

**DIVERSE ROLES OF THE BCL-2 FAMILY PROTEINS IN
HEMOPOIETIC CELL REGULATION**

by

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ABSTRACT

In this thesis, the roles of Bcl-2 family proteins in hemopoietic cell regulation were investigated. We first examined the effects of phosphatidylinositol 3-kinase (PI3K) dependent survival signalling pathways in cytokine dependent hemopoietic cells. Following cytokine withdrawal or PI3K inhibition, there was a loss of FOXO3A phosphorylation, resulting in increased expression of FasL and Fas at the cell surface. However, the Fas mediated signalling did not appear to be involved in apoptosis of cytokine dependent hemopoietic cells. These results support the belief that mitochondrial mediated signals through regulation of Bcl-2 family proteins may play the major role in hemopoietic cell apoptosis.

Amongst the pro-survival Bcl-2 family members, Bcl-2 and Bcl-xL are assumed to have a redundant function. To explore the differential ability of Bcl-2 and Bcl-xL in protecting cells against apoptosis, we over-expressed these proteins in cytokine dependent hemopoietic cell line FDCP-1. Based on our results, Bcl-2 appears to be a more potent pro-survival protein than Bcl-xL against apoptosis induced by cytokine withdrawal.

In addition to their localization at the mitochondria, Bcl-2 family members also localize at ER. To examine the physiological relevance of the membrane targeting of Bcl-xL, we used Rat-1 fibroblast cell lines over-expressing Bcl-xL mutants that were targeted to ER, mitochondrial outer membrane, or wild type Bcl-xL and showed that the ER targeted Bcl-xL was as effective or even more effective than the mitochondrial targeted or wild type Bcl-xL against certain cytotoxic stimuli.

A number of studies have shown involvement of Bcl-2 family proteins in processes other than apoptosis. We explored a role of Mcl-1 in cell cycle regulation, DNA damage checkpoint response, and cellular differentiation and found an interaction between Mcl-1 and the cell cycle

regulatory protein Cdk-1 in nuclear compartment. In addition, Mcl-1 was found to associate with the DNA damage checkpoint regulator, Chk-1, and the hallmark of DNA damage checkpoint response, phospho-histone H2AX. Mcl-1 level also increased in HL-60 cells upon induction of cellular differentiation by PMA. However, over-expression of Mcl-1 in these cells did not appear to enhance cellular differentiation. We, therefore, concluded that Mcl-1 might not play a prominent role in cellular differentiation.

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LIST OF ABBREVIATIONS

α	anti
-/-	knockout
Ab	antibody
ADP	adenosine di-phosphate
AIF	apoptosis inducing factor
ALPS	autoimmune lymphoproliferative syndrome
ANT	adenine nucleotide translocator
ANT	adenine nucleotide translocase
APAF-1	apoptosis protease activating factor 1
Ask-1	apoptosis signal regulating kinase-1
ATM	ataxia telangeictasia mutated
ATP	adenosine tri-phosphate
ATR	ataxia telangeictasia related
Bad	Bcl-2 antagonist of cell death protein
Bcl-2	B cell lymphoma/ leukemia-2
BH	Bcl-2 homology
Bim	Bcl-2-interacting mediator of cell death
Bok	Bcl-2 associated ovarian killer
BrdU	bromodeoxyuridine
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
Ca ²⁺	calcium
CAD	caspase activated DNAase
CARD	caspase recruitment domain
CARD	caspase recruitment domain
Cb5	cytochrome b5
Cdk-1	cyclin dependent kinase-1
CED	cell death protein (<i>C. elegans</i>)
Chk1/ Chk2	checkpoint kinase I/ checkpoint kinase II
CK1/Ck2	casein kinase I/ casein kinase II
CMV	cytomegalovirus
C-terminal	carboxy terminal

CTL	cytotoxic T-lymphocyte
Da	dalton
DD	death domain
DED	death effector domain
DIABLO	direct IAP binding protein with Low PH
DISC	death-inducing signalling complex
DLC	dynein light chain
DNA	deoxynucleic acid
DNA-PK	DNA protein kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
E. Coli	Eschericia coli
eIF	euokaryotic Initiation Factor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorter
FADD	fas associated death domain
FasL	Fas ligand
FLICA	fluorochrome inhibitor of caspases
FLICE	FADD-like IL-1 converting enzyme
FOXO	Forkhead Box
FLIP	FLICE inhibitory protein
GFP	green fluorescent protein
GMCSF	granulocyte macrophage colony stimulating factors
GSK-3	glycogen synthase kinase-3
h	hour
IAPs	inhibitor of apoptosis proteins
ICAD	inhibitor of caspase activated DNAase
IL3	interleukine-3
ILK	integrin-linked kinase
INF-	interferon-
IP	immunoprecipitation
MAC	mitochondrial apoptosis-inducing channel
MAPK-AP	mitogen-activated protein kinase-activated protein

Mcl-1S	short Mcl-1
MEK	map kinase or erk kinase
MEKK1	MEK kinase 1
Min	minutes
Mito	mitochondria
MMP	mitochondrial membrane permeabilization
MOI	multiplicity of infection
MOM	mitochondrial outer membrane
MOM	mitochondrial outer membrane
MPT	mitochondrial permeability transition
Ms	mouse
mTOR	mammalian target of Rapamycin
Na ₃ MoO ₄	sodium molybdate
Na ₃ VO ₄	sodium orthovanadate
NF-AT	nuclear factor of activated T-cells
NK cells	natural killer cells
N-terminus	amino-terminus
P	phospho
P.I.	propidium iodide
PARP	poly ADP ribose polymerase
PBS	phosphate buffer saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDK-1	phosphoinositide dependent kinase -1
PDK-2	phosphoinositide dependent kinase -2
PEST	proline, glutamic acid, serine, threonin
PH	pleckstrin homology
PI	phosphatidylinositol
PI-3 kinase	phosphatidylinositol 3-kinase
PIKKs	PI-3 kinase related kinases
PIP	phosphatidylinositol phosphate
PKB	protein kinase B

