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Regulation of death receptor-mediated apoptosis in B cell malignancies

Presented By

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Declaration

The work in this thesis forms no part of any other thesis and unless otherwise stated was carried out entirely by myself. Some work has been presented in scientific meetings. The work was carried out under the supervision of Dr. Graham Packham at the Ludwig Institute for Cancer Research, Imperial College School of Medicine and the Department of Medical Oncology, University of Southampton.

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOMEDICAL SCIENCES
DEPARTMENT OF MEDICAL ONCOLOGY

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REGULATION OF DEATH RECEPTOR-MEDIATED APOPTOSIS IN B CELL MALIGNANCIES

by Amalia Mouzakiti

Fas and TRAIL receptors are members of the death receptor family and induce apoptosis in susceptible cells when activated by their respective ligands. They play important roles in the maintenance of the immune system and in the pathogenesis of human diseases. In addition, they are being evaluated as targets for novel therapies to treat human cancers.

To examine the biological function of death receptors in B cell malignancies, the responsiveness to Fas- and TRAIL receptor-induced apoptosis was determined in a panel of Burkitt lymphoma (BL) cell lines including Epstein-Barr virus negative and positive lines with type I, II and III latency programmes. The patterns of resistance to either signal differed between distinct groups of BL cell lines and in some cases resistance was independent of the levels of receptor expressed. I examined the role of molecular determinants that modulate death receptor-mediated apoptosis in other cell systems, receptor mutations and the expression of FADD, caspase 8, decoy and soluble receptors as well as the signalling inhibitors FLIPL and FAP-1. However, these did not correlate with the observed patterns of sensitivity in BL. Therefore novel mechanisms may underlie resistance to death receptor-induced apoptosis in these cells. Death receptors have also been implicated in apoptosis induced by chemotherapeutic agents. Although BL cell lines readily underwent apoptosis when treated with the DNA-damaging drug, CDDP, there was no evidence for a major role of Fas and TRAIL receptors in CDDP-induced apoptosis of a BL cell line containing wild type p53.

To compare the results from BL cell lines with primary B cells, the responsiveness to death receptor-mediated apoptosis was also tested in *ex vivo* chronic lymphocytic leukaemia (CLL) cells. The majority of CLL cells analysed were susceptible to spontaneous apoptosis *in vitro*, a significant number was sensitive to Fas-induced apoptosis whereas the entire cell panel were resistant to TRAIL-induced apoptosis. Resistance to TRAIL- and in some cases to Fas-induced apoptosis was receptor-independent consistently with the observations in BL cell lines.

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Abbreviations

Aa	Amino acid
AICD	Activation-induced cell death
AIF	Apoptosis-inducing factor
ALPS	Autoimmune lymphoproliferative disease
APAF-1	Apoptotic protease activating factor-1
APS	Ammonium peroxisulphate
Asp	Aspartate
b	Base
BCR	B cell receptor
BH	BCL-2 homology
BIR	Baculovirus IAP repeat
BL	Burkitt's lymphoma
bp	Base pairs
CAD	Caspase-activated DNase
CARD	Caspase recruitment domain
CD	Cluster of differentiation
CDDP	Cis-diamminochloroplatinum
Cdk	Cyclin-dependent kinases
CLL	Chronic lymphocytic leukaemia
CM	Conditioned media
CRD	Cysteine-rich domain
CTL	Cytotoxic T lymphocyte
Da	Dalton
dATP	Deoxyadenosine triphosphate
DC	Dendritic cell
DcR	Decoy receptor
DD	Death domain
DED	Death effector domain
DISC	Death inducing signalling complex
DNA	Deoxyribonucleic acid
DOC	deoxycholate
DR	Death receptor
DTT	Dithiothreitol
EBER	Epstein-Barr encoded RNA
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetra-acetic acid
EST	Expressed sequence tag
EtBr	Ethidium bromide
Eμ	Immunoglobulin heavy chain enhancer
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death domain
FAP-1	Fas-associated phosphatase-1
FasL	Fas ligand
FasR	Fas receptor
FCS	Foetal calf serum
FDR	Fas decoy receptor
FITC	Fluorescein isothiocyanate

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Germinal centre
gld	Generalized lymphoproliferative disease
GPI	Glycosyl phosphatidyl-inositol
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HHV	Human herpes virus
HIV	Human immunodeficiency virus
hr	Hour
HRP	Horseradish peroxidase
ICAD	Inhibitor of caspase-activated DNase
ICE	Interleukin-converting enzyme
IFN	Interferon
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IL	Interleukin
IP	Immunoprecipitation
JNK	c-Jun-N'terminal kinase
LMP	Latent membrane protein
lpr	Lymphoproliferation
LPS	Lipopolysaccharide
LZ	Leucine zipper
MCL	Mantle cell lymphoma
MDM-2	Mouse double minute-2
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
MM	Multiple myeloma
mRNA	Messenger RNA
MW	Molecular weight
NF-κB	Nuclear factor-kappaB
NGF	Nerve growth factor
NK	Natural killer
NP40	Nonidet P40
OPG	Osteoprotegerin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PARP	Poly-ADP-ribose polymerase
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PI	Propidium iodide
PIG	p53-induced gene
pRB	Retinoblastoma susceptibility protein
PRD	Progressive disease
PS	Phosphatidyl serine
PTP	Protein tyrosine phosphatase
RACE	Rapid amplification of cDNA ends
RIP	Receptor interacting protein
RNA	Ribonucleic acid
RT	Room temperature
RPA	RNase protection assay

RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase-PCR
scid	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
SLE	Systemic lupus erythrematosus
SSCP	Single strand conformation polymorphism
TCR	T-cell receptor
TEMED	N, N, N', N'-tetramethylethylendiamine
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRADD	TNFR-associated death domain
TRAF	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
V	Volts
VH	Heavy chain variable region
WDR	WD-40 repeat
WHO	World Health Organisation
wt	Wild type

Chapter 1. Introduction

Apoptosis is an active, energy dependent, asynchronous, genetically controlled process by which single, unnecessary or damaged/infected cells undergo self-destruction when apoptosis-related genes and/or proteins are activated. It is an evolutionarily conserved mechanism that is critical during the development of an organism for differentiation and morphogenesis, and during adult life for maintenance of tissue homeostasis. At the molecular level the cell death program can be divided into three parts, initiation, execution and engulfment. Ligand binding to the members of a family of death-inducing receptors, the death receptors, is one of the initiating events attracting increasing attention, and the focus of this study. Death receptors, e.g. tumour necrosis factor receptor (TNFR), Fas and TRAIL receptors have direct access to the intracellular apoptotic machinery and therefore a potential for therapeutic utilization. The execution phase of apoptosis is common to most apoptotic pathways, triggered by different stimuli including the death receptors, and is characterized by chromatin and cytoplasmic condensation, and DNA degradation. These events result in the characteristic biochemical profile of apoptosis and *in vivo* end with the engulfment (phagocytosis) of the dying cells by various phagocytic cells. Resistance to apoptosis can clearly be a cause for disease and is a primary genetic event in tumourigenesis. Furthermore, defects in biochemical pathways leading to apoptosis have been detected in tumour cells and can confer resistance to anti-cancer therapies. Recent evidence now suggests not only that cell death receptors may be defective or rendered inoperative in a variety of diseases including cancer, but also that strategies to selectively target the apoptotic machinery in tumour cells may be of therapeutic benefit.

1.1 Apoptosis is a genetically programmed cell death pathway

Although the initial description of events taking place during cell death was first described in humans (Kerr et al., 1972), the idea of genetically programmed cell death was generally accepted following studies on the hermaphrodite nematode worm *Caenorhabditis elegans* (*c. elegans*). Studies on the developmental processes of this organism identified genes dedicated to the regulation and execution of a genetically programmed type of cell death. *C. elegans* is well suited for the study of cell death at

the cellular, genetic and molecular levels because it is both transparent and developmentally invariant. This has permitted the complete lineage description of all 1090 cells born during development of the hermaphrodite worm (Schnabel et al., 1997). Of these 1090 cells, 131 undergo apoptosis during development and thus analyses of worms carrying mutations with specific effects on cell fate and function have lead to the cloning of genes that function in apoptosis execution and ordered these genes in the apoptotic pathway. At the end of 1998, the essentially complete sequence of the *c. elegans* genome was published (consortium, 1998). Homologous genes were subsequently identified in higher organisms including mammals.

Three *c. elegans* genes, egl-1 (egg-laying defective), ced-4 and ced-3 (cell death gene) seem to be required for all somatic cell death to occur. Loss of function mutations of either of these genes results in the survival of nearly all of the 131 cells that would have undergone apoptosis in the wild type nematode. Another gene involved in apoptosis in this animal is ced-9, which protects most cells from undergoing apoptosis during development. Loss of function mutations in ced-9 lead to sterility because of inappropriate cell death in cells that would normally live. These deaths are suppressed by loss of function mutations in either ced-3 or ced-4 (Wu et al., 1997a). By contrast, transgenic worms that overexpress ced-9, or worms with gain of function mutations, fail to activate the death program and cells that should die survive instead. This suggests that the normal function of CED-9 is to antagonize the function of CED-3 and/or CED-4 via an interaction of CED-9 with CED-4 and CED-3 (Wu et al., 1997a). Interaction of CED-9 with CED-4 has been demonstrated *in vitro* and in the yeast two-hybrid system and mutation analysis has identified the domains in CED-9 responsible for the interaction with CED-4 (Wu et al., 1997b). CED-4 also interacts with CED-3, a protease, an interaction that leads to the proteolytic processing of CED-3 and its activation (Chinnaiyan et al., 1997). Furthermore, CED-4 that is complexed with CED-9 is incapable of activating CED-3, further supporting a role upstream of CED-3. By contrast, loss of egl-1 function while preventing cell death just as does loss of ced-4 or ced-3 function does not suppress the lethality resulting from a loss of ced-9 function. This would suggest that EGL-1 functions upstream as a negative regulator of CED-9 (Conradt and Horvitz, 1998).

Based on these and other findings, the current model for apoptosis in *c. elegans* suggests that after activation by upstream signals EGL-1 interacts with CED-9

and releases CED-4 from membrane associated CED-9 (Wu et al., 1997a). Free CED-4 then interacts with and facilitates the processing of inactive CED-3 to an active protease. The active enzyme acts as the mediator of downstream events in cell death, such as proteolytic degradation of protein targets, eventually leading to the destruction of the cell (Metzstein et al., 1998). Release of CED-4 from its binding of CED-9 allows it to oligomerise with other CED-4 molecules; since each CED-4 molecule can also bind a CED-3 molecule these molecules are brought in close proximity. Under these conditions of induced proximity, CED-3, which is thought to be capable of autoprocessing (Hugunin et al., 1996), activates itself. This model is in line with the recently emerged models of caspase activation in mammalian cells, as will be described in later sections, emphasising the conservation of death mechanisms across species.

The protein encoded by the ced-3 gene is homologous to a human protein, interleukin-1-converting enzyme (ICE), the prototypic member of the human caspase family. This discovery played an important role in identifying the important role of caspases in mammalian cell death. In addition, a protein related to CED-4, APAF-1 (apoptotic protease activating factor-1), is involved in the mitochondrial apoptotic pathway in mammalian cells and cloning of the ced-9 gene revealed that its protein product is similar to the human BCL-2 and BCL-XL proteins, both important components of mammalian apoptotic pathways. Mammalian BCL-2 can protect the *c. elegans* cells that carry mutations in ced-9 from cell death (Hengartner and Horvitz, 1994).

Interestingly, mutant *c. elegans* worms with 131 extra cells have a normal life span indicating that in this organism cell death is not essential for either life or ageing. By contrast, more complex animals cannot survive without programmed cell death; mutations that affect apoptosis in the fruit fly *Drosophila melanogaster,* are lethal early in development (Song et al., 1997). Furthermore, knockout mice with deficiencies in apoptosis-related molecules also exhibit embryonic lethality as will be discussed in more detail later on. Moreover, cell death in *c. elegans* seems to be part of the developmental process only, whereas in mammals it forms part of a variety of processes occurring throughout their life and is the result of a variety of intra- and extracellular signals. Metazoan cells appear to be programmed to die by default and they execute apoptosis if they don't receive the necessary signals from their

environment. In addition they have internal sensors that can initiate apoptosis if the cell is unable to repair defects such as damage in DNA (Amundson et al., 1998), (Brown and Wouters, 1999). Higher metazoans and especially mammals have also evolved an additional signalling mechanism that actively directs cells to die by apoptosis via receptor/ligand pathways. Therefore, as might be expected mammals exhibit a much greater complexity in the processes and the controls within the apoptotic pathways. For example, the single BCL-2-type protein in *c. elegans* has its equivalent in a whole family of proteins in mammals, members of which can either be promoters of cell survival or cell death. Similarly, while only a single protease has been characterised in the worm, a cascade of caspases functions in mammals, resulting in a chain reaction of proteolytic events resulting in degradation of essential proteins during apoptosis. The essential molecules participating in *c. elegans* and mammalian death pathways and the basic interactions between them are described in Figure 1.1.

1.2 Morphological and biochemical features of apoptosis and necrosis

In animals cell death can occur via two major mechanisms: apoptosis and necrosis. Apoptosis is the more prominent form and is used for the coordinated death of excess, hazardous or damaged somatic cells. Necrosis is almost always the outcome of severe and acute injury: i.e. abrupt anoxia, sudden shortage of nutrients or extreme physicochemical injury (heat, detergents, strong bases etc.).

The apoptotic process includes mechanisms that organize both packaging and disposal of cell corpses, thereby preventing inflammation of the surrounding tissues. By contrast, during apoptosis, the cell membrane loses its selective permeability and ion pumping capacity as a result of direct membrane damage. This leads to the swelling of the cell and its organelles, including the mitochondria, and leaking of the cellular contents into the extracellular space. Activation of catabolic enzymes such as phospholipases, proteases, RNases and DNases, results in further degradation of membranes, proteins, RNA and DNA, which accelerates the cellular and nuclear degradation. The resulting leakage of cellular components results in damage in neighbouring cells both directly and by promoting inflammation. Necrosis occurs in whole fields of damaged cells, where the leaked cellular debris elicits an inflammatory reaction in the adjacent viable tissues (Kerr et al., 1972). Apoptosis, on the other hand, proceeds as an ordered disassembly of cellular contents and results in a specific pattern

of cellular morphology, avoiding damage of neighbouring tissues. Apoptosis is largely energy dependent; if ATP is present at insufficiently low concentrations, cells die by necrosis (Leist et al., 1997), (Ferrari et al., 1998). During apoptosis, the integrity of the cell membrane and of the mitochondria remains initially intact, the cytoplasm condenses, adherent cells round up and the nucleus coalesces into several large masses before the final disassembly of cellular structures. Apoptotic chromatin condensation, one of the early morphological changes, results from the action of a number of proteins, including apoptosis-inducing factor (AIF) and topoisomerase II as well as the protease-activated chromatin condensation factor called acinus (Susin et al., 1999), (Liu et al., 1998), (Samejima et al., 1998), (Sahara et al., 1999). The nuclear envelope and the nucleolus break apart as chromatin is cleaved into nucleosomal fragments with unit lengths between 120-200 base pairs. The overall result of these processes is a contraction in the nucleus and the overall cytoplasmic volume. Other molecular changes include increased distribution of phosphatidyl-serine (PS) residues between the inner and outer leaflets of the cell membrane, release of cytochrome c from the mitochondria and loss of the mitochondrial membrane potential. Subsequently, the plasma membrane also begins to 'bleb' or form convoluted invaginations and protrusions. *In vitro*, the endoplasmic reticulum transforms into vesicles that fuse with the cytoplasmic membrane and then break up into small vesicles termed apoptotic bodies. The apoptotic bodies enclose fractional parts of the cell compartments and several apparently intact organelles. At this late stage, the cell membrane becomes permeable to dyes such as trypan blue. *In vivo,* apoptotic bodies are not usually observed since engulfment of dying cells occurs earlier. As a result, inflammation occurs in a more localised and controlled way, if at all. Techniques based on detecting some of these changes such as terminal deoxynucleotide transferase-mediated incorporation of labeled nucleotides at DNA breaks (TUNEL), staining of cell surface-exposed PS with annexin V and detection of caspase activation by a variety of methods, have become standard tools for apoptosis research. The main differences between apoptosis and necrosis are summarised in Table 1.1.

Apoptosis	Necrosis
Physiological or pathological	Always pathological
Asynchronous process in single cells	Occurs simultaneously in multiple cells
Genetically controlled	Caused by overwhelming stimuli
Late loss of membrane integrity	Early loss of membrane integrity
Cell shrinkage	Generalised cell and nucleus swelling
Condensation of nuclear contents	Nuclear chromatin condensation
Restricted inflammation	Inflammation in surrounding tissues

Table 1.1: *Major differences between apoptosis and necrosis in advanced organisms.*

1.3 Induction of apoptosis in mammals

Nucleated mammalian cells in both developing and mature organs are able to undergo apoptosis (Ishizaki et al., 1995), and generally express all the protein components required to execute cell death (Weil et al., 1996). The specific signalling pathway activated depends on the cell type and the subcellular element targeted by each type of stress. Apoptosis occurs in three defined phases: initiation (when the cells receive the relevant death signal), execution when, following the decision on the fate of the cell, an irreversible chain of events is set into motion that brings about the cellular disassembly and engulfment of the dying cells by specialised cell types. Various upstream signalling cascades converge on a common final effector mechanism to disintegrate the cell.

Apoptosis can be initiated either at the cell surface, by ligand-dependent triggering of receptors or by the stimulation of intracellular receptor proteins, such as APAF-1, which is activated by mitochondria-released cytochrome c. Apoptotic stimuli fall into four main categories. Firstly, events or insults that will induce DNA damage including ultraviolet (UV) radiation, ionising radiation (IR) and anti-cancer drugs. The second type involves active induction of apoptosis by specialised receptor mechanisms such as receptor activation mediated by glucocorticoids (acting on the thymus), ligand binding on specialised death receptors, or by withdrawal of growth factors such as nerve growth factor (NGF) and IL-3 (interleukin-3). The third type of apoptotic stimuli encompasses biochemical agents that directly engage cytoplasmic components of the

apoptotic pathway and include delivery of granule-associated serine proteases, such as granzyme B, from cytotoxic T lymphocytes into target cells. Finally, another class of apoptotic triggers involves stresses such as heat, and oxidising agents. Excessive production of ROS (reactive oxygen species) such as superoxide, hydrogen peroxide, and the hydroxyl radicals, produces free radicals that damage lipid membranes, proteins, nucleic acids, and extracellular matrix components. The majority of apoptotic triggers, apart from surface receptor activation, primarily engage the mitochondria-related pathway, although subsequent activation of surface receptors can also occur, and *vice versa*.

Among the signalling pathways leading to apoptosis, the one activated by death receptors has been the focus of intense study. Among these, activation of the Fas receptor by its ligand, Fas ligand (FasL) is the most extensively characterised. Initiation of apoptosis by death receptors and the mechanisms of its regulation are the focus of this study and will be discussed in detail in later sections. Regulation of the apoptotic pathways, regardless of the initial trigger, occurs at various levels in the cell, p53 and the BCL-2 family of proteins being the most prominent control mechanisms whereas additional regulatory mechanisms are now emerging (Figure 1.2). They play an important role in the cells' decision to commit to the apoptotic process and deregulation of these mechanisms can disrupt normal apoptosis execution and tissue homeostasis and result in disease.

1.4 The BCL-2 family of proteins

1.4.1 BCL-2 family members and the mitochondrial pathway of apoptosis

The mitochondria were originally deemed minor components of the apoptotic process until one study identified the three factors required for the activation of caspase 3, a caspase important for the proteolysis of essential targets during apoptosis. These factors were mitochondria-derived cytochrome c, caspase 9 and a new protein, APAF-1, which was later recognised as a homologue of the nematode protein CED-4 (Liu et al., 1996a). The new protein contained, in addition to the CED-4 domain, a caspase recruitment domain (CARD) and a C-terminal WD-40 repeat domain (WDR). Binding of APAF-1 to cytochrome c in the presence of dATP exposes its CARD, which then binds to a corresponding motif in caspase 9, resulting in its recruitment to the complex and the formation of a large protein aggregate termed the apoptosome.

This interaction enhances the proteolytic activity of caspase 9 and induces its cleavage through allosteric interactions (Rodriguez and Lazebnik, 1999). Thus, caspase 9 functions as a holoenzyme with APAF-1 as its co-factor initiating a caspase cascade that also involves caspases 3 and 7 followed by caspase 6 (Salvesen and Dixit, 1999), (Zou et al., 1997), (Srinivasula et al., 1998).

Since the release of cytochrome c is such an important step in the formation of the apoptosome, the cell needs to regulate its release from the mitochondria. The BCL-2 family of proteins are important intracellular sensors for cell survival and/or cell death, regulating apoptotic pathways centering on the mitochondria. They function in a similar manner in organisms as diverse as mammals, nematodes and *Drosophila*. The members of the family take their names from the prototypical member, BCL-2 (B-cell leukemia/lymphoma-2), originally cloned as a gene from chromosome 18 that became constitutively expressed following its translocation adjacent to the immunoglobulin locus on chromosome 14 in follicular lymphoma (Yunis et al., 1982). In the years following the initial discovery of BCL-2, at least 20 mammalian protein members have been identified (Table 1.2) and several others in viruses, most of them by sequence homology. All members possess at least one of four conserved motifs termed BCL-2 homology (BH) domains, BH 1-4 while some members also possess a membrane anchor (Adams and Cory, 2001). The BH-domains play a critical role for the functional regulation of BCL-2 family members, being involved in homo- and hetero-dimerisation.

The BCL-2 family comprises both pro- and anti-apoptotic members. The anti-apoptotic group consists of BCL-2 and close relatives such as BCL-XL, A-1, BOO, BCL-W and MCL-1 (Song et al., 1999). BCL-2 and its anti-apoptotic homologues are potent inhibitors of cell death caused by a wide range of inducers, including growth factor deprivation, DNA damage, corticosteroids and staurosporine (Bissonnette et al., 1992), (Boise et al., 1995), (Motoyama et al., 1995), (Lomo et al., 1996). The pro-apoptotic proteins in the family are divided into two subgroups; the first comprises proteins, which possess BH1, BH2 and BH3 regions including BAX, BAK and BOK. The second subgroup encompasses the BH3-only proteins, sharing only the BH3 domain with family members, including BID, BIM, BIK, BAD, HRK, BMF, PUMA and NOXA (Bouillet et al., 1999), (Luo et al., 1998), (Yang et al., 1995). Mutagenesis of the BH3 domain of BAD and BID indicated that this domain is essential for

interactions as well as their killing activity (Luo et al., 1998). In addition, deletion as well as mutagenesis studies have demonstrated a similar role for the BH3 domain in BAX and BAK (Wei et al., 2001).

1.4.2 Mechanism of action of BCL-2 family proteins

An important characteristic of the BCL-2 family members is their ability to form homo- and hetero-dimers; the amphipathic BH3 α-helix of the apoptogenic proteins (e.g. BAX, BAD) can bind to a hydrophobic groove on pro-survival proteins (BCL-2 or BCL-XL), created by α-helices in the BH3, BH1 and BH2 regions. In the BH-3-only group, this short motif of 9-16 residues is necessary and probably sufficient for their killing activity (Kelekar and Thompson, 1998). Most members of the anti-apoptotic subgroup also possess a hydrophilic C-terminal segment, which facilitates their interaction with the endoplasmic reticulum and the outer mitochondrial membrane. Subcellular localisation studies by electron microscopy and biochemical fractionation have established that the bulk of the BCL-2 proteins reside on the cytosolic aspect of these membranes (Green and Reed, 1998), (Hsu et al., 1997), (Zamzami et al., 1998), (Chittenden et al., 1995). Mitochondrial localisation of family members strengthens the current models describing them as regulators of the mitochondrial pathway of apoptosis. Homo- or hetero-dimerisation is important for the apoptosis-regulating function of the BCL-2 proteins, especially the dimerisation that takes place between the pro-apoptotic BAX and anti-apoptotic BCL-2. When BAX is in excess BAX/BAX homodimers are formed which seem to promote apoptosis whereas when BCL-2 is in excess BAX/BCL-2 heterodimers are formed which favours abrogation of apoptosis (Yang and Korsmeyer, 1996). Other family members may influence this balance. Although the precise mechanism by which BCL-2 and its homologues influence apoptotic pathways remains unresolved, currently two (non-exclusive) models are used to explain BCL-2 function. These models describe BCL-2 proteins as ion channels, or as proteins that modulate activation of caspases:

1. A number of apoptogenic factors, such as AIF, cytochrome c, endonuclease G and Smac/DIABLO can be released from the mitochondria to the cytoplasm to initiate apoptosis (Susin et al., 1999), (Li et al., 2001), (Du et al., 2000). The resemblance of the three-dimensional structure of BCL-XL to that of membrane penetrating bacterial toxins, such as diphtheria toxin,

prompted the hypothesis that BCL-2 family members with the hydrophobic BH1-BH2 domains can insert themselves in the mitochondrial membrane leading to the release of apoptogenic factors or acting to prevent this (Liang and Fesik, 1997). In addition to BCL-XL, BCL-2 and BAX are capable of creating channels in liposomes *in vitro* albeit at non-physiological pH (Jurgensmeier et al., 1998), (Shimizu et al., 1999). Furthermore, addition of BAX directly to isolated mitochondria triggers the release of cytochrome c (Shimizu et al., 1999). BAX is also able to interact with the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane and/or the adenine nucleotide translocator, the major components of the permeability transition pore (PTP) to cause permeabilisation of the inner membrane and mitochondrial depolarisation. This process would allow entry of water and solutes into the matrix and lead to mitochondrial swelling and/or rupture (Shimizu et al., 1999). On the other hand, BCL-2 and BCL-XL can prevent release of cytochrome c and AIF from the mitochondria (Shimizu et al., 1999), (Susin et al., 1999). The model suggests they could achieve this either by forming homodimers, or by interacting and forming heterodimers with the pro-apoptotic members (e.g. preventing BAX from forming pores).

2. The BH4 domains of BCL-2 and BCL-XL can bind to or at least immunoprecipitate with a variety of other cellular proteins directly or indirectly involved in cell death regulation including APAF-1 (Hu et al., 1998), (Pan et al., 1998c). These interactions suggest that among the roles of BCL-2 and its family members is to serve as a site onto which other proteins can dock, thereby altering their cellular activities. This process seems to be conserved from nematodes to humans. By analogy with the nematode BCL-2/BCL-XL may inhibit activation of the apical caspase 9 by the CED-4 homologue APAF-1. In such a model, the role of pro-apoptotic BCL-2 family members is to displace BCL-2/BCL-XL from APAF-1 and trigger caspase 9 activation.

BCL-2 family members are subject to transcriptional regulation and post-translational modifications that regulate their functions. The best example of such modification is the regulation of cell death in growth-factor-dependent cell lines by

phosphorylation of BAD. In the presence of the growth factor IL-3, BAD is phosphorylated by the kinase AKT and associated with the cytosolic protein 14-3-3. On removal of IL-3, however, BAD becomes dephosphorylated, dissociates from 14-3-3 and binds to BCL-XL, thus blocking its association with BAX (Yang et al., 1995). This would allow BAX to perform its pro-apoptotic function. The anti-apoptotic members of the family are also subject to phosphorylation events that modulate their functions. BCL-2 and BCL-XL can be regulated by phosphorylation (Maundrell et al., 1997), (Chang et al., 1997). BCL-XL, BCL-2, BAX and BID are also caspase targets (Fujita and Tsuruo, 1998), (Adams and Cory, 2001).

Anti-apoptotic (BH1-4 domains)	**Pro-apoptotic (BH1-3 domains)**	**Pro-apoptotic (BH3-domain only)**
	Mammalian	
BCL-2	BAX	BID
BCL-XL	BAK	BAD
	BCL-XS	
BCL-W	BOK/MTD	BIK/NBK
MCL-1		HRK
A1/BFL1		BIM/BOD
BOO/DIVA		BNIP3
NR13		NIX
		NOXA
		PUMA
		BMF
	C. elegans	
CED-9		EGL-1

Table 1.2: *Pro- and anti-apoptotic BCL-2 family members in mammals and c. elegans.* Mammalian pro-apoptotic BCL-2 family members are assigned to two groups based on sequence conservation: the more fully conserved proteins, containing the BH1-3 domains, and the subset of 'BH3-domain only' proteins (BCL-XS contains BH 3-4 domains).

1.5 Regulation of apoptosis by p53

The p53 tumour suppressor protein was first described as a protein that binds the large T antigen of the SV40 virus. The gene, TP53 encodes a 53 KDa nuclear protein, which functions mainly as a transcription factor, and participates in tumour suppression, cell cycle control, DNA repair, stress responses, cell senescence and apoptosis (Lowe et al., 1993), (Bates and Vousden, 1996). In support of its importance, mutations in p53 have been detected in more than 50% of human cancers and there is growing evidence that additional mutations that disrupt the p53 pathway occur in additional tumours. A germ-line mutation of one allele in humans gives rise to the Li-Fraumeni cancer-susceptibility syndrome (Evan and Littlewood, 1998). In addition, the p53 status of a tumour is critical for the therapeutic response of cancer patients, since the inactivation of p53 in cancer has been associated with poor survival, refractory disease and chemoresistance (Lowe et al., 1993). The main abnormality of p53-knockout mice is the high incidence of spontaneous tumours, mainly sarcomas and lymphomas (Donehower et al., 1992). Along with the fact that these mice are rarely viable, the evidence strongly supports a major role for p53 as a tumour suppressor gene.

p53 inhibits cell growth through induction of cell cycle arrest and apoptosis and the ability to induce apoptosis is thought to be central to its tumour-suppressor function (Bates and Vousden, 1996). When cells are exposed to stress stimuli leading to DNA damage such as UV and treatment with chemotherapeutic agents, but also insults that do not involve obvious DNA damage, such as growth factor deprivation, heat shock and virus infection, p53 levels rise due to, at least in part, increased stability of the protein. Normally the half-life of p53 is approximately 30 minutes in healthy cells but can rise to 150 minutes in cells treated with UV (Liu et al., 1994). The short half-life of p53 is mainly due to degradation mediated by another protein, MDM2, (mouse double minute-2), which is also involved in regulating nuclear export of p53, thereby allowing degradation to occur in the cytoplasm. MDM2 has an intrinsic ubiquitin ligase activity that contributes to p53 degradation (Inoue et al., 2001). Posttranslational changes also cause an increase in p53 transcriptional activity. The mechanisms by which p53 becomes activated include phosphorylation, glycosylation, binding to regulatory proteins and possibly acetylation. Oncogenes, such as the adenovirus E1A oncogene, can also induce p53, leading to increased

apoptosis or premature senescence. C-myc, a transcription factor with oncogenic properties, activates p53 by upregulation of $p14^{ARF}$, an inhibitor of MDM2 (Levine, 1997).

It is not entirely clear how p53 senses DNA damage; a number of proteins have been implicated in delivering the DNA damage signals to p53, including DNA-dependent protein kinase (DNA-PK), ATM (ataxia telangiectasia mutated) and NBS (Nijmegen breakage syndrome). However, the defects in p53 activation in ATM-null and NBS-null cells are selective for certain kinds of DNA damage, suggesting that these proteins are probably involved in some but not all of the pathways signalling to p53 (Kumari et al., 1998). Activation of p53 in response to DNA damage leads to p53-dependent apoptosis or cell cycle arrest in the G1 phase of the cell cycle. This is essential to allow sufficient time for DNA repair prior to subsequent DNA replication. It minimizes the accumulation of genetic errors during DNA replication or chromosome segregation, hence maintaining the integrity of the genome (Levine, 1997). A generally accepted pathway for the induction of cell cycle arrest involves transcriptional activation of one of the major p53 targets, p21, which in turn binds to and inhibits cyclin-dependent kinases, causing hypophosphorylation of retinoblastoma protein (Rb), thus preventing the release of E2F and blocking the transition from the G1 to the S phase of the cell cycle.

In contrast to pathways leading to p53-mediated cell cycle arrest, the mechanism by which p53 triggers apoptosis is not clear, but it is reported to occur via both transcription-dependent and transcription-independent mechanisms. p53's role as a transcription factor has lead to the search for transcriptional targets capable of activating the apoptotic pathway. Analysis of p53-regulated gene expression patterns using cDNA arrays has demonstrated that the nature of the p53 response depends on the levels of the protein, the type of inducing agent or event, and the cell type (Zhao et al., 2000). Several mechanisms have been described by which p53 might signal to the apoptotic machinery:

1. One group of p53-targeted apoptosis-related genes consists of members of the TNFR family, thereby providing a mechanism that directly links p53 to apoptosis signalling cascades. Most of the available evidence suggests the Fas receptor; overexpression of p53 in transformed cell lines can result in enhanced surface expression of Fas and subsequent apoptosis (Bennett et al.,

1998), (Muller et al., 1998). UV and X-ray radiation-induced apoptosis is p53-dependent and is mediated by activation of Fas in some systems (Aragane et al., 1998), (Rehemtulla et al., 1997). Furthermore, a strong correlation between wild type p53 status and induction of Fas in response to DNA damaging chemotherapeutic agents in a variety of tumour cell lines has been reported (Owen-Schaub et al., 1995), (Muller et al., 1998). Finally, identification of p53-responsive elements in the Fas promoter confirmed that p53 can directly regulate the Fas gene (Muller et al., 1998). On the other hand, lpr (lymphoproliferation) mice lacking Fas, have unimpaired responses to p53-mediated apoptosis (Newton and Strasser, 2000). In addition, a study using gld (generalised lymphoproliferative disease) mice lacking functional FasL and cells from p53-/- mice has supported the evidence from lpr mice and shown that p53 and Fas function independently to induce apoptosis (O'Connor et al., 2000).

2. Death receptor 5 (DR5), also a member of the death receptor family, was identified as a novel transcript induced by the anti-cancer agent doxorubicin in cells with wild type p53 (Wu et al., 1997c). Overexpression of a wild type p53 transgene in cells lacking wild type p53 caused induction of endogenous DR5 expression, suggesting that it may form part of a p53 response. Furthermore, ionising radiation but not UV treatment of wild type p53 cell lines upregulated DR5 mRNA in the same way as other known p53 target genes via binding to a intronic DNA-binding site on DR5 (Sheikh et al., 1998), (Sheikh et al., 1999). Recently a mouse homologue of the DR5 gene was cloned (MK) and experiments with p53 conditional mutant cell lines have shown that MK is also inducible by wild type p53 (Wu et al., 1999).

3. BAX is probably the best-known pro-apoptotic p53 target. BAX is upregulated by p53, in response to DNA damage, by interacting with p53-binding sites on BAX promoter (Miyashita and Reed, 1995). BAX links p53 with the apoptotic machinery of the mitochondria, where the BCL-2 family proteins mainly exert their functions. However, it appears to contribute only in part to p53-mediated apoptosis since expression of a BAX transgene only partially restores DNA damage-induced apoptosis in p53-/- thymocytes (Yin et al., 1997). Recently, two new p53 targets belonging to the BH3-only

subgroup of the BCL-2 family were identified; NOXA and PUMA (Nakano and Wousden, 2001), (Oda et al., 2000). It may be that more than one member of the BCL-2 family respond to p53 signals for the induction of apoptosis via disruption of the mitochondria.

4. Serial analysis of gene expression in colon carcinoma cells has identified a set of putative p53 target genes in apoptosis; which were termed PIGs (p53-induced genes). Most of these genes are involved in modulating the redox status of the cell by directly or indirectly stimulating the production of reactive oxygen species which signal mitochondrial release of cytochrome c (Polyak et al., 1997). This further supports a role for p53 in regulating the mitochondrial pathway.

As an added level of regulation, p53 can transcriptionally repress gene targets, particularly in the context of promoters lacking consensus p53 binding sites. For example, the expression of BCL-2 may be reduced in the presence of p53 (Budhram-Mahadeo et al., 1999), (Wu et al., 2001). The molecular mechanisms are poorly understood, but do not appear to involve interaction of p53 with classical p53 DNA binding motifs. Evidence also exists for transcription-independent apoptosis mediated by p53. This comes from observations that p53-dependent apoptosis is resistant to treatment with the transcriptional inhibitor actinomycin D or the translation inhibitor cycloheximide (Asker et al., 1999). Possible transcription-independent pathways may include increase of Fas trafficking from cytoplasmic stores to the cell surface (Bennett et al., 1998). Furthermore, the recently described ability of p53 to induce caspase activation in cell-free extracts suggests that p53 may be able to transduce apoptotic signals by direct protein-protein interactions (Ding et al., 1998).

It is likely that not a single mechanism is responsible for p53-dependent apoptosis induction but a network of interactions varying according to cell type and original stimulus. A schematic diagram of the major pathways originating from p53 is shown in Figure 1.3.

1.6 Execution of apoptosis by caspases

Although a variety of stimuli can initiate apoptosis, the effector stage of apoptosis appears to be shared by different cell systems. Apoptosis is dependent on the activation of a set of highly regulated cysteine proteases termed caspases (cysteine

aspartate-specific proteases). As the name suggests, caspases display a cysteine residue in the active site and specificity for cleavage after aspartic acid (Asp) residues. During apoptosis the caspases are responsible for cleaving vital cellular proteins, to produce the morphological and biochemical features of cell death. The first identified member of the human caspase family was discovered in 1993, after researchers discovered the homology shared between the *c. elegans* enzyme ced-3 and ICE (caspase 1) and the family now comprise 14 members (Table 1.3). Since then caspases have been cloned in *Drosophila*, the lepidopteran *Spodoptera frugiperda* and the frog *Xenopus laevis* (Ahmad et al., 1997), (Nakajima et al., 2000).

1.6.1 Caspase substrate specificity, structure and activation

All caspases hydrolyse peptide bonds on the carboxyl side of an aspartate residue on their substrate, termed the P1 residue. Each active site contains a positively charged S1 subsite that binds the substrate's negatively charged P1 aspartate. The S1 binding site is highly conserved, therefore, all known caspases cleave solely after Asp residues (Thornberry et al., 1997), (Talanian et al., 1997).

Caspases reside inside cells as inactive zymogens with very low intrinsic activity. The active form of the enzyme results from proteolytic cleavage at internal conserved Asp residues. The inactive zymogens contain an N-terminal prodomain of variable length, which is separated from the central large caspase subunit (approximately 20 KDa: termed p20 subunit) by one or two Asp cleavage site(s). The large caspase subunit itself is separated from the C-terminal small subunit (approximately 10 KDa: termed p10 subunit) by one Asp cleavage site or a linker peptide. The large subunit contains the conserved active site pentapeptide (QAC (R, G,Q) G), which forms the S1 site but several residues in the large and small subunits contribute to binding the Asp side chain and to substrate specificity (Cohen, 1997), (Thornberry and Lazebnik, 1998). Activation of the zymogen generally involves the sequential proteolytic cleavage of the interdomain linker between the large and small subunits, followed by the removal of the prodomain. The X-ray crystal structures of caspases 1, 3 and 8 have revealed that active enzymes function as tetramers, consisting of two large/small subunit heterodimers (Wilson et al., 1994), (Blanchard et al., 1999), (Mittl et al., 1997). They contain active sites composed of residues from both the small and large subunits (Figure 1.4).

While caspases share a large degree of similarity in the catalytic domains, there is little similarity in the N-terminal prodomains. The length and sequence of the prodomain varies between individual caspases, ranging from six aa (caspase 14) to more than 220 (caspases 8 and 10) (Earnshaw et al., 1999). The prodomains contain sequence motifs that facilitate their interaction with downstream signalling components. These domains include death effector domains (DEDs) and caspase recruitment domains (CARDs). DED domains are found exclusively on initiator caspases-8 and 10, which are involved in death receptor-initiated apoptosis and are essential for binding to adaptor molecules. CARD motifs are found in caspases activated by intracellular events, as well as caspases involved in cytokine activation. Thus caspases 2 and 9 as well as caspases 1, and 4, all contain CARD domains (Table 1.3). Generally, long prodomain caspases function as signal integrators for apoptotic or pro-inflammatory signals and contain sequence motifs that promote their interaction with activator/adaptor molecules. Thus apoptotic initiators (caspases 2, 8, 9 and 10) function upstream of the small prodomain apoptotic executioners (caspases 3, 6 and 7). By contrast, caspases 1 and 11 function predominantly as cytokine processors (Wang et al., 1998b), (Cohen, 1997). Less is known about caspases 4, 5, 12, 13 and 14, however, these caspases demonstrate a higher degree of sequence similarity to caspase 1 than to the apoptosis-related caspases (Wolf and Green, 1999).

In vitro, caspase activation can result from autoactivation, transactivation and proteolysis by other proteases such as granzyme B. Affinity labelling experiments have demonstrated that caspase zymogens including caspase 8 display an intrinsic low proteolytic activity indicating the potential for caspase autoactivation at close proximities (Muzio et al., 1998). Oligomerisation at the cell membrane is sufficient for caspase 8 autoactivation (induced proximity model) (Martin et al., 1998). The exception to the induced proximity model is procaspase 9 for which, unlike in other caspases, proteolytic processing has only a minor effect on catalytic activity. Rather the key requirement for caspase 9 activation is its association with APAF-1 (Srinivasula et al., 1998) (Stennicke et al., 1999). Less is known about the activation of pro-inflammatory caspases. CARDIAK, a CARD-containing kinase promotes procaspase 1 activation in vitro via a CARD-CARD interaction, suggesting that CARD-mediated oligomerisation may play a role in caspase 1 activation (Thome et al., 1998). Once activated, caspases can activate other caspases, promoting cascade

amplification and positive feedback. Non-caspase proteases can also activate caspases, for example, granzyme B, an aspartate-specific serine protease released by cytotoxic T cells, can activate several caspases and potently induce apoptosis (Stennicke et al., 1998) (Zhou and Salvesen, 1997).

The activation of caspases in apoptosis does not lead to indiscriminate degradation of the dying cell. Rather, specific proteins are cleaved, such as key structural components of the cytoskeleton and nucleus, as well as a number of proteins involved in signalling pathways, DNA metabolism and repair. In most cases, caspase mediated cleavage results in the inactivation of a protein, but caspases can also activate proteins, either directly by cleaving off a negative regulatory domain or indirectly by inactivating a regulatory subunit. One classic caspase substrate is the inhibitor of caspase activated DNase (ICAD). ICAD binds to CAD, the DNase responsible for DNA cleavage during apoptosis, and keeps it in the cytosol. Cleavage of ICAD by caspases allows CAD to migrate to the nucleus where it executes internucleosomal digestion of DNA (Enari et al., 1998). Caspase substrates include structural components of the cytoskeleton (actin, gelsolin, α-fodrin and keratin), nuclear membrane (lamin A, lamin B) to proteins involved in signal transduction (RAF-1, protein kinases) DNA replication and cleavage or in the processing of cytokine precursors (proIL-1β, proIL-16). DNA metabolism and repair-related substrates include: poly-ADP-ribose polymerase (PARP), a classic apoptotic marker, DNA topoisomerase II and RNA polymerase I upstream binding factor (Widmann et al., 1998). Proteins involved in cell cycle regulation, proliferation and regulation of apoptosis that are processed by caspases include: the p53-trascription targets p21, p27 and Rb as well as pro- and anti-apoptotic members of the BCL-2 family. Furthermore, caspases themselves can serve as substrates for other caspases: caspases 3 and 7 are substrates for caspases 6, 8 and 10.

Caspase zymogen	Prodomain length and motif	Function
Caspase-2 (NEDD2, ICH1)	Long, CARD	Initiator
Caspase-8 (FLICE)	Long, DED	Initiator
Caspase-9 (MCH6)	Long, CARD	Initiator
Caspase-10 (FLICE 2)	Long, DED	Initiator
Caspase-1 (ICE)	Long, CARD	Inflammation
Caspase-4	Long, CARD	Inflammation
Caspase-5	Long	Inflammation
Caspase-13 (ERICE)	Long	Inflammation
Caspase-3 (CPP32, YAMA, Apopain)	Short	Executioner
Caspase-6 (MCH2)	Short	Executioner
Caspase-7	Short	Executioner
Caspase 11	Long	Inflammation
Caspase 12	Long	Inflammation
Caspase –14 (MICE)	Short	Inflammation

Table 1.3: *Known members of the human caspase family.* Prodomain characteristics and functions are shown.

1.6.2 The biological role of caspases

One way of determining the biological function of different caspases has been the overexpression of various caspases in different cell lines. In this way, almost all known caspases have been found to be able to induce apoptosis to some extent. However, the understanding of the relevance and contribution of these enzymes in exogenously stimulated and developmentally regulated apoptosis has been helped by

in vivo studies, through production of single caspase-deficient mice. More specifically, the knowledge acquired from knockout studies has helped to elucidate more complex issues such as the position of caspases in different apoptotic pathways, and redundancy between similar caspases. For example, enforced expression of caspase 1 results in induction of apoptosis, but the phenotype of caspase 1 knockout mice argues against a significant role for this caspase in development, since these mice have a normal phenotype and display no embryonic lethality (Li et al., 1995). Furthermore, IL-1β and IL-1α are not proteolytically processed and the mice are resistant to endotoxic shock, a process involving IL-1β. Caspase 11 is required to activate caspase 1 and consequently caspase 11-deficient mice fail to activate caspase 1 and share a similar phenotype (Wang et al., 1998b). These caspases are therefore more important in immune modulation rather than apoptosis.

On the other hand, mice deficient in caspases 3, 8 and 9 show early embryonic lethality. Caspase 8-deficient embryos die at day 11 or 12 of gestation from impaired heart development and abundant haemorrhage in the liver and abdomen (Varfolomeev et al., 1998). Caspase 8 deficiency also results in a complete signalling block through cell surface receptors of the TNF receptor family, known as death receptors, TNFR1, Fas, and DR3 in mouse embryonic fibroblasts. Yet these cells remain sensitive to other apoptotic stimuli indicating that caspase 8 is critical only to selected pathways. The complete resistance to death receptor signalling in the absence of caspase 8 indicates that this caspase is a necessary factor despite previous reports about caspases 2 and 10 interacting with these receptors. Indeed caspase 2-deficient mice have been shown to develop normally and their lymphocytes remain sensitive to Fas and TNF receptor-mediated apoptosis, however that seems to depend on the mouse strain (Bergeron et al., 1998). Caspase 9-deficient mice show gross modifications in the brain accompanied by skull defects with the majority dying embryonically. Caspase 9 deletion prevents activation of caspase 3 in embryonic brains *in vivo* and caspase 9-deficient thymocytes show resistance to a subset of apoptotic stimuli, including absence of caspase 3-like cleavage and delayed DNA fragmentation. Moreover, cytochrome c-mediated cleavage of caspase 3 is absent in the cytosolic extracts of caspase 9-deficient cells. In contrast, cell death from Fas activation occurs normally in the absence of caspase 9 (Fearnhead et al., 1998),

(Hakem et al., 1998), (Kuida et al., 1998). Table 1.4 summarises the phenotypes resulting from targeted deletion of caspase and other apoptosis-related genes in mice.

1.6.3 Caspase inhibitors

Given the importance of caspases in apoptosis, it is not surprising that inhibitors of caspase activity have evolved. A number of viruses encode caspase inhibitors as a means of inhibiting caspase activation and apoptosis in the host cell thus ensuring an appropriate environment for viral replication. Among the best-described viral caspase inhibitors are the cowpox virus product, cytokine response modifier A (CrmA) and the baculovirus protein p35. CrmA is a potent inhibitor of caspases 1 and 8 and a weak inhibitor of other caspases whereas p35 has a wider substrate specificity (Zhou et al., 1997). Both CrmA and p35 appear to act as competitive inhibitors, binding and inactivating caspases as a result (Goyal, 2001).

Baculoviruses also produce another type of caspase inhibitor, the inhibitor of apoptosis proteins (IAP). These proteins are mainly characterised by the baculovirus IAP repeat (BIR) domains near their amino terminus and IAPs containing between one and three BIR domains have been described in a variety of species. These include the fission yeast, *Schizosaccharomyces pombe*, *c. elegans*, *D. melanogaster* and several mammalian species including humans. Seven human IAP-like molecules have been found in humans: XIAP, NAIP, cIAP-1, Survivin, cIAP-2, BRUCE and pIAP (Rothe et al., 1995), (Duckett et al., 1996), (Liston et al., 2001), (Ambrosini et al., 1997). Overexpression of XIAP, cIAP1, cIAP2, NAIP and survivin can suppress apoptosis induced by stimuli including TNF and Fas activation, staurosporin, etoposide, and growth factor withdrawal (Duckett et al., 1996), (Ambrosini et al., 1997), (Deveraux and Reed, 1999). XIAP, cIAP1 and cIAP2 can bind via their BIR domains and inhibit the activation of caspases including caspases 3, 7 and 9 (Deveraux and Reed, 1999), (Roy et al., 1997). Survivin has also been co-immunoprecipitated with caspases 3, 7 and 9 and it suppresses apoptosis induced by overexpression of these caspases (Tamm et al., 1998). Because many RING zinc-finger domains possess E3 ligase activity, some IAPs are capable of ubiquitinating and targeting either their caspase substrates or themselves for proteasome-mediated degradation (Suzuki et al., 2001c), (Yang et al., 2000). Thus in addition to inhibiting specific caspases, certain IAPs may also mediate the removal of caspases from the cell. Because gene knockout studies have not yet

been reported, the *in vivo* physiological roles of individual IAP family genes are unclear. The importance of IAPs in regulating caspase activity appears significant because several proteins have been identified that promote caspase activation by blocking IAP function. In mammalian cells, a mitochondrial factor SMAC/DIABLO is released into the cytoplasm (under conditions of stress), where it displaces XIAP from processed caspases and promotes caspase activity (Du et al., 2000), (Ekert et al., 2001).

Synthetic caspase inhibitors have also been constructed based on the basis of synthetic substrate specificity of the different caspases and are being used in a large number of studies to discriminate between the functions of individual caspases both *in vivo* and in cell-free systems. Because caspases are cysteine proteases, reactive groups successful with cysteine proteases of the apopain family have been tagged into simple peptides in an attempt to convey specificity. These reactive groups include reversible aldehydes, and the less reversible halomethyl ketones, various acyloxymethyl ketones, and diazomethanes (Margolin et al., 1997), (Talanian et al., 1997). The first caspase-1 subfamily inhibitor was based on the tetrapeptide recognition sequence YVAD present in pro-IL-1β, the substrate of caspase 1. Similarly another inhibitor designed around the putative PARP cleavage site, DEVD, aims to be selective for the proapoptotic caspases. However, it should be noted that whereas the inhibitors containing the YVAD frame are reasonably selective for caspases 1 and 4, those based on DEVD show little specificity (Garcia-Calvo et al., 1998). The general trend seems to be that tetrapeptide inhibitors are unlikely to achieve the specificity required to allow inhibition of individual caspases.

Apoptosis-related molecules	Phenotypes of knockout mice
Caspases	
Caspase 1	Viable; resistant to lipopolysaccharide (LPS)-induced endotoxic shock and ischemic brain injury (Li et al., 1995)
Caspase 2	Viable; defects in granzyme B and perforin-mediated apoptosis of B cells, accelerated death of motor neurons (Bergeron et al., 1998).
Caspase 3	Perinatally lethal (depending on mouse strain), neuronal hyperplasia, structural disorganisation and partial resistance of mature T lymphocytes to AICD (Woo et al., 1998).
Caspase 6	Viable
Caspase 7	Embryonic lethality (Zheng and Flavell, 2000)
Caspase 8	Embryonic lethality (E11-13), impaired cardiac muscle development, embryonic fibroblasts resistant to apoptosis (Varfolomeev et al., 1998).
Caspase 9	Perinatal lethality, neuronal hyperplasia (Hakem et al., 1998).
Caspase 11	Viable; resistant to LPS-induced endotoxic shock and ischemic brain injury (Wang et al., 1998b).
Caspase 12	Viable; partially resistant to ER stress inducers (Nakagawa et al., 2000).
Adaptors	
APAF-1	Embryonic lethality (E16); severe craniofacial abnormalities and resistance to genotoxic agents but thymocytes are sensitive to Fas (Yoshida et al., 1998)
FADD	Embryonic lethality; impaired cardiomyogenesis and abdominal haemorrhage, impaired proliferation of lymphocytes and peripheral T cells to mitogens in Rag2-/- chimeras (Yeh et al., 1998).
FADD-DN	Impaired thymocyte proliferation (Newton et al., 1998).
BCL-2 family proteins	
BCL-2	Runted; die within a few months of birth; renal failure, waves of apoptosis in thymus and spleen; melanocyte death; neuronal death in postnatal period (Veis et al., 1993).
BCL-X	Embryonic lethality (E13); extensive neuronal cell death/ apoptosis of haematopoietic cells of liver (Motoyama et al., 1995).
MCL-1	Peri implantation embryonic death (E3.5-4) (Rinkenberger et al.,

	2000).
A1α	Viable; accelerated neutrophil apoptosis (Hamasaki et al., 1998).
BCL-W	Viable; male infertility; no production of mature sperm (Print et al., 1998).
BAX	Viable; modest lymphoid and neuronal hyperplasia, male infertility, increased oocyte lifespan in females (Knudson et al., 1995)
BAK	Viable, fertile (Lindsten et al., 2000).
BAX/BAK	90% perinatal lethality; splenomegaly, embryonic fibroblasts are resistant to staurosporine, etoposide, UV, and serum withdrawal as well as ER stress inducers, apoptosis after exposure to TNFα is fairly normal, adults are resistant to a-Fas-induced hepatocyte apoptosis (Lindsten et al., 2000).
BID	Viable; resistant to a-Fas-induced hepatocyte apoptosis (Yin et al., 1999)
BIM	Partial embryonic lethality before E9.5, perturbed thymic development, lymphadenopathy and splenomegaly with age (1yr) (Bouillet et al., 1999)
Regulatory molecules	
Cytochrome C	Embryonic lethalityE8.5, embryonic cell culture resistant to UV and staurosporine and partially resistant to serum withdrawal (Li et al., 2000a).
AIF	Defective body cavitation during development; ES cells resistant to growth factor withdrawal (Joza et al., 2001)
c-FLIP	Mice have cardiac defects similar to those in caspase-8 or FADD-deficient mice (Yeh et al., 2000).
p53	Viable but high incidence of spontaneous tumours (Donehower et al., 1992)

Table 1.4: *Summary of major phenotypes in mice with deletions in apoptosis-related genes* (adapted from (Ranger et al., 2001)).

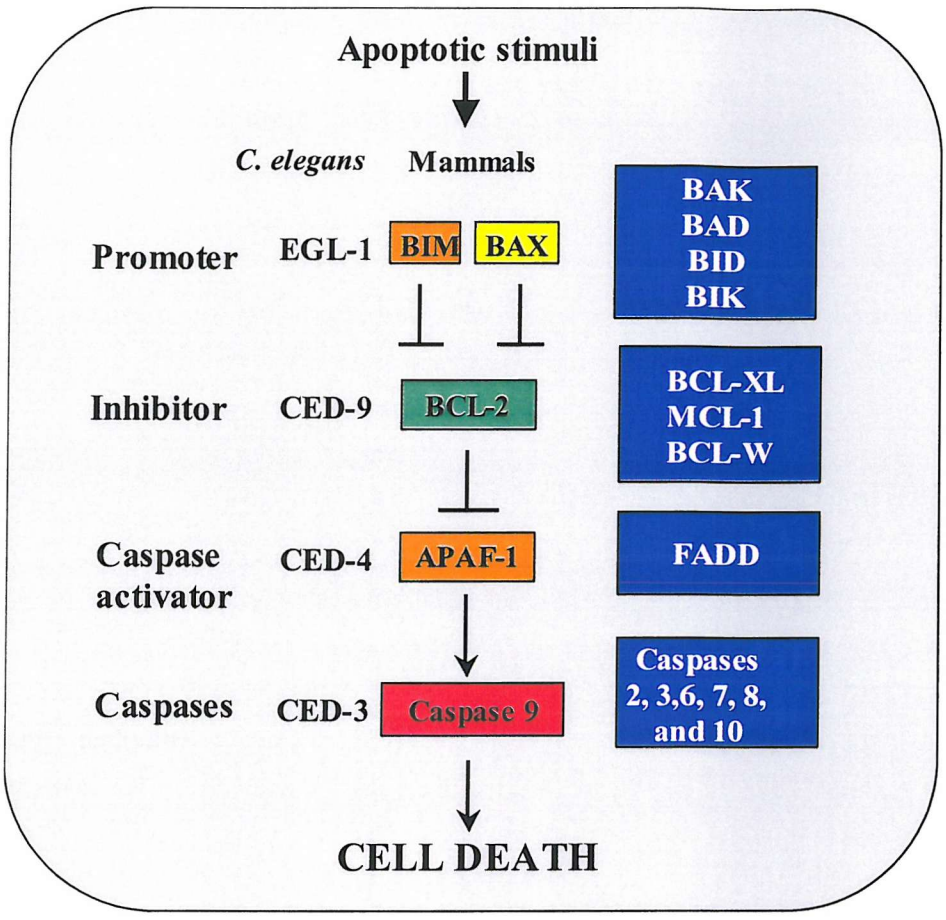


Figure 1.1: *The evolutionarily conserved pathway to cell death in mammalian cells and c. elegans.* Representative mammalian proteins modulating major steps to apoptosis are shown boxed in the center. Related proteins as well as the *c. elegans* homologues are also shown. Promoter proteins facilitate cell death whereas inhibitory proteins inhibit it. Caspase activators facilitate the activation of caspases resulting in proteolytic cleavage of essential cell substrates and cell death

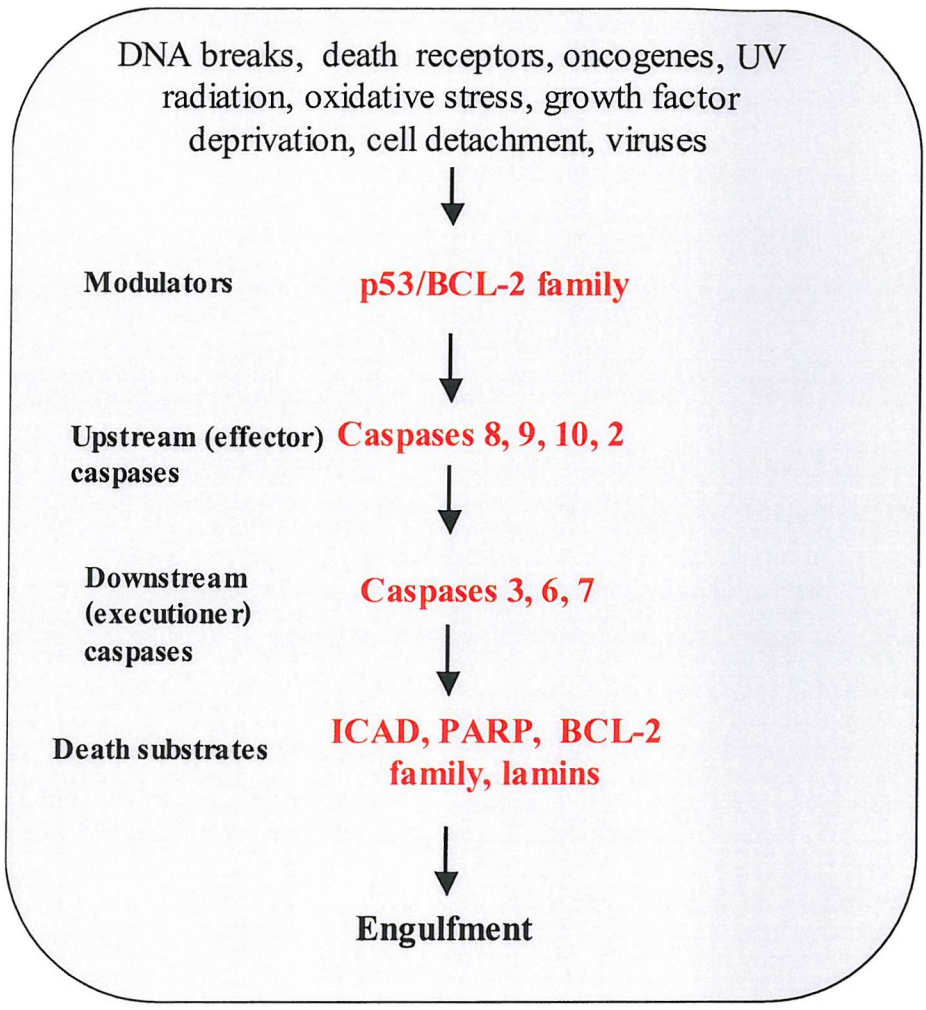


Figure 1.2: *Schematic diagram of the main sequence of events leading to apoptosis in mammals.* Various apoptotic triggers can activate upstream caspases. In turn, activated caspases cleave and activate downstream/ effector caspases. Activation of the effector caspases results in the cleavage of various intracellular target proteins leading to the disassembly of the cell and engulfment by phagocytic cells. Proteins of the BCL-2 family and p53 modulate the cells' responses to apoptotic stimuli during the 'decision' phase.

