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University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

JNK regulates Fas receptor mediated apoptosis in prostate cancer cell lines

A thesis submitted to the National University of Ireland, Cork in

fulfilment of the requirements for the degree of

Doctor of Philosophy

by

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2003

If we work upon marble, it will perish;
If we work upon brass, time will efface it;
If we rear temples, they will crumble into dust;
But if we work upon immortal minds and instill into
them just principles, we are then engraving that
upon tablets which no time will efface,
but will brighten and brighten
to all eternity.

Daniel Webster

**Dedicated to my family and to my
girlfriend for their relentless support
and unfaltering belief in me**

Table of Contents

Declaration

This thesis has not been submitted in whole or in part to this or any university for any degree, and is, unless otherwise stated, the original work of the author.

Signed: _____

        James Curtin

Acknowledgements

Abstract

Prostate Cancer is a disease that primarily affects elderly men. The incidence of prostate cancer has been progressively increasing in the western world over the last two decades. Life expectancy and diet are believed to be the main factors contributing to this increase in prevalence. Prostate cancer is a slowly progressing disorder and patients often live for over 10 years after initially being diagnosed with prostate cancer. However, patients with hormone refractory prostate cancer have a poor prognosis and generally do not survive for longer than 2 or 3 years. Hormone refractory prostate cancer is responsible for over 200,000 deaths each year and current chemotherapeutic regimens are only useful as palliative agents. The long-term survival rate is poor and chemotherapy does not significantly increase this. Cell lines derived from hormone refractory tumours usually display elevated resistance to many cytotoxic drugs.

The Fas receptor is a membrane bound protein capable of binding to a ligand called Fas ligand. Engagement of Fas receptor with Fas ligand results in clustering of Fas receptor on the plasma membrane of cells. A number of proteins responsible for initiating apoptosis are recruited to the plasma membrane and are activated in response to elevated local concentrations. This series of events initiates a proteolysis cascade and that culminates in the degradation of structural and enzymatic processes and the repackaging of cellular constituents within membrane bound vesicles that can be endocytosed and recycled by surrounding phagocytic cells. The Fas receptor is believed to be a key mechanism by which immune cells can destroy damaged cells. Consequently, resistance

to Fas receptor mediated apoptosis often correlates with tumour progression. It has been reported that prostate cancer cell lines display elevated resistance to Fas receptor mediated apoptosis and this correlates with the stage of tumour from which the cell lines were isolated.

JNK, a stress-activated protein kinase, has been implicated both with increased survival and increased apoptosis in prostate cancer. Elevated endogenous JNK activity has been demonstrated to correlate with prostate cancer progression. It has been shown that endogenous JNK activity increases the expression of anti-apoptotic proteins and can increase the resistance of prostate cancer cell lines to chemotherapy. In addition, elevated endogenous JNK activity is required for improved proliferation and transformation of a number of epithelial tumours. However, prolonged JNK activation in response to cytotoxic stimuli can increase the sensitivity of cells to apoptosis. Prolonged JNK activity appears to induce the expression of a separate set of genes responsible for promoting apoptosis.

Our group has recently shown that activation of JNK by chemotherapeutic drugs can sensitise DU 145 prostate carcinoma cells to Fas receptor mediated apoptosis. In order to identify novel targets for treating hormone refractory prostate cancer we have investigated the role of JNK in Fas receptor mediated apoptosis. We have demonstrated that prolonged JNK activation is defective in DU 145 cells in response to Fas receptor activation alone. Co-administering anisomycin, a JNK agonist, greatly enhances the ability of DU 145 cells to undergo apoptosis by increasing the rate of Caspase 8 cleavage. We also investigated the role of endogenous JNK activity in Fas receptor mediated

apoptosis. We found that endogenous JNK activity increased the expression of a kinase HIPK3 that in turn can phosphorylate FADD. Abrogation of JNK activity or HIPK3 was found to restore the interaction between FADD and Caspase 8 and increased the sensitivity of DU 145 cells to Fas receptor mediated apoptosis. Therefore, it appears that JNK can direct both anti-apoptotic and pro-apoptotic signals during early stages of Fas receptor mediated apoptosis in prostate cancer. Although endogenous JNK activity in response to growth factors is elevated, prolonged JNK activation in response to Fas receptor appears to be inhibited in DU 145 cells. It is hoped that by restoring this defective pathway, chemotherapeutic agents will be more effective in treating patients with hormone refractory prostate cancer.

Abbreviations

AICD	Activation induced cell death
ASK1	Apoptosis signal-regulating kinase 1
Asp	Aspartic acid
ATF2	Activation transcription factor 2
BAX	Bcl-2 associated X protein
Bcl-2	B-cell CLL/lymphoma 2
BCL-X	Bcl-2-related gene
BCR-Abl	Break point cluster region - Abelson
BID	BH3-interacting domain death agonist
Bim	Bcl-2 interacting protein
BPH	Benign prostate hyperplasia
CARD	Caspase activation and recruitment domain
CHX	Cyclohexamide
CML	Chronic myeloid leukaemia
CPDD	Cysplatin
CTL	Cytotoxic T lymphocyte
DAXX	Death associated protein
DcR	Decoy receptor
DD	Death domain
DED	Death effector domain
DISC	Death inducing signalling complex
DNA	Deoxyribonucleic acid
DP5	Death protein 5
DR	Death receptor
FADD	Fas associated via death domain
FAF1	Fas-associated factor 1
FAP1	Fas associated protein-tyrosine phosphatase 1
FCS	Foetal calf serum
FLIP	FLICE inhibitory protein
HIPK	Homeodomain interacting protein kinase

HSF1	Heat shock transcription factor 1
HSP27	Heat shock protein 27
IFN	Interferon
IL-1	Interleukin-1
JIP	JNK interacting protein
JNK	c-Jun N-terminal kinase
LIGHT	Homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, areceptor expressed by T lymphocytes
Mcl-1	Myeloid cell leukaemia 1
MEKK	MAP/Erk kinase kinase
MKK	Mitogen activated protein kinase kinase
MKP-2	MAPK phosphatase 2
MMP-7	Matrix metalloproteinase-7
Mst1	Mammalian sterile-20 like 1
ND-10	Nuclear domain-10
NFκ-B	Nuclear factor-kappa B
NK	Natural killer cells
PAR-4	Prostate apoptosis responsive-4
PBS	Phosphate buffered saline
PC	Prostate cancer
PCD	Programmed cell death
PI	Propidium iodide
PKC	Protein kinase C
PML	Acute promylocytic leukaemia, inducer of
PSA	Prostate specific antigen
RAIDD	RIP associated protein with death domain
RIP	Receptor interacting protein kinase
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
Ser	Serine
STAT	Signal transducer and activator of transcription
SUMO-1	Small ubiquitin-related modifier-1

tBID	Truncated BID
TBS	Tris buffered saline
TNF	Tumour necrosis factor
TRADD	TNF receptor 1 associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
TRAF	TNF receptor associated factor
TWEAK	TNF-related weak inducer of apoptosis
UV	Ultraviolet
UVB	Ultraviolet B
VB	Vinblastin
VP16	Etoposide

Chapter 1

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Human judges can show mercy.
But against the laws of nature there is no appeal.

Arthur C. Clarke

Overview of Fas mediated apoptosis

Apoptosis

Apoptosis or programmed cell death is defined by morphological changes including cell shrinkage, chromatin condensation, nuclear fragmentation, membrane blebbing, and apoptotic body formation. More recently, central signalling pathways responsible for apoptosis initiation and progression have been identified. Integral to many forms of apoptosis are a family of at least 14 different cysteine proteases called caspases. Caspases are synthesised as inactive precursors called procaspases. Cleavage and activation of procaspases can occur following a variety of stimuli including DNA damage and death receptor activation (Herr & Debatin, 2001; Joza et al., 2002). Caspases can be classified as initiator or effector in function depending on the role they play in apoptosis. Initiator caspases are responsible for detecting and transducing various apoptotic stimuli by cleaving and activating effector caspases. The preferred cleavage site for caspases is after a four amino acid motif Asp-X-X-Asp where X can be any amino acid. Effector caspases cleave downstream targets that include DNA repair enzymes, cytoskeletal proteins and proteins involved in cell cycle progression (Nicholson, 1999). These targets are responsible for implementing the downstream pathways that culminate in morphological changes associated with apoptosis and loss of cell viability (Wolf & Green, 1999).

Death Receptors

Death receptors belong to a superfamily of receptors involved in proliferation, differentiation and apoptosis called the Tumour Necrosis Factor (TNF) superfamily

(Krammer, 1999). Death receptors are type I integral receptors with a conserved extracellular domain containing 2 to 4 cysteine-rich pseudo-repeats, a single transmembrane region and a conserved intracellular death domain about 80 amino acids in length that binds to adaptor proteins and initiates apoptosis (Golstein, 1997; Griffith & Lynch, 1998; Idriss & Naismith, 2000; Schulze-Osthoff et al., 1998). Each receptor can bind with one or more than one type of ligand expressed on adjacent cells. Binding of ligand to receptor induces receptor trimerisation and clustering on the plasma membrane that is required to initiate apoptosis in cells. At least 6 death receptors have been identified and described to date from homology and by loss of function studies. These are TNFR1 (Gray et al., 1990; Loetscher et al., 1990; Nophar et al., 1990; Schall et al., 1990; Smith et al., 1990), Fas (CD95/Apo1) (Itoh et al., 1991), DR3 (TRAMP/Apo3) (Marsters et al., 1996), DR4 (TRAILR1/Apo2) (Pan et al., 1997), DR5 (TRAILR2/TRICK2) (Screaton et al., 1997; Walczak et al., 1997; Wu et al., 1997), and DR6 (Pan et al., 1998).

In addition to death receptors, 3 decoy receptors (DcR) have been identified. DcR1 (TRAILR3) (Pan et al., 1997) and DcR2 (TRAILR4) (Marsters et al., 1997) are membrane bound receptors that bind with TRAIL. DcR3 (Pitti et al., 1998) is a soluble receptor secreted by cells and binds with Fas ligand. Decoy receptors possess functional extracellular ligand binding domains but do not contain intracellular death domains and cannot recruit adaptor proteins required for apoptosis. The principle function of decoy receptors is modulating the sensitivity to death receptor mediated apoptosis *in vivo* (Ashkenazi & Dixit, 1999).

A number of proteins involved in apoptosis can be recruited to death receptors through intermediate adapter proteins that bind with the death receptors or other components of death inducing signalling complex (DISC). Fas associated death domain (FADD) (Boldin et al., 1995; Chinnaiyan et al., 1995), TNF receptor associated death domain (TRADD) (Hsu et al., 1995), receptor interacting protein kinase 1 (RIP1) (Stanger et al., 1995) and death-associated protein (DAXX) (Yang et al., 1997) contain death domains (DD) that recognise and bind with the corresponding DD on the intracellular surface of death receptors. These proteins function as adaptor proteins and it is believed that they create a scaffold to aid recruitment and binding of various other components of the DISC. The initiator caspases Caspase 8 and Caspase 10 interact with FADD through death effector domains (DED) present on both the Caspase 8 and 10 and also FADD. TRADD can recruit a number of adaptor proteins death receptors including FADD, TNF receptor associated Factor (TRAF) and RIP1. RIP1 interacts with caspase 2 and RIP associated protein with death domain (RAIDD) and DAXX can recruit the Mitogen activated protein kinase kinase kinase ASK1 (Ashkenazi & Dixit, 1999; Sheikh & Fornace, 2000). The interactions between death receptors, their known ligands and intracellular adaptor proteins are summarised in Figure 1.

Role of Fas receptor

Fas mediated apoptosis can be regulated by a variety of signalling pathways in cells and is required for normal cell function. One of the principle roles of Fas receptor is regulating the immune response and this is the most clearly characterised function of Fas

receptor. However, Fas receptor is expressed on most tissues and also plays an important role in regulating the function of many different tissues. A number of studies have illuminated the multiple modes by which Fas receptor signalling can regulate T cell and B cell development, maturation and deletion (Bras et al., 1997; Newton et al., 2000; Rathmell et al., 1996). For example, activation of mature T cells occurs during an adaptive immune response to an infection. Clonal expansion and subsequently deletion of activated T cells results by a process called activation induced cell death (AICD). Increased Fas ligand expression is observed following T cell activation. However, cells are initially resistant to Fas mediated apoptosis. During the course of the infection activated T cells become progressively more sensitive to Fas mediated apoptosis and this is ultimately required for AICD and for regulating the response of the immune system to a pathogen (Van Parijs et al., 1999). In addition, Fas mediated apoptosis regulates other cells involved in adaptive immunity such as natural killer cells (Bjorck et al., 1997; Oshimi et al., 1996) and is a principle mechanism by which cytotoxic T lymphocytes (CTL) induce apoptosis in cells expressing foreign antigens (Medema et al., 1997). Dysfunction of Fas receptor is the underlying cause of autoimmune lymphoproliferative syndrome in humans (Fisher et al., 1995; Rieux-Laucat et al., 1995). Fas receptor mediated apoptosis has been implicated in a number of diseases including post-ischemic neuronal degeneration (Herdegen et al., 1998; Martin-Villalba et al., 1999), during traumatic brain injury (Qiu et al., 2002) and may participate in inflammatory bowel disease (Ueyama et al., 1998) and fulminant hepatitis (Song et al., 2003). Fas receptor may also play a role during developmental apoptosis of various cells including embryonic motor neurons (Raoul et al., 2000) and osteoclast formation (Kitaura et al., 2002).

Progression and metastasis of tumours is associated with resistance to Fas receptor mediated apoptosis (French & Tschopp, 2002). In addition, upregulation of Fas ligand often occurs in tumour cells following chemotherapy and may play a key role in immune evasion by eliminating infiltrating lymphocytes (O'Connell et al., 1996; Pinkoski & Green, 2000; Strand et al., 1996).

Formation of the Fas DISC

Activation of Fas receptor by Fas ligand initiates a caspase cascade culminating in apoptosis in sensitive cells. Effective formation of a protease complex called DISC is required in Fas mediate apoptosis. At least 4 individual steps have been identified in Fas sensitive cells following activation of Fas receptor with Fas ligand. Immediately following Fas receptor ligation with Fas ligand microaggregates of Fas receptor form on the cell surface independent of caspase activity (Algeciras-Schimnich et al., 2002). It has been demonstrated that trimerisation of Fas receptor is the minimal event required for FADD recruitment and effective DISC formation (Schneider et al., 1998). However significant DISC formation requires the presence of a Fas hexamer, consisting of two adjacent trimers (Holler et al., 2003). Subsequent DISC assembly occurs in type I cells and is dependent on reorganisation of cytoskeletal actin filaments. Activation of Caspase 8 occurs following DISC formation and directly regulates the formation of large Fas receptor aggregates on the plasma membrane of cells and increased DISC activity. Finally these large clusters of Fas receptor are endocytosed and recycled (Algeciras-Schimnich et al., 2002). Type II cells have impaired DISC formation and ceramide

production is a necessary step for generation of large receptor aggregates and capping in lipid rafts (von Reyher et al., 1998).

Assembly of the components of the Fas DISC is a highly organised event and involves sequential clustering of adapter and effector proteins at Fas receptor aggregates. FADD is recruited to and binds to the intracellular DD of Fas receptor in response to two adjacent Fas receptor trimers (Holler et al., 2003). Caspase 8 and Caspase 10 bound to FADD are recruited to the plasma membrane and the increased local concentration of these proteases induces autocleavage and activation of Caspase 8 and Caspase 10 *in trans*. Caspase 8 appears to be the principle initiator during apoptosis because cells that were deficient in Caspase 10 expression underwent normal apoptosis while resistance to Fas mediated apoptosis was observed in Caspase 8 deficient cells even when Caspase 10 was overexpressed (Sprick et al., 2002). DAXX can also bind to the intracellular DD of Fas receptor and recruits ASK1. Activation of ASK1 was found to occur following recruitment to the DISC and subsequent JNK activation is believed to promote apoptosis in cells (Chang et al., 1998; Tobiume et al., 2001). In addition, the interaction between DAXX and ASK1 was found to regulate caspase independent cell death and was not dependent on ASK1 kinase activity (Charette et al., 2001; Ko et al., 2001). The DD of RIP1 can also bind to Fas receptor and recruits RAIDD and Caspase 2 to Fas receptor aggregates. Cleavage and activation of Caspase 2 was found to promote the cleavage of effector caspases and regulate apoptosis (Ahmad et al., 1997).

Active Caspase 8 can directly cleave and activate effector caspases in type I cells. However, very little Caspase 8 is activated in type II cells and amplification of apoptosis is required. Cleavage of the Bcl-2 family member BID by Caspase 8 produces the pro-apoptotic tBID fragment that induces cytochrome c release from mitochondria and Caspase 9 activation (Gross et al., 1999; Wang et al., 1996). Expression of anti-apoptotic Bcl-2 family members can regulate the sensitivity of mitochondria to tBID and in turn the sensitivity of type II cells to Fas mediated apoptosis (Scaffidi et al., 1999b). Figure 2 illustrates the principle steps in DISC formation and activation of the caspase cascade.

Modulators of DISC formation

A soluble decoy receptor called DcR3 was identified and shown to bind with Fas ligand (Pitti et al., 1998) and LIGHT (Wroblewski et al., 2003) but not with the other death ligands TNF, TRAIL or TWEAK. The gene coding for DcR3 is located on chromosome 20 and this locus is often amplified in colon cancer (Pitti et al., 1998). About half of all gastrointestinal tract carcinomas and lung cancers overexpress DcR3 suggesting that DcR3 amplification can promote tumour survival (Bai et al., 2000; Pitti et al., 1998; Takahama et al., 2002). DcR3 sequesters and inactivates membrane bound Fas ligand on adjacent cells and infiltrating tumour cells and prevents activation of Fas receptor. Patients with gastrointestinal tract carcinomas that overexpress DcR3 have a significantly shortened mean duration of survival than patients with tumours expressing normal levels of DcR3 (Takahama et al., 2002).   Human keratinocytes overexpress DcR3 and expression is rapidly decreased in cells following ultraviolet B irradiation (Maeda et al., 2001).   The signalling pathways responsible for DcR3 expression have not been identified yet and understanding the pathways responsible for decreasing expression of DcR3 in response to UVB irradiation may identify novel therapeutic targets against gastrointestinal tract carcinomas.

Expression of FADD can also regulate the sensitivity of cells to Fas mediated apoptosis by altering the levels of effector caspases cleaved in response to Fas receptor activation. Even though FADD is a key component of Fas receptor signalling expression it is rarely decreased in tumour cells because expression of FADD is also required for cell cycle progression (Hueber et al., 2000; Zornig et al., 1998). However, a number of cellular

insults including actinomycin D, UV irradiation and heat shock have been shown to increase expression of FADD in hepatocytes (Kim et al., 2002).

Post-translational modification of FADD by PKC has also been shown to regulate Fas receptor mediated apoptosis in cells by inhibiting complete DISC formation following Fas receptor activation (Gomez-Angelats & Cidlowski, 2001; Mueller & Scott, 2000; Ruiz-Ruiz et al., 1997). The atypical Protein Kinase C zeta (PKCξ) appears to play a central role in this process. Phosphorylation of FADD by PKCξ reduced DISC formation in cells following Fas receptor oligomerisation. Inhibition of Fas receptor mediated apoptosis was reversed by overexpressing the PKCξ inhibiting protein prostate apoptosis responsive 4 (PAR-4) (de Thonel et al., 2001). PAR-4 overexpression was also found to enhance the trafficking and activation of Fas receptor and Fas ligand in prostate cancer cells. This suggests that PKCξ may inhibit Fas receptor mediated apoptosis at several stages in the pathway upstream of Caspase 8 activation (Chakraborty et al., 2001). PKCξ activity is implicated in tumour progression within a number of cancers by increasing proliferation and increasing resistance to apoptosis. PKCξ activity was increased in Ras transformed fibroblasts as a direct result of decreased PAR-4 expression (Barradas et al., 1999) and has been implicated as a mediator of a number of mitogenic signals associated with Ras transformation (Bjorkoy et al., 1997; Diaz-Meco et al., 1994). In addition an increase in the activity of PKCξ has been identified in a number of tumours including prostate cancer (Cornford et al., 1999) and hepatocellular carcinomas (Tsai et al., 2000) and expression correlates with invasion in patients with bladder cancer (Langzam et al., 2001).

Another FADD interacting kinase called homeodomain interacting protein kinase 3/ FADD interacting Serine/Threonine Kinase (HIPK3/FIST) has also been implicated in regulating DISC formation *in vivo*. HIPK3 belongs to a family of nuclear kinases with at least two other members, HIPK1 and HIPK2. HIPK3 has been shown to interact with and phosphorylate FADD in cells (Rochat-Steiner et al., 2000) while HIPK2 can associate with TRADD (Li et al., 2000a). This suggests a role for these kinases in regulating death receptor mediated apoptosis. HIPK3 overexpression was shown to inhibit Fas mediated JNK activation but did not affect apoptosis in 293T cells (Rochat-Steiner et al., 2000). Although JNK activation is not required for Fas mediated apoptosis in some cell lines (Abreu-Martin et al., 1999; Herr et al., 1999; Low et al., 1999), the sensitivity to Fas mediated apoptosis increases in other cell lines following JNK activation (Costa-Pereira et al., 2000; Curtin & Cotter, 2002; Le-Niculescu et al., 1999; Zhang et al., 2000). Therefore, overexpression of HIPK3 may inhibit activation of JNK and subsequently decrease the sensitivity to Fas mediated apoptosis in some cell lines. Interestingly, several multi-drug resistant cell lines display increased HIPK3 activity (Begley et al., 1997; Sampson et al., 1993).

In contrast with FADD, the expression of Caspase 8 is often decreased in cells resistant to Fas mediated apoptosis. Hyper-methylation of the caspase 8 gene is frequently accompanied by loss of Caspase 8 expression in a number of tumours including retinoblastomas and neuroblastomas. It is believed that by methylating key residues in the caspase 8 gene that cells can negatively regulate transcription (Harada et al., 2002).

However, while associated with regulating gene expression it is also possible that DNA methylation is just a side effect of decreased transcription. Alternative splicing of Caspase 8 has also been shown to inhibit Fas mediated apoptosis. The splice variant Caspase 8L contains a functional DED but is missing key residues in the catalytic site and is catalytically inactive. Overexpression of Caspase 8L was found to increase resistance of Jurkat cells to Fas mediated apoptosis by interfering with Caspase 8 binding to FADD and functional DISC assembly (Himeji et al., 2002).

Caspase 8L functions in a similar fashion to another inhibitor of Fas mediated apoptosis called FLICE inhibitory protein (FLIP). FLIP was first identified as a viral protein (vFLIP) and was found to inhibit Fas mediated apoptosis when overexpressed in cells (Thome et al., 1997). Cellular homologues were quickly identified and two major isoforms have been characterised, a short ($FLIP_S$) and a long ($FLIP_L$) isoform (Irmler et al., 1997). Although coded by separate genes, FLIP share sequence homology with Caspase 8 and possesses a functional DED that can bind to the DED on FADD in competition with Caspase 8. Like Caspase 8L, $FLIP_L$ and $FLIP_S$ are catalytically inactive and can inhibit Procaspase 8 processing and activation at the DISC when overexpressed *in vivo* (Scaffidi et al., 1999a). Activation of a key transcription factor involved in cell survival called NF-κB can upregulate FLIP expression (Kreuz et al., 2001) and inhibit Fas mediated apoptosis (Chang et al., 2002). Endogenous $FLIP_L$ is expressed at only 1% that of endogenous Procaspase 8 in many cell lines (Scaffidi et al., 1999a) and at low levels of expression $FLIP_L$ appears to enhance and not inhibit Fas mediated apoptosis by enhancing Caspase 8 recruitment and DISC formation (Chang et al., 2002). Procaspase 8

bound to $FLIP_L$ can be partially processed and hetero-dimers of $FLIP_L$ and Caspase 8 have been found to retain some protease activity (Micheau et al., 2002). The preferred substrate for hetero-dimers of $FLIP_L$ and Caspase 8 is a protein kinase called RIP.

RIP1 was initially identified as a death domain containing, Fas receptor interacting protein in two hybrid protein assays. Transient overexpression of RIP1 caused transfected cells to undergo apoptosis (Stanger et al., 1995) and activation of RIP1 may explain in part why endogenous $FLIP_L$ expression enhances Fas mediated apoptosis. RIP1 has been shown to initiate a caspase independent mechanism for Fas mediated cell death in T cells when co-expressed with FADD (Holler et al., 2000). In addition, Caspase 8 mediated cleavage of RIP1 produces a C-terminal fragment that appears to enhance apoptosis through enhanced DISC formation (Kim et al., 2000). RIP1 belongs to a family with at least 3 other members that include RIP2 (Inohara et al., 1998; McCarthy et al., 1998; Thome et al., 1998), RIP3 (Sun et al., 1999; Yu et al., 1999) and RIP4 (Meylan et al., 2002). All 4 members of RIP contain a conserved N-terminal kinase domain and each member contains a unique C-terminal sequence responsible for subcellular localisation and function. A caspase activation and recruitment domain (CARD) is located at the C-terminus of RIP2 and is required for the apoptotic activity of RIP2. RIP2 is recruited to TNFR1 through the family of TRAF adapter proteins and may also be involved in DISC formation of other death receptors (McCarthy et al., 1998). However, recent reports suggest that the principle function of RIP2 *in vivo* is the activation of Caspase 1 and IL-1β production during an innate immune response (Chin et al., 2002; Druilhe et al., 2001; Kobayashi et al., 2002). RIP 3 is also recruited to the

TNFR1 receptor through its unique C-terminus that binds with RIP1. RIP3 inhibits RIP1 mediated NF-κB activation following TNFR1 activation and regulates RIP1 pro-apoptotic function (Sun et al., 2002; Yu et al., 1999). The recently identified RIP4 contains 11 C-terminal ankyrin repeats. The ankyrin repeat domain is believed to regulate the activity of RIP4 and cleavage by caspases during Fas mediated apoptosis has been shown to prevent the activation of the NF-κB survival pathway (Meylan et al., 2002).

Another Fas interacting protein that was identified by two-hybrid screening is DAXX. Transient expression of DAXX increases Fas mediated apoptosis in 293, HeLa, L929 and HT1080 cell lines (Torii et al., 1999; Yang et al., 1997) while a truncated, dominant negative form of DAXX was found to inhibit Fas mediated apoptosis (Charette et al., 2000; Torii et al., 1999). DAXX binds to the death domain of the Fas receptor and can activate the JNK kinase cascade independent of Caspase 8 activation by recruiting and activating the upstream kinase ASK1 (Chang et al., 1998; Ko et al., 2001). Activation of JNK was found to accelerate Fas mediated apoptosis in various cell lines (Costa-Pereira et al., 2000; Curtin & Cotter, 2002; Le-Niculescu et al., 1999; Zhang et al., 2000). Overexpression of the small heat shock protein HSP27 was found to abrogate the interaction between ASK1 and DAXX. Furthermore HSP27 expression can inhibit JNK activation following Fas receptor activation and protects against Fas mediated apoptosis (Charette et al., 2000). Neither DAXX or RIP were found to be necessary for Fas mediated apoptosis in lymphoma cell lines (Villunger et al., 2000). This suggests that both proteins can modulate sensitivity to Fas mediated apoptosis but are not essential

components of Fas mediated apoptosis in all cell lines. By enhancing DISC formation and propagating apoptotic signals these proteins appear to enhance the sensitivity of some cell lines with resistance to Fas mediated apoptosis.

DAXX can bind with and undergo covalent modification by SUMO-1, a ubiquitin-like protein that was found to associate with the death domain of Fas receptor. Overexpression of SUMO-1 can protect against Fas mediated apoptosis in BJAB cells (Okura et al., 1996). Modification of proteins by SUMO-1 can have a variety of effects including changes in stability and subcellular localisation (Hay, 2001). However, modification with SUMO-1 was not found to alter the stability or subcellular localisation of DAXX (Jang et al., 2002; Ryu et al., 2000). Instead modification of PML by SUMO-1 sequesters DAXX in nuclear domains (ND-10 domains) and may inhibit the pro-apoptotic function associated with cytoplasmic DAXX. SUMO-1 can also modify the TRADD binding kinase HIPK2 (Kim et al., 1999). Identification of other proteins modified by SUMO-1 should help explain the mechanism by which SUMO-1 can protect cells from Fas mediated apoptosis.

Like RIP and DAXX, mouse Fas-associated factor 1 (FAF1) was first identified using Fas receptor as bait in a two hybrid screening assay (Chu et al., 1995). Different methods were used in identifying human and quail homologues and these were also found to interact with the intracellular domain of Fas receptor both *in vitro* and *in vivo* (Frohlich et al., 1998; Ryu & Kim, 2001). Overexpression of FAF1 was not found to induce apoptosis in transfected L-cells. Instead the sensitivity of cells to Fas ligand was found to

be enhanced in cells overexpressing FAF1 (Chu et al., 1995). FAF1 has a ubiquitin-like domain but in contrast with SUMO-1 this domain is pro-apoptotic *in vivo* (Ryu & Kim, 2001). Casein kinase 2 can phosphorylate FAF1 at several residues *in vitro* and *in* vivo but the function of this has not yet been identified (Jensen et al., 2001).

Modulators of Fas receptor and Fas ligand expression

A common mechanism employed by cells to increase or decrease the sensitivity to Fas mediated apoptosis is the regulated cell surface expression of Fas receptor and Fas ligand. Alternative splicing, protease-mediated cleavage, gene expression and sequestering of Fas receptor and Fas ligand have been found to regulate Fas mediated apoptosis and are described below and outlined in Figure 3.

Fas receptor is expressed at a single locus on chromosome 10 in human cells and chromosome 19 in mouse cells. At least 8 splice variants and 7 distinct isoforms of Fas receptor have been identified in human cells and arise from alternative splicing of Fas receptor RNA. Only isoform 1 encodes the functional, full-length protein and is 335 amino acids in length. It consists of 3 cysteine-rich pseudo-repeats, a transmembrane domain and an intracellular death domain. Isoform 2 is 314 amino acids in length and encodes 3 cysteine-rich regions, a death domain but is missing a transmembrane region. Isoforms 4 through 7 are also missing a transmembrane region and these soluble isoforms of Fas receptor may sequester and inactivate Fas ligand on adjacent cells and infiltrating cytotoxic T lymphocytes (Cheng et al., 1994). Overexpression of soluble Fas receptor has been implicated with the progression of prostate cancer (Furuya et al., 2001), Melanoma (Ugurel et al., 2001), bladder cancer (Mizutani et al., 1998) breast cancer (Sheen-Chen et al., 2003) and leukaemia (Wood et al., 2003) and is known to antagonise Fas receptor mediated apoptosis *in vitro* (Cheng et al., 1994). Isoform 3 is 220 amino acids long and contains 3 cysteine-rich pseudorepeats, a transmembrane region but the cytoplasmic domain is truncated and does not contain a functional death domain.

Overexpression of this isoform occurs in fetal thymocytes and may account for the high resistance in these cells to apoptosis following Fas receptor aggregation (Jenkins et al., 2000). The mechanisms employed by cells to alter Fas RNA splicing and thus alter the sensitivity to Fas mediated apoptosis appears to be an important process *in vivo* but remain to be characterised.

In contrast with Fas receptor, soluble Fas ligand is not generated by alternative splicing but is instead generated by post-translational modification of membrane bound Fas ligand at the cell surface. Matrix metalloproteinases are external serine proteases that cleave a wide range of extracellular substrates. Membrane bound Fas ligand can be cleaved at a conserved cleavage site by Matrix Metalloproteinase-7 (MMP-7) into a less active soluble form (Powell et al., 1999; Tanaka et al., 1998). MMP-7 expression has been implicated in tumour initiation (Rudolph-Owen et al., 1998; Shigemasa et al., 2000) and invasion (Yamamoto et al., 1999). Expression of MMP-7 in well differentiated tumour cells promotes resistance to Fas mediated apoptosis (Fingleton et al., 2001). In addition, overexpression of MMP-7 is believed to promote mammary tumour initiation and progression in mice by selecting for tumour cells resistant to Fas mediated apoptosis (Vargo-Gogola et al., 2002). Cells expressing low levels of a non-cleavable variant of Fas ligand or inhibition of matrix metalloproteinases increases the sensitivity to Fas mediated apoptosis (Knox et al., 2003). Thus MMP-7 can control the expression of Fas ligand on the surface of adjacent cells and infiltrating lymphocytes by cleaving membrane bound Fas ligand. This appears to be a major mechanism by which MMP-7

regulates the sensitivity of cells to Fas mediated apoptosis. Increased resistance to Fas mediated apoptosis by MMP-7 may also play a role in tumour progression.

