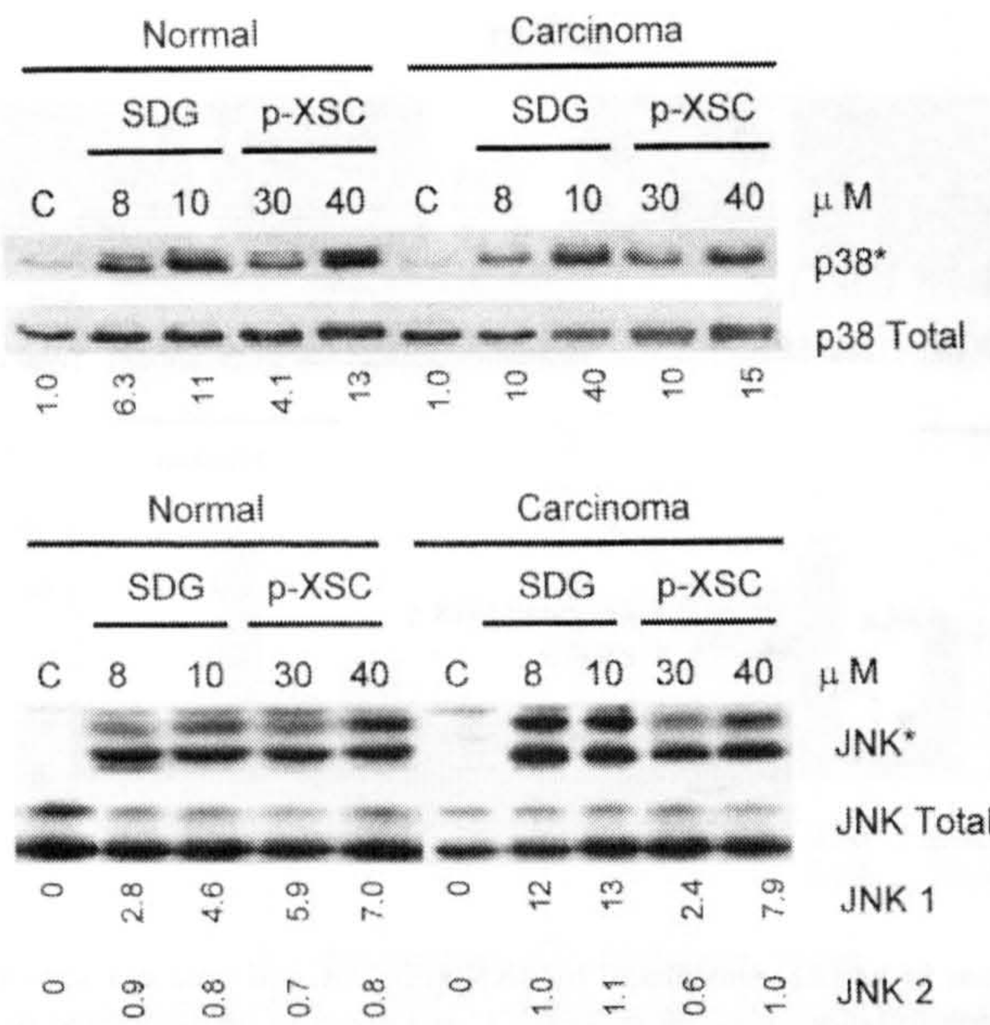

Fig. 6. Activation of JNK and p38 stress kinases by SDG and p-XSC. NOMCs or SCCs were left untreated (*C*) or treated with the indicated concentrations of SDG or p-XSC for 1 h or 30 min, respectively, and cell lysates were prepared and analyzed by SDS-PAGE and Western blotting with antibodies against the activated forms of p38 or JNK (*p38** and *JNK**). As loading controls, the blots were then stripped and Western blotted with antibodies against total p38 or JNK. The figures below the blots indicate the relative levels of p38, JNK1, or JNK2 expression after normalization to the loading control levels.

sufficient numbers, this experiment was performed using HeLa cells because they were found to resemble SCCs in terms of the effects of SDG and p-XSC on JNK, p38, ERKs 1&2, and Akt (data not shown). Thus, a population of HeLa cells expressing TAM67 was obtained by transiently cotransfecting HeLa cells with a GFP vector together with the TAM67 expression vector and analyzing the transfected cells in the GFP-positive fraction by cell sorting (see "Materials and Methods" for details); the transfectants obtained had a level of TAM67 expression at least as high as the endogenous Jun level (Fig. 8*A*). The level of induction of apoptosis by SDG in TAM67-expressing cells was reduced by 40% ($P < 0.001$) compared with cells transfected with a vector alone (Fig. 8, *B* and *C*). TAM67-expressing cells were only marginally protected (by ~15%) from induction of apoptosis by p-XSC, although this was statistically significant ($P = 0.01$; Fig. 8, *B* and *C*). Experiments were also performed to test whether inhibiting both the JNK and p38 kinase pathways together (by treating TAM67-transfected cells with SB203580 to inhibit p38 kinase activity) was more effective at protecting cells from apoptosis by SDG and p-XSC. However, the extent of protection was identical to that afforded by TAM67 overexpression alone (Fig. 8*C*). TAM67 expression in HeLa also reduced the level of induction of Fas-L by SDG, but not p-XSC (Fig. 8*D*), and this correlates with the ability of a dominant negative FADD construct (49) to reduce the extent of apoptosis induced by SDG more than that induced by p-XSC (Fig. 8*E*), though both reductions were statistically significant ($P = 0.014$ and $P = 0.034$, respectively). Overall, these functional transfection experiments with HeLa cells support the conclusion from our data with SCCs based on the use of different concentrations of SB202190 that induction of JNK appears to be the main stress signal transduction pathway functionally required for induction of Fas-L and apoptosis by SDG and p-XSC (although the protection from p-XSC-induced apoptosis by c-Jun inhibition by TAM67 transfection in HeLa cells is not as convincing as that afforded by JNK inhibition by SB202190 in SCCs, for reasons that are presently unclear).

DISCUSSION

The main purpose of this work was to identify the signal transduction mechanisms responsible for apoptosis induced by cancer-protective SE compounds using a relevant human model. Our experimental rationale was based on previous work by ourselves and others that SE compounds with the strongest cancer-protective properties *in vivo,* such as p-XSC, and metabolites of selenite, such as SDG, are also the most effective inducers of growth arrest and apoptosis *in vitro.*

Our first novel finding is that human SCCs are more sensitive to induction of apoptosis by the SDG than NOMCs. This is clearly of considerable relevance to understanding the cancer-protective effect of SE compounds, particularly selenite. It is not clear why p-XSC does not show the tumor-selectivity exhibited by SDG but may reflect the relative importance of JNK/c-Jun pathway in mediating the effects of the two SE compounds (see below). Since the current work and manuscript were completed, a recent report has also shown that the LNCaP prostate carcinoma cell lines are more sensitive to growth inhibition and induction of apoptosis by selenite or selenomethionine than primary prostate cells, though the molecular signal transduction mechanisms responsible were not explored (50).