PKC	protein kinase C
PSR	phosphatidylserine receptor
PtdIns	phosphoinositides
PtdIns (3,4,5)P3	phosphatidylinositol 3,4,5-triphosphate
PtdIns (4,5)P2	phosphatidylinositol 4,5-bisphosphate
PTEN	phosphatase and tensin homolog detected on chromosome
ten	
PTP	permeability transition pore
PUMA	p53 up-regulated mediator of apoptosis
Rb	rabbit
RICTOR	rapamycin-insensitive companion of mTOR
rpm	revolution per minute
s	second
S/ T	serine/ threonine
SCF	stem cell factor
SDS/PAGE	sodium dodesyl sulphate/ polyacrylamide gel
electrophoresis	
Ser	serine
sFasL	soluble fas ligand
SH2	src homology-2
SiRNA	small interfering RNAs
SMAC	second mitochondria-derived activator of caspase
snMcl-1	short nuclear Mcl-1
STAT	signal transducer and activator of transcription
t(14:18)	translocation (14:18)
tBid	truncated Bid
TCLs	total cell lysates
Thr	threonine
TNF	tumor-necrosis factor
TNF-R	tumor necrosis factor receptor
TWEAK	TNF weak inducer of apoptosis
UV	ultra violet
VDAC	voltage dependent anion channels

VDAC

voltage dependent anion channel

W

western blot

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To My Son Bjorn

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CHAPTER 1

INTRODUCTION

1.1 PI-3 KINASE / PKB SURVIVAL SIGNALING PATHWAY

Hemopoietic cell lines that are dependent upon cytokines for their growth and survival have been widely used as model systems for the study of apoptosis. Upon removal of specific cytokines from the growth medium, while still in the presence of serum, the loss of survival signals initiates the processes that lead to death of the cells by apoptosis. One of the important signaling pathways that block apoptosis is the activation of phosphatidylinositol 3-kinase (PI-3 kinase) [Scheid *et al.*, 1995]. The PI-3 kinases are a family of enzymes that phosphorylate phosphatidylinositol (PI) lipids at the 3' position of the inositol ring (Katso *et al.*, 2001). They are divided into 3 classes, based on sequence similarity and biochemical properties. Amongst the PI-3 kinases, the class Ia is primarily responsible for the generation of the D3 phosphoinositides (PtdIns) in response to growth factors and cytokines. The class Ia enzyme is a heterodimer consisting of a regulatory and a catalytic subunit. The p85 regulatory subunit binds to the phosphorylated tyrosine residues on growth factor receptors via its src homology-2 (SH2) domains and, thereby, recruits the p110 catalytic subunit to the membrane. Upon recruitment to the membrane, the class Ia PI-3 kinase phosphorylates phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5) P_2) at the D-3 position of the inositol ring, converting it to PtdIns(3,4,5) P_3 (reviewed by Cantrell, 2001). Studies using pharmacological inhibitors of PI-3 kinase and over-expression of wild type PI-3 kinase as well as its dominant negative forms have established a major role for the PtdIns(3,4,5) P_3 or PIP_3 and possibly its immediate breakdown product PtdIns(3,4) P_2 in the regulation of cellular survival amongst other functions.

In general, the PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 recruit protein kinases that contain PtdIns binding motifs, for example pleckstrin homology (PH) containing proteins, to the plasma membrane, where they interact with other proteins or are themselves activated via binding with PtdIns(3,4,5) P_3 or PtdIns(3,4) P_2 . Of particular interest are the serine-threonine kinases phosphoinositide dependent kinase -1 (PDK-1) and the protein kinase B (also called PKB or Akt). Association with the PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 at the membrane, brings PDK-1 and PKB into close proximity and this leads to phosphorylation of PKB by PDK-1. PDK-1 phosphorylates PKB at the Thr308 residue in the kinase catalytic domain and PKB is additionally phosphorylated on Ser473 residue in the kinase regulatory domain by a kinase termed PDK-2 at the membrane. The identity of the PDK-2 kinase that phosphorylates S473 is not well-known, although there is evidence that both autophosphorylation (Toker and Newton, 2000) and phosphorylation by distinct serine kinases such as the integrin-linked kinase (ILK) [Dedhar *et al.*, 1999], MAPK-AP (mitogen-activated protein kinase-activated protein) kinase-2 (Alessi and Cohen, 1998), the conventional PKC isoform (Kroner *et al.*, 2000), and more recently DNA-PK (Feng *et al.*, 2004) and mTOR-RICTOR (Hresko and Mueckler, 2005) may mediate the phosphorylation of PKB at Ser-473. Phosphorylation of both Thr308 and Ser473 is essential for maximal activation of PKB (Alessi *et al.*, 1996) and inhibitors of PI-3 kinase prevent phosphorylation of both residues (Alessi *et al.*, 1996).

Once activated, PKB phosphorylates multiple substrates, thereby initiating the activation of the downstream signaling events that provide cell survival. The protein substrates that are phosphorylated and negatively regulated by PKB include, the pro-apoptotic Bcl-2 family member, Bad (Downward, 1999), procaspase-9 (Cardone *et al.*, 2000), GSK-3 (Cross *et al.*, 1995), and ASK-1 (Kim *et al.*, 2001). PKB can also regulate

transcription factor activity. Once activated, PKB has been shown to translocate to the nucleus where it can phosphorylate transcription factors such as the FOXO members of the forkhead family which include FOXO1, FOXO3, and FOXO4 at several serine and one threonine site (Kops *et al.*, 1999; Rena *et al.*, 1999; Kops and Burgering, 1999; Brunet *et al.*, 1999; Madema *et al.*, 2000). All 3 FOXO transcription factors have the forkhead DNA binding domain and the conserved PKB phosphorylation sites. Phosphorylation of FOXO proteins by PKB leads to inactivation and nuclear export of these transcription factors.