Regulation of soluble Fas receptor and Fas ligand expression has been implicated in modulating sensitivity of cells to Fas mediated apoptosis. Transcription of the fas receptor and fas ligand genes can also be regulated by a number of signal pathways and this in turn can regulate the extent of cell surface Fas receptor and Fas ligand expression. Natural killer cells (NK cells) possess the ability to kill by Fas mediated apoptosis and this is an important defence mechanism against tumour growth. By directly increasing Fas receptor expression NK cells have been shown to increase the sensitivity of target cells to Fas mediated apoptosis (Screpanti et al., 2001). Cytotoxic T lymphocytes (CTL) can also promote transcription of fas receptor in target cells through an Interferon-γ (IFN-γ) dependent mechanism (Mullbacher et al., 2002). Signal transducer and activator of transcription 1 (STAT1) is required for both IFN-γ mediated upregulation of Fas receptor and Fas ligand expression and also for IFN-γ dependent apoptosis in human tumour cells (Xu et al., 1998). Pretreatment of prostate cancer cells with IFN-γ sensitises these cells to Fas mediated apoptosis (Selleck et al., 2003). Another member of the STAT family STAT3 can negatively regulate transcription of fas receptor. Expression of both STAT3 and another transcription factor c-Jun is required to inhibit fas receptor transcription. Overexpression of either dominant negative STAT3 or dominant negative c-Jun was shown to increase the expression of Fas receptor (Ivanov et al., 2002). Conversely, binding of c-Jun and activation transcription factor 2 (ATF2) to the fas ligand promoter induces fas ligand expression (Faris et al., 1998b). Upregulation of Fas ligand expression

is associated with tumour progression and is believed to act as a defence against infiltrating lymphocytes (Belluco et al., 2002; Cefai et al., 2001; Strand et al., 1996) and overexpression of either dominant negative or dominant active MAP/Erk kinase kinase 1 (MEKK1) demonstrated the role played by the c-Jun N terminal kinase (JNK) cascade in this process (Faris et al., 1998a; Faris et al., 1998b). Activation of JNK has been demonstrated to upregulate Fas ligand expression and promote apoptosis during β-Amyloid induced neuronal apoptosis (Morishima et al., 2001), during AICD after T cell receptor stimulation (Zhang et al., 2000) and following survival factor withdrawal in neurons (Le-Niculescu et al., 1999).

Given the importance of the Fas apoptotic pathway in controlling tumour growth it is not surprising to discover that tumour suppressing proteins and onco-proteins can directly regulate fas receptor expression. Fas receptor can be upregulated following DNA damage by ionising radiation and genotoxic drugs in a P53 dependent manner (Ruiz-Ruiz & Lopez-Rivas, 1999; Sheard et al., 1997). A P53-responsive element was identified within the first intron of the fas receptor gene in subsequent studies and wild type P53 expression was found to be required for Fas receptor upregulation in response to anti-cancer drugs (Muller et al., 1998). Basal expression of Fas receptor is also regulated by the onco-protein Ras. Overexpression of Ras was shown to decrease Fas receptor expression *in vitro* and *in vivo* (Fenton et al., 1998). Inhibition of Ras using Farnesyltransferase inhibitors was found to upregulate Fas receptor expression in ras-transformed cells (Zhang et al., 2002).

In addition to modulating the transcription and alternative splicing of Fas receptor mRNA, the expression of cell surface Fas receptor can be regulated by altering intracellular trafficking of Fas receptor. Fas receptor is a membrane bound protein and subcellular trafficking is an important mechanism for regenerating and recycling Fas receptor. In addition intracellular stores of Fas receptor may translocate to the plasma membrane in response to apoptotic stimuli (Bennett et al., 1998; Sodeman et al., 2000). FAP-1 is also known as Protein-tyrosine phosphatase, nonreceptor-type, 13 (PTNP13) and was found to associate with the carboxy terminal 15 amino acids of human Fas receptor (Sato et al., 1995). *In vitro* inhibition of the interaction between FAP-1 and Fas using synthetic peptides demonstrated that the amino acid motif SLV found at the carboxy terminus of the Fas receptor was both sufficient and necessary for binding to FAP-1 (Yanagisawa et al., 1997). Mouse Fas receptor does not contain this c-terminal motif and does not interact with either mouse FAP-1 (PTP-BL) or human FAP-1 when overexpressed in cells (Cuppen et al., 1997). Overexpression of FAP-1 increased the resistance of Fas sensitive human cell lines to Fas-mediated apoptosis (Li et al., 2000b; Sato et al., 1995; Ungefroren et al., 2001). In addition, Fas resistant memory T cells were found to express higher levels of FAP-1 mRNA than Fas sensitive naïve T cells and resting T cells expressed higher levels of FAP-1 mRNA than activated T cells suggesting that FAP-1 expression regulates the sensitivity of cells to Fas mediated apoptosis (Zhou et al., 1998). FAP-1 was found to sequester Fas receptor in the Golgi complex when overexpressed in pancreatic cancer cells and this prevents the translocation Fas receptor from intracellular stores to the plasma membrane following stimulation with Fas ligand. FAP-1 was not detected at the DISC of Fas stimulated cells suggesting that the

sequestration of Fas receptor and ineffective DISC formation due to low surface

expression of Fas receptor is the principle mechanism by which FAP-1 interferes with

Fas mediated apoptosis (Ungefroren et al., 2001).

Prostate Cancer

Epidemiology of prostate cancer

Prostate cancer is a slowly progressing disease that primarily affects elderly men. In the late 1980's and early 1990's a sharp rise in the incidence of prostate cancer was observed first in the USA and shortly afterwards in northern European countries. This was due to increases in screening for prostate cancer and public awareness of the disease due to media coverage of prostate cancer. In addition, more accurate biomarkers were identified for prostate cancer including prostate specific antigen (PSA) and these markers allowed prostate cancer to be detected before symptoms became evident. This facilitated the screening process for prostate cancer by identifying "at risk" individuals (Potosky et al., 1995).

Prostate cancer is now the third most common cancer identified in men behind lung cancer and stomach cancer with 543,000 new cases identified worldwide in 2000 alone. This accounts for over 10% of the total new cancers identified in males and 5.4% of all cancers detected globally in 2,000. Approximately 204,000 global deaths were directly related to prostate cancer in the same year (Parkin et al., 2001). Prostate cancer is a slow progressing disease and men diagnosed with prostate cancer often are alive for longer than those diagnosed with other cancers. As a result, the global prevalence of prostate cancer is very high and over 1.5 million people are currently alive with prostate cancer at least 5 years after diagnosis. The high incidence combined with the slow progression of the disease makes prostate cancer the most prevalent cancer in men today and the third

most prevalent cancer in both sexes behind breast cancer and colorectal cancer (3.8 million and 2.4 million alive 5 years after diagnosis respectively) (Parkin et al., 2001).

The incidence of prostate cancer is much greater in developed countries such as North America, EU and Australia than in developing countries including South America and Southern Asia. About 400,000 new cases are identified each year in developed countries (75%) while only 150,000 new cases are reported each year in developing countries (25%). Whilst screening methods in developed countries may account for some of this difference, life expectancy in developing countries also plays a role. Prostate cancer is a disease that primarily affects elderly men and the global incidence of prostate cancer is expected to rise to 1 million new cases annually by 2015. This is due primarily to increasing life expectancies in many developing countries (Parkin et al., 2001). Obesity and the adoption of a "western diet" that is high in fats, dairy products and meat has also been implicated in increasing the risk of prostate cancer (Schulman et al., 2001).

Progression and diagnosis of prostate cancer

More than 100 distinct types of cancer have been described in virtually every tissue in the body. Although cancer is a highly heterogenous disease with distinct genetic alterations and mutations all cancer cells can be described as cells that have developed defects in the normal regulatory circuits controlling cell proliferation and location. At least 6 distinct cellular alterations are required during tumorigenesis and progression: 1) self sufficiency for growth signals, 2) insensitivity to growth-inhibitory signals, 3) limitless potential for replication, 4) sustained angiogenesis, 5) metastasis and 6) resistance to apoptosis

(Hanahan & Weinberg, 2000). In prostate cancer each of these barriers between normal and malignant cell growth must be breached before or during disease progression. Figure 4 summarises the principle alterations identified in prostate cancer progression and tumorigenesis.

The earliest genetic alterations detected in prostate cancer to date are chromosomal deletions in normal prostate epithelial cells (Dong, 2001). Subsequent cumulative genetic aberrations transform prostate epithelial cells from a pre-neoplastic state to intraepithelial neoplasia (i.e neoplasia *in situ*) and ultimately to prostate carcinoma. Androgen independence also occurs during prostate cancer progression and this event promotes the successful establishment and proliferation of prostate cancer cells at distal sites (Lara et al., 1999). Progression of prostate cancer from androgen dependence to androgen refractory is an indicator of the severity of the disease and is a major factor when estimating the life expectancy of the patient. Prostate cancer is a slow proliferating disease and the life expectancy for patients diagnosed with localised androgen dependent disease can be up to 10 years (Siemens, 2003). However, patients diagnosed with metastatic, androgen independent prostate cancer have a median life expectancy of only 15 to 20 months (Knox & Moore, 2001). The bone is the principle site of metastases in prostate cancer and skeletal metastases are present in 90% of patients dying from prostate cancer (Bubendorf et al., 2000). As a result, bone pain is the most common symptom associated with advanced prostate cancer. Spinal cord compression, anaemia and edema are other common symptoms displayed by patients with advanced disease (Smith et al., 1999).

The most reliable and commonly used markers for risk definition in prostate cancer are stage (endorectal magnetic resonance imaging), Gleason score (morphology), and serum PSA levels (Oesterling et al., 1997; Partin et al., 1993). In addition, serum PSA levels are widely used to screen for prostate cancer in asymptomatic men over the age of 50. However, elevated PSA may also occur in non-malignant conditions including benign prostate hyperplasia (BPH) (Catalona et al., 1998) and expression of PSA has even been reported in some non-prostatic tissues (Smith et al., 1995; Waheed & Van Etten, 2001). Doubt exists over the accuracy of this test in detecting prostate cancer and studies using microarrays have identified other potential biomarkers for screening prostate cancer (Dhanasekaran et al., 2001).

The clinical value in screening asymptomatic males for prostate cancer is controversial. Although prostate cancer can be detected using serum PSA levels many years before symptoms become evident (Gann et al., 1995), recent studies have not identified any association between PSA screening and decreases in PC mortality (Coldman et al., 2003; Iscoe, 1998). This is most likely because no therapy to date has been demonstrated to significantly prolong patient survival with advanced prostate cancer. In fact palliative care is the principle consideration when dealing with hormone refractory metastatic prostate cancer.

Current treatment of prostate cancer

Treatment of prostate cancer varies depending on the grade (morphology) of the tumour (Repetto et al., 1998). Usually androgen ablation is used to control androgen sensitive tumours. This results in tumour regression and a period of temporary relief in 90% of patients. However, androgen independent prostate cancer invariably develops with a mean time of 12 to 18 months. Androgen independent tumours are usually more aggressive and less responsive to chemotherapy than androgen sensitive tumours. Intermittent androgen ablation can slow but does not prevent the progression of prostate cancer to androgen independence (Akakura et al., 1993).

Radical prosectomy or radical radiotherapy may be administered to patients presenting with localised prostate cancer and both surgery and radiotherapy are believed to increase the mean survival rate after 5 years (Hanks & Lanciano, 1996; Siemens, 2003). However, lymph node metastases are often overlooked before surgery. In patients presenting with metastatic prostate cancer conventional chemotherapeutic agents are only useful as palliative treatments and do not significantly improve long-term survival (Sternberg, 2001). Some new drugs have been identified that slow the progression of prostate cancer including atrasentan (van der Boon, 2002) and epirubicin (Petrioli et al., 2002). However, the ability to control prostate cancer at any stage with chemotherapeutic agents is severely limited and further research is needed to understand the mechanisms by which prostate cancer evades apoptosis.

JNK is a protein with "Jekyll and Hyde" properties in prostate cancer

One protein implicated in prostate cancer progression is c-Jun N-terminal Kinase (JNK) and has been shown to regulate both cell survival and cell death pathways *in vivo*. The threshold of JNK activation appears to be important in determining the fate of the cell. While transient, low levels of JNK activity have been often associated with cell survival, prolonged higher levels of JNK activity are often necessary for proapoptotic effects associated with JNK (Davis, 2000). JNK is usually activated following phosphorylation at both Thr183 and Tyr185 residues by either Mitogen activated protein kinase kinase 4 (MKK4) or MKK7 in response to upstream kinase signals (Figure 5). JNK is activated in response to a number of stress signals including ultraviolet (UV) irradiation, cytotoxic drugs and cytokines (Ip & Davis, 1998; Kyriakis & Avruch, 2001). Various growth factors may also stimulate JNK activity including EGF and IL-1 (Rosette & Karin, 1996).

Translocation of active JNK can occur from the cytoplasm to nuclear extracts where it has been reported to phosphorylate and modulate the activities of numerous transcription factors (Cavigelli et al., 1995; Kyriakis et al., 1994; Mizukami et al., 1997). This in turn alters the expression of genes including transcription factors, growth factors and IFN-γ responsive genes (Han et al., 2002). JNK can also participate directly in signal transduction pathways when it phosphorylates a number of cytoplasmic targets including Bcl-2 (Park et al., 1997) and Bcl-X$_L$ (Kharbanda et al., 2000). JNK interacting protein 1 (JIP-1) acts as a scaffold protein in the JNK kinase cascade and sequesters JNK in the cytoplasm when overexpressed in cells. This prevents phosphorylation of nuclear targets and enhances the phosphorylation of cytoplasmic targets by JNK (Dickens et al., 1997).

JIP-1 is not believed to be involved in regulating survival and death pathways because targets of JNK in the cytoplasm may be either pro-apoptotic or anti-apoptotic and nuclear targets of JNK may also enhance either cell survival or cell death. Instead it is believed to direct JNK between faster acting signal transduction responses and longer lasting gene transcription responses. Interestingly, inactivation of JNK by MAPK phosphatase-2 (MKP-2) was dependent on JNK translocation to the nucleus. In addition, different stress stimuli have been shown to preferentially induce predominantly cytoplasmic or nuclear JNK localisation and presumably different targets for JNK are activated in response to different stimuli *in vivo* (Robinson et al., 2001).

The exact mechanisms utilised by JNK to regulate both cell survival and cell death is poorly understood. However, recent studies have illuminated the novel mechanisms by which JNK can promote either cell survival or apoptosis by targeting different members of the Bcl-2 family. The anti-apoptotic protein Bcl-2 is normally associated with mitochondrial membranes (Gotow et al., 2000) and can be phosphorylated at Ser70 by JNK *in vitro*. This residue resides in a non-structural loop of the Bcl-2 protein. Phosphorylation of Ser70 has been found to stabilise the interaction between Bcl-2 and it's pro-apoptotic partner BAX and has been associated with increased cell survival *in vivo* (Deng et al., 2001). However, prolonged JNK activation has been associated with upregulating the transcription of the BH3-only Bcl-2 family members Bim and Dp5. BH3-only Bcl-2 family members are pro-apoptotic but the expression of either BAX or BAK is required to induce apoptosis in cells. It is believed that Bim and Dp5 bind preferentially with Bcl-2 in place of BAX and cause homodimerisation of BAX and

insertion into mitochondrial membranes. Subsequent pore formation induces cytochrome c release and caspase activation (Harris & Johnson, 2001). In contrast with Bcl-2, phosphorylation of the anti-apoptotic Bcl-2 family members Bcl-X_L (Kharbanda et al., 2000) and Mcl-1 (Inoshita et al., 2002) by JNK is believed to reduce the anti-apoptotic activities and point mutation analysis has shown that cells expressing non-phosphorylatable Bcl-X_L and Mcl-1 mutants are less sensitive to apoptosis following JNK activation (Inoshita et al., 2002; Kharbanda et al., 2000). Thus by altering the expression of various anti-apoptotic Bcl-2 family members cells can increase or decrease their sensitivity to stress induced JNK activation.

Increased basal JNK activity has been implicated in tumour progression and correlates with stage in some tumours and leukaemias. JNK activity is required for the invasion of peripheral organs by BCR-Abl expressing lymphomas and contributes to enhanced disease progression in mouse models of CML. This is primarily due to increasing the resistance of transformed B-lymphocytes to apoptosis (Hess et al., 2002). Many brain tumours display elevated JNK activity in response to EGF receptor overexpression. Elevated JNK activity in brain tumours contributes to anchorage independent cell growth, increased resistance of cells to serum starvation and cytotoxic drugs and also promoted cell growth to a lesser degree (Antonyak et al., 2002). Overexpression of JNK in NIH3T3 fibroblasts conferred some aspects of transformation including increased survival, increased proliferation and anchorage independent cell growth (Rennefahrt et al., 2002). JNK increases the expression of telomerase a key protein in tumour progression (Alfonso-De Matte et al., 2002).

In prostate cancer increased JNK activity and in particular JNK2 has been implicated in promoting proliferation both *in vitro* and *in vivo*. Administering anti-sense directed against JNK1 and JNK2 inhibited prostate tumour growth significantly by increasing the susceptibility of cells to apoptosis (Yang et al., 2003) and was found to sensitise prostate cancer cells to cytotoxic drugs (Potapova et al., 1997). Tumour growth of PC cells was strongly inhibited in mice receiving therapeutic doses of anti-sense JNK oligonucleotides (Gjerset et al., 2001). SAGE analysis of PC3 prostate cancer cells demonstrated that JNK2 increased expression of DNA repair enzymes and drug resistant genes, while it decreased various apoptotic genes (Potapova et al., 2002).

Fas receptor and prostate cancer

Net growth in any tissue is dependent on two opposing factors, the rate of cell proliferation and the rate of apoptosis and normal tissue homeostasis in adults requires that these rates are equal. The regulation of cell proliferation and apoptosis is defective in tumour cells and cell proliferation exceeds apoptosis during tumour progression (Evan & Vousden, 2001; Hardy & Stark, 2002). Inhibition of apoptosis is significant as is increased cellular proliferation in the development of prostate cancer (Tu et al., 1996) and the expression of many proteins implicated in apoptosis may be altered during prostate cancer progression (Figure 4) (Gurumurthy et al., 2001).

One of the key pathways in apoptosis is the Fas receptor mediated pathway and many chemotherapeutic drugs appear to elicit their cytotoxic function by activating various components of the Fas receptor pathway (de Souza et al., 1997; Micheau et al., 1999). In addition, radiotherapy upregulates Fas receptor expression in tumour cells in a P53 independent fashion (Owen-Schaub et al., 1995). Tumour cells develop resistance to Fas receptor mediated apoptosis as a defence against immune surveillance and in response to chemotherapy (O'Connell et al., 1996). However, early stages of prostate cancer display decreased sensitivity to Fas mediated apoptosis even prior to clinical intervention. This is probably due to the reliance of prostate cancer progression on developing resistance to apoptosis above increasing rates of cell proliferation. In addition, cell lines derived from metastatic prostate cancer display increased resistance to Fas mediated apoptosis when compared with cell lines derived from primary tumours and the degree of resistance to

Fas mediated apoptosis has been correlated with clinical progression of prostate cancer (Hedlund et al., 1998).

Chemosensitising prostate cancer cell lines to Fas

A number of groups have studied the ability of various chemotherapeutic drugs to sensitise prostate cancer cell lines to Fas mediated apoptosis. Although resistance to Fas receptor mediated apoptosis correlates with progression of prostate cancer, combined therapy using cytotoxic drugs and Fas receptor agonists is not feasible in patients suffering from cancer because of the severe systemic toxicity associated with anti-Fas antibodies in murine models (Ogasawara et al., 1993). However, it was hoped that the mechanisms by which sublethal doses of chemotherapeutic drugs sensitise cells to would be determined. Such information is vital for developing chemotherapeutic regimens that are effective against prostate cancer and in particular androgen independent, metastatic prostate cancer.

Preliminary studies indicated that prostate cancer cells were resistant to Fas mediated apoptosis even though Fas receptor was expressed on the surface of these cells. Treatment with various cytotoxic drugs sensitised prostate cancer cell lines to apoptosis by both anti-Fas antibodies and cytotoxic T lymphocyte co-cultures (Rokhlin et al., 1997; Uslu et al., 1997). This trend appeared to be independent on the cellular targets of the various chemotherapeutic agents. The Topoisomerase I inhibitor camptothecin was identified as being the most effective drug in sensitising DU 145 prostate cancer cells to Fas mediated apoptosis (Costa-Pereira & Cotter, 1999). Subsequent anti-sense studies

implicated JNK activation by camptothecin as being both necessary and sufficient for sensitising DU 145 cells to Fas mediated apoptosis (Costa-Pereira et al., 2000).

Ligation of Fas receptor with Fas ligand is generally accompanied by a prolonged increase in activity of JNK (Latinis & Koretzky, 1996; Wilson et al., 1996). JNK activation following low levels of Fas receptor crosslinking is dependent on caspase activity (Lenczowski et al., 1997). Cleavage of two upstream kinases in the JNK cascade, Mst1 (Graves et al., 2001) and MEKK1 (Deak et al., 1998) following Fas receptor activation were found to be involved in caspase-dependent JNK activation. Meanwhile caspase-independent JNK activation occurs following higher levels of Fas receptor crosslinking. This requires the recruitment of ASK1 to the DISC through it's association with the Fas binding adapter protein DAXX (Chang et al., 1998; Tobiume et al., 2001). While JNK activity has no effect on Fas mediated apoptosis in some cell lines (Abreu-Martin et al., 1999; Hofmann et al., 2001; Low et al., 1999) others have reported that JNK is required for apoptosis following Fas receptor activation (Costa-Pereira et al., 2000; Le-Niculescu et al., 1999; Zhang et al., 2000). However, the mechanism by which JNK accelerates Fas mediated apoptosis *in vivo* has not been determined yet.

Aims of Project

In light of the recent work highlighting the importance of JNK both in survival and apoptosis in prostate cancer we decided to study the effects of JNK on Fas receptor mediated apoptosis. Although a number of animal models for prostate cancer have been developed including TRAMP mouse model and Dunning rat model (Bostwick et al., 2000) these have not been well characterised yet. We chose to conduct this research using a cell culture based model because of the versatility of these models and also because prostate cancer cell lines have been well characterised in the literature (Hsieh & Chung, 2001).
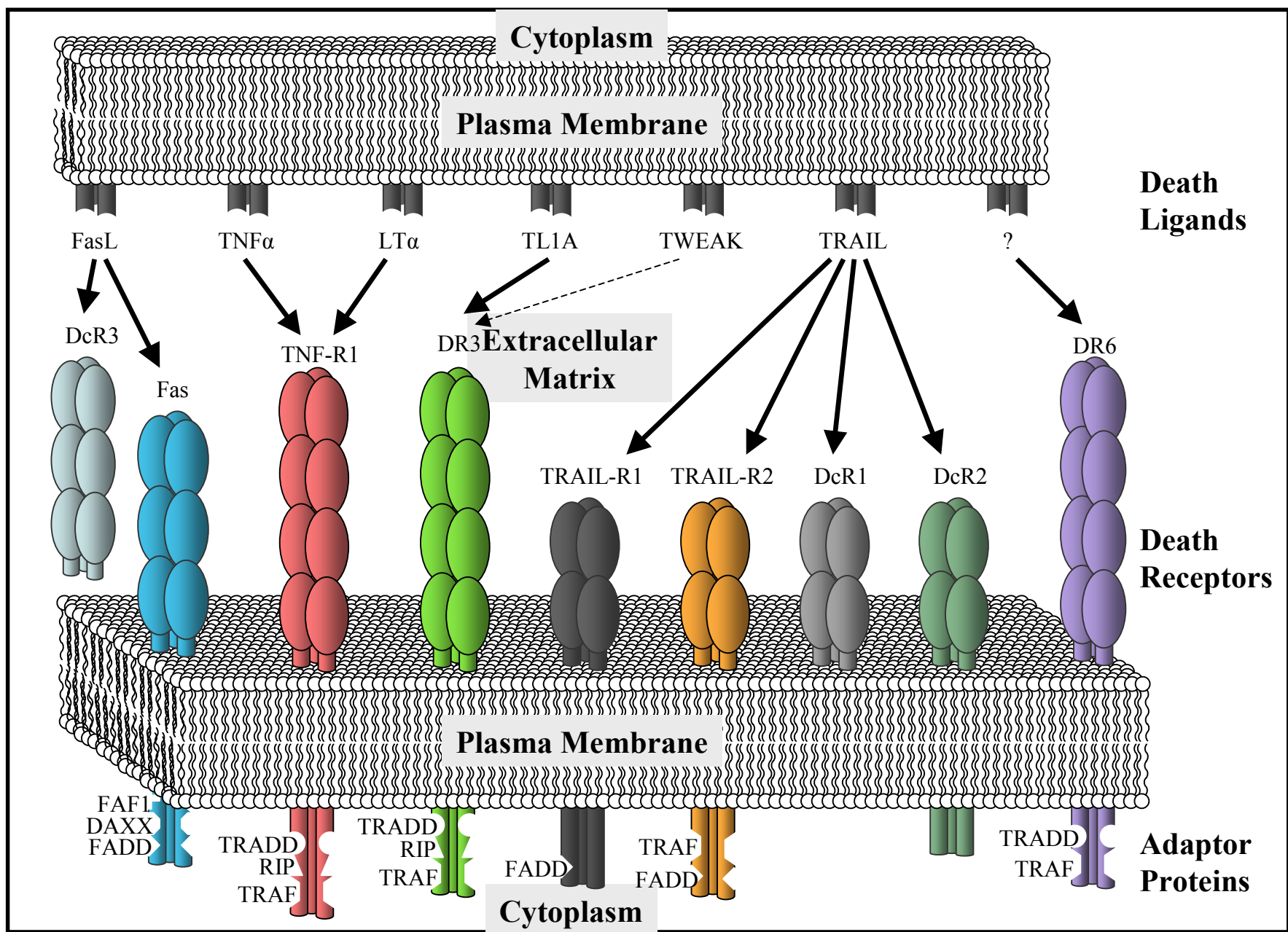
Figure 1:   Schematic diagram depicting the death receptors Fas, TNF-R1, DR3, TRAIL-R1, TRAIL-R2 and DR6 and the decoy receptors DcR1, DcR2 and DcR3. The number of Cysteine-rich pseudorepeats present is indicated by the number of extracellular domains for each receptor. Ligands that are known to bind to these receptors are all shown and are predominantly membrane bound. Some death receptors can bind with more than one ligand and some ligands bind to more than one receptor as indicated. Important adapter proteins that are recruited to each receptor and that are involved in signal transduction are also indicated.

FasL

FasR

Microaggregates

Large Clusters

Receptor
endocytosis
and recycling

FADD

Caspase 8

ASK1

DAXX

RIP1

RAIDD

Caspase 2

JNK
Activation

Mitochondrial
Amplification

Apoptosis
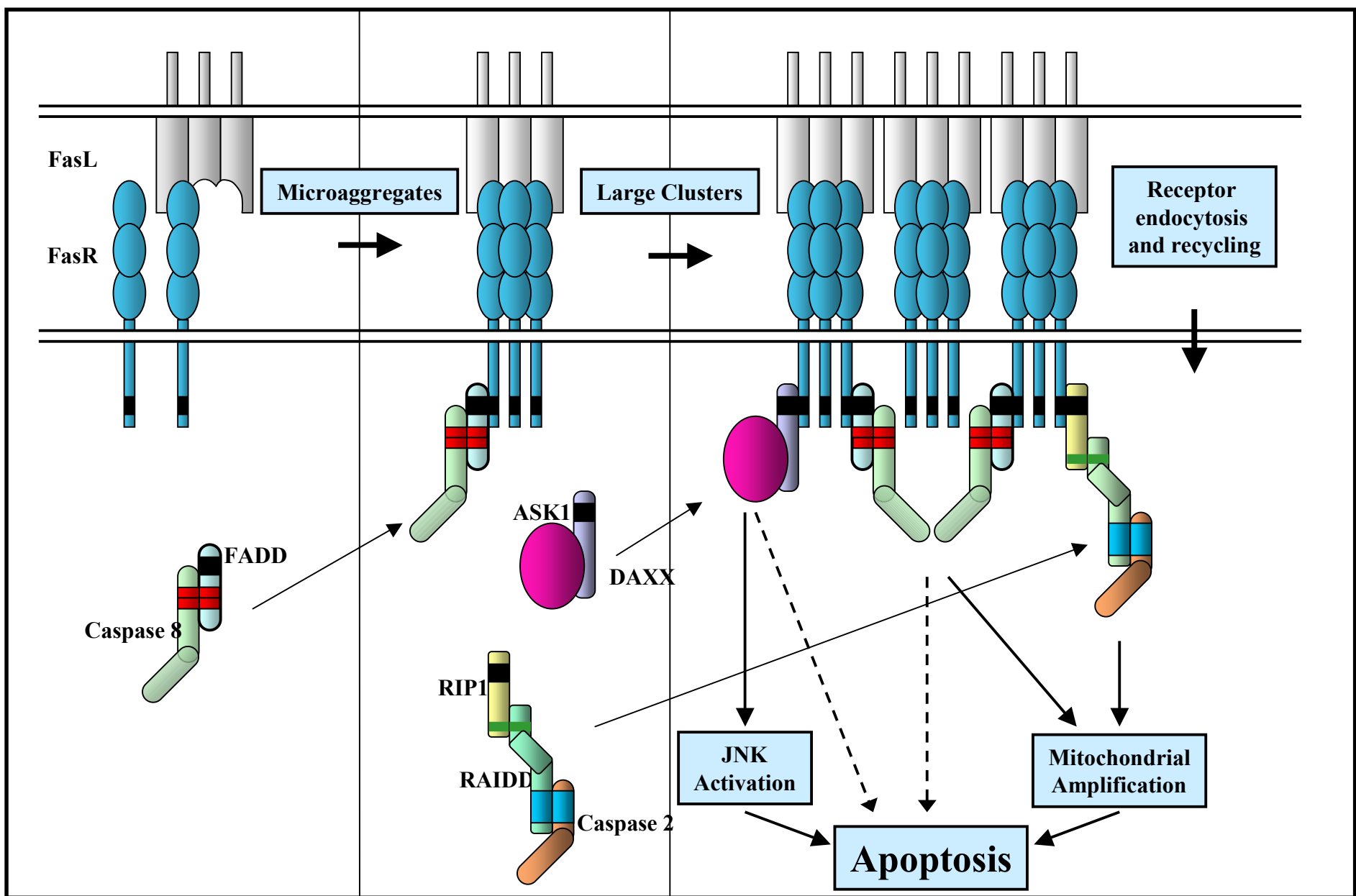
Figure 2: Illustration depicting the major events during DISC formation. Microaggregates of Fas receptor are formed after binding with Fas ligand. Caspase 8 is activated and is required for the formation of large clusters. ASK1 and RIP activation during DISC formation promote apoptosis. Mitochondria serve as an amplification step in type II cells. Finally, receptor clusters are endocytosed and may be recycled.
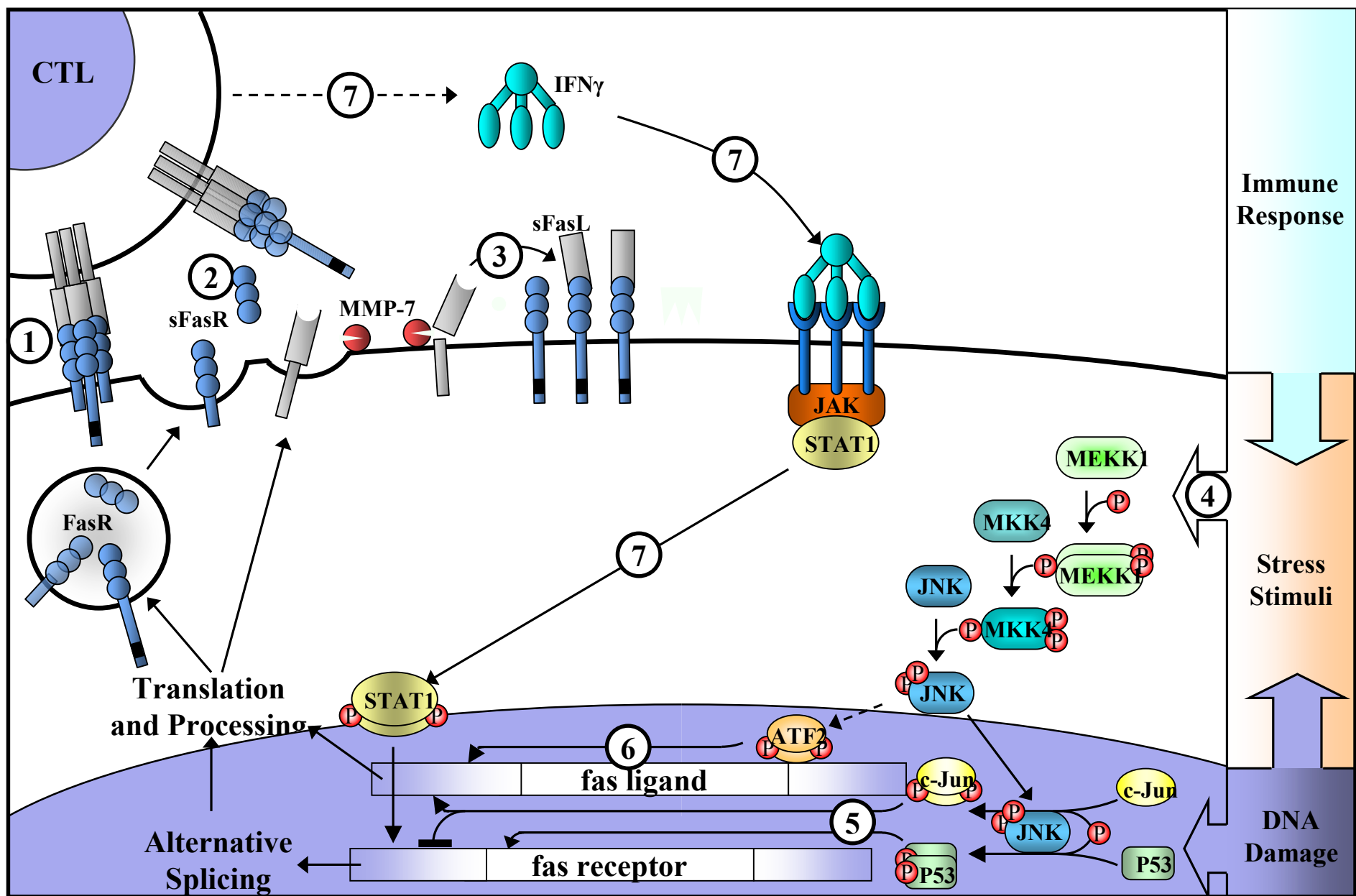
Figure 3: Schematic diagram identifying the major regulatory mechanisms of Fas receptor and Fas ligand expression and activation. Fas receptor is alternatively spliced producing inhibitory isoforms including membrane bound isoform 3 ① and soluble Fas receptor isoforms ② that interfere with Fas receptor activation by Fas ligand. The extracellular protease MMP-7 can cleave Fas ligand at a conserved site. Soluble Fas ligand binds with Fas receptor but inhibits DISC formation ③. Stress stimuli include DNA damage and immune responses and can activate the JNK kinase cascade ④. JNK phosphorylates and activates c-Jun, P53 and ATF2 and increases the expression of Fas receptor and Fas ligand ⑤, ⑥. IFN-γ is secreted by CTL and activates the transcription factor STAT1 ⑦. STAT1 upregulates Fas receptor expression and sensitises cells to CTL mediated cell death.