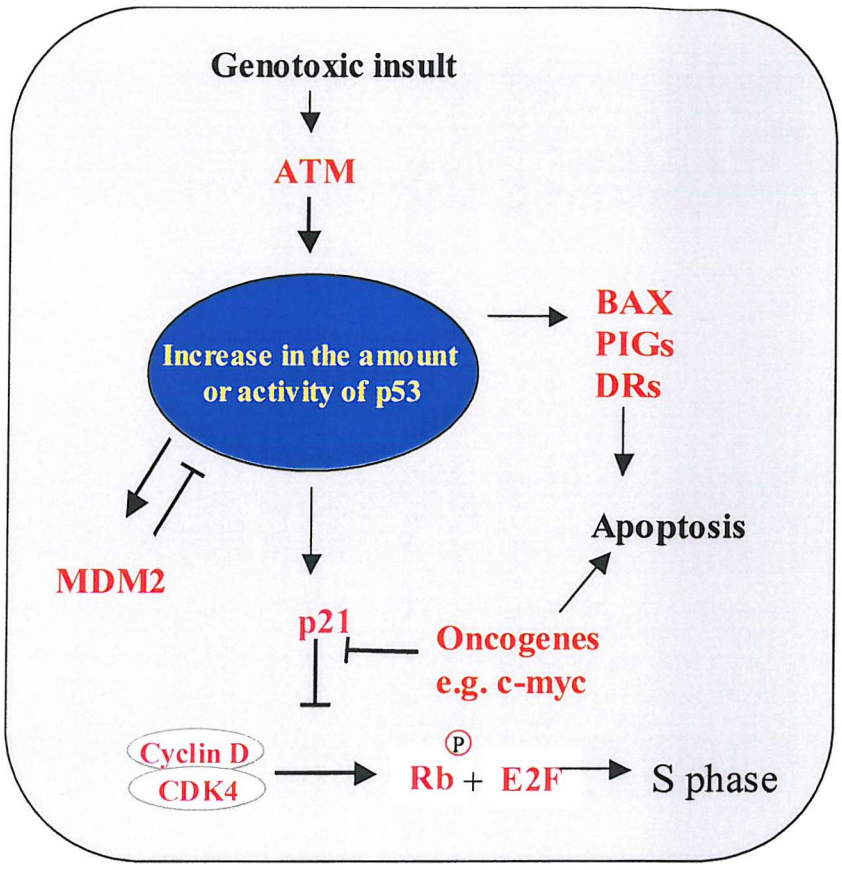


Figure 1.3: *p53-mediated pathways to cell cycle arrest and apoptosis.* Activation of p53 as a result of genotoxic insult results in the transcriptional regulation of target genes such as p21 and BAX which can influence the balance of the p53 response towards either cell cycle arrest or apoptosis.

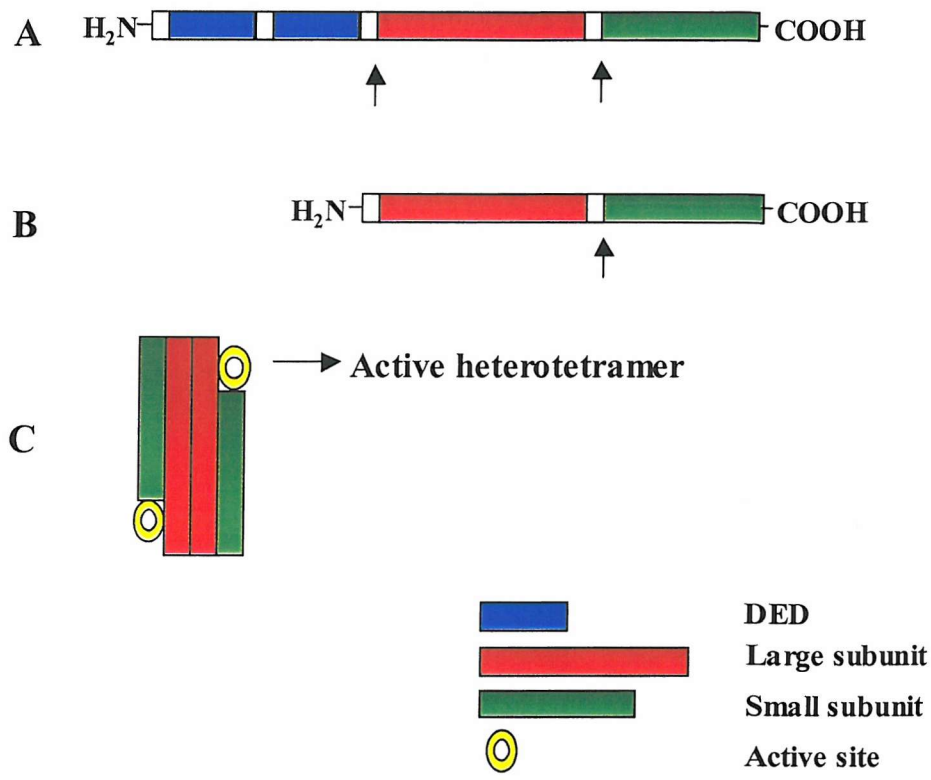


Figure 1.4: *Schematic diagram of the structure of proenzymes of caspases 8 and 3 and an assembled active enzyme.* **(A)** The caspase 8 zymogen contains two DED domains, a large and a small subunit. Other caspases may have CARD domains instead of DED domains. **(B)** Effector caspases like caspase 3 have short prodomains. Inactive zymogens can be activated by cleavage at aspartic acid residues separating the different subunits (indicated by arrows). **(C)** Hetero-tetrameric active enzymes contain two active sites arranged in positions of inverse symmetry.

1.7 Final stage of apoptosis: engulfment of dying cells.

Removal of dead and dying cells and the recycling of degradation products by the surrounding living cells is a key phase in the apoptotic process. This is carried out during engulfment. In mammals, this process generally occurs prior to the late degradative stages of apoptosis that are otherwise observed in isolated cells *in vitro*. This ensures that only minimal leakage of cellular toxic or immunogenic molecules occurs, thus avoiding generalised inflammation. Therefore, engulfment of apoptotic cells may be regarded as a profoundly important aspect of apoptosis without which apoptosis loses its underlying purpose. The cells responsible for engulfment of apoptotic cells in mammals are professional phagocytes, macrophages, as well as fibroblasts and dendritic cells (DC). The process is so efficient that in tissue sections it is difficult to find an apoptotic cell that has not already been phagocytosed. In addition, due to the fact that engulfment requires studying of two cell types, the study and elucidation of its mechanisms is lagging. In *c. elegans* elucidation of the engulfment process relies on genetic defects that interfere with the clearance of dead cells during development. There are six genes believed to regulate engulfment in this organism, ced-1, -6, -7 and ced-2, -5 and –10. Mutations in any one of these genes causes a failure in engulfment in some but not all of the apoptotic cells indicating a redundancy in the gene products (Hoeppner et al., 2001), (Zhou et al., 2001), (Chung et al., 2000).

ced-6 encodes a protein with an apparent phosphotyrosine-binding domain that may act as an adaptor in a tyrosine kinase signalling pathway whereas the ced-7 gene encodes a protein similar to ATP-binding cassette transporters (Horvitz, 1999). It is thought that ced-7 functions to transport molecules that mediate the interaction between dying cells and the cells that engulf them. ced-5 appears to act within the engulfing cells during the process of cell/corpse engulfment and encodes a protein similar to the human protein DOCK 180 and the Myoblast City protein of Drosophila (Wu and Horvitz, 1998). It has been proposed that these three proteins function in the extension of cell surfaces and in particular that CED-5 functions in the extension of the surface of the engulfing cell around the dying cell (Wu and Horvitz, 1998).

Another gene, nuc-1 (nuclease abnormal-1), has been identified as essential for the normal degradation of DNA in dying cells (Hedgecock and Herman, 1995).

Cells undergoing apoptosis display recognition signals on their cell surface to attract phagocytic cells. Such signals include the well-characterised externalisation of PS residues, an established apoptotic marker. Other apoptosis-related cell surface structures include sugar molecules, the adhesion molecule ICAM-3 and complement components. The series of apoptosis-related surface exposed molecules have been recently termed ACAMPs (apoptotic-cells-associated-molecular patterns), however, their nature is poorly defined (Gregory, 2000). In addition, a number of receptors that serve to promote recognition have been described in mammals; they are heterogeneous in nature and include proteins such as integrins, lectins, the PS receptor and several scavenger receptors thought to be involved in the recognition of PS residues. Macrophages, in particular appear to utilise at least five classes of receptor in recognition and phagocytosis of apoptotic cells: the $\alpha_v\beta3$ vitronectin receptor (VnR), SR-A, ABC1, PS-R and CD1 b (Gregory, 2000). Recognition of apoptotic cells is likely to be a co-operative event involving several receptors, which could well function simultaneously or sequentially. Of these, the role of CD14 in recognition and engulfment of apoptotic cells is becoming clear, after its original identification by antibody that could inhibit binding of apoptotic B cells to human macrophages (Gregory, 2000).

1.8 TNF and TNF receptor superfamilies

Mammals have evolved mechanisms that enable them to actively direct individual cells to commit apoptosis and death receptors play a central role in this type of instructive apoptosis. They are directly linked to the intracellular execution machinery and can activate caspases within seconds of ligand binding, causing rapid cell death without the need for transcriptional or translational activity (in susceptible cells). These receptors form part of the TNFR superfamily and their respective ligands form part of the TNF superfamily. This system of apoptosis induction first came to light with the discovery in the 1970s of a bacterially-induced circulating factor that correlated with remission of tumour patients and induced antitumour activity in tumour transplants in mice (Carswell et al., 1975). This factor, designated tumour

necrosis factor, was subsequently cloned and finally emerged as the prototype of a family of similar molecules that are involved in immune regulation and inflammation. The study of cell responses to ligands of the TNF family is one of the most dynamic research areas in the signalling field today. The pattern of activities mediated by this group of ligands and their receptors displays significant complexity as well as distinct effects, which range from destruction of tissues to regulation of immune organogenesis and homeostasis (Smith et al., 1994), (Screaton and Xu, 2000).

With the exception of LT-α and nerve growth factor (NGF), which are secreted, all members of the TNF superfamily are formed as type II transmembrane proteins, with an extracellular C-terminus. They contain a short intracellular segment and a long extracellular region between 140 to 215 amino acids in length (Smith et al., 1994). Some of them are subject to proteolytic processing by metalloprotease enzymes allowing them to act in a soluble form. The so far identified members of the TNF family are: TNF-α and TNF–β (LT-α), LT-β, NGF, CD40L, mouse CD137/4-1BB L, CD134L/OX40L, CD27L, FasL, CD30L, TRAIL and the newest members of the family, APRIL (Yu et al., 2000), BAFF/BlyS (Moore et al., 1999), TWEAK and LIGHT (Chicheportiche et al., 1997). The evidence available so far suggests that TNF family members act in the form of trimers, most of them acting as homotrimers. The only known exception is LT-β, which functions after forming heterotrimers with LT-α, apparently because of its inability to assemble properly on its own. Homotrimers display receptor-binding sites at the boundaries between monomers. The ligand can therefore bind three times its respective receptor at three interaction sites, leading to receptor oligomerisation and initiation of downstream signalling events.

A large variety of signalling pathways are activated by members of the TNF family including activation of caspases, the translocation of the nuclear factor-κB (NF-κB) or the activation of mitogen-activated protein kinases, such as c-Jun N-terminal kinase (JNK). TNF acts as an inflammatory cytokine, coordinating host defences in response to pathogens whereas the LT system is crucial for the development of peripheral lymphoid organs and the organisation of the spleen (Shakhov and Nedospasov, 2001). FasL, TNF and CD30 are responsible for T-cell receptor-mediated apoptosis of T cells and immature thymocytes (Amakawa et al., 1996). Several members in conjunction with the T cell receptor enhance T cell proliferation. Upregulation of family members in T cells is important for the activation and

stimulation of neighbouring cells. CD40L is required for B cell proliferation and differentiation and for isotype switching. VEGI appears to play a role in the physiology of vascular epithelial cells and RANKL/OPGL is involved in osteoclast generation and bone metabolism (Locksley et al., 2001).

Each member of the TNF superfamily binds at least one member of the TNFR superfamily. The known members of the TNFR superfamily are: TNFR1 and 2, CD27, CD30, 4-1BB/CD137, RANK, OPG, Fas/APO1/CD95, death receptor-3 (DR3)/LARD/Wsl-1, TRAIL receptor-1 (TR-1)/DR4, TR-2/DR5, DR6, LT-β receptor, low affinity nerve growth factor receptor (LNGFR/p75), AITR and HVEM, BCMA (Thompson et al., 2000), TACI (Xia et al., 2000) and RELT (Sica et al., 2001). The receptors are type I transmembrane proteins with an intracellular COOH-terminal domain and sequence similarities mainly confined to the extracellular region. They share similar extracellular domain architecture of multiple cysteine-rich repeats (2-6) that constitute the ligand-binding region. Disruption of these repeats generally leads to the loss of ligand binding and effector signalling (Orlinick et al., 1997). The extracellular domains are usually preceded by hydrophobic signal peptides. In addition, soluble receptors can be generated by deletions of the transmembrane and intracellular domains. The death receptor subgroup also contains a cytoplasmic domain responsible for apoptosis induction termed the death domain (DD). Certain ligands and receptors can bind to more than one partner thus enhancing the regulatory flexibility and complexity of the two groups.

A new nomenclature has recently been proposed to provide an integrated picture of the TNF ligand and receptor families and the interactions between them. The new nomenclature system denotes each ligand as TNFSFN and each receptor as TNFRSFN, where N is a specific number. Known members of the TNFR superfamily and their respective ligands including their assignments under the new nomenclature scheme are listed in Table 1.5.

1.8.1 Death Receptors

Currently the most rapidly expanding area of knowledge involves the cell killing activity of the death receptors. The recognition event with their respective ligand is translated, via their DDs, to a death signal that engages the death machinery (e.g. caspases and/or AIF) and leads to apoptosis. The death domain, a 60-80 aa stretch

that is composed of six amphipathic α-helices arranged antiparallel to each other, is essential for the transmission of the death signal. It does not have any enzymatic activities, but instead mediates signalling through protein-protein interactions, mainly with adaptor proteins (Huang et al., 1996), (Baker and Reddy, 1998). The so far identified death domain-containing receptors include TNFR1/p55, Fas, DR3, DR4, DR5 (Wu et al., 1997c) and DR6 (Pan et al., 1998a) among which, Fas is the best characterized. Closely related to the death receptors are the so-called decoy receptors that can bind the respective ligand without delivering a death signal. These receptors can be either membrane bound; TRID (TRAIL-R3/TR3) and TRUNDD (TRAIL-R4/TR4), Fas decoy receptor (FDR), or soluble; osteoprotegerin (OPG) a decoy receptor for OPGL and TRAIL and DcR3 a decoy receptor that binds to Fas and LIGHT (Pitti et al., 1998).

Adaptor proteins linking the cell death receptors, the caspases, and the apoptosis regulators such as the BCL-2 family members, form an essential part of death receptor signalling. These links take the form of physical associations between members of the three classes of molecules, with the adaptor molecules forming the necessary bridge between caspases and the upstream regulators of apoptosis. DED and CARD motifs, as found on caspase prodomains, also provide the basis for interactions with death receptors leading to caspase activation. Following cross-linking of a death receptor, homotypic interactions between its DD and that of the adaptor as well as interactions between DED motifs on the adaptor and caspase molecules allow caspase recruitment and activation. Mammalian procaspases 9 and 2 as well as CED-3 contain CARDs, which are also present in their specific adaptors, APAF-1 and CED-4, and mediate recruitment in the same way as DEDs. In the same way, other molecules containing DED domains can interact with the adaptor molecules and potentially inhibit the interaction with caspases.

1.8.2 Death receptors DR3 and DR6

Very little is currently known on the biological function and physiological importance of two members of the death receptor subfamily, DR3 and DR6. DR6 was identified by homology searching of expressed sequence tag databases using the extracellular, ligand binding domain of TNFR2. Analysis of the predicted open reading frame revealed a death domain (Pan et al., 1998a). DR6 mRNA expression

was found to be abundant in a variety of normal tissues such as heart, brain, pancreas and thymus as well as among human cancer cell lines such as the cervical carcinoma HeLa, colorectal adenocarcinoma SW480 and the lung carcinoma A549. Interestingly, little or no expression was detected in healthy human peripheral blood and tumour cell lines of lymphoid origin. Ectopic expression of this receptor induced apoptosis in HeLa cells, and activation of JNK in a murine cell line (Pan et al., 1998a).

DR3 was reported to be the first member of the death receptor family with a restricted pattern of expression; mainly in lymphoid organs. More recent studies, however, have shown its expression to be less restricted, however such studies are restricted by the presence of at least 13 human and 3 mouse splice variants (Kitson et al., 1996), (Screaton et al., 1997b). Its ligand has not been identified yet, despite previous beliefs (Kaptein et al., 2000), (Kitson et al., 1996). DR3, similarly to TNFR1, recruits both TRADD and FADD as downstream effectors of apoptosis (Bodmer et al., 1997), (Boldin et al., 1995), (Chinnaiyan et al., 1995). It also interacts with TRADD to recruit TRAF2and thus activates NF-κB. Mice deficient in one homologue, show no defects in organ development, lymphoid proliferation, or apoptosis induced by DNA-damaging agents, however negative selection and anti-CD3-induced apoptosis are impaired, suggesting a role for central tolerance *in vivo* (Wang et al., 2001).

1.8.3 The TNF/ TNFR1 system of apoptosis induction

The prototypical member of the TNF family, TNF-α exists in both membrane-bound and soluble form and is a potent immunomodulatory agent. It induces a particularly broad spectrum of events including lymphocyte and leucocyte activation and migration, fever, acute phase response, cell proliferation, differentiation and apoptosis. Soluble TNF-α is released from cell surfaces as the result of metalloprotease cleavage. Although both cell surface and secreted TNF-α appear to be biologically active, it is the soluble TNF-α released into circulation that appears to be primarily responsible for the deleterious physiological responses such as cachexia and endotoxic shock, conditions most often associated with it. TNF-α is expressed mainly on activated macrophages and T cells in response to infection but also on other tissues including mammary and colon epithelia, neurons and the pancreas (Wallach, 1997). Unlike TNF-α, TNF-β/LT-α does not have a membrane bound form and exists as a soluble molecule unless complexed with another ligand, LT.

The extracellular region of the two TNF receptors, TNFR1/p55 and TNFR2/p75, contains four cysteine-rich domains (CRDs). TNFR1 is the death domain containing receptor, which is able to bind onto both TNF-α and -β. Among the numerous cell types known to express TNF-R1 are hepatocytes, monocytes and neutrophils, cardiac muscle and endothelial cells (Screaton and Xu, 2000). TNFR1 has been regarded as the primary signalling receptor for TNF-α inflammatory responses, because TNFR1-deficient mice are resistant to endotoxic shock (Rothe et al., 1993). The crystal structure of the complex between the extracellular domain of TNFR1 and TNF-β (Naismith et al., 1996) first revealed the structural basis of ligand-induced receptor oligomerisation of the TNF receptor family. The complex consists of three soluble TNFR1 (sTNFR1) molecules bound to one TNF-β trimer. Binding of the ligand induces trimerisation and recruitment of several signaling proteins to the cytoplasmic domains of the receptors. The first molecule to be recruited is TRADD (TNF receptor-associated death domain), which serves as a platform for the recruitment of at least three additional mediators, RIP (receptor-interacting protein), FADD (Fas-associated death domain) and TRAF2 (TNF receptor-associated factor 2) (Rothe et al., 1995), (Chinnaiyan et al., 1995). TRAF2 and RIP stimulate pathways leading to the activation of JNK and NF-κB, whereas FADD mediates apoptosis by activating the caspases (Bang et al., 2000). NF-κB, a transcription factor, directs transcription of a large number of genes involved in regulation of cell growth, transcription factors, cytokines and IAPs. Inactive NF-κB is present in the cytoplasm complexed with an inhibitory protein, IκB which is released upon activation, leaving NF-κB to be transported to the nucleus to exert its function. Complete and persistent inhibition of NF–κB has been linked to apoptosis, inappropriate immune development and delayed cell growth. JNK is part of the mitogen-activated MAPK pathway, which leads to the activation of the transcription factor AP-1. Furthermore, JNK activation preferentially promotes cell survival pathways downstream of TNFR (Liu et al., 1996b). Apoptosis activation via FADD is probably the main route to apoptosis downstream of TNFR1 (Bang et al., 2000; Wajant et al., 1998). Signalling through the TNF receptors, however, can have pleiotropic effects and a variety of molecules are proposed to take part in signalling pathways originating in TNF receptor molecules and leading to a wide range of cellular responses.

Interestingly, recent evidence suggests that TNFR chains pre-assemble into complexes on the cell surface prior to ligand binding. The formation of oligomers requires part of the extracellular part of the molecules, including the first CRD. This region termed PLAD for 'pre-ligand assembly domain' is necessary and sufficient for self-assembly. The PLAD is distinct from the ligand binding domain and PLAD interactions are highly specific and allow only receptor homotrimers (Chan et al., 2000).

1.9 Fas-mediated apoptosis

Whereas the prototypic member of the death receptor family, TNFR1 can induce a variety of cellular responses, other members, mainly Fas and the TRAIL receptors, appear to be mainly involved in apoptosis induction and thus constitute more appropriate systems for the study of apoptosis. The ligand for Fas receptor, FasL or CD95L was purified in 1993 from a purified fraction of a cytotoxic T cell line that was observed to kill cells expressing Fas but not the cells that did not (Suda et al., 1993). Under physiological conditions, Fas ligand causes apoptosis via binding to its cognate receptor, Fas receptor (Suda et al., 1993), (Ju et al., 1995). FasL expression is restricted; it has been detected on activated T cells, natural killer (NK) cells, and on immune privileged tissues, like the Sertoli cells of the testis, the anterior chamber of the eye, the placenta and brain (Hahne et al., 1996), (Kayagaki et al., 1995), (Zamai et al., 1998), (Schulze-Osthoff et al., 1998). FasL is a 40 KDa type II membrane protein that acts as a homotrimer and can be cleaved by a metalloprotease to generate a 26-29 KDa soluble fragment (Tanaka et al., 1998). Such post-translational modifications of FasL have profound influence on its activity because only membrane-bound or multimerised Fas ligand can induce cell death, whereas soluble Fas ligand may even inhibit it (Tanaka et al., 1998). *In vivo*, mice injected with sFasL (soluble Fas ligand) survived, showing no signs of sickness. In contrast, injection of sFasL followed by cross-linking antibodies was fatal and the mice died within 3 hours similarly to mice treated with α-Fas antibodies (Ogasawara et al., 1993) or with FasL contained in supernatants of FasL-transfected cells (Rensing-Ehl et al., 1995). The cross-linked soluble FasL induced lethal liver haemorrhages and hepatocyte apoptosis, whereas non cross linked sFasL had no effect on the morphology of hepatocytes (Schneider et al., 1998). Both membrane-bound and naturally processed soluble FasL occur as trimers

suggesting that the aggregation of more than three Fas molecules is required to transduce the death signal efficiently.

Fas/APO-1/CD95, henceforth called Fas, is characterised by three cysteine-rich domains (CRD1-3) in the extracellular region and the presence of the death domain in the intracellular region (Huang et al., 1996). Discovery of Fas was preceded by the discovery of monoclonal antibodies called Fas (Kobayashi et al., 1990) and APO-1 (Trauth et al., 1989), which induced apoptosis when added to various malignant and nonmalignant cell cultures. Two years later, Fas was cloned (Itoh et al., 1991). The gene for Fas is located on chromosome 10q24.1 and comprises 9 exons and 8 introns (Lichter et al., 1992). It encodes 325 amino acids, giving rise to a glycosylated cell surface molecule of approximately 45-52 KDa (Watanabe-Fukunaga et al., 1992b). The primary structure of Fas is shown in Figure 1.5. Compared to FasL, Fas expression is relatively widespread, with abundant levels detected in the thymus and in non-lymphoid tissues such as the liver, heart, lung, kidney and ovary (Watanabe-Fukunaga et al., 1992b) (Schulze-Osthoff et al., 1998). Fas is also highly expressed on mature thymocytes or lymphocytes transformed with HTLV-1, HIV and EBV viruses (Kobayashi et al., 1990).

1.9.1 Activation and signalling via Fas

Fas binds to FasL with the CRD1 and 2 domains providing the major contact surfaces for their interaction (Starling et al., 1997). Fas cross-linking by the trimeric ligand leads to clustering of the receptors' death domains. This is supported by nuclear magnetic resonance structure analysis and mutagenesis studies (Huang et al., 1996). Multimerisation of Fas is a prerequisite for the transduction of the apoptotic signal as Fas dimers do not induce apoptosis (Spencer et al., 1996). The requirement for the formation of large aggregates of Fas for signal transduction is also suggested by the efficacy of agonistic antibodies of either IgM or IgG3 class, which are widely used to induce apoptosis. Recently, use of fluorescence resonance energy transfer has resulted in a new model that challenges the trimeric nature of FasL/Fas complexes. Similarly to TNFR1, extracellular PLADs were described for Fas, thought to lead to receptor aggregation, independently of ligand binding (the PLAD region of Fas is indicated in Figure 1.5). To prevent the premature signalling of pre-associated receptors, intracellular receptor-associated inhibitors of signalling were postulated (Chan et al.,

2000), (Siegel et al., 2000). The new model is supported by observations of dominant negative interfering of Fas mutants that cannot bind FasL since they lack a part of CRD2 (Siegel et al., 2000), however, it does not provide a mechanism for activation of downstream signalling upon ligand binding.

In contrast to the extracellular events initiating Fas receptor signalling, the available evidence supports a single model for translation of the ligand-binding signal into a death signal in the intracellular space. The receptor's death domains are essential for transmitting the death signal to the intracellular machinery. For the apoptotic signal to be propagated, the adaptor molecule FADD, containing a DD and a DED binds to Fas through a DD-DD interaction. The N-terminal region of FADD, containing the DED then recruits the DED-containing caspase 8 to form a multiprotein complex termed DISC (Death-Inducing Signalling Complex) (Kischkel et al., 1995). Despite alternative suggestions regarding the participating molecules in Fas DISC assembly, FADD and caspase 8 are thought to be the essential components of this complex.

FADD/MORT1 was identified from a yeast two-hybrid screen (Boldin et al., 1995), (Chinnaiyan et al., 1995) and it is essential for transmission of the death signal. The evidence comes from knockout mice and transgenic mice expressing a dominant negative form of FADD (FADD-DN) where Fas-induced apoptosis was completely abrogated (Newton et al., 1998), (Yeh et al., 1998). FADD-deficient mice die at day 9 of gestation, with a deformed cardiac muscle (Table 1.4) indicating that FADD is essential for embryonic development. Furthermore, peripheral T cells from FADD-deficient chimeric mice showed reduced proliferation in response to mitogens *in vitro* indicating an involvement of FADD in an as yet uncharacterised survival pathway (Newton et al., 1998). Downstream of FADD, DISC formation also includes caspase 8. Identification of caspase 8 in the Fas DISC directly linked Fas to caspase activation (Muzio et al., 1996). Following Fas activation and under favourable conditions, the entire cytoplasmic caspase 8 is converted into active caspase 8 subunits at the DISC (Medema et al., 1997) (Figure 1.6). Caspase 8 is thought to be auto-proteolytically processed at the DISC, according to the induced proximity model (Muzio et al., 1998), to form two intermediate species of 43 and 41 KDa respectively as well as the active subunits, p10 and p18 (Medema et al., 1997), (Scaffidi et al., 1999a). Although eight different isoforms of caspase 8 are predicted from sequence analyses, studies with

monoclonal antibodies have shown that only two are predominantly expressed in the 13 different cell lines tested, caspases 8a and 8b (approximately 55 and 57 KDa respectively) (Scaffidi et al., 1997). Both isoforms are recruited to the DISC and are processed with similar kinetics.

However, the exact stoichiometry of this system has not been determined, and it may be that a large number of FADD molecules serve to aggregate sufficient numbers of caspase 8 molecules to induce autocatalytic processing. While this process of induced proximity is sufficient for caspase 8 activation in artificial systems, it is possible that factors other than local concentrations (for example, conformational changes induced by binding to FADD) also play a role in Fas-induced pro-caspase 8 activation. Caspase 8 activation is followed by cleavage of other caspases, such as the downstream effector caspases, 3, 6, 7, 2 and 9 in a caspase cascade (Sun et al., 1999) (Holler et al., 2000). Activated caspase 3 and other effector caspases then cleave their apoptosis-specific targets such as PARP, lamins, ICAD, fodrin, gelsolin, and DNA-PK to execute Fas-mediated apoptosis (Lens et al., 1998), (Eischen et al., 1997). A functional role for caspase 10 as part of the complex has also been suggested in early reports (Vincenz and Dixit, 1997); evidence from transgenic mice, however, suggests that caspase 8 is the essential caspase acting proximal to the Fas receptor (Varfolomeev et al., 1998).

Yeast two-hybrid screens with the cytoplasmic domain of Fas as bait also revealed an association with DAXX and overexpression of DAXX activated JNK and potentiated Fas-induced apoptosis (Yang et al., 1997). On the basis of this it has been proposed that Fas engages two independent pathways that induce cell death: one pathway proceeds via FADD and caspase activation and the other via DAXX/JNK activation. However the relevance of this interaction to the apoptotic function of Fas is not established and the same is true for two more molecules previously reported to associate with the Fas receptor, RIP and RAIDD. It is likely that these molecules may participate in Fas-induced apoptosis in certain cell types, but are not needed in the same way that FADD appears to be essential. Furthermore, DAXX seems to play a growth-promoting role with little influence on Fas-induced apoptosis. A study using dominant negative constructs against these molecules showed that their absence makes no difference to sensitivity to Fas in Jurkat, CEM and SKW6 cells whereas absence of FADD markedly reduces Fas-induced apoptosis in these cell lines (Villunger et al.,

2000). Furthermore, mice deficient in DAXX die *in utero* due to abnormally increased apoptosis in critical organs and cells from these embryos are normally sensitive to Fas-mediated apoptosis (Michaelson et al., 1999). Finally, human DAXX seems to be confined at the nucleus where it would not be easily available for Fas signalling (Pluta et al., 1998).

Over the past few years, it has become clear that a cross-talk mechanism links the central Fas signalling pathway, involving caspase 8, and the mitochondrial pathway with caspase 9 as the apical caspase. In comparisons of different cell lines with respect to signalling pathways triggered by α-Fas antibodies, two cell types have been identified, termed type I and type II cells. In type I cells (typified by a number of lymphoid cell lines), induction of apoptosis is accompanied by activation of large amounts of caspase 8 at the DISC. This is followed by rapid cleavage of caspase 3 prior to loss of mitochondrial membrane potential. In contrast, in type II cells, DISC formation is strongly reduced and strong activation of caspases occurs after the loss of mitochondrial membrane potential (Scaffidi et al., 1998). Nevertheless, both types of cells display similar kinetics of apoptosis and the two pathways are initiated regardless of whether Fas activation is induced by agonistic antibodies or by FasL. Furthermore, similar events take place: disruption of the mitochondrial membrane potential with similar kinetics, and cytochrome c release. In both situations the apoptogenic activities of the mitochondria are blocked by overexpression of BCL-2. However, in type II cells BCL-2 overexpression blocked caspase 8 and 3 activation as well as apoptosis. These observations suggest that in Type II cells strong activation of caspase 8 and 3 occurs downstream of the mitochondria and depends on the mitochondrial release of apoptogenic factors such as cytochrome c and perhaps AIF (Scaffidi et al., 1998). In contrast, in Type I cells caspase 8 may be activated directly at the level of the DISC, and proceeds directly in the activation of downstream caspases; therefore, Fas-mediated apoptosis occurs independently of the mitochondrial pathway and BCL-2 proteins. Immunoprecipitation (IP) experiments supported this by showing DISC formation after Fas ligation is reduced in Type II cell lines while in type I cells DISC formation is stronger (Scaffidi et al., 1998).

Type II cell apoptosis after ligation of Fas involves a BCL-2 family member, BID, which provides the intermediate between the two pathways. BID, a 22 KDa BH3-only molecule is cleaved at its amino terminus (Li et al., 1998), (Luo et al.,

1998), (Gross et al., 1999b). Cleavage of cytosolic BID by caspase 8 generates a fragment of approximately 14 KDa that translocates to the mitochondria. Truncated p14 BID (tBID) inserts into the membrane and immunodepletion of tBID from subcellular fractions suggested that tBID is required for cytochrome C release from the mitochondria (Luo et al., 1998). However, apoptosis triggered through Fas was unaffected in lymphoid cells from BID-deficient mice, although liver cells were resistant to α-Fas antibody treatment (Yin et al., 1999). In support of this observation, no major differences in Fas-induced apoptosis were observed in T cells from caspase 9 and APAF-1-deficient mice (Kuida et al., 1998). This evidence suggests that, since activation of BID occurs downstream of caspase activation, the point of irreversible commitment to apoptosis, it may not be essential for apoptosis in all tissues, but may be required to amplify the caspase cascade in cells with low amounts of caspase 8 or a weak initial stimulus. Furthermore, treatment of BID-deficient mice hepatocytes with cycloheximide rendered the previously resistant cells sensitive to Fas-induced apoptosis (Yin et al., 1999) indicating that, in certain cell types, this alternative pathway may be needed to overcome the inhibition caused by a receptor-proximal inhibitory factor with a short half-life. A schematic diagram of the Fas-activated pathways leading to apoptosis is shown in Figure 1.7.

1.9.2 Regulation of Fas-mediated apoptosis

Since Fas expression is widespread regulatory mechanisms protecting cells from inappropriate initiation of apoptosis would be expected. Resistance to Fas signalling has been observed in various cell types that express Fas on their surface, mainly of tumour origin, thus being of interest in therapeutic interventions for cancer. Fas-induced apoptosis can be blocked at various points: at the receptor level, by receptor endocytosis or by generation of soluble FasL molecules, and intracellularly during signal transduction or at the effector stage by caspase inhibition. In addition, Fas expression and function can also be positively regulated by pro-apoptotic signals. Some of the more important regulatory mechanisms proposed to be operating in the Fas pathway, are outlined below:

1. Regulatory factors acting at the level of the receptor include alternatively spliced Fas molecules. Normal human lymphocytes have been reported to express, in addition to the full-length, less abundant and shorter Fas mRNA

species. Most of these variants are expected to code for truncated forms either lacking the intracytoplasmic domain or transmembrane domain and existing in soluble forms (Cascino et al., 1996a). The characterisation of the appropriate antibodies has allowed the detection of such variants in human serum (Cascino et al., 1996a; Cascino et al., 1996b). Elevated levels of soluble Fas variants have been found in over 50% of patients with systemic lupus erythrematosus (SLE) and are capable of inhibiting Fas-mediated apoptosis as well as altering lymphocyte development and proliferation in response to self antigen (Cheng et al., 1994). They are also associated with poorer prognosis in adult T cell leukaemia (Kamihira et al., 1999). More recently, another soluble molecule was described, DcR3 (Pitti et al., 1998). DcR3 is a 35-KDa protein that lacks the transmembrane domain and is secreted into the extracellular space. Although its physiological role in unclear, it binds to FasL with an affinity that equals that of Fas and it is believed to function by competing for FasL binding. A high percentage of DcR3 amplifications were detected in colon and lung carcinomas (Pitti et al., 1998) and it is expressed in the majority of malignant gliomas *in vivo* and *in vitro* and human glioblastoma *in vivo* (Roth et al., 2001). In addition, overexpression of DcR3 in a rat glioblastoma model resulted in reduced immune infiltration (Roth et al., 2001).

2. A membrane-bound Fas decoy receptor (FDR) that lacks a death domain (as a result of alternative splicing), is expressed on human thymocytes and acts as a dominant negative inhibitor of Fas signalling. Unlike most alternative Fas receptors generated by alternative splicing, FDR is not secreted. It can bind FasL with nearly wild type affinity and is expressed predominantly on the cell surface (Jenkins et al., 2000).
3. Soluble FasL can also interfere with Fas signalling. FasL can be shed from the cell surface due to the action of metalloprotease enzymes. Soluble FasL is approximately 1000-times less efficient in apoptosis induction, may compete with the membrane-bound form and cannot induce apoptosis in lymphocytes (Schneider et al., 1998), (Tanaka et al., 1998).
4. Downregulation of receptor expression is another mechanism of biological regulation. Adenoviruses express two proteins, RID and E1B-19K that force

the internalisation of surface Fas and cause its destruction inside lysosomes thus inhibiting apoptosis (Tollefson et al., 1998), (Shisler et al., 1997).

5. Upregulation of Fas surface expression by p53 overexpression or induction by anti-cancer drugs, in transformed cell lines has been reported to result in apoptosis (Bennett et al., 1998) (Muller et al., 1998). Furthermore, p53-responsive elements in the Fas promoter have been identified indicating that p53 can directly regulate Fas expression (Muller et al., 1998). In addition, p53 has been suggested to induce increased expression of Fas on the cell surface by a membrane-trafficking mechanism in vascular smooth cells and fibroblasts in which Fas is located predominantly in the cytoplasm (Bennett et al., 1998). However, other studies have argued against a role for p53 upstream of Fas. For instance, cells from gld and lpr mice have shown unimpaired p53-mediated responses (O'Connor et al., 2000), (Fuchs et al., 1997).
6. A positive regulator of the Fas/FasL system appears to be c-MYC, a transcription factor involved in oncogenesis. Treatment of T cell hybridomas with antisense nucleotides against c-myc or the dominant-negative c-MYC binding protein MAX, completely blocked activation-induced cell death (AICD) and FasL expression (Shi et al., 1992), (Bissonnette et al., 1994). In a more recent study a newly identified microbial metabolite, which specifically inhibits the expression of c-myc in fibroblasts, could inhibit AICD in hybridomas and reduce FasL expression (Wang et al., 1998a).
7. Fas activation may result in the activation of proteins known to be involved in cell proliferation and survival signalling such as NF-κB, JNK and c-fos (Siegmund et al., 2001). NF-κB activation can inhibit Fas-induced apoptosis in Jurkat cells and it may be responsible for the resistance of recently-activated T cells to AICD since it is only expressed transiently during T cell activation (Rivera-Walsh et al., 2000). NF-κB activation can also upregulate FLIPL, a potent inhibitor of Fas signalling (Micheau et al., 2001). However, the precise role of the transcription factor in Fas-induced apoptosis is not very straightforward since NF-κB has also been reported to enhance apoptosis and to upregulate FasL. Two NF-κB sites have been identified on the FasL promoter (Matsui et al., 1998) and activation of NF-κB or

transcription factors such as AP-1 are reported to be involved in the regulation of FasL expression (Kasibhatla et al., 1998).

8. Inhibitors of transcription or translation enhance Fas-induced apoptosis in certain cases, perhaps by removing labile inhibitors with a short half-life. The most likely candidate for this role is c-FLIP, a molecule that disrupts normal DISC function. Similarly to the IAPs, the first c-FLIP homologues the v-FLIPs, were identified in γ-herpesviruses (Hu et al., 1997). v-FLIPs are composed of two DED domains, with a structure that resembles the N-terminal half of caspase 8. Their inhibitory function is mainly carried out via DED-DED interactions at the early stages of signalling; v-FLIPs have been postulated to interact with FADD in such a way as to inhibit recruitment and activation of caspase 8 (Thome et al., 1997), (Djerbi et al., 1999). Analysis of the human molecule has revealed several c-FLIP splice variants at the mRNA level whereas at the protein level, only two isoforms designated FLIP long and short have been detected so far, with FLIPL being the predominant isoform (Irmler et al., 1997), (Goltsev et al., 1997), (Scaffidi et al., 1999b). FLIPL, a 55 KDa protein, contains an entire caspase domain mutated at the catalytic site as well as the S1 binding site, in addition to the DED domains. FLIPL is therefore proteolytically inactive. Similarly to caspases, FLIPL contains a conserved aspartic-acid cleavage site, which can be cleaved *in vivo* in the context of Fas activation. The short form, FLIPS (27 KDa) resembles v-FLIPs in structure and contains two DEDs and a C-terminal extension of approximately 50 amino acids. Both isoforms can be detected at the DISC following Fas receptor activation. In c-FLIP overexpressing cells, both caspase 8 and FLIPL are partially processed at the receptor level, into the small protease subunits of approximately 10 KDa and the remaining 43 KDa portions of either molecule which remain bound to the receptor (Scaffidi et al., 1999b). By contrast, a FLIPS/caspase 8 complex prevents any cleavage of caspase 8 (Krueger et al., 2001). The small amounts of c-FLIP present in most cell lines examined are only able to block a few receptor complexes and caspase 8 is still sufficiently activated at the remaining DISCs, leading to apoptosis (Medema et al., 1997). In fact, the ratio of caspase 8 relevant to FLIPL expression has been suggested to control

susceptibility to Fas-induced apoptosis (Tepper and Seldin, 1999). c-FLIP isoforms are expressed in short-term activated T cells, at the time when they are resistant and disappear when T cells become susceptible to FasL-mediated apoptosis, suggestive of a regulatory role (Irmler et al., 1997). c-FLIP mRNA expression is predominant in the heart, skeletal muscle, spleen and peripheral blood leucocytes. FLIP expression is reduced following T and B cell or macrophage activation, which corresponds to an increase in their sensitivity to death receptor ligation (Irmler et al., 1997), (Algeciras-Schimnich et al., 1999), (Perlman et al., 1999). Studies on the physiological role of FLIP have provided contradicting evidence. Surprisingly, c-FLIP-deficient mice have a cardiac defect similar to mice deficient in FADD or caspase 8 (Yeh et al., 2000) as well as defects in lymphocyte proliferation (Table 1.4). In contrast, c-FLIP deficient embryonic fibroblasts have a specifically enhanced sensitivity to death receptor–induced apoptosis. Thus although FLIP may cooperate with FADD and caspase 8 during cardiac development, it is a negative regulator of death receptor mediated apoptosis in other cell types (Yeh et al., 2000).

9. Following the discovery of the Fas receptor gene, a deletion of 15 amino acids at the C-terminal of the molecule was shown to enhance its apoptotic activity, whereas further deletions inhibited the Fas signal completely (Itoh and Nagata, 1993), (Sato et al., 1995). Later this domain was found to direct binding of FAP-1 (Fas-associated phosphatase-1), a 250 KDa protein tyrosine phosphatase (PTP). FAP-1 is one of the largest known PTPases, with three characteristic features in its primary structure: a leucine zipper (LZ), a membrane-binding domain, and 6 PDZ domains. Binding of FAP-1 to the intracellular portion of Fas correlates with suppression of Fas-induced apoptosis in humans but not in the mouse (Cuppen et al., 1997). FAP-1 can interfere with transmission of the apoptotic signal by Fas via an interaction of its third PDZ domain with the C-terminal three amino acids of Fas. Abrogation of this interaction through microinjection of the corresponding tripeptide has been shown to restore Fas apoptosis in a Fas and FAP-1 expressing colon cancer cell line (Yanagisawa et al., 1997). Furthermore, strong FAP-1 expression was found to correlate with Fas resistance in Fas positive pancreatic tumour cells suggesting that FAP-1 may be involved in

the acquisition of resistance of pancreatic tumour cells to Fas antibody-induced apoptosis (Ungefroren et al., 1998). The same has been suggested for Kaposi sarcoma cells (Mori et al., 1996). Likewise, Jurkat cells overexpressing FAP-1 become resistant to Fas-mediated apoptosis with significantly reduced activation of caspase 8 (Li et al., 2000b). Furthermore, down-regulation of FAP-1 expression correlates with sensitisation to Fas-induced apoptosis in interleukin-2 (IL-2) activated T cells [Zhou, 1998 #929]. However, the mechanisms by which FAP-1 inhibits the apoptotic signal are unclear. It is hypothesised that the PTP activity of FAP-1 may be targeting IκB, the inhibitor of the transcription factor NF-κB (Nakai et al., 2000).

10. Another signalling intermediate that may regulate the activation of caspase 8 is FLASH (FLICE-associated huge protein). FLASH has DED-like domains that seem to facilitate activation of caspase 8 (Imai et al., 1999).

1.9.3 Physiological role of the Fas/FasL system

Naturally occurring Fas mutations provided initial insights into the role of Fas-mediated apoptosis during mammalian development. Although Fas is expressed in a variety of tissues, mutations in Fas in mice and humans cause phenotypic changes mainly associated with haematopoietic cells. Loss of function mutations in lpr and gld mice are characterised by lymphoproliferative diseases with autoimmune features. In humans, a similar syndrome, ALPS, is characterised by massive lymphadenopathy and splenomegaly, accompanied by autoimmune hemolytic anaemia and other autoimmune features (Rieux-Laucat et al., 1995). The patients also exhibit increased numbers of circulating B cells together with a polyclonal hypergammaglobulinaemia. This and other evidence has indicated that Fas and FasL have important functions in the immune system.

Perhaps the best-defined role for Fas in the immune system is in T lymphocytes. Thymocytes have to surmount four major obstacles before evolving into mature T lymphocytes. The first two involve successful rearrangement of the β- and then α-chain of the TCR and the subsequent positive and negative selection, the latter involving T cell receptor (TCR)-mediated signals and the MHC complex. These processes ensure the progression of self-tolerant T cells with a successfully rearranged

TCR that are able to recognise foreign antigen in the context of MHC presentation. A role for Fas in the life of T lymphocytes is thought to follow the completion of these selection processes, however it may also be involved in negative selection of thymocytes after high-dose antigen (Kishimoto et al., 1998). The more clearly defined functions of Fas in the immune system are described below:

1. Fas is important for the peripheral deletion of T lymphocytes following AICD (Singer and Abbas, 1994), (Mogil et al., 1995). Following the primary activation of peripheral T cells, Fas expression remains high whereas FasL is transiently expressed in response to TCR stimulation (Suda et al., 1996). T cells become increasingly sensitive to apoptosis in the presence of IL-2 and are finally deleted by a Fas-dependent mechanism (Brunner et al., 1995), (Dhein et al., 1995), (Ju et al., 1995). Increased expression of Fas and FasL permits T cells to eliminate neighbouring Fas positive cells (fratricide or paracrine death) (Cui et al., 1996). In addition, those cells that secrete FasL can also commit autocrine suicide (Krammer, 2000). Therefore, with the exception of a few that will survive to become memory cells, all antigen-specific T lymphocytes are eliminated at the end of an immune response. This mechanism ensures that homeostasis is maintained as the cytokines produced by effector T cells can trigger inflammation and lead to tissue damage. AICD of peripheral T cells is impaired in lpr and gld mice (Singer and Abbas, 1994), (Zheng et al., 1995).

2. A less-well defined role for FasL is apoptosis induction in antigen-presenting cells (APC) or other cell types, following antigen-driven activation. FasL positive T cells can lyse Fas-expressing antigen presenting cells and/or nearby Fas positive, MHC-mismatched targets (bystander lysis) (Wang et al., 1996). Activation of mature B cells causes the expression of Fas and renders the cells susceptible to Fas-mediated apoptosis (Fukuyama et al., 2002), (Schattner et al., 1996). It has also been suggested that the process of CD4+ T cell activation can result in apoptosis of the stimulating macrophage, a mechanism important for the macrophage homeostasis as well as the elimination of macrophages infected with intracellular organisms (Richardson et al., 1994), (Ashany et al., 1995).

3. Fas also plays a part in cell-mediated cytotoxicity directed against virus-infected cells and cancer cells (Rensing-Ehl et al., 1995), (Kagi et al., 1994), (Lowin et al., 1994). CD4+ T helper cells express FasL and possess Fas-mediated cytotoxicity (and often no granzyme B activity), whereas professional cytotoxic CD8+ T cells express and utilize both mechanisms. Moreover, Fas may be utilised by NK cells for tumour clearance (Screpanti et al., 2001).

4. Contrary to previous beliefs, Fas is now thought to play a role in regulating B cell apoptosis during germinal center (GC) reactions. Recently published data have shown that human GC B cell apoptosis relies on the rapid activation of caspase 8 at the Fas DISC, but does not require the contribution of the caspase 9 apoptosome (Hennino et al., 2001), (van Eijk et al., 2001). Immunoprecipitation analyses showed that GC B cells contain pre-formed Fas DISCs, consisting of FADD, caspase 8 and FLIPL. Upon *in vitro* culture in the absence of exogenous stimuli, FLIPL is lost from the DISC within minutes, resulting in a functional Fas DISC that activates caspase 8 (Hennino et al., 2001). FLIPL decay is inhibited when GC B cells are in contact with follicular dendritic cells or CD40 ligand (CD40L)-transfected cell lines (van Eijk et al., 2001). Furthermore, specific inhibitors of caspase 8 block GC B cell apoptosis, whereas inhibitors of caspase 9 do not (Hennino et al., 2001). Finally, a recent study showed that both clonal selection and affinity maturation of B cells are disrupted in lpr mice (Takahashi et al., 2001).

5. The Fas/FasL system is also thought to be involved in maintenance of immune privilege in tissues such as the cornea of the eye, the placenta and the testis (Bellgrau et al., 1995), (Griffith et al., 1995). These sensitive organs cannot tolerate the tissue damage inherent in inflammatory responses and have evolved strategies to suppress local inflammation, one of which appears to be the expression of FasL. The evidence suggests that FasL expression contributes to the transplantability of immune-privileged tissues and in particular, the success of corneal transplants (Stuart et al., 1997).

A schematic representation of the immune functions of Fas is shown in Figure 1.8.

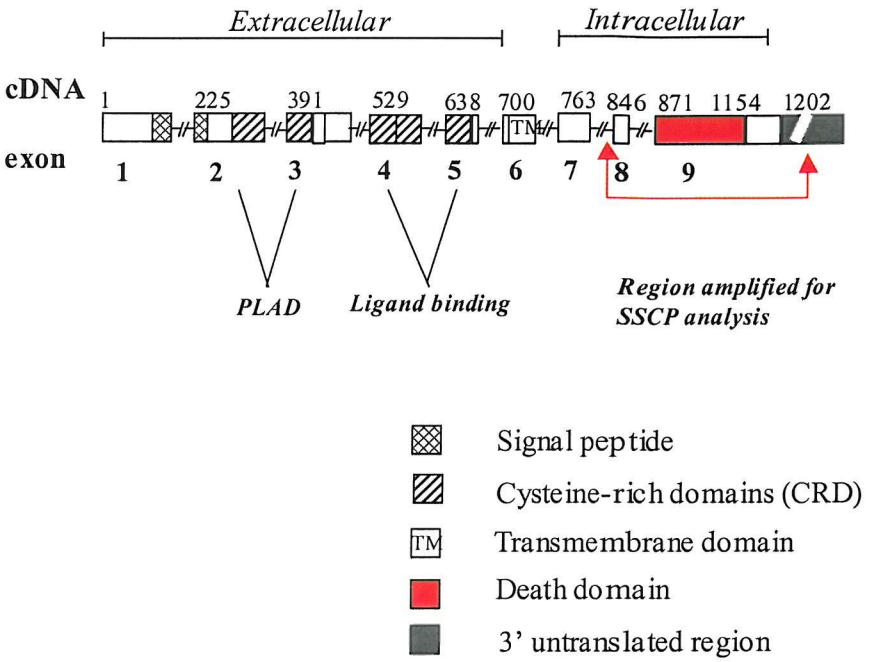


Figure 1.5: *Schematic diagram of the primary structure of Fas.* Distinct domains are indicated. The region amplified for SSCP analysis (817-1300 bp) is also shown.

Ligands			Receptors		
Original name	*New name*	*Comments (phenotypes associated with mutations and functional observations)*	*Original name*	*New name*	*Comments (phenotypes associated with mutations and functional observations)*
LTα	TNFSF1	Binds to mouse and h TNFR1 and R2	TNFR1/ p55	TNFRSF1A	Defective clearance of bacterial pathogens and defective GC formation
TNF-α	TNFSF2/ TNF	Defective clearance of bacterial pathogens and defective GC formation	TNFR2/ p75	TNFRSF1B	Deficiency results in increased sensitivity to bacterial pathogens and reduced antigen induced T cell apoptosis
LTβ	TNFSF3	Defective GC formation	LTβR	TNFRSF3	Defective GC formation
OX40L	TNFSF4	Defective T cell responses	OX40	TNFRSF4	Defective T cell responses
CD40L/CD154	TNFSF5	Defective T cell and IgG responses	CD40	TNFRSF5	Defective Ig class switching and GC formation in knockouts
FasL	TNFSF6	Loss of function results in impaired AICD, autoimmunity and ALPS	Fas/APO1/CD95	TNFRSF6	Loss of function results in impaired AICD, autoimmunity and ALPS
CD27L	TNFSF7	May be capable of inducing apoptosis	DcR3	TNFRSF6B	Secreted receptor for FasL implicated in tumour evasion
CD30L	TNFSF8		CD27	TNFRSF7	Defective T cell responses
4-1BBL	TNFSF9	Defective T cell responses	CD30	TNFRSF8	Marker of Reed-Sternberg cells in Hodgkin's disease
TRAIL	TNFSF10	Involved in anti-tumour and anti-metastatic activities of NK cells and macrophages	4-1BB	TNFRSF9	Involved in T cell responses
OPGL/TRANCE	TNFSF11	Abnormal B-cell development; required for mammary gland development	DR4/TRAILR1	TNFRSF10A	Involved in innate immune responses e.g. NK cell and phagocyte cytotoxicity
TWEAK	TNFSF12	Monocyte and NK cell cytotoxicity	DR5/TRAILR2	TNFRSF10B	Involved in innate immune responses eg. NK cell and phagocyte cytotoxicity
APRIL	TNFSF13	Probable role in B cell responses	TRID/TRAIL-R3	TNFRSF10C	GPI-linked decoy receptor for TRAIL
BAFF/Blys	TNFSF13B	Probable role in B cell responses	TRUNDD/TRAIL-R4	TNFRSF10D	Receptor for TRAIL with truncated death domain
LIGHT/ HVEM-L	TNFSF14		RANK	TNFRSF11A	Deficiency results in abnormal B-cell development; required for mammary gland development
VEGI/ TL1	TNFSF15	Vascular growth inhibitor	OPG	TNFRSF11B	Osteoporosis

NA	TNFSF16		DR3♦	TNFRSF12	
NA	TNFSF17		TACI	TNFRSF13B	
			BAFFR	TNFRSF13C	
TL6/AITRL	TNFSF18	Inhibits T cell receptor-dependent apoptosis	HVEM/LIGHTR/ ATAR	TNFRSF14	Involved in T cell proliferation
				TNFRSF15	
			NGFR	TNFRSF16	Defective sensory neuron innervation
			BCMA	TNFRSF17	Probable role in B cell responses
			AITR	TNFRSF18	Inhibits T cell receptor-dependent apoptosis
			TROY	TNFRSF19	Expressed in hair follicles and epithelium
			RELT	TNFRSF19L	
				TNFRSF20	
			DR6	TNFRSF21	
			SOBa	TNFRSF22	

Table 1.5: *Members of the TNF receptor/TNF ligand superfamilies in mammals.* The new nomenclature for both families is shown (adapted from the online version of the TNFR/L nomenclature scheme supported by the HUGO gene nomenclature committee (HGNC) (1998)).

♦ DR3 is not the receptor for TWEAK Kaptein, A., Jansen, M., Dilaver, G., Kitson, J., Dash, L., Wang, E., Owen, M. J., Bodmer, J. L., Tschopp, J., and Farrow, S. N. (2000). Studies on the interaction between TWEAK and the death receptor WSL-1/TRAMP (DR3). FEBS Lett *485*, 135-141.

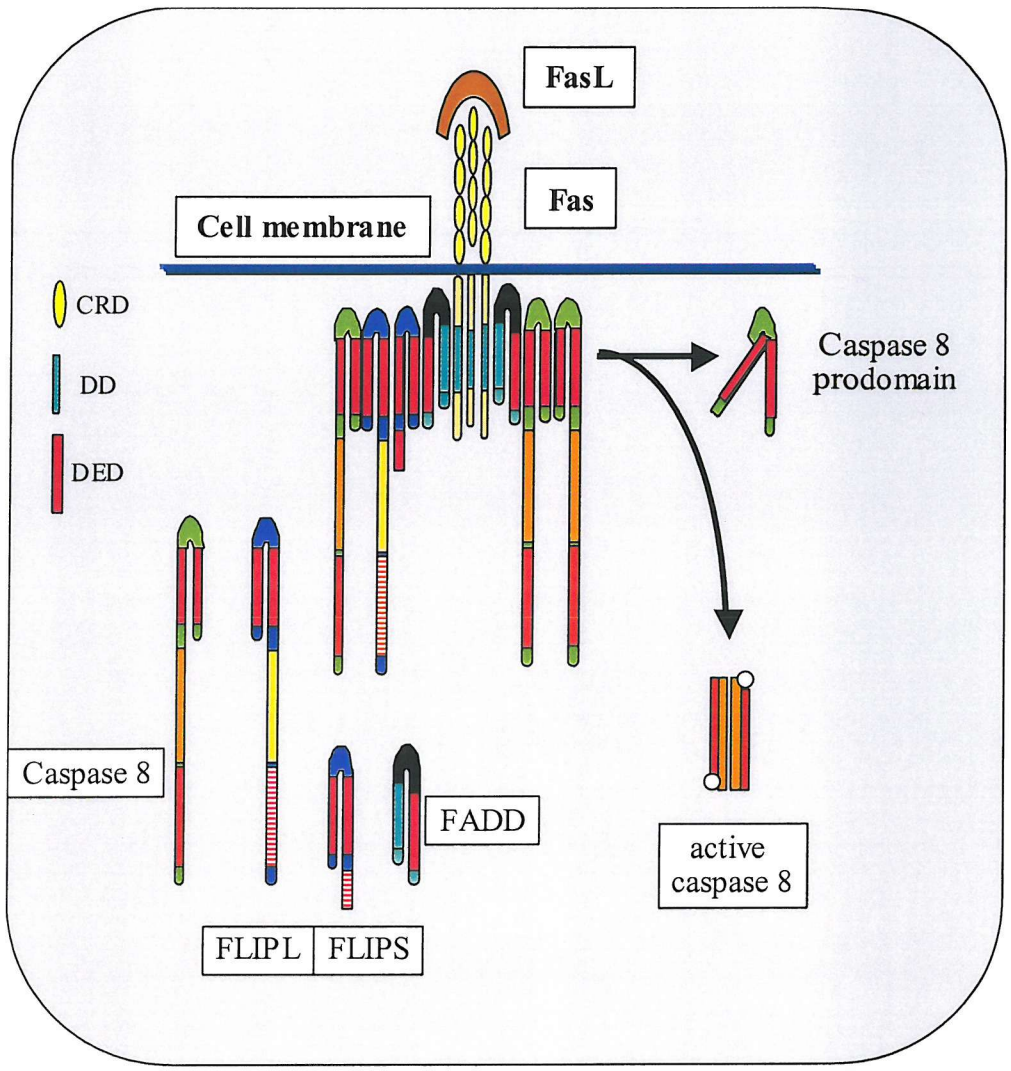


Figure 1.6: *Schematic diagram of Fas DISC assembly and caspase 8 activation.* Cross-linking of Fas by FasL leads to the recruitment of the adaptor molecule FADD via DD interactions and subsequent recruitment of caspase 8 via DED interactions with FADD. Aggregation of caspase 8 molecules in close proximity leads to caspase 8 activation probably by autocatalytic processing as a result of close proximity. C-FLIP can also associate with the DISC via DED domains and inhibit processing of caspase 8.

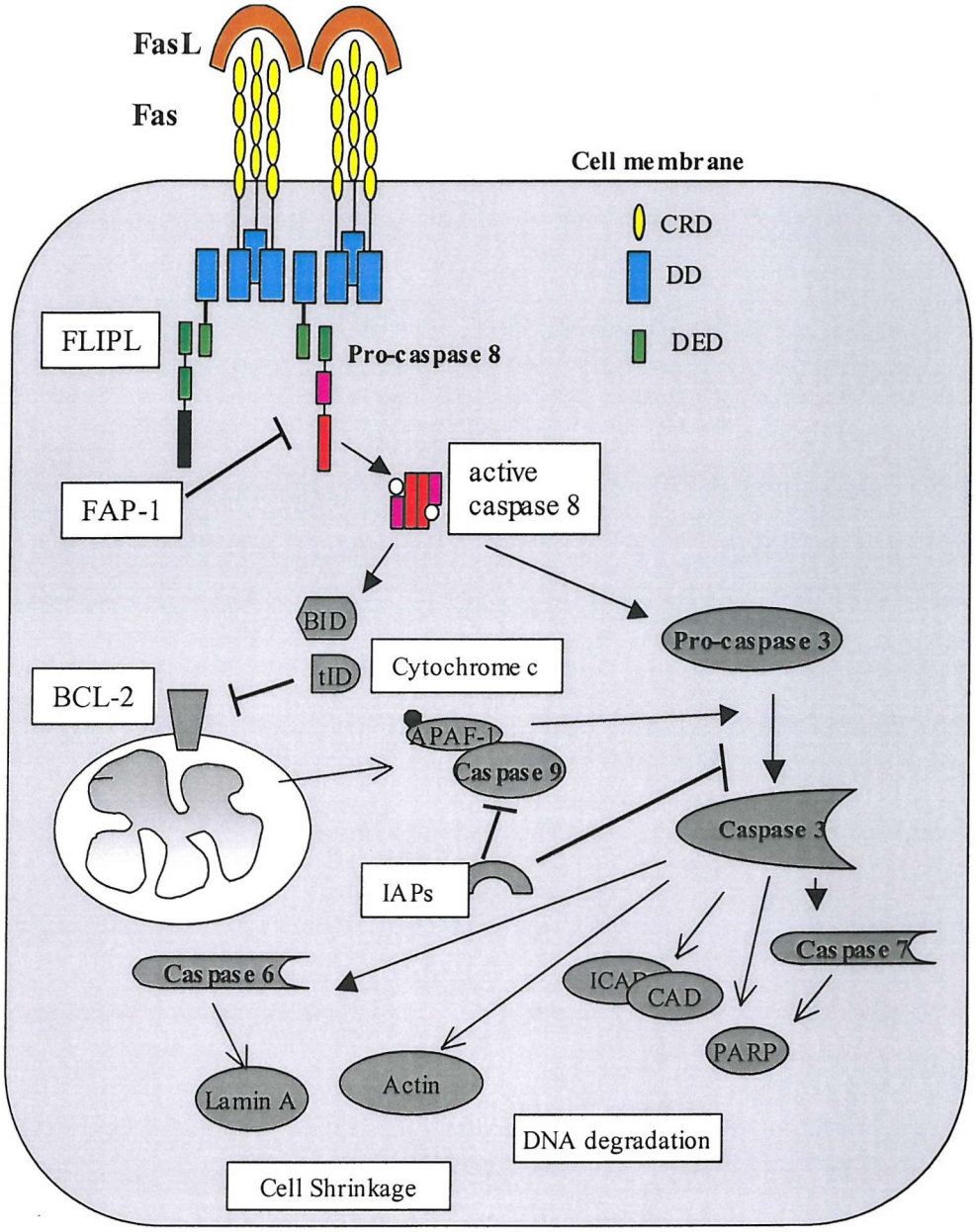


Figure 1.7: *Schematic diagram of apoptotic pathways originating from Fas.* FasL binding to Fas leads to aggregation of the receptors' death domains at the DISC and activation of caspase 8. The death signal can bifurcate at this stage proceeding either via direct activation of the caspase cascade or via cleavage of the BCL-2 family member BID and disruption of the mitochondria. Ultimately the signal cascade results in cleavage of structural proteins, enzymes and other targets and apoptotic cell death. Inhibitory factors such as FAP-1 and FLIPL can disrupt caspase 8 activation.