The FOXO transcription factors have been shown to be involved in the regulation of apoptosis and cell cycle progression through induction of pro-apoptotic genes such as FasL and Bim, and the cell cycle inhibitor P27^{kip1}, respectively. Previous studies have shown that during growth factor withdrawal, FOXO3a activates the death inducing ligand, Fas ligand (FasL), promoter and induces apoptosis (Brunet *et al.*, 1999). Furthermore, the pro-apoptotic Bcl-2 family protein, Bim, was shown to be directly induced by FOXO3a upon cytokine withdrawal in lymphocytes (Dijkers *et al.*, 2000). In addition to Bim, the cell cycle inhibitor, P27^{kip1}, was also up regulated by FOXO3a upon cytokine withdrawal in the cytokine dependent T-cells (Marie *et al.*, 2002). Other studies showed that in mouse fibroblast and B cell lines, both FOXO4 and FOXO1a can arrest cells by inducing the expression of P27^{kip1} (Medema *et al.*, 2000; Dijkers *et al.*, 2000). More recent studies have suggested that PUMA, another pro-apoptotic Bcl-2 family protein, is also partly dependent upon FOXO3a activity, and its up-regulation following loss of PI3K/PKB signalling can contribute to apoptosis (You *et al.*, 2006a; You *et al.*, 2006b; Ekert *et al.*, 2006). Thus, by inhibiting proteins such as, Bad, GSK-3, ASK-1, and the FOXO transcription factors, PKB prevents apoptosis and growth arrest downstream of the PI-3 kinase signaling pathway.

The key functions of the PI3K/PKB pathway in controlling a number of aspects of cell growth, cell cycle and apoptosis, place it in a position as potentially playing a role in development of cancer. Indeed, it has been shown that mutations in many components in the PI-3 kinase/PKB signaling pathway can lead to cellular transformation and oncogenesis, as is the case for many genes that are involved in regulation of cellular viability. For example, amplified or mutationally activated receptor tyrosine kinases, amplification of genes encoding PI3 kinase, over-expression of PKB, or the loss of the phosphatidylinositol-3 phosphatase PTEN, which is the negative regulator of phosphatidylinositol-3 generation, have been detected in many human cancers. Loss of function mutation in PTEN is detected in >75% of glioblastomas, and amplification of PI-3 kinase catalytic domain has been detected in ovarian and other cancers. In addition, PKB over-expression has been found in ovarian, breast, and pancreatic cancers (Schlieman *et al.*, 2003). Inhibition of the PI-3 kinase signaling pathway in selected cancer cells may provide a useful therapeutic intervention.

1.2 PROGRAMMED CELL DEATH (APOPTOSIS)

For many years, it was believed that the demise of all cells was caused by pathological cell death or necrosis. It was Kerr, Wyllie and Currie who first reported that the stereotypic changes observed in physiologic cell death reflected an underlying genetic program which they termed apoptosis (from a Greek word meaning “falling off,” as leaves from a tree) to distinguish it from necrosis (Kerr *et al.* 1972). For many years, this concept was largely overlooked, but in the past ten to fifteen years, apoptosis has become the subject of intense research. There are many observable morphological and biochemical differences between cells undergoing necrosis vs. apoptosis.

Necrosis occurs when cells are exposed to extreme variance from physiological

conditions (e.g., hypothermia, hypoxia) which may result in damage to the plasma membrane. Necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, including the mitochondria, and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response. Apoptosis, in contrast, is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own demise (cellular suicide). It is characterized by the orchestrated collapse of a cell resulting in membrane blebbing, cell shrinkage, condensation of chromatin, and fragmentation of DNA followed by rapid engulfment of the corpse by neighboring cells (Fig. 1.1). It is distinguished from necrotic death by the absence of an associated inflammatory response. Apoptosis is most often found during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, and development of the nervous system. By eliminating redundant, damaged or aged cells, apoptosis shapes the embryo and also maintains tissue homeostasis within the adult.

Key insights into the molecular mechanisms regulating programmed cell death came from the Nobel-prize winning studies of Horvitz and Colleagues (Horvitz, 1999) in the nematode *Caenorhabditis elegans* (*C. elegans*). They traced the lineage of all the somatic cells in *C. elegans* from the fertilized egg to the mature worm and determined that of the 1090 somatic cells generated during development, exactly 131 cells undergo apoptosis. By

Apoptosis (Programmed Cell Death)

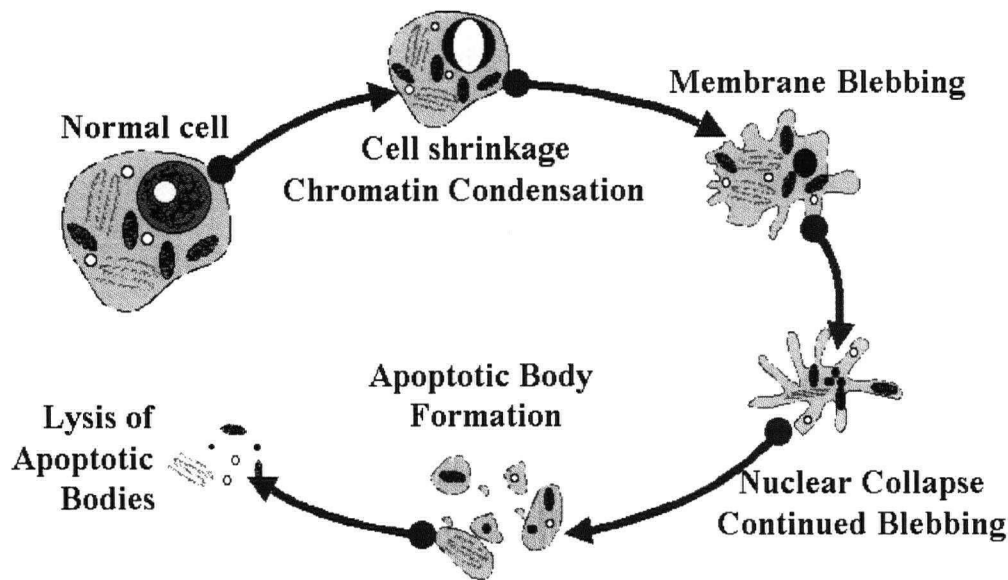


Figure 1.1- Programmed cell death or apoptosis. Apoptosis involves an orchestrated collapse of cells resulting in cell shrinkage, membrane blebbing, nuclear condensation, apoptotic body formation, followed by engulfment and lysis of apoptotic bodies via neighboring cells.

introducing mutations in genes whose proteins played essential role in apoptosis, they showed that CED-3 and CED-4 and Egl-1 genes were required for cell death, whereas, CED-9 suppressed apoptosis, and that the apoptotic pathway could be activated in all cells. Moreover, the finding that cell death did not occur in *ced-9/ced-3* double mutants suggested that CED-9 acts upstream of CED-3 to suppress the apoptotic pathway (Ellis and Horvitz, 1991; Hengartner *et al*, 1992).