Self sufficient in growth signals

Insensitivity to anti-growth signals

Tissue invasion and metastasis

Limitless reproductive potential

Sustained angiogenesis

Resistance to apoptosis

Progression of Prostate Cancer

- ↑ Her2/neu
- ↑ IGF1
- ↑ STAT3
- ↑ hAR

- ↓ P53
- ↓ Rb
- ↓ P27

- ↑ Fas L
- ↑ PTEN
- ↑ Bcl-2
- ↓ Fas R

- ↑ MMP
- ↑ Osteopontin
- ↓ KAI1
- ↓ Integrin
- ↓ Cadherin
- ↓ Selectin

- ↑ VEGF
- ↑ bFGF (?)

- ↑ Telomerase
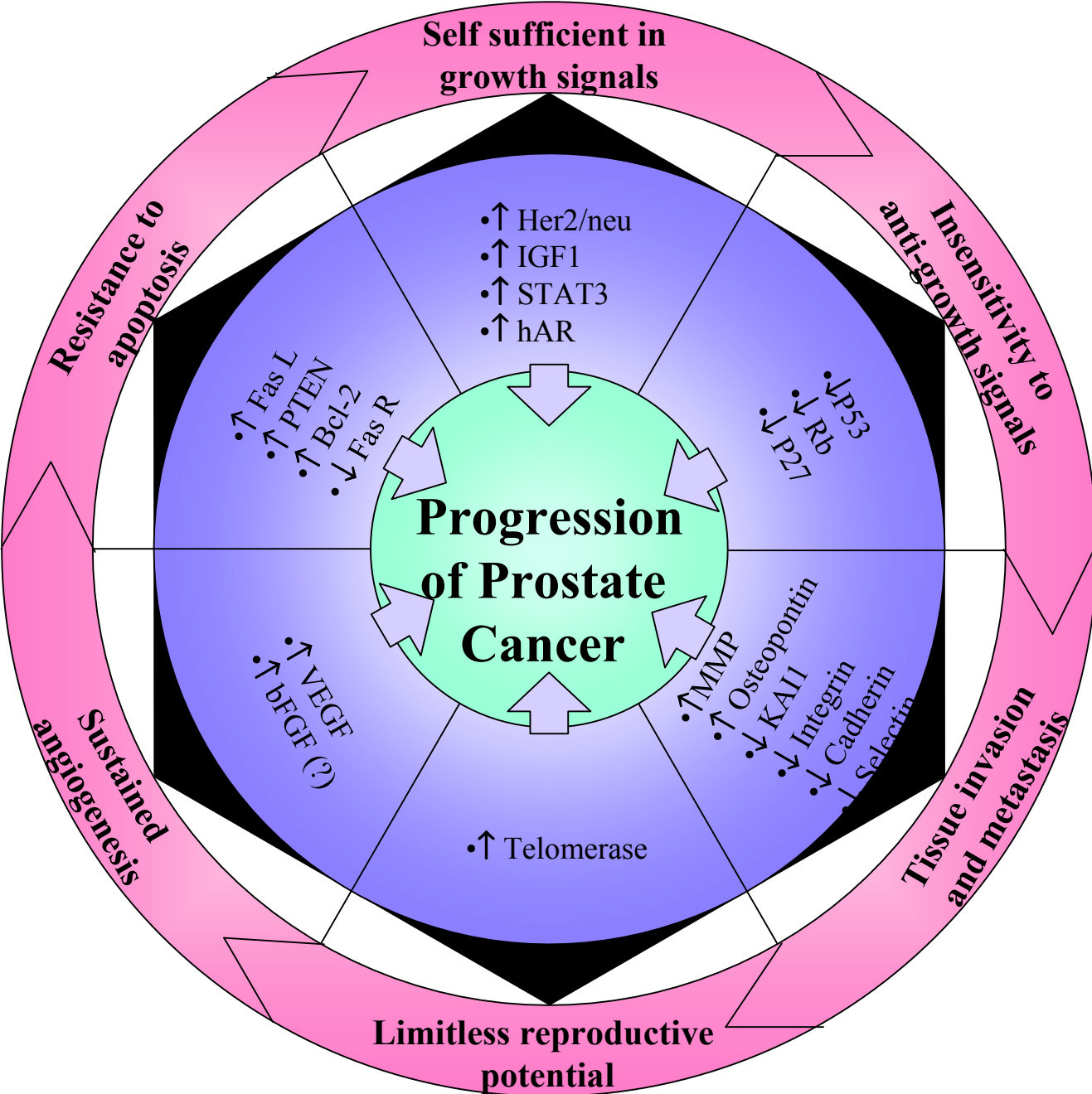
Figure 4; At least 6 independent stages are required for progression of normal prostate epithelial cells into malignant invasive carcinoma cells. This figure summarises these key stages and also highlights the effector proteins required during prostate cancer progression.
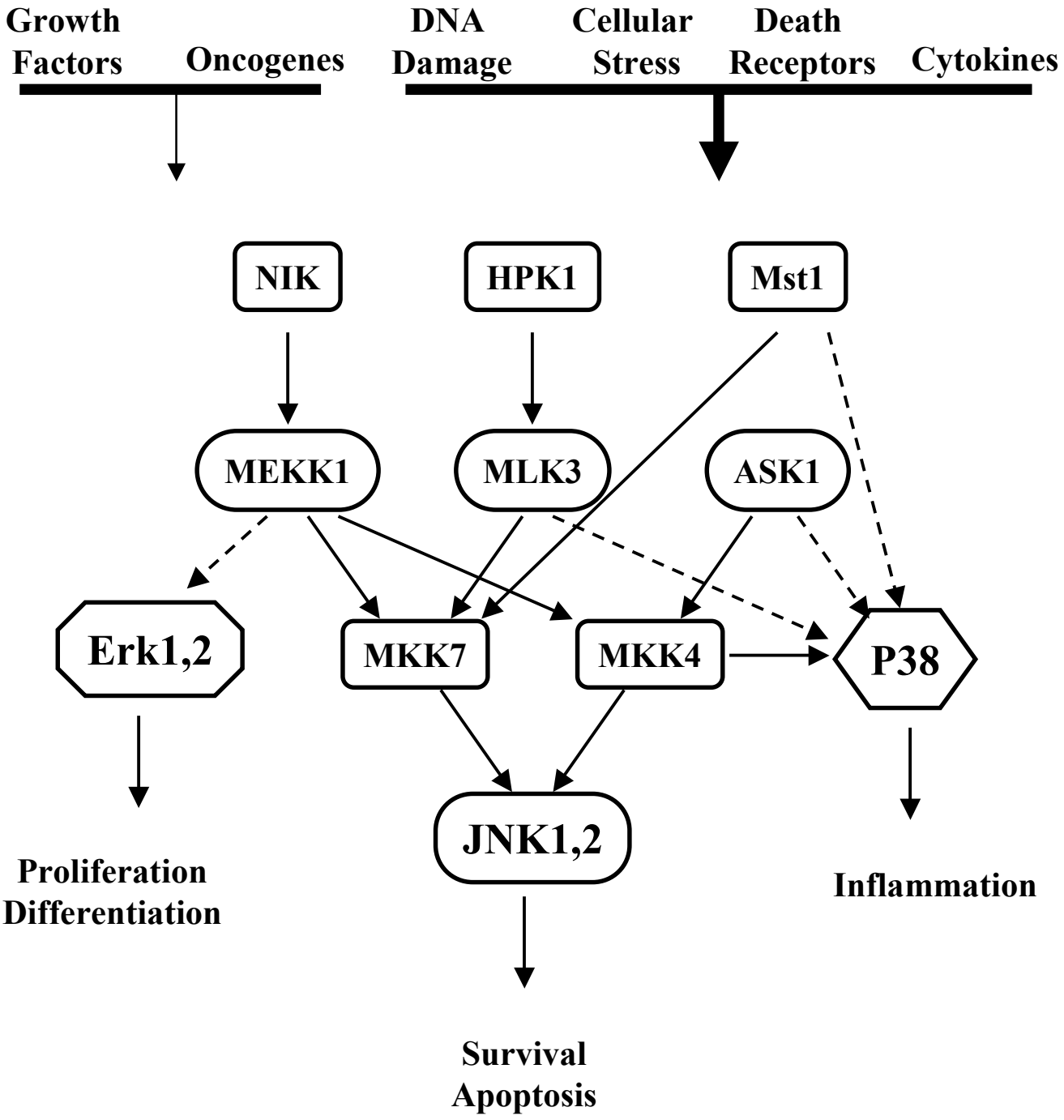
Figure 5;   Kinases upstream of JNK in the JNK cascade display a hierarchal structure and mediate JNK activation in response to numerous signals. MKK4 and MKK7 are immediately upstream of JNK and are called MAP kinase kinases (MAPKK), kinases that phosphorylate MAPKK are called MAPKK kinases (MAPKKK) and so on. The MAPKKK and MAPKKKK are responsible for detecting the various stress stimuli. For example, ASK1 activates JNK in response to death receptor activation and UV irradiation while HPK1 promotes JNK activity in response to the onco-protein Ras. The different scaffold proteins required by each MAPKKK or MAPKKKK for JNK activation may determine the substrate specificity of JNK *in vivo*.

Anisomycin activates JNK and sensitises DU 145 prostate carcinoma cells to Fas mediated apoptosis

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Treatment of the hormone refractory prostate cancer cell line DU 145 with sublethal concentrations of chemotherapeutic drugs has been reported to sensitise these cells to Fas mediated apoptosis. However, the mechanism by which this occurs has not been determined. Our group has shown that inhibition of JNK activity completely abrogates the effects of chemotherapeutic drugs. Using anisomycin, a potent JNK agonist, we have demonstrated a role for JNK in Fas mediated apoptosis in DU 145 cells. Inhibition of Caspase 8 and Caspase 9 completely inhibits this process which suggests that DU 145 cells require mitochondrial amplification of the Fas apoptotic signal. Furthermore, we have shown that inhibition of Fas mediated apoptosis is an early event in DU 145 cells, occurring upstream of Caspase 8 cleavage. It is hoped that identifying the target of JNK will allow novel therapies to be developed for the treatment of hormone refractory prostate cancer. Such therapies are especially important because no single or combined treatment to date has significantly prolonged survival in patients with hormone refractory prostate cancer.
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Keywords: prostate; apoptosis; CD95; Fas; JNK

Prostate cancer is the second most prevalent malignancy in the EU after lung cancer with about 200 000 new cases diagnosed and over 35 000 deaths each year. In England and Wales, 15 000 new cases and 8000 deaths are registered each year (Parkin *et al*, 2001). Although the prognosis is good for individuals with localised tumours, 10 – 20% of patients are diagnosed with metastatic prostate cancer (Crawford *et al*, 1999). These patients are usually treated with hormone ablation therapy which results in immediate tumour regression and temporary relief for the patient. However, hormone refractory prostate cancer invariably develops within 2 – 3 years of hormone ablation (Petrylak, 1999). This slowly proliferating cancer is extremely difficult to treat and the prognosis for the patient is generally poor. Over the past 5 years, chemotherapy has been used to improve the quality of life in patients with metastatic, hormone-refractory prostate cancer. No treatment has yet been found that cures the disease or even significantly prolongs survival (Petrylak, 1999).

Apoptosis, or programmed cell death (PCD), is characterised by morphological features including chromatin condensation, nuclear fragmentation, cell shrinkage, membrane blebbing and apoptotic body formation (Kerr *et al*, 1972). Although a variety of different environmental insults and signalling pathways can stimulate apoptosis in cells, most of these signals converge at a family of cysteine proteases called the caspases. Like many proteases, they are synthesised in an inactive form and cleavage into active caspases is essential for the proliferation of the apoptotic signal. Caspases can be divided into two main subfamilies, initiator caspases and effector caspases (Wolf and Green, 1999). The Fas receptor is a member of the Tumour Necrosis Factor

receptor superfamily and is expressed at the plasma membrane in a variety of tissues. Ligation of Fas ligand or a cross-linking antibody to the Fas receptor induces apoptosis in susceptible cells. Fas receptor clustering results in the recruitment and auto-cleavage of the initiator caspase, Procaspase 8, at the plasma membrane. Active Caspase 8 proceeds to cleave downstream cellular targets including the effector Caspases 3 and 7, and the Bcl-2 family member Bid (Peter and Krammer, 1998). Often an amplification step is required for Caspase 3 cleavage and morphological apoptosis. Caspase 8 cleaves Bid into tBid, a pro-apoptotic Bcl-2 family member that induces cytochrome *c* release and apoptosome formation. This amplification loop through the mitochondrion drives the apoptotic programme in type II cells (Scaffidi *et al*, 1999b).

DU 145 cells, a hormone refractory prostate adenocarcinoma, are highly resistant to Fas mediated apoptosis *in vitro*. In a study performed using cell lines derived from prostate tumours with different pathological stages including DU 145, it was observed that ALVA-31 and PPC-1 were sensitive to Fas mediated apoptosis. These were reported to be isolated from primary prostatic tumours. In contrast, the cell lines LNCaP, DU 145 and PC-3 were resistant. These cell lines were reported to be derived from distant metastases. The authors correlated prostate cancer disease progression with resistance to Fas. Furthermore they suggest that this phenomenon may explain, at least in part, the inability to treat hormone refractory prostate cancer (Hedlund *et al*, 1998). The two other cell lines used in this study, JCA-1 and TSU-Pr1 have since been reclassified as bladder cancer cell lines (van Bokhoven *et al*, 2001).

In order to study the resistance of hormone refractory prostate cancer to chemotherapy, the effects of chemotherapeutic drugs on DU 145 cells was explored (Uslu *et al*, 1997; Costa-Pereira and Cotter, 1999). Our group discovered that sublethal concentrations of camptothecin, a novel topoisomerase I inhibitor, sensitised DU

Experimental Therapeutics

145 cells to Fas mediated apoptosis by 20-fold (Costa-Pereira and Cotter, 1999). Activation of the stress kinase JNK was found to be essential in potentiating Fas mediated apoptosis (Costa-Pereira *et al*, 2000). In this study, we use anisomycin, a potent activator of JNK, to underscore the role played by JNK in Fas mediated apoptosis in DU 145 cells.

MATERIALS AND METHODS

Cell lines and reagents

DU 145 and Jurkat T cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cell culture reagents were purchased from Gibco BRL (UK) with the exception of foetal calf serum (FCS) (Sigma, UK). Anisomycin was purchased from Sigma (UK) and was dissolved in DMSO at a concentration of 5 mg ml^{-1}. A working stock at 10 μg ml^{-1} in RPMI was prepared from the original stock. A FACScan (Beckton Dickinson, BD Biosciences, Germany) and Cell Quest software Version 3.3 (Beckton Dickinson) were used for all flow cytometry assays. Annexin V-FITC was purchased from Bender MedSystems (Germany) and propidium iodide (PI) from Sigma (UK). TUNEL reagents were obtained from Roche (UK) and JC-1 was purchased from Molecular Probes (Netherlands). The antibodies used in this study were mouse anti-Fas IgM clone CH11, mouse anti-Fas IgG for flow cytometry (Bender MedSystems, Germany), phospho-JNK (Thr183/Tyr185) clone G9 and rabbit anti-Caspase 3 (Cell Signalling Technology, New England Biolabs UK), rabbit anti-JNK1 and rabbit anti-Fas ligand (Santa Cruz, USA), rabbit anti-Bid (BioSource International, USA), rabbit anti-human caspase 8 (R&D Systems, UK) and mouse anti-β Actin clone AC-15 (Sigma, UK). All FITC and R-Phycoerythrin conjugated secondary antibodies were purchased from Sigma (UK) and HRP conjugated secondary antibodies from DAKO (Denmark). The Caspase inhibitors z-IETD-fmk and z-LEHD-fmk were obtained from Calbiochem (CN Biosciences UK). Enhanced Chemiluminescence Reagent (ECL) was purchased from Amersham Biosciences (UK).

Cell culture and treatment

DU 145 cells were cultured in RPMI 1640 medium supplemented with 5% FCS, 2 mM L-Glutamine in the presence of 10 IU ml^{-1} penicillin-streptomycin. Jurkat cells were cultured in RPMI 1640 medium containing 10% FCS, 2 mM L-Glutamine and 10 IU ml^{-1} penicillin-streptomycin. Cells were cultured at 37°C in a humidified atmosphere with 5% CO$_2$ and were routinely subcultured every 2–3 days. Prior to every experiment DU 145 cells were grown to 75% confluency and Jurkats were resuspended at 0.5 million ml^{-1}. DU 145 cells were pretreated with 250 ng ml^{-1} anisomycin for 10 min before addition of 200 ng ml^{-1} anti-Fas IgM.

Annexin V binding and propidium iodide uptake assay

DU 145 and Jurkat cells were incubated with 250 ng ml^{-1} anisomycin and 200 ng ml^{-1} anti-Fas IgM for 8 h. The cells were subsequently harvested, washed once in PBS and resuspended in Annexin V binding buffer (150 mM NaCl, 18 mM CaCl$_2$, 10 mM HEPES, 5 mM KCl, 1 mM MgCl$_2$). FITC conjugated Annexin V (1 μg ml^{-1}), which binds specifically to apoptotic cells, was added to each sample and incubated at ambient temperature for 5 min. Propidium iodide (50 μg ml^{-1}), excluded from viable cells, was added immediately prior to reading the samples on the FACScan. Where indicated the Caspase 8 and Caspase 9 inhibitors were incubated for 10 min at 50 μM prior to treating the cells with anisomycin or Fas.

DNA fragmentation assay

TUNEL measures DNA fragmentation using the enzyme Terminal deoxynucleotide Transferase (TdT) to transfer multiple biotin labelled nucleotides to the 3′ hydroxyl groups of DNA. FITC conjugated Avidin can be used to stain this modified label. Using flow cytometry, cells with fragmented DNA in their nuclei display an increased fluorescent signal in the FL-1 channel relative to untreated cells.

DU 145 and Jurkat cells were harvested, washed twice in PBS and fixed slowly in 1% paraformaldehyde (PFA) on ice for 15 min. The cells were washed twice in PBS and resuspended in 25 μl reaction mixture (TdT buffer, 2.5 mM CoCl$_2$, Bio-16-dUTP and TdT enzyme). The DNA labelling reaction was allowed to proceed for 30 min at 37°C. The cells were washed twice in PBS and resuspended in 50 μl staining buffer (5 × SSC, 5% w v^{-1} dry milk, 1 × Avidin-FITC and 1/1000 × Triton X-100). The cells were stained for 30 min at room temperature in the dark and washed twice in PBS. Flow cytometry and Cell Quest were used to collect and analyse the data.

Fas receptor expression

0.5 × 10^6 cells were harvested per sample and washed twice in PBS. They were stained for 1 h at 4°C with 20 μg ml^{-1} of the primary antibody mouse anti-Fas IgG. After another two washes with PBS, the cells were stained with the FITC conjugated secondary antibody sheep anti-mouse IgG for 1 h at 4°C in the dark. Cells stained with secondary antibody alone were used to compensate for intrinsic fluorescence and non-specific binding of the secondary antibody. The cells were washed twice in PBS and the presence of Fas R was detected in FL-1 using a FACScan flow cytometer.

Fas ligand expression

0.5 × 10^6 cells were harvested per sample and were fixed slowly in ice cold 1% PFA for 15 min. The cells were permeabilised overnight in 70% ethanol (-20°C) and stained with 2 μg ml^{-1} rabbit anti-Fas ligand or 2 μg ml^{-1} rabbit irrelevant IgG in IFA$_{TX}$ (4% FCS, 150 mM NaCl, 10 mM HEPES, 0.1% sodium azide, 0.1% Triton X-100) for 1 h at 4°C. Subsequently, the cells were stained for 1 h with 12 μg ml^{-1} FITC-conjugated anti-rabbit IgG in the dark at 4°C. Fas ligand expression was analysed on the FACScan using Cell Quest software.

Mitochondrial membrane depolarisation

The lipophilic cation called JC-1 is cell permeable and selectively accumulates in the mitochondria of live cells. When depolarisation of the mitochondria occurs, the emission spectrum of JC-1 changes from 590 nm (its aggregated form) to 530 nm (its monomeric form) and this can be analysed using flow cytometry. Depolarisation of mitochondria results in an increase in fluorescence in the FL-1 channel, and a concurrent decrease in the FL-2 channel in flow cytometers.

DU 145 cells were harvested, resuspended in RPMI+10% FCS and 2.5 μg ml^{-1} JC-1 was added. The samples were incubated at room temperature for 20 min in the dark, washed twice in PBS and read on the FACScan. Analysis was carried out using Cell Quest software.

SDS–PAGE and Western blot

Protein extracts were prepared from cells using RIPA lysis buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1 mM each of NaF, NaVO$_4$ and EGTA; 1% NP40; 0.25% sodium deoxycholate; 0.2 mM phenylmethylsulphonyl fluoride; 1 μg ml^{-1} each of antipain, apro-

tinin and chymostatin; 0.1 μg ml^{-1} leupeptin; 4.0 μg ml^{-1} pepstatin) and 30 μg of protein was loaded in each lane of an SDS polyacrylamide gel. Electrophoresis and Western blotting was subsequently carried out. Non-specific protein binding sites on the membrane were blocked using 5% dry milk in TBS+0.1% Tween-20 for 1 h at room temperature. The membrane was stained with primary and peroxidase conjugated secondary antibodies according to the manufacturer's recommended protocol and labelled protein was detected using ECL.

RESULTS

### Anisomycin activates JNK and sensitises DU 145 cells to Fas mediated apoptosis

The hormone refractory cell line DU 145 is highly resistant to Fas mediated apoptosis. This appears to be a common event during prostate cancer progression. Cell lines isolated from early stages of prostate cancer are usually sensitive to the Fas activating antibody anti-Fas IgM. Those cell lines derived from secondary tumours after hormone ablation therapy are generally resistant to Fas (Hedlund *et al*, 1998). Our group has previously shown that camptothecin, a Topoisomerase I inhibitor, sensitises DU 145 cells to Fas mediated apoptosis (Costa-Pereira and Cotter, 1999). Additional analysis identified activation of the stress kinase JNK as an integral event in this process (Costa-Pereira *et al*, 2000). In order to understand the mechanisms behind this sensitisation process we have used anisomycin, an agonist of the JNK pathway in mammalian cells, which is often used in studies involving JNK because of its specificity and potency in activating the JNK pathway. As expected, we found that anisomycin can act in synergy with Fas to induce apoptosis in DU 145 cells. Phosphatidylserine flipping, an early event during apoptosis was detected with FITC-conjugated Annexin-V using flow cytometry. Propidium iodide was used as a counter stain to distinguish between early and late apoptosis (Figure 1A). Flow cytometry was also used to detect DNA fragmentation, another hallmark of apoptosis, in TUNEL labelled DU 145 cells following incubation with anisomycin (250 ng ml^{-1}) and anti-Fas IgM (200 ng ml^{-1}) (Figure 1B). This rapid onset of DNA fragmentation in our system is indicative of a stronger apoptotic stimulus when anisomycin is used to sensitise DU 145 cells to anti-Fas IgM in comparison with other cytotoxic drugs. Extensive DNA fragmentation was only observed after 48 h when CPDD and CHX were used (Rokhlin *et al*, 1997; Uslu *et al*, 1997). Numerous reports have been described in the literature of both Caspase 8 dependent and Caspase 8 independent JNK activation during Fas mediated apoptosis. We used an antibody specific to phosphorylated JNK to assess the status of JNK activation in DU 145 cells after 1 and 8 h incubation with anisomycin (250 ng ml^{-1}) and anti-Fas IgM (200 ng ml^{-1}). We verified that JNK is not activated either transiently (1 h) or prolonged (8 h) with anti-Fas IgM (Figure 1c). As expected, anisomycin was found to stimulate prolonged JNK activation in DU 145 cells. An *in vitro* kinase assay using radiolabelled ^{32}P was used to verify the activity of JNK (data not shown).

Fas receptor and Fas ligand are not up-regulated by anisomycin in DU 145 cells

Various reports have shown that down-regulation of Fas receptor or Fas ligand expression occurs in some cancer cells. In addition, expression of Fas ligand has been reported to increase following JNK activation in Jurkat cells (Herr *et al*, 2000). This increase in Fas ligand expression caused an increase in the kinetics of Fas mediated apoptosis. We assessed the expression of both Fas receptor and Fas ligand over an 8 h period (1,2,4 and 8 h) following incubation with anisomycin (250 ng ml^{-1}) or anti-Fas IgM
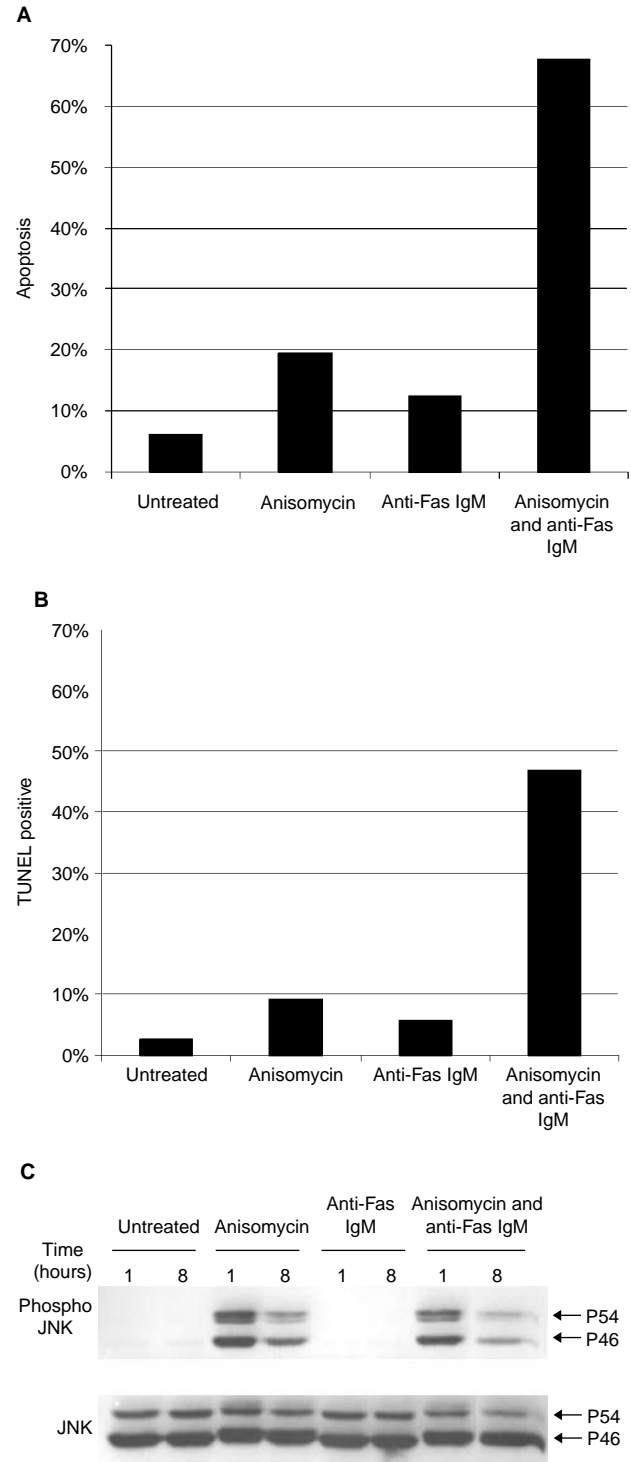
Figure 1 Anisomycin sensitised the androgen independent cell line DU 145 to Fas mediated apoptosis. DU 145 cells were pre-treated with anisomycin (250 ng ml^{-1}) for 10 min before the addition of anti-Fas IgM (200 ng ml^{-1}). Cells were stained with Annexin V and PI (**A**) or TUNEL (**B**) after 8 h incubation as described in the Materials and Methods section. Flow cytometry was used to visualise the extent of apoptosis. Data are representative of at least three independent experiments. (**C**) Anisomycin, not anti-Fas IgM, stimulates prolonged JNK activation in DU 145 cells. Western blot analysis was used to detect active JNK in untreated DU 145 cells, or cells treated with anisomycin (250 ng ml^{-1}), anti-Fas IgM (200 ng ml^{-1}) or both anisomycin and anti-Fas IgM for 1 and 8 h. Total JNK expression was determined to ensure equal protein loading.

A

B
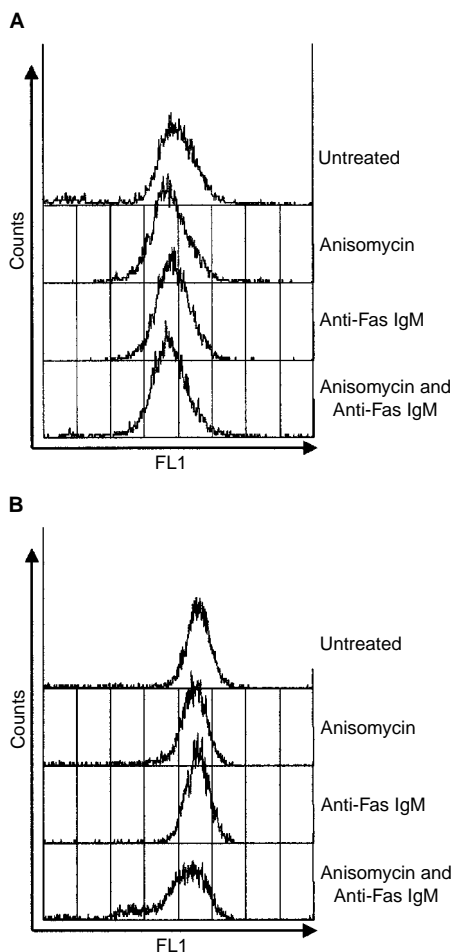
Figure 2 Flow cytometric analysis of Fas receptor (**A**) or Fas ligand (**B**) expression in untreated DU 145 cells or following 8 h incubation with anisomycin (250 ng ml^{-1}), anti-Fas IgM (200 ng ml^{-1}) or both. Data are representative of at least three independent experiments and similar data were obtained for incubations of 1, 2 and 4 h.

(250 ng ml^{-1}). Cell surface Fas receptor was expressed on 95% of DU 145 cells and its expression was not found to change following treatment with anisomycin or anti-Fas IgM (Figure 2A). Similarly, Fas ligand was expressed in 90% of DU 145 cells and expression was not increased in DU 145 cells following incubation with anisomycin or anti-Fas IgM (Figure 2B). Western blot analysis confirmed that the expression of Fas receptor and Fas ligand was not upregulated following drug treatment (data not shown).

Activation of Caspase 3 during Fas mediated apoptosis

In order to delineate the mechanisms by which DU 145 cells are sensitised to Fas mediated apoptosis we analysed the major events during Fas mediated apoptosis. Most apoptotic stimuli converge on Caspase 3, a cysteine protease and the main effecter caspase during Fas mediated apoptosis. Once activated, Caspase 3 cleaves a variety of substrates responsible for the morphological and biochemical changes observed during apoptosis (Nicholson, 1999). We found that both anisomycin and anti-Fas IgM treatment alone were insufficient for Caspase 3 activation in DU 145 cells. However, co-incubation of cells with both anisomycin and anti-Fas IgM clearly potentiates the activation of Caspase 3 in DU 145 cells (Figure 3A). Anti-Fas IgM treated Jurkats are used as a positive control for

Caspase 3 activation. Flow cytometric analysis confirmed that 70% of DU 145 cells expressed the active form of Caspase 3 following treatment with both anisomycin and anti-Fas IgM (data not shown).