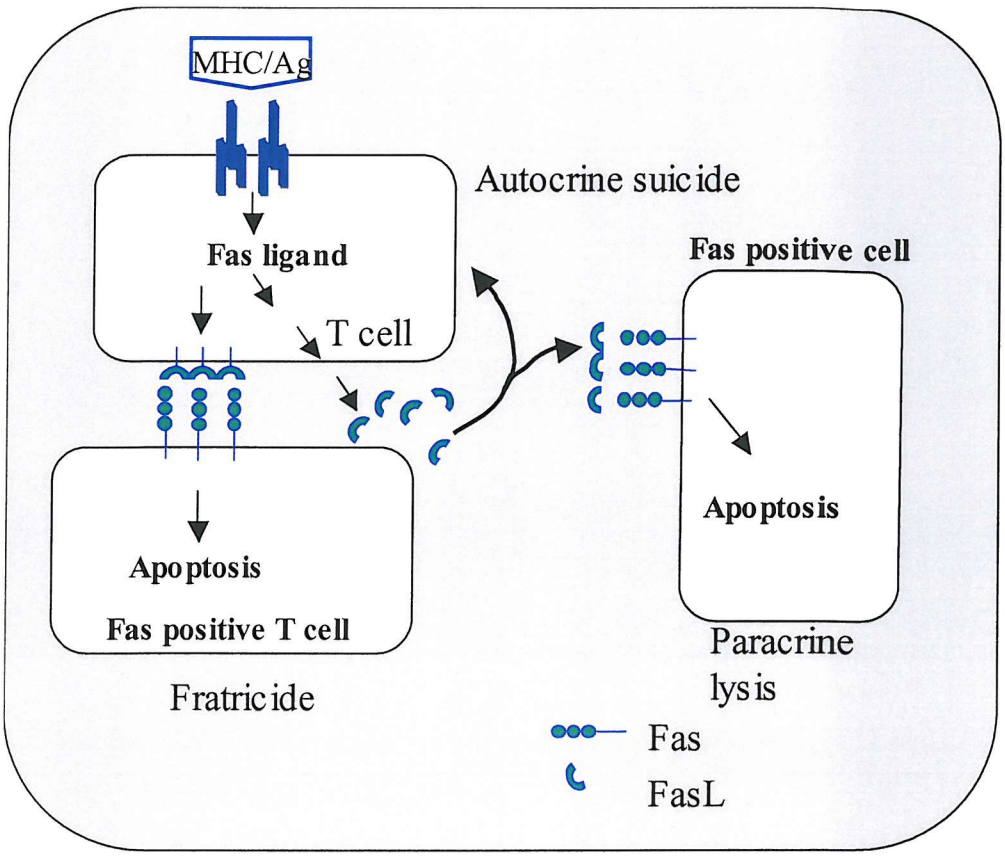


Figure 1.8: *Schematic representation of the function of Fas in the immune system.* Activated T cells stimulated via the CD3/TCR complex through the recognition of antigen presented on the MHC complex are induced to express FasL. FasL molecules cleaved from the T-cell surface by matrix metalloproteinases or still anchored on the cell membrane can induce apoptosis in the same cell (autocrine suicide), in neighbouring T-cells (fratricide), or in Fas positive target cells (paracrine lysis).

1.10 TRAIL-mediated apoptosis

In 1995, TRAIL/APO-2L was identified on the basis of sequence homology with members of the TNF family. Like the rest of the family members, TRAIL is a type II transmembrane protein bearing greatest amino acid homology (28%) with FasL in its extracellular domain (Wiley et al., 1995). In contrast to other death-inducing ligands, it is expressed in a wide range of normal fetal and adult tissues including spleen, prostate, ovary, thymus, small intestine and lung but is absent from the brain, liver and testis (Wiley et al., 1995), (Pitti et al., 1996). A unique feature of TRAIL is an N-terminal insertion of 12 amino acids termed the 'AA loop' that enhances specificity in the interaction between TRAIL and its receptors (Cha et al., 2000). TRAIL binds two death domain-containing receptors, DR4 (TRAILR1) (Pan et al., 1997b) and DR5 (TRAILR2) (Pan et al., 1997a), (Walczak et al., 1997), (Schneider et al., 1997), (Screaton et al., 1997a). TRAIL also binds two additional cell-bound receptors, the decoy receptors; TRID (decoy receptor 1/DcR1) (Degli-Esposti et al., 1997b), (Mongkolsapaya et al., 1998) and TRUNDD (decoy receptor 2 /DcR2) (Degli-Esposti et al., 1997a), (Marsters et al., 1997), (Pan et al., 1998b). TRID is devoid of an intracellular domain and ends immediately after a C-terminal stretch of 15 hydrophobic amino acids, a signal for the addition of glycosyl-phosphatidylinositol (GPI) anchor. TRUNDD resembles DR4 and DR5 but has a substantially truncated cytoplasmic domain. It lacks five out of six amino acids positions that are critical for apoptosis and NF-κB activation by TNFR1, including the position that corresponds to the Fas-inactivating lpr mutation (Marsters et al., 1997). Thus both decoy receptors are unable to transduce the death signal. The genes for DR4, DR5, TRID and TRUNDD are clustered on chromosome 8q21-22, a frequent site of chromosomal translocations where many tumour suppressors are thought to reside (Mitelman et al., 1997). TRAIL can also bind to a soluble receptor, osteoprotegerin (OPG). OPG has a dual specificity and can bind to TRAIL, albeit with a slightly lower affinity compared to the rest of the TRAIL receptors, but it also binds to another member of the TNF superfamily, the osteoclast differentiation factor ODF, OPGL/RANKL which seems to be its

physiological ligand (Emery et al., 1998). The primary structures of the membrane-associated TRAIL receptors are shown in Figure 1.9.

The structure of TRAIL bound to the extracellular domain of DR5 has been determined and revealed a conservation of the 3-fold ligand-receptor complex as seen for LTα/TNFR1 despite little primary sequence similarity. A TRAIL monomer contains two antiparallel β-pleated sheets that form a β-sandwich as a core scaffold and interacts with adjacent subunits in a head to tail fashion to form a homotrimer with an inverted bell shape (Hymowitz et al., 1999), (Hymowitz et al., 2000), (Cha et al., 2000). The described complexes of TRAIL in association with DR5 show three molecules of the receptor bound onto the ligand trimer, with crevices in the central contact region of the receptor accommodating the insertion of the AA loop. The residues of DR5 involved in the complex are mostly identical or highly homologous to DR4, TRID and TRUNDD but not to those of other TNFR family members (Cha et al., 2000). This structure also revealed a novel zinc-binding site that is required for appropriate folding and function. The tetrahedral metal binding site is formed by the side chains of Cys230 from each ligand monomer and is completed by an interior chloride ion. Mutation of the cysteine residues markedly affects the affinity of the ligand for its receptors and its stability, and removal of the zinc ion decreases TRAIL apoptotic activity 90-fold and reduces the thermal stability of the trimer by 20 ^{o}C (Bodmer et al., 2000b).

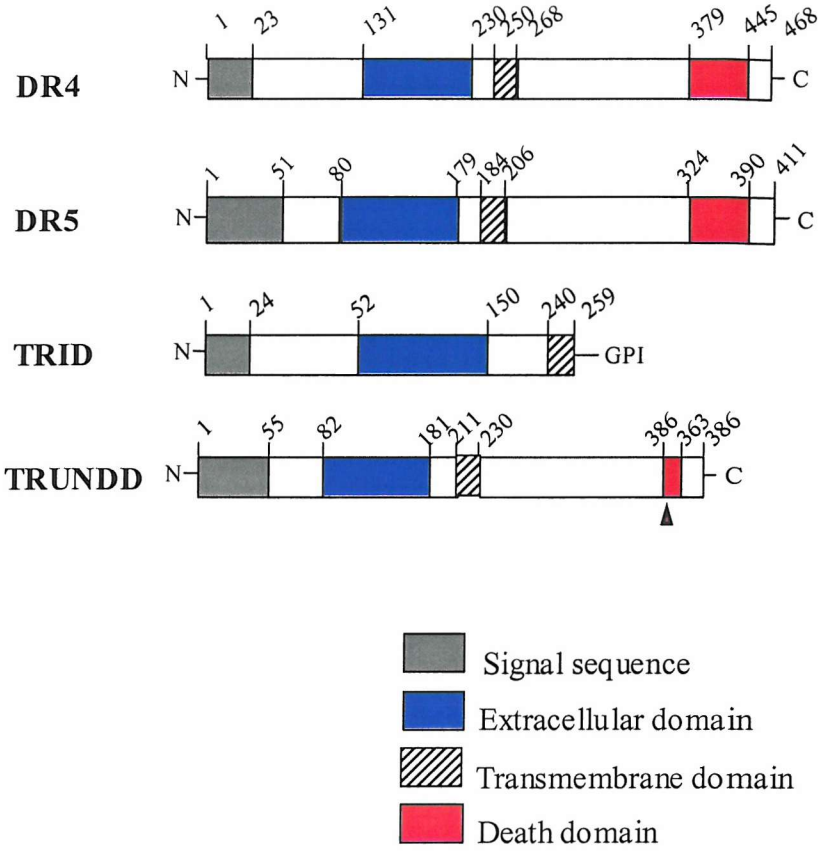


Figure 1.9: *Schematic diagram of the primary structures of membrane-associated TRAIL receptors.*. The locations of the various domains are indicated by amino acid co-ordinates. TRID is a GPI-linked surface molecule without a death domain and TRUNDD contains a partial death domain (truncated segment is indicated by the triangle).

1.10.1 Activation and signalling downstream of the TRAIL receptors

The known signalling pathway downstream of DR4 and DR5 bears many similarities to Fas signalling. Activation occurs after binding of TRAIL and trimerisation of the receptors. In one study, signalling complexes of DR4 and DR5 assembled in response to TRAIL binding but were not detectable before that (Suliman et al., 2001). Furthermore, studies with cross-linking monoclonal antibodies indicate that complexes of one receptor are capable of transmitting the death signal and inducing apoptosis (Griffith et al., 1999a).

FADD and caspase 8 are important components in DR4 and DR5-mediated apoptosis (Schneider et al., 1997), (Wajant et al., 1998). Immunoprecipitation experiments detected FADD as part of the TRAIL-induced DISC complex (Suliman et al., 2001) but this was disputed by others. Genetic studies have been important in establishing the importance of FADD. Thus, dominant negative FADD inhibited TRAIL-induced apoptosis in a number of studies (Schneider et al., 1997), (Walczak et al., 1997), (Wajant et al., 1998). However, transient transfection of DR4 in FADD-/- MEFs could still induce apoptosis (Yeh et al., 1998). However, a later study using a more reliable stable transfection system demonstrated that both DR4 and DR5-mediated apoptosis critically depends on FADD in human and mouse cells (Kuang et al., 2000) and this was later confirmed in Jurkat cells (Bodmer et al., 2000a). In addition, recruitment of FADD may be effected by a novel GTP-binding protein, DAP3 (death-associated protein 3), which is required for TRAIL-induced apoptosis (Miyazaki and Reed, 2001). DAP3 is a putative nucleotide-binding protein originally identified as a mediator of interferon (IFN)-γ-induced cell death (Levy-Strumpf and Kimchi, 1998), (Kissil et al., 1995). DAP3 immunoprecipitated with endogenous DR4 in a ligand-dependent manner and antisense experiments supported a requirement for DAP3 for the association of FADD with TRAIL receptor complexes (Miyazaki and Reed, 2001).

Caspase 8 is the apical caspase in the TRAIL-induced pathway, although some evidence from DCs suggested that caspase 10 may also be important (Wang et al., 1999). No recruitment of caspase 10 was however detected in Jurkat cells in another study and

Jurkat cells that lacked caspase 8 were completely resistant to apoptosis in response to TRAIL, a phenotype which was reversed upon introduction of caspase 8 in this cell line (Kim et al., 2000a), (Suliman et al., 2001). In addition, caspase 8 recruitment and activation can be seen within minutes after the addition of TRAIL to sensitive cells indicating that it is the apical caspase in the TRAIL pathway (Griffith et al., 1998) but this may differ between different cell systems. Activation of the apical caspase results in activation of a less well–defined caspase cascade with evidence for involvement of caspases 2, 3, 7, 8, and 9 (Kim et al., 2000a).

Similarly to Fas signaling, cleavage of BID via caspase 8 activation has also been reported, with a subsequent loss of mitochondrial membrane potential, cytochrome c release and caspase 3 activation (Yamada et al., 1999), (Lacour et al., 2001), (Gong and Almasan, 2000a), (Wen et al., 2000). This implies an involvement of the mitochondrial pathway in TRAIL-mediated apoptosis, the significance of which is unclear (Thomas et al., 2000). Some reports of BCL-2 overexpression in TRAIL-sensitive cell lines have shown protection only against early loss of $\Delta\Psi m$ but not against late disruption of mitochondrial integrity and apoptosis (Walczak et al., 2000), (Suliman et al., 2001). However, others have argued the opposite (Thomas et al., 2000). Again it is likely that distinct cell-type-specific mechanisms may operate in TRAIL as well as Fas-induced apoptosis.

1.10.2 Regulation of TRAIL-induced apoptosis

Shortly after its discovery, the abundance of TRAIL mRNA in a variety of tissue and cell types where its receptors were also frequently expressed suggested strong regulatory mechanisms to avoid damage to healthy cells. In addition, an enhanced cytotoxicity towards transformed cell lines relevant to normal tissues was reported at the same time (Degli-Esposti et al., 1997a), (Gura, 1997), (Kim et al., 2000b). As a result of these observations, it was hypothesized that the decoy receptors opposed the action of TRAIL in resistant cells by scavenging TRAIL or by forming inactive mixed receptor complexes with DR4 or DR5. In support, treatment of TRID-bearing cells with a phospholipase that cleaves the GPI anchor resulted in sensitization to TRAIL-induced apoptosis and transfection of TRUNND in inhibition (Pan et al., 1998b), (Degli-Esposti et

al., 1997a). Later studies, however, using monoclonal antibodies specific for each receptor showed that resistance to TRAIL is more likely to be controlled intracellularly rather than at the cell surface (Griffith et al., 1999a). In one report, neither TRID nor TRUNDD expression levels adequately explained the observed patterns of TRAIL sensitivity in the panel of cancer cells examined (Kim et al., 2000b). This was supported by the fact that endogenous levels of TRID and TRUNDD did not inhibit TRAIL-induced apoptosis in leukemia cells (Wen et al., 2000) and by the detection of decoy receptors in transformed cells (Zhang et al., 2000a), (Kim et al., 2000b). Thus, the mechanism of resistance of normal cells to TRAIL, as well as the functional significance of decoy receptors is still under investigation. Although the number of studies on the regulation of TRAIL-induced apoptosis is small, a number of mechanisms believed to contribute to resistance to TRAIL-mediated apoptosis have emerged:

1. Loss of the genes coding for DR4 and DR5 is one of the proposed regulatory mechanisms. Loss of TRAIL receptor genes has been detected in a small percentage (14%) of melanoma cells [Zhang, 2000 #650]. The genes for all four TRAIL receptors are clustered on chromosome 8 (8p22-21) and it is possible that loss of part of it could be selected in TRAIL-resistant melanoma cells.

2. Use of monoclonal antibodies against the four membrane-bound TRAIL receptors in melanoma cells, has indicated that control of TRAIL-mediated apoptosis is to a large extent exerted by receptor localization (Kim et al., 2000b). DR5, rather than decoy receptor expression, correlated with cell sensitivity to TRAIL in melanoma cell lines (Griffith et al., 1998). Post-translational control mechanisms such as the modulation of intracellular and extracellular distribution of pro- and anti-apoptotic TRAIL receptors may also play a role. For instance, DR4 and DR5 localized predominantly on the cell surface whereas the decoy receptors were predominantly located in the nucleus in two studies of melanoma cell lines (Zhang et al., 1999), (Zhang et al., 2000b). In this cell system, the decoy receptors can be exported to the cytoplasm and the plasma membrane upon exposure to TRAIL by a mechanism that appears to be regulated by the internalization of signaling receptors [Zhang, 2000 #911].

3. TRAIL receptors are targeted by viral gene products that mediate their endocytosis. Human adenovirus type-5 encodes three proteins named RID, E3-14.7K and E1B-19K that independently inhibit TRAIL-induced apoptosis in infected cells. RID protects cells from TRAIL by forcing the internalisation of DR4 that can be detected inside lysosomes where it is probably degraded (Tollefson et al., 2001), (Benedict et al., 2001). In addition, human herpesvirus-7 (HHV-7) also seems to target TRAIL receptor expression. Infection of CD4+ T cells with HHV-7 resulted in the simultaneous upregulation of TRAIL and downregulation of DR4 (Secchiero et al., 2001).

4. Resistance of many types of cancer and healthy cells to TRAIL is reversed by treatment with RNA and protein synthesis inhibitors, suggesting that intracellular labile factors contribute to resistance (Griffith et al., 1998), (Griffith and Lynch, 1998), (Siegmund et al., 2001), (Bretz et al., 1999). As in Fas signalling, c-FLIP is a prime candidate for this role. FLIPL expression correlated with TRAIL resistance in cancer cell lines (Kim et al., 2000b) and in primary versus transformed keratinocytes the levels of FLIPL inversely correlated with sensitivity to TRAIL (Leverkus et al., 2000). By contrast, in leukemia cell lines there was no correlation between intracellular FLIPL levels and sensitivity to TRAIL suggesting cell type-specific regulatory mechanisms (Wen et al., 2000). More recently, genetic screens using retroviral libraries have isolated TRAIL-resistant clones expressing the short form of FLIP, FLIPS, in two independent studies (Burns and El-Deiry, 2001), (Bin et al., 2002). Furthermore, FLIP-knockout embryonic fibroblasts were highly sensitive whereas their wild-type counterparts were resistant to TRAIL-mediated apoptosis (Bin et al., 2002).

5. Although the essential components of the TRAIL DISC are frequently expressed in various cells systems, recent evidence indicates that control can be exerted at the level of caspase 8 expression. In neuroblastoma cell lines, caspase 8 levels were suppressed by methylation of the caspase 8 promoter (Eggert et al., 2001). This resulted in the suppression of TRAIL-induced apoptosis.

1.10.3 Physiological role of TRAIL and TRAIL receptors

Most of the cell types known to express TRAIL belong to the immune system. Among them are monocytes (Griffith et al., 1999b), NK cells (Smyth et al., 2001), dendritic cells (Fanger et al., 1999), CD4+ and CD8+ T cells (Jeremias et al., 1998). TRAIL expression is also upregulated by interferon (IFN)-α (Kayagaki et al., 1999), and to a lesser extent by IFN-γ, IL-2 and IL-15. The available evidence regarding the physiological role of TRAIL points to an immune system-related function and more specifically in innate responses. These functions are mediated through the activation of DR4 and DR5 whereas the function of the decoy receptors remains undefined. Furthermore, the widespread distribution of TRAIL receptors outside the immune system also implies a non-immunological role. The proposed physiological roles of TRAIL are summarized below:

1. TRAIL contributes to anti-tumour innate responses. A study in TRAIL-knockout mice showed that TRAIL contributes to NK cell-mediated protection from tumour development and metastasis (Cretney et al., 2002). Furthermore, interleukins such as IL-2 and IL-15 induced TRAIL expression on murine spleen NK cells (Kayagaki et al., 1999) and murine liver NK cells constitutively expressed TRAIL, which induced apoptosis *in vitro* and suppressed metastases of TRAIL-sensitive tumour cells *in vivo* (Takeda et al., 2001). In another study, administration of therapeutic doses of IL-12, a powerful inducer of IFN-γ production by NK cells, increased TRAIL expression and biological function on spleen, liver and lung NK cells, and suppressed tumour metastases in both liver and lung in a TRAIL-dependent fashion (Smyth et al., 2001). TRAIL may also provide the mechanism for the tumoricidal activity of activated macrophages. Macrophages stimulated with either IFN-α or –γ (two potent mediators of macrophage activation) rapidly upregulated TRAIL on the cell surface and displayed cytotoxic activities against ovarian carcinoma cells *in vitro* (Griffith et al., 1999b). TRAIL may also contribute to the newly identified tumoricidal activities of human immature DCs (Lu et al., 2002).

2. TRAIL also appears to be involved in the control of virally infected cells in association with type I interferons (whose main physiological function lies in their antiviral activities). In this respect, TRAIL may play a role in clearance of virus- as well as intracellular organism-infected cells, a process dependent on type I IFNs but not on perforin or FasL (van den Broek et al., 1995). TRAIL transcription is upregulated upon stimulation of CD3/TCR activated peripheral blood T cells (CD4+, CD8+) with IFN-α and IFN-β. In Jurkat cells, treatment with IFN-α and –β and to a lesser extent –γ, stimulated the activity of the TRAIL promoter, providing further support for transcriptional regulation of TRAIL by interferons (Gong and Almasan, 2000b). This indicates that TRAIL-mediated cytotoxicity is not confined to particular T cell clones but may form part of the TCR/CD3-mediated antigen-specific cytotoxicity of human T cells, especially when they are exposed to endogenous or exogenously administered type I interferons (Kayagaki et al., 1999).
3. TRAIL may constitute a mechanism for T cell-mediated cytolytic activities against either foreign or activated inflammatory host cells. TRAIL is constitutively expressed on transformed murine and human T cells and activated but not on resting normal T cells (Mariani and Krammer, 1998). Following activation, both CD4+ and CD8+ αβ-T cell receptor lymphocytes upregulate TRAIL expression by a process that involves protein synthesis (Jeremias et al., 1998). TRAIL expressed on CD4+ T cell lines and IFN-α-activated peripheral blood T cells induced apoptosis in FasL-resistant melanoma cells and renal cell carcinoma cells respectively (Kayagaki et al., 1999), (Thomas et al., 2000). Furthermore, CTLs deficient in both FasL and perforin retained a residual cytolytic activity thought to depend on TRAIL (Braun et al., 1996).
4. TRAIL and the TRAILRs are expressed on human placentas suggesting a role in the protection of the fetus from immune system attack (Phillips et al., 1999). Immunohistochemical analyses demonstrated that TRAIL and TRID are expressed on the syncytiotrophoblast, a placental cell layer continuously exposed to maternal blood as well as in macrophage-like placental mesenchymal cells where its expression is enhanced by IFN-γ (Phillips et al., 1999).

1.11 Clinical relevance of apoptosis and death receptors.

Cells die by apoptosis in the developing embryo during morphogenesis or synaptogenesis (Barinaga, 1998) and in the adult animal during tissue turnover or at the end of the immune response. The first role of apoptosis is during intrauterine development. It is part of the sculpturing of organ shapes and the formation of interdigital webs of the fingers and toes. In adulthood, around 10 billion cells die each day in order to keep balance with the new cells arising from the bodies stem cell populations. Both the nervous system and the immune system arise through overproduction of cells followed by apoptosis of those cells that fail to establish functional synaptic connections or productive antigen specificities. Since apoptosis is involved in a variety of developmental and homeostatic processes in humans, it is not surprising that its deregulation is of clinical significance. Apoptosis can play a central role in the pathogenesis of human disease when the genes controlling apoptotic processes are suppressed, overexpressed or altered by mutation. There are a significant number of human diseases associated with altered cell survival. There are diseases where cells die prematurely; heart cells die in a heart attack, and brain cells die in a stroke. In these acute conditions of ischemia many cells die of necrosis. But some of the less damaged cells die by apoptosis and research is underway for drugs that block the process in the hope that some of the apoptotic cell deaths can be prevented. Increased apoptosis is a characteristic of AIDS and neurodegenerative diseases such as Alzheimer's disease and Parkinson's' disease. Decreased or inhibited apoptosis apart from being a feature of many malignancies is also involved in autoimmune disorders such as systemic lupus erythrematosus. However, it is the role of apoptosis in cancer that has probably received the greatest attention.

1.11.1 Cancer development and progression and the role of death receptors

Inhibition of apoptosis as well as deregulated proliferation lies at the heart of cancer development; this report is however focused on the mechanisms that act to suppress apoptosis. The processes of carcinogenesis and progression are evolutionary ones in which natural selection acts upon the inherent or acquired diversity of cells and acquisition of mechanisms that suppress apoptosis offer an obvious advantage. There is

now mounting evidence that acquired resistance towards apoptosis is a hallmark of most and perhaps all types of cancer (Hanahan and Weinberg, 2000).

Cellular protooncogenes and tumour suppressor genes have largely been defined by mutations that promote abnormal cell growth, however, mutations that affect apoptosis are becoming increasing involved in carcinogenesis. BCL-2 is a protooncogene and transgenic mice overexpressing BCL-2 in lymphocytes are clearly more prone to tumourigenesis than controls (Korsmeyer, 1999). Although it is a relatively weak transforming oncogene it synergizes potently in tumourigenesis with growth-promoting oncogenes, such as c-myc (Bissonnette et al., 1992), (Evan and Littlewood, 1998). Other proapoptotic members of the BCL-2 family, such as BAX, can act as tumour suppressors (Yin et al., 1997). Given the apparent importance of caspases in apoptosis, mechanisms that inhibit caspase activation are implicated in tumour formation (Teitz et al., 2001), (Juin and Evan, 2000). An example of indirect caspase inactivation is IAP amplification. One IAP family member, survivin, while undetectable in terminally differentiated adult tissues, becomes prominently expressed and in transformed cell lines and in most human cancers including lung, colon, pancreas, prostate and breast (Ambrosini et al., 1997).

More importantly, disruption of death receptor pathways frequently contributes to human disease and this has been best described for Fas. In the naturally occurring lpr mice a splicing defect results in the greatly reduced expression of Fas and in gld mice a point mutation in the C-terminus of FasL impairs its ability to interact successfully with the receptor (Watanabe-Fukunaga et al., 1992a). In humans a similar disease characterised by a dysfunction of the Fas (type Ia 'autoimmune lymphoproliferative syndrome' (ALPS) or FasL (type Ib ALPS) system has been reported. Children with ALPS or Canale-Smith syndrome, show massive non-malignant lymphadenopathy, an altered and enlarged T cell population and a severe autoimmunity (Fisher et al., 1995). Lymphocytes from ALPS individuals are resistant or less sensitive to apoptosis induced by Fas. Fas mutations in ALPS Ia are dispersed among the nine exons of the Fas gene but there is clustering in the death domain (Jackson et al., 1999). The severity of the symptoms depends on the location of the mutation, with mutations in the intracellular domain resulting in a more pronounced pathology than mutations in the extracellular regions. Apart from these

characteristic syndromes, deregulation of Fas function has been associated with various types of human cancer.

1. One study crossed Ipr mice with transgenic mice carrying an L-myc transgene under the control of the immunoglobulin heavy chain enhancer (Eμ-L-myc) resulting in constitutive expression of L-myc in lymphoid tissues (Eμ-L-myc/(lpr/lpr). L-myc transgenic mice are prone to T and B cell lymphoma development that the lack of homeostatic regulation by Fas can influence the process of lymphomagenesis (Zornig et al., 1995).
2. Somatic mutations of Fas have been reported in many types of human cancers. In a screen of 150 different subtypes of B- and T-cell non-Hodgkin's lymphomas, Fas mutations were detected in 11% of cases (Gronbaek et al., 1998). Mutation analysis of the entire coding region of the Fas gene performed in 43 cases of gastric cancer detected missense mutations in 11% of cases, all of which were localised in the death domain (Park et al., 2001). Somatic mutations of Fas were also detected in classical Hodgkin's disease (Muschen et al., 2000). A particularly high incidence of Fas mutations was detected in bladder cancer (Lee et al., 1999) and hepatocellular carcinoma (Lee et al., 2001b).
3. Inhibitory molecules are also implicated in tumour progression. The best example is c-FLIP, which is overexpressed in human melanomas (Medema et al., 1999). Studies have also demonstrated that overexpression of FLIPL leads to immune escape of cancers *in vivo* presumably by blocking Fas-mediated cytotoxic T cell killing (Djerbi et al., 1999).
4. Escape from immune attack can also be mediated by amplification of the decoy receptor, DcR3 that blocks FasL and its killing activity. The DcR3 gene was amplified in about half of 35 primary lung and colon cancers (Pitti et al., 1998).
5. FasL expression is involved in another interesting aspect of tumour biology. Several tumours express FasL having down regulated their own Fas expression. FasL expression on tumours could represent an evasive strategy to avoid immune recognition or an active immunosuppressive function (Hahne et al., 1996), (Song et al., 2001). Expression of Fas ligand has been reported in solid tumours of non-haematopoietic origin, including colon, hepatocellular

carcinoma, melanoma, astrocytoma, lung carcinoma, ovarian carcinoma, oesophageal carcinoma, glioblastoma and renal cell carcinoma (Hahne et al., 1996), (Ungefroren et al., 1998), (Buzyn et al., 1999), (Herrnring et al., 2000). The same mechanism is implicated in establishing immune privilege in certain sites (Kaplan et al., 1999), and it could explain why immune systems fail to eliminate tumours. This is however disputed (Kang et al., 1997). Although the regulation of FasL expression in cancer is poorly understood, the finding that the transcription factor N-myc induces FasL expression in neuroblastoma cells, points to one possible mechanism and is particularly interesting, in view of the increased aggressiveness of neuroblastomas with N-myc overexpression (Tani et al., 2001), (Wang et al., 1998a).

The evidence for involvement of TRAIL receptor mutations in human cancer is more infrequent. Mutations in TRAIL receptors occur at low frequencies with few exceptions. This is partly due to the lack of extensive investigation and the enhanced complexity of the TRAIL system, compared to Fas. However, allelic losses of chromosome 8p21, where the entire family of TRAIL receptors reside, exist in cancers such as colorectal and breast cancer (Arai et al., 1998), (Shin et al., 2001). Inactivating mutations in DR4 and DR5 correlate with metastatic breast cancer (Shin et al., 2001) and nucleotide substitutions in the ligand-binding domain of DR4 (C626G and G422A) were found in 47% and 44% of neck squamous cell cancer and gastric adenocarcinoma samples respectively (Fisher et al., 2001). Furthermore, mutations in the death domain of DR5 have been reported in a small percentage of head and neck cancers where they result in truncation of the death domain and loss of its apoptosis-inducing function (Pai et al., 1998) as well as in non small cell lung cancer (Lee et al., 1999). In addition mutations in one of DR4 or DR5 were detected in a small percentage of non-Hodgkin's lymphomas (Lee et al., 2001a).

1.11.2 Therapeutic applications of apoptosis research and the potential of death receptors for cancer treatment

Deregulated cell proliferation together with suppressed apoptosis, constitute a common platform upon which cancer development and progression occurs. Thus

apoptosis research can provide a useful angle to examining human cancer and identifying differences (from healthy tissues) and to exploit them towards therapeutic goals. Apoptotic pathways are becoming more and more relevant for treatments as well as research towards development of novel treatments or predictive markers.

It is now known that virtually all cytotoxic drugs and radiotherapy programmes induce apoptosis in tumour cells, and resistance to apoptosis is associated with treatment failure. For example, the examination of peripheral blood mononuclear cells from acute leukaemia patients undergoing induction therapy has demonstrated that various agents, including cytarabine, etoposide, paclitaxel, and topotecan cause a marked increase in the number of apoptotic blasts (Li et al., 1994). Characteristic apoptotic changes have also been described in murine solid tumours after treatment of mice with various cytotoxic drugs including cytarabine, 5-fluorouracil (5-FU), fludarabine, doxorubicin, cyclophosphamide, cis-diamminodichloroplatinum (CDDP), etoposide, dactinomycin, and camptothecin (Clarke et al., 1993), (Lowe et al., 1993), (Li et al., 1994), (Bellosillo et al., 1997). The pro-apoptotic effect of the currently used anticancer drugs has important implications for the treatment of human cancer because alterations that make cells more or less susceptible to apoptosis might affect their sensitivity to a wide range of unrelated anticancer agents. In addition, regardless of the cell killing efficiency, the success in repairing the apoptotic response in tumour cells depends on the extent to which such therapies confine death to cancer cells whilst allowing the survival of healthy tissues. Many conventional therapies induce significant toxicity, particularly in tissues that actively maintain a proliferative compartment, such as the gut epithelium and the haematopoietic system. Pro-apoptotic therapies that would specifically target diseased tissues while sparing healthy normal cells would provide a clinical advantage.

The observation that in some cancer patients spontaneous tumour regression was dependent on circulating levels of TNF was the first indication that death receptors are likely anticancer therapy candidates (Carswell et al., 1975). The idea of targeting death receptors to induce apoptosis in tumours is an attractive one since death receptors have direct access to the caspases and operate largely independently of p53, which is frequently inactivated in cancer. Despite these advantages, the clinical use of TNF and Fas targeting reagents has so far been hindered due to the systemic toxicity resulting from their

administration (Rodriguez et al., 1996), (Havell et al., 1988). TNF has been licensed as a drug for the treatment of irresectable soft tissue sarcoma by isolated limb perfusion (restricted administration) and future applications based on the same delivery strategy are being evaluated (Lejeune et al., 2001). However, a number of studies have shown that death receptors are involved in apoptosis induction by a variety of anti-cancer agents, suggesting another potential for utilisation in combination treatments with the potential for lower, less toxic dosages.

In contrast to Fas and TNF, TRAIL is believed to be a promising anti-cancer agent because normal cells appear to be resistant to it whereas the majority of tumour cells examined are sensitive. Furthermore, a number of investigations have established that it can be used with minimal risk of systemic toxicity. A number of studies have been carried out to confirm the cytotoxic capacity of TRAIL *in vivo* as well as to assess the risk of systemic toxicity. Tumourigenic or transformed cells are particularly sensitive to TRAIL-mediated apoptosis (Wiley et al., 1995), (Griffith and Lynch, 1998). Furthermore, TRAIL induces apoptosis in several tumour cell lines including those that resist chemotherapy or ionising radiation because of inactivating mutations in p53 (Marsters et al., 1996), (Ashkenazi et al., 1999), (Walczak et al., 1999).

The anti-tumour potency of TRAIL was confirmed in a severe combined immunodeficiency (scid) mouse model using LZ-TRAIL. Administration of soluble TRAIL to scid mice bearing human tumours reduced tumour size with no apparent toxicity to the animals (Walczak et al., 1999). In gliomas, injected recombinant TRAIL caused complete regression and ablated tumour mass (Roth et al., 2001). Another study used cynomologous monkeys as a preclinical safety model for TRAIL and treatment with different recombinant versions of human TRAIL and found that the animals were not severely affected by TRAIL treatment (Ashkenazi et al., 1999). Later concern was raised by a report suggesting that a polyhistidine-tagged recombinant version of human TRAIL killed human hepatocytes *in vitro* (Jo et al., 2000). This finding, however, was not reproduced in subsequent studies using clinical grade recombinant TRAIL (Lawrence et al., 2001) and is now attributed to non-optimised preparations. Meanwhile, an alternative approach lead to the generation of TRAIL receptor-targeting antibodies. TRA-8 an agonistic monoclonal antibody against DR5 induces apoptosis in tumour cells *in vitro* in

the absence of secondary cross-linking. It also exhibits a strong tumouricidal activity *in vivo* against both leukaemias and solid tumours. More importantly, TRA-8 does not induce hepatocellular cell death in human tissue (Ichikawa et al., 2001). Thus the selective nature of the anticancer activity of TRAIL, as established in mice and non-human primates, has raised strong interest for the upcoming Phase I clinical trials.

In addition, because chemotherapy and radiation therapy on the one hand, and TRAIL on the other hand trigger tumour cell apoptosis primarily via two distinct pathways, other possibilities exist for the use of TRAIL in cancer therapies. Co-treatment of malignancies that still respond to chemotherapy or radiation therapy will potentially diminish the pool of tumour cells that may give rise to an outgrowing resistant clonal variant of the parental tumour. Therefore, concomitant treatment of cancer patients with TRAIL and chemotherapeutic drugs or radiation therapy may reduce the relapse rate in malignancies. An added advantage of a combination therapy would be the decrease in doses of the frequently toxic anti-cancer agents.

Ionising radiation has been reported to exhibit a synergistic effect with recombinant TRAIL in the regression of human breast cancer xenografts in mice by upregulating DR5 (Chinnaiyan et al., 2000). A similar finding was reported in T cell leukaemia cell lines (Gong and Almasan, 2000a). Co-administration of TRAIL and 5-FU has also been shown to act synergistically in tumour eradication and TRAIL co-operated with doxorubicin to kill various cancer cell lines (Kim et al., 2000b), (Lacour et al., 2001, [Wen, 2000 #665). In non-small cell lung cancer (NSCLC) lines, the synthetic retinoid CD437, enhanced TRAIL-induced apoptosis mainly by upregulating DR4 and DR5 (Sun et al., 2000). In addition pre-treatment of prostate cancer cell lines with paclitaxel also enhanced TRAIL-induced apoptosis again via upregulation of DR4 and DR5 levels (Nimmanapalli et al., 2001). Synergistic induction of apoptosis by the combination of TRAIL and chemotherapy was also observed in chemoresistant ovarian cancer cells (Cuello et al., 2001). Furthermore, the combination of TRAIL and CPT-11, greatly enhanced the anti-tumour activity of TRAIL *in vivo*, inducing significant inhibition of murine tumour growth and a high proportion of complete tumour regressions (Gliniak and Le, 1999).

In addition, a synergistic effect and/or requirement for apoptosis induction by anti-cancer drugs have been reported for the Fas/FasL system. The expression of Fas and FasL is important in mediating the cytotoxicity of agents such as CDDP, adriamycin, doxorubicin, and 5-FU, at least in some cells (Friesen et al., 1996), (Uslu et al., 1996). A role in radiation-induced apoptosis has also been shown (Rehemtulla et al., 1997). According to one model, chemotherapy drugs induce expression of either Fas and/or FasL on tumour cells that subsequently die via an autocrine or a paracrine mechanism (Friesen et al., 1996), (Fulda et al., 2000). The role of the Fas/FasL system has been more recently questioned, however, due to the high concentrations of drugs needed to achieve high levels of Fas expression and recent evidence from FADD and caspase 8-knockout mice, which respond to anticancer drugs. Secondly, commercially available Fas-blocking antibodies have not been observed to block drug-induced apoptosis in many cell systems. Thirdly, cells that lack Fas and are therefore unable to respond to a FasL upregulation, can still undergo apoptosis in response to drug treatment (Villunger et al., 1997), (Eischen et al., 1997). Finally, overexpression of c-FLIP or a dominant negative FADD construct has had little effect on drug induced apoptosis in most cases (Wesselborg et al., 1999), (Kataoka et al., 1998). Finally overexpression of CrmA, which inhibits caspase 8 activation, has no effect on drug induced apoptosis (Villunger et al., 1997), (Glaser et al., 1999). It is argued that the majority of reports demonstrating an involvement of Fas in drug-induced apoptosis are based on *in vitro* evidence, however, some *in vivo* evidence also exists (Tani et al., 2001). The most important *in vivo* evidence involves 5-FU, one of the most effective agents against colon cancer, whose active metabolite inhibits thymidylate synthase and causes thymine depletion and subsequent DNA and RNA damage. In this case, 5-FU toxicity does appear to involve Fas signalling (Houghton et al., 1997), (Muller et al., 1997). Death of thymocytes is blocked by FasL antagonistic antibody *in vivo* and lpr mice showed an impaired effect of this drug in the thymus supporting a direct role for the Fas/FasL system in cytotoxicity mediated by 5-FU (Eichhorst et al., 2001).

1.12 B cell malignancies

1.12.1 Introduction

Among the various differentiated lineages of animal cells, B cells undergo a unique differentiation process that generates their essential ability to detect antigens and to respond by antibody production. The process of antibody formation involves several steps in which deliberate alterations of the genome are induced in individual B cells. The B cells' inherent capacity to rearrange and mutate its immunoglobulin genes is critical for generation of specific antibody-mediated immunity. However, this capacity also introduces risks since the processes of immunoglobulin gene rearrangement and mutation can result in rearrangements or mutations of genes involved in cell control. Such mutations are frequently causative agents in the formation of B cell neoplasms.

The B cell malignancies examined in this study were Burkitt's lymphoma and chronic lymphocytic leukaemia. They both fall in the category of mature B cell neoplasms according to the Revised European-American Classification (REAL) and World Health Organisation (WHO) classification scheme for lymphoid neoplasms (Harris et al., 2000). The REAL and WHO classification of malignant lymphomas is based on a further understanding of B lymphocyte biology, careful morphologic evaluation, and application of immunophenotypic and genetic techniques (Harris et al., 2000). According to this new scheme mature B cell neoplasms are clonal proliferations of B cells at various stages of differentiation ranging from naïve B cells to mature plasma cells. They comprise over 90% of lymphoid neoplasms worldwide (Harris, 2001). The morphological and behavioural spectrum of these lymphomas is very diverse, partly because they derive from diverse normal lymphoid cells. In the WHO classification the mature B cell neoplasms are listed according to their major clinical presentations: I. predominantly disseminated, often leukaemic types including CLL, II. primary extranodal lymphomas and III. predominantly nodal lymphomas (which may have extranodal sites as well). BL falls in the latter category (Harris, 2001).

In general, the disseminated B cell neoplasms are relatively indolent whereas nodal lymphomas can be either indolent (follicular lymphoma) or aggressive (BL). Patients with indolent lymphomas such as CLL are considered incurable and may be

observed without treatment until they become symptomatic. More aggressive lymphomas tend to be more susceptible to chemotherapy, for instance BL is often treated successfully with aggressive chemotherapy (Magrath, 1998).

1.12.2 Burkitt's' lymphoma

According to the REAL/WHO classification, BL is a highly aggressive lymphoma often presenting at extranodal sites or as an acute leukaemia, composed of monomorphic medium-sized B cells with basophilic cytoplasm and a proliferation fraction of approximately 100% (Diebold et al., 2001). It is a rare form of disease, typically involving younger patients and is the most common type of paediatric non-Hodgkin's lymphoma (Grogan, 1999). Burkitt's lymphoma displays a germinal centre cell phenotype, and comprises three main subcategories occurring in three clinical settings (Grogan, 1999). These are:

I. Endemic Burkitt's lymphoma: a childhood lymphoma (5-10 year olds), which is prevalent in equatorial Africa and New Guinea
II. Non-endemic/sporadic Burkitt's lymphoma: a worldwide lymphoma affecting slightly older childhood patients and young adults.
III. Immunodeficiency-associated Burkitt's lymphoma (HIV-infection, post-transplant immunosuppression).

Endemic BL is a malignancy of intermediately sized B cells that infiltrate nodal or extranodal sites in a diffuse pattern. The histologic characteristic of BL is the presence of a large number of dense, darkly stained apoptotic cells within scattered unstained macrophages, a feature responsible for the starry sky microscopic appearance at low resolution. BL cells express monotypic Ig, usually IgM and a range of B-cell markers, such as CD10, CD19, CD20, CD77 and CD22. Typically there is also CD21, a receptor for Epstein-Barr virus, at least in the endemic African cases. Normal B cells expressing CD10 and CD77 are found mostly in germinal centres (Gregory et al., 1987). The rate of cell division for BL is very high, as shown by staining for cell cycle-specific markers such as Ki-67, that typically show more than 95% of tumour cells to be progressing through the cell cycle.

Although very similar in histologic and cytologic features, the different forms of BL can have different epidemiological patterns and clinical presentations. However, they all contain one of the three chromosomal translocations that deregulate c-myc, a known oncogene (Harris et al., 1999). The c-myc deregulation is the defining characteristic of BL. Furthermore, in the African type, there is a strong association with the Epstein-Barr virus; all the malignant cells of the great majority (95%) of BL cases carry EBV DNA. This form of the disease is restricted to areas where infection by *Plasmodium falciparum*, the parasite responsible for malaria, is frequent. Unlike endemic BL, only a subset of sporadic and immunodeficiency-associated BLs are EBV-associated. Sporadic BL shows a variable viral association (EBV positive in 15-88% of tumours) whereas in immunodeficiency-associated BL, latent EBV infection occurs in 30-40% of tumours (Cohen, 1999). These variations of EBV content occur even though the great majority (about 95%) of people worldwide are EBV carriers.

In general, BL patients demonstrate a high response rate to chemotherapy, but relapses are frequent, and the prognosis in relapsed disease is poor, with a median overall survival in relapsed disease of around six months [Jaffe, 1999 #1008]. Attempts at intensifying treatment in these patients are promising.

1.12.3 Molecular characteristics of Burkitt's lymphoma: c-myc deregulation and Epstein-Barr virus

Insight into the origin of BL has come from DNA sequence analysis of the Ig variable heavy chain and light chain genes, in endemic sporadic and HIV-associated BL. These analyses have indicated that the cells have undergone somatic hypermutation. In addition, B cells of the endemic form of the disease show evidence of continuing hypermutation. This and other evidence (e.g. surface marker expression) are compatible with a germinal centre origin for BL. Although this is not a unique feature of this disease it helps to distinguish it from certain aggressive B cell lymphomas resembling BL and thus termed Burkitt-like. However the defining feature of BL is the c-myc translocation. The c-myc locus is always reciprocally translocated to one of the immunoglobulin loci. In 80% of cases of BL the translocation partner for c-myc is the IgH locus, resulting from a t8: 14 translocation. In 15% of the cases the translocation partner is the κ chain locus at

chromosome 2p11 whereas in the remaining 5% of cases, the λ locus at chromosome 22q11 is involved. Because Ig enhancer elements are particularly active in mature B cells, their juxtaposition to c-myc in BL drives inappropriately high levels of c-myc-encoded mRNA and protein.

The locations of the breakpoints in many cases of endemic and sporadic BL have been mapped and occur in 5' or 3' of the myc gene. A comparison of tumours from endemic and sporadic areas, did not find a correlation between breakpoint position on the myc gene and EBV status (Gutierrez et al., 1992), however there is a correlation with immunoglobulin breakpoint positions. In the sporadic BLs the breakpoint is normally in switch region, where the switch recombinase mediates isotype switching (Boxer and Dang, 2001). This indicates that the type of translocations characteristic of sporadic BL may be the result of errors in this process in the germinal centres. The breakpoints on the Ig loci in endemic BL are generally in the J region but occasionally in the V or D regions, consistent with the translocation occurring as a result of errors in the normal process of VDJ joining during Ig gene rearrangement, which normally occurs in the bone marrow (Boxer and Dang, 2001). A smaller group of patients is sometimes found to exhibit dual translocation of both c-myc and BCL-2 and they appear to have an extremely poor outcome despite aggressive therapy. Furthermore, 30% of BL biopsies and 60-70% of BL cell lines have been reported to carry p53 mutations (Bhatia et al., 1992), (Farrell et al., 1991), (Vousden et al., 1993). Mutations in BAX have also been suggested to contribute to the molecular pathogenesis of BL (Gutierrez et al., 1999).

1.12.3.1 c-myc deregulation in BL

C-myc is one of a family of related mammalian genes that encode the MYC proteins (L-MYC, B-MYC and N-MYC), which are transcription factors. MYC proteins are expressed in proliferating normal cells responding to mitogenic stimuli, but are absent in terminally differentiated cells. Overexpression of c-myc is sufficient to drive cells into cycle in the absence of external mitogens (Adams et al., 1985). In addition to its well-documented growth promoting effect, MYC is also a powerful inducer of apoptosis, especially under conditions of stress, genotoxic damage or depleted survival factors (Evan et al., 1992), (Askew et al., 1993). In contrast to immortalised cell lines overexpression of

c-myc in primary cells or in vivo results in either cell cycle arrest or apoptosis, which is thought to be one mechanism by which organisms defend against neoplastic cells arising from deregulated oncogenes.

The effects of c-MYC are mediated through the binding of c-MYC/Max heterodimers to the promoter elements of a set of genes, thereby either inducing or repressing their expression. Among these gene targets are cyclin A, lactate dehydrogenase A, and ornithine decarboxylase (Packham and Cleveland, 1995). Furthermore BAX and Fas and/or FasL may also be myc targets (Boxer and Dang, 2001). A recent study with lymphocytes containing conditional myc knockouts demonstrated that myc-null lymphocytes are unable to express Fas or FasL (de Alboran et al., 2001). Furthermore the FasL gene seems to be directly activated by myc through a myc-binding site (Kasibhatla et al., 1998). Mice expressing a c-myc transgene under the control of the Eμ enhancer showed disseminated lymphoma followed by leukaemia within the first year of life (Adams et al., 1985). More recently, mice bearing a c-myc transgene controlled by a reconstructed immunoglobulin lambda locus invariably developed lymphomas, bearing the immunophenotypic characteristics of BL, within 30-130 days (Kovalchuk et al., 2000). Interestingly, lymphocytes from Eμ-myc mice remained dependent on cytokines and in fact died faster than those from normal mice when deprived of cytokines (Adams et al., 1985). The implication was that cells forced to cycle by myc die rapidly when cytokines become limiting. It was thus established that c-MYC promotes apoptosis under adverse growth conditions (Evan et al., 1992). Because of its dual function, c-MYC has only weak oncogenic potential but can co-operate with other oncogenes. One example is BCL-2, which has been shown to inhibit MYC-mediated apoptosis in some systems, while having no effect on its proliferative function (Bissonnette et al., 1992), (Packham and Cleveland, 1995). Furthermore, c-myc overexpression in primary mouse fibroblast cell lines selects strongly for spontaneous inactivation of the ARF-p53 pathway, by inducing ARF, a protein that upregulates p53 expression (Zindy et al., 1998).

1.12.3.2Epstein Barr virus and Burkitt's lymphoma

Epstein-Barr virus is a gamma-1 herpesvirus found throughout human populations, with a prevalence of over 90% in adults. It was originally isolated from a cell

line derived from a Burkitt's lymphoma biopsy (Mitchell et al., 1967). EBV gains entry to B cells by binding, via its envelope glycoprotein gp340/220, to the B cell surface antigen CD21, a broadly expressed antigen throughout B cell development and that is known to act as a receptor for additional ligands including complement fragments and CD23 (Gregory, 1995). It possesses a linear double stranded DNA molecule approximately 172 Kb in length, which upon entry into the host cell circularises and is subsequently maintained in episomal form.

EBV is a B-cell lymphotropic virus that can induce growth transformation in human and primate B cells *in vitro*, and malignant lymphomas in primates. Human infection results in the establishment of a life-long carrier state characterised by stable numbers of latently infected B cells and the shedding of infectious virus into the saliva. Viral expansion is monitored by the immune system, as stable levels of CTLs and antibodies to lytic and latent-stage proteins accompany persistent infection (Rickinson and Kieff, 1996). EBV is associated with several malignant diseases. Apart from Burkitt's lymphoma, other EBV-related malignancies include Hodgkin's disease, nasopharyngeal carcinoma, immunoblastic lymphomas including X-linked lymphoproliferative disease, and even more rarely, natural killer or T cell lymphomas and gastric adenocarcinoma.

Most of our knowledge of EBV-induced B cell proliferation comes from *in vitro* transformation of normal B cells into permanent lymphoblastoid cell lines (LCLs) in which every cell carries multiple episomal copies of EBV. The widely-used for this purpose, B95.8 strain of EBV was the first to be fully cloned and sequenced (Baer et al., 1984). A restricted number of viral proteins, the 'latent' viral gene products are constitutively expressed in LCLs. These include the Epstein-Barr nuclear antigens EBNA 1, 2, 3A, 3B, 3C and LP (leader protein) and three membrane proteins, the latent membrane proteins LMP 1, 2A and 2B. In addition, two untranslated RNAs, the EBER1 and 2, which are small nonpolyadenylated and non-coding transcripts and by far the most abundant viral products in latently infected cells and a family of transcripts from the BamH1A region of the genome are expressed by their functions are unknown (Rickinson and Kieff, 1996). Genetic analyses indicate that only six of the latent proteins are essential for efficient transformation and permanent growth of B cells (Farrell et al., 1997). These

are EBNA-1, 2, 3A, 3C, LP and LMP-1. Furthermore, LMP-1 is highly oncogenic (Farrell, 1998).

The pattern of viral gene expression as seen in LCLs is referred to as latency III or group III phenotype. *In vivo* however, and in cultures of recently isolated BL specimens a distinct phenotype prevails: group I or type I latency is characterised by the EBERs and the BamH1A transcripts as well as EBNA-1 (required for EBV DNA maintenance). Often, after group I BL cells have been serially passaged *in vitro* they acquire new phenotypic characteristics, including expression of the full set of EBV latent proteins, thus displaying latency III phenotype (Rowe et al., 1987). The transition from a group I to group III phenotype is also accompanied by the modification of growth properties such as resistance to apoptosis from serum deprivation and upregulation of endogenous BCL-2 (Henderson et al., 1991), (Silins and Sculley, 1995). This upregulation is principally mediated by LMP-1 and EBNA-2 (Henderson et al., 1991), (Finke et al., 1992). An intermediate pattern of gene expression, latency II, in which EBNA-1 expression is accompanied by LMP-1 expression is also found in certain diseases (Farrell et al., 1997). Group I cells grow as a single cell carpet and display none of the B cell activation markers consistently present in LCLs such as Ki-24, CD23, CD39. Group III BL, on the other hand, show a more marked progression associated with growth in large clumps, and the expression of the activation antigens CD23 and CD39 as seen in LCLs (Rowe et al., 1987).

Notably, all LCLs regardless of the B cell maturation stage at the time of infection display a consistent array of differentiation antigens on their surface. These antigens include molecules involved in survival signalling, CD23 and CD40 as well as intercellular adhesion molecules LFA-1, ICAM-1 and LFA-3, typical activation markers. Among the latent genes products EBNA 2 and 3C as well as LMP1 are thought to be responsible for the upregulation of these markers (Gregory, 1995).

The evidence linking EBV to Burkitt's lymphoma is statistical and perhaps circumstantial. Considering the high incidence of EBV infection worldwide EBV's association with human disease is rare. The disparate levels of EBV expression in the different forms of the disease indicate that EBV might be an accidental passenger during

the malignant transformation of an EBV positive BL. On the other hand, it would be surprising if the episomal viral genome was maintained (every tumour cell in EBV positive BL contains the virus) without offering an advantage to cell growth and survival. EBV's role was at first seen as enhancing the size of the precursor B lymphocyte pool where Ig rearrangements are occurring thus increasing the possibility of an aberrant rearrangement. However the lack of expression of most of the immortalising genes in BL cells and failure to detect the LCL-like EBV infected cells in lymph nodes makes this explanation difficult to sustain. Because of the location of Ig breakpoints in endemic BL (VDJ region) it is possible that the c-myc translocation may have occurred as an early event, which was followed by EBV infection. EBV infection would then complement c-myc by a growth-permitting function, enhancing the possibility of an early cancer cell growing out into a tumour.

1.12.4 Chronic lymphocytic leukaemia

Chronic lymphocytic leukaemia is one of the four major small B cell diseases in the REAL scheme of lymphoma classification. It is a neoplasm of small B lymphocytes in the peripheral blood, bone marrow and lymph nodes, usually expressing CD5 and CD23. CLL is the most common leukemia of adults in the western world where it accounts for 25-30% of adult leukaemias (Muller-Hermelink and Catovsky). However, the true incidence of CLL is difficult to assess, since patients may be asymptomatic prior to an incidental diagnosis (Warnke, 1999).

CLL is defined as a neoplasm composed of monoclonal lymphocytes that have the cytological appearance of unstimulated lymphocytes. Cells appear small; they are slow-growing and accumulate in the body predominantly in the G_0 phase of the cell cycle. In addition a low mitotic index is generally associated with the disease (Andreeff et al., 1980). CLL is distinguished by the expression of B cell surface markers such as CD19, CD20 and CD23 together with the T-cell antigen CD5 in the absence of other T cell markers. This co-expression only occurs in one other disease, mantle cell lymphoma (MCL), however MCL cells can be distinguished from CLL cells from the lack of CD23 and the high expression of cyclin D1. Furthermore, the typical CLL cell expresses faint amounts of surface immunoglobulins, mostly of the IgM of IgD subtype (Kalil and

Cheson, 1999). There are no clearly defined risk factors for CLL although several lines of evidence such as increased prevalence of CLL among first-degree relatives suggest a genetic component.

1.12.4.1 Clinical features and treatment of CLL

The clinical features of CLL are directly related to the gradual and progressive accumulation of the leukaemic B cells in the lymphoid tissues and bone marrow. One clinical feature of CLL is the occurrence of a variable degree of humoral immunodeficiency in up to 70% of these patients, as evidenced by the appearance of hypogammaglobulinemia, diminished humoral response to antigen immunization, and frequent infections, mainly bacterial. There is also a high prevalence of autoimmune phenomena.

The disease course in CLL is also variable and the impact on survival is heterogeneous with some patients generally experiencing a prolonged phase of indolent disease and eventually dying from causes unrelated to CLL whereas other patients show a rapidly progressive disease (PRD) with death directly due to CLL or its direct complications. Although raised lymphocyte counts and high tumour burden are generally present in both phases, tumour growth rapidly accelerates in PRD with bone marrow failure, poor general conditions and urgent need for treatment.

The expansion of the total tumour burden occurs without any apparent reaction of the immune system against it. This may be caused by an impaired T cell-mediated response, including a depressed function of NK cells and antibody-mediated cellular cytotoxicity, a reduced susceptibility of the tumour cells towards the effector cells, or an inability of the tumour cells to function as APCs. This is despite the strong expression of major histocompatibility complex class I and class II molecules (Nouri et al., 1998), (Guy et al., 1986). Failure to express adequate co-stimulation, particularly B7-1 molecules, may be a factor in this, since it would lead to anergy or tolerance of effector T cells (Buhmann et al., 1999).

The proliferation, survival and antigen presenting capacity of normal B cells depend on signals transduced through their sIg receptors and through CD40. As in most B cell tumours, CLL cells express CD40. CD40L-stimulated CLL cells also exhibit

enhanced costimulatory activity in mixed lymphocyte reactions (Buhmann et al., 1999). Triggering CD40 in B cell lymphoma lines has been reported to suppress growth and induce apoptosis in some cases (An et al., 1997), (Wang et al., 1997). However, CD40 can also play an important role in promoting both normal and neoplastic B cell proliferation and survival. Given the complex role of CD40 either as a promoter or suppressor of apoptosis, it is important to understand more about its function in CLL as well as other B cell malignancies. In addition, the potential for the clinical use of CD40L to increase the immunogenicity of CLL cells has attracted interest, and pre-clinical trials to evaluate CD40L therapy are underway. In addition to co-stimulatory antigens, CD40L also upregulates expression of Fas on activated normal B cells, providing a means of eradicating B cells that fail to receive complementary signals through their antigen receptors(Schattner et al., 1995).

The most widely used agents in the treatment of CLL are alkylating agents such as chlorambucil and cyclophosphamide and purine analogs, mainly fludarabine. Fludarabine is an adenosine deaminase-resistant nucleotide analogue and is the most active agent in the treatment of CLL available today (Keating et al., 1998, {Rai, 1994 #1048). Several of the drugs used in CLL therapy such as glucocorticoids, chlorambucil, fludarabine and theophylline, induce apoptosis of B CLL cells (Keating, 2001). However, after initial responsiveness to these drugs, the majority of patients become resistant to treatment and cures are rarely achieved (Muller-Hermelink et al., 2001). In fact, CLL is one of the best examples of a malignancy that primarily involves defects in apoptosis rather than cell proliferation. Most investigations on the ability of CLL cells to escape apoptosis have been centred on the BCL-2 protein family. However, additional factors such as abnormal p53 function or cytokine deregulation are implicated in drug resistance (Pepper et al., 1998).

Since CLL is currently untreatable the timing of treatment depends on factors such as the stage of the disease, and the presence/absence of recurrent infections determine the onset of treatment. Prognostic variables considering clinical and laboratory parameters also help to provide guidelines for management and treatment. These include clinical staging systems, lymphocyte doubling time, BM histology, cell morphology,

molecular genetics serum levels of LDH, b2-microglobulin, soluble CD23 and cytokine levels such as IL-2 receptors or ICAM molecules.