The second novel point of interest is that induction of apoptosis by

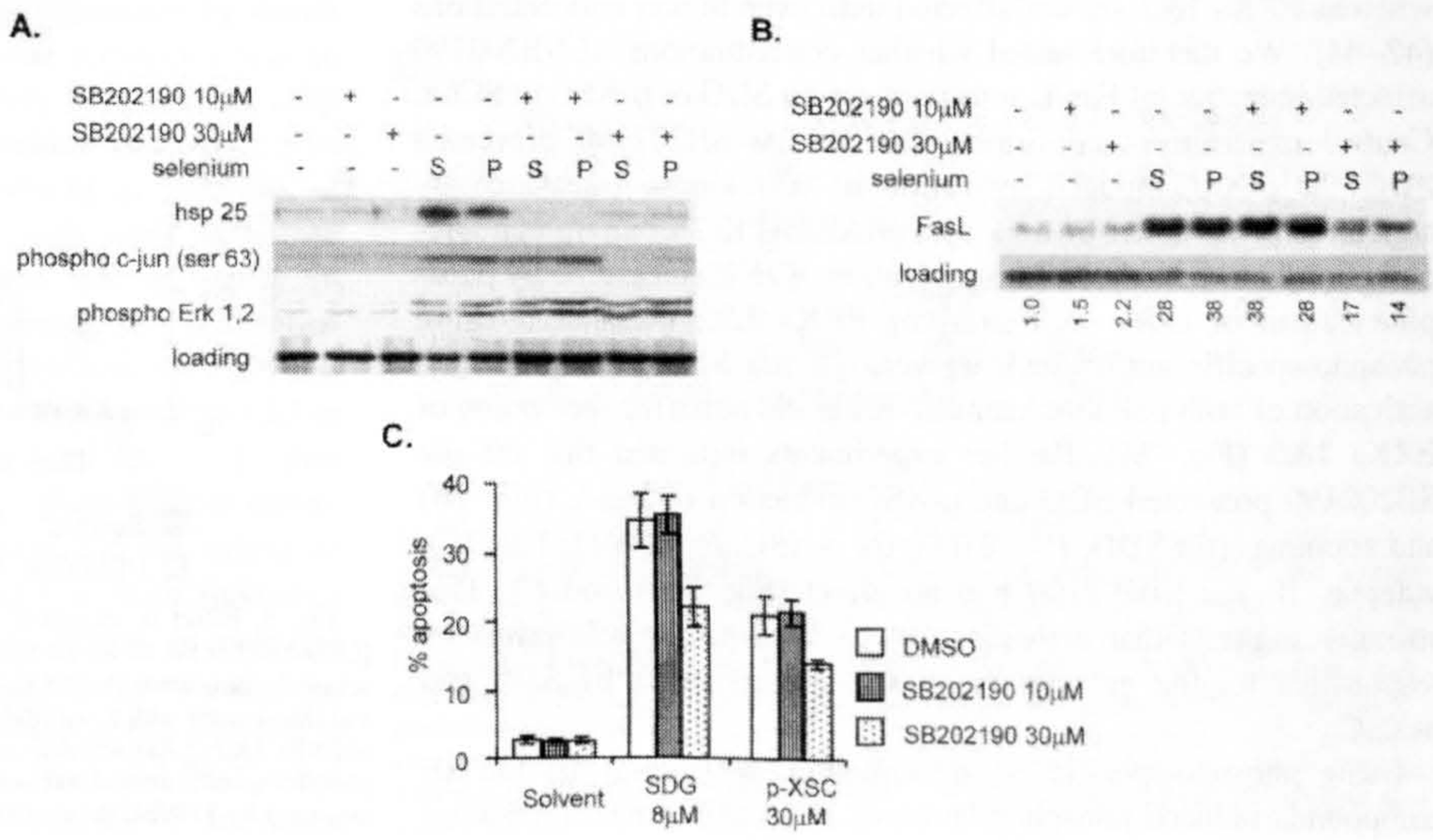

Fig. 7. Effect of inhibition of p38 kinase and JNK activation by SB202190 on induction of Fas-L and apoptosis by SDG and p-XSC in SCCs (*A* and *B*). Effect of varying concentrations of SB202190 on the activation of p38 kinase, JNK, and ERKs 1&2 (*A*) and induction of Fas-L by 8 μM SDG (*B*) or 30 μM p-XSC. In *A,* p38 kinase activity was measured by activation of its direct substrate MAPKAPK-2 in an immunoprecipitation kinase assay using Hsp25 as MAPKAPK-2 substrate. Activation of JNK and ERKs 1&2 was assayed by phosphorylation using a Ser63-phospho-specific anti-Jun or ERKs 1&2 antibodies. Total p38 levels were used as loading controls. *B* and *C,* SCCs were pretreated for 30 min with the indicated concentrations of SB202190, followed by 8 μM SDG or 30 μM p-XSC for 16 h. In *B,* Fas-L levels were measured by Western blotting as in Fig. 3*A*. The figures below the blots indicate the fold increase in Fas-L expression above control level after SE treatment. In *C,* apoptosis was measured by TUNEL staining.