Mitochondrial membrane depolarisation

It has been shown that Fas receptor activation is often not sufficient for direct activation of Caspase 3. These cells, known as type II cells, require an amplification signal through the mitochondrion. Caspase 8 cleaves and activates Bid, a pro-apoptotic Bcl-2 family member. This results in mitochondrial membrane depolarisation, cytochrome c release and amplification of the Fas apoptotic signal through Caspase 9 (Kim *et al*, 2000). Here we used the voltage sensitive, lipophilic fluorescent probe JC-1 to analyse the extent of mitochondrial membrane depolarisation in DU 145 cells. Depolarisation of the mitochondrion causes an increase in FL-1 fluorescence and a concomitant decrease in FL-2 fluorescence when analysed by flow cytometry (Cossarizza *et al*, 1993). We found that stimulation of DU 145 cells for 8 h with anisomycin (250 ng ml^{-1}) or anti-Fas IgM (200 ng ml^{-1}) alone did not result in permeability transition of the mitochondria (Figure 3B). This suggested that Fas mediated apoptosis is inhibited up-stream of mitochondrial depolarisation in DU 145 cells. Incubation of DU 145 cells with both anisomycin (250 ng ml^{-1}) and anti-Fas IgM (200 ng ml^{-1}) resulted in mitochondrial depolarisation. We found that incubation of DU 145 cells with 50 μM z-LEHD-fmk (a Caspase 9 specific inhibitor) completely abrogated apoptosis when incubated with anisomycin (250 ng ml^{-1}) and anti-Fas IgM (200 ng ml^{-1}) (Figure 3C). This suggested that mitochondrial membrane depolarisation and cytochrome c release are essential events for Fas mediated apoptosis in DU 145 cells.

Caspase 8 activation in DU 145 cells

The proximal caspase in the Fas apoptotic pathway is Caspase 8. Recruitment and auto-cleavage of Procaspase 8 occurs following Fas receptor activation in sensitive cells. Active Caspase 8 is tetrameric, consisting of two P14 and two P10 subunits. Using Western blot analysis, we found that Caspase 8 is not cleaved in DU 145 cells following treatment with anti-Fas IgM (200 ng ml^{-1}) (Figure 4A). Therefore, we concluded that inhibition of Fas mediated apoptosis occurred upstream of Caspase 8 activation in DU 145 cells. Caspase 8 cleavage products were only evident following incubation with both anisomycin (250 ng ml^{-1}) and anti-Fas IgM (200 ng ml^{-1}). This was also true for the Caspase 8 substrate Bid (Figure 4B). Z-IETD-fmk (50 μM), an irreversible inhibitor specific to Caspase 8, was found to completely protect against apoptosis induced by anisomycin (250 ng ml^{-1}) and anti-Fas IgM (200 ng ml^{-1}) (Figure 4C). Interestingly, this inhibitor also abolishes apoptosis associated with anisomycin alone. This suggests that low levels of Caspase 8 activation occur in DU 145 cells following incubation with anisomycin alone. A dose titration of z-IETD-fmk confirmed that this inhibitor specifically inhibits Caspase 8 at 50 μM (data not shown). FLIP is a family of proteins structurally related to Caspase 8 that inhibit Fas mediated apoptosis when overexpressed in cells (Scaffidi *et al*, 1999a). Two main isoforms of FLIP are expressed in cells, a long splice variant (FLIP$_L$) and a short splice variant (FLIP$_S$). Using Western blot analysis we found that DU 145 cells express FLIP$_S$. However, expression of this caspase 8 inhibitor does not appear to decrease following incubation with anisomycin (data not shown).

DISCUSSION

The sensitivity of prostate cancer cell lines to Fas mediated apoptosis has been shown to correlate with tumour stage, grade and

Experimental Therapeutics
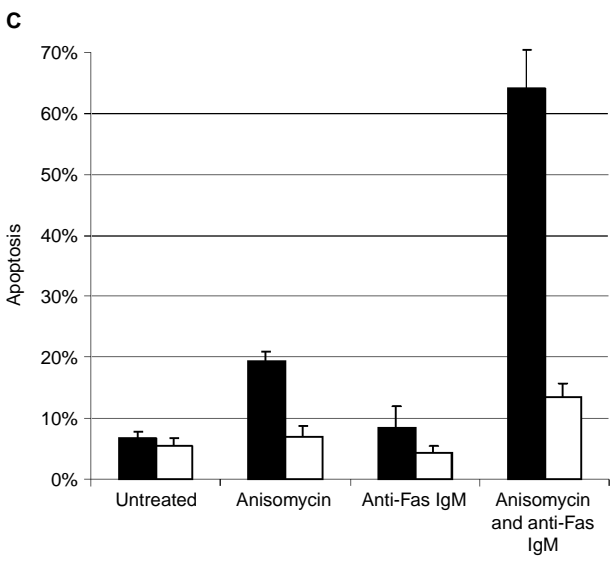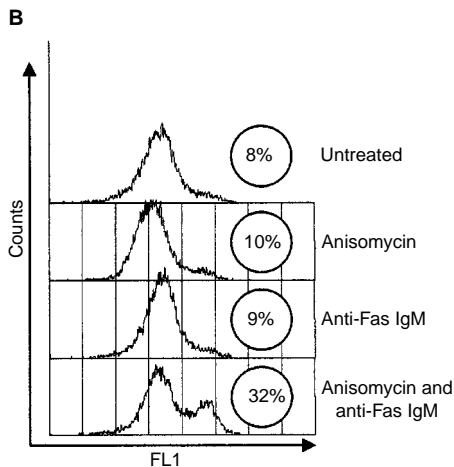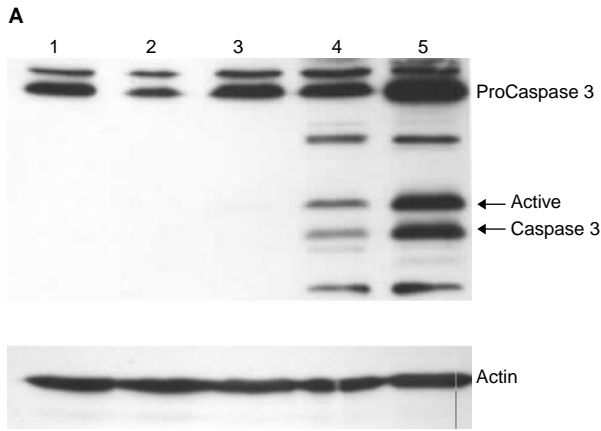
Figure 3 (**A**) Untreated DU 145 (**1**) cells or cells treated for 8 h with anisomycin (250 ng ml⁻¹) (**2**), anti-Fas IgM (200 ng ml⁻¹) (**3**) or both (**4**) were stained for Caspase 3 cleavage. Jurkats treated with anti-Fas IgM (200 ng ml⁻¹) for 4 h were used as a positive control (**5**). (**B**) Mitochondrial membrane depolarisation (ΔΨ) was detected in DU 145 cells using the lipophilic JC-1 probe, as described previously. ΔΨ was only observed in DU 145 cells treated for 8 h with both anisomycin (250 ng ml⁻¹) and anti-Fas IgM (200 ng ml⁻¹). (**C**) DU 145 cells were pre-treated with 50 μM z-LEHD-fmk (white columns) or a DMSO control (black columns) for 10 min before treating with anisomycin (250 ng ml⁻¹)
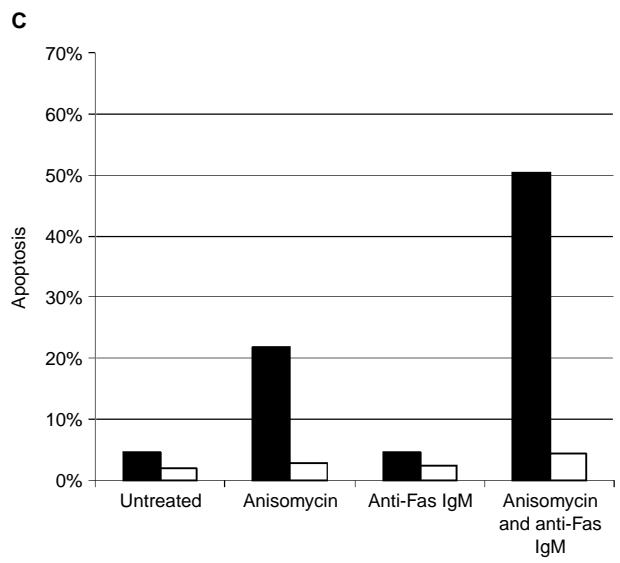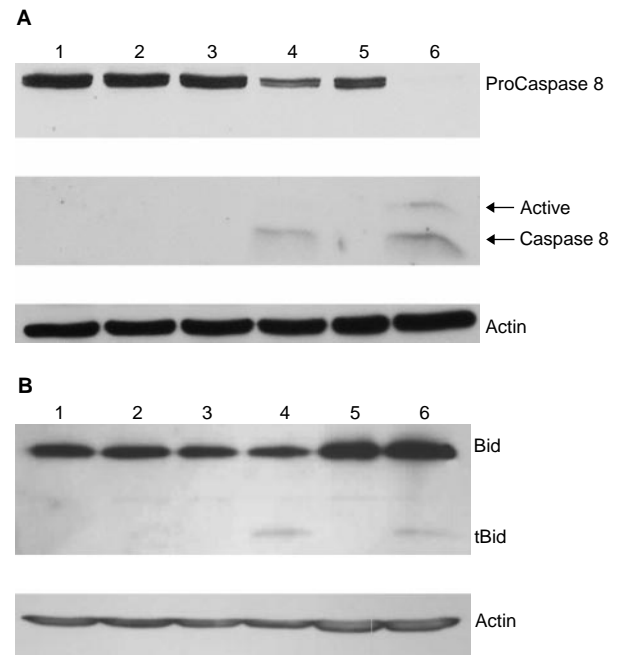
Figure 4 Inhibition of Fas mediated apoptosis occurs upstream of Caspase 8 cleavage in DU 145 cells. (**A**) Western blot analysis of Caspase 8 in untreated DU 145 cells (**1**) or following incubation with anisomycin (250 ng ml⁻¹) (**2**), anti-Fas IgM (200 ng ml⁻¹) (**3**) or both (**4**) for 8 h. Untreated (**5**) and anti-Fas IgM treated (200 ng ml⁻¹ anti-Fas IgM, 4 h) (**6**) Jurkat cells were used as a positive control for the P14 and P10 Caspase 8 cleavage products. β-Actin was also probed to ensure equal protein loading. (**B**) Bid expression and cleavage was analysed by Western blot in untreated DU 145 cells (**1**) or in cells incubated with anisomycin (250 ng ml⁻¹) (**2**), anti-Fas IgM (200 ng ml⁻¹) (**3**) or both (**4**) for 8 h. Jurkats untreated (**5**) or treated with anti-Fas IgM (200 ng ml⁻¹) for 4 h (**6**) are used as a positive control. (**C**) DU 145 cells were pre-treated with 50 μM z-IETD-fmk (white columns) or a DMSO control (black columns) for 10 min before treating with anisomycin and anti-Fas IgM as before. Apoptosis was determined by staining with both Annexin V and PI after 8 h. Data is representative of three independent experiments.

and anti-Fas IgM (200 ng ml⁻¹) for 8 h. Apoptosis was assessed by staining the cells with Annexin V-FITC and PI. The error bars represent standard deviation after three independent experiments.

resistance to chemotherapeutic drugs (Hedlund et al, 1998). Our group and others have shown that DU 145 cells are highly resistant to Fas mediated apoptosis. Co-treatment with sublethal concentrations of chemotherapeutic drugs including cyclohexamide (CHX), cisplatin (CPDD), etoposide (VP16) and camptothecin was found to sensitise these cells to Fas mediated apoptosis (Uslu et al, 1997; Rokhlin et al, 1998; Costa-Pereira and Cotter, 1999) independently of new protein synthesis (Frost et al, 1999). Our group subsequently identified a key role for JNK in this process. DU 145 cells were co-treated with camptothecin and anti-Fas IgM and were completely protected from apoptosis by anti-sense oligonucleotides specific for JNK (Costa-Pereira et al, 2000). In addition, camptothecin is a potent activator of JNK and sensitises DU 145 cells to Fas mediated apoptosis to a much greater extent than CHX, CPDD and VP16 (Costa-Pereira and Cotter, 1999). We have shown that anisomycin, a potent activator of JNK in mammalian cells, sensitises DU 145 cells to Fas mediated apoptosis to a similar extent as camptothecin. We felt that because camptothecin is also a topoisomerase I inhibitor and the mechanisms by which it activates JNK are unclear, anisomycin would present a better option for delineating the effects of JNK during Fas induced apoptosis.

Binding of Fas ligand, or Fas activating antibodies, to Fas receptor results in DISC formation and prolonged JNK activation by either Caspase 8 dependent or Caspase 8 independent mechanisms (Chang et al, 1998; Rudel et al, 1998; Charette et al, 2001; Graves et al, 2001). We have shown that stimulating Fas R with anti-Fas IgM alone does not result in JNK activation in DU 145 cells. We found that mitochondrial membrane depolarisation only occurs in DU 145 cells co-stimulated with anisomycin and anti-Fas IgM. In addition Caspase 8 and Bid were only cleaved following incubation with both anisomycin and anti-Fas IgM. This suggests that anisomycin sensitises DU 145 cells to Fas mediated apoptosis at a point upstream of Caspase 8 cleavage, probably during DISC formation.

There are some reports of caspase independent cell death following Fas R activation. These are mediated through kinases such as RIP and ASK1 (Holler et al, 2000; Charette et al, 2001). However, we have shown that both Caspase 8 and Caspase 9 inhibitors completely abrogate apoptosis induced by anisomycin and anti-Fas IgM in DU 145 cells. Therefore, anisomycin sensitised DU 145 cells to apoptosis mediated by Fas that is dependent on both Caspase 8 activity to initiate the pathway and Caspase 9 activity as an amplification step required for Caspase 3 activation and apoptosis.

The principal apoptotic pathway activated by many anti-cancer drugs is the Fas apoptotic pathway. DU 145 cells incubated with toxic concentrations of 9-amino camptothecin were found to increase Fas receptor and Fas ligand expression and decrease c-FLIP$_S$. Apoptosis could be inhibited by transient overexpression of c-FLIP$_S$, suggesting that 9-amino camptothecin induces apoptosis through the Fas apoptotic pathway (Chatterjee et al, 2001). However, we found no evidence for increased Fas receptor or Fas ligand expression following incubation with anisomycin. We did observe a decrease in Fas ligand expression after apoptotic body formation in cells incubated with both anisomycin and anti-Fas IgM. This is most probably due to the shedding of membrane bound Fas ligand and is irrelevant to the initiation of apoptosis in these cells. We also analysed FLIP$_S$ expression but no changes were observed following treatment with anisomycin. Chemotherapeutic drugs have also been reported to activate the Fas apoptotic pathway without upregulation of Fas ligand or Fas receptor. Apoptosis induced by CPDD, VP16 and vinblastine (VB) was shown involve Fas receptor clustering and Caspase 8 activation and was independent of Fas ligand in various colon cancer cells and leukaemia cells (Micheau et al, 1999). It is possible that anisomycin is inducing Fas receptor aggregation independently of anti-Fas IgM but this is highly unlikely in light of our results. It seems more probable that JNK activation enhances Fas receptor aggregation and DISC formation through its interaction with some key regulator of DISC formation in DU 145 cells.

Conventional chemotherapy has been unsuccessful in treating prostate cancer. No single or combined chemotherapy regime has been identified that significantly enhances long term survival. This may be due, at least in part, to the resistance developed to Fas mediated apoptosis in hormone refractory prostate cancer. We have sensitised DU 145 cells to Fas mediated apoptosis using the JNK agonist anisomycin. In addition we have traced the effects of JNK to a point upstream of Caspase 8 cleavage. It is hoped that by understanding this process novel drug targets may be identified that improve the treatment of hormone refractory prostate cancer.

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Experimental Therapeutics

CHAPTER 3

This chapter has been submitted for publication at the time of writing (2003)

Defects in Death Inducing Signalling Complex (DISC) formation prevent JNK activation and Fas mediated apoptosis in DU 145 prostate carcinoma cells

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Abstract

Androgen independent prostate carcinomas are resistant to chemotherapy and cell lines derived from androgen independent prostate carcinomas such as DU 145 cells are highly resistant to Fas mediated apoptosis. Incubation of DU 145 cells with anti-Fas IgM agonistic antibody of Fas receptor fails to activate JNK, a stress kinase involved in regulating apoptosis. We have previously shown that JNK activation is sufficient and necessary to promote Fas mediated apoptosis in DU 145 cells. We investigate the mechanisms by which JNK activation and apoptosis are abrogated. HSP27 is overexpressed in DU 145 cells and has previously been reported to sequester DAXX and prevent JNK activation in cells treated with anti-Fas IgM. However, we find no evidence that HSP27 interacts with DAXX in DU 145 cells. Instead we find that FADD does not interact with Caspase 8 and this results in defective death inducing signalling complex (DISC) formation following Fas receptor activation.

Introduction

Prostate cancer is the second most common malignancy in the EU with about 200,000 new cases diagnosed each year. It is a disease that affects primarily elderly men and is responsible for over 35,000 deaths each year (Parkin et al., 2001). Androgen dependent prostate cancer is generally a slowly progressing tumour and treatment with androgen ablation therapy results in tumour regression and an improvement in the quality of life for most patients. However, androgen refractory tumours subsequently develop with a median asymptomatic period of 18 months following androgen ablation (Petrylak, 1999). Androgen independent tumours are generally more aggressive than androgen dependent tumours and chemotherapy is only used as a palliative agent (Sternberg, 2001). No single or combined chemotherapeutic regimen has been shown to significantly enhanced long-term survival in patients presenting with invasive, hormone refractory prostate cancer (Petrylak, 1999).

The Fas apoptotic pathway has been extensively studied in a variety of tissues and cell types. Tumour cells often develop resistance to Fas receptor mediated apoptosis as a defense mechanism against the immune system and also against conventional chemotherapeutic agents (Micheau et al., 1999; O'Connell et al., 2001). Engagement of Fas receptor with Fas ligand or Fas activating antibodies causes recruitment of Procaspase 8 to the DISC through the adapter protein FADD in cell lines sensitive to Fas receptor mediated apoptosis. Auto-cleavage and activation of Caspase 8 occurs in the DISC and this in turn cleaves a variety of cellular targets, culminating in Caspase 3 cleavage and apoptosis (Boldin et al., 1996; Muzio et al., 1996).

Engagement of Fas receptor with Fas ligand also results in the recruitment of a variety of proteins not directly involved in Caspase 8 recruitment and cleavage. These proteins are responsible for initiating other signal transduction pathways from Fas receptor. One protein recruited to the DISC following Fas receptor activation is DAXX, normally present in the nucleus of cells. DAXX binds to Fas receptor at a different site to FADD and is responsible for the recruitment and activation of the kinase ASK1. ASK1 in turn activates the MAPK cascade resulting in JNK activation (Chang et al., 1998; Tobiume et al., 2001). JNK can also be activated by a Caspase 8 dependent mechanism involving cleavage of Mst1 (Graves et al., 2001) or MEKK1 (Deak et al., 1998) and JNK activation has been reported to enhance Fas receptor mediated apoptosis in some cell lines (Brenner et al., 1997; Costa-Pereira et al., 2000; Le-Niculescu et al., 1999; Yang et al., 1997; Zhang et al., 2000).

Upregulation of Heat Shock Transcription Factor-1 (HSF1) was reported to occur in metastatic prostate cancer cell lines. This results in increased expression of HSP27 (Hoang et al., 2000) and is invariably associated with poor clinical outcome in patients with advanced prostate cancer (Cornford et al., 2000). HSP27 can protect cells from a variety of apoptotic insults including Fas mediated apoptosis and various chemotherapeutic drugs (Mehlen et al., 1996; Samali & Cotter, 1996) by sequestering cytochrome c after it is released from the mitochondria and preventing Caspase 9 activation (Garrido et al., 1999). It can also prevent cytochrome c release by inhibiting Bid translocation to the mitochondrion (Paul et al., 2002). HSP27 can bind to and

prevent the translocation of DAXX to the plasma membrane following Fas receptor activation, thus inhibiting JNK activation and the pro-apoptotic function associated with DAXX (Charette et al., 2000).

DU 145 prostate carcinoma cell lines are highly resistant to Fas mediated apoptosis. This resistance can be overcome by co-administering sub-lethal concentrations of various chemotherapeutic drugs (Costa-Pereira & Cotter, 1999; Rokhlin et al., 1997). Our group has previously demonstrated that JNK activation is sufficient to sensitise DU 145 cells to Fas receptor mediated apoptosis (Costa-Pereira et al., 2000; Curtin & Cotter, 2002). However, we found that engagement of Fas receptor with Fas activating antibodies could not activate JNK in DU 145 cells. In order to understand the mechanism by which DU 145 cells are resistant to Fas we explored the events inhibiting JNK activation. We found that DAXX did not translocate from the nucleus to the cytoplasm following stimulation of Fas. Although HSP27 was highly overexpressed it did not appear to play a role in this process by sequestering DAXX. Procaspase 8 was not cleaved following Fas receptor activation and further investigation demonstrated that defective DISC formation was the underlying cause by which Fas receptor activation failed to activate either JNK or Caspase 8.

Materials and Methods

Cell Lines and Reagents

DU 145 and Jurkat T cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cell culture reagents were purchased from Sigma (UK). The fluorescent probes used to detect apoptosis were FITC conjugated Annexin V (IQ Products, The Netherlands) and propidium iodide (PI) (Sigma, UK). SB203580 and ZVAD-fmk were purchased from Calbiochem (UK). The antibodies used in this study were Fas activating mouse anti-Fas IgM clone CH-11 and rabbit anti-FADD (Upstate Biotechnology, UK), mouse anti-Fas IgG (Bender Med Systems), phospho-JNK (Thr183/Tyr185) clone G9 and mouse anti-Caspase 8 (Cell Signalling Technology, UK), rabbit anti-JNK1 and rabbit anti-DAXX (Santa Cruz, USA), mouse anti-PARP (PharMingen, BD Biosciences, UK), mouse anti-HSP27 (Stressgen, UK) and mouse anti-Actin (Sigma, UK). The HRP labelled anti-rabbit IgG and anti-mouse IgG antibodies were obtained from DAKO (Denmark) while FITC conjugated anti-rabbit IgG was purchased from Sigma (UK). Protein G agarose slurry was obtained from Peirce (UK) and all other reagents were from Sigma (UK).

Cell Culture

DU 145 cells were cultured in RPMI 1640 medium supplemented with 5% FCS, 2mM L-Glutamine and 10 IU ml^{-1} penicillin-streptomycin. Jurkat cells were cultured in RPMI 1640 medium containing 10% FCS, 2mM L-Glutamine and 10 IU ml^{-1} penicillin-streptomycin. Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 and were routinely subcultured every 2-3 days. DU 145 cells were grown to 75% confluency

and Jurkat cells were resuspended in fresh media at 0.5×10^6 per ml on the day of each experiment. Cells were incubated with 200 ng ml^{-1} anti-Fas IgM for 1 h, 4 h or 24 h as indicated in the figure legends and pretreated for 1 h with 25 μM z-VAD-fmk or 5 μM SB203580 where used.

Fas Receptor Expression

0.5×10^6 cells per sample were harvested and washed twice in PBS. They were stained for 1 h at 4 °C with 20 μg ml^{-1} of mouse anti-Fas IgG. After another two washes with PBS, the cells were stained with the FITC conjugated sheep anti-mouse IgG for 1 h at 4 °C in the dark. Cells stained with secondary antibody alone were used to compensate for intrinsic fluorescence and non-specific binding of the secondary antibody. The samples were read on a FACScan flow cytometer and the data was analysed using Cell Quest software (Beckton Dickenson, UK).

Annexin V binding and PI uptake assay

DU 145 and Jurkat cells were incubated with 200 ng ml^{-1} anti-Fas IgM for 4 h and 24 h as indicated. The cells were subsequently harvested, washed once in PBS and resuspended in Annexin V binding buffer (150 mM NaCl, 18 mM CaCl$_2$, 10 mM HEPES, 5 mM KCl, 1 mM MgCl$_2$). 1 μg ml^{-1} FITC conjugated Annexin V, which binds specifically to external phosphatidyl serine on apoptotic cells, was added to each sample and incubated at room temperature for 5 minutes. 50 μg ml^{-1} PI was added immediately prior to reading the samples on the FACScan. Viable cells exclude PI and stain negative

on FL-2. Apoptotic cells are labelled with annexin V and stain positive on FL-1.
Analysis was carried out using Cell Quest Software.

SDS-PAGE and Western Blot

Protein extracts were prepared from cells using RIPA lysis buffer (50 mM Tris, pH 7.4,
150 mM NaCl, 1 mM each of NaF, NaVO$_4$ and EGTA, 1% NP40, 0.25% sodium
deoxycholate, 0.2 mM phenylmethylsulphonyl fluoride, 1 µg ml^{-1} each of antipain,
aprotinin and chymostatin, 0.1 µg ml^{-1} leupeptin, 4.0 µg ml^{-1} pepstatin) and 30 µg of
protein was loaded in each lane of an SDS polyacrylamide gel. Electrophoresis and
western blotting was subsequently carried out. Non-specific protein binding sites were
blocked and the membrane was stained with primary and peroxidase-conjugated
secondary antibodies according to the manufacturer's recommended protocol. Labelled
protein was detected using ECL (Amarsham, UK).

Immunofluorescent staining of DAXX

DU 145 cells were seeded on glass coverslips and grown to confluency over 48 hours.
They were incubated with 200 ng ml^{-1} anti-Fas IgM for 4 h or left untreated. The media
was aspirated and the coverslips were washed in PBS. The cells were fixed for 15
minutes at room temperature in 3% PFA in PBS. They were washed in PBS and
incubated for 15 minutes at room temperature in quenching buffer (50 mM NH$_4$CL in
PBS). Cells were permeabalised with 0.1% Triton X-100 in PBS for 5 minutes at room
temperature and washed in PBS before incubating with primary antibody (1:100 in PBS)
with 5% FCS for 1 h at room temperature. The primary antibody was aspirated off and

cells were washed in PBS before incubating for 1 h at room temperature in FITC-conjugated secondary antibody (1:80 in PBS) with DAPI and 5% FCS. Cells were then washed in PBS and mounted on glass slides.

Isolation of Nuclear and Cytoplasmic Enriched Fractions

A minimum of 5 x 10^6 cells were incubated with 200 ng ml^{-1} anti-Fas IgM for 4 h. The cells were harvested and resuspended in 250µl homogenising buffer (210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES, 1 mM EGTA, 0.5% BSA, 1 mM DTT, 0.2 mM PMSF, 5 µg ml^{-1} each of antipain, aprotinin and chymostatin, 0.5 µg ml^{-1} leupeptin, 20 µg ml^{-1} pepstatin). The sample was then transferred to a 2 ml tissue grinding tube (Kontes Glass Company, New Jersey) and homogenised with 100 strokes of the low clearance pestle. The homogenate was centrifuged at 3000 xg for 5 minutes. The supernatent (cytoplasmic fraction) was washed 3 times at 3000 xg. The pellet (nuclear fraction) washed 3 times in PBS, and the protein extract was prepared using RIPA lysis buffer. SDS-PAGE and western blotting was performed as described previously.

Immunoprecipitation

A minimum of 500 µg of protein was used per sample. DU 145 cells were treated and harvested as described in the figure legends. The cells were gently lysed on ice in lysis buffer (10 mM Tris pH 7.5, 50 mM NaCl, 10 mM Sodium Pyrophosphate, 50 mM NaF, 1 mM NaVO$_4$, 1% NP40, 0.2 mM PMSF, 5 µg ml^{-1} each of antipain, aprotinin and chymostatin, 0.5 µg ml^{-1} leupeptin, 20 µg ml^{-1} pepstatin) and centrifuged at 20000 xg for 15 min to remove insoluble material. Total cell protein was quantitated and diluted to 1

μg ml^{-1} in PBS. Protease inhibitors were added (1 μg ml^{-1} each of antipain, aprotinin and chymostatin, 0.1 μg ml^{-1} leupeptin, 4 μg ml^{-1} pepstatin) and samples were incubated with 10 μg ml^{-1} rabbit anti-DAXX or 10 μg ml^{-1} rabbit anti-FADD overnight at 4 °C. Protein G-agarose conjugated beads were prepared according to the manufacturers recommended instructions and incubated with the samples for a further 1 h at 4°C. DAXX was immunoprecipitated from total protein by centrifugation at 1000 xg for 3 min. The beads were washed 4 times in PBS and boiled in SDS-PAGE loading buffer for 5 min. The agarose beads were precipitated out of solution by centrifugation at 20000 xg for 2 min and the supernatent was loaded onto an SDS-polyacrylamide gel and analysed by western blot as described previously.

Results

DU 145 prostate carcinoma cells are resistant to Fas mediated apoptosis.

Flow cytometry was used to determine the expression of cell surface Fas receptor in DU 145 cells and Jurkat T cells. We found that the expression of Fas Receptor was comparable between the two cell lines (Figure 1a). However, the sensitivity of these two cell lines to Fas mediated apoptosis was found to be markedly different. Using Annexin V-FITC to detect cells at early stages in apoptosis, Jurkat cells were found to undergo extensive apoptosis in less than 4 h following incubation with 200 ng ml^{-1} anti-Fas IgM. By contrast, no increase in apoptosis was observed in DU 145 cells treated with 200 ng ml^{-1} anti-Fas IgM even for 24 h (Figure 1b). Morphological assessment of apoptosis was used to confirm this observation (data not shown). JNK activation has been reported to accompany Fas receptor activation. Some studies found that JNK was not required for Fas receptor mediated apoptosis (Abreu-Martin et al., 1999; Hofmann et al., 2001; Low et al., 1999) but others have shown that JNK activation accelerates Fas mediated apoptosis in a number of cell lines (Brenner et al., 1997; Le-Niculescu et al., 1999; Yang et al., 1997; Zhang et al., 2000). Our group has previously identified JNK activation as necessary for Fas mediated apoptosis in DU 145 cells (Costa-Pereira et al., 2000; Curtin & Cotter, 2002). As a result we determined the extent of JNK activation in DU 145 cells and Jurkat cells following incubation with 200 ng ml^{-1} anti-Fas IgM for 1 h and 4 h. We found that JNK was only extensively phosphorylated in Jurkat cells treated for 4 h with anti-Fas IgM (Figure 1c). No increase in JNK phosphorylation was observed in DU 145 cells even after 24 h (data not shown).

DAXX is expressed in the nucleus of DU 145 cells.

Fas receptor is believed to activate JNK by caspase dependent and independent mechanisms. Activation of Fas receptor can recruit DAXX, a nuclear protein, to the plasma membrane during DISC formation. DAXX subsequently binds to and activates ASK1, an upstream kinase in the JNK signalling pathway (Chang et al., 1998; Tobiume et al., 2001). Immunofluorescence was used to determine DAXX subcellular localisation in DU 145 cells. We found that DAXX was predominately located in the nucleus of DU 145 cells and the staining pattern appeared to be punctated. This is in agreement with other reports that localized DAXX to ND-10 domains in the nucleus (Charette et al., 2000; Torii et al., 1999). No change in DAXX subcellular localization was observed following incubation for 4 h with 200 ng ml^{-1} anti-Fas IgM (Figure 2a). Expression of DAXX was also determined in nuclear and cytoplasmic enriched fractions by western blot. No increase in cytoplasmic DAXX was identified in cells incubated with 200 ng ml^{-1} anti-Fas IgM for 4 h confirming the immunofluorescence data (Figure 2b). We also assessed the extent of DAXX translocation after 8 h and 24 h incubation with anti-Fas IgM and did not observe any increase in the cytoplasmic fraction of DAXX (data not shown).

HSP27 is overexpressed in DU 145 cells but does not interact with DAXX.

HSP27 has previously been found to bind and inhibit DAXX translocation and apoptosis in response to Fas receptor activation (Charette et al., 2000). In addition, overexpression of HSP27 correlates with prostate cancer progression (Cornford et al., 2000). Therefore we analysed whether HSP27 inhibited JNK activation following Fas receptor activation

in DU 145 cells. HSP27 expression was determined in nuclear and cytoplasmic enriched fractions from DU 145 cells and Jurkat cells. We found that HSP27 was highly overexpressed in DU 145 cells and was predominantly located in cytoplasmic enriched fractions. No change in subcellular expression was observed following treatment with 200 ng ml^{-1} anti-Fas IgM for 4 h (Figure 3a). Although HSP27 is expressed mainly in the cytoplasm and DAXX is present primarily in the nucleus it is possible that the cytoplasmic DAXX is important for binding Fas receptor or that HSP27 prevents recruitment of nuclear DAXX to the Fas DISC. As a result we immunoprecipitated DAXX from cell lysates of DU 145 cells incubated for 4 h with 200 ng ml^{-1} anti-Fas IgM or left untreated. We could not detect any HSP27 expressed in the immunoprecipitate suggesting that either HSP27 and DAXX do not interact in DU 145 cells or the percentage HSP27 that interacts with DAXX is extremely small in comparison with total HSP27 expression in these cells (Figure 3b). It was reported that endogenous P38 activity maintained HSP27 in active dimers. Inhibition of P38 resulted in multimeric complexes of HSP27 and this abrogated the interaction between HSP27 and DAXX. In addition, SB203580 sensitised cells to Fas receptor mediated apoptosis by allowing DAXX translocation from nucleus to cytoplasm (Charette et al., 2000). We did not observe any increase in cytoplasmic DAXX (data not shown) or increase in apoptosis in cells pre-incubated with SB203580 (Figure 3c). This supports our conclusion that HSP27 does not prevent DAXX translocation and JNK activation in DU 145 cells.