1.12.4.2 Molecular features and prognostic factors in CLL

Chromosomal abnormalities are a common feature of CLL. The most common genetic alteration is deletion of 13q14 (more than 50% of patients), followed by deletion of 11q22-23 (19% of patients), and trisomy 12 (15% of patients) (Juliusson and Merup, 1998). Thus in contrast to other B-cell neoplasms and Burkitt's lymphoma the most common chromosomal abnormalities are likely to involve tumour suppressor genes rather than oncogenes. Deletions at 13q14 are associated with a more aggressive clinical behavior in patients with an early form of the disease (Bullrich et al., 2001). 11q22-23 deletions are also associated with disease progression and reduced survival (Starostik et al., 1998). Furthermore, two recently-emerged markers for disease progression and outcome in CLL that may aid in predicting the development of progressive disease and the time of treatment, are now being investigated:

1. The origin of the CLL lymphocyte seems to be more heterogeneous than previously recognised. Recent studies have shown that CLL may originate either from a 'naïve' pre-germinal center cell with unmutated Ig heavy chain variable region (VH) genes or from a memory post-germinal center cell having acquired point mutations in the Ig VH genes (Fais et al., 1998). This finding was independently confirmed demonstrating that the subset derived from naïve B cells corresponds to progressive disease with a median survival of 8 years. The second (memory B cell subset) corresponds to approximately 60% of CLL cases, and has a median survival of 25 years (Damle et al., 1999), (Hamblin et al., 1999). Moreover, patients with 13q14 deletions often have somatic mutations of the VH region whereas Trisomy 12 patients often lack somatic mutations (Naylor and Capra, 1999). The prognostic significance of the absence of mutated VH chains in CLL is substantial, with recent publications also noting an inferior survival and high predisposition to early treatment for symptomatic disease (Damle et al., 1999), (Hamblin et al., 1999). Although the presence of somatic mutations is more likely to be a marker of the origin of the malignant clone, it

allows us to analyse the critical genetic features that distinguish these two populations.

2. CD38 is a surface antigen that mediates variable functions including cell adherence and proliferation. Increased levels of CD38 expression have been associated with poor outcome in CLL (Ibrahim et al., 2001). In addition, cells from patients with unmutated immunoglobulin genes were also positive for CD38 expression (30% or more of the cells) whereas cells from patients with mutated Ig genes had low CD38 expression and indolent disease with significantly longer survival times (Hamblin et al., 2000).

1.12.4.3 Apoptosis deregulation in CLL

The gradual accumulation of CLL cells in the patients' blood and organs despite the low proliferative index cannot be explained by cell proliferation and seems to be the result of prolonged survival. CLL not only prolongs the physiological life span of the cells but also renders them resistant to the cytotoxic effects of many anticancer drugs. Therefore, CLL is a model for apoptosis failure and a variety of biochemical mechanisms by which CLL cells avoid apoptosis have been proposed, some of which are described below.

1. The BCL-2 family proteins are overexpressed in 90% of B-CLL cells although in the large majority of cases there is no chromosomal translocation of the bcl-2 gene as in follicular lymphomas (Bannerji and Byrd, 2000). BCL-2 protein levels however, are comparable to those from a t(14:18)-containing cell line probably as a result of promoter hypomethylation (Hanada et al., 1993). Furthermore, the BCL-2: BAX interaction seems to be a more significant factor rather than BCL-2 levels. In chemosensitive CLL cells BAX levels are rapidly increased following chlorambucil treatment (Pepper et al., 1998) and an increased BCL-2:BAX ratio was found in patients refractory to standard chemotherapy (Pepper et al., 1998). Another study has shown MCL-1 to be associated with failure to achieve complete remission in response to chlorambucil or fludarabine therapy (Kitada et al., 1998).
2. Mutations of p53 are moderately frequent in CLL cells (10-20%), however they become more frequent in progressive disease and correlate with

unresponsiveness to treatment with alkylating or purine analogue-based agents in some studies but not in others (Cordone et al., 1998), (Byrd et al., 1998). Reduced expression and mutations of the ATM protein have been described in CLL and are associated with impaired p53 function. They can also cause loss of cell cycle control and DNA damage-induced responses. Mutations across the entire coding sequence have been found in a moderate proportion of CLL patients (Starostik et al., 1998). Loss of heterozygosity in tumours with the 11q22-23 deletion may indicate gene amplification or loss of tumour suppressor by mutation of the ATM gene in germ-line cells indicating a possible inherited predisposition to CLL (Bullrich et al., 1999), (Stankovic et al., 2002). Amplification of the mdm2 gene has also been described in the leukaemic cells of a high proportion of CLL patients and partial or total replication of chromosome 12 could be responsible for this (Juliusson and Gahrton, 1987). However no correlation has yet been shown between mdm2 expression and karyotype.

3. CD40 expression is normal in CLL cells but CD40 signal transduction is defective and high levels of plasma CD40L have been described in some patients (Laytragoon-Lewin et al., 1998). CD40L is thought to promote CLL cell survival and to rescue cells from FasL-induced apoptosis. In addition, Fas appears to be down regulated on the membrane of CLL cells which may enable the tumour cells to avoid recognition by CTLs (Laytragoon-Lewin et al., 1998).

1.13 Aims

The aim of this study was to determine the sensitivity to Fas and TRAIL-mediated apoptosis in BL cell lines as a model for a B cell malignancy. In addition, it aimed to examine molecular determinants that regulate death receptor-mediated apoptosis and determine the extent of their influence in BL. Finally, this study also aimed to compare the results from this cell system of established cell lines to primary B cell malignancies.

Chapter 3 describes the examination of responsiveness to Fas and TRAIL receptor signalling in a panel of BL cell lines. Regulation at the level of receptor expression was also analysed.

Chapter 4 examines the role of Fas and TRAIL receptors in BL cell apoptosis induced by the DNA-damaging anti-cancer drug, CDDP.

Chapter 5 examines the role of known molecular determinants in the regulation of Fas-induced apoptosis in BL cell lines.

Chapter 6 investigates the role of signalling components of the TRAIL pathway as well as signalling inhibitors in the regulation of TRAIL-induced apoptosis in BL.

Chapter 7 describes spontaneous as well as Fas and TRAIL receptor-mediated apoptosis in *ex vivo* CLL cells and the role of CD40L.

Chapter 2. Materials and Methods

All chemicals and solvents were supplied by Merck Ltd. (BDH laboratory supplies, UK) unless otherwise stated.

2.1 Solutions and Buffers

Agarose gel Loading Buffer (6X)	0.25% (w/v) Bromophenol Blue 15% (w/v) Ficoll 400
2x Tryptone-Yeast (2x TY) Extract medium	16 mg/ml Tryptone (Difco, USA) 10 mg/ml Yeast Extract (Difco, USA) 5 mg/ml NaCl NaOH to pH 7.4
Formamide buffer for single strand conformation polymorphism (SSCP) assay	95% (v/v) Formamide 10 mM NaOH 0.25% (w/v) bromophenol blue 0.25% (w/v) xylene xyanol
Lysozyme solution	50 mM glucose 10 mM Ethylenediaminetetra-acetic acid (EDTA) 25 mM Tris-HCl pH 8.0
Phosphate buffered saline	125 mM NaCl 16 mM Na_2HPO_4 11 mM NaH_2PO_4 HCl to pH 7.2
Ponceau staining solution	0.5% Ponceau S (w/v) 1% acetic acid glacial (v/v)
Protein running buffer	25 mM Tris Base

	200 mM Glycine 0.1% (w/v) Sodium dodecyl phosphate (SDS)
RIPA lysis buffer	150 mM NaCl 50 mM Tris-HCl pH 8.0 5% (v/v) Nonidet-P40 0.5 % (w/v) Deoxycholate (DOC) 0.1 % (w/v) SDS
Tris-Borate-EDTA buffer (TBE)	80 mM Tris Base 80 mM Orthoboric acid 1 mM EDTA
Tris-Buffered Saline (TS)	10 mM Tris-HCl pH 8.0 150 mM NaCl
Tris-EDTA buffer (TE)	10 mM Tris-HCl pH 7.6 2 mM EDTA
Tryptone-Yeast Extract (TYE) plates	10 mg/ml Tryptone 5 mg/ml Yeast extract 8 mg/ml NaCl 15 mg/ml Agar (Difco, USA)
WASH buffer (for flow cytometry)	1% (v/v) Foetal calf serum FCS 0.1% (v/v) NaN_3 in PBS

2.2 Cell culture and treatments

2.2.1 Cell culture and maintenance

All cell lines were cultured in RPMI 1640 growth medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, penicillin G (100 μg/ml) and streptomycin (100 μg/ml). They were maintained in a humidified atmosphere of 10% CO_2 and 37 ^{0}C. All cell culture media were warmed to 37^0 C before addition to the cells. The human Burkitt's lymphoma (BL) cell lines BL40, BL41, BL2, Ramos and Louckes are Epstein Barr Virus (EBV)-negative (Ben-Bassat et al., 1977), (Lenoir et al., 1985). Akata 6 (Takada et al., 1991) and Elijah are EBV-positive group I cell lines (Rowe et al., 1987). Namalwa and Raji are EBV-positive group II cells (Farrell et al., 1997), (Klein and Dombos, 1973) whereas Jijoye, MUTU III clone 148, MAK III and BL 72 are EBV-positive group III BL cell lines (Rowe et al., 1987), (Lenoir et al., 1985). IB4, LCL3 and X50-7 are EBV-immortalized lymphoblastoid cell lines (LCLs), generated by infection of peripheral blood B cells with EBV (strain B95.8) (Wilson and Miller, 1979) (Table 2.1).

To analyse the effect of EBV on sensitivity to TRAIL-induced apoptosis, I used clonal EBV positive and negative Akata cells, derived from the original BL cell line, Akata. Although the original line was described as EBV positive, some clones lost EBV spontaneously in culture while others retained it (Takada et al., 1991). The EBV negative Akata clones used were Akata 1, 23 and 31 and EBV positive Akata clones were Akata 11, 18 and 33 (gift of Prof. P. Farrell) (Inman et al., 2001). Jurkat is a human T cell leukaemia line. K562 is a chronic myeloid leukaemia cell line, HeLa is a cervical carcinoma cell line and the MCF-7 cell line is derived from a breast adenocarcinoma.

Peripheral blood from 11 cases of B-CLL was obtained with informed consent. CLL was diagnosed according to standard clinical and laboratory criteria and peripheral blood was obtained from the Department of Haematology in Royal Bournemouth Hospital. Mononuclear cells were isolated by centrifugation on a Ficoll/Hypaque gradient and used immediately in the assays.

Cell line	EBV status
BL2	EBV negative
BL40	EBV negative
BL41	EBV negative
Ramos	EBV negative
Louckes	EBV negative
Akata 6	EBV positive, group I
Elijah	EBV positive, group I
Namalwa	EBV positive, group II
Raji	EBV positive, group II
BL72	EBV positive, group III
Jijoye	EBV positive group III
MAK III	EBV positive, group III
MUTU III c. 148	EBV positive group III
IB4	LCL
X50-7	LCL
LCL3	LCL

Table 2.1: *EBV status and gene transcription profiles in Burkitt's lymphoma cell lines and LCLs.* Groups I, II and III denote different EBV transcription programmes, see Chapter 1: Introduction for details. The LCL gene expression profile is very similar to group III.

2.2.2 Cell treatment

All cells were diluted to a density of 0.2 x 10^6 cells per ml on the day before the experiment was started, to ensure they were in exponential growth phase and were cultured overnight in 75 cm^2 flasks. CDDP was from Roger Bull Laboratories and was used at 10 μg/ml unless otherwise stated. In experiments where CDDP was added to cells untreated (control) samples were included at the start and end of the experiment. These untreated controls are denoted as 36-CDDP or 48-CDDP throughout. Sensitivity to TRAIL-induced apoptosis was determined by addition of human recombinant TRAIL (rTRAIL), composed of the extracellular domain of human TRAIL fused at its N-terminus to a FLAG-tag domain via a linker peptide (Alexis Corporation). rTRAIL was used with an activity-enhancing antibody (Enhancer) against the FLAG-tag which

cross-links TRAIL (Alexis Corporation). The enhancer was normally used at 5 times the concentration of rTRAIL. The IgM class Fas-specific agonistic antibody (α-Fas: clone CH-11; Upstate Biotechnology) was used to determine cell sensitivity to Fas activation. This antibody is commonly used *in vitro* to mimic the effects of Fas ligand. The IgM class trinitrophenol (TNP)-specific antibody (Pharmingen) was used as an isotype control for CH-11. Both rTRAIL and α-Fas were added directly to the cell culture medium and mixed to disperse.

Target	**Antibody (clone)**	**Use**	**Isotype**
Fas	CH-11	Receptor activation	IgM
	ZB4	Neutralisation	IgG1
	DX2	Flow cytometry	IgG1
FasL	NOK-2	Ligand neutralisation	IgG2

Table 2.2: *Antibodies against Fas and* FasL. The Fas and FasL-specific antibodies and their experimental applications are shown. CH-11 is an IgM class antibody and therefore effectively cross-links Fas whereas ZB4 is IgG class and prevents Fas activation by FasL.

To block signalling via the TRAIL receptors, recombinant chimeric proteins consisting of the extracellular domain of human TRAIL receptors DR4 and DR5 coupled to the constant region (Fc) of human IgG1 (rhFc:DR4 or rhFc:DR5) were used. The recombinant receptors act via sequestering TRAIL. rhFc:DR3 is a chimeric protein comprising the extracellular domain of the death receptor family member DR3, and was used as a negative control since the DR3 receptor does not bind TRAIL. The chimeric receptors were a kind gift from Calvin Roff (R&D Systems). The reagents used to block signalling via Fas were the Fas-specific antibody ZB4, (Immunotech) and the FasL-specific antibody NOK-2 (Pharmingen). All Fas specific antibodies and the FasL-blocking antibody are listed in Table 2.2. When more than one reagents were used to treat the cells, the reagents were mixed together at the appropriate concentration before addition to the tissue culture media.

Human CD40-specific monoclonal antibody purified from supernatant of the hybridoma cell line G28-5 (American type culture collection) (purified by Dr. L.

McCarthy-Morrogh) or trimeric (leucine zipper mediated) human CD40L were used to activate CD40 in CLL cells.

2.3 Cell proliferation and apoptosis assays

2.3.1 Cell viability assay

Viable cell numbers were determined at various times during experiments by dilution in an equal volume of 0.4% (w/v) trypan blue in 0.81% (v/v) NaCl and 0.06% (v/v) K_2HPO_4 (Sigma). Non-viable cells that have lost membrane integrity take up the dye and appear blue under the light microscope whereas viable cells exclude the dye. A total number of at least 100 cells (viable and dead) per sample were counted using a haematocymeter.

2.3.2 MTS assay

The Cell Titer-96 Aqueous Cell Proliferation Assay (Promega) was used routinely to investigate the effects of reagents on the survival of B cells. This approach was chosen because of the expense of the reagents and has been used extensively in the literature (Cuello et al., 2001). It is an indirect way of determining cell proliferation and comprises a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl) (MTS), and an electron coupling reagent, phenazine methosulfate (PMS). The conversion of MTS into a coloured, water-soluble formazan product is catalysed by dehydrogenases in metabolically active cells (Cory et al., 1991). The formazan product is then detected by spectrophotometry. Direct comparisons between ^{3}H-thymidine incorporation and tetrazolium conversion have demonstrated less than 5% difference between the two bioassays (Promega, Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay, Technical Bulletin). The cells were plated at a density of $1x10^5$ cells per well (100 µl total volume) in a 96-well plate and allowed to recover for 2 - 4 hours before addition of test reagents. CLL cells were plated at a density of 2×10^5 cells/ well. Following the required treatment period, 20 µl of a solution containing both MTS and PMS were added to the tissue culture medium and the cells incubated for a further 3 - 4 hours at 37 °C until the absorption of the coloured product was measured in an ELISA plate reader using the 490 nm filter. Typically, triplicate determinations were made and the results are presented as the mean absorbance value for each set of triplicate wells minus the mean obtained from three wells with media alone (corrected absorbance). Standard error of the mean and

standard deviation were calculated using the data analysis tools in Excel (Version, 2000; Microsoft, USA).

2.3.3 Long-term survival assays

To determine whether α-Fas or rTRAIL affected the long-term growth potential of cells, a serial dilution assay was performed (Illidge et al., 2000). Cells were treated with α-Fas (500 ng/ml) or rTRAIL (500 ng/ml) or left untreated as a control and then plated into individual wells of a 96-well plate at a density of 0.98 x 10^4 cells per 100 µl. This initial cell stock was serially diluted (three-fold dilutions; eight dilutions) to give a final cell density of approximately 5 cells per well. After 14 days the fraction of wells containing no surviving cells was determined by visual inspection using the light microscope. The dilution factor that resulted in 70 % empty wells was recorded.

2.3.4 Propidium iodide (PI) staining of DNA

Cellular DNA content was analysed by PI (Sigma) staining and flow cytometry. Typically, $1x10^6$ cells were harvested by centrifugation for 3 minutes at 1300 rpm (Sorvall RC3C with H-6000A rotor), washed once in ice-cold PBS and fixed in 80% ice-cold ethanol with vortexing. Fixed cells were kept for at least 1 hour at 4^0 C before being collected by centrifugation for 2 minutes in a microfuge. The ethanol was removed by aspiration and the cells were resuspended in 1 ml of PBS with 100 µg/ml RNase A and 50 µg/ml PI and incubated in the dark for 30 minutes at RT. The cells were analysed on a flow cytometer (FACSCalibur, Becton Dickinson), using the FL2-A channel. 10000 events per sample were acquired. The percentage of dying cells in a sample was defined as the fraction of nuclei with hypodiploid DNA content on PI fluorescence histograms.

2.3.5 PI exclusion assay

A PI exclusion assay was used to analyse CLL cell viability. PI is excluded from viable cells (if not fixed) whereas in dead cells it is taken up and incorporated into DNA. 1 x 10^5 CLL cells were resuspended in ice-cold flow cytometry wash buffer and PI was added to a final concentration of 2 µg/ml. Cells were incubated for 5 minutes at RT and PI uptake was determined by analysis on a flow cytometer using the FL2A channel. 10000 events per sample were acquired.

2.4 CD40, Fas, DR4 and DR5 receptor surface expression

Cell surface expression of CD40, Fas, DR4 and DR5 receptors on live cells was measured by flow cytometry. The CD40-specific monoclonal antibody was prepared from supernatant from the hybridoma G28-5. The Fas-specific antibody, DX2 (Pharmingen), was used to detect cell surface Fas expression. DR4 and DR5-specific antibodies were a gift from Calvin Roff (R&D Systems). An equivalent concentration of isotype-matched control immunoglobulins was used to control for non-specific staining (Table 2.3).

1x 10^6 cells per sample were collected by centrifugation at 1300 rpm for 3 minutes (Sorvall RC3C with H-6000A rotor) and washed once in wash buffer (containing 1% (v/v) heat-inactivated FCS to block surface Fc receptors). They were incubated on ice for 45 minutes in wash buffer with 1 μg of primary antibody (100 μl total volume). The cells were subsequently washed twice in wash buffer and incubated with 1 μg/ml FITC-conjugated rat anti-mouse antibodies (Pharmingen) or FITC-conjugated streptavidin (Stressgen, UK) for 45 minutes on ice. Cells were washed once in wash buffer and analysed on a FACSCalibur flow cytometer. Fluorescence signals were recorded in logarithmic mode. For each sample 10000 events were acquired. Analysis of flow cytometer data was carried out using the CellQuest software. The relative fluorescence intensity (RFI) values for CD40, DR4 and DR5, representing the antigen density, were determined as follows:

RFI: Mean fluorescence intensity of experimental samples / Mean fluorescence intensity of isotype-matched antibody-stained samples.

Antigen	Primary antibody	Isotype-matched control	Secondary reagents
CD40	Mouse monoclonal (G28-5), IgG1	α- TNP, mouse monoclonal IgG1	FITC-conjugated rat α-mouse IgG1 immunoglobulins
Fas	Mouse monoclonal, IgG1, κ chain	α-TNP, mouse monoclonal IgG1, ..κ chain	FITC-conjugated rat α-mouse IgG1 immunoglobulins
DR4	Biotinylated goat α-human DR4	Biotinylated goat immunoglobulins	FITC-conjugated streptavidin
DR5	Biotinylated goat α-human DR5	Biotinylated goat immunoglobulins	FITC-conjugated streptavidin

Table 2.3: *Antibodies used for the detection of cell surface antigens by flow cytometry.* The antibodies used to detect CD40, Fas, DR4 and DR5, the corresponding isotype controls and detection reagents are shown.

2.5 Western blotting

2.5.1 Protein extraction for western blotting

Cells were collected by centrifugation for 3 minutes at 1300 rpm (Sorvall RC3C with H-6000A rotor) and washed once in ice-cold PBS. The cells were then lysed in RIPA buffer containing a protease inhibitor cocktail comprising (4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane, bestatin, leupeptin and aprotinin) (Sigma) with broad specificity for the inhibition of serine, cysteine and aspartic proteases and aminopeptidases. Protein lysates were clarified by centrifugation at 12000 rpm for 15 minutes at 4° C in a microcentrifuge and the protein content of the supernatant determined using the Protein Assay Reagent (BioRad Laboratories) with bovine serum albumin (Sigma) as standard. An equal volume of 2x SDS sample buffer containing 82 mM dithiothreitol (DTT) (New England Biolabs) was added to the protein extract and the lysate was heated at 95 ^{0} C for 4 minutes before loading onto an SDS-polyacrylamide gel. Broad range protein markers (New England Biolabs) were used as molecular weight standards. Unless otherwise stated a total protein content of 20 μg was used in each analysis. When only small numbers of cells were available for

western blotting analysis, whole cell lysates were prepared by resuspending the cell pellets in 100 µl of 1x SDS sample buffer with 41 mM DTT, sonicating for 30 seconds at 4 ^{0}C and heating at 95 ^{0}C for 4 minutes before analysis.

2.5.2 Western blotting

This was performed in accordance with the methods of Laemmli (Laemmli, 1970). Gels were prepared using acrylogel 2.6 solution (30% acrylamide: bisacrylamide 37.5:1; BDH). Mini-gels (120 mm x 82 mm, Bio-Rad mini protean II unit) were run at 20V/ cm for 1 hour in protein running buffer. Different concentrations of acrylamide were used (10% or 12% total acrylamide content) according to the molecular weight of the protein investigated.

Proteins were then 'wet' electroblotted onto nitrocellulose membrane (Schleicker & Schuell) in protein running buffer with 25% (v/v) ethanol in a mini protean II transfer unit (Bio-Rad) for 1 hour. Non-specific protein binding sites were blocked by incubating the membrane in a 5% (w/v) non-fat milk solution (Marvel) in TS buffer for approximately 1 hour at RT. Filters were usually incubated overnight with primary antibodies diluted in binding buffer (TS with 0.05% (v/v) Tween-80 and 5% (w/v) non-fat milk). The nitrocellulose membranes were washed three times in TS and incubated for 1 - 2 hours with the appropriate secondary antibody solution (horse radish peroxidase (HRP)-conjugated, Amersham), typically at a dilution of 1:2000. The membranes were washed three times in TS and bound immunocomplexes were detected by enhanced chemiluminescence (Amersham) on autoradiography film or a fluorescence imager (Bio-Rad). Unless otherwise stated western blotting experiments were repeated twice.

2.5.3 Antibodies used for western blotting

Antibody	Immunogen	Supplier	Dilution/ concentration[a]
BCL-2 (mouse monoclonal-clone 124)	Amino acids (aa). 41-54	DAKO	1 µg/ml
Caspase 8 (rabbit polyclonal)	aa. 2-20	Pharmingen	1:1000
DR4 (goat polyclonal)	extracellular domain	R&D Systems	1 µg/ml
DR5 (goat polyclonal)	extracellular domain	R&D Systems	1 µg/ml

FADD (mouse monoclonal)	death domain	Transduction Laboratories	1:1000
FAP-1 (rabbit polyclonal)	Human FAP-1	Gift from Prof. Taka-Aki Sato	1 μg/ml
FLIP (rabbit polyclonal)	aa.447-464	Upstate Biotechnology	1:1000
MCL-1 (rabbit polyclonal-clone S-19)	internal region	Santa Cruz	1:500
p53 (mouse monoclonal-clone DO-1	aa. 11-25	Gift from Dr. Xin Lu	1:50
PARP (mouse monoclonal-clone C2.10)	Bovine PARP	TCS Biologicals	1:1000
PCNA (mouse monoclonal-clone PC-10)	Rat PCNA	Gift from Dr. Xin Lu	1:50

Table 2.4: *Antibodies used in western immunoblotting.* The antibodies used for Western blotting and the relevant immunogens are shown.

[a]Dilution factor is given where concentrations are not known

2.6 DNA extraction and manipulation

DNA cloning was performed using standard procedures (Sambrook et al., 1989) and TOPO-TA cloning systems for PCR products (Invitrogen, USA).

2.6.1 Restriction endonuclease digestion

All restriction endonucleases were obtained from New England Biolabs. A typical double stranded DNA digestion reaction was set up as follows:

10X Restriction Endonuclease buffer	1.5 μl
Restriction Endonuclease	1 μl (units variable)
Double stranded DNA in sterile water	Up to 1 μg (2-6 μl)
Sterile water	To a final volume of 15 μl

The restriction reactions were generally incubated at 37 ^{0}C (or the appropriate temperature for the restriction enzyme used) for 2 - 4 hours. In order to achieve total digestion, for example when linearising a vector for use in RNase protection assay (RPA), the reactions were incubated overnight. Digestions of small-scale preparations of DNA (Miniprep) were supplemented with 0.1 mg/ml of ribonuclease (RNase) A and incubated at 37 ^{0}C for two hours. The resulting fragments were subjected to agarose gel electrophoresis and visualised under UV transillumination.

2.6.2 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was performed using 1.0% (v/v) agarose gels (Seakem CTC Agarose, FMC BioProducts) containing 1 μg/ml ethidium bromide in TBE buffer.

2.6.3 Phenol:chloroform extraction and ethanol precipitation of nucleic acids

In order to purify DNA or RNA from enzymatic reactions, proteins were extracted by diluting the reaction in sterile water or appropriate buffer to at least 100 μl, adding an equal volume of Phenol:chloroform (1:1) and vortexing. The emulsion was then centrifuged in a microfuge at 13000 rpm for 3 minute at RT to separate aqueous and organic phases. The upper aqueous phase was removed and residual phenol was extracted by adding an equal volume of water-saturated chloroform and repeating the procedure. To precipitate DNA, 1/10 volume of 3M sodium acetate, pH 4.8 and 2 volumes of 100% ethanol were added and incubated at 4 ^{0}C overnight or at –20 ^{0}C for 30 minutes to 1 hour. The DNA was then collected by centrifugation in a microfuge at 13000 rpm for 15 minutes at 4 ^{0}C, washed once in ice-cold 70% (v/v) ethanol and allowed to air dry. The DNA was then resuspended in TE buffer.

To precipitate RNA, a similar procedure was followed but RNA was precipitated by adding 2.5 volumes of 100% ethanol. The reactions were incubated at 20 ^{0}C for 20 minutes to 1 hour, and the RNA was precipitated by centrifugation, washed in 90% (v/v) ethanol, air dried and resuspended in water or the appropriate buffer.

2.6.4 Gel purification of DNA

In order to purify DNA fragments, DNA was electrophoresed on a 1% (w/v) TBE agarose gel, the bands were excised and purified using the Freeze and Squeeze Gel Extraction Spin columns (BioRad, USA) according to the manufacturer's instructions.

2.6.5 Polymerase chain reaction (PCR) amplification of DNA fragment

A typical PCR reaction would be:

10x Reaction Buffer	5 µl
Deoxynucleotide mix (1:1:1:1 of dATP, dTTP, dCTP, dGTP) (each at 2.5 mM)	4 µl
$MgCl_2$ (2.5 mM)	3 µl
Primer 1 (25 µM)	1 µl
Primer 2 (25 µM)	1 µl
Taq polymerase (10 units/µl)	1 µl
Sterile water	35 µl

All components of the PCR reactions were purchased from Promega (UK).

The cycling parameters of a typical PCR reaction using a Perkin Elmer Cetus DNA Thermal Cycler were:

Initial denaturation	95^0 C for 4 minutes	1 cycle
Denaturation	95^0 C for 1 minute	
Primer annealing	Variable	30 cycles
Extension	72^0 C for 45 seconds	
Final extension	72^0 C for 5 minutes	1 cycle

Primer sequences and specific annealing temperatures are described in the following section.

2.6.6 PCR primers

Primers	Sequence	Annealing conditions
PTP-BAS1 (FAP-1)	5'-GAATACGAGTCAGACATGG	50 ^{0}C for 60 seconds
PTP-BAS2 (FAP-1)	5'-AGGTCTGCAGAGAAGCAAGAATAC	
Fas-DD (Fas death domain1)	5'-AGAAAGCACAGAAAGGAAAACC	50 ^{0}C for 60 seconds
Fas-DD2 (Fas death domain)	5'-ACAGCCAGCTATTAAGAATC	

Table 2.5: *PCR primers for the amplification of FAP-1 and the death domain of Fas are shown.*

2.6.7 TOPO-TA cloning

The TOPO-PCR II vector (Invitrogen, USA) was used to subclone PCR products. The plasmid vector is supplied linearised with single 3' deoxythymidine overhangs. These allow the efficient ligation of PCR products generated by Taq polymerase (which adds single deoxyadenosine residues to the 3' ends of PCR products). The vector contains covalently attached topoisomerase I to facilitate ligation of the PCR products. TOPO cloning reactions were carried out according to the manufacturer's recommendations. A typical ligation reaction would be:

Fresh PCR product	0.5 - 3 μl
Salt solution	1 μl
Sterile water	1 μl
TOPO vector	To a final volume of 5 μl

2.6.8 Bacterial strains

The *Escherichia coli* strain TOP10 OneShot (Invitrogen, USA) was used in both small and large -scale preparation of plasmid DNA.

2.6.9 Transformation of competent bacteria

For TOPO-TA based cloning of PCR products using the TOPO-PCR II vector (Invitrogen, USA), TOP10 OneShot bacteria were used for transformation. 2 μl of the ligation reaction were added to a vial of chemically competent cells, mixed gently and left on ice for 5 minutes. The cells were then heat-shocked at 42 ^{0}C for 30 seconds and immediately transferred to ice. SOC medium (250 μl) (Invitrogen, USA) was added to the vial and the cells were incubated at 37 ^{0}C with shaking for 1 hour prior to plating on TYE agar plates supplemented with the appropriate selecting antibiotic (usually 50 μg/ml ampicillin) and coated with 40 μl of a 40mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactoside solution for blue-white screening. This bacterial strain does not overexpress the lac repressor and therefore no isopropylthio-beta-D-galactoside is required.

2.6.10 Plasmid constructs

pBSDR4 and pBSDR5 (gift of Dr. G. Packham) contain fragments of the human DR4 and DR5 genes cloned into pBluescript vector and were used to generate templates for RPA analysis.

pFAP-1 contains cDNA from human FAP-1 (4390-4996 base pairs (bp)) and was used as template for RPA analysis. cDNA was amplified by RT-PCR from total RNA isolated from BL72 cells as described in Sections 2.6.5 and 2.7.2 using primers PTP-BAS1 and PTP-BAS2 (Section 2.6.6). The PCR product was ligated into the EcoRI site of TOPO-PCR II (Section 2.6.7) and a clone was chosen oriented with the 5' end proximal to the T7 promoter sequence. For RPA analysis the plasmid was digested with XhoI and the antisense probe was transcribed using SP6 RNA polymerase.

pTRUNDD Contains an XmnI restriction digestion fragment of the human TRUNDD cDNA prepared from pCMVFLAG1-TRUNDD (gift of Dr. C. Vincenz). The restriction digestion fragment was purified from agarose gel using Spin and Squeeze DNA columns as described in section 2.6.4. To ligate the product into TOPO-PCR II a 'tailing' reaction to insert 3' deoxyadenosine residues (A) residues was carried out as follows:

DNA	10 µl (5 µg)
10x Reaction buffer	4 µl
Taq polymerase	1 µl (10 units)
$MgCl_2$ (2.5 mM)	3 µl
dNTPs (each at 2.5 mM)	4 µl
Sterile water	To a total volume of 40 µl

The 'tailing' reaction was incubated at 72 ^{0}C for 10 minutes and the product was ligated into the EcoRI site of TOPO-PCR II as described in section 2.6.7 and a clone was chosen oriented with the 5' end proximal to the T7 promoter sequence. For use in RPA analysis the plasmid was linearised by restriction digestion using StyI and the antisense probe was transcribed using SP6 RNA polymerase.

2.6.11 Large scale preparation of plasmid DNA (Maxiprep)

Alkaline lysis of competent bacteria and caesium chloride (CsCl) gradient isolation of plasmid DNA was carried out as previously described (Birnboim, 1983). A 500 ml culture of bacteria was allowed to grow overnight with shaking at 37 ^{0}C. The cells were harvested by centrifugation at 5000 rpm for 10 minutes (IEC PR7000 centrifuge with GS-3 rotor) and lysed in lysozyme solution (20 ml) supplemented with 20 mg lysozyme (Sigma) at RT for 5 minutes. 40 ml of a solution containing 1% (w/v) SDS and 0.2 M NaOH was added and the lysate incubated for 5 minutes at RT. 20 ml of 3M of sodium acetate (trihydrate salt) (pH 4.8) was added, mixed by gentle shaking and cellular debris was subsequently removed by centrifugation at 7000 rpm for 20 minutes (IEC PR 7000 Centrifuge with GS-3 rotor). The resultant supernatant was filtered through a muslin cloth and plasmid DNA was precipitated and harvested by addition of 50 ml propan-2-ol and centrifugation at 7000 rpm for 20 minutes (Sorvall RC5C with SLA1500 rotor). The DNA was resuspended in 20 ml sterile water and 25 g CsCl was added and allowed to dissolve completely before the addition of 7 mg of ethidium bromide. The preparation was clarified by centrifugation at 10000 rpm for 10 minutes (Sorvall RC5C with SS34 rotor). The supernatant was removed and the DNA was banded by centrifugation in an ultracentrifuge (Beckman L8-M with 70Ti rotor; 50000 rpm for16 hours at 20 °C followed by 40000 rpm for 1 hour at 20 °C) and then

halted without the brake. Plasmid DNA was removed from the caesium gradient using a peristaltic pump and the ethidium bromide removed by repeated extraction with water-saturated butan-1-ol. The DNA was then ethanol precipitated, harvested by centrifugation, air-dried and resuspended in TE buffer.

2.6.12 Miniprep

Minipreps were performed using alkaline lysis essentially as described before (Sambrook et al., 1989). 2 ml of 2x TY medium were inoculated with individual bacterial colonies from an agar plate and grown overnight at 37 °C with shaking. The cells were harvested by centrifugation in a microfuge at 13000 rpm and the supernatant discarded. 100 µl of lysozyme solution supplemented with 2 mg/ml lysozyme was added to the cell pellet, the cells were resuspended by vortexing and incubated at RT for 5 minutes. 200 µl of 0.2 M NaOH, 1% (w/v) SDS was added and after thorough mixing, 150 µl of 3 M sodium acetate were added followed by incubation at –20 °C for at least 10 minutes. Cellular debris was removed by centrifugation at 13000 rpm in a microfuge and the supernatant retained. Plasmid DNA was precipitated by addition of 900 µl of 95% (v/v) ethanol at 20 °C and harvested by centrifugation at 13000 rpm in a microfuge. DNA was air-dried and resuspended in 50 - 60 µl of TE buffer.

2.7 RNA extraction and RPA Assays

2.7.1 Extraction of total RNA

Total RNA was extracted from cells using the RNazol reagent (AMS Biotechnology, USA) as recommended by the manufacturer. Briefly, 30 x 10^6 cells were harvested by centrifugation at 1300 rpm (Sorvall RC3C with H-6000 rotor) washed in ice-cold PBS and lysed in 6 mls of RNazol B. RNA was extracted and precipitated with isopropanol.

2.7.2 cDNA synthesis

A master reaction mix was first prepared:

MMLV Reverse transcriptase	
5X Buffer	12 µl
dNTP mix (each at 2.5 mM)	24 µl
RNasin (RNase inhibitor) (40 units/ µl)	1.2 µl
AMV Reverse Transcriptase	
(20 units/ µl)	2.4 µl
Oligo $(dT)_{15}$ primers (500 µg/ml)	1.2 µl
Sterile water	5 µl

5 µg of total RNA were vacuum dried and resusepended in 5 µl of sterile water, heated to 95 ^{0}C for 4 minutes and cooled rapidly on ice. 15 µl of reaction mix was then added to the RNA to give a total volume of 20 µl. cDNA synthesis reactions were carried out at 42 ^{0}C. All reaction components were from Promega, UK. Control reactions lacking reverse transcriptase were also performed to monitor DNA contamination.

2.7.3 Radiolabelled probe generation

In order to generate an anti-sense riboprobe for RPA analysis, 10 µg of plasmid DNA containing the target cDNA sequence were linearised by restriction digestion. The restriction site was chosen so as to yield an antisense transcript of 200-300 bp. The digested plasmid DNA was purified by phenol:chloroform and chloroform extraction, precipitated and resuspended in water at a final concentration of 1 µg/µl. The digested plasmid DNA was then used as the template in an *in vitro* transcription reaction:

5x Transcription optimised buffer	4 µl
T7 or T3 RNA Polymerase (20-40 units)	1 µl
RNasin (20-40 units)	1 µl
Ribonucleotide mix (1:1:1 of rATP, rCTP, rGTP, each at 2.5 mM)	4 µl
DTT at 100 mM	2 µl
α-[^{32}P]UTP (740 MBq/ml or 30 TBq/mmol)	2 µl

Sterile water	5 μl
Linearised plasmid DNA (1 μg)	1 μl

All reagents were from Promega, UK.

The transcription reactions were incubated at 37 ^{0}C for 1 hour. 1 μl of RQ1 DNase (1 unit) was then added to digest the DNA template and the reaction incubated at 37 ^{0}C for a further 15 minutes. 20 μl of denaturing gel loading dye (Ambion RPA III assay kit) were added and the preparation heated to 95 ^{0}C for 4 minutes. The reaction was loaded onto a denaturing gel (Sequagel-6, National Diagnostics) and electrophoresed in 1x TBE for approximately 1.5 hours at 20 W. The position of the full length probe was visualised by autoradiography and the region excised and eluted at 65^0 C in 100 μl Riboprobe Elution Buffer (Ambion RPA II assay kit). After 1 – 2 hours, the activity of the eluted probe was determined by Cerenkov scintillation counting (Beckman LS3801).

In addition to probes for TRUNND, DR4, DR5 and FAP-1, the hAPO3C Multi-probe template Set (Pharmingen) was used as a template to generate probes to simultaneously detect caspase 8, FasL, Fas, TRID, DR3, DR4, DR5, TRAIL, TNFR1, TRADD and RIP as well as two housekeeping gene products, L32 and GAPDH. In this case, the probes were not gel purified and were extracted by phenol:chloroform and ethanol precipitation. The precipitated probe was resuspended in 50 ml of RPA III hybridisation solution (Ambion, USA) by vortexing and the activity of the probe determined by Cherenkov counting. The probe was always used at the optimal concentration of 1x 10^5 cpm/ μl/ hybridisation reaction as recommended by the manufacturer. The transcription reaction to generate the multiple probes was carried out as follows:

5x Transcription buffer	4 μl
T7 RNA Polymerase (20-40 units)	1 μl
RNAsin (20-40 units)	1 μl
Ribonucleotide mix (ATP, CTP, GTP at 2.5 mM each)	4 μl

DTT at 100 mM	2 µl
α-[32^P]UTP (740 MBq/ml or 30 TBq/mmol)	5 µl
Sterile water	2 µl
hAPO3C multi-probe template	1 µl

All reagents were from Promega, UK.

2.7.4 RPA analysis

RPAs were carried out using the RPA III kit, and following the manufacturer's instructions (Ambion, USA). Typically 5-10 µg of RNA was hybridised with 25000 counts per minute of radiolabelled riboprobe. Probe was added to the RNA and the mixture (if less than 5 µl total volume) was then resuspended in 20 µl of hybridisation buffer (Ambion RPA III kit). A negative control reaction was also set up using the same amounts of probe and yeast total RNA to control for non-specific hybridisation. If the sample RNA and probe mixture was more than 5 µl in volume, it was first dried under vacuum (Savant DNA Speedvac 100) for approximately 20 minutes and then resuspended in hybridisation buffer. The reactions were heated to 95 ^{0}C for 4 minutes to denature the RNAs and then incubated at 45 ^{0}C for 16-20 hours.

Following incubation, free probe and other single stranded RNA molecules were digested by adding 200 µl of RNase digestion buffer containing 0.01 units of RNase A and 2 units of RNase T1 (Ambion RPA III kit) and incubation at 37 ^{0} C for 30 minutes. The RNA was then precipitated by adding 300 µl of RNA precipitation buffer (Ambion RPA III kit) and incubating at -20 ^{0}C for at least 15 minutes. RNase-protected fragments were collected by centrifugation at 13000 rpm in a microfuge, at 4 ^{0}C for 20 minutes and the supernatant was discarded. Pellets were then resuspended in 6 µl of denaturing loading dye, the mixture heated to 95 ^{0}C and electrophoresed on a denaturing gel (Sequagel-6, National Diagnostics/ prepared according to the manufacturer's instructions) on a Bio-Rad Sequi-gen apparatus in 1x TBE at 25 W maintaining the tank temperature at 50^0 C. The gel was then vacuum dried and exposed on autoradiography film. Analysis of RNAs for the housekeeping gene, GAPDH (or L32 when the multi-probe template set was used) was performed as a control.

2.8 SSCP analysis

SSCP was used to screen for mutations in the death domain of Fas. The Fas death domain was first amplified from cDNA using primers and conditions shown in Section 2.6.6. The PCR products were digested with an appropriate enzyme to generate fragments smaller than 300 bases. Digested PCR products were diluted 1:10 in formamide buffer and heated at 95 ^{0}C for 3 minutes followed by immediate cooling on ice. Fragments were resolved using the FMC Mutation Detection Enhancement (MDE) gel matrix (Intermountain Scientific, USA), at 11-12 W for 12-14 hours. After electrophoresis the gel was fixed in fixative (10 % (v/v) acetic acid and 10% (v/v) ethanol), transferred onto 3MM Whatman paper, dried and the DNA fragments visualised by autoradiography.

Chapter 3. Sensitivity to death receptor-mediated apoptosis in Burkitt's lymphoma cell lines

3.1. Introduction

Death receptors provide one of the most direct means of activation of the cellular apoptotic machinery. The death receptor family members and most importantly the Fas and TRAIL receptors, are capable of delivering a potent apoptotic signal upon binding to their natural ligand or cross-linking antibodies. The physiological role of Fas in the immune system is well characterised and an important role for the TRAIL receptors in immune system homeostasis and innate immune responses is now emerging. Death receptor functions also have important implications for the establishment and treatment of human diseases, most importantly cancer. Resistance to signalling through Fas in particular has been implicated in the establishment and progression of tumours. In addition, TRAIL and Fas pathways may contribute to anti-cancer drug toxicities and can co-operate with anti-cancer agents to induce apoptosis in some settings. Death receptors are also the focus of research into the development of novel anti-cancer strategies with enhanced efficiency and specificity but with reduced toxicity. The proven efficacy in reducing tumour burden in animal models and the observed lack of systemic toxicity (Walczak et al., 1999), (Ashkenazi et al., 1999) indicates that TRAIL in particular may prove important in tumour therapy. The latest formulations of recombinant TRAIL are now being considered for clinical trials (Lawrence et al., 2001). In addition, Fas-activating reagents are currently considered as part of combination treatments only, due to hepatotoxicity resulting from systemic treatment.

Given the current interest in using Fas and TRAIL receptor-activating agents in therapeutic regimens and the impact that they may have on existing chemotherapeutic strategies, it was important to determine the sensitivity of BL cells to TRAIL. Indeed, sensitivity of BL to Fas stimulation has been shown to be variable whereas TRAIL-mediated apoptosis in BL has not yet been investigated. Since the Fas and TRAIL pathways share signalling intermediates it was interesting to directly compare their effects in BL cells of various phenotypes.

3.2. Burkitt's lymphoma cell lines are resistant to TRAIL-mediated apoptosis

A panel of BL and lymphoblastoid cell lines were screened for sensitivity to TRAIL-induced apoptosis. The BL cell lines analysed were selected to include EBV positive and negative cells with different EBV transcription programmes. Three LCL cell lines, derived from normal B lymphocytes infected by EBV *in vitro* (Falk et al., 1992), were used as controls. Cell growth assays were performed using rTRAIL to treat the panel of BL cells and LCLs in 96-well plates and the MTS assay to measure cell growth. rTRAIL is FLAG-tagged and was always used in combination with the enhancer (an anti-FLAG antibody that induces cross-linking of the ligand and thus TRAILR activation). The enhancer was typically used at 5 times the concentration of rTRAIL. The MTS assay measures viable cell numbers using a soluble reagent, which is bioreduced in metabolically active cells into a soluble formazan product. This product is soluble in tissue culture media, thus allowing absorbance measurement in an ELISA plate reader. The absorbance value of each well is proportional to the number of metabolically active cells in that well. The absorbance of media-only wells was subtracted from experimental values to provide the corrected absorbance values. Since this assay can be performed at a small scale, it is ideal for experiments using expensive reagents and therefore is frequently used in studies of TRAIL as well as Fas-induced apoptosis.

The cells were incubated with increasing concentrations of rTRAIL (5, 50 or 500 ng/ml) combined with enhancer or with enhancer alone (at the highest concentration, i.e. 2.5 μg/ml) as a control. The concentration range of rTRAIL was based on previous reports (Schneider et al., 1997). The cells were treated for 24 (data not shown), 48 hours or left untreated as a control before cell growth was measured by the MTS assay. Jurkat cells were used as a positive control for responsiveness to rTRAIL (Mariani et al., 1997). The 96-well plates were also examined visually under the light microscope to assess cell viability prior to addition of the MTS reagent. Cells were considered sensitive to rTRAIL if the absorbance values of rTRAIL-treated cells were equal to or less than 75% of the absorbance values of control (untreated) cells (Figures 3.1, 3.2 and 3.3).

Jurkat cells were sensitive even at the lowest dose of rTRAIL. Among the BL cell lines, the EBV negative BL2, BL40, BL41 and Loukes cells were sensitive at the

highest doses of rTRAIL whereas BL40 cells were sensitive even at the lowest dose of rTRAIL. One EBV negative cell line, Ramos, did not respond to rTRAIL. The majority of EBV positive BL cells were resistant to rTRAIL with the exception of MAK III and MUTU III c. 148 cells. rTRAIL clearly inhibited cell growth in one LCL, IB4, but not in the remaining two cell lines, X50-7 and LCL3. A summary of the results of these assays is shown in Table 3.1. The results from the MTS assays were consistent with the visual examination of the 96-well plates at the end of the experiment where characteristic features of cell death could clearly be seen in the wells containing BL2 and BL40 cells. Therefore, the results from this initial screen were contradictory to the prevailing concept at the time of these experiments; i.e. that TRAIL can act as a potent anti-tumour agent against cells of both haematopoietic and non-haematopoietic origin.

Treatment with enhancer only appeared to cause a moderate increase in cell growth in some cells. This control has not been performed in previous studies and the mechanism of the growth-enhancing property of the enhancer is not understood, however it may be Fc receptor-mediated (the enhancer is an IgG1 class antibody). Nevertheless, the modest effects of the enhancer could not have interfered with the conclusions from this assay, i.e. that EBV positive BL cells are resistant to TRAIL-induced apoptosis except maybe in Akata 6 cells, where it could have concealed a TRAIL-sensitive phenotype. The same may be true in X50-7 cells. Since the growth-promoting effect of the enhancer was not shared by the majority of cells, or even between cells with a similar phenotype, it was deemed a non-specific effect of the antibody and its mechanism was not further investigated.

3.3. rTRAIL induces PARP cleavage in EBV negative BL cell lines

To confirm the results of the MTS assays and to demonstrate apoptosis induction by rTRAIL in BL cells, a more reliable apoptosis assay was used. Cleavage of PARP was examined by western blotting in a subset of the cell lines. PARP is a well-characterised caspase target and a classic apoptotic marker. During apoptosis, it is cleaved from the 116 KDa full-length form, producing two fragments of approximately 89 and 27 KDa in size. The antibody used in these experiments recognizes the full-length protein and larger cleavage product. EBV negative, BL2, BL40, BL41, Ramos and Loucke as well as the EBV positive BL cell lines, Akata 6

and Elijah were treated with rTRAIL (250 (H) or 100 (L) ng/ml) for 12 or 18 hours. The cells were also treated with CDDP (10 μg/ml), which is known to induce apoptosis and PARP cleavage in BL cells and LCLs, as a control (Allday et al., 1995).

CDDP treatment induced various levels of PARP processing in the cell lines tested (Figures 3.4 and 3.5). Consistent with the MTS assays, complete processing of PARP was observed in BL2 and BL40 cells, from as early as 12 hours following addition of rTRAIL (even at the low dose, 100 ng/ml). This is shown by the disappearance of full-length PARP and the appearance of the large cleavage product, confirming the induction of apoptosis. PARP cleavage also occurred in BL41 and Louckes cells following treatment with the high dose of rTRAIL (250 ng/ml), however, processing was not as efficient as in BL2 or BL40 cells. Contrary to their response in MTS assays, Ramos cells also cleaved PARP following treatment with rTRAIL and are therefore considered sensitive. The reason for the discrepancy between MTS and PARP cleavage assays is unclear, and may be due to the distinct nature of events determined by each assay. By contrast, processing of PARP in rTRAIL-treated Akata 6 and Elijah cells was minimal, consistent with the results of the MTS assays, whereas the addition of CDDP resulted in more efficient cleavage (Figure 3.5). The reduced rates of PARP cleavage in group I BL cells, implied the existence of a mechanism that attenuates TRAIL-induced apoptosis in these cell lines. The difference in responsiveness between the two groups of cells is surprising given that EBV negative and EBV positive BL cell lines with a group I phenotype are phenotypically similar. The differential responsiveness could be due to lack of TRAIL receptor expression or the expression of a signalling inhibitor. To begin to investigate the basis of the differential responses to rTRAIL between EBV negative and EBV positive cells, I examined the levels of pro-apoptotic TRAIL receptors in the panel of cell lines tested.

3.4. TRAIL receptor expression in BL lines and LCLs

To determine whether resistance to rTRAIL is receptor-dependent or independent I first determined the expression of the pro-apoptotic TRAIL receptors, DR4 and DR5. Receptor expression was first examined by western blotting and when the relevant reagents became available, by flow cytometry. Jurkat cells were used as

positive controls for DR5 expression in both investigations (Bodmer et al., 2000a). I initially confirmed the specificity of DR4 and DR5-targeting antibodies by western blotting analysis, using recombinant receptor molecules consisting of the extracellular domains of human DR4 and DR5 fused to the Fc region of IgG: rhFc:DR4 and rhFc:DR5 (data not shown).

Western blotting analysis showed that DR5 expression is shared by the BL lines, including Akata 6 and Elijah cells as well as LCLs at levels that were not dissimilar to Jurkat cells (Figure 3.6). DR4 expression was more variable and was expressed at low levels in Jurkat cells consistently with previous studies (Bodmer et al., 2000a). Low levels of DR4 were detected in Louckes, IB4, X50-7 and LCL3 cells whereas it was undetectable in MAK III, MUTU III c. 148 and BL72 cells. The remaining cell lines expressed comparable levels of DR4. The variability in DR4 expression is consistent with previous analyses (Kim et al., 2000b) and was not due to loading variation as determined by western blotting for the proliferating cell nuclear antigen (PCNA) (data not shown). Since the literature indicates that the two TRAIL receptors are redundant in apoptosis induction, low levels or lack of DR4 is unlikely to be independently responsible for resistance to TRAIL-induced apoptosis (as all cell lines express at least one TRAIL receptor). Furthermore, TRAIL receptor expression in BL did not correlate with EBV status and/or gene transcription pattern in BL.

However, endogenous protein levels do not necessarily correlate with cell surface expression, so it was important to confirm the presence of the two TRAIL receptors on the cell surface. Surface expression was determined by indirect immunofluorescence staining and flow cytometry analysis. Two groups of cells were selected for this analysis, the EBV negative cell lines and the EBV positive group I cell line, Akata 6. Jurkat cells were examined as controls. Based on the RFI values, cells were arbitrarily defined as positive for TRAILR expression if the corresponding RFI values were higher than 1.5.

Consistently with the western blotting analysis, Jurkat cells were positive for DR5 but negative for DR4 (Figure 3.7). By contrast, Ramos cells did not express DR5 on the cell surface. BL2, BL40 and Akata 6 cells express both TRAIL receptors and surprisingly Akata 6 cells displayed the highest levels (Figure 3.7 B). Therefore, FACS analysis of DR4 and DR5 in selected EBV negative and EBV positive group I cell lines, demonstrated some variability in receptor expression, but did not show any correlation between receptor expression and sensitivity to rTRAIL. Both western

blotting and flow cytometry analyses showed that low receptor levels or lack of either DR4 or DR5 does not have a significant impact on cell sensitivity to rTRAIL, as cells with low DR4 or DR5 levels can respond to rTRAIL (e.g. Ramos and Jurkat). Therefore, TRAIL receptor expression patterns in BL cells indicated that resistance to TRAIL-mediated apoptosis is receptor-independent, implying intracellular mechanisms of resistance.

3.5. Long-term growth potential in EBV negative and EBV positive group I cell lines treated with rTRAIL

Short-term apoptosis assays do not always reflect the outcome of long-term survival assays, as the lack of cell death in a short-term assay does not establish the cells' ability to replicate. For instance, it is possible that apparently TRAIL-resistant BL cells actually underwent delayed cell death or lost the ability to replicate after rTRAIL treatment. To determine whether rTRAIL altered the long-term growth potential of TRAIL receptor-positive group I BL cells, I performed long-term growth assays. The EBV negative cells, BL2, Ramos and the EBV positive group I cell line Akata 6 were treated with rTRAIL (500 ng/ml) for 24 hours or left untreated as controls. Cells were then transferred to 10 wells in a row in 96-well plates and serially diluted (3-fold dilutions). The cell concentration range was from approximately 1x 10^4 cells/well to 5 cells/ well. Cells were maintained without medium changes for 14 days and on day 14 the cell growth was visually assessed under the light microscope. The highest serial dilution at which cell growth was detected in at least 70% of the wells was recorded (Figure 3.8). BL2 cells, included as positive controls, required a cell density of at least 6x 10^3 in order to grow. However, the long-term growth potential of Akata 6 and Ramos cells was not altered by rTRAIL. Therefore, long-term growth assays of Akata 6 and Ramos cells were consistent with the short-term MTS assays similarly to a previous report (Kim et al., 2000b).

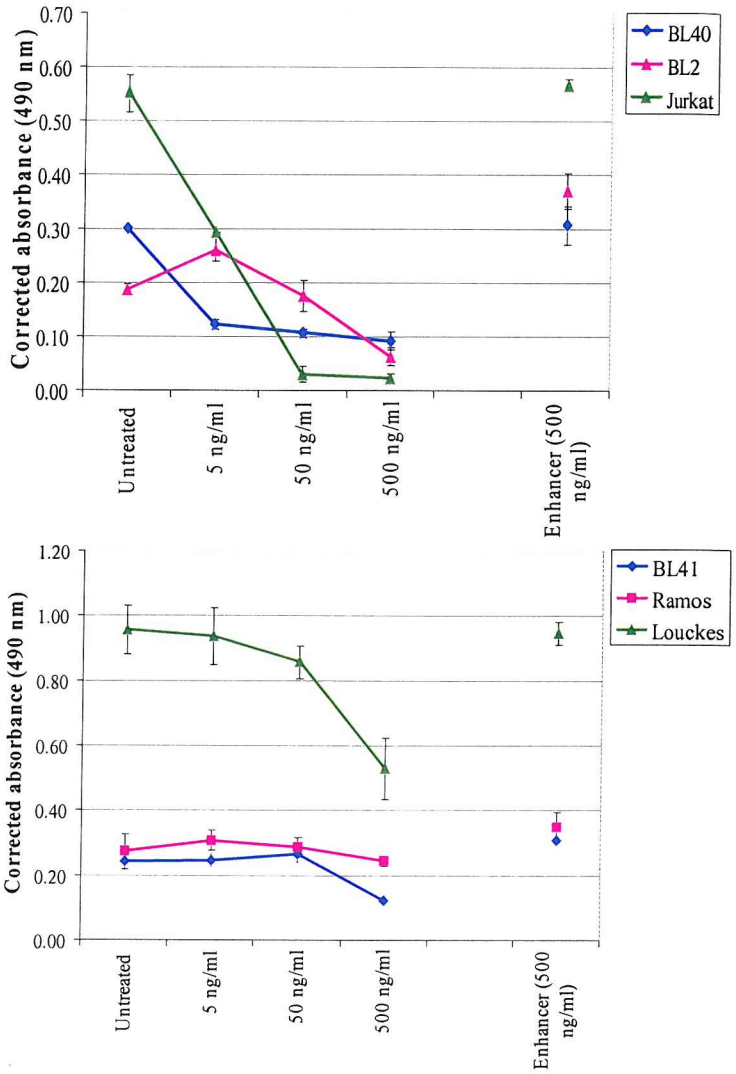


Figure 3.1: *Sensitivity to rTRAIL in EBV negative BL cell lines.* Cells were cultured in 96 well plates in the absence or presence of rTRAIL (500, 50, 5 ng/ml) or enhancer only (500 ng/ml) for 48 hours and cell growth was determined by the MTS assay. Jurkat cells were used as a positive control for sensitivity to rTRAIL. Data points presented here are the average of triplicate wells +/- standard error of the mean. Some errors are too small to show.

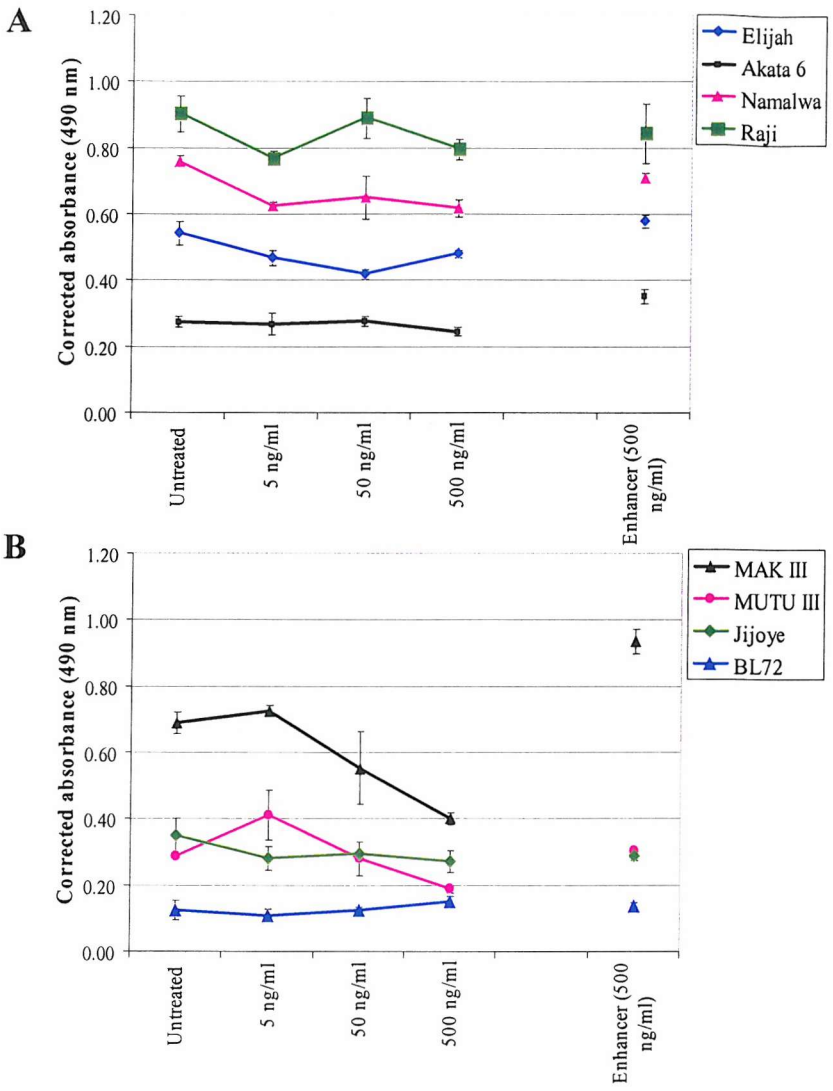


Figure 3.2: *Sensitivity to rTRAIL in EBV positive group I, II and III BL cell lines.* Group I and II **(A)** and group III BL cell lines **(B)** were cultured in 96-well plates in the absence or presence of rTRAIL (500, 50, 5 ng/ml) or enhancer only (500 ng/ml) for 48 hours. Cell growth was measured by the MTS assay. Data points presented here are the average of triplicate wells +/- standard error of the mean. Some errors are too small to show.