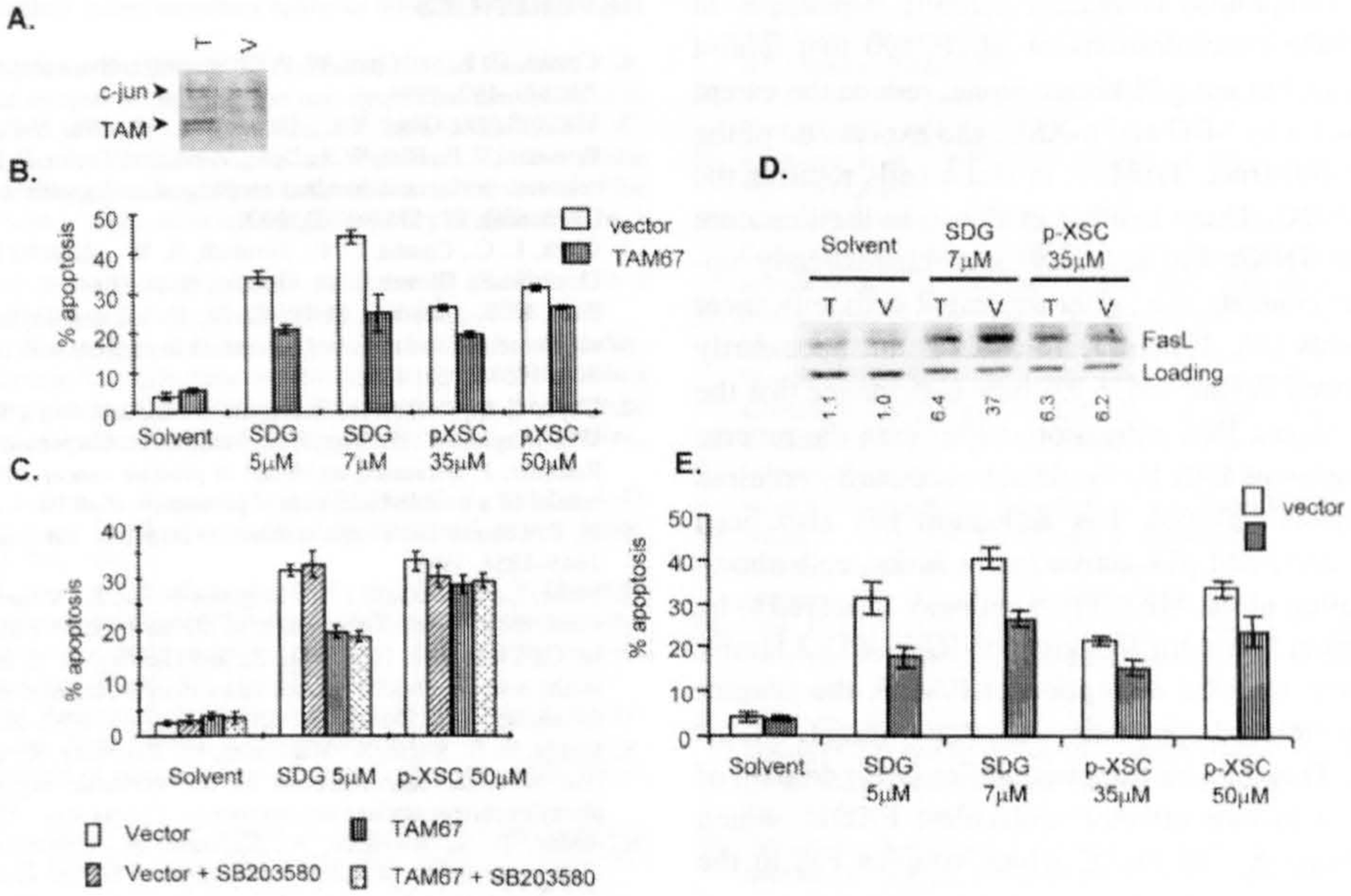

Fig. 8. Effect of overexpressing dominant negative c-Jun (*TAM67*) on induction of apoptosis and Fas-L by SE compounds. HeLa cells were transiently transfected with a TAM67 expression vector (*T*) or empty vector (*V*) together with a GFP-expression vector and the GFP-positive cells purified by cell sorting. In *A,* Western blotting experiments with a pan-Jun antibody showed that the cells transfected with the TAM67-expression vector express TAM67 at as least as high a level as endogenous normal Jun. In *B,* cells were then treated with 5 μM SDG or 50 μM p-XSC for 18 h, and the percentage of apoptotic cells was determined by measuring the percentage of sub-G_0-G_1 cells. Similar differences between vector- and TAM67-expressing cells were found in two other experiments, using annexin V staining as the criterion for apoptosis (data not shown). In *C,* TAM67-transfected cells were preincubated with 10 μM SB203580 for 30 min followed by 5 μM SDG or 50 μM p-XSC for 18 h, and the percentage of apoptotic cells was determined as in *B*. In *D,* vector (*V*)- or TAM67 (*T*)-transfected cells, cotransfected with a GFP-expression vector, were treated with the indicated concentrations of SDG or p-XSC for 8 or 18 h, respectively, and then analyzed for Fas-L expression. The percentages of transfected cells, determined from the percentage of GFP-expressing cells, were 85 and 83% for vector and TAM67 transfections. The figures below the blots indicate the fold increase in Fas-L expression above control level after SE treatment. In *E,* an "empty" expression vector or the same vector encoding a dominant negative FADD-GFP fusion protein was transiently transfected into HeLa cells and treated with the SE compounds indicated for 16 h, and the GFP-positive population was assayed for apoptosis by the proportion of sub-G_0-G_1 cells.

both SE compounds is associated with a large induction in Fas-L expression above the low basal level in untreated cells; the form of Fas-L produced is the soluble form which could therefore be secreted and act intercellularly. The extent of Fas-L induction by the SE compounds correlates closely with the level of apoptosis induced in normal mucosa or carcinomas, and inhibition of the Fas pathway in SCCs by a Fas/Fc chimera attenuates the induction of apoptosis by SDG and p-XSC. Fas-L expression in biopsies of squamous carcinomas of the head and neck has also been demonstrated recently in another report and shown to be biologically active in inducing apoptosis in cocultivated activated T lymphocytes (51). This is of considerable interest because the Fas-L/Fas receptor interaction is one of the major pathways initiating apoptosis by a variety of agents (46), including alkylating agents (52) and drugs used in chemotherapy (53, 54). Because both NOMCs and SCCs express the Fas receptor constitutively (data not shown), induction of Fas-L may explain why SE compounds induce apoptosis in human oral cells. *In vivo,* the soluble Fas-L produced by carcinoma cells could also enhance immunological responses that could target the carcinoma cells (55). Activation of Fas pathway by SE compounds could therefore be a factor explaining their effects in enhancing antitumor immune responsiveness (1, 56). Our data show that Fas-L induction by the SE metabolite, SDG, is readily detectable at a SE concentration within the range of plasma concentrations found in humans, although higher than the average level (57). The concentrations of the synthetic SE derivative, p-XSC, required for Fas-L induction are higher than for SDG, but it is well established in animal models that p-XSC has a higher chemopreventive index than selenite, exerting its maximum cancer-protective effect at high dietary levels (about 30 ppm compared with 2 ppm for selenite) because of its lower toxicity (58).

Thirdly, both SDG and p-XSC induce activation of the p38 and JNK stress kinase pathways at concentrations that induce apoptosis, confirming and extending a previous report demonstrating activation of JNK by p-XSC (59). SDG and p-XSC also induce ERKs 1&2 and Akt, but these effects are relatively small (except for the significant effect of p-XSC on ERKs 1&2). Our functional intervention experiments show that activation of the JNK pathway seems to be most important, because inhibition of the JNK pathway, but not the p38 kinase pathway, with appropriate concentrations of SB202190 or using a dominant negative c-Jun construct, reduces SE-induced apoptosis, especially with SDG. Conversely, inhibition of the P-I-3 kinase pathway by LY294002 did not reduce the sensitivity of SCCs to induction of apoptosis by the SE compounds; in fact, it increased the extent of apoptosis induced by SDG, suggesting that the observed induction of Akt in response to SDG was an (unsuccessful) protective response of cells to induction of apoptosis by SDG. Finally, PD98059, a specific inhibitor of ERKs 1&2 activation, did not affect the sensitivity of SCCs to induction of apoptosis by SDG or p-XSC. Moreover, our experiments exclude the possibility that SDG or p-XSC act by inhibiting activation of the ERKs 1&2 or Akt pathways by mitogens, such as EGF or serum which would have biased cells toward cell death.

Induction of JNK by SDG in SCCs is somewhat more marked than in NOMCs, which may therefore explain the increased sensitivity of SCCs to induction of Fas-L/apoptosis by SDG. This effect of micromolar levels of SE in inducing apoptosis via activation of the JNK pathway has to be distinguished from the ability of submicromolar levels of selenite to inhibit UV-induced apoptosis and activation of JNK (60), presumably by increasing the detoxification of reactive oxygen species by increasing the activities of one or more of the known selenoproteins.

The final point of interest is our evidence indicating that induction

of Fas-L by selenium compounds is at least partially dependent on induction of JNK because concentrations of SB202190 that inhibit both JNK and p38 kinase, but not p38 kinase alone, reduce the extent of Fas-L induction in SCCs by SDG and p-XSC, and expression of the dominant negative Jun construct, TAM67, in HeLa cells reduces the induction of Fas-L by SDG. There is other evidence in the literature also indicating that the JNK/c-Jun pathway is mechanistically upstream of Fas-L in other contexts, *e.g.*, after treatment with anticancer drugs or alkylating agents (46, 47). However, this is not necessarily the case (recently reviewed in Ref. 45); *e.g.*, there is evidence that the Fas/Fas-L interaction induces JNK activation, rather than the reverse (61, 62), and that activation of JNK by Fas is not necessarily required for induction of apoptosis (63–65). Fas activation has also been shown to activate both JNK and p38 activation in Jurkat cells; however, in this case, activation of the MKK3/p38 pathway appeared to be important as a downstream target for Fas-induced ICE/CED-3 family proteases (66). Moreover, targeted disruption of FADD, the adaptor protein required for Fas-induced apoptosis, does not eliminate stress-induced apoptosis (67). This may be because the Fas death domain of Fas binds independently to two effector molecules: FADD, which couples Fas to pro-caspase-8, and Daxx, which couples Fas to the JNK pathway (62).

Exactly how SE compounds induce the stress kinase pathways is not yet clear. It has been postulated that SE compounds induce oxidative stress by production of superoxide radicals or hydrogen peroxide (68–70). However, we have previously presented evidence that SE compounds do not induce apoptosis in the same way as hydrogen peroxide (71). More recently, we have shown that the apoptosis-inducing effects of SDG and p-XSC in animal cell lines are prevented by antioxidants, such as NH_2-acetylcysteine and GSH; however, putative hydroxyl radical scavengers, such as mannitol and pyrrolidine dithiocarbamate, are more effective in protecting against apoptosis induced by p-XSC than SDG (33). This is another suggestion that SDG and p-XSC do not act by precisely the same mechanism. One hypothesis to explain induction of apoptosis by SDG is that it may alter the redox status of the cells by manipulating the level of a cellular reducing agent, such as thioredoxin, which has been implicated in growth control in various contexts and is overexpressed in many tumors (reviewed in Ref. 72). This may be a plausible idea because SDG has been shown to be a specific inhibitor of thioredoxin and thioredoxin reductase in cell-free systems (73, 74). Alternatively, continuously high dietary levels of SE result in a reduction in thioredoxin reductase activity, postulated to be attributable to the formation of an irreversible diselenide "trap" at the selenocysteine in its active site (19); this would also result in a reduction in the level of reduced thioredoxin. Of particular interest in this context is the fact that ASK1, one of the upstream activators of both JNK and p38 (75), is inhibited by binding of reduced thioredoxin to its NH_2-terminal region (76); thus, depletion of reduced thioredoxin by SE compounds by one of the mechanisms described above might be expected to activate ASK1 and induce apoptosis. ASK1 is also regulated by the level of intracellular GSH (77), which may also be affected by the level of SE metabolites. We are currently attempting to test both these hypotheses.