Fas mediated caspase activation is required for JNK activation in Jurkat cells but is defective in DU 145 cells.

In order to study caspase dependent JNK activation, we incubated DU 145 cells and Jurkat cells with 200 ng ml^{-1} anti-Fas IgM in the presence and absence of the general caspase inhibitor z-VAD-fmk for 4 h. Cleavage of Procaspase 8 was subsequently assessed by western blot analysis. We found that Procaspase 8 was extensively cleaved into the intermediate P41/P43 products and active P18 subunit only in Jurkat cells following incubation with 200 ng ml^{-1} anti-Fas IgM. Z-VAD-fmk was found to completely abrogate the cleavage of Caspase 8. Procaspase 8 was not cleaved following treatment of DU 145 cells with 200 ng ml^{-1} anti-Fas IgM. This indicates that inhibition of Fas mediated apoptosis is an early event in these cells, possibly during DISC formation (Figure 4a). The effect of caspase inhibition on JNK activation was also assessed in Jurkat cells. Z-VAD-fmk was found to completely abrogate JNK activation in Jurkat cells following incubation with 200 ng ml^{-1} anti-Fas IgM. This suggests that in this cell line the principle mechanism of JNK activation is caspase dependent (Figure 4b).

DISC formation following Fas receptor activation is defective in DU 145 cells.

Although cell surface Fas receptor expression is similar in DU 145 cells and Jurkat cells we found that neither JNK or Caspase 8 are activated in DU 145 cells. This may be due to defective DISC formation following Fas receptor activation in DU 145 cells. We immunoprecipitated FADD from cells before and after incubation with 200 ng ml^{-1} anti-Fas IgM for 1 h. Expression of Caspase 8 was assessed in these immunoprecipitates to determine the extent of FADD-Caspase 8 aggregation in the DISC. While FADD was found to associate with Caspase 8 in Jurkat cells incubated with anti-Fas IgM, no interaction was evident in DU 145 cells (Figure 5). These data suggest that the

interaction between Caspase 8 and FADD is defective and prevents DISC formation following Fas receptor activation in DU 145 cells. This appears to be the principle mechanism by which DU 145 cells are resistant to anti-Fas IgM and also why JNK is not activated in these cells.

Discussion

Activation of the Fas receptor in cells by Fas ligand results in Caspase activation and morphological apoptosis in a variety of cell lines. Fas receptor plays a critical role in the homeostasis of the immune system and may be involved in immune surveillance and clearance of defective cells (O'Connell et al., 2001; Pinkoski & Green, 2000). Although the events initiated by Fas receptor culminating in Caspase activation and apoptosis are well understood, the mechanisms by which tumour cells alter signalling pathways and become resistant to Fas mediated apoptosis are not. DU 145 cells are androgen independent prostate carcinoma cells and are resistant to a variety of chemotherapeutic drugs *in vitro*. We found that while cell surface expression of Fas receptor was comparable to Jurkat cells, DU 145 cells were highly resistant to Fas mediated apoptosis. Activation of JNK using chemotherapeutic drugs or anisomycin was sufficient to sensitise these cells to Fas (Costa-Pereira & Cotter, 1999; Costa-Pereira et al., 2000; Curtin & Cotter, 2002).

JNK activation has been reported to accompany Fas receptor activation and appears to be involved in regulating Fas mediated apoptosis in various cell lines. While JNK is not pro-apoptotic in every cell line, it appears that certain cell lines resistant to Fas mediated apoptosis require JNK activation to promote apoptosis. We found that treatment of DU 145 cells with anti-Fas IgM alone did not stimulate JNK activation. In order to better understand the resistance of DU 145 cells to Fas mediated apoptosis, we investigated JNK activation following Fas receptor stimulation in DU 145 cells. JNK activation following Fas receptor activation may be Caspase 8 dependent or Caspase 8 independent.

60

During Caspase 8 independent JNK activation DAXX is recruited to the plasma membrane and binds to the intracellular C-terminus of Fas receptor independent of FADD. ASK1, a JNK kinase kinase is recruited to the plasma membrane and binds to DAXX. Activation of ASK1 *in trans* results in MKK4/JNKK1 phosphorylation and ultimately JNK phosphorylation at Thr183/Tyr185 and activation (Tobiume et al., 2001). DU 145 cells were found to express DAXX predominantly in ND-10 domains in the nucleus and this is consistent with previous reports (Charette et al., 2000; Torii et al., 1999). We found that a small fraction of DAXX was present in the cytoplasmic fraction, although the levels of cytoplasmic DAXX were not found to increase following Fas receptor activation. In addition, no clustering of DAXX at the plasma membrane was evident in cells treated with anti-Fas IgM.

HSP27 overexpression has been associated with prostate cancer progression and can independently predict the clinical outcome of prostate cancer, suggesting it plays an important role in the resistance of prostate cancer to chemotherapy (Cornford et al., 2000; Thomas et al., 1996). HSP27 inhibits apoptosis by a variety of mechanisms including sequestering cytosolic pro-apoptotic Cytochrome c, inhibiting pro-apoptotic tBID translocation from cytosol to the mitochondrion and preventing DAXX association with Fas receptor and subsequent JNK activation (Concannon et al., 2003). We found that DU 145 cells overexpress HSP27 and is predominantly found in the cytoplasmic fraction. A small fraction present in the nucleus was also evident. However, we could not identify any physical interaction between HSP27 and DAXX in DU 145 cells either before or after Fas receptor activation. This suggests that is not involved in regulating DISC

formation and JNK activation in DU 145 cells. It is likely that HSP27 regulates sensitivity of mitochondria to apoptosis signals and can prevent cytochrome c release in response to cytotoxic drugs because HSP27 overexpression correlates with poor clinical outcome (Cornford et al., 2000). However, another mechanism inhibits Fas receptor mediated apoptosis and JNK activation in DU 145 cells.

Caspase 8 activation results in cleavage and constitutive activation of MEKK1 and Mst1, kinases that can phosphorylate and activate JNK. We found that Caspase 8 is expressed at similar levels in DU 145 cells and Jurkat cells but Fas receptor engagement with Fas activating antibodies was only found to cleave Procaspase 8 into active fragments in Jurkat cells. This cleavage could be completely abrogated using ZVAD-fmk, an irreversible Caspase inhibitor. JNK phosphorylation was also completely inhibited in cells lacking Caspase 8 active fragments suggesting that Caspase 8 mediated JNK activation was the predominant pathway in Jurkat cells, at least after 4 h. It is possible that Caspase 8 independent JNK activation can also occur here, but progresses more slowly.

In light of our data, defective DISC formation following Fas receptor activation appeared to be the mechanism by which DU 145 cells were resistant to Fas mediated apoptosis. We immunoprecipitated FADD, the adaptor protein required for Caspase 8 recruitment to Fas receptor, to determine the extent of interactions between FADD and Caspase 8 in DU 145 cells and Jurkat cells before and after Fas stimulation. Although DU 145 cells appear to express higher levels of FADD than Jurkat cells, no interaction between FADD and

caspase 8 was evident before or after Fas receptor stimulation. By contrast, Caspase 8 was found to immunoprecipitate with FADD in both untreated and anti-Fas IgM treated Jurkat cells. Increased Caspase 8 in anti-Fas IgM treated Jurkat cell immunoprecipitates was consistently observed and this is probably due to stable interactions between FADD and Caspase 8 in Fas receptor aggregates.

Numerous Fas receptor and FADD interacting proteins have been identified and a number of these have been shown to regulate DISC formation following Fas receptor engagement with Fas ligand and Fas activating antibodies. These include FAP-1, FAF-1, FLASH, HIPK3 and PKCξ (Peter & Krammer, 2003). It is likely that one or more of these proteins are differentially regulated in prostate cancer and as a result increase the treshold required for Fas receptor activation and apoptosis following engagement of Fas receptor with Fas ligand. Further studies are ongoing with the aim of identifying these components. It is hoped that by identifying the dysfunctional elements in Fas receptor mediated apoptosis in DU 145 cells that novel therapeutic targets may be identified for prostate cancer.

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C
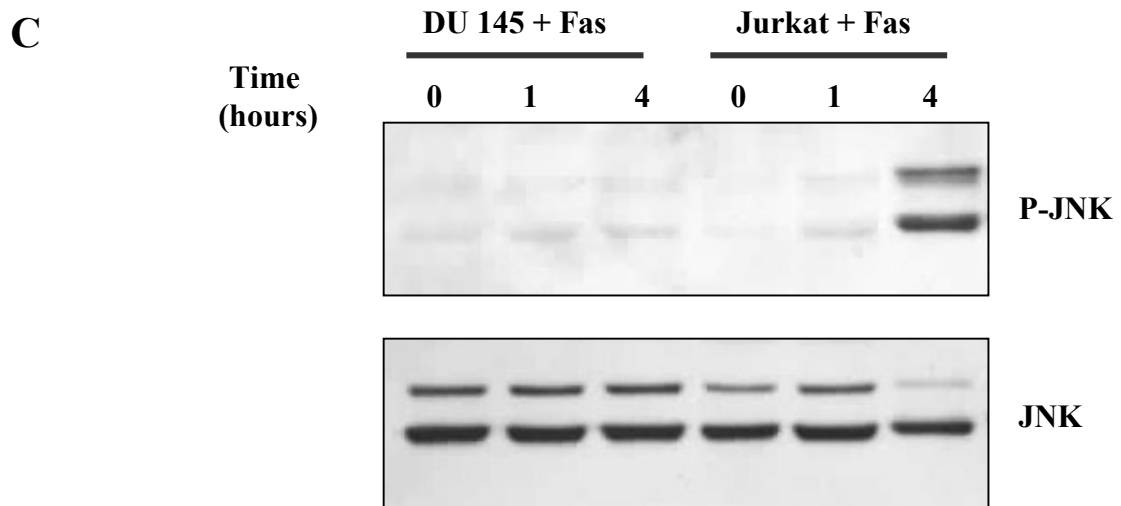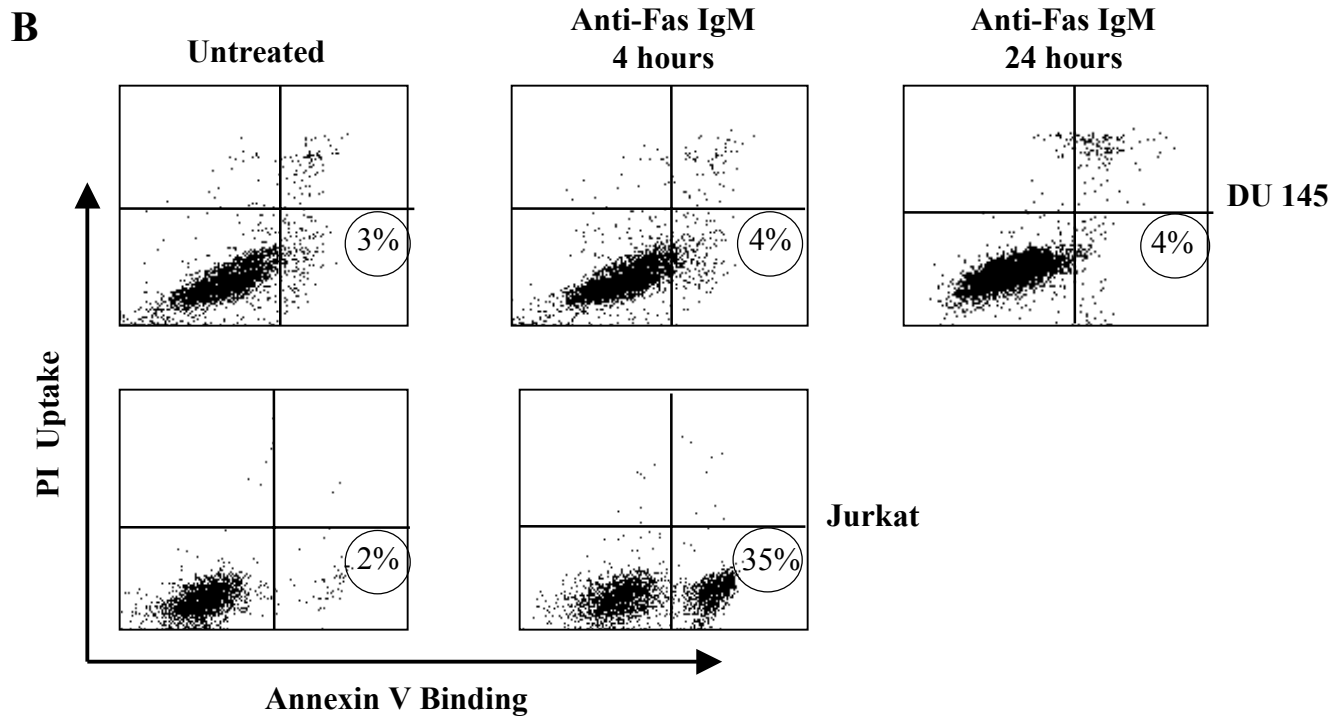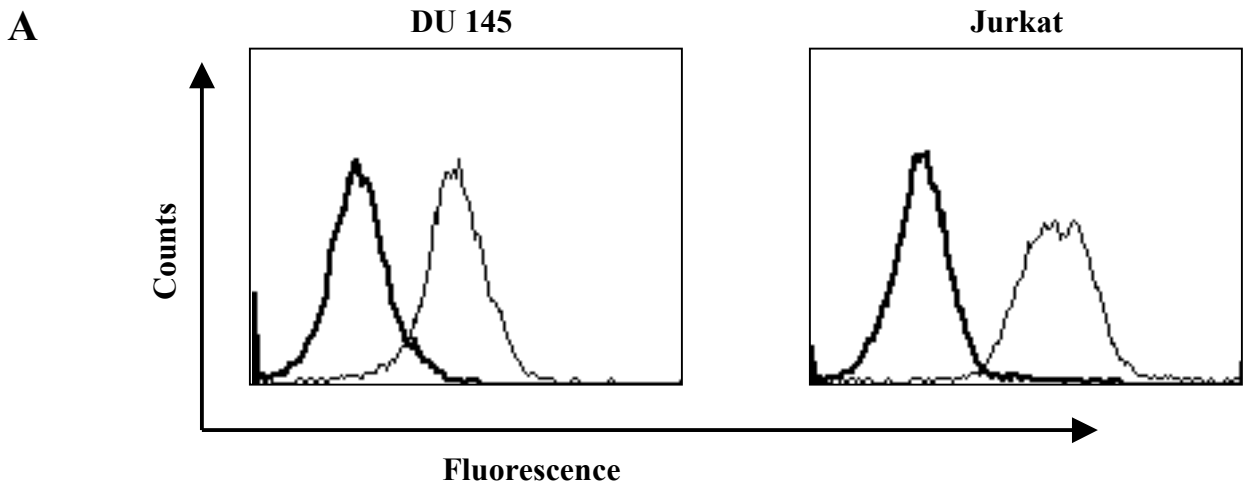
Figure 1: DU 145 cells express Fas receptor at the plasma membrane but are highly resistant to Fas induced apoptosis. (**A**) Cell surface expression of Fas receptor (—) was assessed in DU 145 cells and Jurkat cells as described in the materials and methods section. Intrinsic fluorescence of cells labelled with secondary antibody alone (━) is also shown. Data are representative of three independent experiments. (**B**) Jurkat cells were incubated for 4 h and DU 145 cells were incubated for 4 h and 24 h with 200 ng ml^{-1} anti-Fas IgM. Apoptosis was subsequently determined by staining with Annexin V-FITC and Propidium Iodide. The percentage of apoptotic cells is shown in the bottom right quadrant of each plot. Data are representative of 3 independent experiments. (**C**) Phosphorylation of JNK at residues Thr 183 and Tyr 185 was assessed in DU 145 cells and Jurkat cells following treatment with 200 ng ml^{-1} anti-Fas IgM for 1 h and 4 h where indicated. Total JNK expression was also determined to demonstrate equal protein loading.

A

Untreated	α-Fas IgM 4 h

α-Daxx
FITC

DAPI
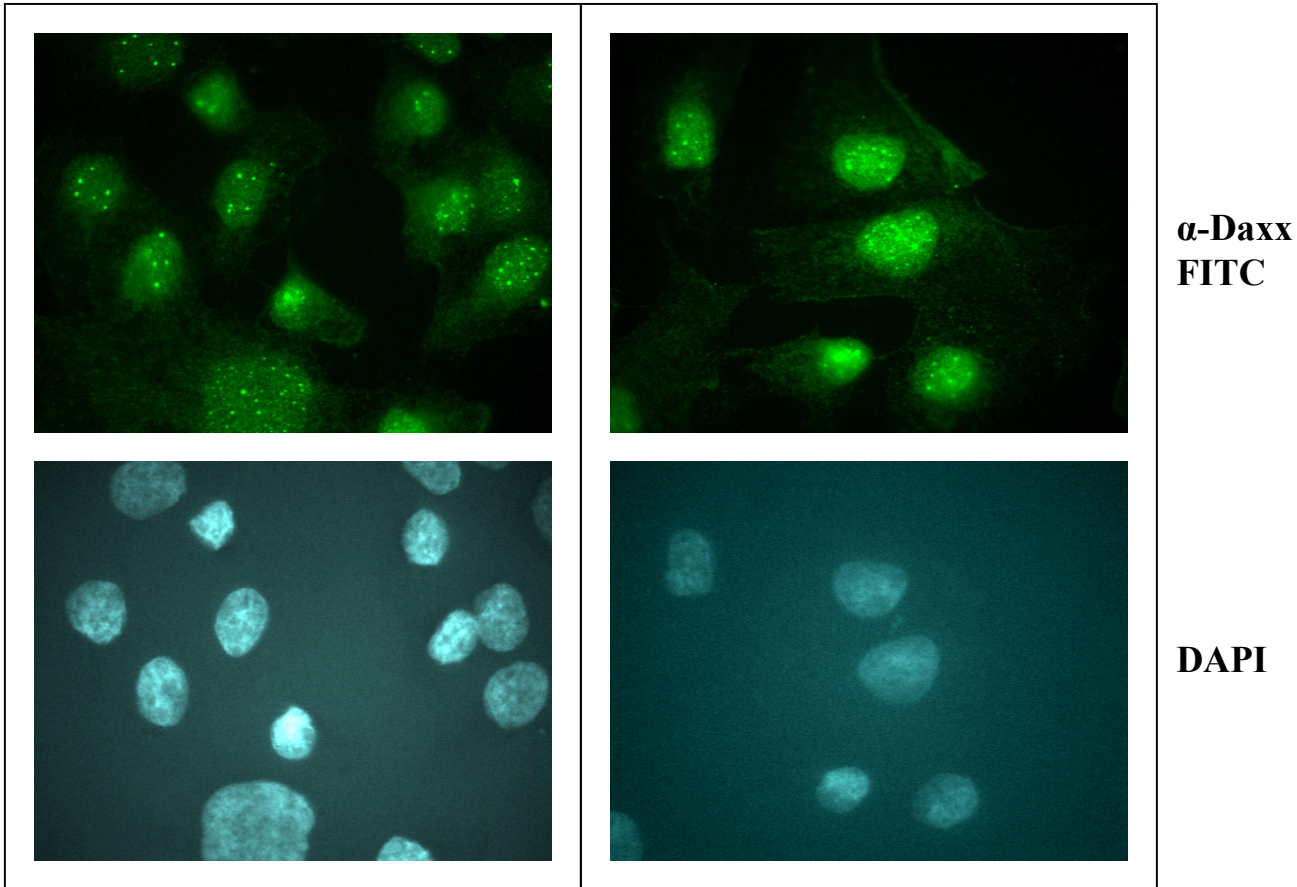
B

| Anti-Fas IgM | - | - | + | + |
| Fraction | N | C | N | C |

DAXX

PARP
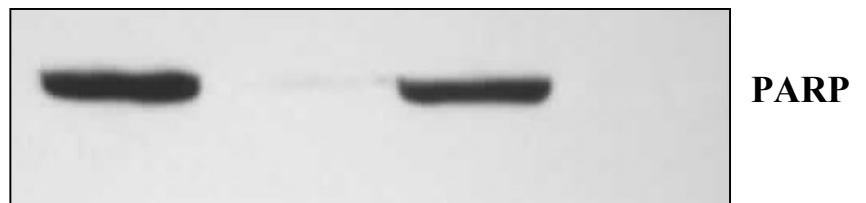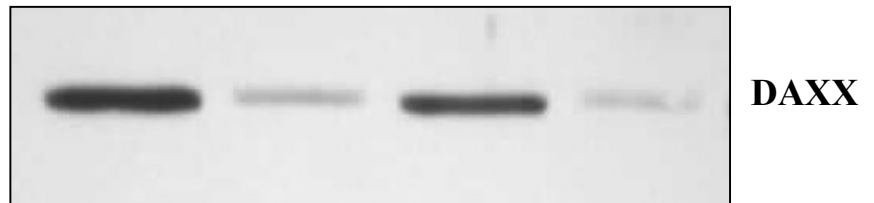
Figure 2: DAXX expression and subcellular localisation in DU 145 cells. (A) Immunofluorescent analysis of DAXX was performed on DU 145 cells incubated for 4 h with 200 ng ml^{-1} anti-Fas IgM. Samples were counterstained with DAPI to visualise the nuclei of cells. (B) Western blot analysis of DAXX expression in nuclear and cytoplasmic enriched fractions obtained from DU 145 cells incubated for 4 h with 200 ng ml^{-1} anti-Fas IgM or without. PARP was also stained to assess the purity of the fractions.

A

DU 145   Jurkat

| Anti-Fas IgM | − | − | + | + | − | − | + | + |
| Fraction | N | C | N | C | N | C | N | C |

HSP27

PARP

B

5%WCE   IP DAXX

| Anti-Fas IgM | − | − | + |

WB HSP27

WB DAXX
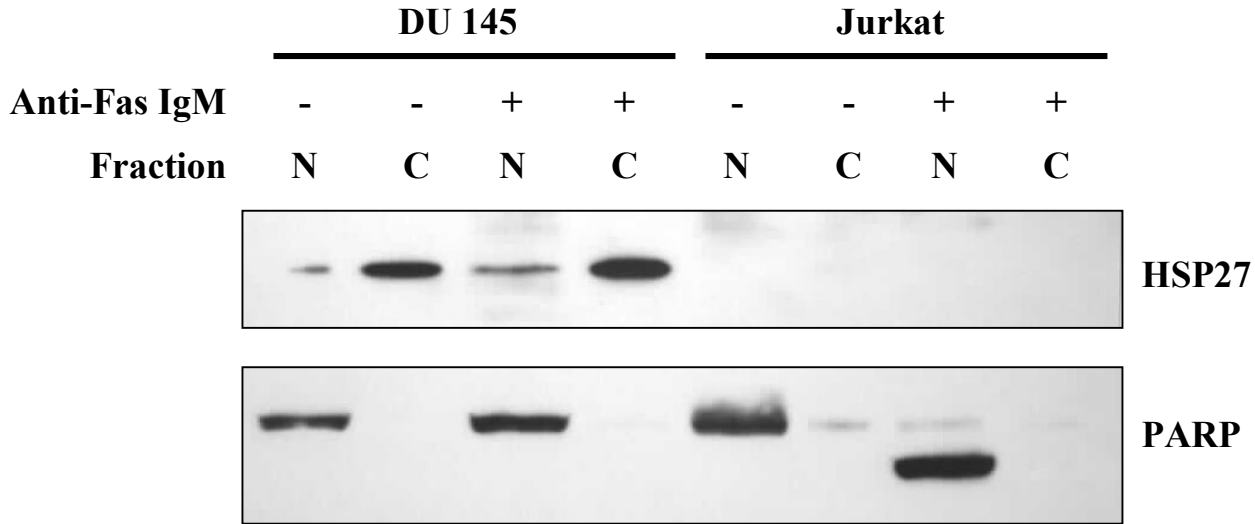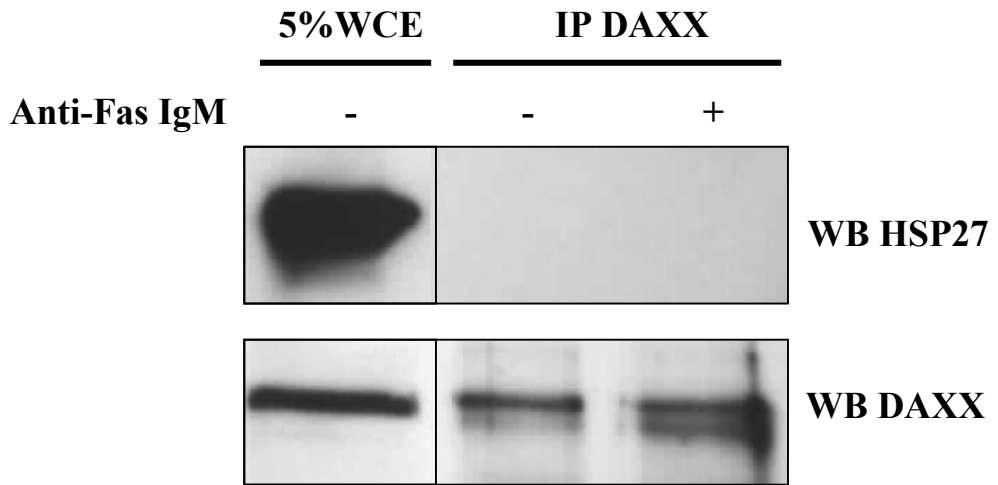
C
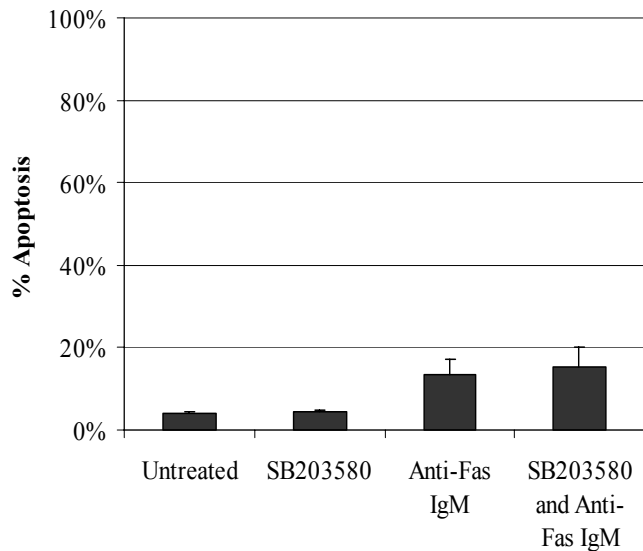
Figure 3: HSP27 is overexpressed in DU 145 cells but is localised predominantly in the cytoplasm and does not co-immunoprecipitate with DAXX. (A) Expression of HSP27 was analysed in nuclear-enriched and cytoplasmic-enriched fractions isolated from DU 145 cells and Jurkat cells before and after incubation with 200 ng ml^{-1} anti-Fas IgM for 4 h. PARP was also probed to determine the purity of the fractions. (B) DU 145 cells were treated for 4 h with 200 ng ml^{-1} anti-Fas IgM or left untreated. Cells were lysed gently and DAXX was immunoprecipitated as described in the materials and methods. Immunoprecipitated complexes and 5% whole cell extract from untreated DU 145 cells were subsequently analysed for HSP27 expression by western blot. DAXX was probed to determine equal protein loading. (C) DU 145 cells were stained with Annexin V-FITC and PI to determine the extent of apoptosis following incubation for 24 h with 5 μM SB203580 and 200 ng ml^{-1} anti-Fas IgM. Error bars represent the standard deviation from the mean for 3 independent experiments.

A
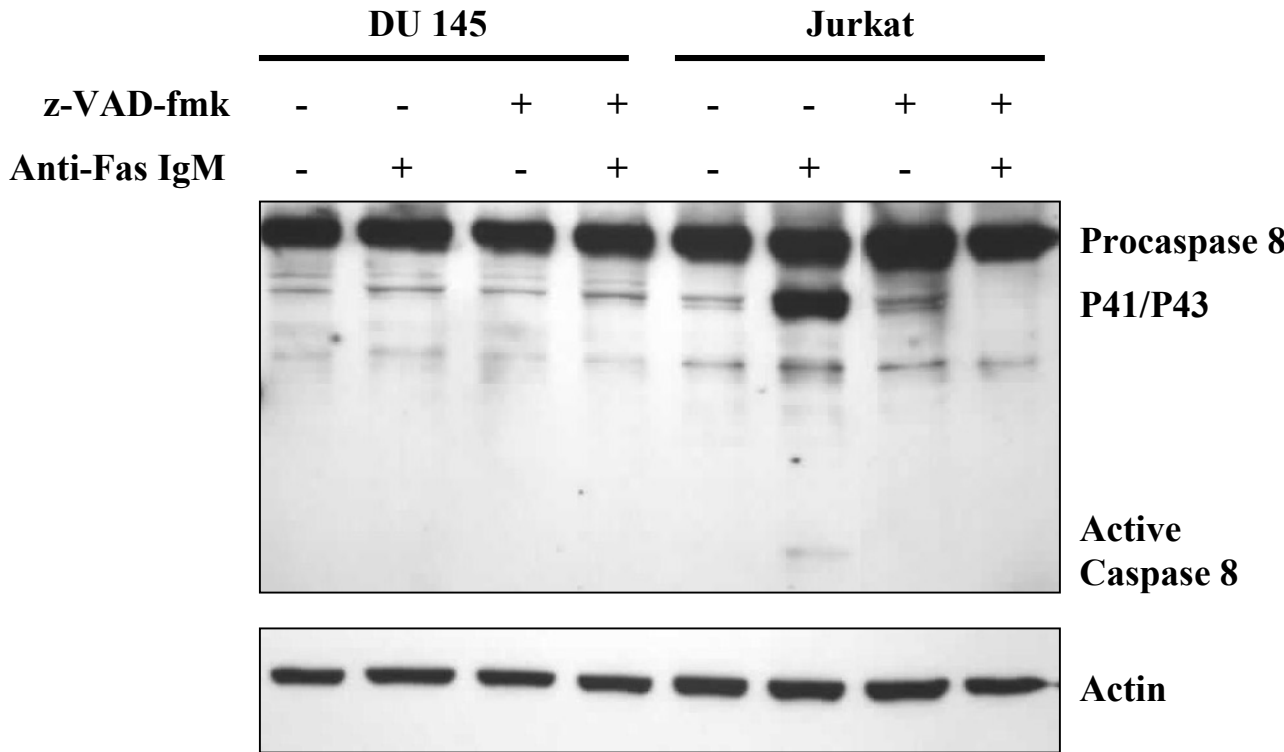
B
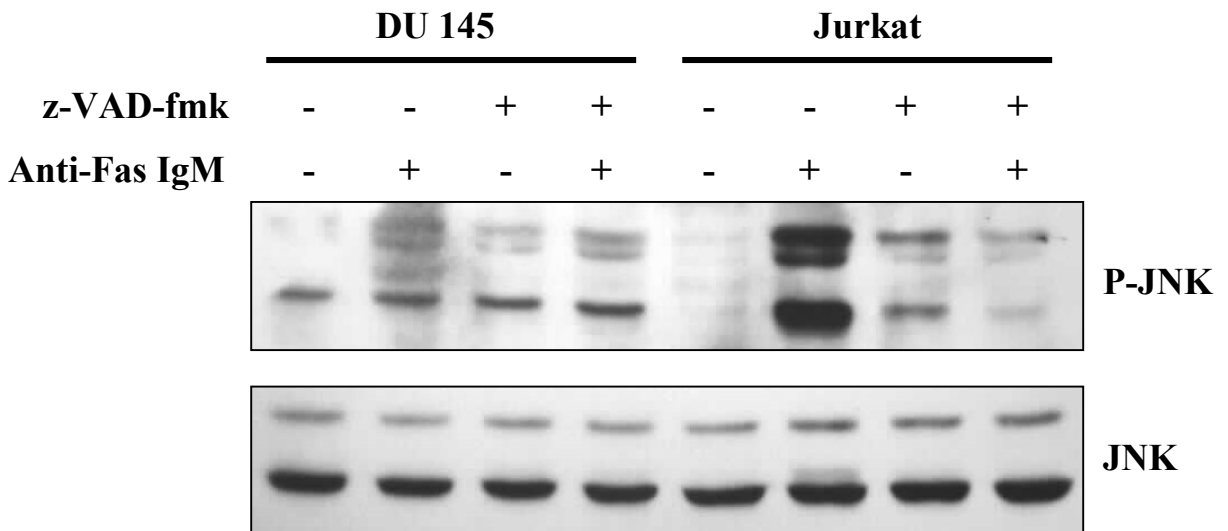
Figure 4: Inhibition of Caspase activity with z-VAD-fmk completely abrogates JNK activation in Jurkat cells. (A) DU 145 cells and Jurkat cells were incubated with 25 μM z-VAD-fmk and 200 ng ml^{-1} anti-Fas IgM for 4 h as outlined above. Cell lysates were subsequently probed for Caspase 8 expression and cleavage. Cleavage of Procaspase 8 into intermediary P41/P43 and active P18 Caspase 8 subunits was only evident in Jurkat cells incubated with 200 ng ml^{-1} anti-Fas IgM alone. Actin was also probed to assess equal protein loading. (B) DU 145 cells and Jurkat cells were incubated with 25 μM z-VAD-fmk and 200 ng ml^{-1} anti-Fas IgM for 4 h. Phosphorylation of JNK was assessed in cell lysates by western blot. Total JNK expression was also assessed to demonstrate equal protein loading.