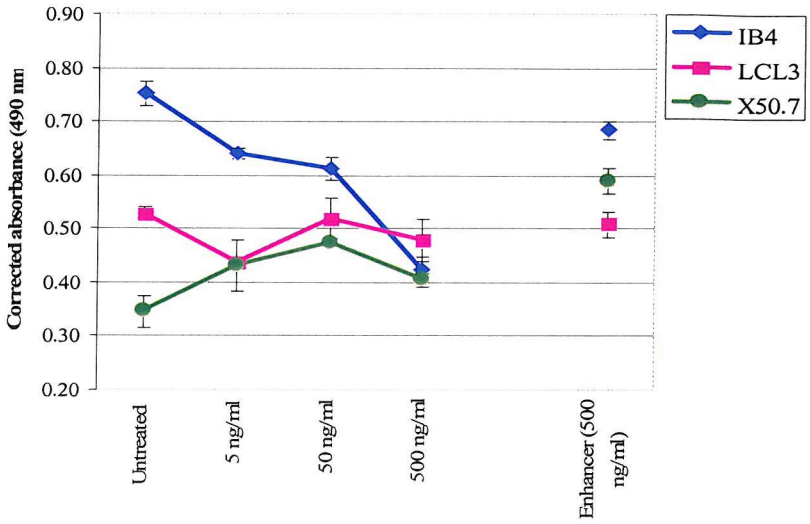


Figure 3.3: *Sensitivity to rTRAIL in LCLs.* Cells were incubated in the absence or presence of rTRAIL (500 ng/ml) or enhancer alone (500 ng/ml) for 48 hours. Cell growth was measured by the MTS assay. Data points presented here are the average of triplicate wells +/- standard error of the mean. Some errors are too small to show.

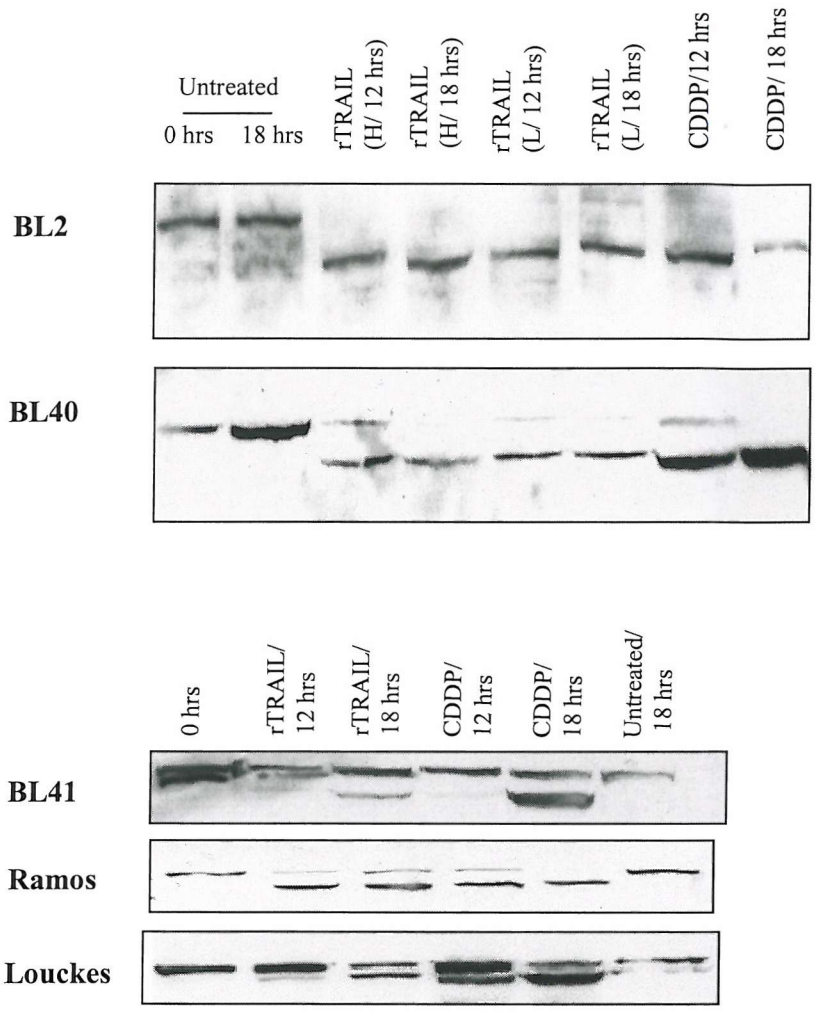


Figure 3.4: *Western blotting analysis of PARP cleavage in EBV negative BL cell lines treated with rTRAIL.* BL2 and BL40 cells were treated with two doses of rTRAIL; 250 ng/ml (H) or 100 ng/ml (L) for 12 or 18 hrs, or left untreated as controls. BL41, Ramos and Loukes cells were treated with one dose of rTRAIL; 250 ng/ml or left untreated as controls. Cells were also treated with CDDP (10 μg/ml) as a positive control for PARP cleavage. Equal volumes of whole cell lysates were resolved on 10% polyacrylamide gels and transferred onto nitrocellulose. Filters were probed with a PARP-specific mouse monoclonal antibody (clone C2-10).

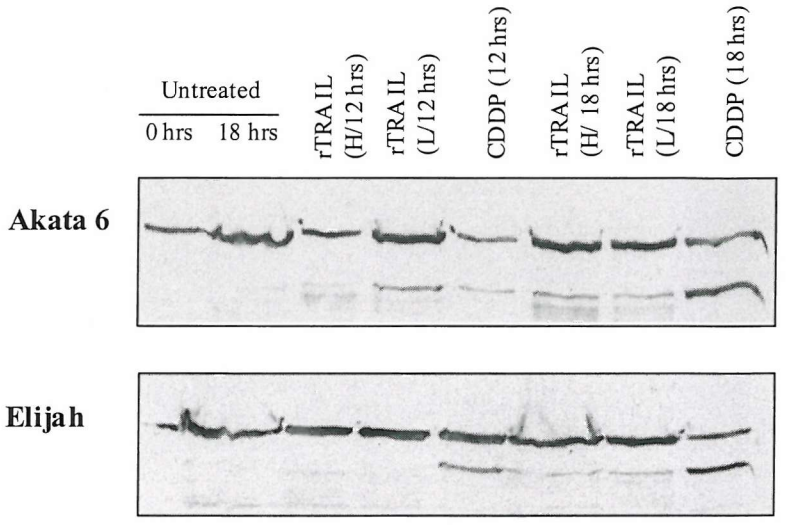


Figure 3.5: *Western blotting analysis of PARP cleavage in EBV positive group I BL cell lines treated with rTRAIL.* Cells were treated with two doses of rTRAIL; 250 ng/ml (H) or 100 ng/ml (L) for 12 or 18 hrs, or left untreated as controls. Cells were also treated with CDDP (10 μg/ml) as a positive control for PARP cleavage. 20 μg of protein from each time point were resolved on 10% polyacrylamide gels and transferred onto nitrocellulose. Filters were probed with a PARP-specific mouse monoclonal antibody (clone C2-10).

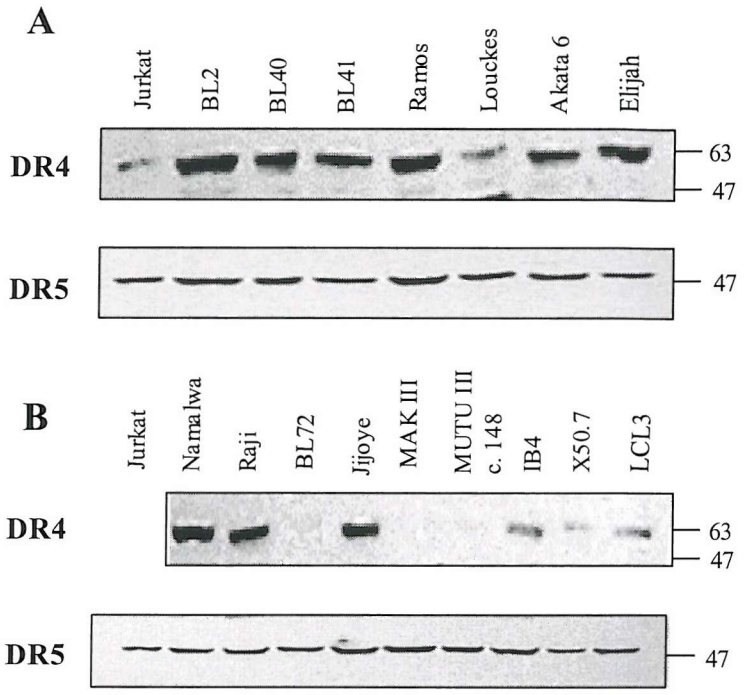
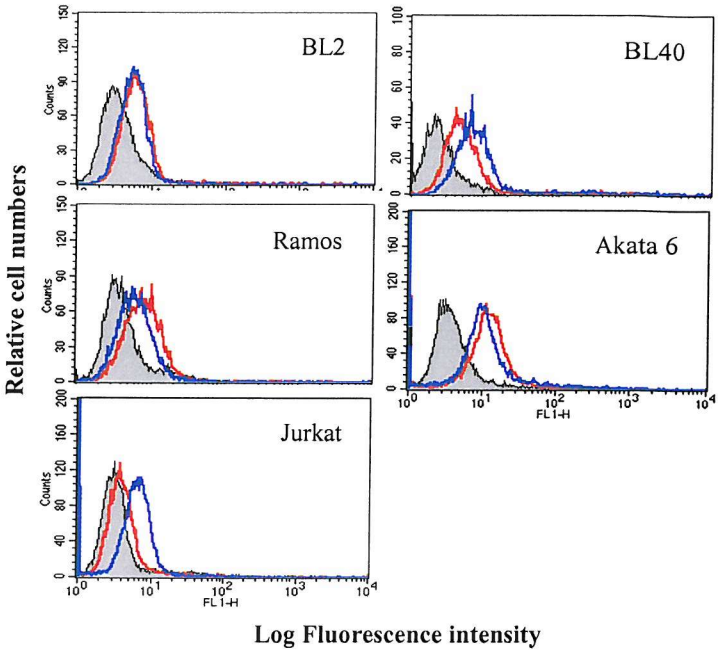


Figure 3.6: *Western blotting analysis of DR4 and DR5 in BL cell lines and LCLs.* 20 µg of protein from EBV negative and EBV positive group I BL cells **(A)** and from EBV positive group II, III BL cell lines and LCLs **(B)** were resolved on 10% polyacrylamide gels and transferred onto nitrocellulose. Jurkat cells were used as positive control for DR5 expression. The filters were probed with DR4 and DR5-specific rabbit polyclonal antibodies and a PCNA-specific antibody as a loading control (data not shown). The position of the molecular weight markers is indicated (KDa).

A



Cell line	DR4 expression	DR5 expression
BL2	1.7	1.5
BL40	1.9	3
Ramos	1.8	1.4
Akata 6	3.8	2.6
Jurkat	1.1	1.7

Figure 3.7: *FACS analysis of DR4 and DR5 expression on EBV negative and EBV positive group I BL cell lines.* **(A)** DR4 (red) and DR5 (blue) were analysed by flow cytometry using DR4- and DR5-specific goat polyclonal antibodies and biotinylated goat immunoblobulin (filled histogram) as isotype-matched control. 10000 events per sample were acquired using the FL1 channel. **(B)** The RFI values were determined using CellQuest software.

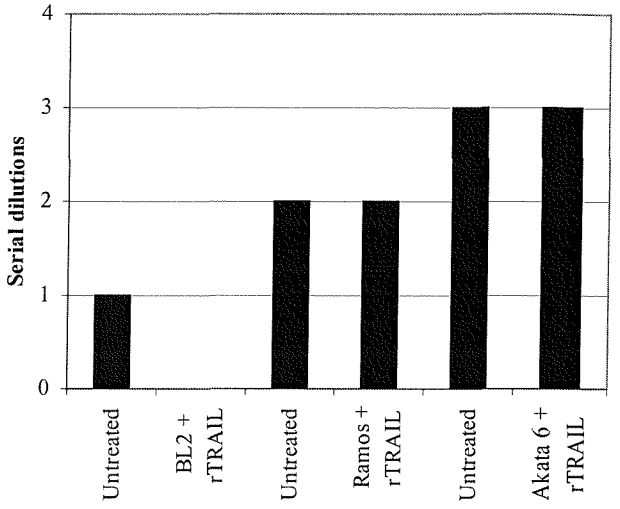


Figure 3.8: *Long-term growth of EBV negative and EBV positive group I BL cell lines treated with rTRAIL.* Cells were treated with rTRAIL (500 ng/ml) for 24 hours or left untreated as a control. On day 0, the cells were plated out on 96-well plates and serially diluted by three-fold dilutions to a final density of approximately 5 cells/ well. On day 14, cell growth was assessed under the light microscope. The highest serial dilution at which cell growth was detected in at least 70% of wells was recorded.

3.6. Resistance to Fas-mediated apoptosis in Burkitt's lymphoma cell lines

TRAIL receptor and Fas-originating pathways are known to share signalling components; therefore the sensitivity of BL cell lines to Fas-induced apoptosis was investigated in order to compare BL cell responses to both stimuli. The panel of BL cell lines and LCLs were screened for sensitivity to α-Fas, a Fas specific IgM class monoclonal antibody that induces apoptosis by cross-linking of Fas (Itoh et al., 1991). This antibody is well characterised and often used in studies of Fas signalling (Fadeel et al., 1998), (Li et al., 2000b). BL lines and LCLs were incubated in 96-well plates with various concentrations of α-Fas (500, 50 or 5 ng/ml), isotype-matched IgM immunoglobulin (500 ng/ml), or left untreated as controls. The concentration range of α-Fas was based on previous reports (Falk et al., 1992). The cells were incubated for 48 hours before addition of the MTS reagent and measurement of absorbance in an ELISA plate reader. Jurkat cells were used as positive control for sensitivity to α–Fas (Dhein et al., 1995). The 96-well plates were also examined under the light microscope to visually assess cell viability prior to the addition of the MTS reagent. Cells were considered to be sensitive to α–Fas, if the mean absorbance values of α-Fas-treated cells (at the highest dose, 500 ng/ml) were equal to or lower than 75% of the absorbance values of untreated (control) cells (Figure 3.9, 3.10 and 3.11). The threshold values are indicated in Figures 3.9 and 3.10.

Jurkat cells and LCLs responded to α-Fas in a dose responsive manner (Figure 3.11). As expected, Jurkat cells were sensitive even at the lowest dose of α-Fas and were not greatly affected by IgM treatment. IB4 cells were the most sensitive LCL demonstrating a significant reduction in absorbance values even at the lowest dose of α–Fas. The majority of the BL cell lines were resistant with only Louckes demonstrating a specific reduction in cell growth in response to α-Fas (Figures 3.9 and 3.10). Visual examination of the cells under the light microscope was consistent with the results of this assay particularly in the LCLs, which displayed the characteristic morphological features of cell death. Whereas it was felt important to use a control, IgM antibody to confirm the specificity of the effects of α-Fas, this reagent gave surprising results in some cases. Although a dose response assay using this control in Jurkat cells performed prior to these analyses showed no evidence of cytotoxicity in this cell line (data not shown), IgM had a growth-inhibitory effect in some BL cell

lines. Although the reasons for growth inhibition induced by IgM treatment are not clearly understood, a similar finding has been reported from another study (Owen-Schaub et al., 1994) whereas many other studies have not performed this control (Falk et al., 1992). IgM-mediated cytotoxicity is likely to be a non-specific effect of the antibody and thus the mechanism was not investigated further. However, with the possible exception of Jijoye, Raji and MUTU III c. 148 cells, it does not alter the conclusion that BL cell lines were resistant to α-Fas. By contrast, sensitivity to α-Fas in the LCLs was clearly specific. Therefore BL and LCL responses to α-Fas did not correlate with their sensitivity to rTRAIL, although the two pathways are known to share the majority of their signalling components. The observed resistance to Fas activation in BL is consistent with previous studies (Falk et al., 1992).

3.7. α–Fas induces PARP cleavage in LCLs but not in BL cell lines

To confirm the observations from the MTS assays, it was important to use a more reliable assay of apoptosis to test the effect of α-Fas in BL cells as well as the LCLs. Thus, selected cell lines were treated with α-Fas and were subsequently tested for PARP cleavage by western blotting. This analysis included the EBV positive BL lines Jijoye, MUTU III c.148, MAK III, Raji, Namalwa and the LCLs, IB4 and X50-7. Cells were treated with α-Fas (500 ng/ml) for 12 or 18 hours or left untreated as controls. The cells were also treated with CDDP (10 μg/ml) as a control.

Consistent with their responses in the MTS assays, α-Fas-treated IB4 cells underwent apoptosis accompanied by cleavage of PARP within 12 hours with complete processing of PARP within 18 hours of treatment (Figure 3.12 B). X50-7 cells also underwent apoptosis following addition of α-Fas but cleavage of PARP was reduced relevant to IB4 cells. By contrast, α-Fas treatment did not induce PARP cleavage in the BL cell lines identified as Fas-resistant by the MTS assay, namely Jijoye, Namalwa, Raji (data not shown), MAK III and MUTU III c. 148 (Figure 3.12 A). This was not due to a general inability to activate caspases since PARP was cleaved in most BL cells after addition of CDDP (with the exception of MAK III cells). CDDP did not induce cleavage of PARP in the LCLs. Burkitt's lymphomas and BL cell lines are known to be highly chemosensitive (Magrath, 1998) and readily undergo apoptosis in response to CDDP (Allday et al., 1995). The LCLs also undergo

CDDP-induced apoptosis (Allday et al., 1995), thus the lack of PARP cleavage in these assays was probably due to the time-scale of the experiments. Importantly, the IgM control did not induce PARP cleavage indicating that the reduction in viability in Jijoye and MUTU III c. 148 cells as seen in the MTS assay, is probably the result of a non-specific effect of the antibodies. Therefore, PARP cleavage analysis confirmed apoptosis induction by α-Fas in the LCLs and the resistant phenotype of EBV positive BL cell lines, indicating the presence of an inhibitory mechanism as previously reported (Falk et al., 1992).

3.8. Fas surface expression in BL and lymphoblastoid cell lines.

Inhibition of α-Fas-induced apoptosis in BL cells could be due to the lack of Fas or the expression of an inhibitor. To begin to address the reasons behind BL cell resistance to α-Fas, I determined the cell surface expression of Fas using indirect immunofluorescent staining and flow cytometry. A Fas-specific antibody raised against the extracellular region of Fas that binds Fas without activating it was used for this analysis (clone DX2). An isotype-matched antibody was used to control for non-specific binding and the Fas sensitive LCLs, IB4 and X50-7, were used as positive controls.

FACS analysis demonstrated that the Fas resistant EBV negative (BL40, BL41, Loukes, BL2 (data not shown)) and positive BL cells with a group I phenotype (Akata 6 and Elijah (data not shown)), lack Fas on the cell surface. By contrast, cells with a group III phenotype expressed Fas at levels similar to the Fas-sensitive LCLs (Figure 3.13). Of the group II BL lines, Namalwa cells lacked Fas whereas Raji were Fas positive. Therefore, similar to the results of (Falk et al., 1992) using a different panel of cells, expression of Fas on BL cell lines and LCLs correlated with the EBV gene transcription pattern (Fas expression correlated with complete latent gene expression as seen in group III BL and LCLs) but did not correlate with responsiveness to α-Fas as previously reported (Falk et al., 1992).

3.9. Examination of the long-term growth potential in EBV positive group III BL cell lines treated with α-Fas

To determine whether α-Fas altered the long-term growth potential of Fas positive BL cell lines, which did not respond to α-Fas in the MTS assays and did not cleave PARP after α-Fas treatment, long-term growth assays were performed. MAK III, MUTU III c.148 and Jijoye cells were treated with α-Fas (500 ng/ml) for 24 hours or left untreated as controls. The cells were then transferred to 10 wells in a row in 96-well plates and serially diluted (3-fold dilutions). The cell concentration range was from approximately 1 x 10^4 cells/well to 5 cells/well. Cells were maintained without medium changes for 14 days. On day 14, the plates were examined under the light microscope for cell growth. The highest dilution factor at which cell growth was recorded in at least 70% of the wells was recorded and is shown here (Figure 3.14). IB4 cells were included in the assay as a positive control, but did not grow at any cell dilution. The BL cells did not grow at the lower cell densities, even when untreated, and required between 1 x 10^3 and 3 x 10^3 cells per well in order to grow. With the possible exception of MAK III cells, where α-Fas treatment reduced cell growth by one dilution factor, α-Fas did not affect the long-term growth potential of BL cell lines. The effect in MAK III cells is unlikely to be significant, since a similar increase in cell growth (compared to untreated cells) was detected in α-Fas-treated Jijoye cells. Therefore, the long-term growth assays provided no indication that treatment with α-Fas had an effect in the long-term growth potential of Fas positive BL cells with a group III phenotype, namely MAK III, MUTU III c. 148 and Jijoye.

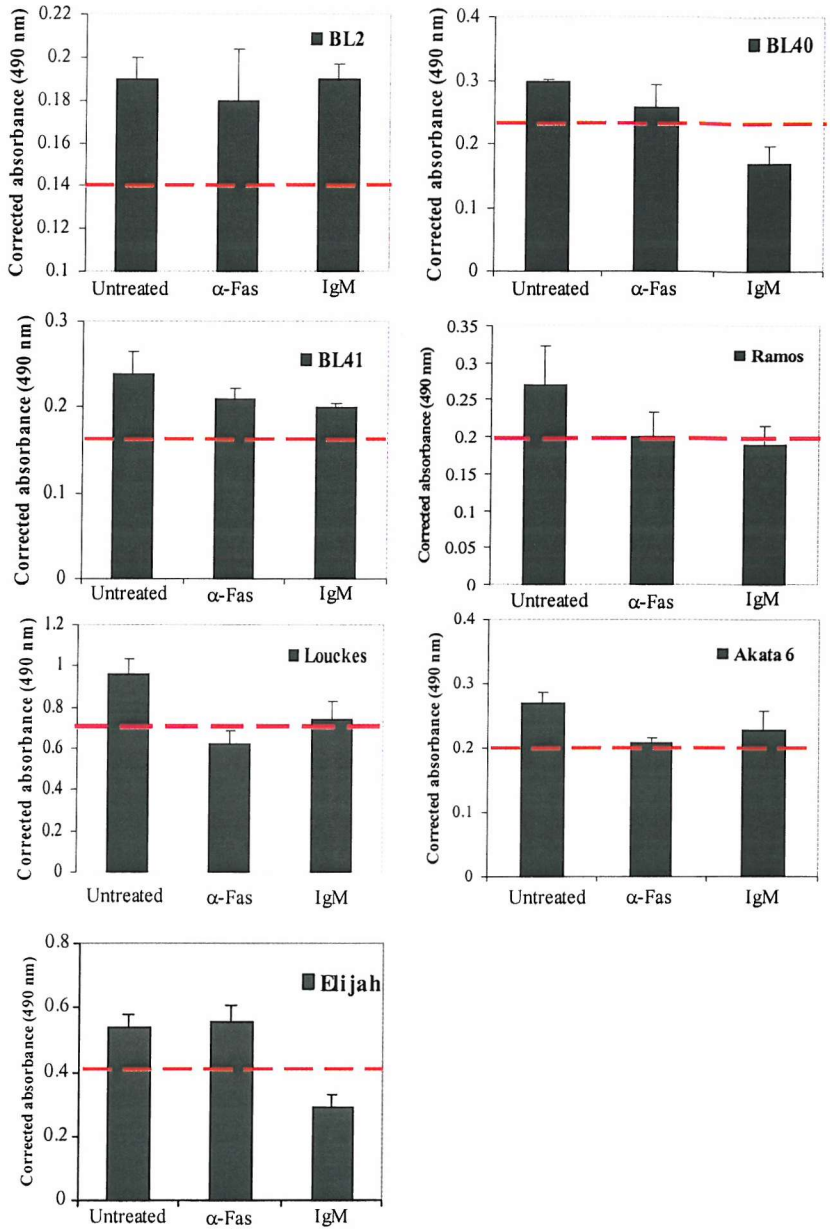


Figure 3.9: *Sensitivity to α-Fas in EBV negative and EBV positive group I BL cell lines.* Cells were cultured in 96-well plates in the absence or presence of α-Fas (500 ng/ml) or IgM control (500 ng/ml) for 48 hours. Cell growth was determined by the MTS assay. Data points presented here are the average of triplicate wells +/- standard error of the mean. The threshold for sensitivity to α-Fas was set to 75% of the absorbance value of untreated cells as indicated (red line).

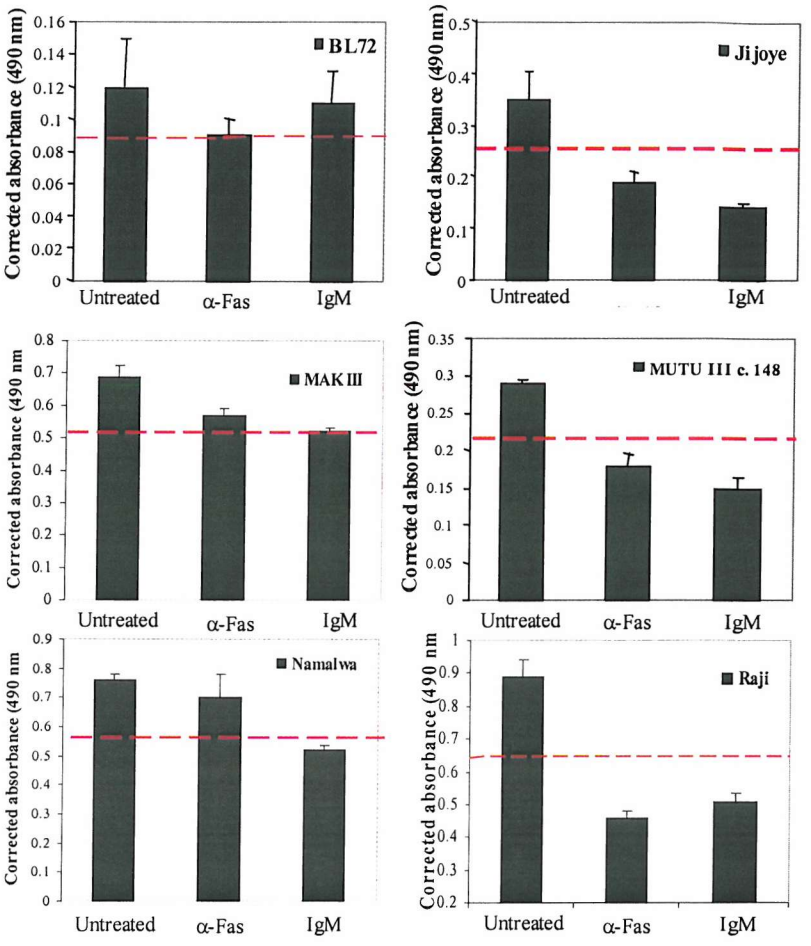


Figure 3.10: *Sensitivity to α-Fas in EBV positive group II and III BL cell lines.* Cells were cultured in 96-well plates in the absence or presence of α-Fas (500 ng/ml) or IgM control antibody (500 ng/ml) for 48 hours. Cell growth was determined by the MTS assay. Data points presented here are the average of triplicate wells +/- standard error of the mean. The threshold for sensitivity to α-Fas was set to 75% of the absorbance value of untreated cells as indicated (red line).

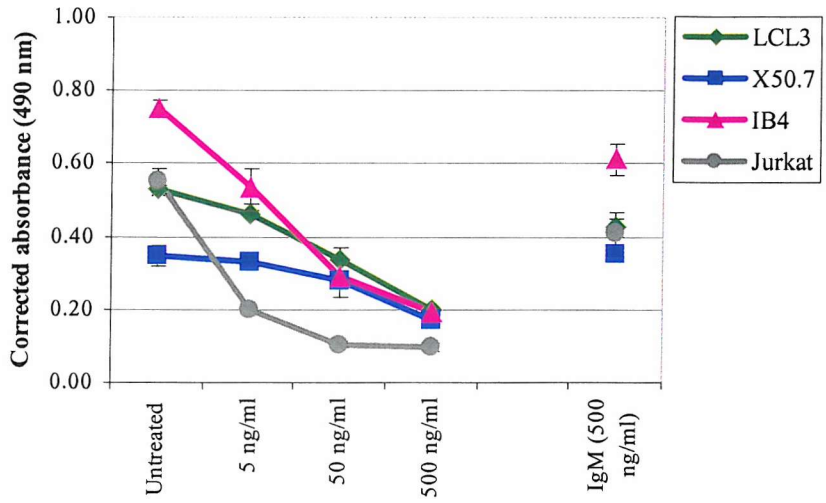


Figure 3.11: *Sensitivity to α-Fas in LCLs.* Cells were cultured in 96 well plates in the absence or presence of α-Fas (500, 50 or 5 ng/ml) or isotype-matched IgM control (500 ng/ml) for 48 hours and cell growth was measured by the MTS assay. Jurkat cells were used as a positive control. Data points presented here are the average of triplicate wells +/- standard error of the mean. Some errors are too small to show.

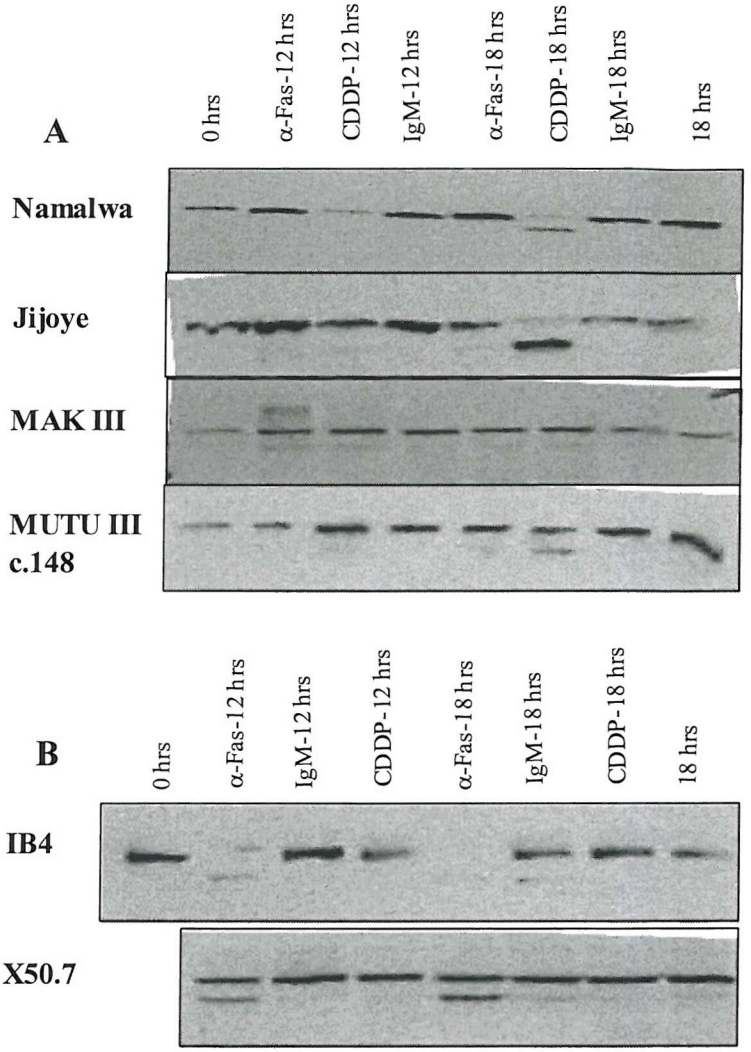


Figure 3.12: *Western blotting analysis of PARP cleavage in group II and III BL cell lines and LCLs treated with α–Fas.* ***(A)*** BL cell lines and **(B)** LCLs were treated with α-Fas (500 ng/ml), isotype-matched IgM control (500 ng/ml) or CDDP (10 μg/ml) for 12 or 18 hours or left untreated as controls. Cell lysates were resolved on 10% polyacrylamide gels and transferred onto nitrocellulose. Filters were probed with a PARP-specific mouse monoclonal antibody (clone C2-10).

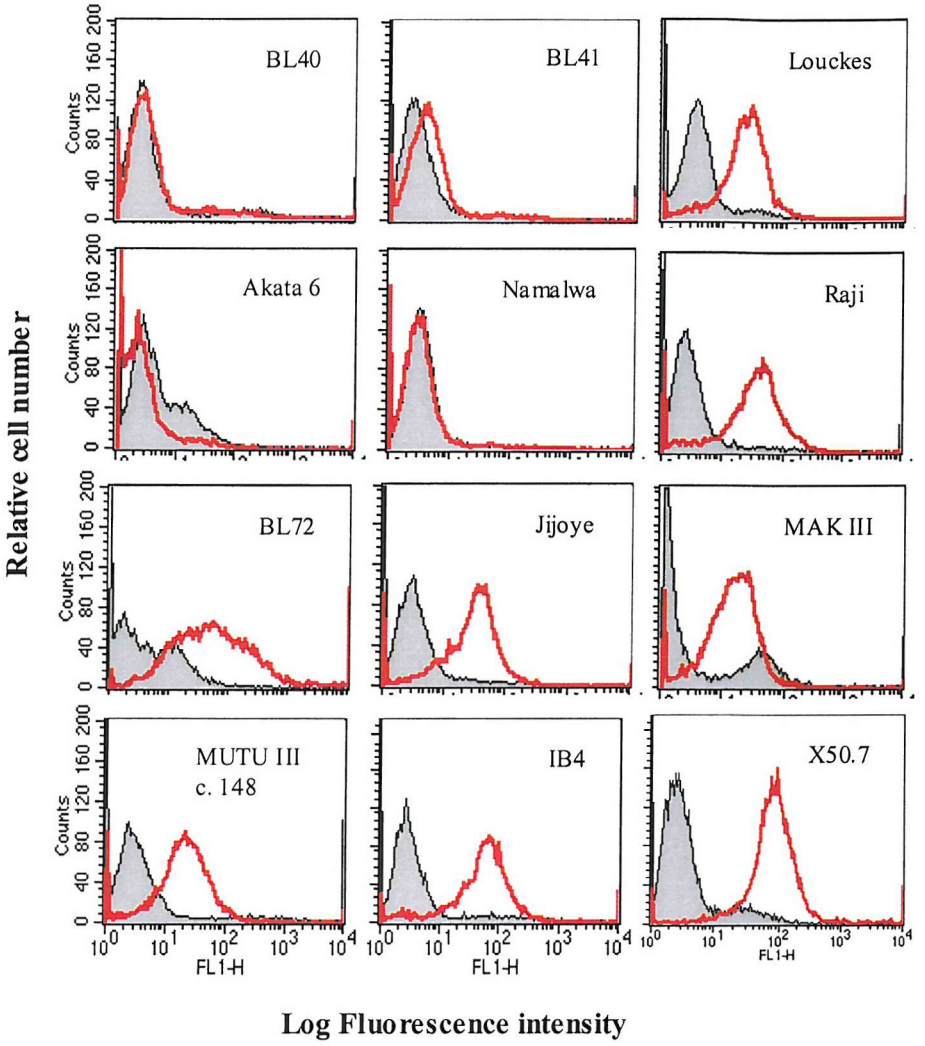


Figure 3.13: *FACS analysis of Fas surface expression on BL cell lines and LCLs.* Cells were incubated with a Fas-specific mouse monoclonal antibody against the extracellular domain of Fas (clone DX2) (red line) or with an isotype-matched antibody (α-TNP) (filled histogram). Antibody binding was detected by a FITC-conjugated rat mouse-specific antibody. 10000 events per sample were acquired using the FL1 channel and the data analysed using CellQuest software.

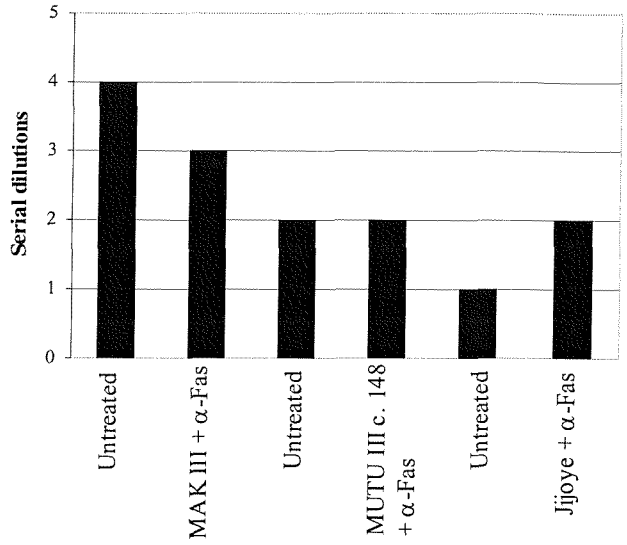


Figure 3.14: *Long-term survival of group III BL cell lines treated with α-Fas.* Cells were treated with α-Fas (500 ng/ml) for 24 hours or left untreated as controls. On day 0, 1 x 10^4 cells were plated on 96 well plates and serially diluted by three-fold dilutions to a final density of approximately 5 cells/ well. On Day 14 cell growth was visually assessed under the light microscope. The highest serial dilution at which cell growth was recorded in at least 70% of the wells is shown.

EBV status	Cell line	Sensitivity to α-Fas	Sensitivity to rTRAIL	Fas expression	TRAILR expression (protein)	TRAILR expression (cell surface)
EBV negative BL	BL2	-	+	-	DR4, DR5	DR4, DR5
	BL40	-	+	-	DR4, DR5	DR4, DR5
	BL41	-	+	-	DR4, DR5	NA
	Ramos	-	+	NA	DR4, DR5	DR4, DR5
	Louckes	+	+	+	DR4, DR5	NA
EBV positive BL, group I	Akata 6	-	-	-	DR4, DR5	DR4, DR5
	Elijah	-	-	-	DR4, DR5	NA
EBV positive BL, group II	Namalwa	-	-	-	DR4, DR5	NA
	Raji	-	-	+	DR4, DR5	NA
EBV positive BL, group III	BL72	-	-	+	DR5	NA
	Jijoye	-	-	+	DR4, DR5	NA
	MAK III	-	+	+	DR5	NA
	MUTU III c. 148	-	+	+	DR5	NA
LCLs	IB4	+	+	+	DR4, DR5	NA
	X50-7	+	-	+	DR4, DR5	NA
	LCL3	+	-	NA	DR4, DR5	NA
Negative (T-cell leukemi)	Jurkat	+	+	+	DR4, DR5	DR5

Table 3.1: *Sensitivity to Fas and TRAIL receptor-induced apoptosis in BL cell lines and LCLs.* Sensitivity was determined by MTS (at the highest dose 500 ng/ml) and PARP cleavage assays. Fas and TRAIL receptor expression is shown. TRAIL receptor expression was analysed by western blotting and flow cytometry in some cell lines. Fas expression was analysed by flow cytometry. Jurkat cells were used as positive controls in some assays (NA; not analysed).

3.10. Discussion

The aim of this study was to extend the current knowledge on Fas-mediated apoptosis in BL and to compare the responsiveness of BL cells to the two major death receptor pathways, Fas and TRAIL receptors. This was important since previous analyses of BL cell sensitivity to Fas signalling have examined a small number of BL cell lines without confirming apoptosis using specific assays (Falk et al., 1992), or concentrated on selected phenotypic groups of BL cell lines (Henriquez et al., 1999). In addition, sensitivity to TRAIL in BL has not yet been investigated. In this study, a large number of BL cell lines (selected to include different EBV transcription programmes) and LCLs were screened for sensitivity to α–Fas and rTRAIL. Although the general understanding regarding the normal counterpart of the BL cell is that it is a GC B cell (Hecht and Aster, 2000), this is not universally accepted. This and the restricted availability of normal GC B cells has meant that only a small number of investigations have used this cell type as a control in studies of apoptosis in BL whereas LCLs are frequently used as controls.

The initial cell screening for sensitivity to death receptor-mediated apoptosis was performed by MTS assays. The main advantage of this cell growth assay is that it can be performed at a small scale, using a small number of cells, reducing the required amounts of expensive reagents such as recombinant TRAIL. In addition, the 96 well plate format of these assays also allows direct comparisons to be made with the visual examination of the cells under the light microscope at the time of the reading of absorbance. Thus initial screening using this assay demonstrated that responses to α-Fas and rTRAIL are distinct between phenotypically different BL cell groups (the results from these assays are summarised in Table 3.1). The results from the initial screening were subsequently confirmed by PARP cleavage analysis and long-term growth assays in selected cell lines.

Contrary to the widely-accepted view at the time; rTRAIL was deemed a potent cytotoxic treatment against the majority of transformed cells, sensitivity to rTRAIL was a prominent feature of EBV negative BL cells but was absent from the majority of cell lines tested. The differential responsiveness between EBV negative and EBV positive cells with a group I phenotype was surprising since the two groups of cells are phenotypically similar and display similar responses to apoptotic stimuli such as growth factor deprivation {Henderson, 1991 #179;Gregory, 1991 #799}.

Interestingly, the evidence from BL cells suggested that resistance to TRAIL-induced apoptosis was independent of the levels of TRAIL receptor expression. This was demonstrated using more reliable and quantitative assays in contrast to early reports, which used RT-PCR analyses to detect TRAIL receptor expression. The entire panel of cell lines expressed at least one pro-apoptotic TRAIL receptor at the protein level whereas BL2, BL40, Ramos and Akata 6 cells were also positive for TRAIL receptor expression on the cell surface. Interestingly, although Ramos cells express both DR4 and DR5 at the protein level, only DR4 was detected on the cell surface. This indicated a degree of regulation of receptor translocation to the cell surface, which is similar to previous findings in melanoma cells (Zhang et al., 2000a). Since either receptor should be sufficient for apoptosis signalling, as for example in Jurkat cells, the patterns of TRAIL receptor expression implied novel mechanisms of resistance to rTRAIL, which are perhaps intracellular.

By contrast, the majority of both EBV-negative and positive BL cells were resistant to α-Fas-induced apoptosis. It should be noted that the IgM antibody used as a control for α-Fas had a growth-inhibitory effect in the majority of cell lines tested, sometimes exceeding the effects of α-Fas. Thus, PARP cleavage assays provided a more reliable means of establishing resistance to α-Fas in certain cell lines (e.g. in Jijoye, Raji and MUTU III c. 148 cells). However, in LCLs and Jurkat cells, the reduction in cell growth due to IgM was negligible and thus the cytotoxic effect of α-Fas was clear. To my knowledge, this control has been performed in one previous study using ^{3}H-thymidine-based proliferation assays to determine cytotoxicity, where a similar effect was observed (Owen-Schaub et al., 1994). It was therefore clear from this study that the lack of the IgM control in studies where the CH-11 clone of Fas agonistic antibody is used, may impact on the validity of the results.

In addition, Fas surface expression analysis showed that resistance to Fas-induced apoptosis was due to lack of Fas in EBV negative and EBV group I BL cells, consistently with previous studies (Schattner et al., 1996). The fact that the two groups are representative of BL biopsies, suggests that the Fas-negative phenotype may be a common feature of Burkitt's lymphoma *in vivo*. Lack of surface Fas would contribute to a successful immune evasion strategy, protecting BL cells from T cell-mediated cytotoxicity and allowing BL cells to enter the long-lived memory B cell pool. By contrast, in group III BL cells and the group II cell line, Raji, resistance to α–Fas was

receptor-independent since these cells expressed high levels of Fas, similar to the Fas-sensitive LCLs. The Fas-resistant phenotype of group III cell lines was confirmed by long-term growth assays in MAK III, MUTU III c. 148 and Jijoye cells. Analysis of the remaining cell lines, BL72, Raji and the LCLs was not feasible, as they did not grow at low-density conditions. To my knowledge, long-term growth assays have not been performed in similar studies using cells growing in suspension.

The correlation of responsiveness to Fas-mediated apoptosis as well as Fas expression with EBV transcription pattern has been described previously. Indeed it was shown that the phenotypic shift from group I to group III latency in BL cell lines is accompanied by upregulation of Fas on the cell surface (Falk et al., 1992) and LMP-1 was later identified as responsible for mediating this (Gutierrez et al., 1999). The study by (Falk et al., 1992) also noted that the LCLs are sensitive to α-Fas and suggested that an undefined mechanism possibly associated with the tumour origin of BL cell lines may be responsible for lack of responsiveness in the BL cell lines. Resistance to Fas-induced apoptosis was also described in other systems of Fas-expressing tumour-derived cell lines (Ungefroren et al., 1998), (Muschen et al., 2001), (Owen-Schaub et al., 1994). Since Fas and TRAIL-triggered apoptotic pathways are thought to be similar, sharing known intermediates (e.g. caspase 8 and FADD) and acting on common targets, it was also surprising to find that among the Fas-sensitive LCLs only IB4 cells were sensitive to rTRAIL. This is suggestive of the existence of an inhibitory mechanism, specific for the TRAIL pathway, in X50-7 and LCL3 cells. It should also be noted that two Fas-resistant, group III BL cell lines, MUTU III c. 148 and MAK III were sensitive to rTRAIL, again suggesting a divergence between the two death receptor-activated signalling pathways. Since not many studies have compared the biological function of the two death receptor-activated pathways in the same cell system, the nature/identity of the alternative component(s) or the point of divergence is not clear.

In conclusion, BL cells have acquired resistance to Fas-mediated apoptosis by distinct mechanisms that include lack of receptor expression (EBV negative and group I cells) or expression of regulatory mechanisms (group III BL cell lines and Raji cells). Since the LCLS express the same set of EBV latent genes as group III BL cell lines, yet they are Fas-sensitive, these mechanisms are unlikely to be EBV-dependent and may reflect an additional event acquired during the process of tumourigenesis. By

contrast, resistance to TRAIL-induced apoptosis in BL is unlikely to be regulated at the level of the pro-apoptotic TRAIL receptors, since they are widely expressed, but it may be controlled by EBV. Since TRAIL is now believed to play a role in host anti-viral responses exerted by cytotoxic immune cells, it is possible that EBV may have evolved a strategy enabling it to evade TRAIL-dependent immune attack.

Chapter 4. Analysis of the role of Fas and TRAIL receptors in CDDP-induced apoptosis in BL cell lines

4.1 Introduction

Death receptors play an important role in modulating anti-cancer drug-induced apoptosis in a variety of cell systems (Eichhorst et al., 2001), (Friesen et al., 1996), (Muller et al., 1998). Activation of death receptor pathways can enhance drug cytotoxicity in a synergistic manner and render previously chemoresistant cell lines sensitive to drug treatment (Morimoto et al., 1993), (Micheau et al., 1997), (Wen et al., 2000). Furthermore, Fas and DR5 are induced in a p53-dependent manner in response to drug treatment by both transcription-dependent and independent mechanisms (Wu et al., 1997c), (Muller et al., 1998). However, the contribution of Fas to UV and anti-cancer drug cytotoxicity has recently been disputed in certain cell systems, particularly as part of a p53-induced response (O'Connor et al., 2000), (Newton and Strasser, 2000), (Fuchs et al., 1997). Since the pro-apoptotic function of p53 is likely to involve an integrated network of interactions that depend on cell type and apoptotic stimulus, it is likely that death receptor pathways may contribute to p53-induced apoptosis in a cell type-specific manner. BL is generally responsive to drug treatment, thus the most effective treatment available today is aggressive chemotherapy, which although effective can also have toxic side effects. At the moment, novel cancer treatments under examination with the aim of optimising clinical efficiency include apoptosis-modulating regimens, some of which are death receptor-related.

The examination of a panel of BL cell lines for sensitivity to Fas and TRAIL receptor-induced apoptosis showed that BL cell lines are generally resistant to Fas- but EBV negative cells are sensitive to TRAIL-induced apoptosis. The fact that EBV negative BL cells are sensitive to rTRAIL suggests that the "core" apoptotic machinery is intact in these cells. Furthermore, EBV negative BL cells can upregulate Fas and become sensitive to Fas-induced apoptosis when treated with B cell activating agents such as CD40L (Schattner et al., 1996). Therefore, it is possible that chemotherapy and death receptor-activated pathways may interact to enhance apoptosis induction in these cells, or at least BL cells with wild type p53, as in other cell systems. Although such a mechanism would have significant clinical implications, it has not yet been investigated.

Therefore, to begin to investigate the contribution of death receptor pathways to anti-cancer drug-induced apoptosis in BL, I characterised CDDP-induced apoptosis in a BL cell line, BL40, using IB4 cells as normal B cell controls. CDDP is a DNA-damaging drug, used in chemotherapy and an efficient means of inducing apoptosis in BL cells *in vitro* (Chapter 3) and (Allday et al., 1995). BL40 cells are representative of the EBV negative BL, as it appears in both sporadic and immunodeficiency-associated tumours and more importantly contain wild type p53.

4.2 Characterisation of CDDP-induced apoptosis in BL cells expressing wild type p53

Previous examination of PARP levels in CDDP-treated IB4 and BL40 cells showed that both cell lines undergo apoptosis following the addition of CDDP (Chapter 3). I therefore examined the kinetics of CDDP-induced cell death in BL40 and IB4 cells. Both cell lines contain wild type p53 (Farrell et al., 1991). CDDP is a potent anticancer drug with a well-defined mechanism of action. Upon entry into the cell it undergoes hydrolysis, resulting in the formation of a bifunctional electrophilic compound, which causes the formation of intra- and inter-strand cross-links between neighbouring purine residues on DNA and RNA (Pilch et al., 2000). Drug binding affects both replication and transcription of DNA as well as the mechanisms of DNA repair. Thus, CDDP treatment results in the activation of p53 in BL and other cell systems (Wade and Allday, 2000), (Allday et al., 1995), (Muller et al., 1998). The concentration of CDDP used in the experiments described here was selected from the literature and falls within a clinically relevant range (Allday et al., 1995).

To measure cell death in this system, I used established biochemical and morphological assays such as trypan blue exclusion and flow cytometric analysis of PI-stained nuclei. To confirm that cell death was apoptotic, I analysed the levels of PARP by western blotting. These assays allow the identification of distinct phases in apoptosis and were carried out in parallel. BL40 and IB4 cells were diluted to a density of 0.2 x 10^6 cells/ml 24 hours prior to treatment, to ensure they were at the logarithmic phase of growth (Rowe et al., 1987). On day 0, they were treated with CDDP (10 μg/ml) or left untreated as controls and cell viability was measured at the indicated time points by trypan blue exclusion, counting at least 100 cells per sample. This assay detects the loss of membrane permeability in dead cells, a late apoptotic

event. Dead cells take up the dye and appear blue under the light microscope, whereas viable cells exclude the dye. Trypan blue exclusion identified a marked reduction in viability in both BL40 and IB4 cells, which occurred in a time-dependent manner (Figure 4.1). Incubation with CDDP resulted in large numbers of trypan blue-positive cells within 24 hours, which increased significantly after 36 hours. Cell death was dependent on the addition of CDDP and did not result from growth factor depletion, since the viability of untreated cells cultured in parallel for 36 hours was unaffected.

A characteristic feature of cell death is the fragmentation of nuclear DNA, which is lost from the cell, giving rise to cell populations with hypo-diploid DNA content. DNA loss can be detected using a DNA-binding dye, such as PI, and flow cytometry analysis to measure cellular DNA content. Although this is not a reliable assay for apoptosis, it can support other apoptosis assays (e.g. PARP cleavage analysis) and provide information on the kinetics of cell death. In experiments parallel to trypan blue exclusion assays, BL40 and IB4 cells were incubated with CDDP (10 μg/ml) and samples were collected for PI staining and western blots of PARP. Cells were also left untreated as controls.

The DNA profiles of untreated cells at 0 hours are characteristic of cell populations at different stages of the cell cycle with the G1 and G2/M phases, with 2N and 4N DNA content respectively, indicated for untreated controls (Figures 4.2 and 4.3). A noticeable increase in the number of sub diploid cells was detected after 24 hours in IB4 and 36 hours in BL40 cells and loss of DNA was further augmented in both cell lines 48 hours after addition of CDDP (Figures 4.2 B and 4.3 B). The late appearance of sub diploid cells is probably due to the fact that loss of DNA occurs at the late stages of apoptosis. The DNA content profile of untreated IB4 cells at the end of the experiment is distinct, due to data having been acquired under different parameters, however, it is clear that the number of hypodiploid cells in this sample is low, indicating that DNA loss was CDDP-specific. The same was true for BL40 cells. In addition, DNA fragmentation to nucleosomal size fragments was detected in CDDP-treated IB4 cells by gel electrophoresis of genomic DNA (data not shown). Following, the kinetics of PARP cleavage was examined by western blotting analysis. CDDP treatment induced PARP cleavage in IB4 (after 12 hours) as well as in BL40 cells (after 24 hours) (Figure 4.4). In BL40 cells, processing of PARP was detected at

24 hours whereas in IB4 at 12 hours after the addition of CDDP, thus preceding the loss of DNA.

4.3 Analysis of p53 transcription targets in IB4 and BL40 cells undergoing apoptosis induced by CDDP

p53 is an important modulator of apoptosis in response to DNA damage. Although the precise mechanism linking DNA damage and p53 activation to apoptosis is not fully understood and likely to differ between different cell systems, several molecules directly involved in apoptosis are transcriptional targets of p53. Although disputed, p53-dependent upregulation of death receptors has been demonstrated *in vitro* and found to be important for apoptosis after genotoxic drug treatment in some systems. Furthermore, p53 can upregulate pro-apoptotic members of the BCL-2 family such as BAX and PUMA but also transcriptionally repress anti-apoptotic members such as BCL-2 (Zhan et al., 1994), (Nakano and Wousden, 2001), (Budhram-Mahadeo et al., 1999).

p53 is a protein with a short half life; however, DNA damage leads to the stabilisation of the wild type protein, which accumulates in the cells. I therefore examined the levels of p53 in CDDP-treated IB4 and BL40 cells by western blotting (Figure 4.5). The levels of p53 in untreated cells were negligible or below the level of detection of this assay, however, addition of CDDP lead to rapid upregulation of p53 in both cell lines from as early as 6 hours. This is consistent with the wild type p53 status of IB4 and BL40 cells. p53 levels continued to rise with time after addition of the drug in BL40 cells, whereas in IB4 they remained constant following the initial upregulation. The nature of the additional smaller molecular weight bands in IB4 cells is not clear, they could be break down products or cross-reactive proteins. The upregulation of p53 preceded caspase activation as shown by PARP cleavage in both cell lines (Figure 4.4).

Since the rise in p53 is an indication of CDDP-induced activation of p53, I examined the levels of previously reported pro-apoptotic p53 targets, Fas and TRAIL receptors as well as several members of the BCL-2 family.

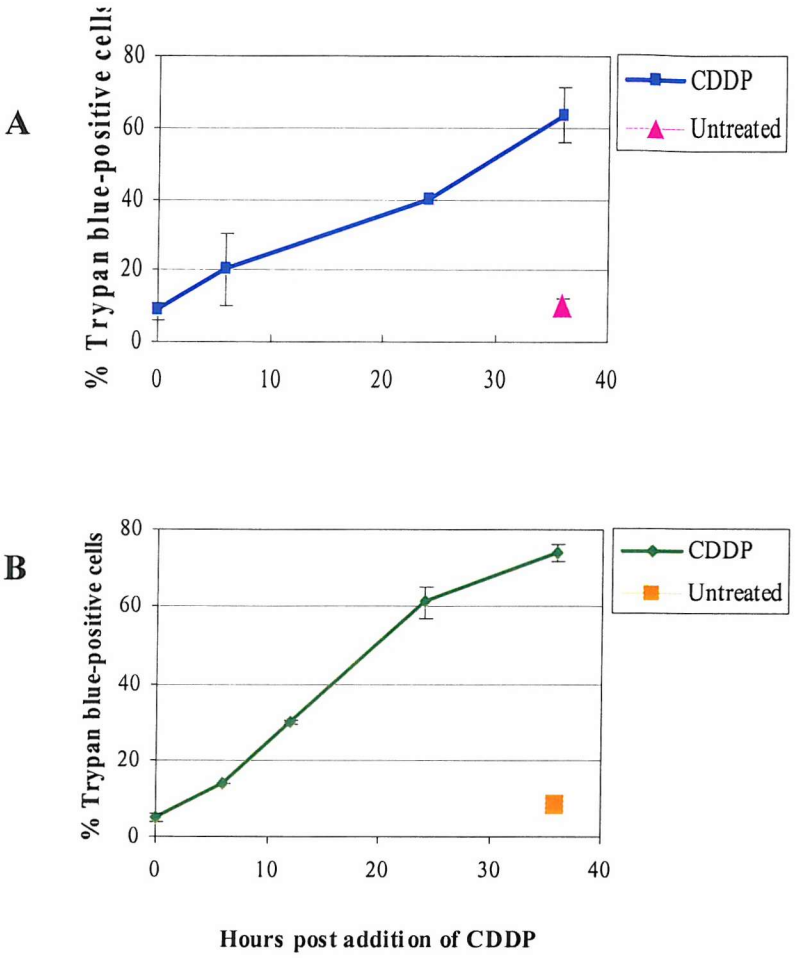
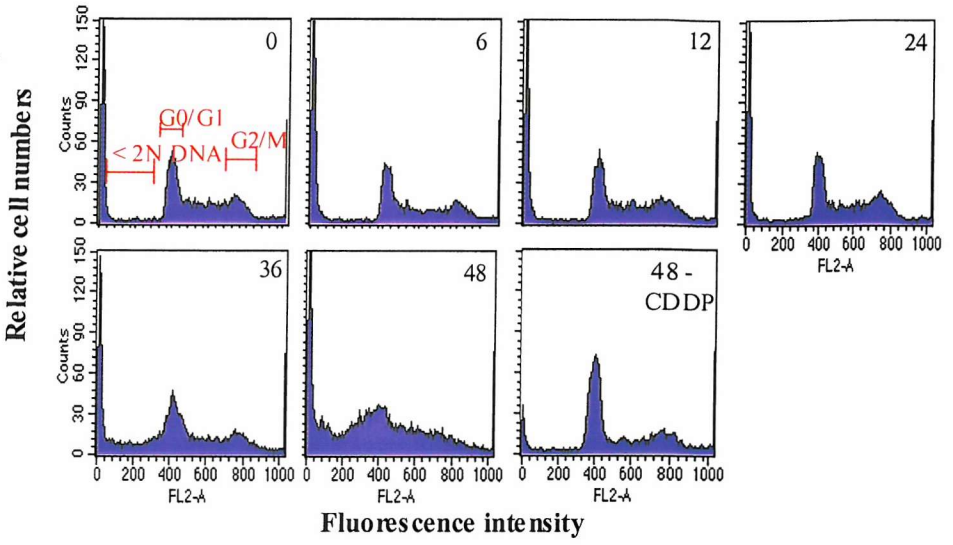


Figure 4.1: *Viability in CDDP-treated BL40 and IB4 cells.* Duplicate cultures of IB4 **(A)** and BL40 **(B)** cells were treated with CDDP (10 μg/ml) or left untreated for up to 36 hours. Cells were also left untreated as a control. Viability was measured at the indicated time points by trypan blue exclusion counting at least 100 cells. Data points indicate the average of duplicate cultures +/- standard error. Some errors are too small to show. These experiments were performed at least three times.

A

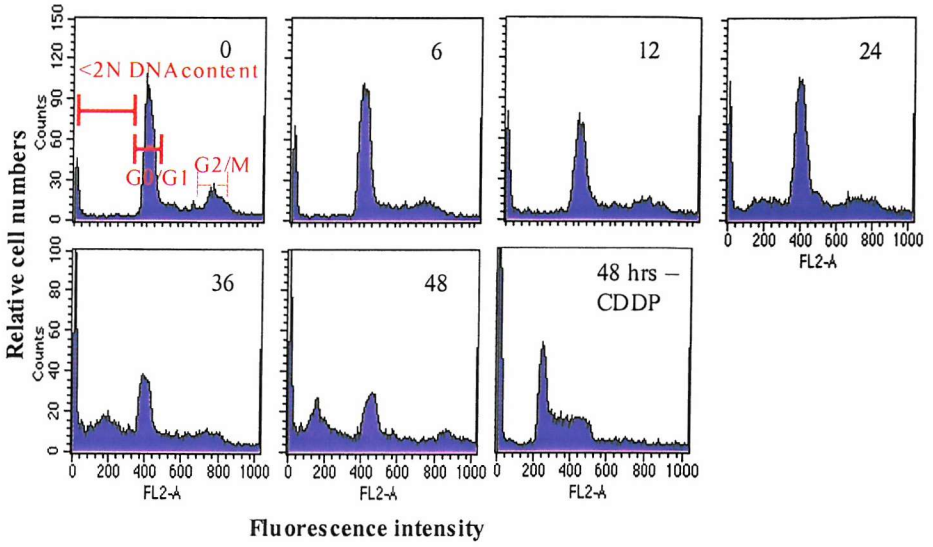


B

Times (hours)	% of cells with hypodiploid DNA content
0	4
6	5
12	3
24	4
36	16
48	30
48-CDPP	3

Figure 4.2: *FACS analysis of PI incorporation in CDDP-treated BL40 cells.* **(A)** BL40 cells were treated with CDDP (10 μg/ml) for the indicated times (hours) or left untreated as controls. Cells were subsequently incubated with PI (50 μg/ml) and analysed on a flow cytometer using the FL2-A channel. The regions of hypodiploid DNA content and the G0/G1 and G2/M peaks are indicated for untreated cells at the start of the experiment. 10000 events/sample were collected. **(B)** Percentages of cells with hypodiploid DNA content were calculated using CellQuest software.

A



B

Time (hours)	% of cells with hypodiploid DNA content
0	2
6	3
12	5
24	15
36	28
48	26
48 - CDDP	3

Figure 4.3: *FACS analysis of PI incorporation in CDDP-treated IB4 cells*. **(A)** IB4 cells were treated with CDDP (10 μg/ml) for the indicated times or left untreated as controls. Cells were subsequently incubated with PI (50 μg/ml) and analysed on a flow cytometer using the FL2A channel. The regions of hypodiploid DNA as well as G0/G1 and G2/M peaks are indicated. 10000 events/ sample were acquired. **(B)** The percentage of cells with hypodiploid DNA content was calculated using CellQuest software.