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Molecular mechanisms of cancer prevention by selenium compounds

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Abstract: *Based on the observed correlation between the effectiveness of a variety of selenium compounds as chemopreventive agents in animal models and their ability to inhibit cell growth and induce apoptosis in vitro, one hypothesis to explain the cancer protective effects of selenium compounds is that they reduce the outgrowth of tumour cells in vivo. Our recent work has therefore investigated the effects of selenium compounds on signalling pathways responsible for growth and apoptosis in biopsies of normal oral mucosa and oral carcinomas, using a primary culture system. Interestingly, oral carcinomas are significantly more sensitive than normal oral mucosa to induction of apoptosis by a natural selenium metabolite (selenodiglutathione, SDG) and this is associated with induction of Fas ligand (Fas-L), a well known mediator of apoptosis in other contexts, and activation so-called stress kinase signalling pathways, of which the Jun terminal kinase (JNK) pathway appears to most important, as judged by functional intervention experiments. Heme oxygenase (HO), another marker of stress responses, is also induced by selenite and SDG. The selective activation of the Fas pathway in carcinomas could be responsible directly for their destruction by apoptosis or target them for attack by immunological responses. In contrast, although the potent pharmacological selenium chemopreventive agent, 1,4-phenylenebis(methylene)selenocyanate (p-XSC), also induces Fas-L, HO and stress kinase pathways, the mechanism of is clearly different to that of SDG since apoptosis/Fas induction is not JNK-dependent and p-XSC does not show tumour-selectivity. These differences in mechanism between SDG and p-XSC may be due to the manner in which they induce redox changes in the cells, since, although the effects of SDG and p-XSC are both prevented by anti-oxidants such as glutathione or N-acetyl cysteine(NAC), hydroxyl radical scavengers such as mannitol or pyrrolidine dithiocarbamate (PDTC) only protect against the effects of p-XSC.*

Animal evidence for a cancer protective effect of selenium

There is very convincing evidence that a high dietary level of naturally-occurring selenium, usually in the form of sodium selenite, substantially reduces the incidence of a wide variety of animal cancers under conditions where animal growth and health are not affected (1-3). Most studies have observed the maximum cancer-protective effect at non-toxic levels considerably higher than normal nutritional levels but there is also more limited evidence that sub-nutritional selenium deprivation increases cancer risk (recently reviewed by Ip (4) and Combs & Gray (5)). In animal models, the most effective chemopreventive agents seem to be those that are metabolised directly to selenols (6-9), including aliphatic or benzyl selenocyanates (10-12), particularly p-XSC (13-15), and Se-methylselenocysteine, which may be the major non-volatile form of selenium in plant foods (16). In particular, selenium derivatives like p-XSC are effective in animal carcinogenesis models in which selenite is not so effective, for example, NNK-induced lung cancers (13).

Epidemiological evidence for a cancer protective effect of selenium

Human epidemiological evidence also indicates a statistically significant inverse relationship between selenium level and risk of cancer overall, particularly in men (17-28; recently reviewed in (5)). In terms of individual cancer types, the evidence from the largest studies is strongest for upper respiratory tract, prostate, oesophageal and stomach cancers (27,29-32) but there is little evidence of any link with colon (23,27,28,32) or breast (34-36) cancer risk in such cohort studies, although the length of follow-up in some of these studies was quite short. Three intervention trials have also investigated whether selenium supplementation reduces the risk of cancer. An early randomised intervention trial in China, with about 1000 subjects in each of the

treatment and placebo arms, reported that dietary supplementation with 200 μg/d selenium as selenised yeast for 2 y reduced the incidence of liver cancer in a high risk area by 45% ($p = 0.05$) (37). A joint Chinese/US randomised factorial design study in a nutritionally poor population in Linxian County, China, showed that dietary supplementation for 5 y with a cocktail of three antioxidants (β-carotene, vitamin E and selenium) at about twice their RDA values produced a statistically significant reduction in overall cancer rate of 13 % and a 21 % reduction in stomach cancer rate, whereas supplementation with other cocktails of vitamins or trace elements had no effect (38,39). It seems plausible that selenium was responsible for the effect of the antioxidant cocktail since evidence from intervention trials suggests that neither β-carotene nor vitamin E reduce cancer risk generally (40-43): indeed, although there may be a significant inverse relationship between prostate cancer and vitamin E intake (44,45), β-carotene supplementation was consistently associated with a higher risk of lung cancers in subjects who were current smokers (46-48). But the case for selenium supplementation reducing cancer risk has received greatest support from the recent randomised placebo-controlled intervention trial by Dr. Larry Clark and his colleagues involving 1312 men with a prior history of skin basal or squamous cell carcinoma (49,50): although this found no evidence for a reduction in risk of recurrence of skin cancer with 200 μg/d selenium as selenised yeast, total cancer rates in the treatment arm were reduced significantly (by 37 %), as were the individual rates of lung, colorectal and prostate cancers (49,50). The strongest treatment effect was observed in subjects with the lowest plasma selenium levels prior to supplementation.

Possible molecular mechanisms

The molecular mechanism(s) responsible for reduction in cancer risk by selenium are presently unclear. The early hypothesis that the chemopreventive effect of selenium might be mediated by selenoproteins, such as the glutathione peroxidases, eliminating tumor-promoting reactive oxygen species now seems unlikely since the activities of known selenoproteins seem to be saturated in animals at a much lower dietary selenium level (0.1-0.4 p.p.m.) (51-53) than that required for the maximum chemopreventive effect of selenium (2-4 p.p.m.) (1-4). There is further evidence that at least the cytosolic glutathione peroxidase (GPX1) is not involved since GPX1-null mice do not show any abnormal histopathologies up to 15 months age (54,55) (although they are more sensitive to exogenous oxidative stress (56)) and GPX1-transgenic mice are actually more sensitive to DMBA/TPA-induced skin cancer, rather than the reverse, hypothetically because the generation of tumour-promoting lipoxygenase-derived peroxides is increased (57). However, thioredoxin reductase remains a possible candidate since it has been shown to be inhibited by continuous high selenium levels, possibly due to diselenide bond formation at the selenocysteine in the active site (58). New selenoproteins have recently been discovered (59,60) but their functions and selenium saturation levels are presently unknown.

Although selenite and p-XSC can reduce DNA damage by carcinogens (4,61—63), they are also effective if given after carcinogen in the early phase of tumour progression (1,4), suggesting that they may act primarily as anti-promotion agents. This is also suggested by the fact that, in general, the relative efficacies of selenium derivatives as chemopreventive agents in vivo parallel their growth inhibitory effects in vitro and their ability to induce apoptosis (64-68). Moreover, induction of apoptosis

by selenium metabolites in vitro is readily detectable at levels towards the upper limit of plasma concentrations found in humans, i.e. about 5 μM, although higher than the average level (Figure 1). Thus, understanding the mechanisms mediating the growth inhibitory effects in vitro may be relevant to the mechanism of chemoprevention in vivo and so identifying the molecular target(s) of selenium action may suggest avenues for devising novel compounds that may be useful as cancer-protective agents, for example in high-risk groups. We have previously shown that treatment of cells with the selenite metabolite, selenodiglutathione (SDG) induces p53 accumulation in cells containing wild type p53 (65). However, as we and others have shown, selenium compounds induce growth arrest/apoptosis of cells expressing mutant p53 (65) or lacking p53 completely (69): thus, the mechanism whereby selenium compounds inhibit cell growth and induce apoptosis does not necessarily require a functional wild-type p53 pathway. Recent work showing that both inorganic and organic forms of selenium induce gadd34, gadd45 and gadd153 (69) suggests that interference with cell cycle checkpoint controls is associated with growth arrest/apoptosis induced by selenium compounds.