	5% WCE				IP FADD				
	DU 145		Jurkat		DU 145		Jurkat		
anti-Fas IgM	−	+	−	+	−	+	−	+	

Caspase 8

P41/P43
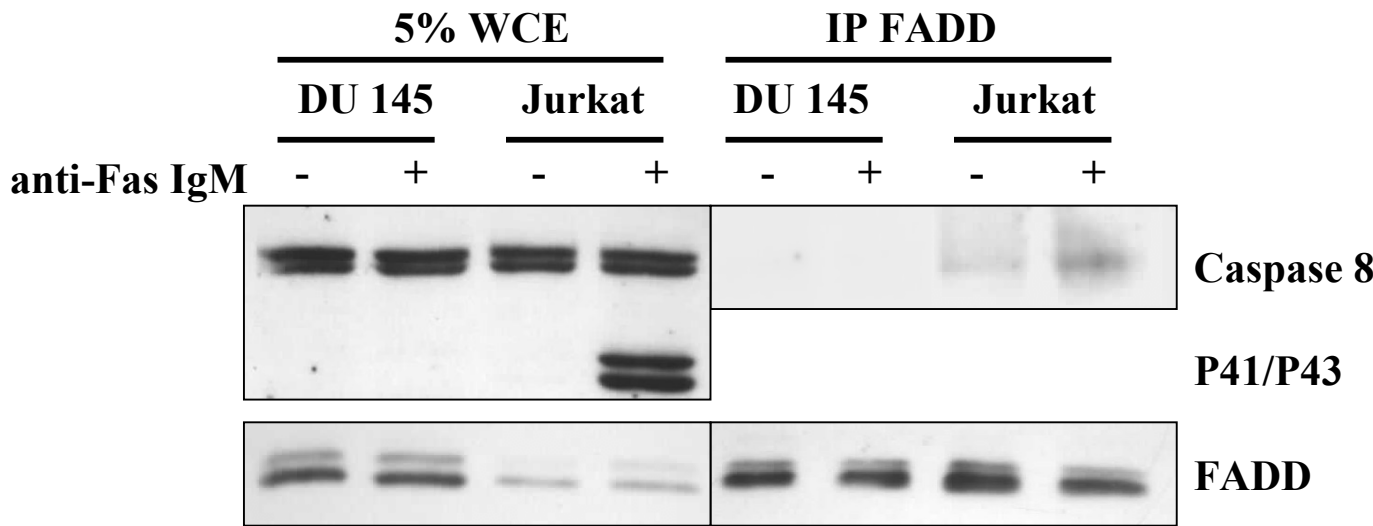
FADD

Figure 5: The interaction between FADD and Caspase 8 is defective in DU 145 cells. FADD was immunoprecipitated from DU 145 and Jurkat cells before and after incubation with 200 ng ml^{-1} anti-Fas IgM. The samples were probed for Caspase 8 and FADD expression by western blot. The endogenous expression of FADD and Caspase 8 in 5% whole cell extracts was also determined.

CHAPTER 4

This chapter has been submitted for publication at the time of writing

(2003)

HIPK3 expression is regulated by endogenous JNK activity and promotes resistance to Fas mediated apoptosis in DU 145 prostate carcinoma cells

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Abstract

Elevated endogenous JNK activity has recently been implicated in prostate cancer tumourigenesis and can promote resistance to apoptosis in response to chemotherapeutic drugs. In addition, JNK has been demonstrated to promote transformation of epithelial cells by increasing proliferation and survival. In this study we examine the effects of endogenous JNK activity on Fas receptor mediated apoptosis in DU 145 prostate carcinoma cells. Inhibiting JNK activity with SP600125 abrogates FADD phosphorylation and increases the sensitivity of DU 145 cells to Fas receptor mediated apoptosis. This event was found to occur at an early stage in the Fas receptor signalling pathway, upstream of Caspase 8 cleavage. Subsequent analysis identified an increase in the interaction between FADD and Caspase 8 in response to incubation with SP600125. We find that the expression of HIPK3 is elevated in DU 145 cells and we found that FADD phosphorylation was dependent on HIPK3 expression using RNA interference. In conclusion elevated JNK activity was found to promote the expression of HIPK3 and intefere with effective DISC formation by reducing the interaction between FADD and Caspase 8 in DU 145 cells.

Introduction

Apoptosis was first defined in 1972 as a morphologically distinct form of cell death and is characterised by changes in cell morphology including cell shrinkage, plasma membrane blebbing, nuclear condensation, nuclear fragmentation and apoptotic body formation (Kerr et al., 1972). Within the last decade the central biochemical pathways in apoptosis have been elucidated and perhaps the most extensively studied pathway is Fas mediated apoptosis. The Fas receptor is a member of the Tumour Necrosis Factor superfamily of receptors and is expressed in many tissues (Nagata, 1997). Ligation of Fas receptor with Fas ligand or Fas activating antibodies results in Fas receptor clustering at the plasma membrane, recruitment and activation of Caspase 8 via the adapter protein FADD and subsequent cleavage of a number of downstream targets, culminating in apoptosis (Chinnaiyan et al., 1995; Muzio et al., 1996). Fas mediated apoptosis is believed to be a mechanism by which the immune system destroys defective cells or cells expressing abnormal surface proteins (O'Connell et al., 2001). In addition, various anti-cancer drugs are dependent on Fas receptor activation in order to induce apoptosis (de Souza et al., 1997; Micheau et al., 1999). As a result there is a selective pressure on tumour cells to inhibit the Fas pathway and resistance to Fas is a common event during cancer progression.

One of the most prevalent cancers in the world today is prostate cancer (Parkin et al., 2001) and progression from localised tumours to metastatic, hormone-refractory prostate cancer correlates with an increase in resistance to Fas receptor mediated apoptosis (Hedlund et al., 1998). Although hormone sensitive tumours respond well initially to

androgen ablation and chemotherapy, hormone-refractory tumours invariably develop within a median of 12 to 18 months (Petrylak, 1999). Hormone-refractory prostate cancer usually displays increased malignancy, proliferation and metastatic potential over androgen-sensitive tumours and can survive in the absence of androgen. Transformation of prostate epithelial cells from a pre-neoplastic state into an intra-epithelial neoplasm requires a number of sequential genetic alterations that have not been fully characterised yet (Dong, 2001). Some of these aberrations in protein function increase the resistance of prostate cancer cells to apoptosis while others decrease the dependence of prostate cancer cells on growth factors for survival and proliferation. One protein that has been implicated in transformation and progression in numerous tumours including prostate cancer (Potapova et al., 2002), breast cancer (O'Hagan & Hassell, 1998) and lung cancer (Bost et al., 1997) is a stress activated protein kinase called JNK. Overexpression of JNK conferred a partially transformed phenotype on fibroblasts by regulating the response of these cells to survival and proliferative signals (Rennefahrt et al., 2002). It also greatly enhanced the transformation potential of Ras (Pruitt et al., 2002) and BCR-Abl (Raitano et al., 1995).

Endogenous JNK activity in response to growth factors and other signalling molecules generally bestows survival and proliferative advantages on cells. However, prolonged and excessive stimulation of JNK often accompanies stress signals in cells and is pro-apoptotic. Prolonged JNK activation has been reported to accompany the engagement of Fas receptor with Fas ligand during Fas receptor mediated apoptosis (Cahill et al., 1996; Yang et al., 1997) and excessive stimulation of JNK can accelerate Fas receptor mediated

apoptosis (Brenner et al., 1997; Costa-Pereira et al., 2000; Le-Niculescu et al., 1999; Yang et al., 1997; Zhang et al., 2000). In prostate cancer, our group has previously demonstrated that activation of JNK is defective following engagement of Fas receptor with anti-Fas IgM. In addition, we have shown that prolonged activation of JNK using camptothecin or anisomycin sensitises DU 145 prostate cancer cells to Fas mediated apoptosis (Costa-Pereira et al., 2000; Curtin & Cotter, 2002).

In this study we investigate the role of endogenous JNK activity in Fas receptor mediated apoptosis in prostate cancer using the specific JNK inhibitor SP600125. In contrast with our recent publications where we show that prolonged overactivation of JNK is pro-apoptotic in prostate cancer cells, we demonstrate here that endogenous JNK activity can promote survival in DU 145 prostate cancer cells. We show that inhibition of endogenous JNK activity decreases the expression of the FADD associated kinase HIPK3 (FIST/PKY/DYRK6). The interaction between FADD and Caspase 8 is defective in DU 145 cells but incubation with SP600125 restores the affinity of FADD for Caspase 8 and increases the sensitivity of DU 145 cells to anti-Fas IgM. Finally, we propose a mechanism through which endogenous JNK activity is anti-apoptotic and prolonged overactivation of JNK is pro-apoptotic in DU 145 cells.

Materials and Methods

Cell Lines and Reagents

DU 145, PC-3, HL-60 and Jurkat T cells were obtained from American Type Culture Collection (ATCC, Rockville MD, USA). PPC-1 and ALVA 31 cells were a gift of Gary and Heidi Miller (University of Colorado, USA). DU 145 cells were cultured in DMEM (Life Technologies, UK) supplemented with 5% foetal calf serum (FCS) and 4 mM L-Glutamine (all from Sigma, UK). PC-3, PPC-1 and ALVA 31 cells were grown in DMEM supplemented with 10% foetal calf serum (FCS) and 4 mM L-Glutamine. Jurkat T cells and HL-60 cells were cultured in RPMI 1640 supplemented with 10% FCS, 4 mM L-Glutamine and 10 IU ml^{-1} penicillin/streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C and routinely subcultured every 2 to 3 days. Unless otherwise stated, cells were grown to 75% confluency before treating with various drugs and inhibitors.

The probes used for the apoptosis assays were Annexin V-FITC (IQ Products, The Netherlands), Propidium Iodide (Sigma, UK), and JC-1 (Molecular Probes, The Netherlands). The primary antibodies used in this study were rabbit anti-cJun (Calbiochem, CN Biosciences, UK), mouse anti-Actin clone AC-15 (Sigma, UK), mouse anti-phosphoJNK (Thr183/Tyr185) clone G9, mouse anti-Caspase 8 clone IC12 and rabbit anti-Caspase 3 (Cell Signalling Technology), rabbit anti-JNK and rabbit anti-PKCζ (Santa Cruz, CA, USA), rabbit anti-BID (BioSource International, CA, USA), mouse anti-PARP (PharMingen, UK), mouse anti-FADD clone IF7 and rabbit anti-ERK2 (Upstate Biotechnology, UK) and rabbit anti-rat HIPK3 (a gift of Jorma Palvimo,

University of Helsinki, Finland). All Peroxidase-conjugated secondary antibodies were from DAKO (Denmark). Rabbit anti-FADD (Upstate Biotechnology, UK) was used for immunoprecipitations and Protein G-Agarose slurry was purchased from Peirce (UK). The Fas activating mouse anti-Fas IgM (clone CH11) was obtained from Upstate Biotechnology (UK). The JNK inhibitor SP600125, the PKCζ pseudosubstrate inhibitor and the caspase inhibitors z-IETD-fmk and z-LEHD-fmk were from Calbiochem (UK). Small interfering RNA oligonucleotides against HIPK3 and control oligonucleotides were purchased from Dharmacon (USA) and oligofectamine was purchased from Life Technologies (UK). Primers used to amplify target sequences in HIPK3 and GAPDH by RT-PCR were designed using GeneFisher software and were purchased from MWG (UK). Other reagents required for RT-PCR were bought from Promega (UK) and all other chemicals were purchased from Sigma (UK).

Apoptosis Assays

Annexin V-FITC and Propidium Iodide were used to detect apoptosis in DU 145 cells by flow cytometry. Phosphatidylserine is exposed early during apoptosis in cells and binds specifically to Annexin V-FITC. This causes an increase in FL-1 fluorescence in cells undergoing apoptosis when compared with normal viable cells. Counterstaining with propidium iodide is used to assess plasma membrane integrity. Loss of the plasma membrane integrity results in an increased FL-2 fluorescence and occurs later in apoptosis (Vermes et al., 1995). Unless otherwise indicated DU 145 cells were incubated with 200 ng ml^{-1} anti-Fas IgM for 24 h. Cells were preincubated for 1 h with 0.5% DMSO in the presence or absence of 50 µM SP600125. Caspase inhibitors were added

to cells 10 min prior to treating with anti-Fas IgM. After incubation with anti-Fas IgM the cells were then harvested with Trypsin and incubated with 1 μg ml^{-1} Annexin V-FITC for 5 min at room temperature in Annexin V binding buffer (150 mM NaCl, 18 mM CaCl$_2$, 10 mM HEPES, 5 mM KCl, 1 mM MgCl$_2$). Cells were incubated with 50 μg ml^{-1} propidium iodide for a further 5 min before reading the sample on a FACScan flow cytometer (Beckton Dickenson, BD Biosciences, Germany).   Cell Quest software (Beckton Dickenson) was used to analyse the data.

Mitochondrial membrane depolarisation can be measured in intact cells using the fluorescent cationic cell permeable probe JC-1 (Salvioli et al., 1997). DU 145 cells were incubated for 24 h with SP600125 and anti-Fas IgM as described above. The cells were harvested and resuspended in RPMI supplemented with 10% FCS and 2.5 μg ml^{-1} JC-1. The cells were incubated for 20 min at room temperature in the dark, washed once in PBS and analysed by flow cytometry. A decrease in FL-2 fluorescence is indicative of mitochondrial membrane depolarisation.

SDS-PAGE and Western Blot Analysis

Cells were treated as described in the figure legends. The cells were then harvested and lysed in RIPA buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1 mM each of NaF, NaVO$_4$ and EGTA; 1% NP40; 0.25% sodium deoxycholate; 0.2 mM AEBSF; 1 μg ml^{-1} each of antipain, aprotinin and chymostatin; 0.1 μg ml^{-1} leupeptin; 4 μg ml^{-1} pepstatin) for 20 min on ice. The lysates were centrifuged at 20,000 xg for 15 min to remove insoluble debris and protein concentrations were determined. At least 30 μg protein was loaded into each

lane of an SDS-polyacrylamide gel. Electrophoresis of the samples and transfer to a nitrocellulose membrane was carried out. Staining of the membrane with the various antibodies was performed using the manufacturers recommended protocol.

Cell Cycle analysis

DU 145 cells were treated with 50 µM SP600125, 8 mM hydroxyurea and 2 µg ml^{-1} nocodazole for 24 h. At least 200,000 DU 145 cells were washed in PBS-EDTA and fixed in ice-cold 70% ethanol overnight at –20 °C. Cells were resuspended in PBS-EDTA with 40 µg ml^{-1} Propidium iodide and 200 µg ml^{-1} DNase free RNase A (Sigma, UK) in dark for 30 min and DNA content of cells was analysed on FACScan flow cytometer.

RNA interference

Cells were transfected with HIPK3 siRNA (5'-AAU ACU UAC GAA GUC CUU CAU-3') or control siRNA (5'-AAA AAU UUC CAC CCC CCG GGC-3') using oligofectamine following the manufacturers protocol exactly. Expression of HIPK3 was determined every day after transfection and 4 to 5 days post transfection was found to be optimal for silencing HIPK3 in DU 145 cells. RNA and protein were extracted taken on day 4 and apoptosis assays were begun on day 4 and completed by day 5.

Reverse transcriptase - PCR

RNA was extracted from 100,000 DU 145 cells using Triazol reagent and 0.5 µg was converted to cDNA using MMLV reverse transcriptase. Primers were subsequently used

to amplify up target sequences on HIPK3 cDNA (forward 5'-ACA TTG GAA GAG CAT GAG GCA GAG A-3', reverse 5'-CTG CTG AAA AGC ATC ACC ACA ACC A-3') and GAPDH (forward 5'-ACC ACA GTC CAT GCC ATC AC-3', reverse 5'-TCC ACC ACC CTG TTG CTG TA-3') cDNA using PCR and DNA bands were visualised using agarose gel electrophoresis.

Immunoprecipitation of FADD

A minimum of 500 μg of protein was used per sample. DU 145 cells were treated and harvested as described in the figure legends. The cells were lysed gently using lysis buffer (10 mM Tris pH 7.5, 50 mM NaCl, 10 mM Sodium Pyrophosphate, 50 mM NaF, 1 mM NaVO$_4$, 1% NP40, 0.2 mM PMSF, 5 μg ml^{-1} each of antipain, aprotinin and chymostatin, 0.5 μg ml^{-1} leupeptin, 20 μg ml^{-1} pepstatin) and centrifuged at 20,000 xg for 15 min to remove insoluble material. Total cell protein was diluted to 1 μg ml^{-1} in PBS and incubated with 10 μg ml^{-1} rabbit anti-FADD overnight at 4°C. Protein G-agarose conjugated beads (Peirce) were prepared according to the manufacturers recommended instructions and incubated with the samples for a further 2 h at 4°C. FADD was immunoprecipitated by centrifugation at 1,000 xg for 3 min. The beads were washed 4 times in PBS and boiled in SDS-PAGE loading buffer for 5 min. The agarose beads were precipitated out of solution by centrigugation at 20,000 xg for 2 min and the sample was loaded onto an SDS-polyacrylamide gel and analysed by western blot.

Results

SP600125 inhibits endogenous JNK activity in DU 145 prostate carcinoma cells

JNK can activate the transcription factor c-Jun by phosphorylating two key residues, Ser63 and Ser73. Once phosphorylated, active c-Jun can alter the expression of a number of genes, including itself. We analysed the extent of normal c-Jun expression and phosphorylation in DU 145 cells incubated with and without 50 μM SP600125, a recently described anthrapyrazole inhibitor of JNK that has been demonstrated to specifically inhibit JNK activity both *in vitro* and *in vivo* (Bennett et al., 2001). In addition, the effects of SP600125 on the high levels of JNK activity that accompanies incubation with anisomycin were also analysed. We found that c-Jun expression was reduced when incubated with 50 μM SP600125 for 4 h. Furthermore, anisomycin mediated phosphorylation of c-Jun was partially inhibited in cells pre-treated with 50 μM SP600125 for 4 h. These data suggested that 50 μM SP600125 inhibits endogenous JNK activity in resting DU 145 cells and can also reduce the activity of JNK following treatment with anisomycin (Figure 1a). The upstream JNK kinase MKK4 can be inhibited when high concentrations of SP600125 are used (Bennett et al., 2001). We investigated if MKK4 was significantly inhibited in DU 145 cells using 50 μM SP600125 and found that phosphorylation of JNK at Thr183 and Tyr185 by anisomycin was not affected in cells co-incubated with 50 μM SP600125. This suggests that inhibition of JNK signalling pathway by SP600125 in DU 145 cells is due entirely to inhibition of JNK activity and not through inhibiting other upstream kinases in the JNK cascade (Figure 1b).

SP600125 sensitises DU 145 prostate carcinoma cells to Fas mediated apoptosis.

Therefore, we used 50 μM SP600125 to decrease the endogenous activity of JNK and observed a concomitant increase in the sensitivity of DU 145 cells to Fas mediated apoptosis. Treatment with the Caspase 8 specific inhibitor z-IETD-fmk completely abrogated apoptosis demonstrating that apoptosis in response to SP600125 and anti-Fas IgM was entirely dependent on Caspase 8 activity (Figure 1c). This result agrees with the current theory that Caspase 8 is the apical caspase in the Fas receptor pathway and is absolutely required for the subsequent caspase cascade and apoptosis following Fas receptor engagement with Fas ligand. In contrast, inhibition of Caspase 9 activity with the specific inhibitor z-LEHD-fmk did not completely abrogate apoptosis in response to treatment with SP600125 and anti-Fas IgM. Therefore, mitochondrial amplification of Caspase 9 activity may accelerate but is not absolutely required for apoptosis in response to SP600125 and anti-Fas IgM (Figure 1c). We also decreased the endogenous activity of ERK and P38 using U0126 and SB203580 but we did not detect any increase in the sensitivity of DU 145 cells to anti-Fas IgM (data not shown). Consequently, we hypothesized that a target specifically regulated by JNK may be responsible for the observed increase in sensitivity to Fas receptor engagement.

Endogenous JNK activity increases the resistance of DU 145 cells to Fas mediated apoptosis by inhibiting Caspase 8 activation by Fas receptor.

We analysed the major events that occur during Fas mediated apoptosis in order to gain some insight into the anti-apoptotic potential of endogenous JNK activity in DU 145 cells. Caspase 8 is only cleaved and activated when FADD and Caspase 8 are recruited

together to the death inducing signalling complex (DISC) following Fas receptor engagement with anti-Fas IgM. Detectable cleavage products of Caspase 8 were only evident in cells co-incubated with both SP600125 and anti-Fas IgM (Figure 2a). As a result the recruitment and cleavage of Caspase 8 in the death inducing signalling complex (DISC) is defective in resting DU 145 cells and inhibition of JNK activity can increase the cleavage of Caspase 8 in response to anti-Fas IgM. We also analysed downstream events during Fas mediated apoptosis and observed cleavage and activation of Bid only in DU 145 cells incubated with both SP600125 and anti-Fas IgM (Figure 2b). In addition, we only detected mitochondrial membrane depolarisation and Caspase 3 cleavage products in response to anti-Fas IgM when endogenous JNK activity had been decreased (Figure 2c and 2d). These results supported out hypothesis that inhibition of JNK using SP600125 facilitated DU 145 cells to undergo Fas mediated apoptosis primarily by enhancing either recruitment of or subsequent cleavage of Caspase 8 at the DISC.

FADD phosphorylation is regulated by endogenous JNK in DU 145 cells.

SP600125 appears to decrease the treshold required for Fas mediated apoptosis in DU 145 cells. In order to understand the mechanisms, we analysed the expression of the major components of Fas DISC. We did not observe any significant alterations in expression of Fas receptor, Fas ligand, FADD, Caspase 8 or FLIP in response to SP600125 treatment (data not shown). However, we did observe a decrease in FADD phosphorylation after incubating DU 145 cells with SP600125. No change in FADD phosphorylation was evident following treatment with anti-Fas IgM alone (Figure 3a).

FADD phosphorylation has previously been described and correlates with cell cycle progression. Cells arrested in G1 phase of the cell cycle display predominantly unphosphorylated FADD whereas cells arrested during mitosis display predominantly phosphorylated FADD (Scaffidi et al., 2000). We confirmed that phosphorylation of FADD is regulated by cell cycle progression in DU 145 cells using 8 mM hydroxyurea to arrest cells during G1 phase and 2 μg ml^{-1} nocodazole to arrest cells during mitosis (Figure 3b). Although JNK has previously been implicated in cell cycle progression during DNA synthesis (Potapova et al., 2000), incubation with SP600125 did not arrest DU 145 cells in the S phase of the cell cycle (Figure 3c and 3d). In addition, the rate of proliferation of cells incubated with SP600125 was not significantly altered compared with untreated DU 145 cells (data not shown). These data suggest that JNK does not indirectly regulate FADD phosphorylation in DU 145 cells by regulating cell cycle progression.

Protein kinase C zeta (PKCζ) is not responsible for FADD phosphorylation in DU 145 cells.

We hypothesised that JNK directly regulates the activity of a FADD kinase in DU 145 cells. A number of FADD interacting kinases have been identified (Kennedy & Budd, 1998) and one kinase that has been shown to associate with and phosphorylate FADD *in vivo* is PKCζ, an atypical member of the PKC family (de Thonel et al., 2001). We found that PKCζ is more highly expressed in the Fas resistant prostate cancer cell lines DU 145 and PC-3 when compared with the Fas sensitive PPC-1 and ALVA 31 prostate cancer cell lines (Figure 4a). Moreover we noted that FADD phosphorylation was more

extensive in DU 145 cells and PC-3 cells than in PPC-1 and ALVA 31 cells (data not shown). However, the extent of FADD phosphorylation was not found to change when a pseudosubstrate inhibitor of PKCζ was incubated in DU 145 cells (Figure 4b). Phosphorylation of ERK in response to PKCζ activity was significantly decreased using 15 μM and 20 μM PKCζ pseudosubstrate inhibitor (Figure 4c) and this confirmed that PKCζ was not the kinase responsible for FADD phosphorylation in DU 145 cells.

HIPK3 phosphorylates FADD and increases the resistance of DU 145 cells to Fas mediated apoptosis.

Another protein kinase known to interact with FADD is HIPK3, a 170kDa kinase that can regulate DISC formation *in vivo* (Rochat-Steiner et al., 2000). The expression of HIPK3 was found to be elevated in Fas resistant DU 145 and PC-3 cells in comparison with more sensitive PPC-1 and ALVA 31 prostate carcinoma cells (Figure 5a). Little is known about the function of HIPK3 in cells and we used RNA interference to reduce the expression of HIPK3 in DU 145 cells as outlined in the materials and methods section. We found that incubation with anti-HIPK3 RNA oligonucleotides for 4 days was sufficient to reduce the expression of HIPK3 mRNA (Figure 5b). Next we analysed the extent of FADD phosphorylation in DU 145 cells after incubation with HIPK3 RNA oligonucleotides and that FADD phosphorylation was significantly reduced after 4 days (Figure 5c). The sensitivity of DU 145 cells to Fas receptor mediated apoptosis was found to increase in cells with reduced levels of HIPK3 expression in comparison with control oligonucleotides (Figure 5d).

SP600125 decreases transcription of HIPK3 and regulates the interaction between FADD and Caspase 8 in DU 145 cells.

We found that incubation of DU 145 cells with 50 μM SP600125 decreased the expression of HIPK3 mRNA (Figure 6a) and also decreased the expression of HIPK3 protein (Figure 6b). These data strongly indicate that JNK can regulate the activity of HIPK3 by altering the rate of transcription at the HIPK3 gene locus. We immunoprecipitated FADD from DU 145 cell lysates and probed for Caspase 8 expression. Although Caspase 8 is believed to associate with FADD in unstimulated cells, we did not detect any interaction between FADD and Caspase 8 in resting DU 145 cells. No interaction between FADD and Caspase 8 was evident even after 24 h stimulation with 200 ng ml^{-1} anti-Fas IgM and co-incubation with SP600125 and anti-Fas IgM for 24 h was required for Caspase 8 association with FADD (Figure 6c). Thus it appears that JNK interferes with FADD and Caspase 8 binding in DU 145 cells by upregulating the expression of HIPK3. Reducing the expression of HIPK3 using RNAi or SP600125 increases the sensitivity of DU 145 cells to Fas mediated apoptosis by increasing the affinity of FADD for Caspase 8.

Discussion

The role of JNK in both survival and apoptosis has been well documented (Harper &
LoGrasso, 2001; Lin, 2003). However, much work is required to identify the exact
mechanisms employed by JNK during these very different responses. We have
previously highlighted the requirement of JNK activity for promoting apoptosis in
prostate cancer cells in response to treatment with anti-Fas IgM. We noted that the
targets of JNK appeared to be upstream of Caspase 8 activation in these cells and were
independent of alterations in Fas receptor, Fas ligand and FLIP expression (Curtin &
Cotter, 2002). In this study we focus on the relationship between endogenous JNK
activity and Fas receptor mediated apoptosis for 2 principle reasons. Firstly very little
work has been done on the role of endogenous JNK activity in Fas mediated apoptosis
and secondly there is a growing consensus that elevated JNK activity is important in
prostate cancer development and progression (Potapova et al., 2002).

We found that endogenous JNK signalling confers survival advantages against Fas
mediated apoptosis in DU 145 cells and inhibition of JNK augments Fas mediated
apoptosis by a mechanism upstream of Caspase 8 activation. Consequently, we analysed
the expression of various components of the Fas receptor DISC and discovered that the
inhibition of JNK activity reduces the levels of FADD phosphorylation. A number of
kinases have been reported that phosphorylate FADD *in vitro* and *in vivo*. Two of these
kinases that have been demonstrated to inhibit the effective formation of the DISC in
cells are PKCξ and HIPK3.

PKCξ interacts with and phosphorylates FADD in haematopoietic cells and it has been found that overexpression of PKCξ abrogates Fas receptor mediated apoptosis by interfering with effective DISC formation (de Thonel et al., 2001). Overexpression of PAR-4, the cellular inhibitor of PKCξ activity, has been shown to sensitise prostate cancer cell lines to Fas mediated apoptosis and this may implicate PKCξ in the resistance of DU 145 cells to treatment with anti-Fas IgM (Chakraborty et al., 2001). JNK1 activity has been reported to decrease the expression of PAR-4 in epithelial cells (Han et al., 2002) and this may lead to an increase in the activity of PKCξ. In addition, increases in PKCξ expression have been reported during prostate cancer progression (Cornford et al., 1999). Although we confirmed that the expression of PKCξ is elevated in DU 145 cells we did not observe any decrease in the phosphorylation of FADD when we incubated cells with the PKCξ pseudo-substrate inhibitor. In addition, no increase in the sensitivity of cells to anti-Fas IgM was detected and these data suggest that PKCξ may not be the principle kinase of FADD in DU 145 cells.

A second FADD interacting kinase called HIPK3 was first identified as a putative multi-drug resistant protein from studies of cancer cells (Begley et al., 1997; Sampson et al., 1993). Further studies demonstrated that HIPK3/FIST can interact with FADD and has been shown to phosphorylate FADD when overexpressed in cells (Rochat-Steiner et al., 2000). Although HIPK3 did not interfere with apoptosis in these cells, it did prevent JNK activation and this suggests that HIPK3 can interfere with DISC formation. We found that HIPK3 was expressed at higher levels in the Fas resistant prostate cancer cell lines DU 145 and PC-3. RNA interference reduced the expression of HIPK3 in DU 145

cells and this was accompanied with a decrease in the extent of FADD phosphorylation. In addition, an increase in the sensitivity of cells to Fas mediated apoptosis was observed in cells with reduced expression of HIPK3. We found that HIPK3 was significantly reduced in cells treated with SP600125. This suggests that increases in endogenous JNK activity during prostate cancer progression may increase the expression of HIPK3 and this in turn may increase the resistance of prostate cancer cells to Fas mediated apoptosis. Inhibition of JNK activity using SP600125 was also found to correlate with an increase in the interaction between FADD and Caspase 8. The association of FADD and Caspase 8 was found to be defective in normal DU 145 cells and interaction between these two proteins was only restored after co-incubation with SP600125 and anti-Fas IgM. Therefore, elevated JNK activity and HIPK3 expression can affect the interaction between FADD and Caspase 8. This may explain, at least in part, the association between multi-drug resistance in cancer cells and HIPK3 activity.