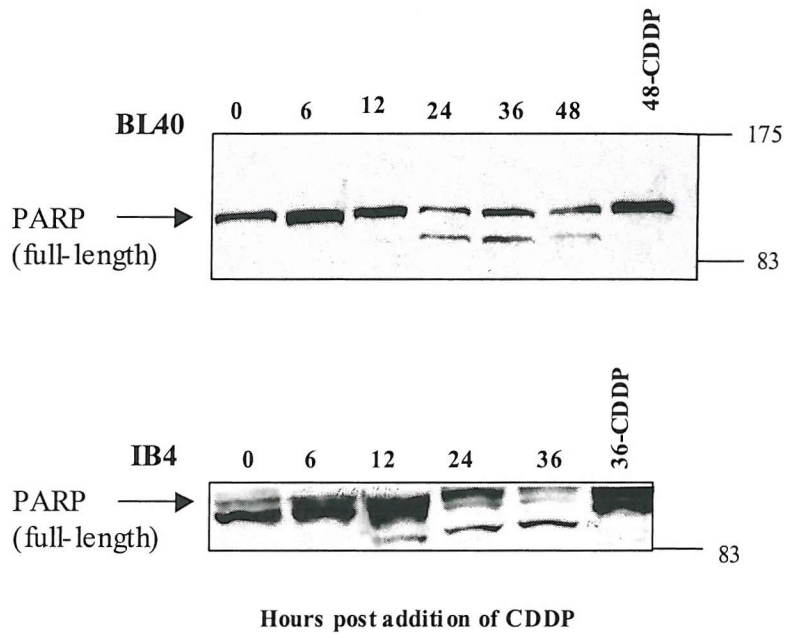


Figure 4.4: *Western blotting analysis of PARP in CDDP-treated IB4 and BL40 cells.* IB4 and BL40 cells were treated with CDDP (10 μg/ml) for the indicated times (hours) or left untreated as controls. 20 μg of protein lysate from each time point were resolved on 10% polyacrylamide gels and transferred to nitrocellulose. Filters were probed with a PARP-specific mouse monoclonal antibody. The position of the molecular weight markers is indicated (KDa).

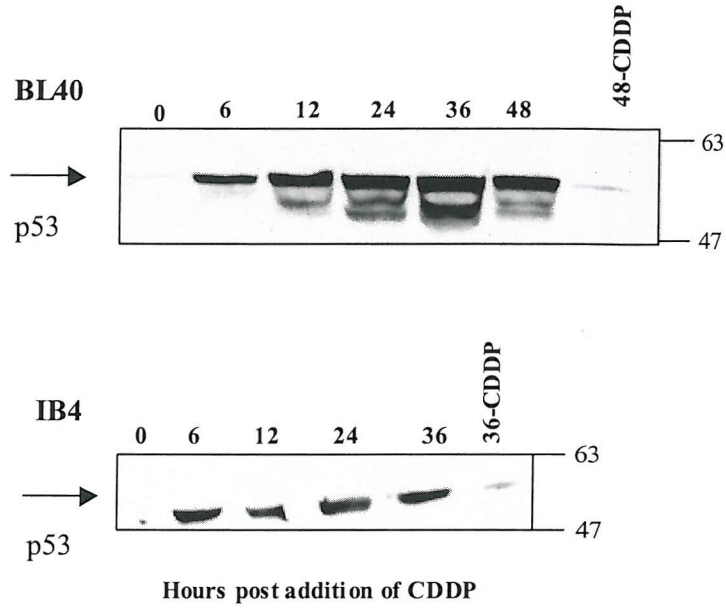


Figure 4.5: *Western blotting analysis of p53 in CDDP-treated IB4 and BL40 cells.* IB4 and BL40 cells were treated with CDDP (10 μg/ml) for the indicated times or left untreated as controls. For western blotting analysis, 20 μg of protein lysate from each time point were resolved on 10% polyacrylamide gels and transferred to nitrocellulose. Filters were probed with a p53-specific mouse monoclonal antibody (clone DO-1). The position of the molecular weight markers is indicated (KDa).

4.4 Expression of Fas in IB4 and BL40 cells treated with CDDP

The levels of Fas during CDDP-induced apoptosis in BL40 and IB4 cells were analysed using indirect immunofluorescence staining and FACS analysis. Cells were cultured in the absence or presence of CDDP (10 μg/ml) for up to 36 hours and cell viability was determined by the trypan blue exclusion assay, to confirm cell death, prior to flow cytometry.

IB4 cells constitutively express Fas as shown earlier (Chapter 3) and treatment with CDDP for 24 hours did not induce any changes in Fas (data not shown). Moreover, the addition of CDDP did not alter the Fas negative phenotype of BL40 cells (Figure 4.6). Although treating the cells with CDDP for 36 hours seemed to result in increased Fas levels, an equal increase in isotype-matched antibody binding was observed, suggestive of a non-specific effect of CDDP. Non-specific antibody binding is often seen in FACS analyses of apoptotic cells, and it was the reason for using the isotype-matched control antibody with every time point. Furthermore, untreated cells at 0 and 36 hours were consistently negative for Fas expression. Therefore, treatment of IB4 and BL40 cells with CDDP did not alter Fas levels on either cell line.

4.5 TRAIL receptor expression in CDDP-treated IB4 and BL40 cells

Since TRAIL receptors are upregulated in a p53-dependent manner in response to anti-cancer drugs in some cell systems (Chinnaiyan et al., 2000; Sheikh et al., 1998; Sun et al., 2000), I examined the levels of DR4 and DR5 in IB4 and BL40 cells treated with CDDP. Initially, TRAIL receptor transcripts were measured by RPA. This assay is based on the solution hybridisation of a single-stranded, discrete size, ^{32}P-labelled antisense probe to a sample of total RNA. After hybridisation, any remaining unhybridised probe and sample RNA are removed by digestion with a mixture of ribonucleases. Labelled probe that is hybridised to complementary RNA from the sample is protected from ribonuclease digestion, and is resolved on a polyacrylamide gel and visualised by autoradiography. When the probe is present in molar excess over the target in the hybridisation reaction, the intensity of the protected fragment is directly proportional to the amount of target RNA in the sample mixture. Thus RPAs are sensitive as well as quantitative assays.

In BL40 cells, DR4 transcripts remained constant up to 6 hours after addition of CDDP but gradually fell to levels below detection after 36 hours (Figure 4.7). DR5 transcripts were not examined in BL40 cells due to technical difficulties. Consistent with previous reports, RPA analysis showed a transient increase in the levels of both DR4 and DR5 transcripts in IB4. DR4 and DR5 levels rose rapidly 6 hours after addition of CDDP but subsequently declined rapidly and became undetectable after 36 hours (Figure 4.8). The upregulation of TRAIL receptor transcripts was consistent with the upregulation of p53. The signals for both DR4 and DR5 were specific since there was no probe protection in the absence of homologous sequence (yeast RNA controls). Furthermore, the levels of either transcript were unchanged in untreated cells at the start and the end of the experiment, indicating that transcript upregulation was CDDP-specific. Transcripts of the 'house-keeping' gene, GAPDH, analysed in parallel as a loading control, appeared fairly constant up to 12 hours following addition of CDDP, after which time they progressively decreased (Figures 4.7 and 4.8). This was probably due to mRNA degradation or transcriptional impairment in cells undergoing apoptosis. However, the kinetics of the GAPDH response was slower compared to the sharp decline in death receptor transcripts in either cell line, indicating that the initial upregulation in TRAIL receptor transcripts in IB4 cells and the subsequent decline in IB4 and BL40 cells were CDDP-specific.

Both DR4 and DR5-protected probes consistently appeared as double bands in these analyses. This is probably due a phenomenon common with this assay when using probes with A-U-rich 3' ends. Transient strand separation or 'breathing' allows RNase digestion at the 3'end of the probe giving rise to protected probe fragments of slightly lower molecular weight than expected (Ambion, 2002).

To test whether the changes in the mRNA levels were reflected at the protein levels of the TRAIL receptors, I analysed DR4 and DR5 in CDDP-treated cells by western blotting. Both DR4 and DR5 were easily detectable in either cell line and their levels were not altered by CDDP treatment (Figure 4.9). The disparity between RPA and western blotting analyses, may be due to inhibition in protein translation (due to the drug) during the initial phase when transcript levels rose or a reduction in the protein's half-lives so that any increase in transcription would be balanced by protein degradation resulting in the absence of an overall increase in protein levels.

4.6 Analysis of BCL-2 family proteins during CDDP-induced apoptosis in BL40 and IB4 cells

BCL-2 family members can modulate cell sensitivity to anti-cancer drugs by regulating the release of apoptogenic factors from the mitochondria (Clem et al., 1998), (Gross et al., 1999a). In addition, certain members are transcriptional targets of p53 and can influence death receptor-induced apoptosis in some systems. For instance, overexpression of BCL-2 and BCL-XL can inhibit Fas-mediated apoptosis in Type II cells (Scaffidi et al., 1998). Since BCL-2 family members can regulate drug-induced apoptosis but also indirectly influence death receptor signalling, I examined the levels of the prototypic family member, BCL-2, as well as several pro- and anti-apoptotic homologues, namely BAX, BCL-XL and MCL-1 in CDDP-treated IB4 and BL40 cells.

CDDP treatment was carried out as previously described and the expression levels of BCL-2 homologues were determined by western blotting. BCL-2, BAX and BCL-XL levels were unaffected by the drug consistent with previous reports (Allday et al., 1995) (Figures 4.10 and 4.11). BCL-2 was not readily detectable in BL40 cells and required increased amounts of protein (40 μg) and longer exposures on autoradiography film in order to be detected. This was consistent with previous reports (Spender et al., 1999). By contrast the anti-apoptotic family member, MCL-1, declined significantly following addition of CDDP. In IB4 cells the decline was observed after 12 hours whereas in BL40 cells MCL-1 levels dropped after 24 hours. MCL-1 was undetectable in IB4 cells 24 hours and in BL40 cells 36 hours after addition of CDDP consistently with the kinetics of PARP cleavage. The levels of MCL-1 in untreated cells at the start and end of the experiment remained constant indicating that the change in MCL-1 levels was CDDP-specific.

It is not clear from this evidence whether the downregulation of MCL-1 is a p53-mediated event. However, since MCL-1 contains two PEST motifs in its primary structure, a feature characteristic of proteins with a short half-life (Kozopas et al., 1993), the rapid decline may be the result of transcriptional inhibition. In addition, the timing of MCL-1 downregulation, which correlates with caspase activation as indicated by cleavage of PARP, was also suggestive of caspase-mediated processing.

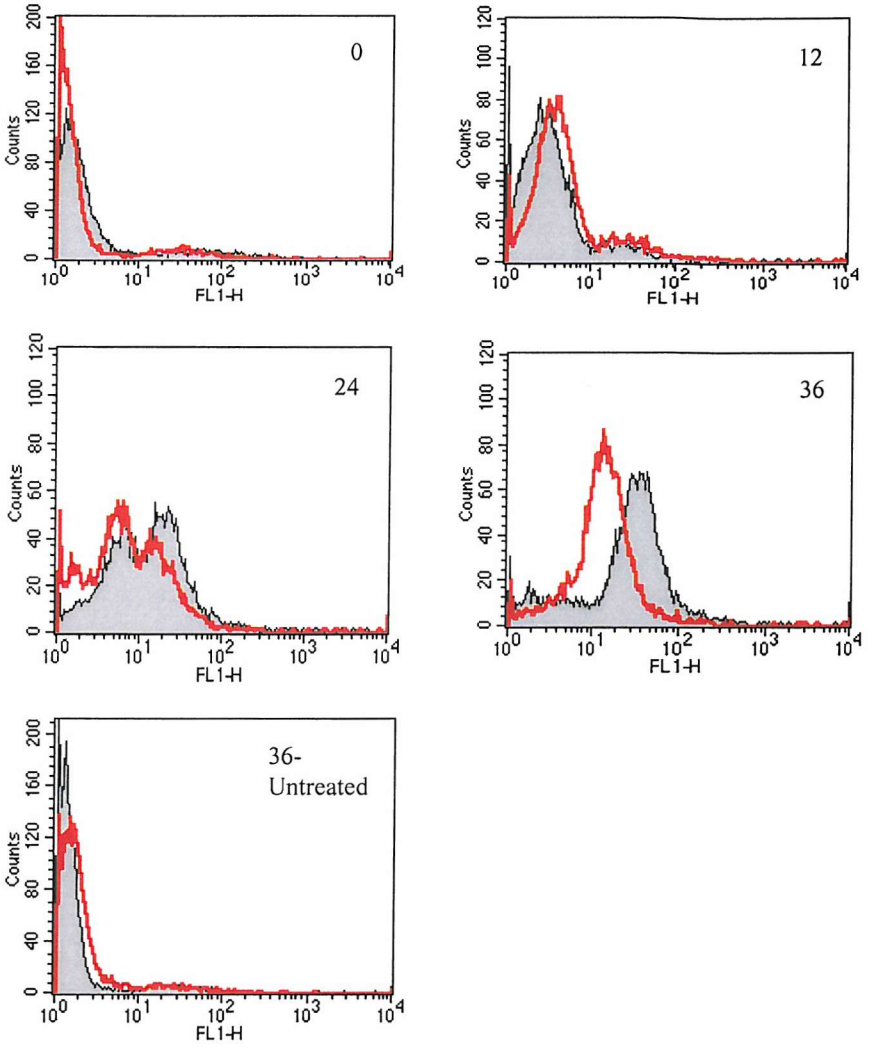


Figure 4.6: *Fas surface expression in CDDP-treated BL40 cells.* BL40 cells were treated with CDDP (10 μg/ml) for the indicated time points (hours) or left untreated as controls. Cells were incubated with a Fas-specific antibody (clone DX2) (red histogram) or an isotype-matched antibody (α-TNP) (filled histogram) as a control for background staining. 10000 events per sample were acquired on a flow cytometer using the FL1 channel and analysed using CellQuest software.

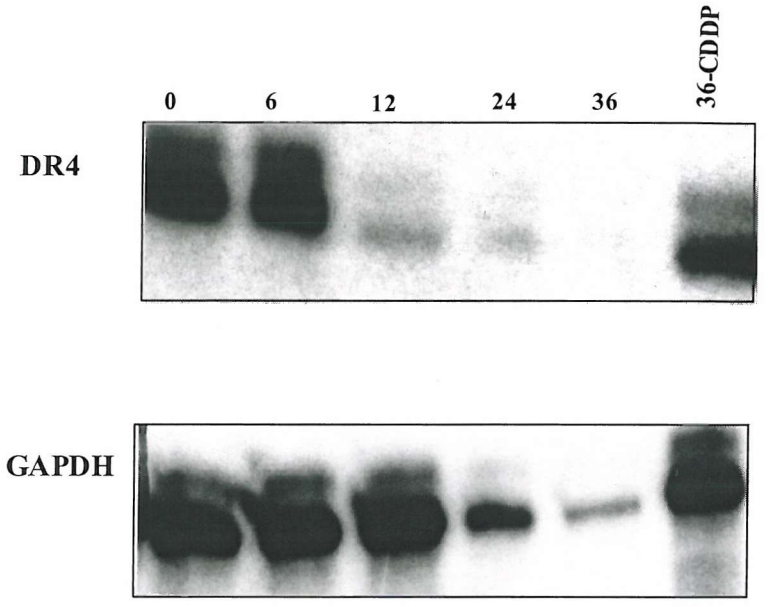


Figure 4.7: *RPA analysis of DR4 in BL40 cells treated with CDDP.* BL40 cells were treated with CDDP (10 μg/ml) for the indicated times (hours) points or left untreated as controls (36- CDDP). 10 μg of RNA from each sample was hybridised to a DR4-specific ^{32} P-labelled antisense riboprobe and subjected to RNase digestion. RNase-protected probe fragments were resolved on a denaturing poly-acrylamide gel and visualised by autoradiagraphy. The level of the house-keeping gene GAPDH, was analysed as a loading control.

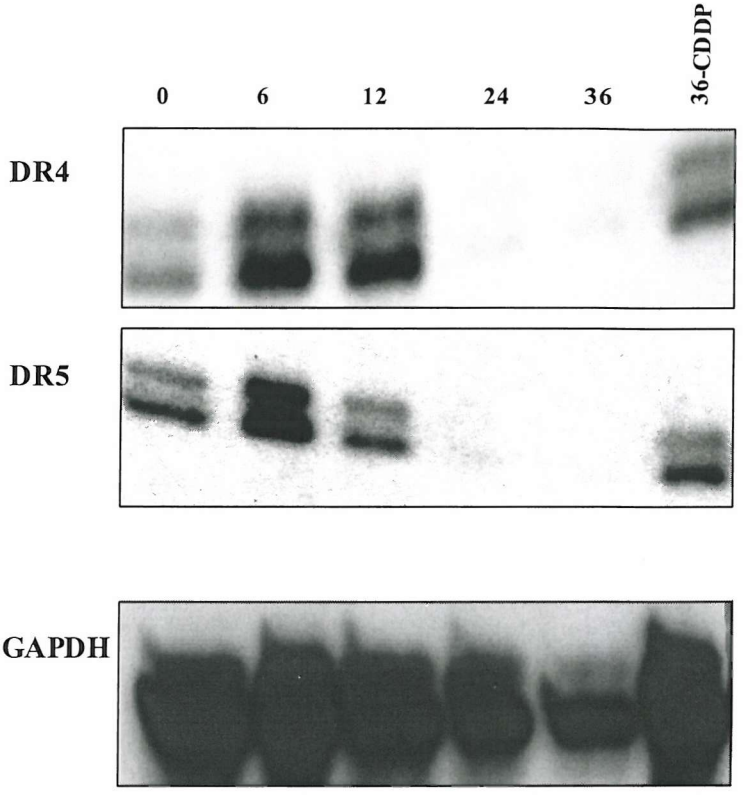


Figure 4.8: *RPA analysis of DR4 and DR5 in IB4 cells treated with CDDP.* IB4 cells were treated with CDDP (10 μg/ml) for the indicated times (hours) or left untreated as controls. 10 μg of RNA from each sample were hybridised to DR4 or DR5-specific ^{32}P-labelled antisense riboprobes and subjected to RNase digestion. Protected probe fragments were resolved on denaturing poly-acrylamide gels and visualised by autoradiagraphy. The house-keeping gene GAPDH was also analysed as a positive control.

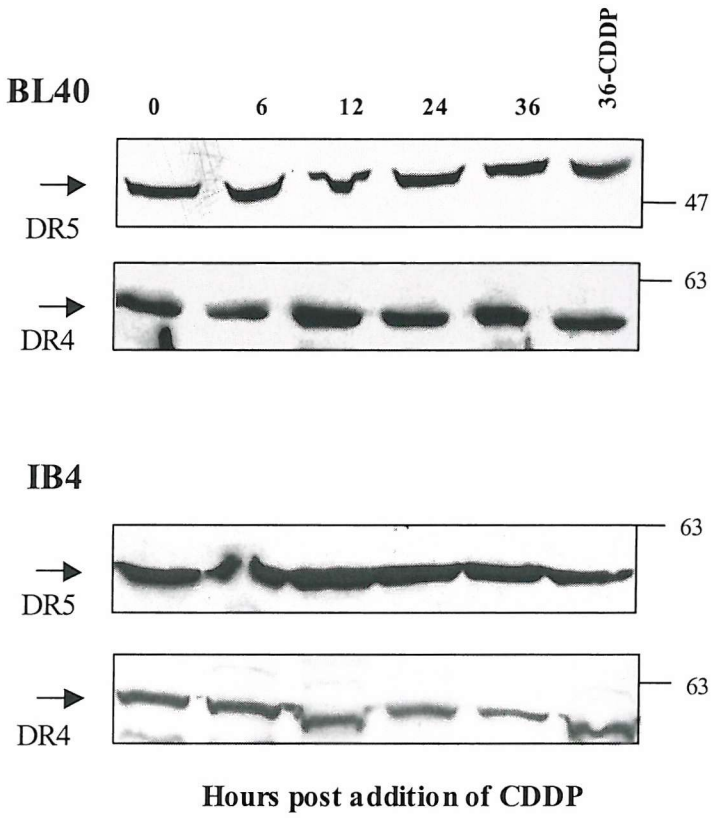


Figure 4.9: *Western blotting analysis of DR4 and DR5 in IB4 and BL40 cells treated with CDDP.* IB4 and BL40 cells were treated with CDDP (10 μg/ml) for the indicated times (hours) or left untreated as controls. 20 μg of protein from each time point were resolved on 10% polyacrylamide gels and transferred onto nitrocellulose. Filters were probed with DR4- and DR5-specific rabbit polyclonal antibodies. Filters were also probed with PCNA-specific antibody as a loading control (data not shown). The position of the molecular weight markers is indicated (KDa).

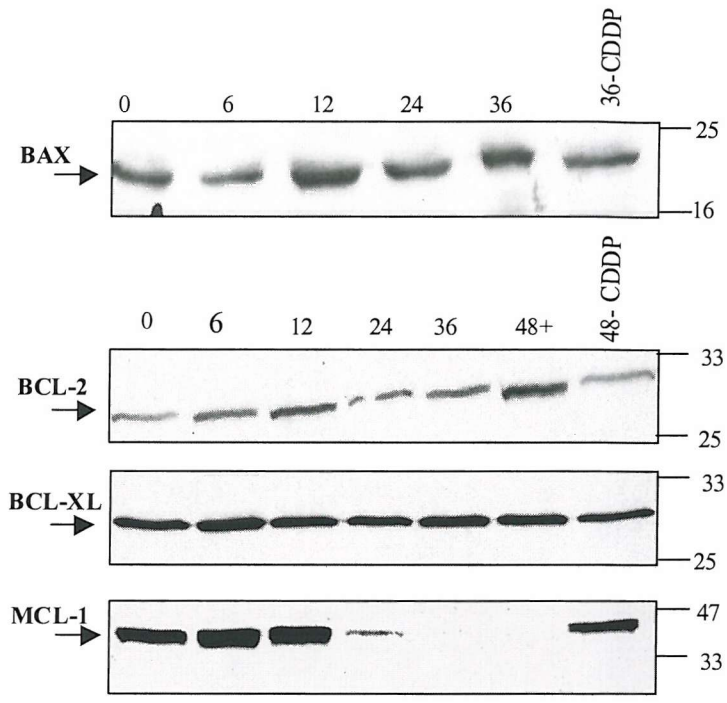


Figure 4.10: *Western blotting analysis of BCL-2 family proteins in CDDP-treated BL40 cells.* BL40 cells were treated with CDDP (10 μg/ml) for the indicated times (hours) or left untreated as controls. 20 μg (40 μg for analysis of BCL-2) of protein from each time point were resolved on 12% polyacrylamide gels and transferred onto nitrocellulose. Filters were probed with a BCL-2-specific mouse monoclonal and BCL-XL, MCL-1 and BAX-specific rabbit polyclonal antibodies. Filters were also probed with PCNA-specific antibody as a loading control (data not shown). Slight variations in BCL-2 protein levels are due to loading variation. The position of the molecular weight markers is indicated (KDa).

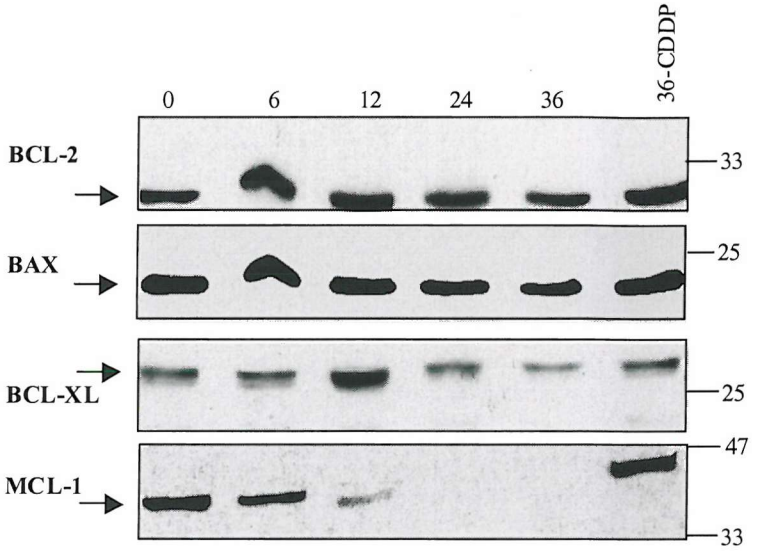


Figure 4.11: *Western blotting analysis of BCL-2 family proteins in CDDP-treated IB4 cells.* IB4 cells were treated with CDDP (10 μg/ml) for the indicated times (hours) or left untreated as controls. 20 μg of protein from each time point were resolved on 12% polyacrylamide gels and transferred onto nitrocellulose. Filters were probed with a BCL-2-specific mouse monoclonal and BCL-XL, MCL-1 and BAX-specific rabbit polyclonal antibodies. Filters were also probed with PCNA-specific antibody as a loading control (data not shown). The slight variation in BCL-XL levels is due to loading variation. The position of the molecular weight markers is indicated (KDa).

4.7 Neutralisation of Fas and TRAIL receptor pathways does not inhibit CDDP-induced apoptosis in IB4 and BL40 cells

Although there was no alteration in the levels of either Fas or TRAIL receptors following treatment of IB4 and BL40 cells with CDDP, it was possible that death receptors still contributed to CDDP-induced apoptosis. This was tested in a functional manner, by neutralising Fas and TRAIL pathways at the same time as treating cells with CDDP and measuring cell growth.

I first tested the efficiency of a panel of recombinant TRAIL receptors comprising rhFc:DR4 and rhFc: DR5 as well as rhFc:TRID and rhFc:TRUNDD, in neutralising TRAIL-mediated apoptosis in Jurkat cells. The recombinant TRAIL receptors should bind onto TRAIL and therefore inhibit apoptosis in sensitive cells by reducing the effective concentration of the ligand. rhFc:DR3 should not bind TRAIL and was used as a negative control. Jurkat cells were treated with three doses of rTRAIL (500, 50 and 5 ng/ml) in the presence or absence of rhFc:DR3, rhFc:DR4, rhFc:DR5, rhFc:TRID and rhFc:TRUNDD (500 ng/ml) for 24 (data not shown) and 48 hours and cell growth was determined by the MTS assay. Cells were also left untreated as controls. Jurkat cell growth was not affected by the addition of recombinant receptors alone whereas cells died in response to rTRAIL. rhFc:DR5 effectively neutralised rTRAIL even at the highest dose whereas rhFc:DR4 inhibited rTRAIL-induced apoptosis at the low dose of rTRAIL. rhFc:TRID and rhFc:TRUNDD molecules were not effective in blocking rTRAIL in this assay (Figure 4.12 and data not shown). The reasons were not investigated but may indicate lower affinity of the decoy receptors for rTRAIL. Therefore rhFc:DR5 was used in the following experiments to block rTRAIL-induced apoptosis in IB4 and BL40 cells. rhFc:DR3 did not neutralise TRAIL-induced apoptosis (as expected) and was used as a negative control.

Two well-characterised neutralising reagents, a Fas-specific antagonistic antibody (ZB4) and a FasL-specific antibody (NOK-1) were used to neutralise Fas. Both reagents have been used interchangeably in studies of Fas signalling (Houghton et al., 1997), (Eischen et al., 1997). To inhibit Fas signalling in IB4 cells, I used the NOK-1 antibody to neutralise FasL on these cells since any Fas-mediated effect would be produced by the interaction of Fas with its ligand. BL40 cells were treated with the

antagonistic antibody, ZB4, to block any Fas molecules perhaps present at low levels below the level of detection of FACS analysis.

The neutralising reagents rhFc:DR5, ZB4 and NOK-1 were used separately or combined, with or without CDDP, to treat IB4 and BL40 cells for 24 (data not shown) or 48 hours. The cells were also treated with CDDP (10 μg/ml), α-Fas (500 ng/ml) or rTRAIL (500 ng/ml) as positive controls. IB4 cells were sensitive to CDDP, rTRAIL and α-Fas whereas BL40 cells were sensitive to rTRAIL and CDDP but not α-Fas as expected (Figures 4.13 and 4.14). However, the neutralising reagents ZB4, NOK-1, and/or rhFc:DR5 did not hinder CDDP-mediated apoptosis in either cell line. In addition, NOK-1 had a growth-promoting effect in IB4 cells (perhaps Fc receptor-mediated) and therefore the modest increase in cell growth when CDDP and NOK-1 were combined was deemed a non-specific effect of this antibody (Figure 4.14). Therefore, in contrast to previous reports, the evidence indicated that neutralisation of Fas and/or TRAIL receptors did not inhibit CDDP-induced apoptosis suggesting that the death receptor pathways did not play a significant role in CDDP-induced apoptosis in IB4 and BL40 cells.

4.8 CDDP and α-Fas or rTRAIL act additively and not synergistically to induce apoptosis in IB4 and BL40 cells

Although there was no evidence for an increase in Fas and TRAIL receptor expression during apoptosis induced by CDDP in IB4 or BL40 cells, it is possible that the respective pathways co-operated with CDDP-activated pathways to induce apoptosis as suggested by previous reports (Uslu et al., 1996), (Muller et al., 1998). To investigate the possible co-operation between the two pathways, cells were treated with CDDP combined with α-Fas or rTRAIL and the effect of individual as well as combined treatments on cell viability was measured.

Duplicate cell cultures were incubated with CDDP (1, 3, 10 μg/ml), α-Fas (50, 250, 500 ng/ml) and rTRAIL (500 ng/ml) or combinations of CDDP (1 or 3 μg/ml) with α–Fas (50 or 250 ng/ml) or with rTRAIL (500 ng/ml) for up to 48 hours. Cell viability was determined at various times, 12, 24, 36 and 48 hours by the trypan blue exclusion assay (Figures 4.15-4.18 and data not shown). The cells were treated with intermediate and low doses of CDDP and α-Fas to avoid excessive rates of cell death

and to facilitate the detection of a synergistic effect when the two reagents were combined. IgM antibody was also used as isotype-matched control for α-Fas. The criterion for synergy was set at values equal to or exceeding the observed additive values by 25%.

In BL40 cells, CDDP-induced apoptosis was time and dose-dependent whereas BL40 cells were not significantly affected by α-Fas as expected (Figure 4.15 A and data not shown). The positive controls for this experiment were IB4 cells as shown in Figure 4.17 (the experiments were performed in parallel). The IgM control did not seriously affect BL40 cell growth and the combination of CDDP with α-Fas resulted in a reduction of cell viability that did not exceed the sum of individual treatments, therefore the effect of CDDP and α-Fas reagents was deemed additive. BL40 cells were also incubated with rTRAIL in the absence or presence of CDDP. Although high levels of spontaneous apoptosis were observed in untreated cells, CDDP did not potentiate the effect of rTRAIL under these conditions; therefore there was no evidence for synergy between CDDP and rTRAIL in this cell line (Figure 4.16). In IB4, apoptosis mediated by CDDP or α-Fas was both time and dose-dependent whereas IgM did not affect cell growth (Fig.4.17 and data not shown). In addition, the combination of CDDP and α-Fas potentiated apoptosis in an additive manner. For example, CDDP treatment (1 μg/ml) resulted in 36% of cells becoming trypan blue positive whereas 45% of cells incorporated the dye after addition of α-Fas (50 ng/ml). The combination of the two reagents at the same concentrations resulted in 83% of cells becoming trypan blue positive, which is only slightly higher than the sum of individual treatments (Figure 4.17, A). Therefore, their effect was deemed additive. Although IB4 cells responded to rTRAIL, the extent was less pronounced than previously shown (Chapter 3) (Figure 4.18 A). However, rTRAIL induced a marked reduction in the viability of Jurkat cells used as controls in a parallel experiment (Figure 4.19, B). The discrepancy may be due to differences in the assays used to determine sensitivity; the MTS assays, used previously, measure the reduction in the cellular metabolism whereas trypan blue uptake measures the loss of membrane integrity, which is a late event in apoptosis. This assay showed that the combination of CDDP and rTRAIL induced marginally higher rates of apoptosis compared to CDDP alone. Thus, there was no evidence for synergy between CDDP and α-Fas or rTRAIL in IB4 cells.

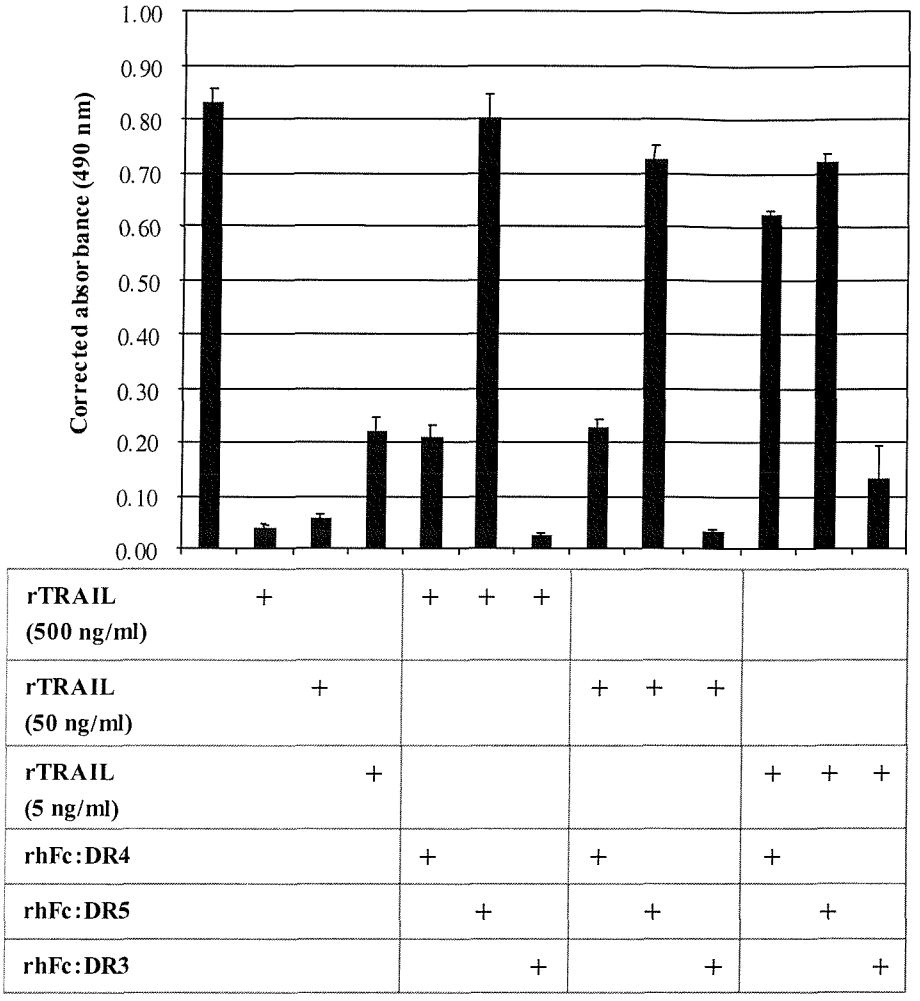
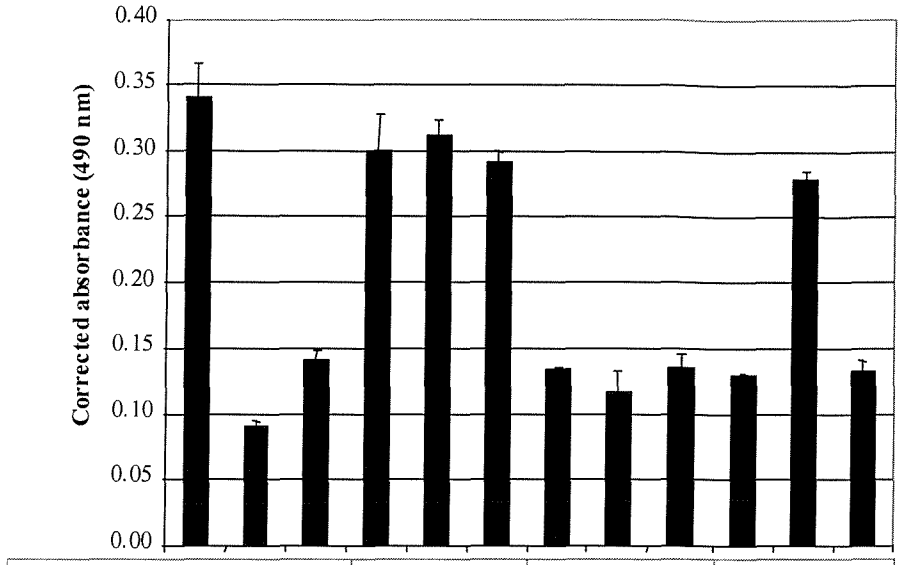


Figure 4.12: *Neutralisation of rTRAIL in Jurkat cells.* Jurkat cells were treated with rTRAIL (500, 50, 5 ng/ml) in the presence or absence of chimeric TRAIL receptors rhFc:DR4, rhFc:DR5, rhFc:DR3 (500 ng/ml) for 48 hours. Cells were also left untreated as a control and cell growth was measured by the MTS assay. Values presented here are the average of corrected absorbance values of triplicate wells +/- standard error of the mean. Some errors are too small to show.



CDDP (10 μg/ml)	+			
CDDP (3 μg/ml)	+		+ + +	+
rhFc:DR5		+	+	+
rhFc:DR3		+	+	+
ZB4		+	+	+
α-Fas				+
rTRAIL				+

Figure 4.13: *The neutralisation of death receptors does not inhibit CDDP-induced apoptosis in BL40 cells.* BL40 cells were treated with CDDP (3 μg/ml) in the presence or absence of rhFc:DR5 (500 ng/ml) and/or ZB4 antibody (500 ng/ml) for 24 hours. rhFc:DR3 (500 ng/ml) was used as a negative control. Cells were also treated with CDDP (10 μg/ml), α-Fas (500 ng/ml), rTRAIL (500 ng/ml) or left untreated as controls and cell growth was determined by the MTS assay. Data points presented here are the average of corrected absorbance values of triplicate wells +/- standard error of the mean. Some errors are too small to show. This analysis was repeated twice.

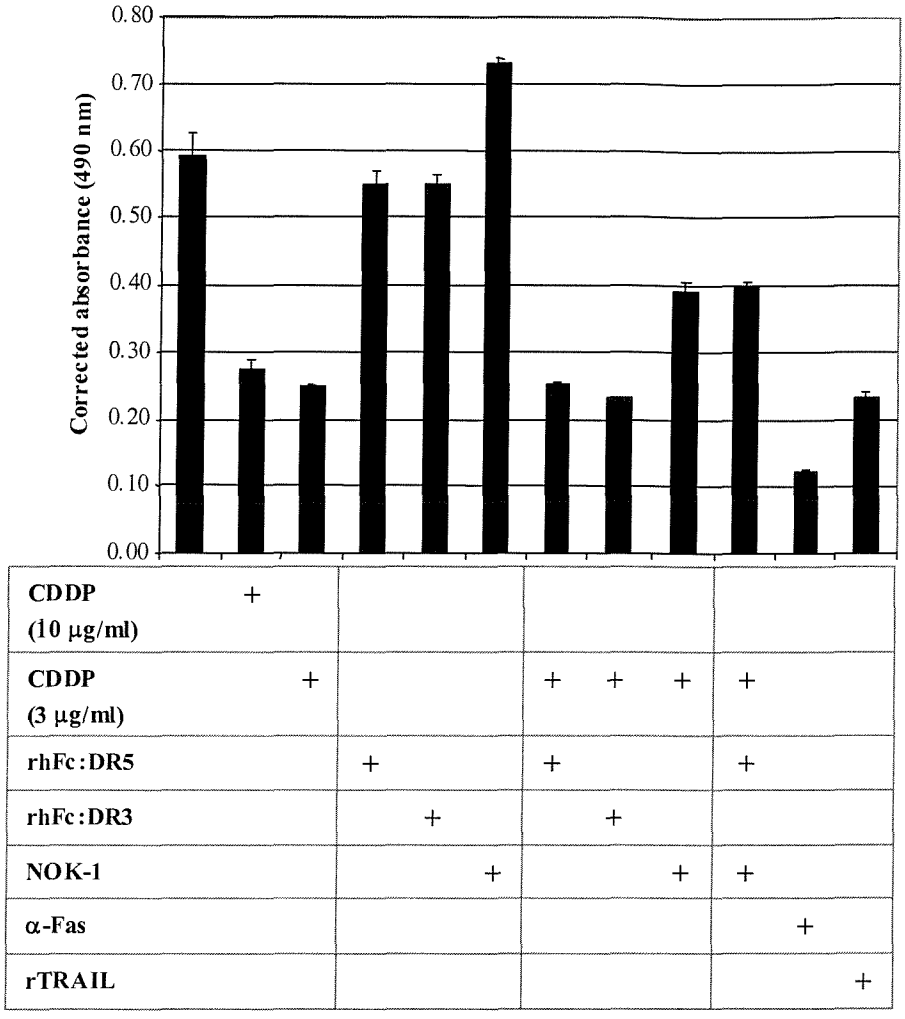
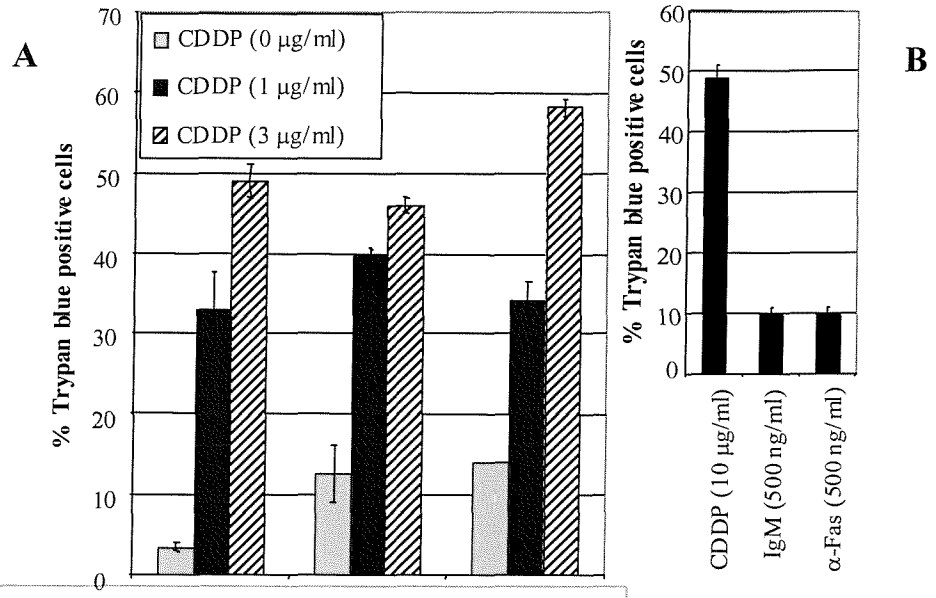
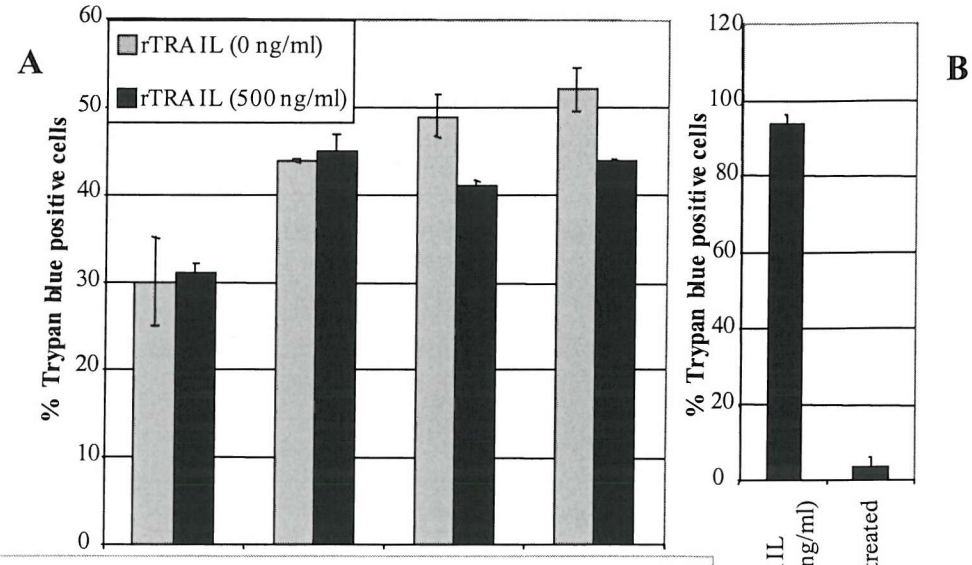


Figure 4.14: *The neutralisation of death receptors does not inhibit CDDP-induced apoptosis in IB4 cells.* IB4 cells were treated with CDDP (3 μg/ml) in the presence or absence of rhFc:DR5 (500 ng/ml) and/or FasL-neutralising antibody NOK-1 (500 ng/ml) for 48 hours. rhFc:DR3 was used as a negative control. Cells were also treated with CDDP (10 μg/ml), α-Fas (500 ng/ml), rTRAIL (500 ng/ml) or left untreated as controls and cell growth was determined by the MTS assay. Data points presented here are the average of corrected absorbance values of triplicate wells +/- standard error of the mean. Some errors are too small to show. The experiment was repeated twice.



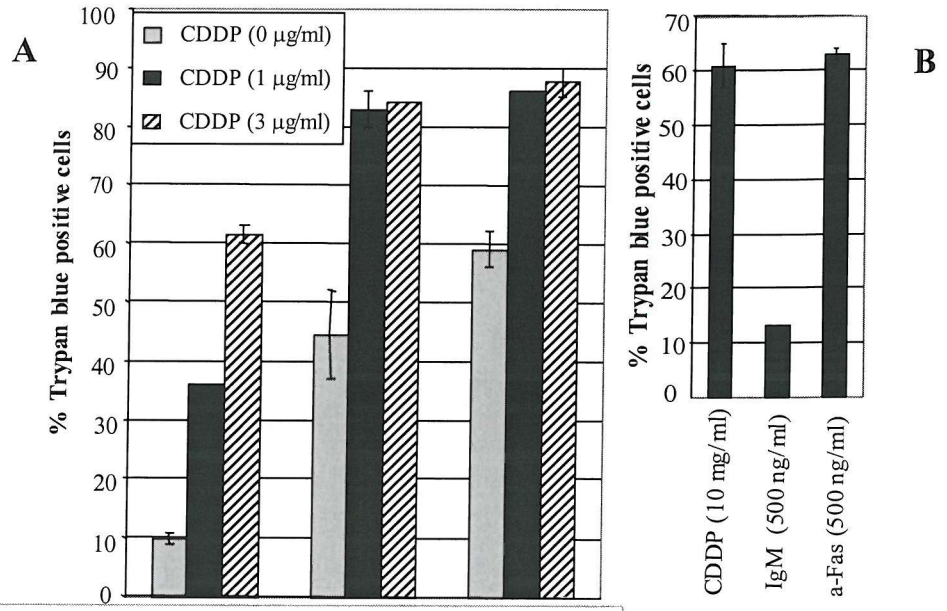
	α-Fas (0 ng/ml)	α-Fas (50 ng/ml)	α-Fas (250 ng/ml)
CDDP (0 µg/ml)	4	14	14
CDDP (1 µg/ml)	34	40	34
CDDP (3 µg/ml)	49	46	58

Figure 4.15: *CDDP does not co-operate with α–Fas to kill BL40 cells.* **(A)** BL40 cells were incubated with CDDP (1 or 3 µg/ml) in the absence or presence of α-Fas (50 or 250 ng/ml) or left untreated as controls for 36 hours and cell viability was measured by the trypan blue exclusion assay. The percentages of trypan blue-positive cells for each treatment are shown in the table. **(B)** BL40 cells were incubated with CDDP (10 µg/ml), α-Fas (500 ng/ml) or isotype-matched IgM immunoglobulin (500 ng/ml) as controls. Viability was measured by the trypan blue exclusion assay after 36 hours. The values shown here are the average of duplicate cultures +/- standard error. Some errors are too small to show.



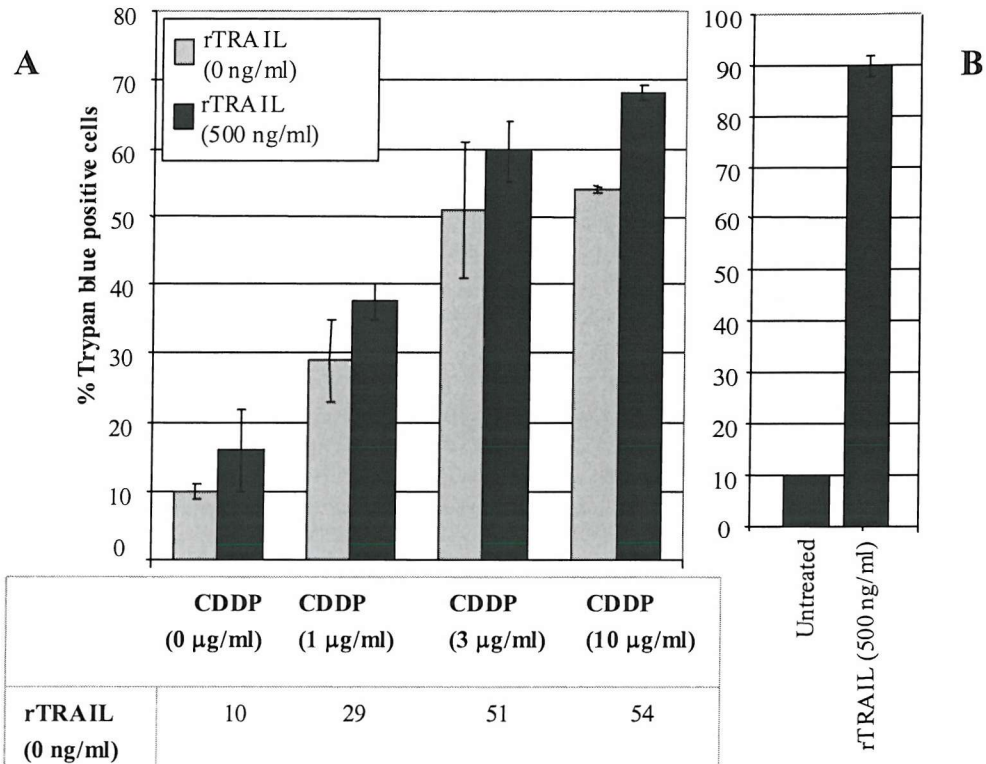
	CDDP (0 µg/ml)	CDDP (1 µg/ml)	CDDP (3 µg/ml)	CDDP (10 µg/ml)
rTRAIL (0 ng/ml)	30	44	49	52
rTRAIL (500 ng/ml)	31	45	41	44

Figure 4.16: *CDDP does not co-operate with rTRAIL to kill BL40 cells.* **(A)** BL40 cells were incubated with CDDP (1, 3, 10 µg/ml) in the presence or absence of rTRAIL (500 ng/ml) or left untreated as controls for 36 hours before cell viability was measured by trypan blue exclusion. The percentages of trypan blue positive cells are shown. **(B)** Jurkat cells were incubated in the absence or presence of rTRAIL (500 ng/ml) as a positive control for rTRAIL cytotoxicity and cell viability was measured by the trypan blue exclusion assay after 36 hours. The points shown here are the means of duplicate cultures +/- the standard error. Some errors are too small to show.



	α-Fas (0 ng/ml)	α-Fas (50 ng/ml)	α-Fas (250 ng/ml)
CDDP (0 µg/ml)	10	45	59
CDDP (1 µg/ml)	36	83	86
CDDP (3 µg/ml)	62	84	88

Figure 4.17: *CDDP does not co-operate with α–Fas to kill IB4 cells.* **(A)** IB4 cells were incubated with CDDP (1 or 3 µg/ml) in the absence or presence of α-Fas (50 or 250 ng/ml) or left untreated as controls for 36 hours. Cell viability was measured by trypan blue exclusion. Percentages of trypan blue positive cells for each treatment are shown. **(B)** IB4 cells were incubated with CDDP (10 µg/ml), α-Fas (500 ng/ml) or isotype-matched IgM immunoglobulin (500 ng/ml) as controls and viability was measured by trypan blue exclusion after 36 hours. Values shown are the average of duplicate cultures +/- standard error. Some errors are too small to show.



	CDDP (0 µg/ml)	CDDP (1 µg/ml)	CDDP (3 µg/ml)	CDDP (10 µg/ml)
rTRAIL (0 ng/ml)	10	29	51	54
rTRAIL (500 ng/ml)	16	38	60	68

Figure 4.18: *CDDP does not co-operate with rTRAIL to kill IB4 cells.* **(A)** IB4 cells were incubated with CDDP (1, 3 or 10 µg/ml) in the presence or absence of rTRAIL (500 ng/ml) or left untreated as controls for 36 hours and cell viability was measured by trypan blue exclusion. The percentages of trypan blue positive cells are shown in the table. **(B)** Jurkat cells were incubated with rTRAIL (500 ng/ml) as a positive control for rTRAIL cytotoxicity. Cell viability was measured after 36 hours by the trypan blue exclusion assay. Values shown are the average of duplicate cultures +/- standard error. Some errors are too small to show.

4.9 Discussion

This study aimed to investigate anti-cancer drug-induced apoptosis in BL cells with wild type p53 and to examine the role of death receptors in this process. The majority of anti-cancer treatments act mainly by inducing apoptosis, as a consequence apoptotic defects in the target tissue often result in chemoresistance. Therefore, the elucidation of mechanisms by which anti-cancer treatments induce apoptosis could improve our understanding of cancer therapy. This knowledge may also help in the identification of therapeutic strategies that increase the efficacy and specificity while reducing the toxicity of treatments. In this respect, the attention on death receptor pathways and their role in anti-cancer treatments is justified. The evidence so far suggests that they may mediate and/or contribute to the cytotoxic effects of cancer-targeting treatments, particularly as part of p53-dependent responses.

This study demonstrated that treatment of two cell lines containing wild type p53, the EBV negative BL40 and the LCL, IB4, with the DNA-damaging agent CDDP, resulted in dose and time-dependent apoptosis and CDDP-specific upregulation of p53. Although the majority of BL cells *in vivo* express wild type p53 (Bhatia et al., 1992), mutations in p53 are very frequent in BL cell lines (Farrell et al., 1991). For this reason, EBV positive BL cells containing wild type p53 were not easily available and therefore not examined. In addition, it should be noted that stabilisation of p53 in IB4 and BL40 cells following treatment with CDDP is not sufficient evidence of a functional involvement. However, CDDP has been previously shown to functionally activate p53 in LCLs under similar conditions (Allday et al., 1995). However, the analysis of previously reported p53 transcription targets, which may also regulate apoptosis, did not show changes in their expression levels in BL40 cells.

CDDP did not induce a detectable change in the levels of Fas in the Fas-positive IB4 or the Fas-negative BL40 cells. However, upregulation of FasL instead of Fas would be another means of engaging the Fas pathway and such a mechanism has been shown to operate in leukaemia cells (Friesen et al., 1996). Unfortunately, I was unable to repeat previous analyses of FasL surface expression using flow cytometry and the validity of western blotting analyses performed was questioned when a later study demonstrated cross-reactivity of the FasL-targeting antibody with unrelated proteins (Smith et al., 1998). By contrast, a CDDP-specific transient upregulation of

DR4 and DR5 transcripts was detected in IB4 cells by RPA analysis. In BL40 cells, however there was no change the levels of DR4 mRNA. In both cell lines, TRAIL receptor transcripts declined rapidly, from as early as 12 hours following addition of CDDP. However, the subsequent examination of TRAIL receptors by western blotting showed that the changes in mRNA were not reflected at the protein level. The decline in transcript levels may have been the result of a reduction in the half-life of the transcripts during apoptosis. Therefore, the evidence indicated that the upregulation of TRAIL receptor expression is unlikely to be an important mechanism of CDDP-induced apoptosis in this cell system. Examination of DR4 and DR5 expression on the cell surface would perhaps be required to confirm this, particularly as more recent studies in melanoma cell lines have demonstrated TRAIL receptor relocation from intracellular stocks to the cell surface and *vice versa* as a mechanism of regulating sensitivity to TRAIL (Zhang et al., 2000a). This investigation was not possible at the time of these experiments (due to lack of reagents) and the evidence from functional analyses suggested it was not necessary, so it was not pursued further.

An important class of molecules with a significant role in the regulation of death receptor-induced apoptosis is the BCL-2 protein family. It was possible that CDDP-induced changes in the levels of BCL-2 homologues could indirectly influence death receptor signalling by modulating caspase activation. I therefore analysed several BCL-2 family proteins namely BAX, BCL-2, MCL-1 and BCL-XL. Loss of function of BAX had been associated with resistance to α-Fas in BL (Gutierrez et al., 1999), whereas BCL-2 and BCL-XL can inhibit Fas- and to an extent TRAIL-induced apoptosis in other cell systems (Srinivasan et al., 1998), (Scaffidi et al., 1998). Finally, MCL-1 expression was also investigated since its levels are intriguingly high in BL and other cell types of GC origin (Kitada et al., 1998). However, with the exception of MCL-1, CDDP did not alter the expression levels of the remaining BCL-2 homologues. However, the CDDP-specific decline in the levels of MCL-1 that coincided with the kinetics of PARP cleavage was of interest.

Enhanced expression of this anti-apoptotic protein in BL is suggestive of a role in maintaining cell viability, implying that its decline may be a requirement and/or an indication of the cells' commitment to apoptosis. The latter is supported by studies on cultured human neutrophils, which lose MCL-1 during spontaneous apoptosis whereas all tested agents that delay this entry into apoptosis also maintain MCL-1 levels

(Moulding et al., 1998). Furthermore, increased expression of MCL-1 has been associated with maintenance of cell viability and decreased expression with cell death of normal peripheral blood B lymphocytes (Lomo et al., 1997). It was possible that the downregulation of MCL-1 was a consequence of apoptosis induction, perhaps a result of targeting by caspases as has been described for other family members, e.g. BID. This would be consistent with the timing of its decline but preliminary examination of the primary sequence of MCL-1 did not show consensus caspase cleavage sites. However, there is no evidence for a role of MCL-1 in the regulation of death receptor pathways, so its mechanistic significance in this system is unclear. Finally, the downregulation of this protein may simply be due to its short half-life combined with transcriptional downregulation during apoptosis (Kozopas et al., 1993). The lability of MCL-1 makes it an ideal target for antisense depletion, an approach that may clarify its role in this system.

The investigation on the role of Fas and TRAIL receptors in CDDP-induced apoptosis in BL40 and IB4 cells was extended to the functional analysis of their role using neutralising reagents. However, the combination of Fas and/or TRAILR-neutralising reagents with CDDP did not hinder CDDP-induced apoptosis in BL40 and IB4 cells, contrary to the reports from other cell systems. I also tested the potential for synergism between death receptor pathways and CDDP-induced responses. Co-operation between the two systems could result in the activation of the two central caspase cascades (mitochondria and receptor-initiated) and amplification of apoptosis. However, CDDP did not co-operate with α-Fas to induce apoptosis in either cell line, rather the two reagents displayed an additive effect and the same was true with the combination of CDDP and rTRAIL. Therefore, the examination of apoptosis induced by CDDP in BL40 and IB4 cells, containing wild type p53, did not provide evidence of a functional interaction with Fas and/or TRAIL receptor pathways.

Chapter 5. Molecular determinants of resistance to Fas-mediated apoptosis in Burkitt's lymphoma cell lines

5.1 Introduction

The majority of Burkitt's lymphoma cell lines were identified as resistant to Fas-induced apoptosis (Chapter 3) and the mechanism of resistance was independent of Fas levels in a BL subset, the EBV positive group III cell lines and Raji cells (group II). By contrast the EBV-immortalised LCLs that express the same set of EBV-encoded proteins as group III BL cells were Fas-sensitive. This indicated that the Fas-resistant phenotype in BL cells does not reflect a B-cell-specific phenotype suggesting that the mechanism of resistance may be associated with the tumour origin of BL cell lines.

Resistance to Fas-mediated apoptosis has been described in a variety of cell systems including haematological and non-haematological cancers and a number of mechanisms have been proposed to account for it. Apart from lack of receptor expression, apoptosis can be inhibited by functional inactivation of the receptors, e.g. by mutations, production of alternatively spliced variants lacking the transmembrane domain (soluble receptors) or the death domain (Landowski et al., 1997) (Cascino et al., 1996a). In addition, intracellular mechanisms acting downstream of Fas can also interfere with the apoptotic process, e.g. by interacting with effector molecules such as caspases. Such mechanisms include the expression/overexpression of inhibitory molecules, such as c-FLIP and FAP-1 (Li et al., 2000b) and anti-apoptotic members of the BCL-2 family, for example BCL-2 and BCL-XL (Scaffidi et al., 1998). Defects in signalling components can also inhibit Fas-mediated apoptosis.