Induction of stress responses by interference with redox control

The main purpose of our recent work has been to identify the signalling mechanisms responsible for apoptosis induced by cancer-protective selenium compounds. Our experimental rationale was based on previous work by ourselves and others that selenium compounds with the strongest cancer-protective properties in vivo, such as p-XSC, and metabolites of selenite, such as SDG, are also the most effective inducers of growth arrest and apoptosis in vitro. The reason for using SDG is based on our observation that selenite freshly added to cells only inhibited their growth after a lag

period of 24 h, whereas conditioned medium from such cells transferred to fresh cells to give the same selenium concentration inhibited their growth rapidly and the active agent was shown to be low molecular weight by passage through filters with different pore sizes (data not shown). The lag period was also reduced by the addition of glutathione (GSH) together with selenite (Figure 2). Both these findings suggested that the active inhibitor might be SDG, which would be formed by reaction of selenite with GSH either intra- or extra-cellularly and this was consistent with the fact that purified SDG inhibited cell growth without a significant lag period (Figure 2). This growth arrest was also shown to be associated with apoptosis as determined by the TUNEL assay (data not shown).

Exactly how selenium compounds induce apoptosis is not yet clear. It has been postulated that selenium compounds induce oxidative stress by production of superoxide radicals or hydrogen peroxide (70-72), although our previous work suggested that SDG does not induce apoptosis in the same way as hydrogen peroxide (73) and this is supported by our new evidence that selenium compounds do not induce the same large scale changes in phosphorylation of tyrosine residues in proteins characteristic of oxidants such as hydrogen peroxide and diamide (Figure 3). Nevertheless, selenite, SDG and p-XSC induce heme oxygenase (HO) (Figure 4), a well-established indicator of a variety of cellular stresses, for example by oxidants, irradiation and sulphydryl reagents, as well as heme (74) and this occurs at selenite and SDG concentrations of 3-5 μM, which is within the range of plasma concentrations found in humans (Figure 1). We also present further evidence here that, although the growth-inhibitory effects of both SDG and p-XSC in animal cell lines are prevented by the antioxidant N-acetylcysteine (NAC), putative hydroxyl radical

scavengers, such as mannitol and PDTC are more effective against p-XSC than SDG (Figure 5). This suggests that SDG and p-XSC do not act by precisely the same mechanism. One hypothesis to explain induction of growth arrest/apoptosis by SDG is that it may alter the redox status of the cells by manipulating level of a cellular reducing agent, such as thioredoxin which has been implicated in growth control in various contexts and is over-expressed in many tumours (recently reviewed in (75)).

Tumour-selective induction of apoptosis by the selenium metabolite, SDG.

In view of the fact that lung and esophageal cancers are two of the cancer types in which the epidemiological evidence for a cancer protective effect of selenium is strongest, in our recent work (76) we have focussed on human oral cancers since they share similar risk factors to lung and other head and neck cancers and represent one of the few experimental models where biopsies of both normal tissue and lesions at various stages of cancer progression can be obtained and studied in primary culture (77, 78). One of the novel findings to emerge from this work (76) is that human oral carcinomas are more sensitive to induction of apoptosis by the SDG than normal oral mucosa cells. This is clearly of considerable relevance to understanding the cancer-protective effect of selenium compounds. p-XSC does not show this tumour-selectivity: the reasons for this difference between SDG and p-XSC are unclear but appears to be connected with the relative importance of the various kinase signalling pathways that mediating the effects of the two selenium compounds (discussed further below). Another recent report has also shown that the LNCaP prostate carcinoma cell lines is more sensitive to growth inhibition and induction of apoptosis by selenite or selenomethionine than primary prostate cells, though the molecular signalling mechanisms responsible were not explored (79).

Involvement of the Fas and stress kinase pathways in induction of apoptosis by selenium compounds.

Another important finding we have made is that induction of apoptosis by both SDG and p-XSC is associated with a large induction in Fas-L expression above the low basal level in untreated cells (76): the form of Fas-L produced is the soluble form which could therefore be secreted and act inter-cellularly. The extent of Fas-L induction by the selenium compounds correlates closely with the level of apoptosis induced in normal mucosa or carcinomas. Fas-L expression in biopsies of squamous carcinomas of the head and neck has also been demonstrated recently in another report and shown to be biologically active in inducing apoptosis in co-cultivated activated T lymphocytes (80). Since both normal oral mucosa and oral carcinomas express the Fas receptor constitutively (data not shown), induction of Fas-L may explain why selenium compounds induce apoptosis in human oral cells. In vivo, the soluble Fas-L produced by carcinoma cells could also enhance immunological responses that could target the carcinoma cells (81). Activation of Fas pathway by selenium compounds could therefore be a factor explaining their effects in enhancing anti-tumor immune responsiveness (5,82). Our data show that Fas-L induction by the selenium metabolite, SDG, is readily detectable at a concentration which is within the range of plasma concentrations found in humans, although higher than the average level (Figure 1). The concentrations of the synthetic selenium derivative, p-XSC, required for Fas-L induction are higher than for SDG but it is well established in animal models that p-XSC is less toxic and exerts its maximum cancer-protective effect at high dietary levels (about 30 p.p.m. compared to 2 p.p.m. for selenite (83)).

Another recent finding is that induction of the so-called JNK and p38 stress kinases, members of the MAP kinase family of kinases, is associated with induction of apoptosis by SDG and p-XSC (76). However, functional intervention experiments show that the JNK stress kinase pathway specifically is most important for induction of Fas-L by SDG (76) (but this is not the case for p-XSC, for reasons that remain unclear (76)). There is other evidence in the literature also indicating that the JNK/c-Jun pathway is mechanistically upstream of Fas-L in other contexts, for example after treatment with anticancer drugs or alkylating agents, but this is not necessarily the case (recently reviewed in (84)). How selenium metabolites, such as SDG, might activate the JNK/Fas-L pathway is not yet clear but one possibility, in view of our evidence that changes in redox balance may be important (Fig. 4), is that redox regulation of the upstream kinase (ASK1) that regulates JNK/p38 kinases (85) is involved since ASK1 is known to be inhibited by reduced thioredoxin (86) and glutathione (87) and SDG has been shown to inhibit of thioredoxin and thioredoxin reductase (58,88,89). We are currently attempting to test this hypothesis.