The duration of JNK activation in individual cells and not the intensity of activation is believed to be the deciding factor between survival and apoptosis signalling (Bagowski et al., 2003; Chen et al., 1996). Targets of JNK have been identified that either promote or inhibit Fas mediated apoptosis at two key stages (Figure 7). JNK can phosphorylate and alter the activity of a number of Bcl-2 family members and this in turn modulates the sensitivity of the mitochondrion to apoptotic signals. JNK may also modulate early events during Fas mediated apoptosis such as decreasing PAR-4 expression (Chakraborty et al., 2001; Han et al., 2002) and enhancing the clustering of Fas receptor in response to Fas ligand (Reinehr et al., 2003). Our results have illuminated another target of JNK in

Fas receptor mediated apoptosis. By regulating HIPK3 expression and FADD phosphorylation, JNK appears to regulate the interaction between FADD and Caspase 8 and increases the treshold of Fas receptor activation required to promote apoptosis in prostate cancer cells. However, HIPK3 is not the sole mechanism that increases resistance of DU 145 cells to Fas mediated apoptosis. Bcl-2 family members and HSP27 may regulate other components of the Fas apoptotic pathway. These mechanisms are semi-redundant and act independently to increase the treshold of Fas receptor activation required for apoptosis induction. As a consequence, it is likely that any therapy directed against Fas receptor for prostate cancer will target multiple inhibitory effects in order to maximise apoptosis and reduce the tumour burder in patients.
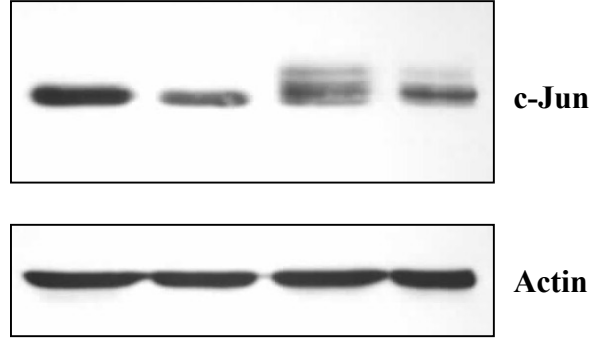
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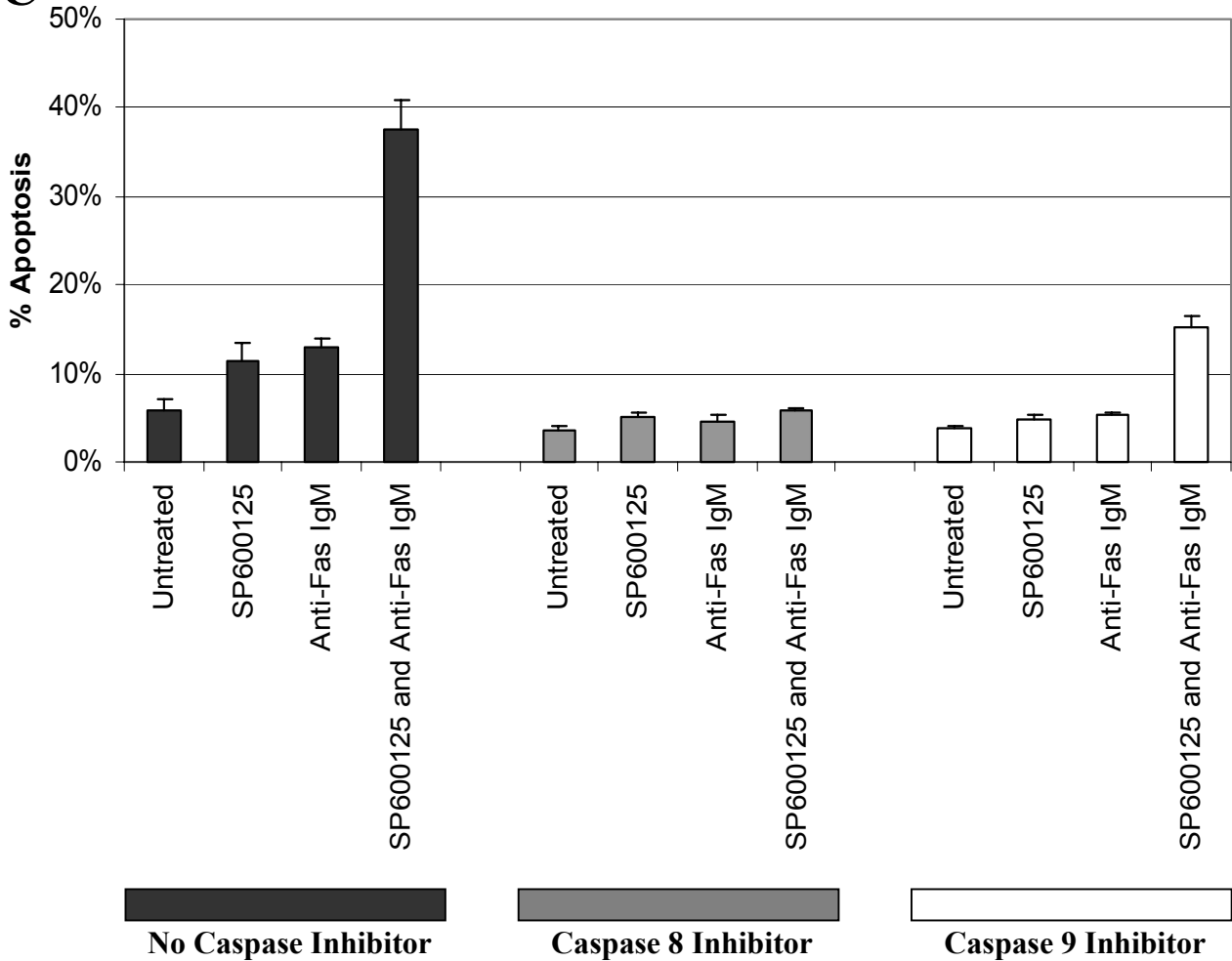
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A

Anisomycin   -   -   +   +
SP600125   -   +   -   +

c-Jun

Actin

B

Anisomycin   -   -   +   +
SP600125   -   +   -   +

P-JNK

JNK

C

Curtin JF and Cotter TG, 2003 Figure 1

Figure 1: Inhibition of endogenous JNK activity with SP600125 sensitises DU 145 prostate carcinoma cells to Fas mediated apoptosis. (A) JNK was activated with 250 ng ml^{-1} anisomycin in the presence or absence of 50 μM SP600125. Western blotting was used to determine the expression and phosphorylation of c-Jun in DU 145 cell lysates after 4 h treatment. β-Actin was also probed to demonstrate equal protein loading in the lanes. (C) JNK phosphorylation at Thr183 and Tyr185 was assessed by western blot in cells incubated with 250 ng ml^{-1} anisomycin in the presence and absence of 50 μM SP600125 for 4 h. Total JNK expression was also analysed to determine equal protein loading. (C) DU 145 cells were incubated for 1 h with 50 μM SP600125 as outlined in the materials and methods section before addition of 200 ng ml^{-1} α-Fas IgM agonistic antibody. Cells were stained 24 h after adding α-Fas IgM with Annexin V-FITC and Propidium iodide to visualise the extent of apoptosis. 25 μM Caspase 8 inhibitor z-IETD-fmk and Caspase 9 inhibitor z-LEHD-fmk were added 10 min before α-Fas IgM where indicated and error bars represent the standard deviation of 3 independent experiments.

A

	DU145				Jurkat	
Anti-Fas IgM	−	−	+	+	−	+
SP600125	−	+	−	+	−	−

Procaspase 8

Active Caspase 8

Actin

B

	DU145				Jurkat	
Anti-Fas IgM	−	−	+	+	−	+
SP600125	−	+	−	+	−	−

BID

tBID

Actin

C

4% — Untreated

8% — SP600125

6% — Anti-Fas IgM

30% — SP600125 and Anti-Fas IgM

Counts

FL-2

D

	DU145				HL-60	
UV	−	−	−	−	−	+
Anti-Fas IgM	−	−	+	+	−	+
SP600125	−	+	−	+	−	−

Procaspase 3

Active Caspase 3

Actin

Curtin JF and Cotter TG, 2003 Figure 2
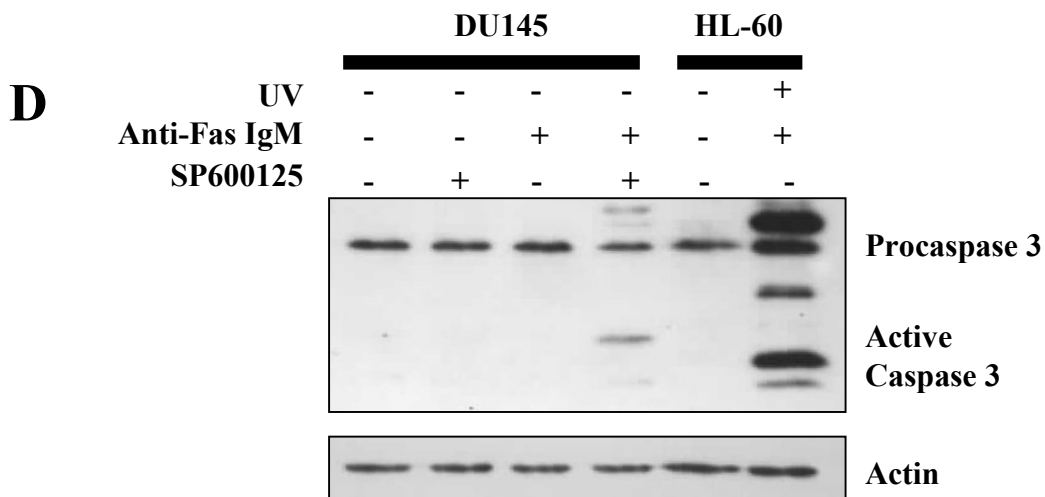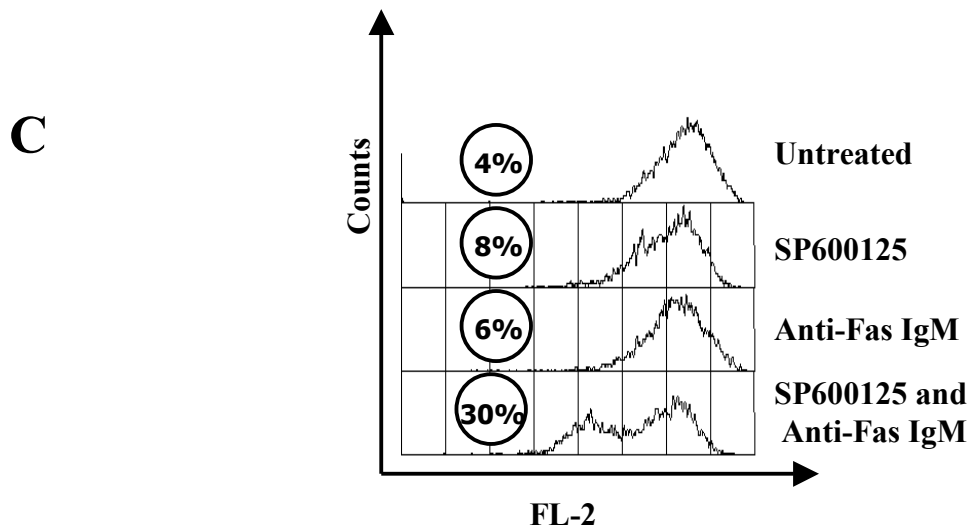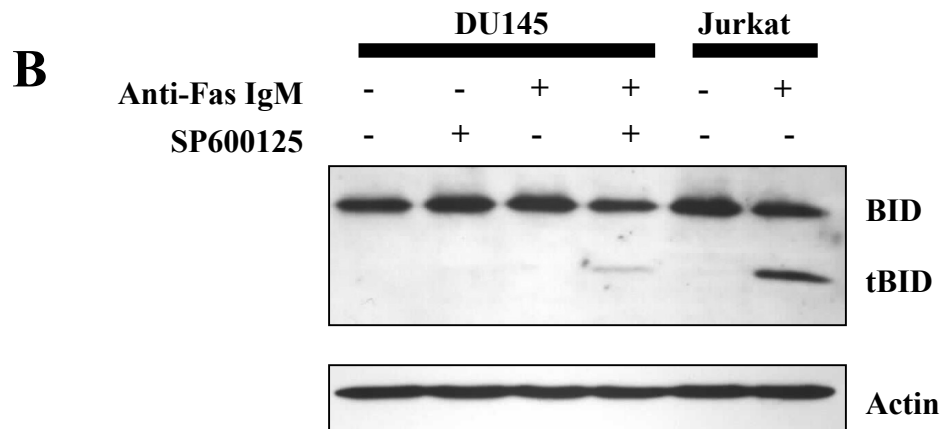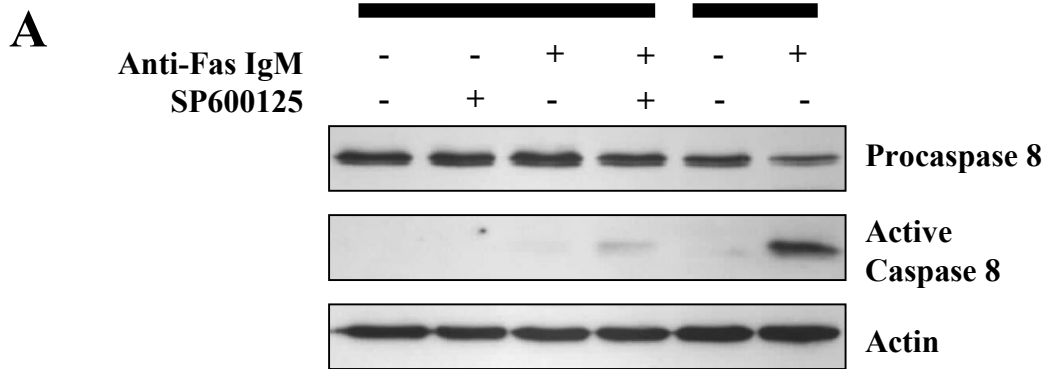
Figure 2: **SP600125 sensitises DU 145 cells to Fas induced apoptosis upstream of Caspase 8 activation.** (A) Expression of Procaspase 8 (55/57 kDa) and the large Caspase 8 active subunit (18 k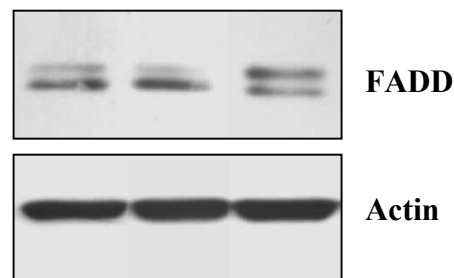Da) were analysed by western blot in lysates from DU 145 cells treated with 50 µM SP600125 and 200 ng ml^{-1} anti-Fas IgM for 24 h. Untreated and Fas treated Jurkats were used as a positive control for Caspase 8 cleavage products and actin was also probed to demonstrate equal protein loading. (B) The cleavage of BID (22 kDa) into the pro-apoptotic tBID (15 kDa) was analysed in DU 145 cells treated with 50 µM SP600125 and 200 ng ml^{-1} anti-Fas IgM for 24 h. Untreated and Fas treated Jurkat T cells were used as a positive control for BID cleavage following Fas R activation. β-Actin was probed for equal protein loading. (C) Mitochondrial membrane depolarisation was assessed in DU 145 cells treated for 24 h with 50 µM SP600125 and 200 ng ml^{-1} anti-Fas IgM using the cationic probe JC-1. A decrease in FL-2 fluorescence indicates depolarisation of the mitochondrial membrane in the cells. Data are representative of three independent experiments. (D) Western blot analysis of Procaspase 3 expression and cleavage in DU 145 cells treated for 24 h with 50 µM SP600125 and 200 ng ml^{-1} anti-Fas IgM. UV irradiated HL-60 cells were used as a positive control for Procaspase 3 cleavage. Procaspase 3 (35 kDa) and various cleavage products including active caspase subunits are evident. β-Actin was also probed to ensure equal protein loading.

A

Anti-Fas IgM − − + +
SP600125 − + − +

FADD

Actin

B

Hydroxyurea − + −
Nocodazole − − +

FADD

Actin

C

Untreated SP600125 Hydroxyurea Nocodazole

SubG1 G1 S G2/M   SubG1 G1 S G2/M   SubG1 G1 S G2/M   SubG1 G1 S G2/M

Counts

DNA Content

D

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%

Untreated SP600125 Hydroxyurea Nocodazole   Untreated SP600125 Hydroxyurea Nocodazole   Untreated SP600125 Hydroxyurea Nocodazole   Untreated SP600125 Hydroxyurea Nocodazole

SubG1   G1   S   G2/M
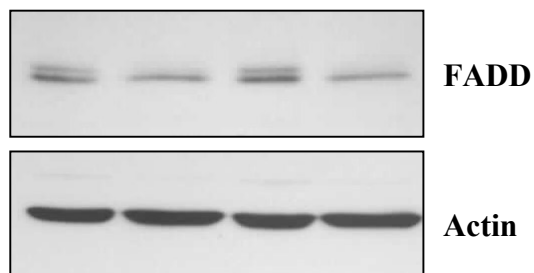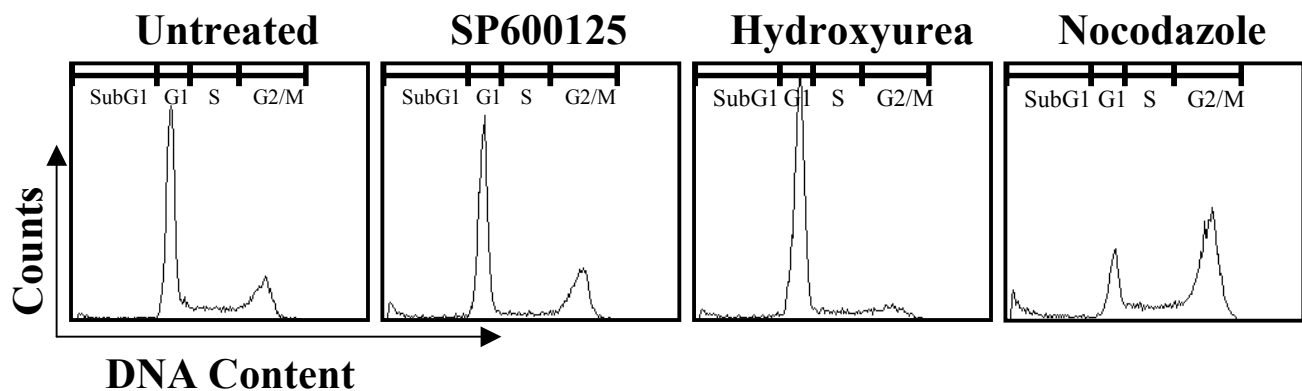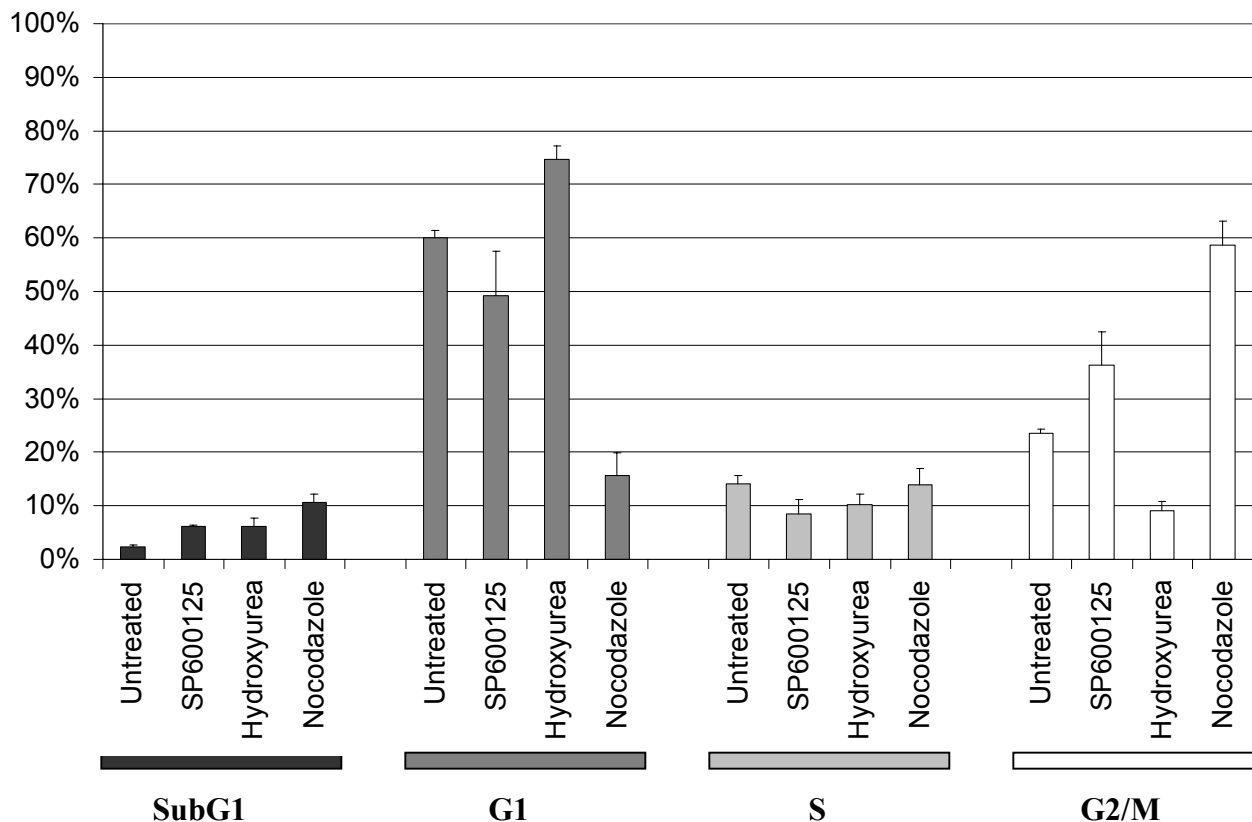
Figure 3: SP600125 inhibits FADD phosphorylation in DU 145 cells. (A) Western blot analysis of FADD in DU 145 cell lysates following treatment for 24 h with SP600125 and anti-Fas IgM as described earlier. The doublet band present in untreated and anti-Fas IgM treated cells represents unphosphorylated and Ser 194 phosphorylated FADD. Actin was also probed to ensure equal protein loading. (B) Effects of the cell cycle inhibitors Hydroxyurea and Nocodazole on FADD phosphorylation are investigated in DU 145 cells. Actin was probed to verify equal protein loading. (C) Cell cycle analysis in DU 145 cells incubated with 50 μM SP600125, 8 mM Hydroxyurea and 2 μg ml^{-1} Nocodazole for 24 h. Propidium iodide was used to assess the DNA content of cells by flow cytometry as described in the materials and methods section. (D) Bar chart representing DNA content and cell cycle phase for DU 145 cells incubated with SP600125, hydroxyurea and nocodazole. Error bars represent the standard deviation from the mean after 3 independent experiments.

A

DU 145   PC3   PPC-1   ALVA 31

PKCζ

PARP

B

SP600125   -   -   -   -   50
PKCζ pseudosubstrate   -   20   15   10   -

FADD

Actin

C

PKCζ pseudosubstrate   -   20   15   10

pERK

ERK2

Curtin JF and Cotter TG, 2003 Figure 4
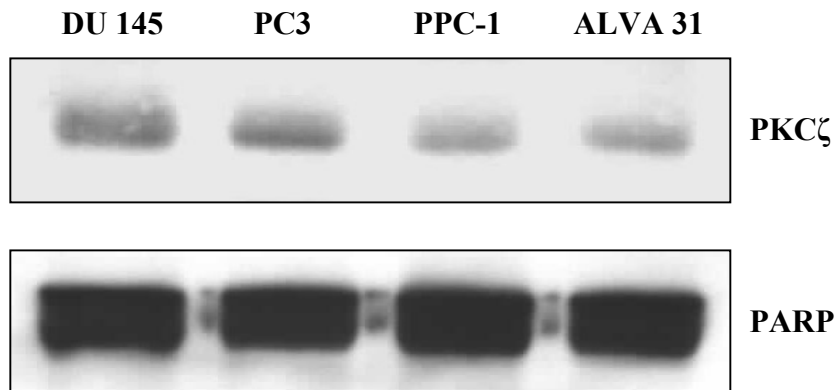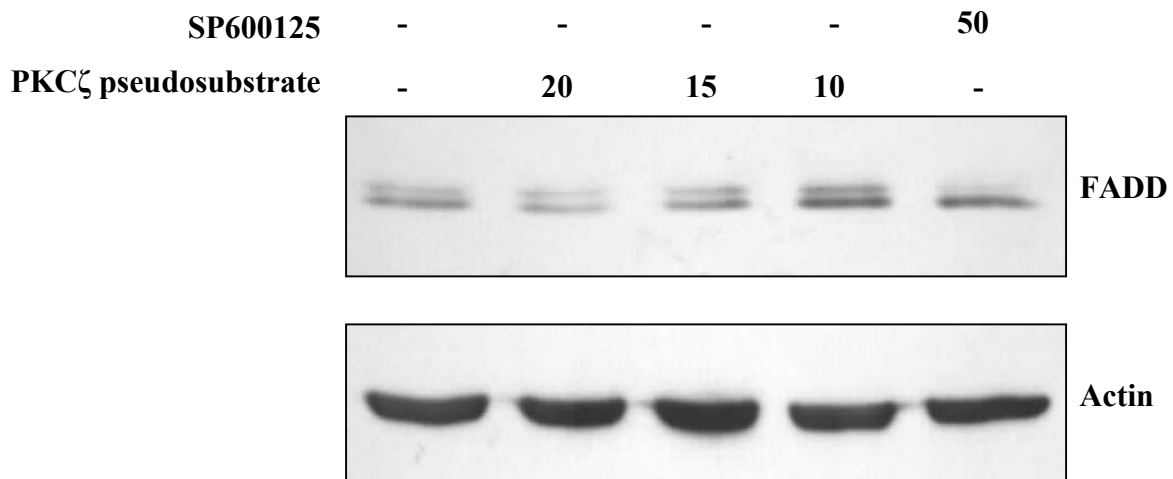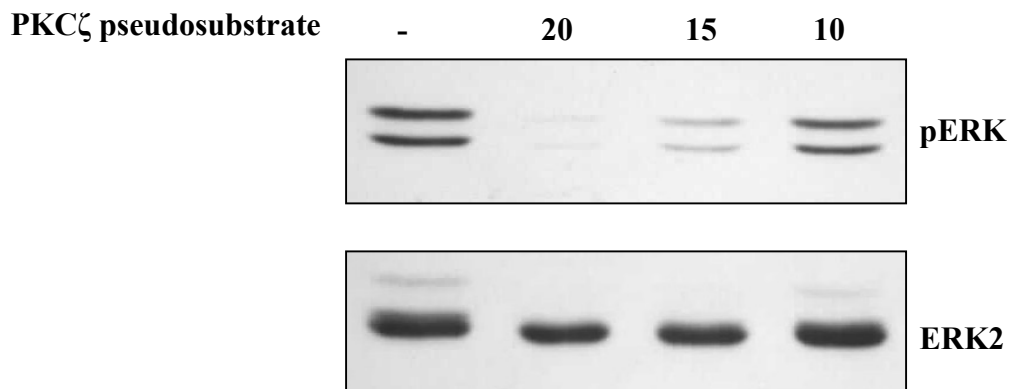
Figure 4: PKCζ does not phosphorylate FADD in DU 145 cells. (A) Total expression of PKCζ is investigated in 4 prostate cancer cell lines by western blot. Equal loading was verified by probing for PARP. (B) DU 145 cells were incubated with 20 μM, 15 μM and 10 μM PKCζ pseudo-substrate inhibitor for 24 hours and the extent of FADD phosphorylation was subsequently determined by western blot. Cells were also incubated with 50 μM SP600125 for comparative purposes and actin demonstrated equal protein loading. (C) Expression of phosphorylated ERK was determined for the cell lysates used above to verify that effective concentrations of PKCζ pseudo-substrate inhibitor were used. Equal protein loading was verified by determining total expression of ERK2.

A

DU 145   PC3   PPC-1   ALVA 31

HIPK3

Actin

B

Control   +   -
HIPK3   -   +

HIPK3

GAPDH

C

Control   +   -
HIPK3   -   +

FADD

Actin

D

Control siRNA   HIPK3 siRNA
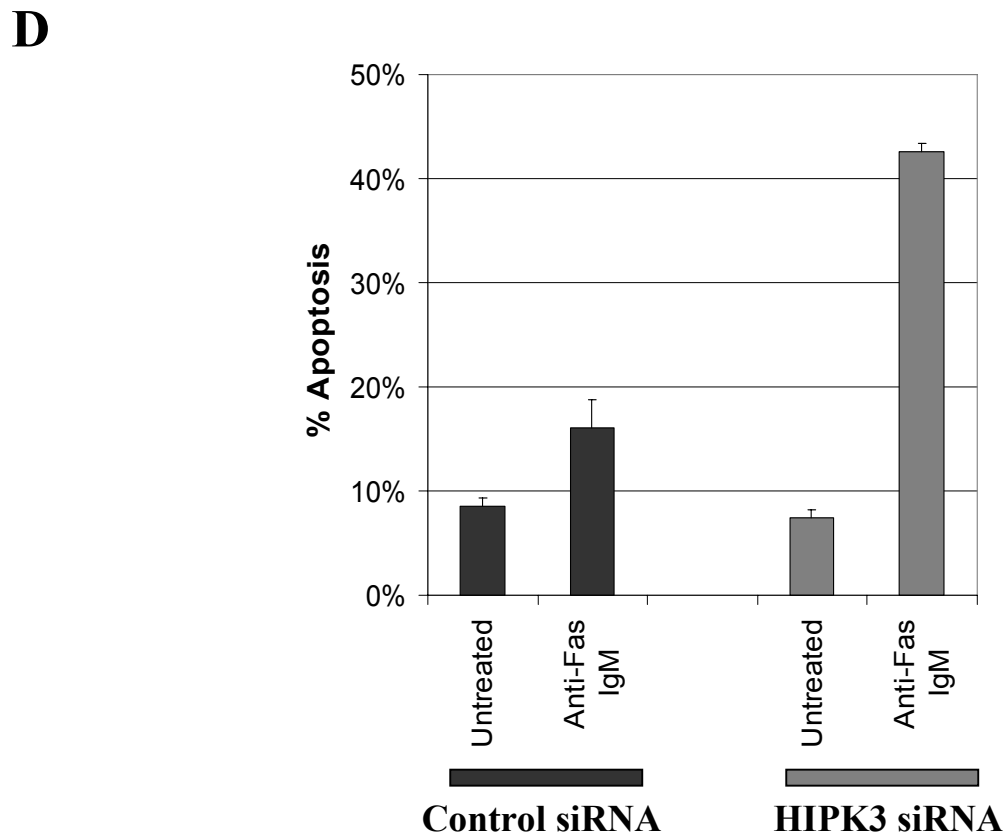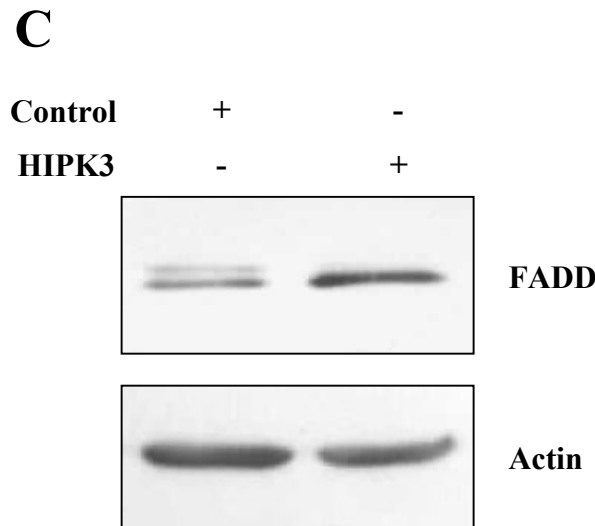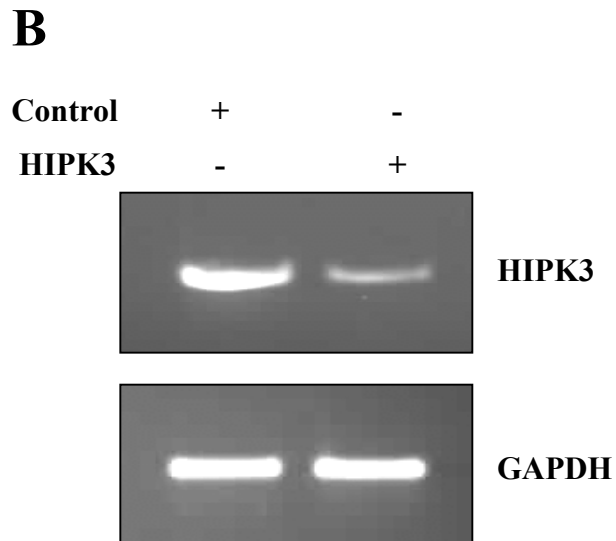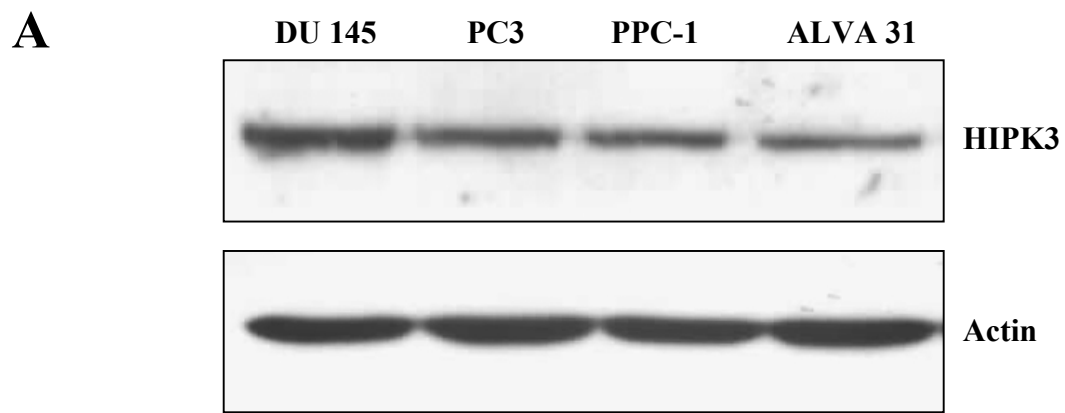
Figure 5:   HIPK3 phosphorylates FADD and increases the resistance of DU 145 cells to Fas mediated apoptosis. (A) Total expression of HIPK3 was determined in the 4 prostate cancer cell lines. Expression of HIPK3 was found to be highest in DU 145 cells. Actin was also probed to verify equal protein loading. RNA interference was used to decrease the expression of HIPK3 mRNA over 4 days (B) and a decrease in FADD phosphorylation was also observed in DU 145 cells in comparison with control oligonucleotides (C). GAPDH (B) and Actin (C) were also probed to demonstrate equal loading. (D) DU 145 cells were incubated with control or HIPK3 small interfering RNA for 4 days and subsequently treated with 200ng ml^{-1} anti-Fas IgM for 24 h. Apoptosis was measured using Annexin V-FITC and Propidium iodide staining as described in the materials and methods section. Error bars represent the standard deviation from the mean for 3 independent experiments.