A distinguishing feature of the BL cell system, that could be influencing Fas-induced apoptosis, is the overexpression of c-myc. This is suggested by the differential responsiveness between group III BL cell lines and LCLs (which lack the c-myc translocation while expressing the same set of EBV latent genes). However, the deregulated c-myc is known to display a dual function encompassing both pro- and anti-apoptotic properties. In fact, c-MYC is a potent activator of apoptosis, and in some instances, it induces apoptosis via the Fas/FasL pathway. c-MYC sensitises cells to Fas-induced apoptosis (Rohn et al., 1998), probably by triggering the release of cytochrome c (Juin et al., 1999) and c-MYC-induced apoptosis in fibroblasts requires

interaction of Fas with FasL (Hueber et al., 1997). In addition, ligation of the TCR on activated T cells induces the expression of FasL by a c-MYC-dependent mechanism suggesting a role for c-MYC in promoting AICD (Shi et al., 1992), (Bissonnette et al., 1994). Furthermore, CD40 ligation on EBV negative BL cell lines (which also overexpress c-myc) has been reported to result in the upregulation of Fas and susceptibility to Fas-induced apoptosis *in vitro* (Schattner et al., 1996). Therefore, the existing evidence suggests that c-myc overexpression in group III BL cell lines would be more likely to facilitate apoptosis rather than hinder it and that it is unlikely that c-myc overexpression alone would be responsible for the Fas-resistant phenotype in group III BL cell lines.

To investigate the mechanisms of resistance to Fas-induced apoptosis in group III BL cell lines, I determined the expression of previously described inhibitory mechanisms that disrupt Fas-mediated apoptosis in other cell systems. LCLs were also examined for comparison and Raji cells (group II BL) were also included in some assays.

5.2 Analysis of mutations in the death domain of Fas in group III BL cell lines and LCLs

A number of mutations that result in loss of Fas function have been described in mice and humans. At least 20 distinct mutations have been reported in humans, some associated with different forms of cancer (Landowski et al., 1997), (Gronbaek et al., 1998), (Tamiya et al., 1998). The majority of mutations occur in the death domain of Fas, they are usually heterozygous and can affect Fas function in a dominant negative manner (Cascino et al., 1996a). Since the DD essentially acts as a binding interface allowing the death signal to be passed onto the intracellular effectors of apoptosis, mutations in this part of the molecule would clearly disable its function.

Thus, group III BL cell lines, LCLs and Raji cells were screened for mutations in the DD of Fas by PCR-SSCP analysis. Jurkat cells were used as wild type controls in this assay. SSCP analyses provide a sensitive and efficient method of mutation detection, allowing the examination of a relatively large number of samples. To screen for mutations, ^{32}P-labelled RT-PCR products containing the entire DD-encoding region of Fas were amplified (the region amplified is shown in Figure 1.5). The primers used span the region between 817-1300 bp of the human Fas gene and are

positioned on different exons (exons 7 and 9) to prevent any genomic DNA influence in the PCR reaction (Itoh and Nagata, 1993). Since SSCP assays are more efficient in resolving fragments up to 300 bp long, the amplified fragments were subjected to restriction endonuclease digestion with an appropriate enzyme (BglII), in order to generate two fragments of appropriate sizes: 291 bp (Fas-DD1) and 192 bp (Fas-DD2).

The basis of SSCP assays is that mutations or polymorphic differences in the primary gene structure will result in altered intramolecular interactions that will generate a sequence-specific three-dimensional structure under the conditions of the assay. Wild type and polymorphic/mutant molecules will thus move at different rates through a non-denaturing gel matrix. To this end, RT-PCR fragments were first denatured to single strands by heating and rapidly cooling at 0^0 C, a procedure that allows the denatured strands to refold, gaining a sequence-specific conformation. The samples were subsequently resolved on a specially formulated gel matrix with high sensitivity to DNA conformational differences. The matrix structure allows DNA separation to occur on the basis of both size and conformation, thus increasing the probability of detecting sequence differences to approximately 80% relevant to standard matrices (BioWhittaker Molecular Applications, technical information sheet).

To confirm the sensitivity of this assay, I also amplified exon 4 of bax from Daudi cells as a positive control for mutations (Peng et al., 1998). This region carries a heterozygous amino acid substitution (resulting from a single nucleotide change): glycine to valine at position 108 (G108V) (Meijerink et al., 1998) and therefore will migrate to a different position compared to the wild type (wt) exon 4 from Jurkat cells and normal human genomic DNA used as controls. Since the size of this PCR product was appropriate for this assay (209 bp), it wasn't subjected to restriction endonuclease digestion and thus it produced a single set of bands. The amplified fragments were resolved on the same gel as Fas-DD1 and Fas-DD2 fragments and visualised by autoradiography (data not shown) and on a phosphoimager.

The mutation in Daudi cells was indicated by a mobility shift (relevant to Jurkat and normal genomic DNA), confirming the ability of this assay to detect single base changes. The PCR products containing the DDs of Fas gave rise to two major sets of bands and some faint bands, each major band corresponding to one allele from the DD1 and DD2 fragments (Figure 5.1). The death domain region failed to amplify from two cell lines, Jijoye and LCL3, both of which express Fas on the cell surface as determined by FACS analysis (Chapter 3). This was probably due to technical

variation. However, the SSCP analysis did not provide evidence for mutations in the DD of the remaining cell lines as no mobility shifts were detected in the remaining BL cell lines or the LCLs.

5.3 Analysis of the role of soluble Fas molecules in modulating resistance to α-Fas in group III BL cell lines

Alternative splicing variants of human Fas often encode molecules that lack the transmembrane domain and are therefore soluble. Such molecules have been detected in sera from patients with autoimmune diseases, adult T cell leukaemia and CLL (Cheng et al., 1994), (Kamihira et al., 1999), (Osorio et al., 2001). Soluble Fas molecules can bind ligand with an affinity comparable to their membrane-bound counterparts, thus interfering with normal Fas activation by sequestering the ligand (Cascino et al., 1996b; Cheng et al., 1994).

I investigated the role of soluble Fas molecules in resistance to Fas-mediated apoptosis by transferring conditioned media (CM) from Fas-resistant cell lines to Jurkat cells, which were incubated with α-Fas. The rationale behind this approach was that any soluble Fas molecules in the tissue culture media of resistant cells would be transferred and inhibit Fas-induced apoptosis in Jurkat cells in the same way as they would be expected to function in Fas-resistant cell lines. Conditioned culture media (where cells had been growing for at least 24 hours) were collected from Fas-resistant Jijoye and MAK III cells and used to supplement Jurkat cell cultures. The transferred media were diluted in an equal volume of Jurkat CM so as not to disturb Jurkat cell growth but also to avoid excess dilution of any soluble molecules. Jurkat cells were treated with α–Fas in the presence or absence of CM from either MAK III or Jijoye BL cells for 48 hours, and cell growth was determined using the MTS assay.

Jurkat cell growth was not significantly affected by the presence of the transferred media and was markedly inhibited by α-Fas. However, the media transfer did not reverse Fas cytotoxicity in Jurkat cells as α-Fas was equally potent in the presence of transferred media. This indicates that soluble Fas molecules are unlikely to be major determinant of resistance to Fas-mediated apoptosis in MAK III and Jijoye cells.

5.4 Expression of essential Fas DISC components in group III BL cell lines and LCLs

The expression of the essential components of the Fas DISC, FADD and caspase 8, was subsequently determined. Following cross-linking of the receptors by FasL or α-Fas, FADD and caspase 8 are recruited to Fas aggregates via DD and DED-mediated interactions respectively, to form the Fas DISC (Kischkel et al., 1995). Binding of the adaptor molecule FADD to the cross-linked Fas receptor is essential for the recruitment and efficient activation of caspase 8, an event that precedes and limits the initiation of the caspase cascade. As caspase 8 is the caspase acting proximally to Fas, its presence at the DISC is also essential for signal transmission and there is no evidence for redundancy at this level. The importance of these two signalling intermediates was illustrated by studies with knockout mice (Yeh et al., 1998), (Varfolomeev et al., 1998).

The expression of FADD and caspase 8 in group III BL cells and LCLs was determined by western blotting using Jurkat cells as positive controls (Tang et al., 1999). Although several isoforms of caspase 8 have been predicted from sequence analyses, only two endogenous forms (with a similar function) are usually detected: caspase 8a and 8b (55 and 57 KDa respectively) (Scaffidi et al., 1997). Both isoforms were detected at levels similar or higher than in Jurkat cells (Figure 5.3). In addition, the adaptor molecule FADD was easily detectable and was expressed at similar levels between the cell lines examined. Therefore, there was no correlation between DISC component expression and susceptibility to Fas-induced apoptosis in the cell lines tested.

5.5 Expression of Fas signalling inhibitors, FAP-1 and FLIPL and BL cell lines and LCLs

Since there was no evidence for defects in the DD of Fas or lack of FADD and/or caspase 8, I subsequently investigated the expression of inhibitory molecules known to attenuate Fas function. Possibly the most important negative regulators of Fas-induced apoptosis are FAP-1 and c-FLIP. FAP-1 interacts with a 15 aa-long domain downstream of the death domain and in so doing inhibits apoptosis by an

undefined mechanism (Itoh and Nagata, 1993), (Yanagisawa et al., 1997). Overexpression studies in Jurkat cells showed a correlation between FAP-1 levels and resistance to Fas-mediated apoptosis (Sato et al., 1995) and microinjection of a tripeptide corresponding to the region of Fas responsible for binding to FAP-1 reversed the Fas-resistant phenotype in a colon cancer cell line (Yanagisawa et al., 1997). c-FLIP has been implicated in the regulation of Fas-induced apoptosis during T cell activation, and probably contributes to it by blocking caspase 8 activation during the initial resistant phases. Alternative splicing generates two isoforms; FLIPL and FLIPS. Association of FLIPL (the best studied isoform) with the DISC interferes with the activation of caspase 8 and complete processing to the smaller molecular weight subunits that comprise the active enzyme (Scaffidi et al., 1999b).

The expression of FAP-1 transcripts was initially examined by RPA. FAP-1 had been previously detected in BL72 cells by RT-PCR (data not shown). Thus, the RT-PCR product from BL72 cells was subcloned into an *in vitro* transcription plasmid (pFAP-1) to use as template. Restriction analysis and DNA sequencing confirmed the identity and orientation of the insert. Prior to RPA analysis, the pFAP-1 was linearised with an appropriate enzyme (XhoI) to yield a transcription unit of appropriate size for this assay (305 bp) (Figure 5.4 A). Using this probe, FAP-1 transcripts were identified in both Fas-resistant and sensitive cell lines; BL72, MUTU III c. 148, IB4, X50.7 and LCL3 cells (Figure 5.4 B). The transcript levels were higher in MUTU III c. 148, IB4 and X50-7 cells. Since FAP-1 is believed to exert a direct inhibitory effect on Fas signalling, the identification of FAP-1 expression in Fas-sensitive cells was surprising. It was therefore felt important to investigate the corresponding protein levels. When a FAP-1-specific antibody became available, FAP-1 protein expression was investigated using MCF-7 and Jurkat cells as positive and negative controls respectively (Li et al., 2000b). Western blotting analysis showed that the approximately 220 KDa FAP-1 protein was present at low levels in MCF-7 cells but was absent from Jurkat cells (Figure 5.5 A). In addition, FAP-1 was easily detectable in IB4 and BL72 cells but not in the remaining cell lines where FAP-1 transcripts were previously detected. Additional bands of lower molecular weight (approximately 180 KDa) were detected in most cells. At this point, it is not clear whether the additional bands represent alternative isoforms or degradation products, however, they are also detected by the authors of the original report who provided the FAP-1-specific antibody used in this analysis (Sato, 2001). The same authors have identified a number of alternative

transcripts of the FAP-1 gene but their function is still unknown (Sato, 2001). Therefore, FAP-1 protein was more restricted than mRNA expression in BL cell lines but it did not correlate with resistance to α-Fas since it was expressed in IB4 cells. This indicates that the observed levels of FAP-1 may not be sufficient to inhibit Fas-induced apoptosis in this cell system.

To assess the role of c-FLIP in resistance to Fas-induced apoptosis in group III BL cells, FLIPL expression was also analysed by western blotting. Moderate levels of FLIPL were detected in BL cells as well as the LCLs and Jurkat cells with no significant variations (Figure 5.5 B). It is therefore, unlikely that the existing levels of FLIPL are responsible for attenuating Fas signalling in this system. Analysis of the second FLIP isoform, FLIPS, was not performed due to the lack of commercial antibodies specific for this isoform at the time of these experiments.

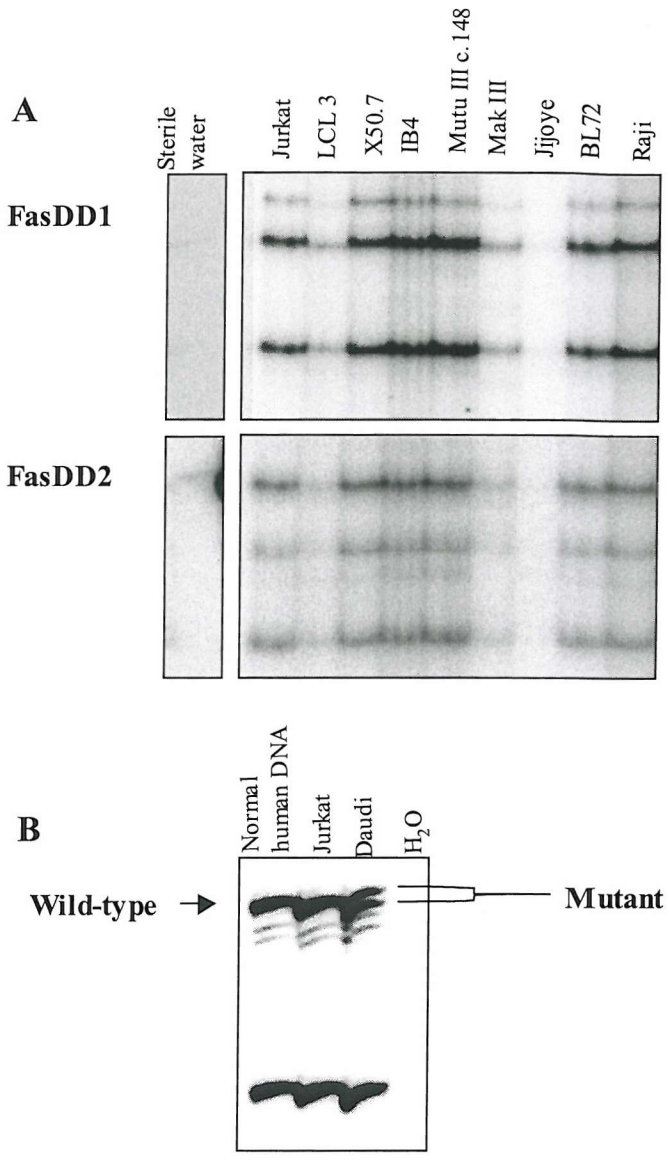


Figure 5.1: *PCR-SSCP analysis of the DD of Fas in group III BL lines and LCLs.* **(A)** The DD of Fas was amplified by RT-PCR and subjected to restriction endonuclease digestion before being resolved on a high-resolution gel matrix. Resolved fragments were visualised on a phosphoimager. **(B)** The exon 4 of bax was amplified from Daudi (G108V), Jurkat (wt) and normal human (wt) genomic DNA by RT-PCR and resolved on the same gel as the DDs of Fas, as a positive control.

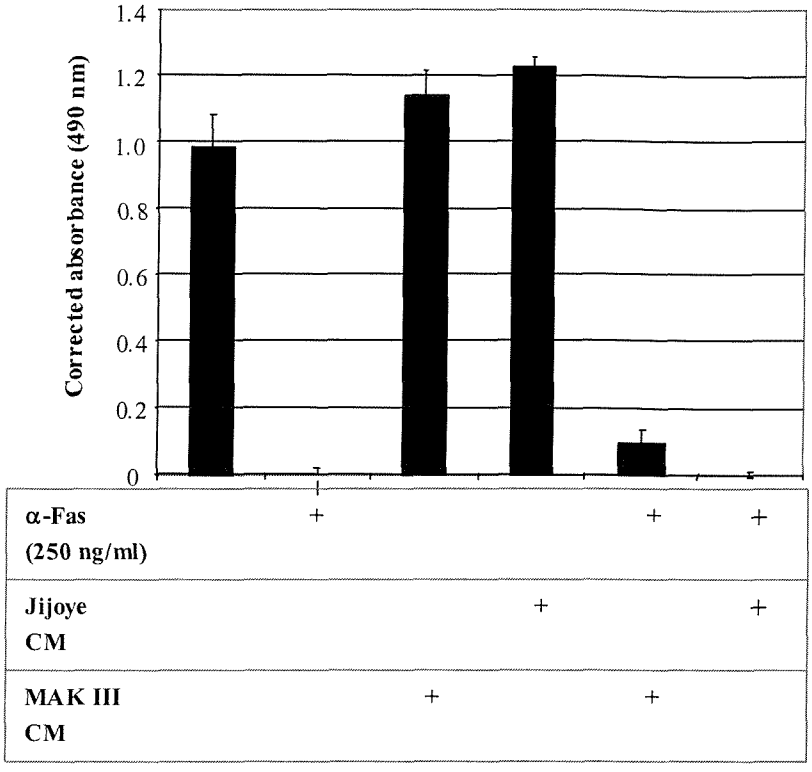


Figure 5.2: *Transfer of conditioned media from Fas-resistant BL cell lines does not reverse the Fas-sensitive phenotype in Jurkat cells.* Jurkat cells were cultured in the absence or presence of α–Fas (250 ng/ml) alone or combined with CM from MAK III and Jijoye BL cells for 48 hours. Cell growth was measured by the MTS assay. The values presented here are the average of the corrected absorbance of triplicate wells +/- standard error of the mean. The assay was repeated twice.

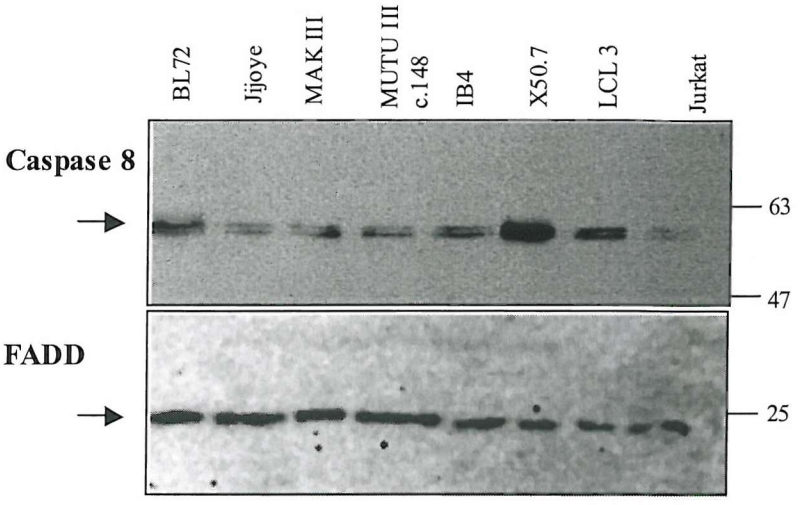


Figure 5.3: *Western blotting analysis of FADD and caspase 8 in group III BL cells and LCLs.* 20 μg of protein from each cell line were resolved on 12% polyacrylamide gels and transferred onto nitrocellulose. Jurkat cells were used as a positive control. Filters were probed with a FADD-specific mouse monoclonal antibody and a caspase 8-specific rabbit polyclonal antibody. Filters were also probed with a PCNA-specific antibody as a loading control (data not shown). The position of the molecular weight markers is indicated (KDa).

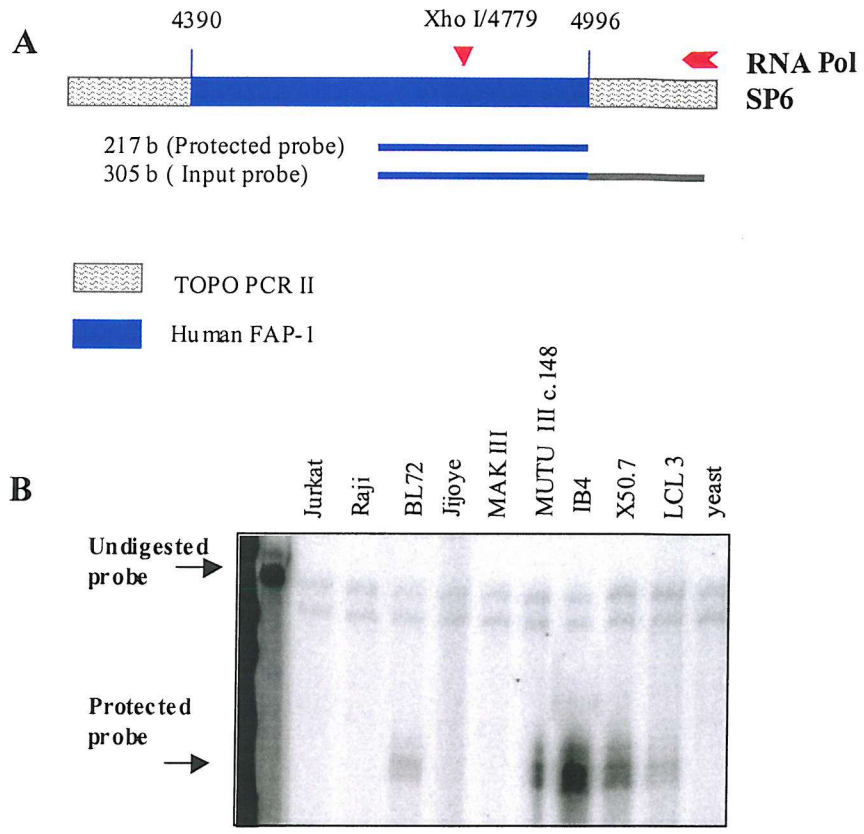


Figure 5.4: *Generation of an antisense riboprobe specific for FAP-1 and RPA analysis.* **(A)** A FAP-1 riboprobe was generated by *in vitro* transcription using pFAP-1 as a template. The regions corresponding to undigested and protected probe are shown. **(B)** RPA analysis of FAP-1 in group III BL cell lines and LCLs. 10 µg of RNA from each cell line were hybridised to the ^{32}P-labelled anti-sense riboprobe and subjected to RNase digestion. Protected fragments were resolved on a polyacrylamide gel and visualised using ImageQuant software on a Storm phosphoimager. Undigested probe was also resolved on the same gel as a control for RNA degradation. GAPDH transcripts were analysed as a loading control (data not shown).

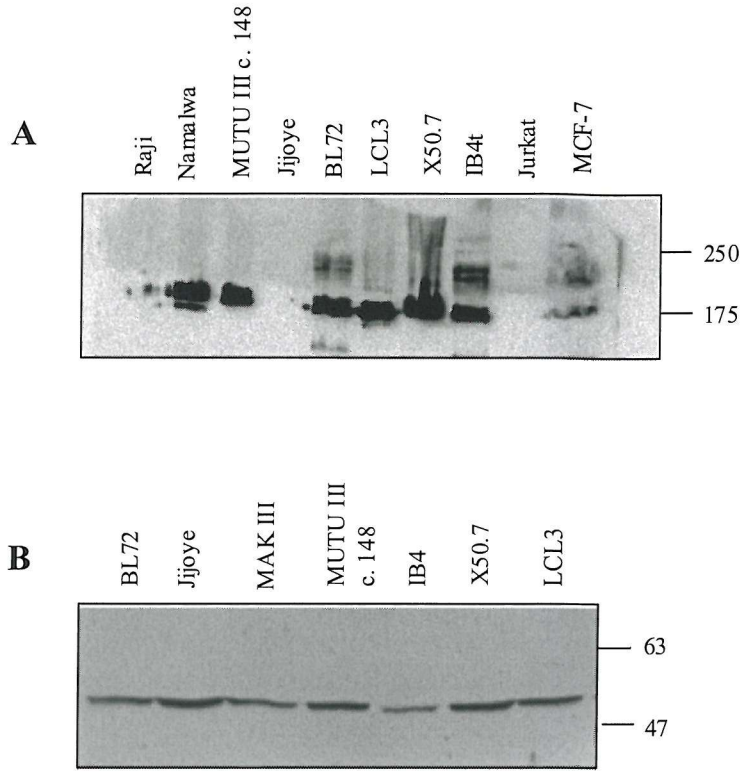


Figure 5.5: *Western blotting analysis of Fas signalling inhibitors, FAP-1 and FLIPL, in group III BL cell lines and LCLs.* **(A)** 40 μg of protein from each cell line were resolved on an 8% polyacrylamide gel and transferred onto nitrocellulose. The filter was probed with a FAP-1-specific rabbit polyclonal antibody. MCF-7 and Jurkat cells were used as positive and negative controls respectively. Filters were stained with Ponceau solution as a loading control. **(B)** 20 μg of protein from each cell line were resolved on a 10% polyacrylamide gel and transferred onto nitrocellulose. The filter was probed with a FLIPL-specific mouse monoclonal antibody. Filters were also probed with PCNA-specific antibody as a loading control (data not shown). The position of the molecular weight markers is indicated (KDa).

5.6 Discussion

The dissociation of Fas receptor expression from its apoptosis-inducing function in a subset of BL cell lines became clear from analyses of α-Fas cytotoxicity and Fas expression in Chapter 3. The resistance to α-Fas in group III BL lines was contrasted by the marked sensitivity of LCLs, expressing the same set of EBV latent genes. Since resistance to α-Fas in the BL cell lines did not reflect a B cell-specific phenotype and was receptor-independent, I examined molecular determinants that may confer resistance to α-Fas in group III BL cell lines.

Mutations in the DD of Fas are a common mechanism of resistance and their presence was investigated by SSCP analyses. These provide a sensitive screening method, however they do not provide any information on the nature of the mutations, so sequencing of the irregularly migrating fragments is needed after detection. However, the lack of evidence for mutations in the panel of cell lines examined did not make this necessary. This was consistent with a previous report in BL cell lines (Gutierrez et al., 1999). Notably, the lack of evidence for mutations in the death domain does not ascertain the integrity of the primary structure of Fas, since mutations that could interfere with its function could still exist in other parts of the molecule, for example in the ligand-binding region. According to the PLAD model, mutations in CRD1 (extracellular region) could also compromise normal receptor function. Nevertheless, the majority of studies have indicated that the death domain is the 'hot-spot' for mutations in Fas.

Soluble Fas receptor molecules can be encoded by alternative transcripts that often lack the region encoding the transmembrane domain. The presence of soluble Fas molecules was investigated by a functional assay based on conditioned media transfers from resistant cell lines to Jurkat. These assays did not provide any evidence for soluble receptors that may attenuate Fas-induced apoptosis in at least two Fas-resistant cell lines, Jijoye and MAK III, consistent with the findings of an earlier study (Gutierrez et al., 1999).

The majority of group III BL cell lines undergo apoptosis in response to the CDDP, accompanied by PARP cleavage (Chapter 4). This is indicative of a functional apoptotic proteolytic cascade, implying that a disruption in early signalling events proximally to Fas may be responsible for resistance to α-Fas. To begin to investigate

the role of inhibitory mechanisms acting proximally to Fas, I determined the expression of FLIPL and FAP-1 and confirmed the expression of FADD and caspase 8. Comparable levels of FADD and caspase 8 were found in Fas-sensitive as well as resistant cell lines, indicating that sufficient levels of Fas DISC components are expressed in the Fas-resistant BL cell lines. However, it is possible that DISC formation and activation of caspase 8, fails to occur in this cell system as has been suggested by the earlier study of (Tepper and Seldin, 1999). The most prominent inhibitor of caspase 8 activation at the DISC is FLIPL; therefore its levels were determined by western blotting. FLIPL was expressed in the entire panel of cell lines tested (including Jurkat), at similar levels, indicating that it cannot be independently responsible for resistance to α-Fas in the cell lines tested. Perhaps, even higher levels of this inhibitor are required for the efficient suppression of the death signal. This finding is in disagreement with an earlier report by (Tepper and Seldin, 1999) in which the authors used competitive PCR and densitometry analysis to compare the levels of caspase 8 and FLIPL in a panel of Fas-sensitive and resistant BL cell lines. Their study suggested a strong correlation between a low caspase 8/ FLIPL ratio and resistance to Fas-induced apoptosis in group III BL cells. This finding was supported by western blotting analysis showing increased levels of FLIPL in Fas-resistant cells. Clearly, the data in Figures 5.3 and 5.5B are in disagreement; for instance, BL72 cells appear to display a high caspase 8 / FLIPL ratio but they were resistant to α-Fas. The fact that the previous report relied heavily on PCR analysis, is probably one of the reasons for the disagreement. Western blotting (used in this report) is a more quantitative assay. In addition, the earlier study used a small number of group III cell lines and LCLs, the majority of which were different to the ones used here, therefore the molecular characteristics of BL cells with a group III phenotype may not be accurately reflected. Finally, although the authors of the previous report used overexpression of caspase 8 to alter the FLIPL/caspase 8 ratio and induce apoptosis, the reverse approach would perhaps have been more informative, i.e. introduction of an anti-sense FLIPL construct. It has become clear from a large number of previous reports that overexpression of apoptosis-related signalling components, particularly those containing recruitment domains such as DD and DED can often result in induction of apoptosis through non-specific mechanisms.

Another inhibitor that specifically disrupts Fas signalling is FAP-1, a protein phosphatase known to influence Fas signalling since the cloning of the receptor, but with a so far undefined mechanism of action. Since neither the expression nor the role of FAP-1 has been investigated in BL, I determined its expression by RPA and western blotting analyses. FAP-1 transcripts were detected in a large proportion of BL cell lines and LCLs, however the protein expression was more restricted and only found in BL72 and IB4 cells. The discrepancy between mRNA and protein expression is intriguing and may be due to translation inhibition. It is possible that higher levels of FAP-1 are required for efficient inhibition of Fas signalling or that more than one isoforms exist. A more recently-described inhibitor, reported to act proximally to Fas, is SADS (small accelerator of death signalling), a molecule reported to facilitate the interaction of FADD and caspase 8 and to be upregulated in patients with colon carcinoma (Suzuki et al., 2001b). This was a promising finding, implying additional regulatory element acting at the level of the DISC; I therefore investigated its expression using RT-PCR analysis. However, this approach failed to detect this molecule in BL cells (data not shown) and the original report was later retracted (Suzuki et al., 2001a).

Therefore, the examination of previously reported molecular determinants that may be controlling the Fas pathway, did not show a correlation with the pattern of Fas sensitivity in BL cell lines and LCLs, suggesting that novel mechanisms may be responsible for the regulation of Fas-mediated apoptosis in BL.

Chapter 6. Molecular determinants of resistance to TRAIL-mediated apoptosis in Burkitt's lymphoma cell lines

6.1 Introduction

The potency of rTRAIL in tumour regression and apoptosis in a wide variety of cell types as well as the lack of normal tissue toxicity *in vivo* has prompted significant interest and pre-clinical studies to evaluate its potential in cancer therapy. However, other reports also highlighted the lack of responsiveness to rTRAIL-induced apoptosis in a number of tumour cell systems and have provided evidence for a variety of mechanisms regulating sensitivity to rTRAIL in primary tumours as well as cell lines and normal cells.

Since the BL cell lines examined displayed distinct responsiveness to rTRAIL that correlated with their EBV status (Chapter 3), it was important to investigate the mechanisms that control sensitivity. The elucidation of the mechanisms that inhibit TRAIL-induced apoptosis may reveal a way to overcoming them but may also provide information on apoptotic defects associated with tumourigenesis. Furthermore, BL may also benefit from rTRAIL-based therapies. This study concentrated on two phenotypically similar groups of BL cell lines (EBV negative and positive BL cells with a group I phenotype). Both cell groups retain the morphologic features of BL biopsies and are similar in their growth patterns and their enhanced sensitivity to apoptosis-inducing stimuli such as ionomycin and growth factor deprivation (Rowe et al., 1987), yet displayed distinct responsiveness to rTRAIL.

6.2 Investigation of the roles of p53 and EBV in regulating TRAIL-mediated apoptosis of BL cell lines

To begin to investigate the molecular determinants that may modulate sensitivity to rTRAIL in BL cells, I tested the role of EBV and wild type p53. Type I latency in BL cell lines is characterised by limited expression of the EBV latent genes; EBNA-1 is the only EBV-encoded protein expressed, which serves in maintaining the EBV episome. In addition, the EBER RNAs and BamA transcripts, present in group I cell lines, have been recently implicated in tumourigenicity and resistance to apoptosis (Ruf et al., 2000; Ruf et al., 1999). It is therefore possible that EBV gene products may hinder TRAILR signalling in group I BL cells. To test this I examined sensitivity to

rTRAIL in a panel of isogenic Akata cells with different EBV status. These are single cell clones, derived from the original EBV positive BL cell line, Akata. Some cells maintained EBV while in culture but EBV negative clones have also emerged, which retain the group I phenotype of the parental clones (Takada et al., 1991). Similar Akata cell systems have been used to investigate the role of EBV in tumourigenicity (Komano et al., 1998), (Ruf et al., 1999).

The clones used in this analysis were the EBV negative, Akata 1, 23 and 31 and the EBV positive, Akata 6, 11, 18 and 33 (Inman et al., 2001). BL40 cells were included as a positive control of sensitivity to rTRAIL. Cells were treated with rTRAIL (500 ng/ml), or enhancer only (2.5 μg/ml), for 48 hours. CDDP (10 μg/ml) was also added to cells as a control and cell growth was measured by the MTS assay. BL40 cells were clearly sensitive to rTRAIL, whereas the Akata cell lines, irrespective of their EBV status, were resistant (Figure 6.1). By contrast, the entire panel of Akata clones were sensitive to CDDP. Combined with the fact that Akata 6 cells were previously shown to undergo CDDP-induced apoptosis (Chapter 3), this indicated that the Akata clones were specifically resistant to rTRAIL. Importantly, the fact that EBV negative as well as positive Akata cells were equally resistant to rTRAIL indicates that EBV is not likely to be a major determinant of resistance to TRAIL-induced apoptosis in group I BL cell lines.

In addition to the wild type p53 content of EBV negative cell lines BL2 and BL40 (Farrell et al., 1991), the group I cell lines Akata 6 and Elijah both lack wild type p53. Akata 6 is p53 null (Farrell et al., 1991) whereas Elijah cells express constitutively high levels of p53 that do not increase in response to DNA damage indicative of deregulated p53 (G. Packham, personal communication). Thus it was possible that p53 could be potentiating TRAIL-induced apoptosis in the EBV negative cell lines. p53-specific responses are generally accompanied by stabilisation of the otherwise low half-life protein, which can be detected by western blotting. Therefore, to begin to address the role of wild type p53 in TRAIL-mediated apoptosis, I examined the levels of p53 in BL40 cells treated with rTRAIL under the same conditions that induced PARP cleavage. Thus BL40 cells were treated with two doses of rTRAIL (100 and 250 ng/ml) both sufficient to induce apoptosis (Figure 3.4) for 12 or 18 hours. BL40 cells were also treated with CDDP (10 μg/ml) as a positive control for p53 induction. p53 was analysed using a p53-specific antibody that recognises the N-

terminal region of both wild type and mutant p53 (clone DO-1). Western blotting analysis demonstrated that p53 is expressed at low levels in control (untreated) cells, both at the start and at the end of the experiment, whereas p53 was induced in CDDP-treated cells as expected. However, addition of rTRAIL did not upregulate p53 in BL40 despite the fact that the cells displayed microscopical features of apoptosis as well as PARP cleavage (Figure 6.2 and data not shown). In fact, p53 levels were lower in rTRAIL-treated cells perhaps as a result of the proteins' low half-life combined with translational impairment and enhanced proteolytic activity during apoptosis. This was consistent with the levels of the "house-keeping" gene, PCNA, which were significantly reduced in rTRAIL and CDDP-treated cells (data not shown). The failure of rTRAIL to induce p53 is not a definitive indication of p53-independent apoptosis in BL40 cells (this would need to be supported by a functional analysis of p53 activation, for instance by western blotting analysis of p21 induction). However, it indicates that it is unlikely that p53 is an important regulator of rTRAIL-induced apoptosis in BL40 cells.

6.3 Decoy receptor expression in EBV negative and EBV positive BL cell lines with a group I phenotype

Decoy receptors are thought to inhibit TRAIL-induced apoptosis by two major mechanisms: the first involves sequestration of TRAIL making it unavailable for binding and activation of the pro-apoptotic receptors whereas the second involves the induction of growth-promoting signals, as for instance activation of NF-κB (Degli-Esposti et al., 1997a). To test whether the decoy receptors may be responsible for the control of TRAIL-induced apoptosis in this cell system, I first determined the expression of decoy receptor transcripts by RPA analysis.

TRID expression was analysed using a multiple-probe template that generates anti-sense probes specific for a variety of apoptosis-related molecules including TRID and the death receptors DR4 and DR5, Fas, TNFR1 as well as caspase 8. Probes for the 'house-keeping' genes GAPDH and L32 are included in the construct as loading controls (L32-protected probes shown here). Protected fragments can be readily identified since undigested as well as the corresponding protected probe sizes are provided by the manufacturer. DR4 and DR5 transcripts were easily detected in the

majority of cells examined whereas only DR5 was detected in Jurkat cells as expected (Figure 6.3). TNFR1 expression was shared by all cell lines with some variation and caspase 8 transcripts were also ubiquitously expressed. Fas expression was more variable and was detected in Louckes and Akata 6 and to a lesser extent Ramos cells but was absent from BL2, BL40 and BL41 cells consistently with FACS analyses (Chapter 3). The signals were specific since yeast RNA used as a control did not hybridise with the probes. However, the decoy receptor, TRID, was undetectable by this assay even after longer exposures.

TRUNDD expression was investigated using pTRUNDD as template. This template was generated by sub cloning a cDNA fragment excised from pCMVFLAG1-TRUNDD and subcloned in an *in vitro* transcription vector. The identity and orientation of the insert was confirmed by sequencing and the plasmid was linearised by an appropriate restriction enzyme to yield a transcription unit of 287 bp (Figure 6.4 A). This RPA analysis showed low levels of TRUNDD transcripts in two EBV negative cell lines; BL40 and Ramos but failed to detect TRUNDD in the remaining cell lines (Figure 6.4 B). Surprisingly, both BL40 and Ramos cells are TRAIL-sensitive (BL40 responded to rTRAIL in both the MTS and PARP cleavage assays whereas Ramos cells cleaved PARP after rTRAIL treatment). In addition, TRUNDD was undetectable in the TRAIL-resistant cell lines, Akata 6 and Elijah. Therefore, expression of the decoy receptors did not correlate with resistance to rTRAIL-induced apoptosis in group I BL in these analyses.

Since both DR4 and DR5 are expressed at relatively high levels, small amounts of TRID and/or TRUNDD would be unlikely to play a major role in regulating apoptosis. Thus, they would be unlikely to effectively sequester TRAIL, participate in a sufficient number of mixed receptor complexes or even induce sufficient levels of NF-κB in order to counteract apoptosis triggered by DR4 and DR5.

6.4 Analysis of the role of soluble TRAIL receptors in modulating resistance to apoptosis

Since decoy receptor expression did not correlate with the observed pattern of TRAIL sensitivity in BL cell lines, I examined the role of soluble TRAIL receptors. The best-known soluble TRAIL receptor is OPG, a molecule with dual specificity that can also bind OPGL, another member of the TNFR family. OPG is involved in

osteoclastogenesis and increases bone density *in vivo* but can also inhibit TRAIL-mediated apoptosis, for instance in Jurkat cells (Emery et al., 1998). Thus, it was possible that OPG or other, so far unidentified, soluble receptors may interfere with the interactions between TRAIL and the pro-apoptotic receptors and attenuate TRAIL-induced apoptosis.

This was tested in a functional assay using transferred conditioned media. CM from the TRAIL-resistant cell lines, Akata 6 and Elijah, were collected, filtered and added to BL40 cell cultures in a 1:2 dilution. BL40 cells were cultured in the absence or presence of rTRAIL (250 ng/ml) with or without CM from either Akata 6 or Elijah cells, for 48 hours and cell growth was measured by the MTS assay.

The transfer of conditioned media did not hinder cell growth in BL40; instead it appeared to have a modest growth-enhancing effect. However, when combined with rTRAIL it did not reverse BL40 cell sensitivity; the reduction in cell growth in wells containing the combination of rTRAIL with transferred CM was similar to the cell growth reduction induced by rTRAIL alone (Figure 6.5). This indicated that soluble TRAIL receptors are unlikely to play a major role in resistance to rTRAIL-induced apoptosis in Akata 6 or Elijah cells.

6.5 Expression of essential DISC components, FADD, caspase 8 and the inhibitor molecule FLIPL in EBV negative and EBV positive group I cell lines

DISC formation following TRAIL binding onto DR4 and DR5 is an essential event for the transmission of the apoptotic signal, required to activate caspase 8. Although studies of TRAIL signalling have so far been limited, the TRAILR DISC complex is believed to be similar to Fas, comprising FADD and caspase 8. Both DISC components were detected in DR5 signalling complexes by IP analyses (Bodmer, 2000) and Jurkat cells that lack either FADD or caspase 8 are resistant to TRAIL-mediated apoptosis (Kuang et al., 2000). Furthermore, sensitivity to TRAIL correlated with expression of caspase 8 in neuroblastoma cells (Eggert et al., 2001). To test whether deficiencies in these molecules might be responsible for resistance to rTRAIL in Akata 6 and Elijah cells, I analysed FADD and caspase 8 expression by western blotting. The western blots showed that both TRAIL-sensitive and TRAIL-resistant cell lines express similar levels of FADD and caspase 8, both isoforms of which were

easily detectable (Figure 6.6). Caspase 8 protein expression was consistent with transcript expression as detected by RPA analysis. Therefore, both DISC components were expressed at levels sufficient for signalling.

FLIPL is mostly associated with the regulation of Fas signalling; however it can also inhibit TRAIL-induced apoptosis by associating with the DISC in a manner that inhibits the efficient activation of caspase 8. FLIPL expression correlated with susceptibility to TRAIL in studies of melanoma cell lines (Griffith and Lynch, 1998) but not in other cell systems (Wen et al., 2000). To test the role of FLIPL in TRAIL-induced apoptosis in BL, I compared the levels of FLIPL in EBV negative cell lines and Akata 6 and Elijah cells by western blotting.

The majority of cells expressed comparable levels of FLIPL, with the exception of BL2, which express lower levels (Figure 6.7). This was not due to loading variation as determined by blotting for PCNA (Figure 6.6). Low levels of FLIPL in BL2 cells may be facilitating TRAIL-induced apoptosis, however, this is unlikely since BL40 cells express higher levels of FLIPL yet they also are TRAIL sensitive. In conclusion, there was no evidence that defects in the essential DISC components of the TRAIL receptors or the levels of FLIPL may be independently responsible for the control of TRAIL-mediated apoptosis in EBV positive cell lines with a group I phenotype.

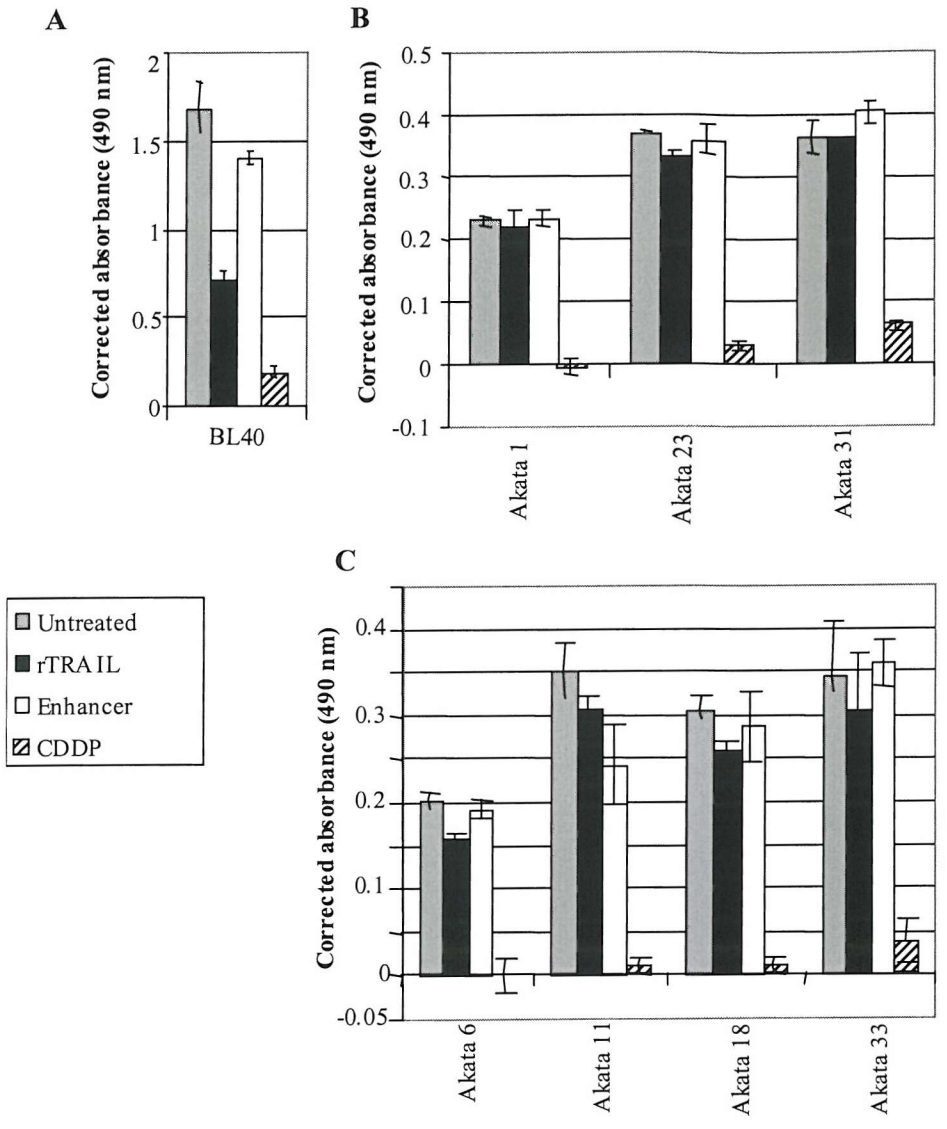


Figure 6.1: *Sensitivity to rTRAIL in EBV negative and EBV positive Akata clones.* **(A)** BL40 cells **(B)** EBV negative and **(C)** EBV positive Akata clones were cultured in the absence or presence of rTRAIL (500 ng/ml), enhancer only (2.5 μg/ml) or CDDP (10 μg/ml) for 48 hours. Cell growth was measured by the MTS assay. The data points shown here are the average of triplicate wells +/- standard error of the mean.

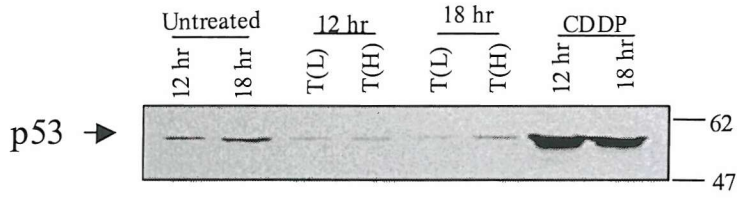


Figure 6.2: *Western blotting analysis of p53 in rTRAIL-treated BL40 cells.* BL40 cells were treated with two doses of rTRAIL, 100 ng/ml (L) and 250 ng/ml (H) or CDDP (10 μg/ml) for 12 or 18 hours. 20 μg of protein from each time point were resolved on a 10% polyacrylamide gel and transferred onto nitrocellulose. The filter was probed with a p53-specific mouse monoclonal antibody (clone DO-1). Filters were also probed with a PCNA-specific antibody as a loading control (data not shown). The position of the molecular weight markers is indicated (KDa)

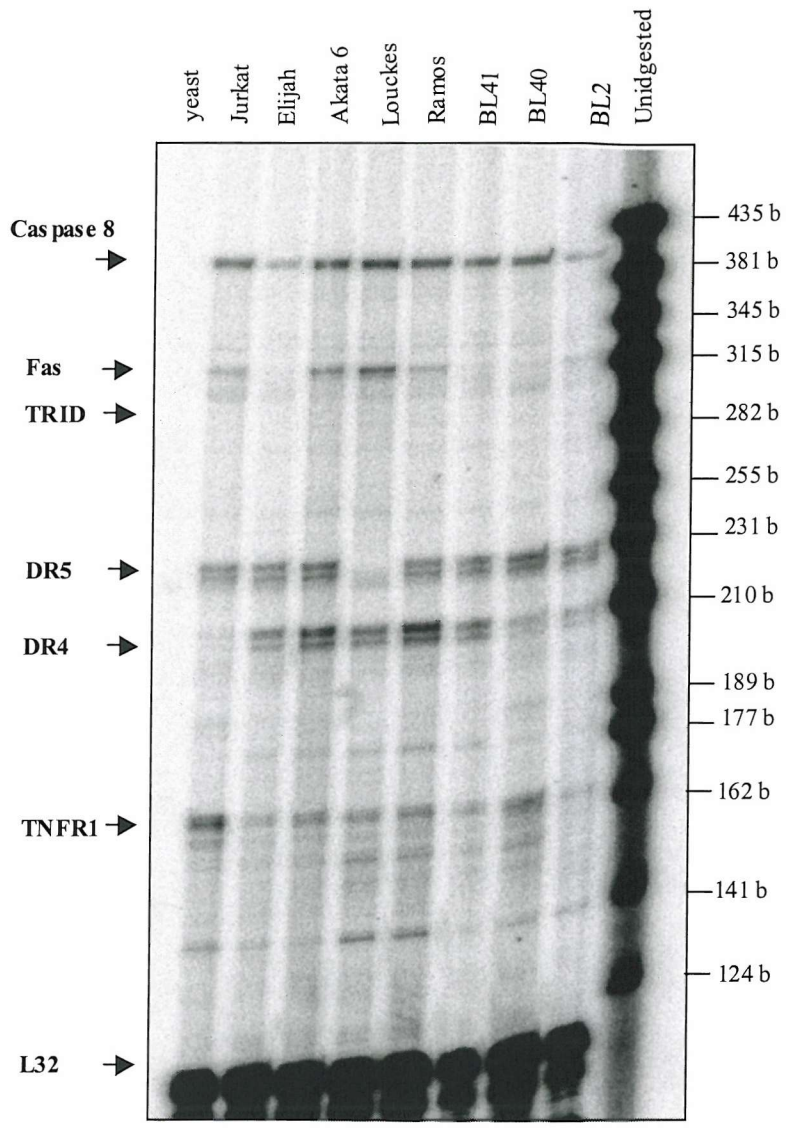


Figure 6.3: *RPA analysis of TRAIL receptors and other apoptosis-related proteins in EBV negative and EBV positive group I BL cell lines.* 10 μg of total RNA were hybridised to multiple ^{32}P-labelled probes transcribed from the same template set and subjected to RNase digestion. Digested probes were resolved on a polyacrylamide gel and undigested probe was used as a molecular weight marker. A probe for the housekeeping gene L32 is included as a loading control and yeast total RNA was also used as a control. Protected probes were visualised using Image Quant software on a STORM phosphoimager.

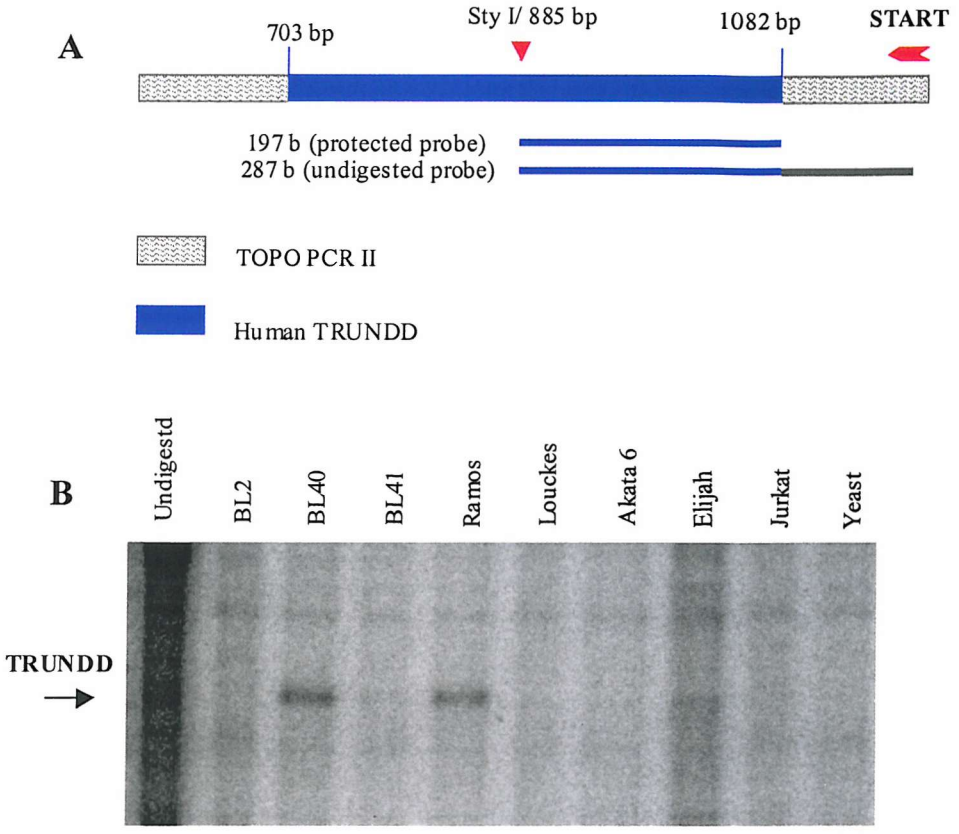


Figure 6.4: *Generation of an antisense riboprobe for TRUNDD and RPA analysis.* **(A)** A TRUNDD riboprobe was generated by an inverse transcription reaction using pTRUNDD as a template. The sizes of the undigested and protected probes are indicated. **(B)** For RPA analysis 10 µg of RNA from each cell line were hybridised with the ^{32}P-labelled riboprobe and subjected to RNase digestion. Digested probes were resolved on a polyacrylamide gel. Undigested probe was also resolved on the same gel as a control. Protected fragments were visualised using the ImageQuant software on a STORM phosphoimager.

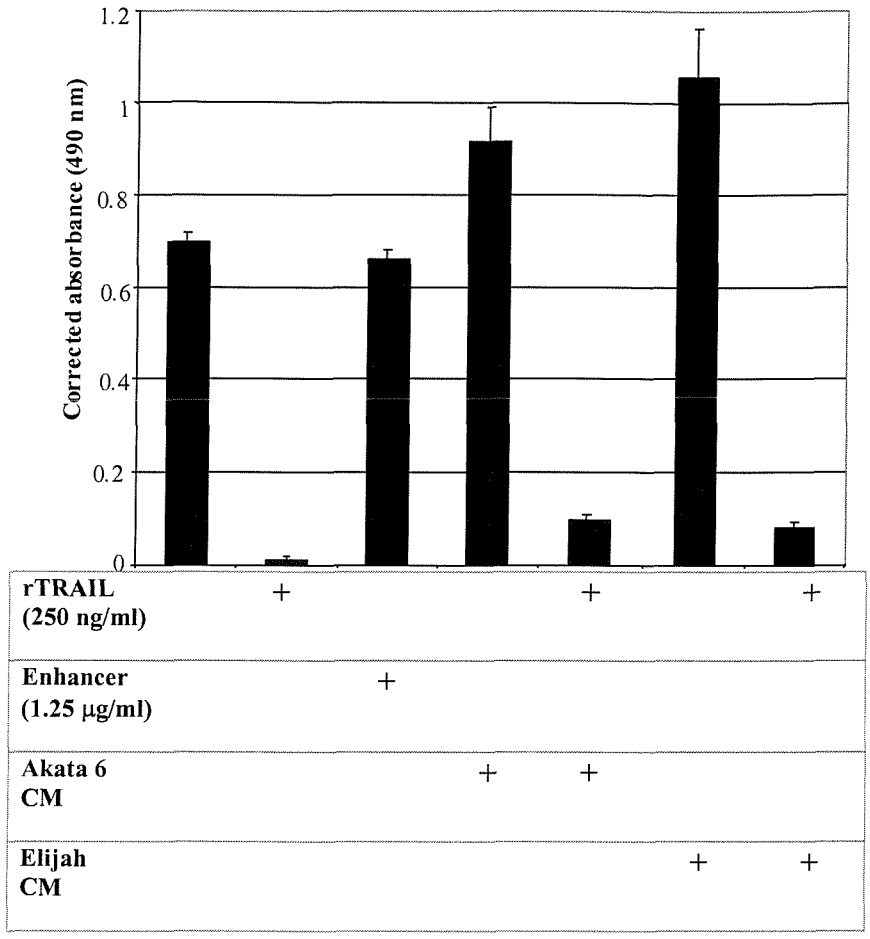


Figure 6.5: *Transfer of conditioned media from Akata 6 and Elijah does not reverse the TRAIL-sensitive phenotype in BL40 cells.* BL40 cells were cultured in the absence or presence of rTRAIL (250 ng/ml) or enhancer only (1.25 µg/ml) in the presence or absence of conditioned media (CM) from Akata 6 and Elijah cells (1:2 dilution). Cell growth was determined after 48 hours by the MTS assay. The values presented here are the means of corrected absorbance values) of triplicate wells +/- standard error of the mean.

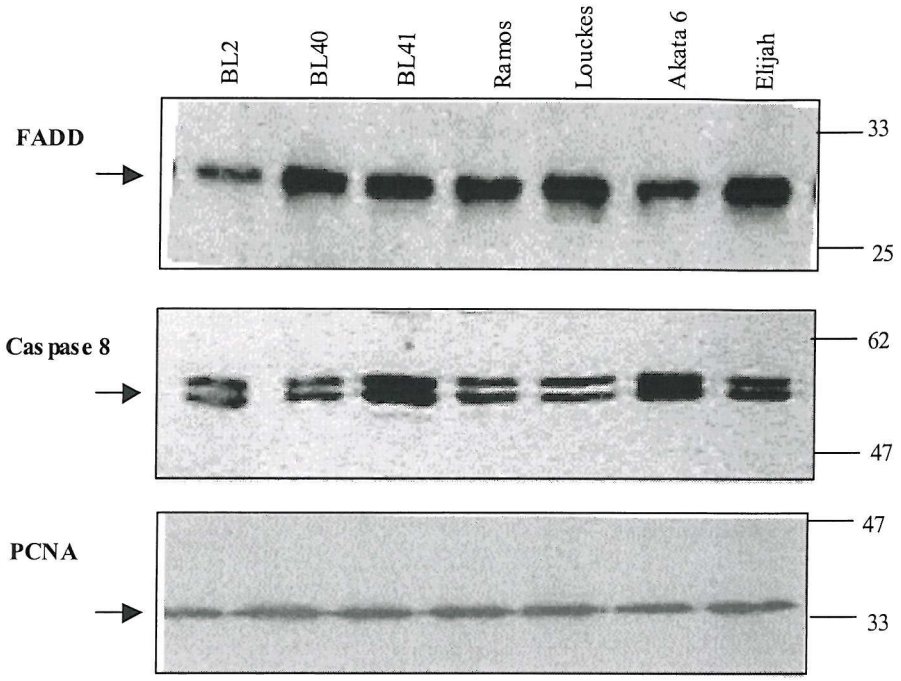


Figure 6.6: *Western blotting analysis of FADD and caspase 8 in EBV negative and EBV positive group I BL cell lines.* 20 μg of protein from each cell line were resolved on 12% polyacrylamide gels and transferred onto nitrocellulose. Filters were probed with a FADD-specific mouse monoclonal antibody and a caspase 8-specific rabbit polyclonal antibody. Filters were also probed with a PCNA-specific antibody as a loading control. The positions of the molecular weight markers are indicated (KDa).

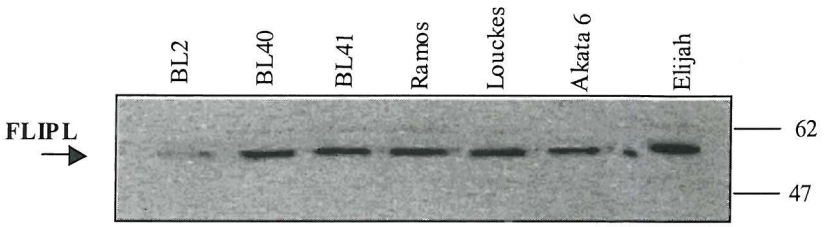


Figure 6.7: *Western blotting analysis of FLIPL in EBV negative and EBV positive group I BL cell lines.* 20 μg of protein from each time point were resolved on a 12% polyacrylamide gel and transferred onto nitrocellulose. Filters were probed with a FLIPL-specific rabbit polyclonal antibody and anti-PCNA antibody as a loading control (data not shown).

6.6 Discussion

Since resistance to rTRAIL-induced apoptosis in the BL cell lines examined in this study occurred independently of TRAIL receptor levels, alternative mechanisms proposed to regulate TRAIL-mediated apoptosis in other cell systems were investigated. This analysis concentrated on two groups of BL cell lines, the EBV negative and EBV positive cells with a group I phenotype because they are phenotypically similar yet display distinct responses to rTRAIL.