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LEGENDS TO FIGURES

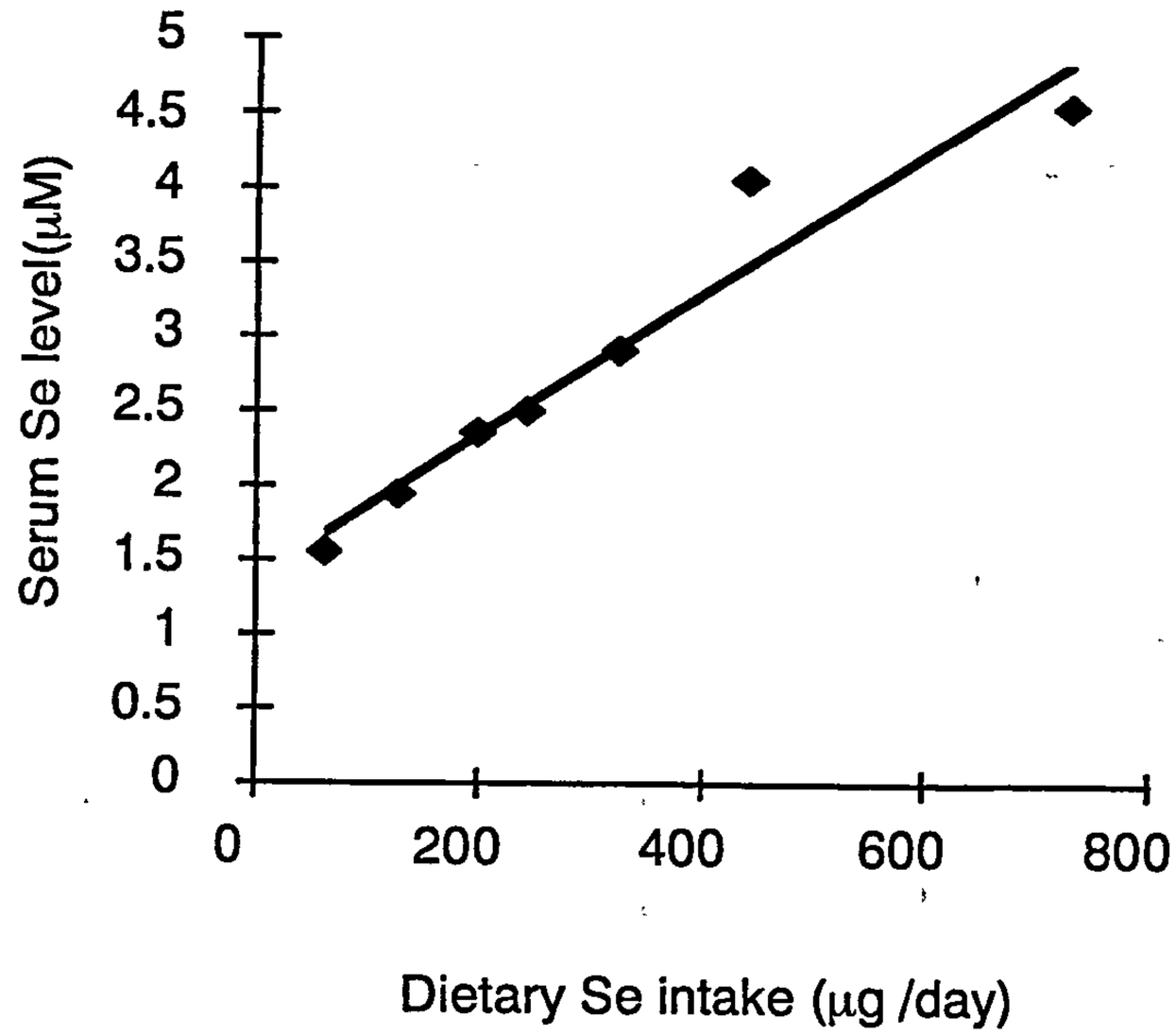
Figure 1. Range of selenium levels in vivo (compiled from data in Longnecker et al.(90)). Plotting the selenium intake of people (μg/day) living in seleniferous areas against the measured serum selenium levels (μM) revealed that serum concentrations of 3-5 μM selenium can be achieved, in vivo, by people taking high doses of selenium without any apparent adverse effects.

Figure 2. Potentiation of the growth inhibitory effects of selenite by glutathione: comparison with SDG. Growth of C57 cells, a mouse mammary epithelial cell line, in response to treatment with 50 μM sodium selenite (Na Se), 50 μM glutathione (GSH), 50 μM Na Se plus 50 μM GSH and 5 μM SDG for 1-4 days. Cell viability was assayed using the MTT assay (Promega, UK) according to the Manufacturer's protocol.

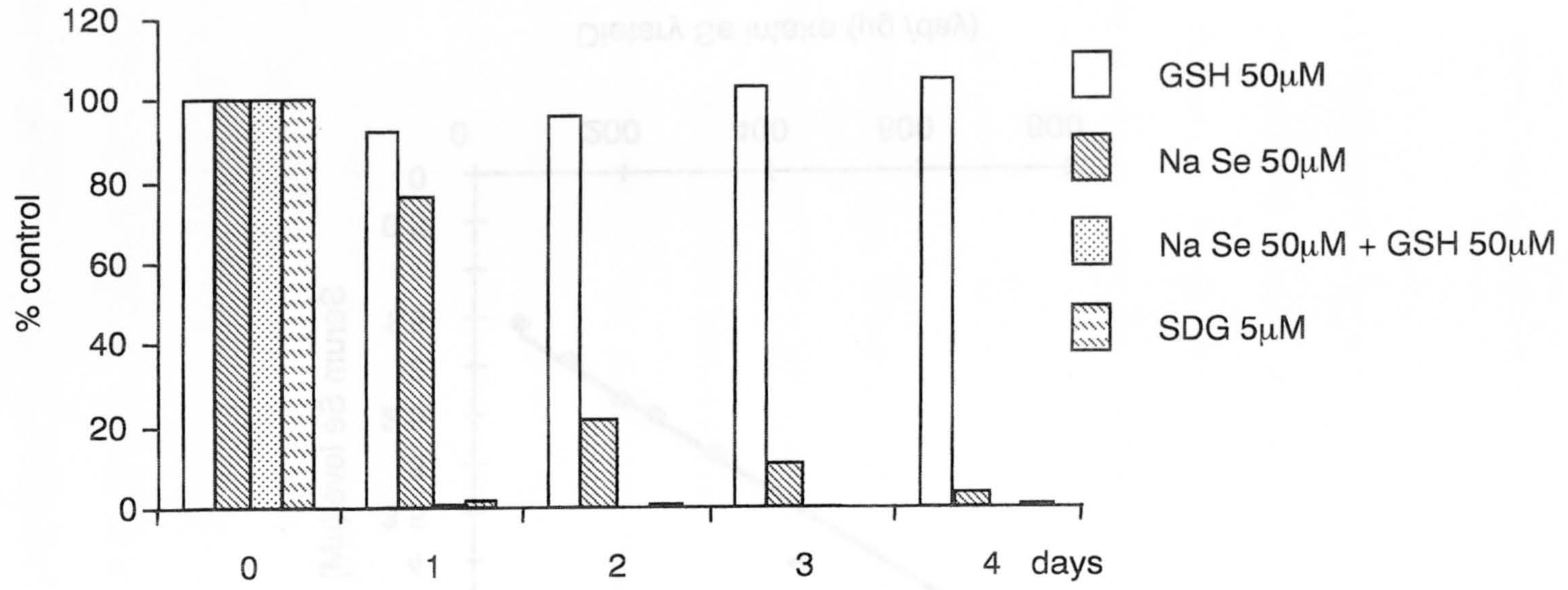
Figure 3. Tyrosine phosphorylation patterns in response to treatment with oxidants and selenium compounds. Cells were treated as indicated with H_2O_2, diamide, selenite, SDG or p-XSC, protein extracts prepared and immunoblot analysis performed using a phospho-tyrosine specific antibody (Santa Cruz Biotech., CA) .

Figure 4. Induction of HO mRNA by selenium compounds. C57 cells were treated with the indicated concentrations of sodium selenite (Na Se), SDG or p-XSC for 4, 6 or 18 h, RNA extracted and analysed by Northern transfer and hybridisation with a HO-1 cDNA probe (74) or 7S ribosomal RNA probe as a loading control.

Figure 5. Effects of antioxidants on inhibition of growth by selenium compounds. Cells were pretreated for 18h with 20 mM NAC or 100 mM mannitol; or for 2 h with 80 μM PDTC and then with the indicated concentrations of SDG or p-XSC in continued presence of antioxidant for 4 h (NAC or PDTC) or 18 h (mannitol). Cells were then washed in PBS and cell growth measured using the MTT assay. The results are expressed relative to the cells treated with antioxidant alone.