These exciting and detailed studies in *C. elegans* helped in the later discovery that apoptosis is a conserved evolutionary pathway with astonishing genetic similarities between worm and human. One of the very first molecular regulators of apoptosis identified in human was Bcl-2 (B Cell Lymphoma-2). It was identified as a breakpoint rearrangement (translocation 14:18) in human follicular lymphomas (Tsujimoto *et al.*, 1984) and Bcl-2 was shown to act as a

proto-oncogene that promoted cell survival rather than cell proliferation (Vaux *et al.*, 1988). Not only are the Bcl-2 and CED-9 proteins homologous (Hengartner and Horvitz, 1994), but a bcl-2 transgene can block the extensive cell death found in ced-9 mutant worms (Vaux *et al.*, 1992). Thus both proteins act as regulators that suppress the apoptotic pathway. Moreover, as will be discussed later in this section, the proteins encoded by CED-3, CED-4, and Egl-1 all have genetic counterpart in mammals as well.

Apoptotic death can be induced by a wide variety of cytotoxic stimuli. Among the more studied death stimuli is DNA damage (by irradiation or drugs used for cancer chemotherapy), which in many cells leads to apoptotic death via a pathway dependent on p53. In other cases, cells appear to have a default death pathway which must be actively blocked by a survival factor such as cytokines or growth factors which bind to their cell surface receptors and trigger cell survival pathways. When the survival factor is removed, the default apoptotic death program is triggered.

Once cells commit to suicide, tightly controlled biochemical changes take place in the cells. The first biochemical hallmark of apoptotic death is the activation of caspases, which are cysteine proteases related to ced-3, the "death gene" of the nematode *C. elegans*. Upon activation, caspases irreversibly cleave and inactivate many cellular substrates resulting in both biochemical and morphological changes in the cells undergoing apoptosis, as discussed in the following section. Finally, the changes in the apoptotic cells trigger phagocytosis by non-activated macrophages. The apoptotic pathway and the engulfment process are part of a continuum that helps ensure the non-inflammatory nature of this death paradigm. Phagocytes recognize the surface of the dying cell most likely through an 'engulf me' signal. In mammalian systems, the best characterized signal is phosphatidylserine (PS) displayed on the

plasma membrane of dying cells (Fadok *et al.*, 2001). A number of lines of evidence exist for the participation of multiple engulfment receptors including CD91, CD14, CD36, and $\alpha\text{v}\beta 3$ integrin, as well as the phosphatidylserine receptor (PSR) (Savil and Fadok, 2000).

1.2.1 Caspases

Most of the morphological changes observed in apoptotic cells are caused by a family of cysteine-aspartic acid proteases called caspases. The essential role of cysteine proteases in apoptosis was first discovered in *C. elegans* with the discovery of the caspase homologue CED-3 (Yuan *et al.*, 1993) and since then it has been demonstrated that caspases are, in fact, highly conserved throughout evolution and are found in species as diverse as flies (*Drosophila*), worms (*C. elegans*), mice, and humans. So far more than a dozen of this highly homologous family of proteases have been identified, two-thirds of which function in apoptosis and the rest appear to be important in the regulation of inflammatory response (Budihardjo *et al.*, 1999; Earnshaw *et al.*, 1999; Fesik and Shi, 2001; Salvesen and Dixit, 1997). Caspases are initially synthesized as enzymatic ally inert zymogens with an N-terminal pro-domain and a C-terminal protease domain (Cohen *et al.*, 1997; Earnshaw *et al.*, 1999). Most caspases are activated by proteolytic cleavage of the zymogen between P20 and P10 subunits. An active caspase is believed to be a homodimer, with each monomer consisting of a larger p20 and a smaller p10 subunits.

The apoptotic caspases are divided in to two groups based on their structural similarities and substrate specificities: the initiator caspases which include caspases 2, 8, 9, 10, and the executioner/ effector caspases that include caspases 3, 6, and 7. The initiator caspases have a long N-terminal pro-domain, whereas this domain is very short in executioner caspases. The initiator caspases contain in their pro-domains distinct protein-

protein interaction modules, for example, the Death Effector Domain (DED) in pro-caspase 8 and 10, and the Caspase Activation and Recruitment Domain (CARD) in pro-caspase 2 and 9. Similar modules, also present on the oligomerized adaptor/regulator molecules that are present upstream of these initiator caspases, are involved in the homotypic protein-protein interaction between the adaptors and the caspases, which help bring multiple initiator caspase molecules into close proximity and presumably lead to their autocatalytic activation. Multiple pro-caspase 8 molecules, for example, are recruited to the death receptor, Fas, through interaction between the DED on caspase-8 and a similar DED on the oligomerized adaptor molecule FADD on the activated Fas receptor. According to the “induced proximity” model, this brings multiple pro-caspase molecules into close proximity which results in their cleavage and subsequent activation by “cross proteolysis”, meaning that one caspase molecule could digest another molecule resulting in the formation of processed active caspases (Salvesen and Dixit, 1999). Alternatively, caspase 8 activation may occur as a result of dimerization rather than processing (Chen *et al.*, 2002), and the cleavage and processing might merely be involved in stabilizing the caspase-8 dimers (Boatright *et al.*, 2003). The induced proximity model is also applied to the mechanism of activation of caspase-9 which initiates the mitochondrial mediated caspase activation cascade. In addition, similar to caspase-8, the proteolytic cleavage of procaspase-9 appears to be unnecessary for its activation (Srinivasula *et al.*, 2001).

Once activated, the initiator caspases cleave and activate the downstream executioner caspases resulting in a cascade of caspase activation. The executioner caspases then cleave a number of cellular substrates, leading to demise of cells. For example, DNA fragmentation which is a major hallmark of apoptosis takes place downstream of caspase activation through

cleavage of ICAD (Inhibitor of Caspase Activated DNAase) by effector caspases. In healthy growing cells, ICAD interacts with CAD (Caspase Activated DNAase) and this complex resides in the cytoplasm. This ensures that the chromosomal DNA remains protected from degradation by CAD. During apoptosis, ICAD is cleaved off by the effector caspases and CAD, with its nuclear translocation sequence (NLS) exposed by caspase cleavage, translocates to the nucleus where it cleaves off chromosomal DNA into ~200 base pair fragments, a hallmark of DNA fragmentation during apoptosis.