EBV and other herpesviruses possess various mechanisms to prevent apoptosis in infected cells thus allowing the establishment and maintenance of latent infection and their survival. However, the role of EBV in the regulation of TRAIL-mediated apoptosis has not yet been investigated. It was possible that EBV-encoded gene products expressed in group I BL cell lines, namely EBNA-1, the EBERs and BamA transcripts might confer resistance. (Ruf et al., 1999) have recently suggested that the type I latency program can contribute to long-term survival and resistance to apoptosis in Akata cells probably by downregulating c-myc. The exact mediators of resistance were not defined, however, a later study suggested that the EBERs may be responsible (Ruf et al., 2000). The authors used EBV negative and positive isogenic Akata clones to investigate the role of EBV. I used a similar cell system to examine the role of EBV in resistance to rTRAIL-induced apoptosis in the group I BL cell lines, Akata 6 and Elijah. However, this examination found no evidence for EBV being a major determinant of rTRAIL resistance. It is possible that the EBV negative Akata clones, may have acquired additional anti-apoptotic mechanisms to allow them to survive the loss of EBV, which may have affected their responses to rTRAIL. The re-infection of an EBV negative clone with EBV (for example the well-characterised strain B95.8) might help elucidate this by allowing the comparison between transfected cells and the parental EBV negative cells for sensitivity to TRAIL.

p53 was not stabilised in response to rTRAIL in BL40 cells. This is suggestive of lack of functional activation, however, specific assays of transcriptional activation would be essential to confirm this, for instance analysis of the well-defined p53 target, p21. Since p53 can also transcriptionally repress targets during a p53-dependent response, e.g. BCL-2, p21 analysis would provide limited information on p53 function. To clarify this a more thorough investigation would be needed. However,

more recent studies have also shown that TRAIL-mediated apoptosis is p53-independent (Kim et al., 2000b). In addition, another report has demonstrated that p53 responses in the majority of BL cell lines are deregulated despite the expression of wild type p53. The disruption is due to alterations in two components of p53 pathways, MDM2 and p14ARF (Lindstrom et al., 2001). Taken together, the evidence indicates that p53 is unlikely to be a major determinant of sensitivity to rTRAIL in the EBV negative BL cells.

Although the available evidence for the biological function of the decoy receptors and their role in TRAIL-mediated apoptosis is incomplete, decoy receptor expression has correlated with TRAIL resistance in some cell systems (Zhang et al., 2000a) but not in others (Kim et al., 2000b), (Zhang et al., 1999). The TRAIL/TRAILR system is now emerging as a highly complex and tightly regulated system in contrast to initial hypotheses. To begin to investigate its regulation by decoy receptors, I determined the expression of TRID and TRUNDD transcripts by RPAs. Previous studies used PCR to analyse decoy receptor expression, but I chose RPA assays. RPAs are sensitive assays but also quantitative and thus more informative. Although DR4 and DR5 transcripts were easily detected by a multi-probe RPA analysis, TRID expression was undetectable in the cells examined. Furthermore, TRUNDD mRNA expression was restricted in two TRAIL-sensitive cell lines, BL40 and Ramos, thus showing no correlation with the observed pattern of sensitivity to rTRAIL-induced apoptosis. Generally, the low levels of decoy receptors in both EBV negative and EBV positive group I cell lines are in marked contrast with the abundant expression of the pro-apoptotic TRAIL receptors. It is therefore unlikely that they would have a significant functional impact in this system if expressed at such low levels. An additional level of regulation of TRAIL-mediated apoptosis is provided by the soluble TRAIL receptor, OPG, which has a low affinity for the ligand but its overexpression can inhibit apoptosis in Jurkat cells (Emery et al., 1998). However, media transfer experiments, carried out to investigate the role of OPG or any other as yet unidentified soluble TRAIL receptors did not provide evidence for such a mechanism in BL cell lines.

Therefore, it is possible that intracellular inhibitory mechanisms may have been responsible for the attenuation of TRAIL-mediated apoptosis in Akata 6 and Elijah cells. Defects in caspase 8 and/or FADD or the overexpression of FLIPL may have played a role. This was indicated by *in vivo* studies with knockout mice and *in vitro*

analyses, which have shown an essential and non-redundant role for caspase 8 in TRAIL-mediated apoptosis. Furthermore, transcriptional repression of caspase 8 expression by promoter methylation correlated with resistance of neuroblastoma cells to TRAIL (Eggert et al., 2001). DN-FADD also inhibited TRAIL-mediated apoptosis and treatment of TRAIL-resistant melanoma cells with protein synthesis inhibitors, such as cycloheximide resulted in conversion to a TRAIL-sensitive phenotype (Griffith et al., 1998), indicative of an inhibitor with a short half-life. High levels of c-FLIP correlated with resistance to TRAIL in tumour cell lines (Kim et al., 2000b). However, FADD, caspase 8 and FLIPL were detected in both TRAIL sensitive as well as resistant cells with only modest variations (with the exception of BL2 cells) and so did not correlate with resistance.

The evidence from these analyses indicates that novel regulatory mechanisms may be controlling responsiveness to rTRAIL in BL cell lines. For instance, activation of survival-promoting proteins such as NF-κB, in response to TRAIL signalling is a currently emerging regulatory mechanism that warrants investigation. In addition, examination of the alternative isoform of c- FLIP, FLIPS, recently identified in genetic screens as an inhibitor of TRAIL signalling, may also be informative and is now possible with the arrival of FLIPS-specific antibodies (Bin et al., 2002).

Chapter 7. Spontaneous and death receptor-mediated apoptosis in *ex vivo* CLL cells

7.1 Introduction

The investigation of the function of Fas and TRAIL receptor-activated pathways in BL cell lines and LCLs demonstrated a differential sensitivity to death receptor signalling among phenotypically similar groups of cells. Moreover, there was no correlation with the expression of known inhibitors of Fas and/or TRAIL-mediated apoptosis in the BL cell lines where resistance was receptor independent. Therefore, the molecular mechanisms controlling sensitivity to death receptor signalling in BL could not be defined, but it was evident that resistance to death receptor-induced apoptosis is not an inherent feature of B lymphocytes. Since clonal variations resulting from prolonged *in vitro* culture may have introduced novel mechanisms of resistance in the cell lines tested, it was important to compare death receptor-mediated apoptosis between BL cell lines and primary B cell malignancies. B cells derived from BL biopsies would make an ideal system for this analysis; however, obtaining such specimens is not practical due to the rarity of BL (particularly EBV positive BL) in this country. Therefore the study of death receptor-mediated cell death was extended to a different B cell malignancy, B cell chronic lymphocytic leukaemia. CLL is the most common leukaemia among adults in the western world and importantly, it is a suitable model of cancer where deregulation of apoptosis plays a major part in tumourigenesis. In addition, acquiring CLL patient samples was feasible due to an ongoing study in this laboratory. Furthermore, CLL and BL cell lines (or at least certain subsets) share certain features such as their normal equivalent cell types, i.e. germinal centre B cells as well as some molecular features (e.g. high levels of MCL-1 and BCL-2).

However, in contrast to BL, CLL is a low-grade malignancy with a characteristic accumulation of small non-dividing B lymphocytes. Intriguingly, CLL can progress with two strikingly different clinical courses (indolent or highly aggressive) and is currently incurable. Although some CLL cases have been shown to respond well to treatment with fludarabine, relapse is a frequent event and therefore new treatments are needed and are being investigated (Keating et al., 1998). The

identification of a new genetic marker, the presence of somatic mutations in the variable region of the heavy chain gene, has demonstrated that CLL comprises two disease entities; one with a naïve and the other with a post-germinal centre B cell phenotype. This molecular dichotomy is clinically relevant as the median survival of CLL patients of the naïve B cell type is substantially shorter than the survival of CLL patients of the memory B cell phenotype (Hamblin et al., 1999). This discovery has attracted attention for its clinical use as a prognostic marker to help differentiate between disease subsets, facilitate the decision on the timing and thus help achieve a more efficient course of treatment.

The current knowledge on the regulation of cell growth and death in CLL is incomplete but like in normal B cells, the BCR and CD40 influence malignant cell survival. CD40 ligation can direct the T cell-mediated rescue of germinal centre B cells (van Essen et al., 1995) as well as enhance proliferation, differentiation into antibody-secreting cells and isotype switching. Currently attention is focused on CD40L (CD154)-based gene therapies for CLL, which have produced promising results in Phase I clinical trials (Wierda et al., 2000), (Vonderheide et al., 2001). Although the mechanism responsible for the reduction in tumour burden was not defined it is possible that upregulation of Fas made cells susceptible to T cell cytotoxicity. However, previous quantitative and qualitative analyses of Fas function in CLL have provided conflicting results.

Resting B cells express no or low levels of Fas and are resistant to Fas-mediated apoptosis but after activation by various signals including anti-IgM antibodies, IL-4, and CD40 ligation, Fas is induced and the B cells become sensitive (Daniel and Krammer, 1994), (Schattner et al., 1995), (Foote et al., 1998). Fas expression was detected on a minority of *ex vivo* CLL B cells, which displayed an intrinsic resistance to agonistic stimulation. Although Fas levels rose upon CD40L stimulation, the resistant phenotype was altered in only a small percentage of cells (Wang et al., 1997). Similar results were reported elsewhere (Panayiotidis et al., 1995), (Buhmann et al., 1999). In another study, only type I cytokines such as IL-12 or IFN-α were able to increase Fas expression and biological function (Williams et al., 1999). Finally a recent study reported transient resistance to Fas-induced apoptosis shortly after CD40 ligation followed by increasing responsiveness after 72 hours (Chu et al., 2002). Importantly, the biological function of Fas in the two recently identified disease subsets has not yet been analysed and it may be of clinical significance.

Furthermore, the function of the TRAIL pathway in CLL is only now beginning to be investigated, therefore the examination of TRAIL-mediated apoptosis in CLL is important as it may also have clinical implications.

The VH status of CLL cells is probably a reflection of different pathogenic mechanisms involved in the development of this malignancy. It is conceivable that the molecular differences are the result of distinct molecular events, which could involve the death receptors. Fas, for instance, may play a role in B cell selection in the GC (Takahashi et al., 2001; van Eijk et al., 2001). Therefore, the investigation of the susceptibility of the two distinct CLL subsets to death receptor-mediated apoptosis would be of interest in extending our understanding of the underlying mechanisms leading to the two clinical courses but may also be of therapeutic potential in identifying intact apoptotic pathways that could be exploited for treatment. Furthermore, as CLL is currently incurable novel treatments are required and death receptor pathways may provide the basis for such treatments.

An ongoing study in this laboratory has characterised a large number of CLL patient samples with respect to the mutational status of the immunoglobulin (Ig) heavy chain variable region genes (VH). VH gene sequences amplified from patient lymphocytes were analysed from cDNA with direct sequencing and assessment of somatic mutations was made by alignment with germ line sequences available in EMBL/GenBank and VBASE. Based on sequence homology, the samples were divided into two categories: 'mutated' and 'unmutated'. Unmutated cases were defined as those with more than 98% homology to the closest germ line gene. Mutated cases were defined as those in which the B cells displayed less than 98% homology to the closest homologue, as was described previously (Hamblin et al., 1999). I investigated Fas and TRAIL-mediated apoptosis in CLL B lymphocytes from 5 patients with unmutated VH genes and six patients carrying VH gene mutations. The effect of CD40L on induced and spontaneous apoptosis was investigated in parallel. All samples were CD19 positive B cells co-expressing CD5, and CD23 at the time of initial diagnosis.

7.2 Examination of spontaneous apoptosis and the effect of CD40L in CLL patient samples

High rates of spontaneous apoptosis have been reported in *ex vivo* CLL cells indicating that the malignant cells are highly dependent on *in vivo* signals for survival (Younes et al., 1998). In order to investigate the responsiveness of CLL cells to Fas and TRAIL-induced apoptosis, it was important to determine the rates of spontaneous apoptosis, since this would impact on cytotoxicity assays. For these analyses, mononuclear cells were isolated from peripheral blood of CLL patients by centrifugation on a density gradient and used immediately in the assays. At the start of the experiment, the viability of CLL cells was determined by FACS analysis of PI exclusion and surface expression of CD40 was also determined by flow cytometry.

CLL cells were also incubated in the absence or presence of trimeric CD40L (1 μg/ml) or an agonistic CD40-specific antibody that activates the receptor (α-CD40) (5 μg/ml). α-CD40 was used before CD40L became available. After 48 hours in culture, both stimulated and unstimulated samples were harvested and cell viability as well as surface expression of Fas was determined by FACS analysis. In parallel with this, the cells were plated in 96-well plates and treated with rTRAIL (500 ng/ml) or α-Fas (500 ng/ml) in the absence or presence of CD40L or α-CD40 as above. Cell growth was measured after 48 hours using the MTS assay. FACS analysis of PI exclusion was used to measure cell viability and is based on a principle similar to trypan blue exclusion. Live cells with intact membranes do not take up the dye and show low levels of fluorescence. By contrast, cells whose membrane integrity has been compromised and are dead are permeable to the dye and display high levels of fluorescence. Thus, the numbers of dead cells at 48 hours were used to arbitrarily define CLL cells as susceptible to spontaneous apoptosis if they exceeded the numbers of dead cells at the start of the experiment by 25% or more. Similarly, CD40 ligation was arbitrarily considered to 'rescue' cells from spontaneous apoptosis, if CD40L or α-CD40 reduced cell death by 25% or more (compared to untreated cells).

Maintenance of CLL cells *in vitro* resulted in spontaneous apoptosis in the majority of samples (82%) irrespective of their mutation status. By contrast, a single CLL sample from each group (mutated and unmutated) was unaffected. CD40 ligation resulted in decreased rates of spontaneous apoptosis in the majority of CLL cells tested

(70%) consistent with previous reports. Interestingly, CD40 ligation resulted in rescue of all unmutated samples otherwise susceptible to spontaneous apoptosis whereas in the mutated group, 2 out of 4 CLL samples were rescued. Treatment with α-CD40 also rescued one of three tested samples. The enhanced survival of CD40L-treated cells indicates that CD40 signals can enhance the viability CLL cells *in vitro* and may be one of the factors contributing to the extended life span of CLL cells *in vivo*. Representative FACS profiles of PI exclusion are shown in Figure 7.1 and the results from the viability assays are summarised in Table 7.1.

Patient	**VH status**	**Spontaneous apoptosis *in vitro***	**Rescue by CD40L**
1	Unmutated	+	+
2*	Unmutated	+	+
3	Unmutated	-	-
4	Unmutated	+	+
19	Unmutated	+	+
10*	Mutated	+	-
11	Mutated	+	NA
5	Mutated	+	+
6*	Mutated	+	-
7	Mutated	+	+
8	Mutated	-	-

Table 7.1: *Spontaneous apoptosis in ex vivo CLL cells and rescue by CD40L.* 'Mutated' and 'unmutated' CLL patient samples were maintained *in vitro* and the rates of spontaneous apoptosis were measured by FACS analysis of PI exclusion (* indicates the use of α-CD40 instead of CD40L, NA/ not analysed).

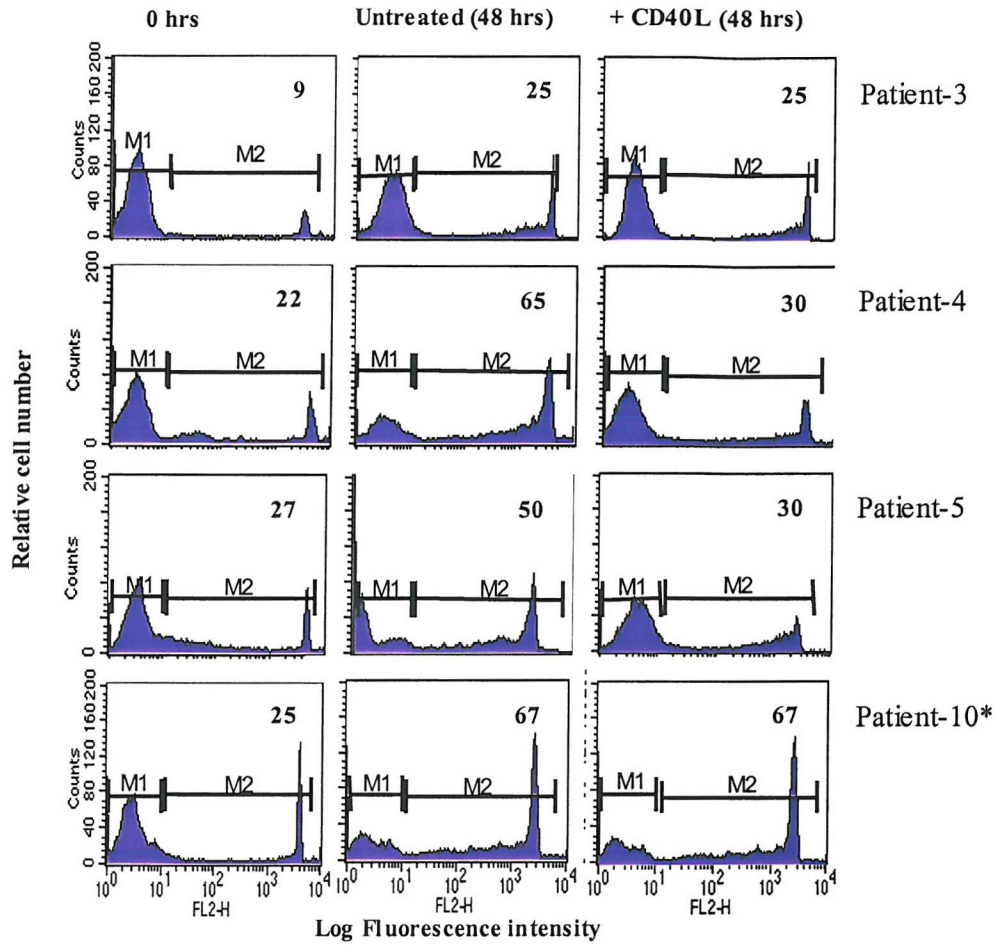


Figure 7.1: *Spontaneous apoptosis in CLL patient samples and rescue by CD40L.* CLL cells were maintained *in vitro* in the absence or presence of CD40L (1 μg/ml) or α-CD40 antibody (5 μg/ml) (indicated by *) or left untreated as controls. Viability was determined at 0 and 48 hours by FACS analysis of PI exclusion. 10000 events were acquired from each sample and analysed using CellQuest software with the M1 and M2 markers denoting areas of live and dead cells respectively as used in statistical analyses (the percentage of dead cells for each treatment is indicated in the upper right hand corner of each histogram).

7.3 Constitutive and induced expression of Fas on CLL patient samples

CLL cells constitutively express low levels of Fas and treatment with CD40L frequently upregulates it. However, the upregulation of Fas however is not predictive of biological responsiveness (Younes et al., 1998), (Wang et al., 1997), (Chu et al., 2002). Prior to determining the responsiveness of CLL cells to α-Fas, I investigated the constitutive and CD40L-induced levels of Fas on CLL patient samples with or without VH gene mutations. To confirm the specificity of CD40L, I also examined the expression of CD40 on the malignant cells by indirect immunofluorescence staining and FACS analysis. Jurkat or IB4 cells and the BL cell line Akata 6 were used as positive controls for Fas and CD40 expression respectively in each analysis (data not shown). Cells were arbitrarily defined as positive for CD40 or Fas expression if the corresponding RFI values were higher than 1.5. Fas-positive cells were further divided into two groups according to these values: RFI values below 3 were defined as corresponding to low levels whereas RFI values in excess of 3 were defined as corresponding to high levels of Fas.

CD40 expression is a common feature of B cells including CLL cells (Buhmann et al., 1999) and this was confirmed here as all CLL samples tested were CD40 positive. On the other hand, expression of Fas was restricted; 4 out of 11 CLL samples displayed low levels of Fas (Table 7.2 and Figures 7.2, 7.3). Following stimulation via CD40, the pre-existing levels of Fas increased and previously Fas-negative CLL cells became positive. CD40L treatment resulted in upregulation of Fas in 7 out of 11 samples (65%); this included three out of five (60%) unmutated and four out of six (67%) mutated samples. α-CD40 antibody also induced Fas expression in two out of three tested samples. However, Fas expression on CLL cells was consistently lower compared to Jurkat or IB4 cells analysed as positive controls (data not shown). Furthermore, following the 48-hour incubation period, the numbers of cells binding antibody in a non-specific manner increased probably due to extensive spontaneous apoptosis in these cells resulting in loss of membrane integrity (Figures 7.2 and 7.3, upper left regions of each plot).

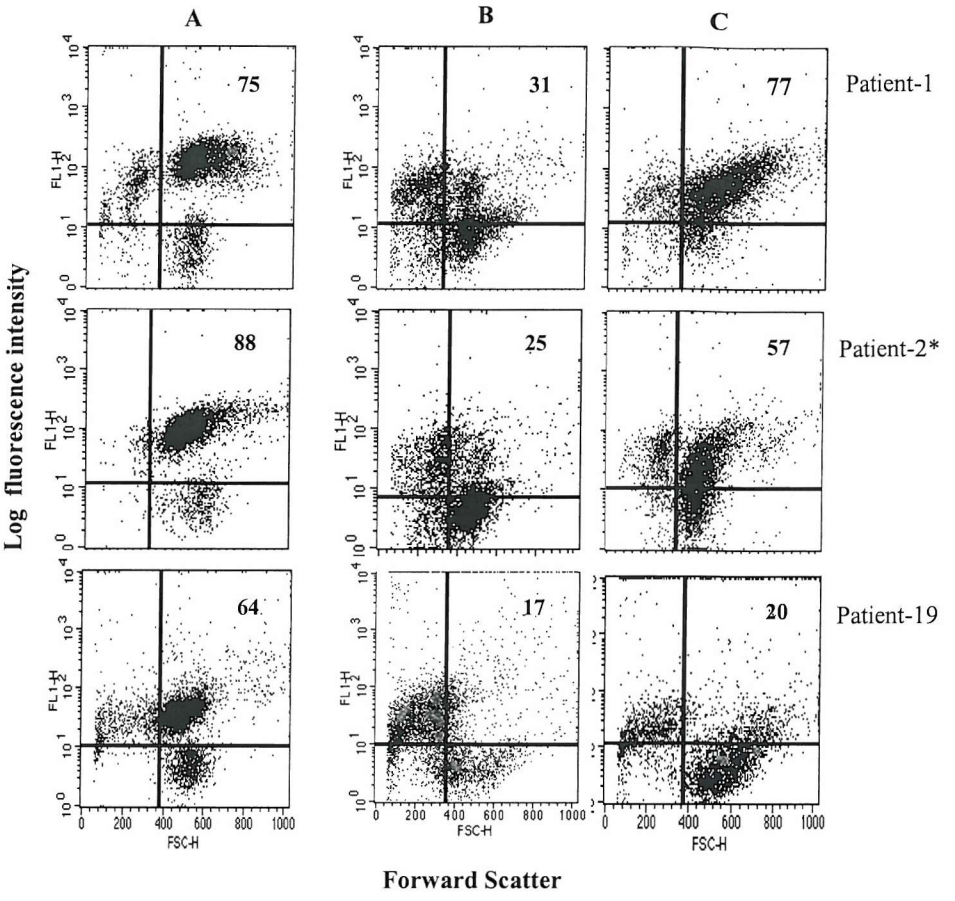


Figure 7.2: *Representative FACS profiles of CD40 and Fas expression on CLL patient samples with unmutated Ig VH genes.* **(A)** CD40 surface expression was determined at 0 hours. Following this cells were maintained *in vitro* in the absence **(B)** or presence **(C)** of CD40L (1 μg/ml) or α-CD40 antibody (5 μg/ml) (denoted by *) for 48 hours before analysis of Fas expression. CD40 and Fas surface expression were measured by indirect immunofluorescent staining and FACS analysis using CD40- and Fas-specific mouse monoclonal antibodies. Cells were also stained with an isotype-matched antibodies as controls for non-specific staining. 10000 cells/sample were acquired and analysis was performed using CellQuest software. Percentages of CD40 and Fas-positive cells are shown in the upper right hand corners.

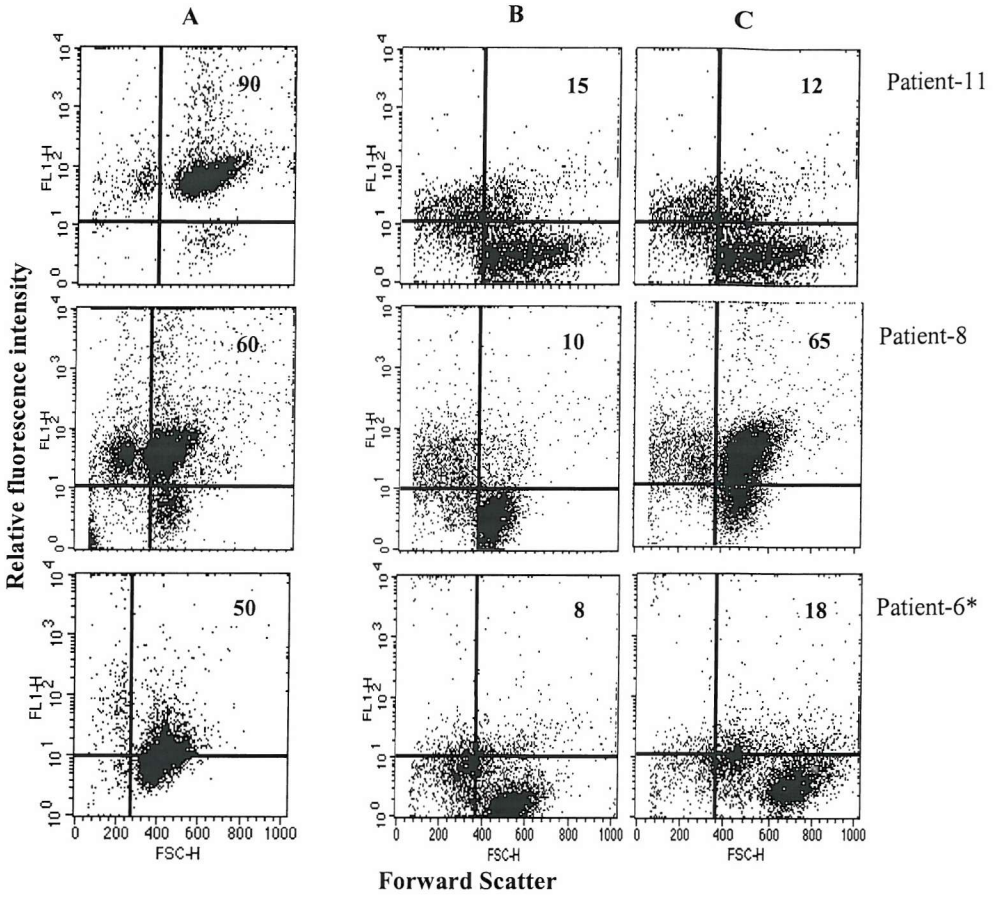


Figure 7.3: *Representative FACS profiles of CD40 and Fas surface expression in CLL patient samples with mutated VH genes.* **(A)** CD40 expression was determined at 0 hours. Cells were also maintained *in vitro* in the absence **(B)** or presence **(C)** of CD40L (1 μg/ml) or α-CD40 antibody (5 μg/ml) (denoted by *) for 48 hours prior to the analysis of Fas expression. CD40 and Fas surface expression were measured by indirect immunofluorescent staining and FACS analysis using CD40- and Fas-specific mouse monoclonal antibodies. Cells were also stained with isotype-matched antibodies as controls for non-specific staining. 10000 cells/sample were acquired and analysis was performed using CellQuest software. The percentages of CD40 or Fas-positive cells are indicated in the upper right hand corners.

Patient	CD40 expression	Constitutive expression of Fas	Fas expression after stimulation with CD40L
1	+	L	H
2*	+	L	H
3	NA	L	-
4	+	-	H
19	+	-	-
10*	+	-	-
11	+	-	-
5	+	-	L
6*	+	-	L
7	+	L	H
8	+	-	H

Table 7.2: *CD40 and Fas surface expression in CLL patient samples.* CD40 and Fas expression was measured by FACS analysis and the RFI values were used to determine expression levels (H/ high, L/low, NA/not analysed, * indicates the use of α-CD40).

7.4 Analysis of CLL cell sensitivity to Fas and TRAIL-induced apoptosis

To extend the current understanding of the responsiveness to death receptor-mediated apoptosis in CLL and in the two disease subsets in particular, I determined the sensitivity of CLL patient samples to α-Fas and rTRAIL-induced apoptosis using MTS assays. The biological effect of CD40L-induced upregulation of Fas was also examined. Since there was no evidence for an interaction between the TRAILR and CD40-triggered pathways, the effect of the combination of rTRAIL and CD40L treatments was not investigated. α-Fas and TRAIL-sensitive Jurkat cells were included in these assays as positive controls. In experiments parallel to the ones described here, the effect of CD40L on CLL cell growth was analysed by S. Langham as part of an ongoing study using the same experimental methods and MTS assays for measurement of cell growth. According to these analyses, CD40L treatment enhanced cell growth in

the majority of samples tested to variable degrees, although never in excess of 100% relevant to untreated cells.

CLL cells were considered sensitive to α-Fas if the growth reduction caused by α-Fas exceeded the growth reduction (due to spontaneous apoptosis) in untreated cells by 25%. The cells were also incubated with IgM antibody as a control for α-Fas, but contrary to the previous observation in BL cell lines, IgM treatment did not affect CLL cell growth (data not shown). Agonistic stimulation with α-Fas inhibited cell growth in more than 60% of CLL samples including mutated (80%) and unmutated (50%) samples. Simultaneous treatment with CD40L or α-CD40 did not have an effect on cell sensitivity to α-Fas with the exception of one sample where CD40L reversed the Fas sensitive phenotype (Patient 19) (Table 7.3). Thus consistently with some previous studies CD40 ligation did not significantly alter CLL cell responses to α-Fas. However, a significant proportion of CLL cells were constitutively sensitive to Fas-induced cell death in this study. Interestingly, α-Fas killed 3 CLL samples identified as Fas negative by FACS analysis (Patient-19, 5 and 6), indicating that low levels of Fas (below the threshold value of 1.5) are perhaps sufficient to induce cell death in a responsive cell line. Furthermore, CD40L-induced upregulation of Fas on the cell surface did not reflect in the cells' sensitivity to the biological effects of α-Fas. Therefore, the control of sensitivity to Fas-mediated apoptosis was independent of receptor levels in certain CLL patient samples.

To examine CLL cell sensitivity to TRAIL-mediated apoptosis, cells were treated with rTRAIL (500 ng/ml) or left untreated as a control and cell growth was measured by the MTS assay. Surprisingly rTRAIL had no effect on CLL cell growth even though the growth reduction in Jurkat cells consistently exceeded 60% (compared to controls) (Figure 7.5 and Table 7.3 and data not shown). Unexpectedly, CLL cells that responded to α-Fas were resistant to rTRAIL indicating that CLL cells may lack functional TRAIL receptors or that they express inhibitors specific for the TRAIL pathway. Unfortunately not enough patient samples were available for FACS analyses of TRAILR expression at the time therefore this was not determined.

Finally, it should be noted that CLL cell growth rates (as indicated by absorbance values), were consistently lower compared to Jurkat cells analysed in parallel, despite the fact that CLL cell numbers in each well were doubled. Low

absorbance values were probably a reflection of the resting phenotype of the malignant B cells.

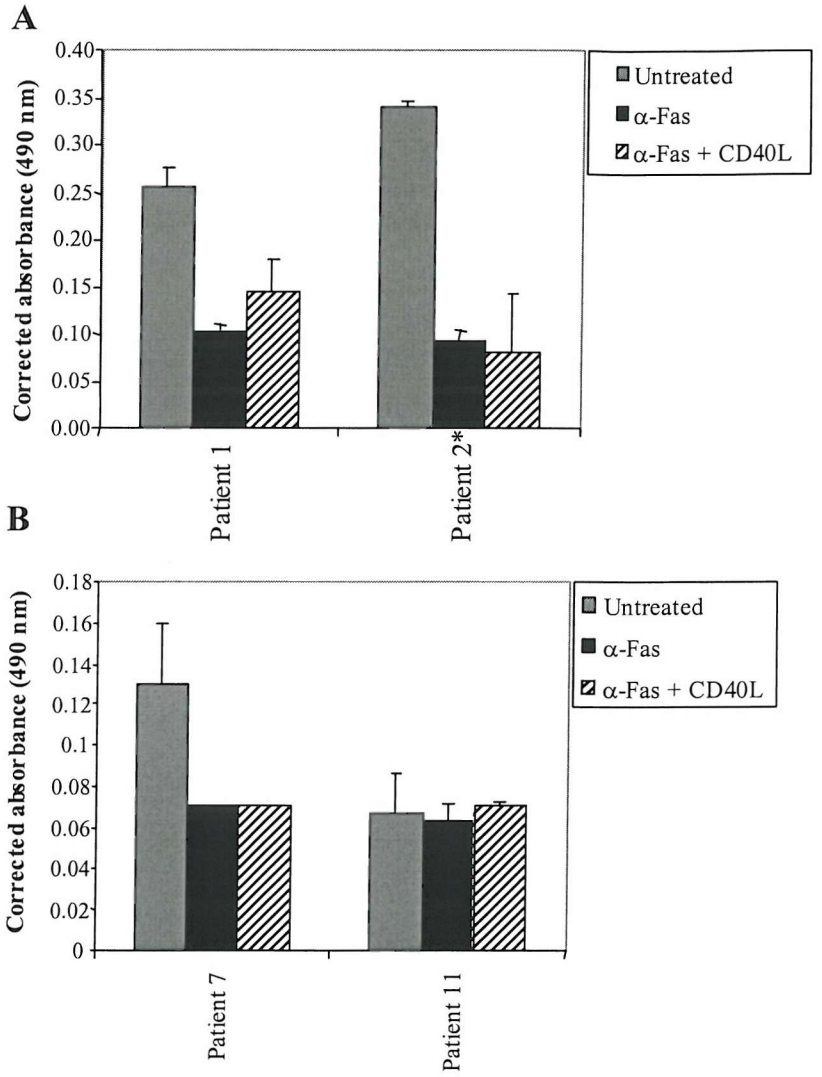


Figure 7.4: *a-Fas-mediated growth inhibition of CLL cells. U*nmutated **(A)** or mutated **(B)** CLL patient samples were treated with α-Fas (500 ng/ml) in the absence or presence of CD40L (1 μg/ml) of α-CD40 (5 μg/ml) (denoted by *) or left untreated for 48 hours. Cell growth was measured by the MTS assay. Data points represent the average of corrected absorbance values of duplicate wells +/- standard error. Some errors are too small to show.

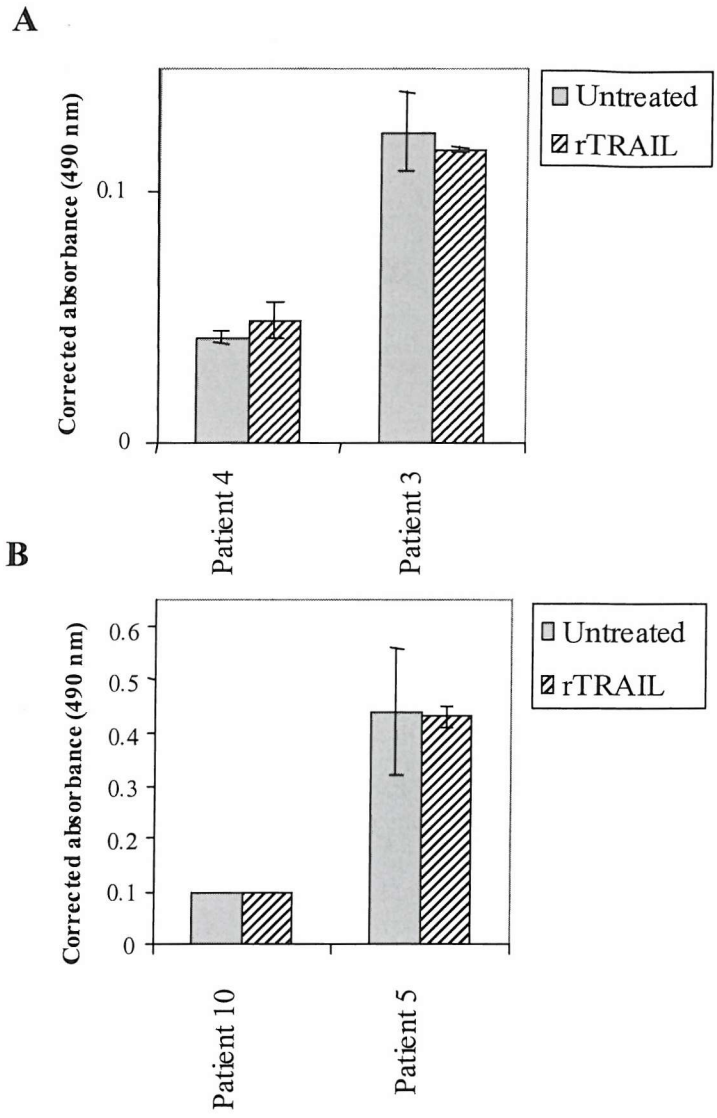


Figure 7.5: *rTRAIL-mediated growth inhibition of CLL cells.* Unmutated **(A)** or mutated **(B)** CLL patient samples were treated with rTRAIL (500 ng/ml) or left untreated for 48 hours. Cell growth was measured by the MTS assay. Data points represent the average of corrected absorbance values of duplicate wells +/- standard error. Some errors are too small to show.

Patient	Growth reduction (α-Fas)	Growth reduction (α-Fas + CD40L)	Growth reduction (rTRAIL)
1	+	+	NA
2*	+	+	NA
3	+	+	-
4	-	-	-
19	+	-	-
10*	-	-	-
11	-	-	NA
5	+	+	-
6*	+	+	-
7	+	+	NA
8	-	-	-

Table 7.3: *Sensitivity to rTRAIL and α-Fas-induced cell death and the effect of CD40L in CLL patient samples.* CLL cells were treated with α-Fas with or without CD40L (or α-CD40, indicated by *) or rTRAIL and cell growth was measured by the MTS assay (NA/ not analysed).

	Mutated (%)	Unmutated (%)
Spontaneous apoptosis	84	80
Rescue by CD40L	40	100
Constitutive Fas expression	67	60
Fas expression after CD40L stimulation	17	60
Sensitivity to α-Fas-induced apoptosis	80	50
Sensitivity to α-Fas after CD40L stimulation	60	50
Sensitivity to rTRAIL-induced apoptosis	0	0

Table 7.4: *Summary of sensitivities to spontaneous and induced apoptosis in 11 CLL patient samples.* The percentages of responsive cells in each subset of CLL (mutated and unmutated) are shown. Fas expression is also shown.

7.5 Expression of TRAIL receptors and other molecular determinants of apoptotic sensitivity in CLL patient samples

Resistance to α-Fas-induced cell death was receptor independent in some CLL patient samples. In addition, the entire panel of cells tested were resistant to rTRAIL. To begin to investigate the molecular determinants that control death receptor-induced cell death in CLL, I examined the expression of FADD and caspase 8 as well as BCL-2 in a small number of samples. DR4 and DR5 expression was also investigated by western blotting. Unfortunately, not all patient samples could be examined due to restricted availability. IB4 cells were used as a positive control for BCL-2, FADD and caspase 8 analysis whereas rhFc:DR4 and rhFc:DR5 were used to confirm the specificity of the antibodies used in analyses of DR4 and DR5 expression.

FADD was easily detectable in the CLL samples tested whereas the expression of caspase 8 was more variable (Figure 7.6). One CLL sample (Patient 17) completely lacked caspase 8 whereas low levels of the protein were detected in two more samples (Patient 5 and Patient 8). Between these two samples, one (Patient 5) responded to α-Fas in the MTS assays whereas the other (Patient 8) was resistant. The difference in caspase 8 levels was not due to protein loading as indicated by Ponceau staining of the nitrocellulose filters but also by the signals from BCL-2 and DR5 blots (Figure 7.7). Analysis of PCNA, used as loading control in BL cell lines, was not appropriate for CLL cells as this antigen is expressed in actively proliferating cells, whereas CLL cells are predominantly arrested in the G_0 phase of the cell cycle. CLL cells are known to overexpress BCL-2 (Kitada et al., 1998) and this was confirmed here as BCL-2 expression levels in the majority of samples examined were higher compared to IB4 cells where BCL-2 expression is controlled by EBV (Finke et al., 1992). BCL-2 was expressed at similar levels in most CLL patient samples with few exceptions (Patients 1 and 2).

To investigate the basis of resistance to rTRAIL, I examined DR4 and DR5 expression. Due to the limited availability of CLL samples, the nitrocellulose filters were initially probed with the DR5-specific antibody and re-probed without stripping with the DR4-specific antibody. The signals from the recombinant receptor controls confirmed that there was no cross-reactivity associated with the antibodies used or at least the anti-DR5 antibody did not cross-react with DR4 (Figure 7.7). Since the antibodies were produced in the same host, the signal from DR5 was also detected

when the filters were probed with the anti-DR4 antibody. However, the size difference between DR4 and DR5 facilitated the interpretation of the results. DR5 was detected in all cells tested. Re-probing showed a higher molecular weight band corresponding to DR4, with a more restricted expression pattern, expressed in Patient 1, 4 and 3. Since the CLL patient samples tested expressed at least one pro-apoptotic TRAIL receptor, the resistant phenotype of CLL patient samples appears to be receptor-independent. FACS analysis of surface expression would be needed to confirm this. In addition, there was no correlation between responsiveness to Fas and/or TRAIL-induced apoptosis and expression of FADD, caspase 8 and/or BCL-2 in CLL patient samples.

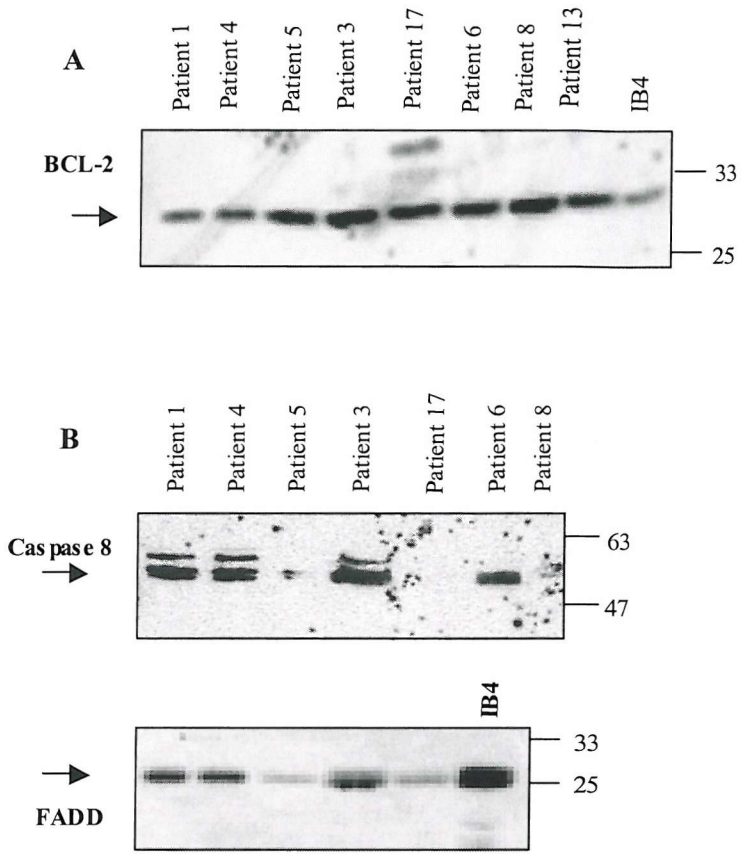


Figure 7.6: *Western blotting analysis of BCL-2, FADD and caspase 8 in CLL patient samples.* 40 µg of protein from each patient sample and IB4 cells (used as controls) were resolved on 12% polyacrylamide gels and transferred onto nitrocellulose. Filters were probed with BCL-2 and FADD-specific mouse monoclonal and caspase 8-specific rabbit polyclonal antibodies. Filters were also stained with Ponceau solution as a loading control. The position of the molecular weight markers is indicated (KDa).

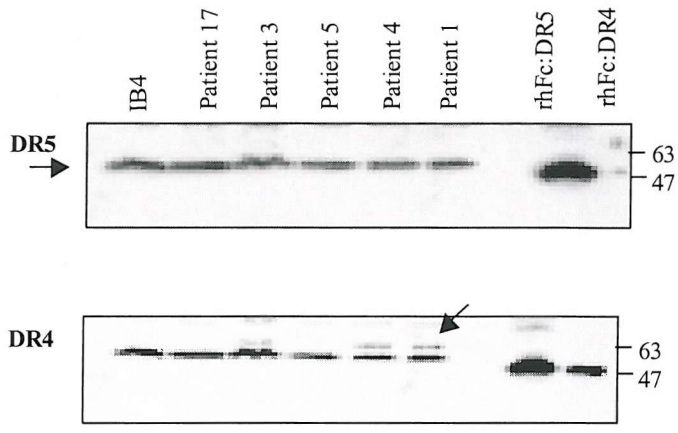


Figure 7.7: *Western blotting analysis of TRAIL signalling receptors in CLL patient samples.* 40 μg of protein from IB4 cells and each CLL sample and 50 ng of recombinant proteins (rhFc:DR4 and rhFc:DR5) were resolved on 10% polyacrylamide gels and transferred onto nitrocellulose. Filters were probed with DR4 and DR5-specific goat polyclonal antibodies. The filters were also stained with Ponceau solution to estimate loading. The position of the molecular weight markers is indicated (KDa)

VH status	Patient	Spontaneous apoptosis	Rescue by CD40L	Fas expression	CD40L-induced Fas expression	Cell death (α-Fas)	Cell death (α-Fas + CD40L)	Cell death (rTRAIL)
Unmutated	1	+	+	L	H	+	+	NA
	2*	+	+	L	H	+	+	NA
	3	-	-	L	-	+	+	-
	4	+	+	-	H	-	-	-
	19	+	+	-	-	+	-	-
	10*	+	-	-	-	-	-	-
	11	+	NA	-	-	-	-	NA
Mutated	5	+	+	-	L	+	+	-
	6*	+	-	-	L	+	+	-
	7	+	+	L	H	+	+	NA
	8	-	-	-	H	-	-	-

Table 7.5: *Summary of the results of cell growth assays in CLL cells.* The VH gene mutation and Fas expression status is shown (L/ low level, H/high level, NA/not analysed, * indicates the use of α-CD40)

7.6 Discussion

To investigate responsiveness to Fas and TRAIL-mediated apoptosis in CLL, I examined the sensitivity of 11 CLL patient samples to α-Fas and rTRAIL. CD40 signals provide an additional stimulus that modulates the growth, differentiation and apoptosis of B cells *in vivo*. CD40 also regulates Fas sensitivity in CLL cells. I therefore investigated the effect of CD40 ligation on susceptibility to spontaneous apoptosis as well as sensitivity to α-Fas in CLL cells. Although CLL cells are characterised by prolonged survival *in vivo*, they die rapidly in culture. Thus, significant rates of spontaneous apoptosis were observed in *ex vivo* CLL cells following a 48-hour culture period. The susceptibility to spontaneous apoptosis indicates that survival signals from the malignant cell microenvironment may be controlling their prolonged survival *in vivo*.

In this study, CD40L or agonistic α-CD40 antibody substantially reduced the levels of spontaneous apoptosis in 60% of the CLL cells tested. However, it failed to rescue a significant number of cells (37%) indicating that although it can provide a strong survival signal *in vitro* and perhaps *in vivo*, it is not a 'universal' survival stimulus. The reason may be that the *in vitro* stimulus in not strong enough to detect all responsive cells in this system. In addition, CD40L may be one of a number of micro-environmental factors able to promote growth in CLL and since CLL is a diverse group of diseases, different/additional requirements may exist. Interestingly, CD40L-mediated rescue was more pronounced in the unmutated subset with complete reversal of spontaneous apoptosis in all tested samples (Table 7.4). By contrast only 40% of mutated samples were rescued by CD40L. This indicates that the unmutated subset may be more dependent on CD40 signals for survival. However, the limited scope of this study should be emphasized, as analysis of a larger number of patient samples is required for any conclusions to be drawn.

CLL cells have also been reported to be Fas negative or to express low levels of Fas and reduced responsiveness to Fas signalling, whereas *in vitro* stimulation with B cell mitogens can upregulate Fas in some cases. Consistent with previous reports, Fas was constitutively expressed on a small percentage of cells at low density (RFI values up to 3) but was upregulated upon CD40 stimulation in the majority of cells. Surprisingly, a small number of samples previously identified as Fas-negative by

FACS analysis, were found to be sensitive to α–Fas in the MTS assays (Table 7.5). This indicated that low levels of the receptor, below the set threshold for Fas expression were sufficient to induce apoptosis in these cells. The high concentration (relevant to previous studies) of activating antibody may have enhanced cell death. Interestingly, treatment with CD40L did not alter the Fas-responsive profiles of CLL cells even though it induced an increase in receptor levels both in terms of cell numbers (data not shown) as well as antigen density. Thus, consistently with the previous analyses in BL cell lines, CLL can also display receptor-independent control of Fas-mediated apoptosis.

The lack of responsiveness in Fas-expressing CLL cells was indicative of an inhibitory mechanism. The small-scale analysis of signalling components performed here, did not show any evidence for defects in FADD and/or caspase 8 that may correlate with resistance. Although surprisingly low levels of caspase 8 were detected in a Fas-sensitive sample (Patient 5), this was deemed an indication that such low levels may be sufficient for apoptosis, particularly as B cells have been described as type II cells (where the original signal is amplified at the mitochondria) (Scaffidi et al., 1998). Interestingly, a very recent study reported that CLL cells although initially resistant to Fas signalling become sensitive after prolonged culture (longer than 72 hours) in the presence of CD40L possibly through shifts in the balance of FADD and FLIP (Chu et al., 2002). The expression of c-FLIP as well as other inhibitors may be investigated in the future.

Notably, CLL cells appear to be one of limited cell types displaying resistance to TRAIL-mediated apoptosis. To my knowledge, the only available study on this subject reported a modest sensitivity to rTRAIL in CLL, which was significantly enhanced by the translation inhibitor actinomycin D, indicating the presence of a labile inhibitory factor, probably c-FLIP (Olsson et al., 2001). However, although more extended analysis is required, the study presented here has shown widespread resistance to rTRAIL which appeared to be receptor-independent, as both DR5 and DR4 (in some cells) were detected by western blotting. The reason for the discrepancy with the previous report is not clear. However, the observations in CLL cells support the previous findings of receptor-independent resistance to rTRAIL in BL. Since some of the TRAIL-resistant cells were killed by α-Fas, an inhibitory mechanism possibly specific for the TRAIL pathway is suggested similarly to the LCLs. In addition, a

previous study has reported functional expression of TRAIL on CLL cells, suggesting that CLL cells may use TRAIL as a mechanism of 'immune counter-attack' (Zhao et al., 1999). Thus, CLL cells would be expected to develop strategies for self-protection from apoptosis induced in an autocrine manner.

Therefore, this study identified receptor-independent resistance to Fas and TRAIL-induced apoptosis, confirming the results of previous analyses in BL cell lines. However, it failed to find any correlation between susceptibility to death receptors and VH mutation status. A larger sample size needs to be examined for useful conclusions to be made and for the existing results to be validated. However, the results imply that rTRAIL-based therapeutic regimens are unlikely to be successful in CLL treatment. On the contrary, there is potential for Fas and CD40L-based treatments if the issue of systemic toxicity is resolved.

Summary

This study has examined death receptor-mediated apoptosis in malignant B lymphocytes including BL cell lines and *ex vivo* CLL cells and has demonstrated the following:

0 EBV negative BL cell lines are sensitive to TRAIL-mediated apoptosis whereas the majority of EBV positive BL cells are resistant. Resistance is independent of TRAIL receptor levels and does not correlate with EBV transcription programmes. EBV is unlikely to be independently responsible for resistance as isogenic clones with varying EBV status displayed similar responsiveness to rTRAIL.

1 Fas surface expression correlated with EBV status and transcription programme in BL cell lines. Thus EBV negative and EBV positive BL cells with a group I phenotype (except Louckes) were Fas negative. EBV positive group III BL cells that express the full set of EBV latent genes as well as Raji cells (group II), were Fas positive. Fas-positive group II and III cell lines were resistant to α-Fas whereas the LCLs (which express the same set of EBV genes as group III cells) were sensitive. Resistance to α-Fas in Raji and III BL cells was therefore Fas and EBV-independent.

2 Fas and the TRAIL signalling receptors, DR4 and DR5, do not play a major role in CDDP-induced apoptosis in BL40 and IB4 cells nor do they co-operate or enhance CDDP-induced apoptosis. Downregulation of the anti-apoptotic protein, MCL-1, during CDDP-induced apoptosis in both cell lines coincided with PARP cleavage.

3 There was no evidence for a role of mutations in the DD of Fas, defects in the signalling components FADD and caspase 8, expression of the inhibitory molecules FLIPL and FAP-1 or soluble receptor molecules in determining resistance to α-Fas in group III BL cell lines.

4 There was no evidence for a role of p53, EBV, decoy or soluble receptors, defects in FADD and caspase 8 expression or FLIPL in determining resistance to rTRAIL-induced apoptosis in BL cell lines with a group I phenotype.

5 *Ex vivo* CLL cells are susceptible to spontaneous apoptosis *in vitro*, a large proportion is sensitive to α-Fas but all tested samples were resistant to TRAIL-induced apoptosis. Resistance to rTRAIL and in certain cases to a-Fas-induced apoptosis was receptor independent in CLL samples.

Chapter 8. Discussion and future directions

The study presented here has examined death receptor-mediated apoptosis and the mechanisms of its regulation in B cell malignancies, concentrating primarily on Burkitt's lymphoma but also examining chronic lymphocytic leukaemia. The function of two death receptor pathways, Fas and TRAIL, was examined since they provide essential signals for the maintenance of homeostasis in haematopoietic cells as well as being part of the causative mechanisms of carcinogenesis. Of equal importance is the involvement of death receptor pathways in existing anti-cancer therapies as well as their potential in the development of novel treatments.

Several classes of BL cell lines were used in this analysis; this permitted the examination of important aspects of this system without genetic manipulation, namely the consequence of EBV infection and latency programmes for the biological function of death receptor pathways. By contrast, the number of CLL samples analysed was limited and did not allow definitive conclusions to be made, however it served to illustrate features that are shared with BL cells. The results from these analyses demonstrated receptor-independent regulation of Fas and TRAIL-mediated apoptosis in both types of malignancies. Furthermore, it was shown that sensitivity to Fas-mediated apoptosis does not necessarily correlate with responsiveness to rTRAIL in B cells including BL cell lines, *ex vivo* CLL cells and immortalised B cell lines (LCLs). In addition, the ability of the majority of BL cell lines to readily undergo DNA damage-induced apoptosis indicated that apoptosis can be initiated by mechanisms distal to the death receptors, whereas the effector mechanisms carrying it out to completion are intact. Furthermore, in contrast to a number of reports from other cell systems, this study failed to detect a major role for death receptor pathways in apoptosis induced by the DNA-damaging drug, CDDP, in a BL cell line with wild type p53.

To address the basis for the lack of biological function of Fas and TRAIL receptors in receptor-positive BL cell lines, I examined the expression of several molecular determinants acting upstream of caspase activation and believed to inhibit

death receptor-mediated apoptosis in other cell systems. The lack of clear correlation between inhibitor expression and the observed patterns of sensitivity to either α-Fas and/or rTRAIL indicated cell type-specific function of such inhibitory mechanisms as well as illustrating the complexity of the pathways examined. Thus, by indicating the potential for death receptor-based treatments but also the need for prior determination of apoptotic responsiveness for each disease entity, this study has contributed to the better understanding of apoptosis regulation in B cell malignancies.

Although resistance to α-Fas in group III BL cells had been previously described (Falk et al., 1992) (Gutierrez et al., 1999) (Tepper and Seldin, 1999), this study has been more extensive in terms of examining a large panel of BL cell lines and using a reliable assay of apoptosis to support the existing evidence. The use of an isotype control for α-Fas (IgM) has been useful in helping to evaluate the results from these analyses and in indicating that the non-specific cytotoxicity observed may be an underlying reason for some inconsistencies in the literature. Furthermore, this is one of the first studies to examine TRAIL-induced apoptosis both in BL and in CLL and to demonstrate distinct responsiveness to Fas and TRAIL-induced apoptosis. TRAIL-induced apoptosis in particular, has so far been more extensively described in melanoma cell systems (Griffith et al., 1998), (Thomas et al., 2000; Zhang et al., 1999; Zhang et al., 2000a), but not much is known about the biological function of TRAIL in lymphoid cells with few exceptions (Jeremias et al., 1998; Zhao et al., 1999). Although TRAIL receptors and Fas share structural and functional properties, they also appear to have unique properties. The distinct patterns of responsiveness have indicated that additional signalling components and/or inhibitory mechanisms may be involved in TRAIL-induced apoptosis and that TRAIL receptor pathways do not necessarily mirror signalling events downstream of Fas. This information may be useful in future analyses. To my knowledge comparative analysis of the two pathways has been described by one publication (Mariani et al., 1997). In addition, this is one of a limited number of studies to examine most of the major molecular determinants controlling death receptor-mediated apoptosis in lymphocytes.

Contrasting with the oldest and most extensively studied death receptor family member, TNFR1, a great deal is lacking from the current knowledge of Fas and TRAILR signalling pathways. The TRAIL-activated pathways in particular, constitute a relatively novel research area. At the start of this investigation, death receptors were believed to have a distinctively pro-apoptotic function contrasting with the more complex function of TNF receptors. TRAIL-induced apoptosis, in particular, was an exciting but largely unexplored field with simplistic prevailing views regarding the mechanisms of its regulation; i.e. the decoy receptors were considered the main regulators of resistance (Degli-Esposti et al., 1997a; Degli-Esposti et al., 1997b; Gura, 1997; MacFarlane et al., 1997; Marsters et al., 1997; Pan et al., 1997a; Sheridan et al., 1997). Since this study began, however, a more complex picture has emerged with survival pathways emerging as part of both Fas and TRAIL receptor pathways. For instance, PKC, JNK and NF-κB have been implicated in Fas and TRAIL receptor-induced signalling events in murine and human cells (Mueller and Scott, 2000), (Degli-Esposti et al., 1997a), (Micheau et al., 2001), (Choi et al., 2002), (Rivera-Walsh et al., 2000), (Baetu et al., 2001). Their significance is unclear but their involvement further complicates the task of elucidating the molecular mechanisms controlling responsiveness. FADD has also emerged as a dual function component, participating in mitogenic as well as apoptosis pathways (Newton et al., 1998). Furthermore, the physiological role of the TRAIL/TRAILR system is still unclear and although TRAIL appears to play a role in immune system homeostasis, it may also have a function in areas outside the immune system. Elucidation of the essential function of the TRAIL receptors may also help reveal the mechanisms that control apoptosis.

In addition, new evidence has highlighted the qualitative differences in the study approaches employed so far. Variation in the reagents used in analyses of Fas signalling may have been responsible for the observed differences in cell responses, for instance the use of FasL may result in different stoichiometry and/or composition of the DISC compared with the use of various Fas-activating antibodies (Thilenius et al., 1997). Moreover, the newly-emerged model of the PLAD domain that describes receptor pre-association highlights the need for elucidation of the structure of

Fas/FasL complexes (Chan et al., 2000). Furthermore, a large part of the available evidence at the start of this investigation was based on overexpression studies, an approach now seen as likely to provide erroneous results due to unpredicted aggregations of DD, DED and CARD-bearing molecules.

Therefore, in contrast to previous beliefs, death receptor-mediated apoptosis may not be actively suppressed by the expression/overexpression of a single inhibitory molecule, but may be the result of an established equilibrium involving the simultaneous or sequential activation of both apoptotic and survival pathways. Therefore, the understanding of the regulatory pathways that influence the outcome of Fas and TRAIL receptor activation in BL and CLL cells would benefit from additional work that may include:

- Examination of DISC formation following α–Fas and/or rTRAIL treatment. IP analyses would elucidate the recruitment of FADD and/or caspase 8 to the DISC and determine caspase 8 activation proximally to the receptors. This approach would also help determine whether the inhibitory molecules FAP-1 and FLIPL are recruited to the Fas DISC or whether DAP3 and FLIPL participate in DISC formation downstream of the TRAIL receptors in BL cells. The presence of the short isoform of c-FLIP, FLIPS, can now be examined since specific antibodies have now become available.
- If the analysis of DISC formation suggests that efficient processing of caspase 8 is inhibited by one of the above mentioned inhibitory molecules, anti-sense expression constructs may be introduced in Fas or TRAIL-resistant cell lines and their effect on cell viability assessed.
- The integrity of the primary structure of DR4 and DR5 may also be investigated i.e. by screening for mutations in the death domains of either receptor by SSCP, similarly to the analyses of Fas DD.
- If caspase 8 is activated at the DISC following the addition of α-Fas, it may be useful to determine which category (with respect to the events downstream of Fas activation) BL cells fall into; Type I or II. This can be investigated by analysis of BID cleavage and /or cytochrome c release from the mitochondria by western

blotting and flow cytometry analyses respectively. Examination of the timing of these events would also be required, for example does BID cleavage and cytochrome c release precede or follow downstream caspase activation and cleavage of caspase targets? Caspase activation can be determined by either flow cytometric analysis, e.g. caspase 3 or by western blotting e.g. caspases 6 and 7.

- Analysis of a larger number of CLL cell samples is required to confirm and extend the findings of this report. Preliminary studies have already been carried out with additional CLL patient samples and these may be completed and extended to include the analysis of signalling components and inhibitory molecules.
- However, since a larger number of molecular determinants and regulatory mechanisms that play a role in the control of death receptor signalling have become known, making the detailed examination of each a time-consuming task, it is felt that novel approaches may be more suitable, for instance genetic screening. Functional screening of a Fas and/or TRAIL sensitive cell line transfected with a cDNA library from a Fas and/or TRAIL-resistant BL cell line may enable the identification of a perhaps novel molecular determinant that is specifically responsible for attenuation of Fas and/or TRAIL-mediated apoptosis in BL.

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