Fleming et al. Fig 1



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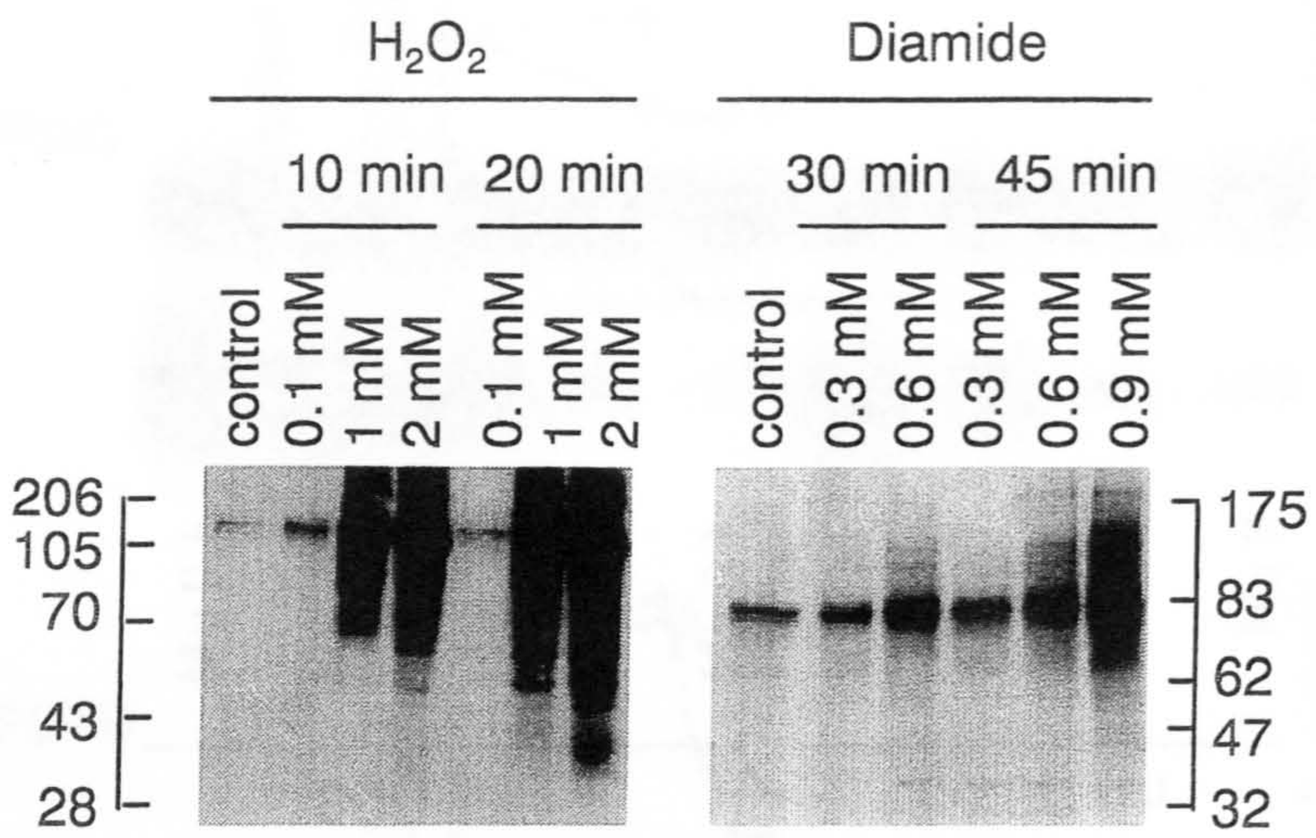
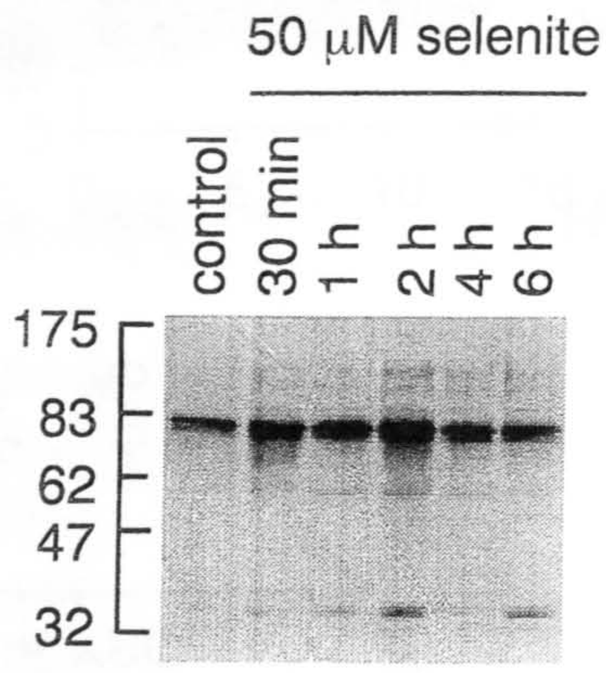
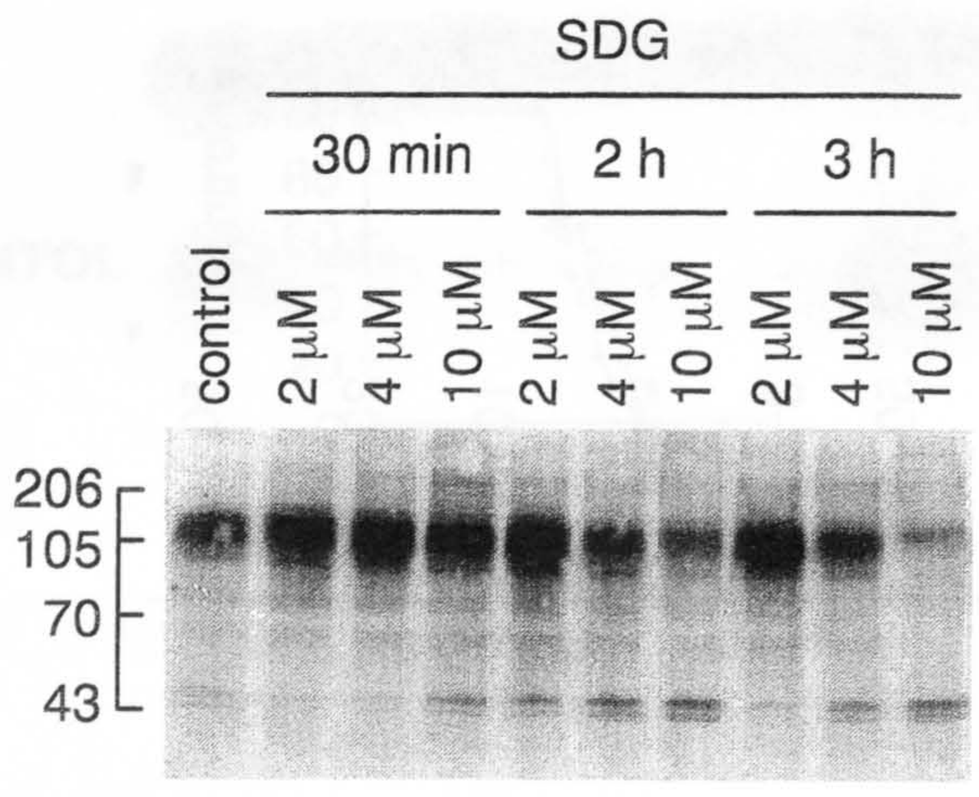
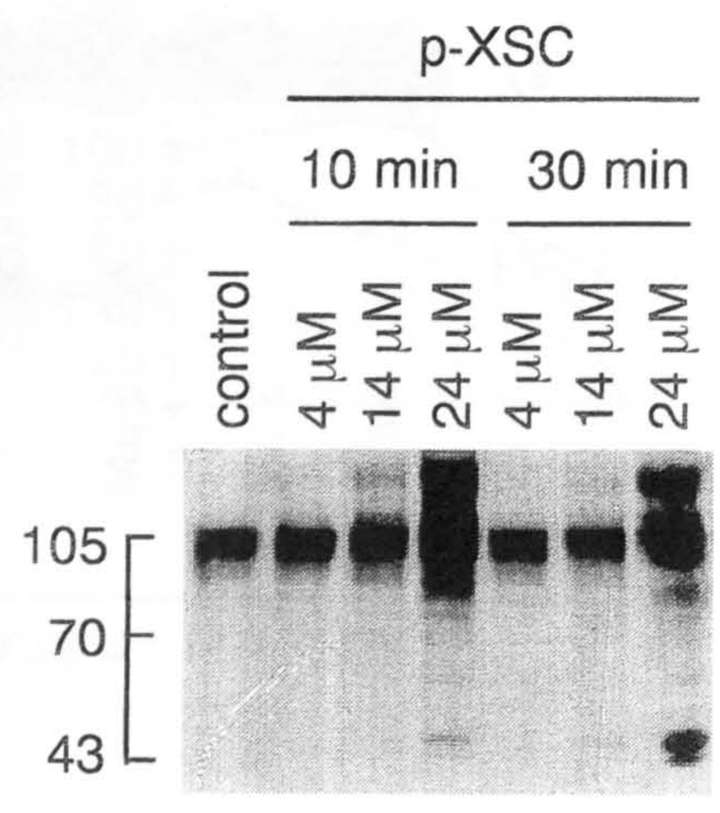
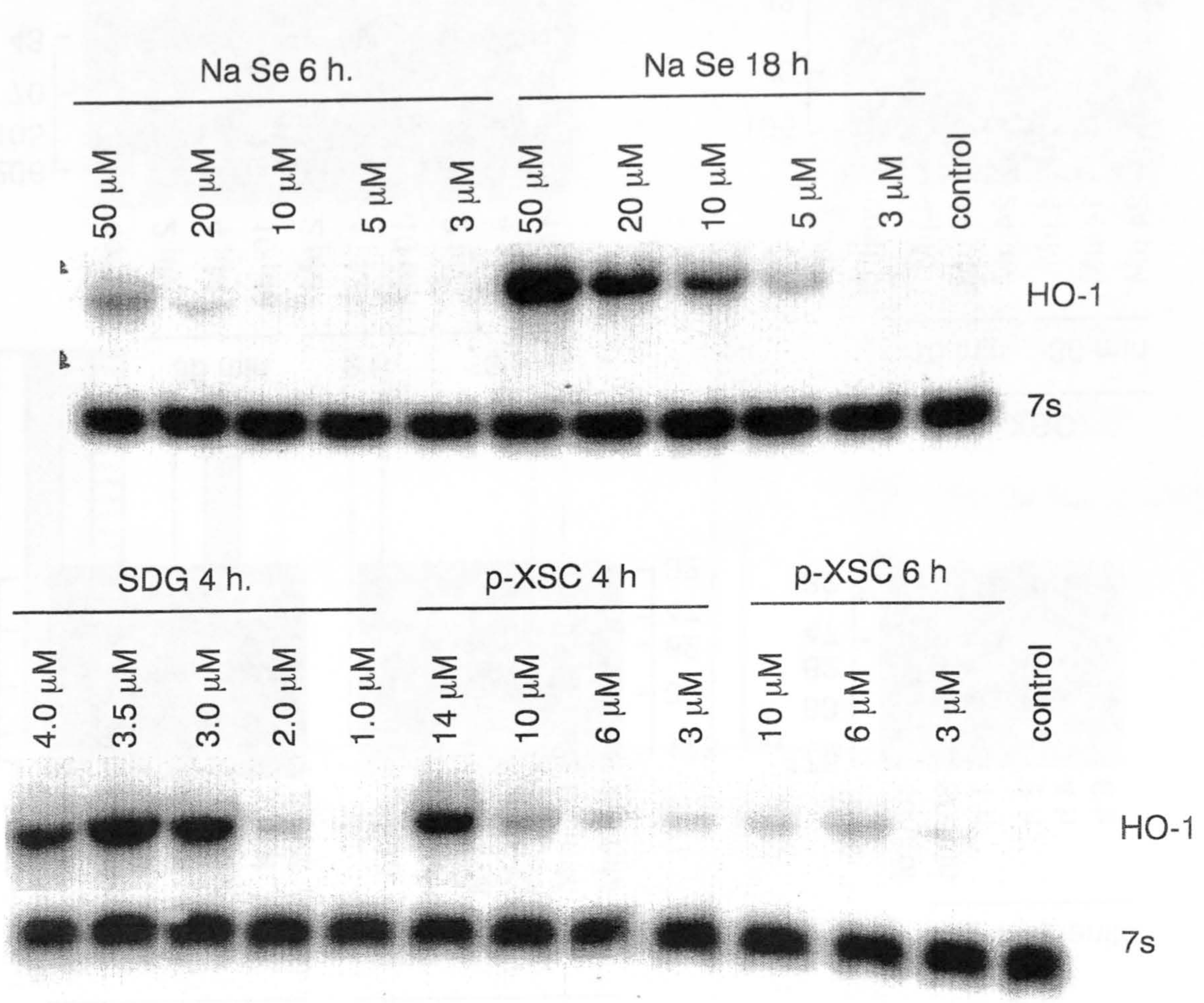