A

SP600125   −   +

HIPK3

GAPDH

B

SP600125   −   +

HIPK3

Actin

C

			1h		6h		24h	
SP600125	−	+	−	+	−	+	−	+
Anti-Fas IgM	−	−	+	+	+	+	+	+
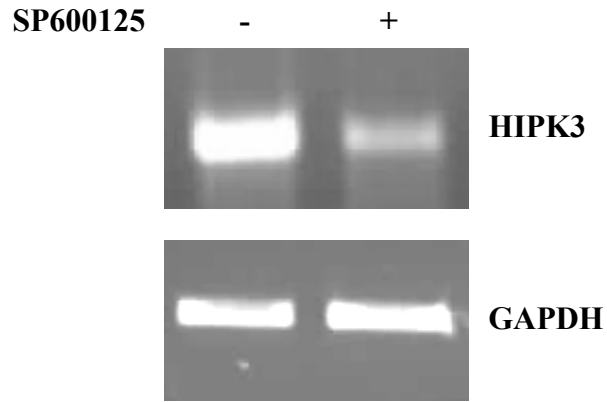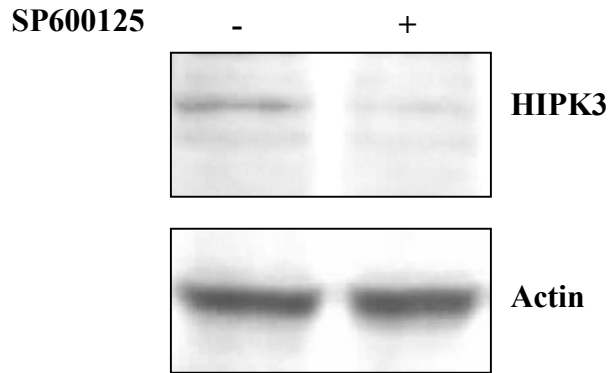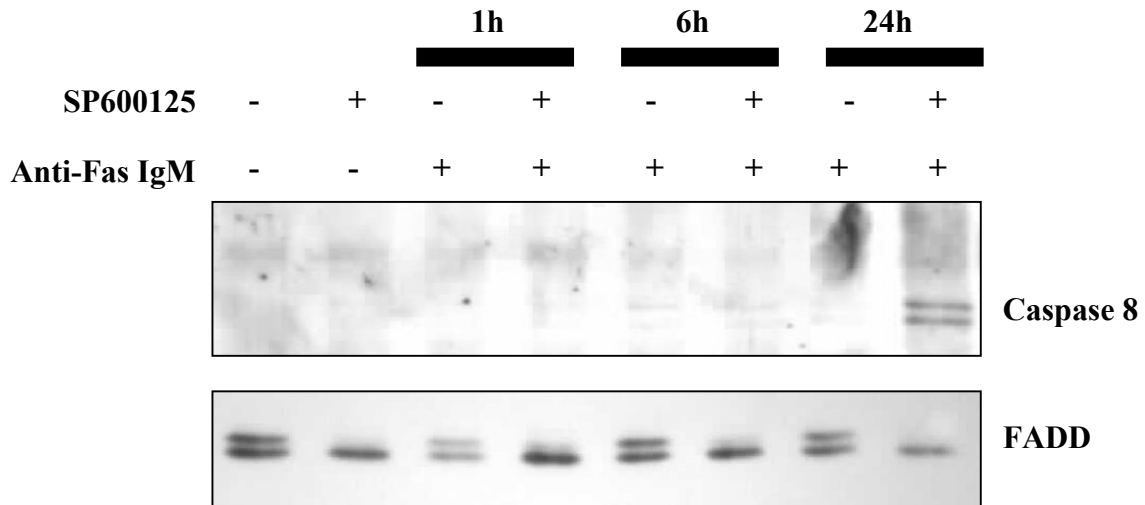
Caspase 8

FADD

Figure 6:   SP600125 decreases expression of HIPK3 in DU 145 cells and increases the affinity of FADD for Caspase 8. (A) RT-PCR analysis of HIPK3 expression in DU 145 cells incubated with or without 50 µM SP600125 for 24 h. Expression of GAPDH was also determined to demonstrate equal loading of cDNA. (B) Western blot analysis of HIPK3 expression in DU 145 cells following 24 h incubation with SP600125. Actin was also probed for equal protein loading. (C) Immunoprecipitation of FADD from DU 145 cell lysates incubated with 50 µM SP600125 for 24 h and 200 ng ml^{-1} for 1 h, 6 h and 24 h where indicated. Co-precipitation of Caspase 8 is evident in cells incubated with both SP600125 and anti-Fas IgM for 24 h. FADD was also probed to demonstrate equal protein loading.
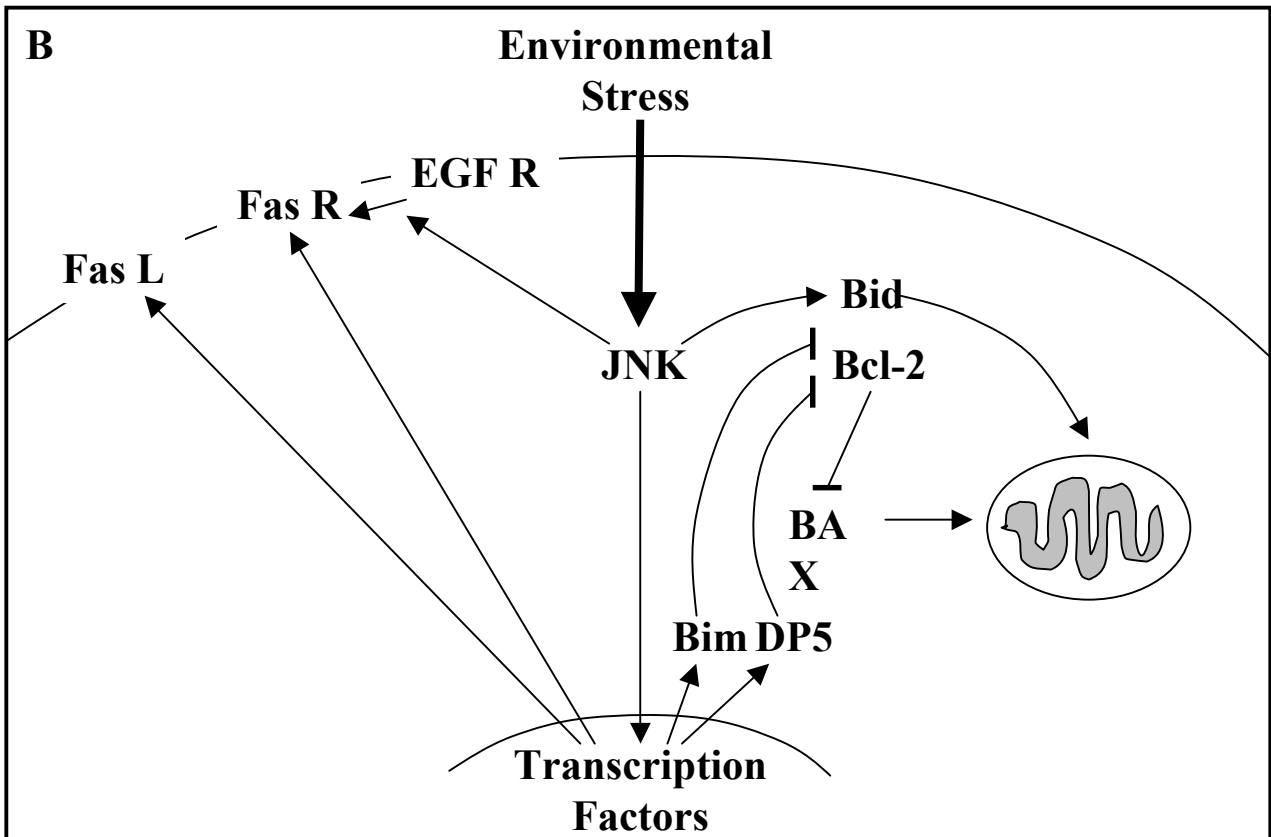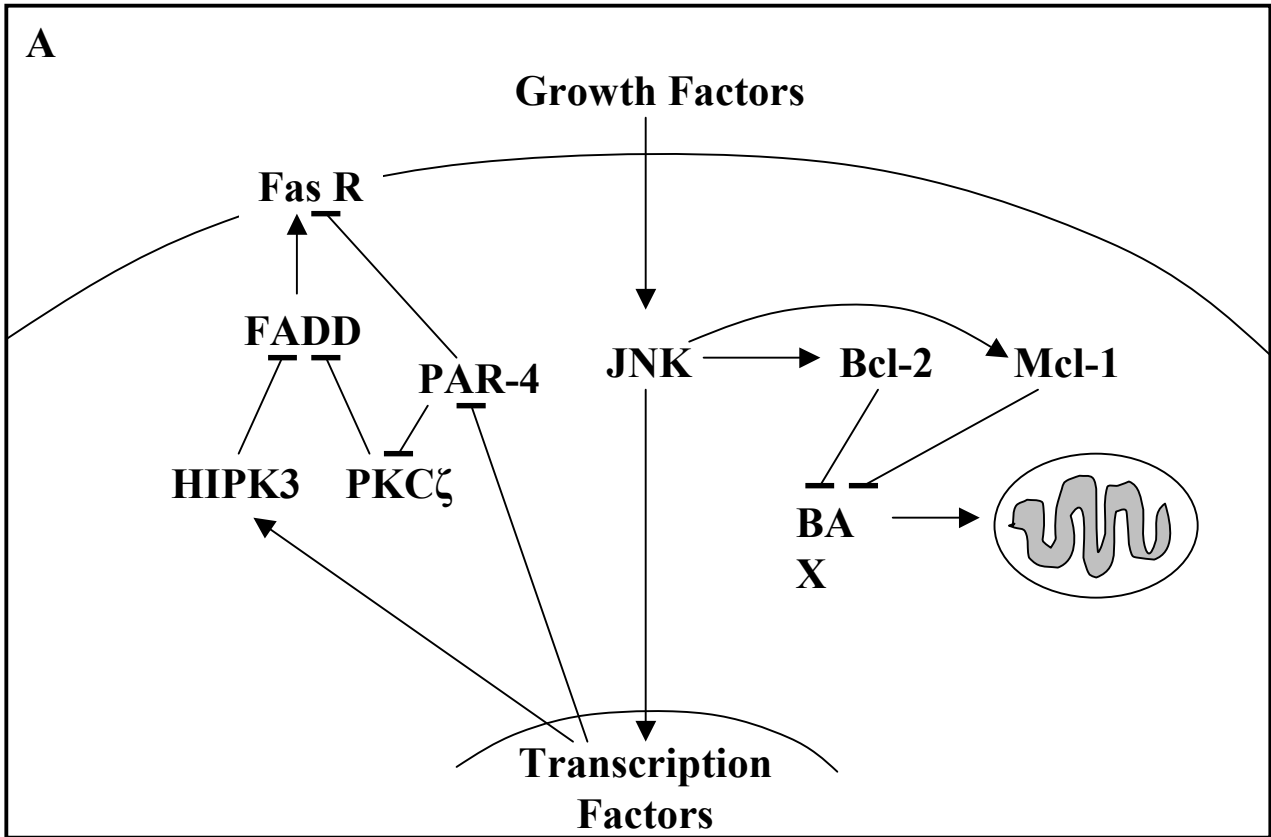
Figure 7:   Targets of JNK during Fas receptor mediated apoptosis. (A) Endogenous JNK activation promotes survival by phosphorylation of Bcl-2 family members and altering the activity of PKCξ and HIPK3. (B) Stress signals induce prolonged JNK activation and pro-apoptotic targets of JNK include increased processing of Bid, increased expression of Bim, DP5, Fas receptor and Fas ligand and tyrosine phosphorylation of Fas receptor by EGF receptor.

DISCUSSION

There are many hypotheses in science which are wrong. That's
perfectly all right; they're the aperture to finding out what's right.
Science is a self-correcting process. To be accepted, new ideas
must survive the most rigorous standards of evidence and scrutiny.

Carl Sagan

Discussion

Prolonged JNK activation sensitises DU 145 cells to Fas receptor mediated apoptosis.

Jun N-terminal kinase (JNK) is a member of the mitogen activated protein kinase (MAPK) family that also includes extracellular signal-regulated kinase (ERK) and P38. JNK is activated in response to environmental and cellular stresses, growth factors, inflammatory cytokines and G protein coupled receptor agonists (Davis, 2000; Kyriakis & Avruch, 2001). Prolonged JNK activation has been observed following engagement of Fas receptor with Fas ligand or with agonistic antibodies that can cross-link Fas receptor (Latinis & Koretzky, 1996; Wilson et al., 1996). Further studies demonstrated that Fas receptor activates JNK by either caspase dependent or caspase independent mechanisms. Cleavage of caspase 8 has been shown to correlate with cleavage of two upstream kinases in the JNK cascade called Mst1 (Graves et al., 2001) and MEKK1 (Deak et al., 1998). The kinase fragments relocate to soluble cellular fractions and induce prolonged JNK activation. Caspase independent JNK activation has also been reported and requires the translocation of DAXX from the nucleus to the cytoplasm where it associates with the intracellular surface of Fas receptor. DAXX can bind with Fas receptor independently of FADD and Caspase 8 (Yang et al., 1997). ASK1 is another upstream kinase of JNK and associates with DAXX in the cytoplasm. When ASK1 is recruited to Fas receptor aggregates local elevated concentrations of ASK 1 induce auto-phosphorylation and activation *in trans*. Active ASK1 can subsequently activate both JNK and P38 by phosphorylating and activating MKK4 and MKK6 (Chang et al., 1998; Tobiume et al., 2001).

JNK activation has been demonstrated to promote Fas mediated apoptosis in a number of cell lines (Costa-Pereira et al., 2000; Le-Niculescu et al., 1999; Zhang et al., 2000). It is not believed to be required for apoptosis in every cell line (Abreu-Martin et al., 1999; Hofmann et al., 2001; Low et al., 1999) and may instead serve to lower the treshold required for activation of Fas receptor. JNK can phosphorylate a wide variety of cellular targets and many of these promote apoptosis. Perhaps the most widely studied targets of JNK are the Bcl-2 family of proteins that play an integral role in regulating the sensitivity of mitochondria to cellular insults. The anti-apoptotic Bcl-2 family members Bcl-2, Bcl-X_L and Mcl-1 can all be phosphorylated by JNK (Deng et al., 2001; Inoshita et al., 2002; Kharbanda et al., 2000; Yamamoto et al., 1999). JNK can regulate the expression of pro-apoptotic Bcl-2 family members including Bim and Dp5 and these proteins are believed to displace Bax from Bcl-2 causing Bax homo-dimerisation and cytochrome c release from mitochondria (Harris & Johnson, 2001). JNK activity can also promote the cleavage and activation of another pro-apoptotic Bcl-2 family member called Bid by Caspase 8 (Gabai et al., 2002).

JNK can also regulate early events in Fas receptor mediated apoptosis. Previous reports have demonstrated that Fas ligand expression increases following JNK activation and promotes Fas mediated apoptosis in Jurkat cells (Herr et al., 2000). The promoter region of the Fas ligand gene is positively regulated by the transcription factors c-Jun and ATF-2 that are in turn regulated by JNK (Faris et al., 1998b). JNK can increase Fas receptor expression through increasing the stability of the transcription factor P53. A P53

responsive element is present in the first intron of Fas receptor gene that positively regulates Fas receptor expression (Muller et al., 1998).

In this study, we discovered that JNK was not activated following Fas receptor engagement with anti-Fas IgM in DU 145 cells. In addition, DU 145 cells were completely resistant to anti-Fas IgM at relevant physiological concentrations that can induce apoptosis in a wide variety of Fas sensitive cell lines. We found that stimulation of JNK using anisomycin was not toxic alone but significantly enhanced the rate of apoptosis in DU 145 cells when co-administered with anti-Fas IgM antibodies. We also found that Caspase 3 was not cleaved in response to anti-Fas IgM or anisomycin alone, but extensive cleavage was observed in cells co-incubated with anisomycin and anti-Fas IgM. Similarly, depolarisation of mitochondrial membranes was only evident in cells treated with both anti-Fas IgM and anisomycin. We did not observe any cleavage products of Caspase 8 or Bid in cells incubated with anti-Fas IgM or anisomycin alone. These data indicate that JNK affects Fas receptor mediated apoptosis upstream of Caspase 8 activation. Therefore, modulation of the Bcl-2 family members does not appear to be necessary for Fas receptor mediated apoptosis in DU 145 cells (Chapter 2).

We analysed the expression of Fas ligand and Fas receptor and we did not observe any increase in expression following JNK activation (Chapter 2). Other chemotherapeutic drugs have also been used to sensitise DU 145 cells to Fas receptor mediated apoptosis. Our group demonstrated that camptothecin, a topoisomerase I inhibitor, sensitised DU 145 cells to anti-Fas IgM mediated apoptosis by a JNK dependent mechanism (Costa-

Pereira & Cotter, 1999; Costa-Pereira et al., 2000). An analogue of camptothecin called 9-nitrocamptothecin was also found to sensitise prostate cancer cells to Fas mediated apoptosis over a longer time period (48 h and 72 h) (Chatterjee et al., 2001). The authors established that an increase in Fas receptor and Fas ligand expression and a decrease in the expression of the apoptosis inhibitor c-FLIP were responsible for the increase in apoptosis observed. Induction of apoptosis in our system is rapid and usually occurs within 8 h. We did not observe any increase in either Fas receptor or Fas ligand expression when using camptothecin or anisomycin. We also studied the expression of c-FLIP in DU 145 cells and did not observe any decrease in either $FLIP_S$ or $FLIP_L$ following incubation with anisomycin. Therefore, anisomycin and 9-nitrocamptothecin appear to sensitise DU 145 cells to Fas mediated apoptosis by different mechanisms. In fact, the short incubation required to induce apoptosis in our system suggests that *de novo* gene and protein expression may not be the principle effector of anisomycin in DU 145 cells (Chapter 2).

We can use the observations made already to narrow down the list of potential targets of JNK in DU 145 cells. We know that this target is involved in early events in Fas mediated apoptosis somewhere downstream of Fas receptor engagement with anti-Fas IgM and upstream of Caspase 8 activation. We can also speculate that a direct phosphorylation event is more likely the mediator than altered gene expression in light of the short time period before apoptosis is evident. Finally, no post translational modification of Fas receptor, Fas ligand, FADD, Caspase 8, FLIP or DAXX was noted.

This leaves us with a number of possible targets of JNK. Perhaps the most obvious is the ability of cytotoxic drugs to activate the Fas receptor pathway independent of Fas ligand. This is believed to be a principle mechanism employed by cytotoxic drugs to induce apoptosis in cancer cells (Micheau et al., 1999). A more recent report has described a caspase-independent, serine protease-dependent pathway that is regulated by FADD in prostate epithelial cells. Activation of a serine protease by FADD was found to occur in normal prostate epithelial cells but not in DU 145 prostate cancer cells. This serine protease induced cell death is independent of Caspase activation (Thorburn et al., 2003). Recently it has also emerged that the Fas receptor can associate with EGF receptor under conditions of cellular stress including hypo-osmolarity. EGF receptor phosphorylates Fas receptor at tyrosine residues and this was found to significantly enhance Fas receptor oligomerisation in response to Fas ligand engagement. The authors demonstrated that while JNK activity did not alter the interaction between EGF receptor and Fas receptor, it was absolutely required for phosphorylation of Fas receptor by EGF receptor (Reinehr et al., 2003). Therefore JNK can promote the trimerisation of Fas receptor and subsequent DISC formation in response to anti-Fas IgM.

Our results argue against the first two of these three possibilities. The low cytotoxicity of anisomycin and the synergy observed between anisomycin and anti-Fas IgM suggests that anisomycin does not activate Fas receptor. Instead, our data suggest that components required for Fas mediated apoptosis are defective in DU 145 cells and JNK activity either restores or bypasses these early events. We have shown that Caspase 8 activity is absolutely required for apoptosis in response to anisomycin and anti-Fas IgM using the

specific inhibitor z-IETD-fmk. In addition, we have observed DNA fragmentation and

membrane blebbing after 8 h treatment, which do not occur in response to the serine

protease induced cell death. Therefore, we can assume that Caspase 8 and not a serine

protease is the central mediator of apoptosis when DU 145 cells are co-incubated with

anti-Fas IgM and anisomycin. The third hypothesis is more promising. We believe that

either Fas receptor clustering or DISC formation may be defective in DU 145 cells. In

addition, JNK is not activated in response to anti-Fas IgM alone. Consequently, we

chose to study the events preventing Fas receptor mediated JNK activation in more detail.

Prolonged JNK activation in response to Fas receptor activation is inhibited in DU 145 cells.

JNK can be activated by either DAXX or Caspase 8 dependent pathways in response to Fas receptor engagement with Fas ligand and anti-Fas IgM antibodies but both of these pathways are defective in DU 145 cells. Our data suggested that restoring DAXX dependent JNK activation would be sufficient to activate Caspase 8 and initiate apoptosis in response to anti-Fas IgM. Consequently, we studied the mechanisms inhibiting JNK activation in order to further comprehend the inhibition of Fas receptor mediated apoptosis in prostate carcinoma cells.

The absence of Caspase 8 independent JNK activation suggested that a fundamental defect exists in DISC formation following Fas receptor activation in DU 145 cells. A number of possibilities may explain this. Firstly, Fas receptor must form trimers at the cell surface following engagement of Fas ligand or anti-Fas IgM antibodies in order to recruit functional DISC components (Holler et al., 2003; Schneider et al., 1998). Inefficient clustering of Fas receptor may help explain both the absence of JNK and Caspase 8 activation in DU 145 cells following treatment with anti-Fas IgM antibodies. Alternatively, FADD and DAXX might be sequestered within DU 145 cells and unable to bind with Fas receptor even after engagement of Fas receptor with Fas ligand. HSP27 has been reported to sequester DAXX in nuclei and overexpression of HSP27 prevents DAXX translocation from the nucleus to the cytoplasm in response to anti-Fas IgM. In addition, the authors discovered that only HSP27 dimers could bind with DAXX. Inhibition of MAPKAP2 activity using the P38 inhibitor SB203580 prevents HSP27

dimer formation from larger oligomers. This abrogated the interaction between DAXX and HSP27 and in turn sensitised cells to Fas receptor mediated apoptosis (Charette et al., 2000). HSP27 overexpression is associated with poor clinical outcome in prostate cancer (Cornford et al., 2000)

We did not observe translocation of DAXX from nuclear to cytoplasmic fractions. This suggested that either DAXX is sequestered in the nucleus or Fas receptor aggregation is defective and prevents DAXX translocation. We also found that HSP27 is overexpressed in DU 145 cells. However, we could not co-immunoprecipitate DAXX and HSP27 from DU 145 cell lysates. We did not observe any translocation of DAXX from nuclear to cytoplasmic extracts when cells were co-incubated with SB203580 and anti-Fas IgM and no increase in the sensitivity of cells to anti-Fas IgM was observed following incubation with SB203580. These data strongly suggest that HSP27 is not involved in sequestering DAXX in DU 145 cells (Chapter 3).

DAXX has also been reported to associate with PML at PML oncogenic domains (POD's/ND-10) in the nuclei of cells. Modification of PML by the small ubiquitin-like protein (SUMO-1) has been reported to sequester DAXX in ND-10 domains. This may prevent DAXX translocation from nuclear to cytoplasmic fractions in response to engagement of Fas receptor with anti-Fas IgM or Fas ligand (Li et al., 2000a). Overexpression of SUMO-1 can protect cells from Fas receptor mediated apoptosis (Okura et al., 1996). SUMO-1 appears to be a likely candidate for sequestering DAXX in DU 145 cells.

Next, we analysed the Caspase dependent pathway that mediates JNK activation in DU 145 cells. We had already discovered that Caspase 8 was not activated in DU 145 cells following Fas receptor engagement with anti-Fas IgM (Chapter 2). After further analysis, we demonstrated that the interaction between Caspase 8 and FADD was defective in DU 145 cells (Chapter 3). This would prevent Caspases 8 recruitment to Fas receptor clusters on the plasma membrane, which is a necessary event for cleavage and activation of Caspase 8 (Donepudi et al., 2003). Consequently, DISC formation and activation of Caspase 8 and JNK in response to Fas receptor mediated apoptosis would not occur.

Binding of Caspase 8 with FADD occurs in untreated cells and is mediated by homophyllic interactions between 2 death effector domains (DED) on FADD and on Caspase 8. Disruption of the interaction between FADD and Caspase 8 has been reported by a number of authors and this can abrogate Fas receptor mediated apoptosis. Early studies suggested that Caspase 8 resides primarily in mitochondria of resting cells and translocates into the cytosol in response to Fas receptor engagement with Fas ligand. Inhibition of the permeability transition pore was found to prevent release of Caspase 8 and prevent apoptosis (Qin et al., 2001). However, more recent reports that have disputed this claim and it is now believed that Caspase 8 resides predominantly in the cytosol in untreated cells where it is free to interact with FADD (van Loo et al., 2002).

FLIP is a cellular homologue of Caspase 8 that contains two DED and is capable of binding to FADD in place of Caspase 8. Although endogenous FLIP expression is

usually only 1% of endogenous Caspase 8 expression, overexpression of FLIP can protect cells from Fas receptor mediated apoptosis by preventing Caspase 8 binding with FADD (Scaffidi et al., 1999a). We analysed the expression of FLIP in Fas resistant DU 145 cells and Fas sensitive Jurkat cells. DU 145 cells were found to express $FLIP_L$ at similar levels to Jurkats and did not express $FLIP_S$. Expression of FLIP was not found to change following incubation with anisomycin or anti-Fas IgM. Caspase 8 was found to co-immunoprecipitate with FADD in untreated Jurkat cells demonstrating that FLIP expression alone is not responsible for abrogating the homophyllic binding between FADD and Caspase 8 in DU 145 cells (Chapter 3).

PKCξ overexpression and activation has been shown to phosphorylate FADD at Ser194 and prevents the interaction between FADD and Caspase 8 in the haematopoietic cell line KG1a. Treating cells with anti-Fas IgM was found to induce Fas receptor clustering on the plasma membrane and recruitment of FADD to Fas receptor. However, Caspase 8 was absent from these DISC immunoprecipitates when compared with Jurkat cells. Inhibition of PKCξ restored the interaction between FADD and Caspase 8 and promoted Fas receptor mediated apoptosis (de Thonel et al., 2001). Interestingly, we found that DU 145 cells express high levels of phosphorylated FADD (Chapter 4) and PKCξ expression has been shown to increase during prostate cancer tumorigenesis (Cornford et al., 1999).

Endogenous JNK activity inhibits Fas receptor mediated apoptosis.

As our work to understand the defects in Fas receptor signalling that inhibit prolonged JNK activation in response to Fas receptor activation progressed, we attempted to inhibit JNK activity in response to anisomycin using a newly synthesised inhibitor SP600125. Although we could not fully inhibit JNK activation in response to anisomycin using SP600125, we did abrogate endogenous and transient JNK activity and observed a significant increase in the sensitivity of these cells to Fas mediated apoptosis (Chapter 4). This was completely unexpected because we had already demonstrated that JNK activity promotes Fas receptor mediated apoptosis in DU 145 cells. However, while prolonged JNK activity is associated with events that induce or promote apoptosis, transient JNK activity is implicated with increased cell survival. In fact, elevated JNK activity has been reported during prostate cancer progression and inhibiting JNK expression using anti-sense oligonucleotides reduced tumour growth and sensitised prostate cancer cells to cytotoxic drugs *in vitro* and *in vivo* (Gjerset et al., 2001; Potapova et al., 1997; Yang et al., 2003). In addition, JNK is the major effector of Ras and BCR-Abl transformation *in vitro* (Hess et al., 2002; Pruitt et al., 2002) and overexpression of JNK confers a partially transformed phenotype on fibroblasts (Rennefahrt et al., 2002).

In order to comprehend how JNK activation can elicit both survival and apoptosis inducing signals, it is important to understand the kinetics of JNK activation in cells. Recent studies have demonstrated that JNK activation in cells is usually an "all or none" response to initial signals. In contrast, when analysing JNK activation in a population of cells such as by western blot a graded response is usually observed. This simply

represents the percentage of cells expressing active JNK at the time of lysis and it appears that the length of time that JNK is activated in a particular cell is the most important factor that determines the fate of that cell (Bagowski et al., 2003; Bagowski & Ferrell, 2001). Anisomycin induces prolonged JNK activation and promotes apoptosis. However, transient JNK activation in response to survival factors may only activate JNK for short periods at a time and is generally associated with cell survival. Because the time that JNK is active in each cell is much shorter, fewer cells express active JNK at any given time and the activity of JNK in that population of cells is substantially lower when analysed by western blot.

In many instances, JNK activity can mediate both pro-apoptotic and anti-apoptotic signals at the same cellular targets. This is best characterised in the Bcl-2 family of proteins where transient JNK activity can phosphorylate and alter the anti-apoptotic activity of Bcl-2 and Mcl-1 (Deng et al., 2001; Inoshita et al., 2002). It has been suggested that the regulation of pro-apoptotic Bcl-2 family members is more important in determining the response of cells to JNK (Lei et al., 2002). Sustained activation of JNK in a cell induces the expression of pro-apoptotic Bcl-2 family members such as Bim and DP5 and thus overcomes the previous anti-apoptotic signals (Harris & Johnson, 2001). Another well-characterised example is the modulation of gene expression by altering activity of transcription factors. Transient JNK activity is associated with expression of a number of genes involved in cell growth, proliferation and tumour progression (Alfonso-De Matte et al., 2002; Potapova et al., 2002). In contrast, sustained JNK activity alters

the expression of many apoptosis related genes including Fas ligand and PAR-4 (Han et al., 2002; Zhang et al., 2000).

There is a paucity in the literature regarding endogenous JNK activity and Fas mediated apoptosis. As a result we decided to study the mechanism by which transient JNK activity inhibited Fas receptor mediated apoptosis in DU 145 cells and discovered that endogenous JNK activity was required for FADD phosphorylation. A number of FADD interacting kinases have been identified and two of these, PKCξ and HIPK3 have been shown to inhibit DISC formation during Fas receptor mediated apoptosis (de Thonel et al., 2001; Rochat-Steiner et al., 2000).

JNK has been reported to decrease the expression of PAR-4, a negative regulator of the FADD interacting kinase PKCζ (Han et al., 2002). In addition, PKCζ expression increases during prostate cancer progression (Cornford et al., 1999). We used a pseudo-substrate inhibitor of PKCζ to inhibit the activity in DU 145 cells but we did not observe any change in the extent of FADD phosphorylation. This suggests that PKCζ is not the dominant FADD kinase in DU 145 cells. Next, we abrogated the expression of HIPK3 using RNA interference and observed a decrease in the phosphorylation of FADD. In addition, cells incubated with HIPK3 siRNA but not with control oligonucleotides were sensitive to anti-Fas IgM mediated apoptosis. Inhibition of JNK using SP600125 was found to reduce the expression of both HIPK3 mRNA and protein. This suggested that endogenous JNK activity promotes HIPK3 expression in prostate cancer. We also found that interaction between FADD and Caspase 8 was restored in cells incubated with

SP600125 and anti-Fas IgM, suggesting that HIPK3 plays a role in inhibiting the interaction between FADD and Caspase 8 (Chapter 4).

HIPK3 was first identified in tumour cells where increased expression and activity was implicated with tumorigenesis (Begley et al., 1997; Sampson et al., 1993). Subsequently, it was demonstrated that HIPK3 interacts with and phosphorylates FADD and prevents caspase dependent and independent JNK activation (Rochat-Steiner et al., 2000). We found that endogenous JNK activity increases the expression of HIPK3 (Chapter 4), an event that can prevent prolonged activation of JNK in response to Fas receptor (Rochat-Steiner et al., 2000). Such negative feedback loops are common in biochemical pathways and one such loop has been described previously during anisomycin-mediated JNK activation. However, the exact cause of this negative feedback loop was never identified in this study (Hazzalin et al., 1998).

HIPK2, another member of the homeodomain interacting protein kinase family, has been reported to interact with TRADD, and adapter protein similar to FADD that binds to TNFR1 and DR6 (Li et al., 2000b). In addition, HIPK2 has been shown to phosphorylate P53 in response to UV irradiation and enhances the transcription of P53 inducible genes. Unlike HIPK3, however, overexpression of HIPK2 promotes apoptosis and administration of anti-sense oligonucleotides against HIPK2 abrogates UV-induced apoptosis (D'Orazi et al., 2002; Hofmann et al., 2002). The third member of this family is called HIPK1 and phosphorylation of DAXX by HIPK1 promotes DAXX redistribution within nuclei by disrupting the interaction between PML and DAXX

(Ecsedy et al., 2003). Although this may promote DAXX translocation into the cytoplasm in response to Fas receptor oligomerisation and therefore counteract the effects of HIPK3, the role of HIPK1 in apoptosis has not yet been determined. Little is known about the role of the HIPK family of protein kinases in Fas mediated apoptosis. However, each member of the family has been found to interact with and phosphorylate targets involved in death receptor mediated apoptosis. This suggests that these protein kinases may play a key role in regulating death receptor mediated apoptosis.

Future Perspective

As life expectancy increases and the incidence of prostate cancer and mortality from prostate cancer increase globally, it is worth remembering that no chemotherapeutic regimen to date has been demonstrated to significantly improve long-term survival. This failure must be addressed and identifying novel chemotherapeutic targets in prostate cancer is paramount to this. Our studies have demonstrated that inhibition of Fas receptor mediated apoptosis occurs at multiple, semi-redundant stages in the Fas apoptotic pathway. The next stage in this study should be to determine the efficacy of various chemotherapeutic agents on *in vivo* models of prostate cancer. In particular, new technologies including gene therapy and RNA interference could be utilised to evaluate the role of various anti-apoptotic proteins on prostate cancer survival and proliferation *in vivo*. These studies should give a clearer insight into the mechanisms employed by prostate cancer cells to evade apoptosis. Identifying these inhibitory alterations in Fas receptor signalling and developing therapeutic drugs that target these components should enhance the efficacy of chemotherapy and increase the life expectancy of patients with hormone refractory prostate cancer.

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Publications

Induction of apoptosis in prostate carcinoma cells by BH3 peptides which inhibit Bak/Bcl-2 interactions.

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In my end is my beginning.

T. S. Eliot