Effector caspases also cleave major structural components of the nuclear envelope such as nuclear lamin, leading to morphological changes observed in the nucleus during apoptosis. The cytoskeletal proteins such as actin and formin are also degraded by caspases leading to cell shrinkage, membrane blebbing, and altered cell signaling pathways. Other cellular substrates of caspases include Poly ADP Ribose Polymerase (PARP), DNA proteinase K (a DNA repair enzyme), translation initiator factors (e.g., eIF 4G, eIF 4B, and eIF 2a), and cleavage of signaling and apoptotic regulatory proteins such as PKC, MEKK1, Bcl-2 and Bcl-xL. In addition, executioner caspases can further cleave the initiator caspases such as procaspase-8 and 9 through a positive feed back loop, ensuring that sufficient caspase molecules are activated for completion of apoptosis.

Caspases are regulated in many different ways including transcriptionally and via post-translational modifications. In addition, due to the presence of a positive feed back loop in the caspase cascade, the enzymatic activity of caspases has to be tightly regulated with buffers and dampeners, to prevent, for example, an exaggerated cellular response in an unlikely event when very small numbers of caspase molecules are accidentally activated. The Inhibitor of Apoptosis Proteins (IAPs) act as one set of such regulators. IAPs are found in

species as diverse as baculovirus and mammals. They interact with caspases and block caspase activity. In turn, IAPs, themselves, are inhibited by Smac/Diablo and Omi/HtrA2, mitochondrial proteins which are released into cytosol during apoptosis (review by Van Gurp *et al.*, 2003)

1.2.2 Two ways to die by apoptosis: Mitochondrial-mediated (intrinsic) and the death receptor-mediated (extrinsic) cell death

So far, two principal pathways for activating caspases have been discovered (Fig. 1.2). The more ancient, mitochondrial mediated cell death, which is induced by diverse intracellular stresses, including cytokine deprivation and genotoxic damage, is regulated by Bcl-2 and its relatives on the mitochondrial membrane. A more recently evolved pathway, death receptor-mediated cell death, is triggered when 'death receptors' of the tumor-necrosis factor (TNF) family engage with their cognate receptors on the plasma membrane. Both pathways lead to activation of downstream caspases followed by apoptosis.

1.2.2.1 Mitochondrial-mediated or intrinsic cell death

In addition to its role as the cellular energy producer, the mitochondrion is also actively involved in the regulation of program cell death. The Bcl-2 family proteins which are established regulators of apoptosis either reside at the mitochondrial outer membrane (MOM) or are targeted to the mitochondria during apoptosis. These proteins are composed of members with opposing functions. Some family members are anti-apoptotic, while others are pro-apoptotic. The exact mechanism by which the Bcl-2 family proteins regulate the mitochondrial membrane integrity is not quite well established but it is well known that the pro-survival members maintain the mitochondrial membrane integrity whereas the pro-

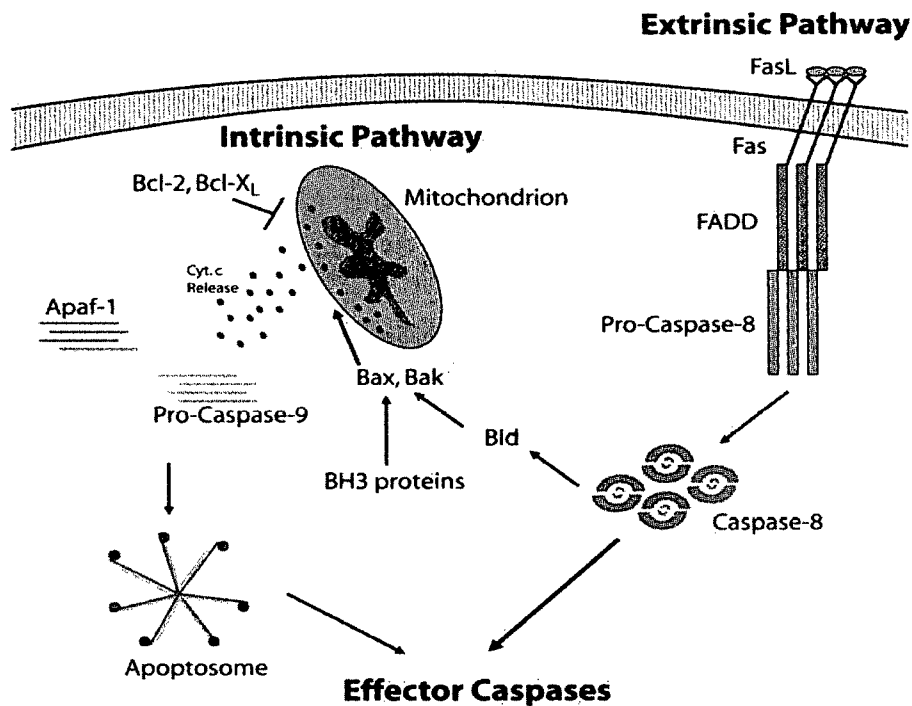


Figure 1.2- Caspase activation via the intrinsic and extrinsic pathways of apoptosis. The activation of caspases during apoptosis proceeds through two distinct mechanisms. Activation of caspases through the extrinsic pathway involves the binding of extracellular ligands (e.g., FasL) to their cognate receptors (e.g., Fas) and the recruitment of intracellular adaptor proteins (e.g., FADD) to activate caspase 8 and subsequent downstream effector caspases. The intrinsic pathway involves the release of cytochrome c from the mitochondria into the cytosol. This leads to the formation of the apoptosome, the activation of caspase 9, and the cleavage of effector caspases.

apoptotic members induce loss of its integrity. The balance between the pro-apoptotic and pro-survival Bcl-2 family proteins at the MOM thus determines if the cells die or survive (Fig. 1.3). In addition, a number of pro-apoptotic proteins are housed within the inter-membrane space of the mitochondria where they are sequestered away from their cytosolic targets. Upon induction of apoptosis followed by the loss of mitochondrial membrane integrity, these proteins are released into the cytosol where they activate the downstream effectors of apoptosis, the caspases.