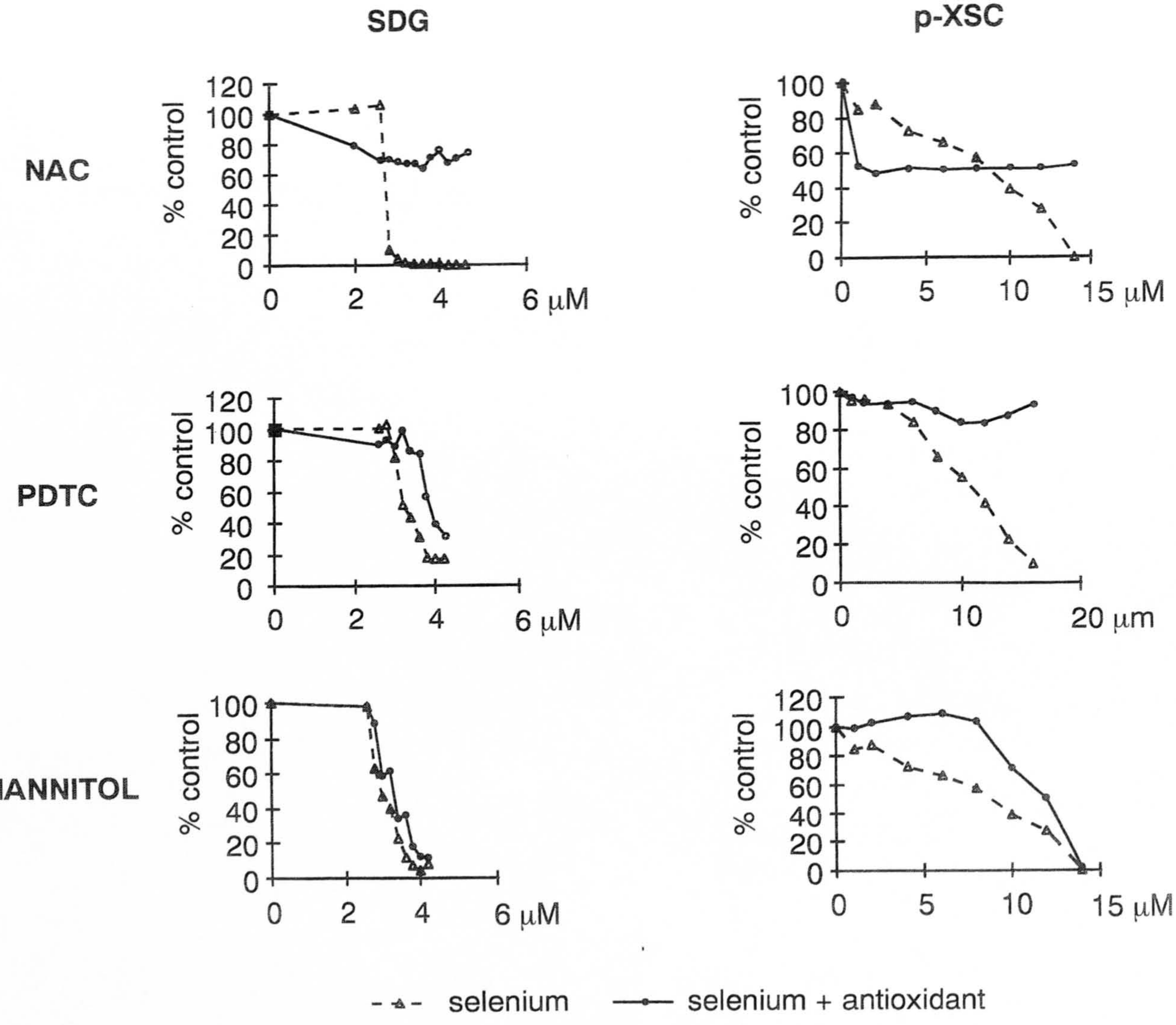




Fleming et al. Fig 3



Fleming et al. Fig 4



Fleming et al. Fig 5