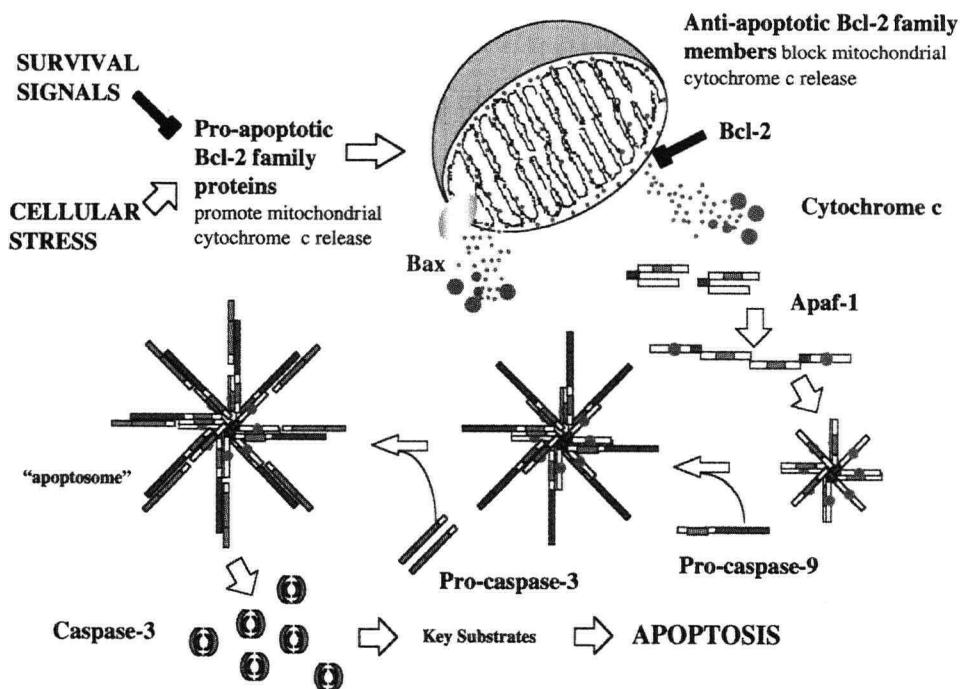


Figure 1.3- Induction of apoptosis via the mitochondrial pathway. Cellular stress induces pro-apoptotic Bcl-2 family members to translocate from the cytosol to the mitochondria, where they induce the release of cytochrome c. Cytochrome c catalyzes the oligomerization of Apaf-1, which recruits and promotes the activation of procaspase 9. This, in turn, activates procaspase 3, leading to apoptosis.

1.2.2.1.1 The mitochondria

Mitochondria also referred to as the power plant of the cell, is an organelle normally found in eukaryotic cells. The mitochondria is composed of four compartments: an outer membrane, an inner membrane, an inter-membrane space, and a matrix. The outer membrane is semi-permeable to small molecules of only up to 5000 Dalton (Da). The high permeability of the outer membrane is mediated through the Voltage Dependent Anion Channels (VDAC) embedded within this compartment. The inner membrane surrounds the matrix, and is folded to form cristae which contain the components of the electron transport chain. This electron transport chain generates the hydrogen ion gradient across the inner-membrane needed for ATP production. In order to maintain this hydrogen ion gradient, the passage of proteins and

metabolites across the inner-membrane is tightly regulated. The adenine nucleotide translocator (ANT) which is also embedded within the inner membrane is the site where the newly formed ATP in the matrix is exchanged for ADP in the inter-membrane space. The matrix contains mitochondrial DNA, ribosomes and RNA plus some enzymes. The inter-membrane space contains cytochrome c which is an electron carrier that shuffles electrons between the cytochrome c oxidase and the cytochrome c reductase, two membrane bound compartments within the electron transport chain. Further, the inter-membrane space also contains many metabolites, ions, and proteins required for energy production. In addition to its role in cellular ATP production, cytochrome c is also involved in induction of apoptosis. Other pro-apoptotic proteins within the inter-membrane space include, the apoptosis inducing factor (AIF), endonuclease G, Smac/Diablo, and HtrA2/Omi.

Following stress-induced apoptosis and the loss of mitochondrial outer membrane integrity, cytochrome c leaks into the mitochondria where it interacts with APAF-1 (apoptosis inducing factor-1) through its WD40 motif. Apaf-1 has also a nucleotide binding domain which binds with high affinity to ATP upon binding with cytochrome C. These interactions induce a conformation change in Apaf-1 exposing its caspase recruitment domain (CARD) and also allow one molecule of Apaf-1 to interact with six other Apaf-1 molecules of the same configuration generating a heptameric wheel-like structure referred to as the "apoptosome". The CARD domain on APAF-1 interacts with the CARD domain on procaspase 9 allowing for seven molecules of procaspase 9 to be recruited to each apoptosome complex. The procaspase 9 molecules are then activated through induced proximity with other procaspase 9 molecules. The activated caspase 9 then cleaves and activates the downstream effector caspases including caspases 3, 6, or 7. The effector

caspases then cleave a wide variety of cellular substrates, as mentioned above, leading to cell shrinkage, chromatin condensation, nuclear blebbing, formation of apoptotic bodies (membrane embedded cellular contents), followed by engulfment of apoptotic cells or apoptotic bodies by macrophages through a non-inflammatory process.

AIF and endonuclease G are other pro-apoptotic proteins released from the inter membrane space during apoptosis. Upon release into the cytosol, they translocate into the nucleus where both molecules induce large scale DNA fragmentation independent of caspase activation (Daugas *et al.* 2000). AIF apparently does not have nuclease activity (Susin *et al.*, 1999) but it is hypothesized that once bound to the chromosomal DNA, AIF recruits other nucleases to the site (Ye *et al.*, 2002). AIF also induces chromosomal DNA condensation which is an early hallmark of apoptosis (Susin *et al.*, 2000).

Smac/Diablo and Omi/HtrA2 are other mitochondrial proteins which are released into cytosol, along with cytochrome C, during apoptosis (Fig. 1.4). While cytochrome C activates apaf-1, Smac/Diablo and Omi/HtrA2 relieve the inhibition on caspases by binding to the Inhibitor of Apoptosis Proteins, IAPs, and disrupting their associations with processed caspases, for example caspase 9. This would, then, allow caspase 9 to activate caspase 3 leading to apoptosis (Van Gurp *et al.*, 2003; Du *et al.*, 2000; Zimmermann *et al.*, 2001). As mentioned earlier, IAPs are cytosolic proteins which bind to caspases and inhibit caspase activity. They are inactivated during apoptosis to allow cell death mediated by caspases to proceed.

1.2.2.2 Death receptor-mediated, or extrinsic, cell death

In addition to the mitochondrial-mediated, or intrinsic cell death, higher organisms have evolved other mechanisms to eliminate unwanted cells. One such mechanism is the cell

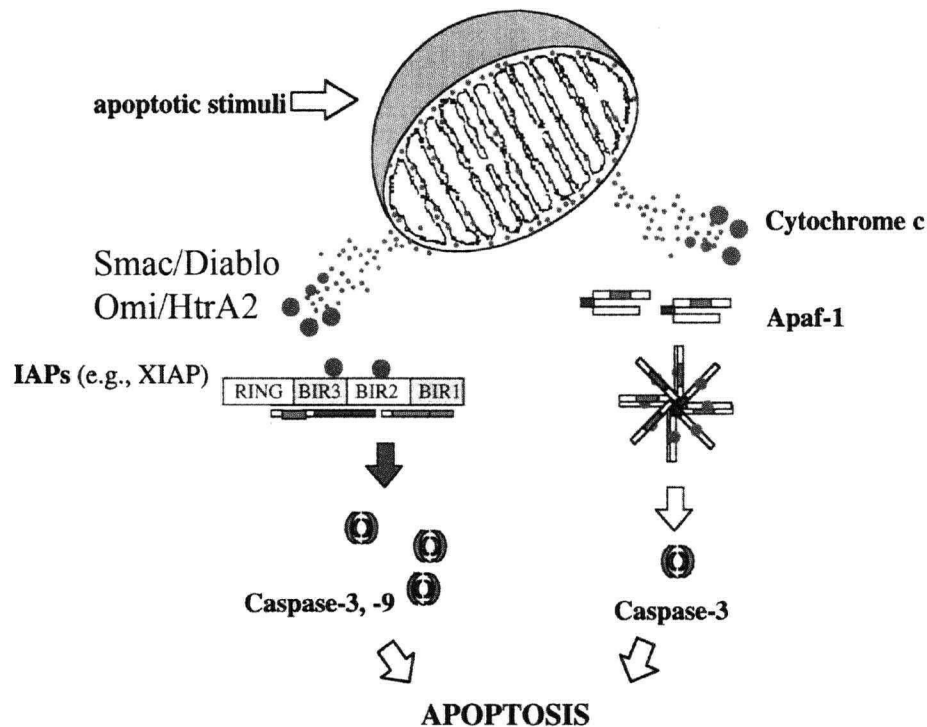


Figure 1.4- Second mitochondrial activator of caspases (Smac/DIABLO) and Omi/HtrA2. Cytochrome c, Smac/Diablo, and Omi/HtrA2 are coordinately released from the mitochondria upon induction of apoptosis. While Cytochrome c activates Apaf-1, Smac/DIABLO and Omi/HtrA2 relieve the inhibition on caspases by binding to the IAPs, thereby disrupting the association of IAPs with processed caspase 9, allowing caspase 9 to activate caspase 3, leading to apoptosis.

death mediated by receptors, which is also known as extrinsic cell death. This involves members of the tumor necrosis factor receptor (TNF-R) family also known as the “death receptors”, since they transduce death signals upon engagement with their cognate receptors at the cell surface. Six members of this family are known so far, including TNF-R1, CD95 (Fas, APO-1), DR3 (APO-3), TRAIL-R1 (APO-2, DR4), TRAIL-R2 (DR5), and DR6 (Schulze-Osthoff *et al.*, 1998).

Fas or CD95 is the best characterized member of this family and it plays a major role in the immune function. It is widely distributed throughout the body with a particularly higher expression in the thymus, liver, kidney, and heart. Fas is a glycosylated type I

transmembrane receptor with a molecular mass of ~ 45-52 KDa (Itoh *et al.*, 1991; Oehm *et al.*, 1992) but soluble forms of Fas generated by alternative splicing also exist which are thought to act as scavengers for the endogeneous ligand (Cascino *et al.*, 1996). The mature membrane bound Fas receptor is composed of 3 domains: a cysteine rich extracellular domain which is involved in ligand binding, a transmembrane domain, and an intracellular domain containing the 80 amino acid long Death Domain (DD) which is necessary for transducing death signals.

The Fas-mediated apoptosis pathway is activated by cross-linking of Fas by binding of its natural ligand, FasL or CD95L, which is a type II transmembrane protein of 40 KDa belonging to the TNF family of ligands. Other ligands belonging to this family of proteins include tumor necrosis factor alpha (TNF α), TNF-related apoptosis-inducing ligand (TRAIL/APO-2), and TNF weak inducer of apoptosis (TWEAK) (Ashkenazi *et al.*, 1998). FasL is mainly expressed on activated T- lymphocytes and Natural killer (NK) cells, although it is also constitutively expressed on the tissues of "immune-privilege sites" such as the testis and the eyes. The mature FasL molecule is composed of 3 domains: an extracellular domain containing both the receptor binding motif and the oligomerization motif which is required for self assembly of the ligand (Orlinick *et al.*, 1997), a single transmembrane domain, and an intracellular domain containing a proline rich motif which is responsible for sorting FasL to secretory lysosomes (Blott *et al.*, 2001). The membrane bound FasL maybe cleaved off by the action of matrix metalloproteases generating a soluble form, sFasL, which is less effective in inducing apoptosis in Fas expressing cells even though it sheds as a trimer and binds Fas (Cheng *et al.*, 1994; Tanaka *et al.*, 1995, Schneider *et al.*, 1998).

Productive interaction between Fas and FasL requires oligomerization, most likely

trimerization, of both the receptor and the ligand (Holler *et al.*, 2003). The intracellular events triggered by ligation of the Fas receptor with its ligand include induction of apoptosis (Peter and Krammer, 2003) but in some context this ligation might instead induce cellular proliferation through activation of nuclear factor κ B (NF- κ B) signaling pathway (Ahn *et al.*, 2001; Desbarats and Newell, 2000). Induction of apoptosis by a Fas mediated pathway involves a series of orchestrated events that starts with cross-linking of Fas with its ligand and ends with caspase activation and cell death. Upon cross-linking with FasL, the activated Fas receptor interacts with the DD containing adaptor molecule, FADD (Fas Associated Death Domain), via DD-DD interaction. In addition to the death domain, FADD also has a Death Effector Domain (DED) which subsequently recruits the DED containing procaspase 8 (FLICE) and/or procaspase 10 to the receptor via the DED-DED interactions. The resulting complex which is known as the Death-Inducing Signaling Complex (DISC) forms within seconds of receptor engagement (Kischkel *et al.*, 1995). The procaspase 8 and/or procaspase 10 are activated upon recruitment to the DISC. Initially it was thought that induced proximity of these caspases at the DISC leads to their cleavage and subsequent activation by cross proteolysis as mentioned earlier. However, recent data suggests that caspase 8 activation occurs as a result of dimerization rather than processing (Chen *et al.*, 2002), and the cleavage and processing is merely involved in stabilizing the caspase-8 dimers (Boatright *et al.*, 2003). Whether it's by processing or dimerization, activated caspase8/10 in turn activate effector caspases such as caspase 3 or caspase 6 causing the cells to undergo apoptosis. This caspase 8 activation can be blocked by the DED containing catalytically inactive enzyme, c-FLIP (FLICE Inhibitory Protein) [Golks *et al.*, 2005]. It is believed that c-FLIP prevent Fas-mediated apoptosis by competing with pro-caspase 8 for binding to FADD at the DISC.

Fas-induced apoptosis can follow two pathways (Fig 1.5). In type I cells (Barnhart *et al.*, 2003) such as T-lymphocytes, large amount of caspase-8/10 is activated at the DISC. This is followed by rapid cleavage of the effector caspases such as caspase 3 prior to the loss of mitochondrial membrane potential, suggesting direct caspase cascade activation. In type II cells (Barnhart *et al.*, 2003) such as hepatocytes, since insufficient amount of caspase 8/10 is activated at the DISC to carry out the direct caspase activation cascade, the mitochondria are used as 'amplifiers' to initiate the executionary caspase cascade. This mitochondrial or intrinsic cell death pathway is activated by caspase 8 mediated cleavage of the BH3 only containing Bcl-2 family protein Bid (Luo *et al.*, 1998). The pro-apoptotic truncated Bid, t-Bid, then translocates to the mitochondria where it can induce activation of the pro-apoptotic Bcl-2 family proteins Bax and Bak resulting in loss of mitochondrial membrane potential, release of apoptogenic factors such as cytochrome C from the mitochondria, followed by activation of executioner caspases and subsequent apoptosis as discussed earlier. Thus Bid forms a critical link between the extrinsic and intrinsic cell death pathways. In type II cells, the pro-survival Bcl-2 protein could protect cells against Fas-mediated apoptosis, whereas, in type I cells, Bcl-2 is refractory (Krammer, 2000). This is consistent with the susceptibility of thymocytes but resistance of hepatocytes to Fas mediated apoptosis in Bid deficient and Bax/Bak double deficient mice (Lindsten *et al.*, 2000; Wei *et al.*, 2001)

Apoptosis mediated through the death receptor pathway could be regulated in several ways. For example, glycosylation status of the Fas receptor at the cell surface has been shown to modulate Fas mediated apoptosis (Keppler *et al.*, 1999). Inside the cell, caspase 8

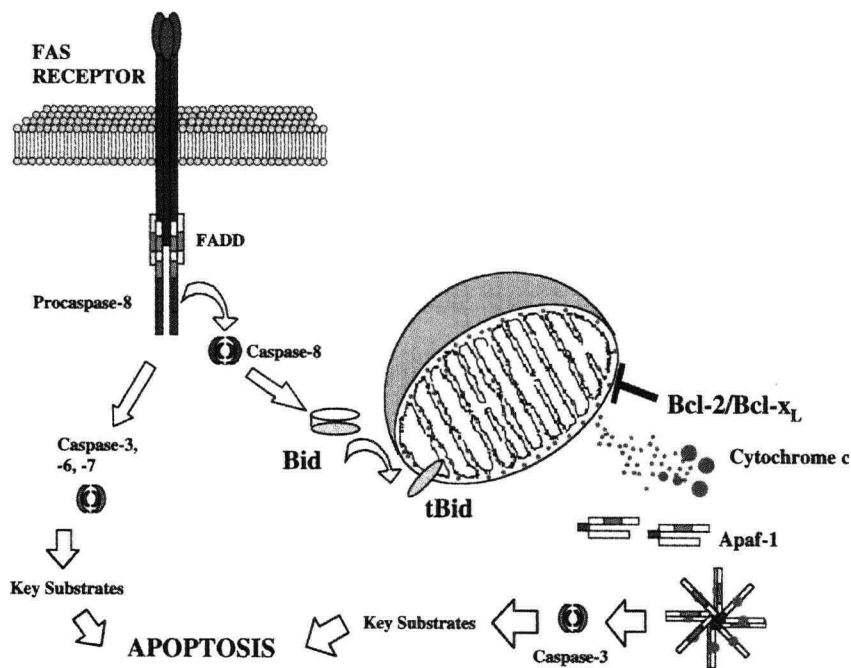


Figure 1.5- Signaling from the Fas receptor via activation of caspase 8. FasL recruits FADD to the intracellular region, which, in turn, recruits procaspase-8. The procaspase-8 transactivates. In type I cells, the mature caspase 8 now can cleave and activate procaspase-3, leading to apoptosis. In type II cells, signaling from the Fas receptor to mitochondria involves cleavage of the BH3-only protein Bid by caspase-8. Bid subsequently induces cytochrome c release and downstream apoptotic events.

activation can be blocked by over-expression of c-FLIP. An increase in c-FLIP has been linked to cancer and over-expression of c-FLIP can protect cells against Fas-mediated apoptosis (Tschopp *et al.*, 1998; Krueger *et al.*, 2001). The death receptor (Fas) pathway can be further regulated at a transcriptional level. For example, Fas (CD95) expression is induced by the tumor suppressor, P53, during DNA damage induced apoptosis (Muller *et al.*, 1998), and STAT1 is required for INF- γ mediated up-regulation of Fas (Xu *et al.*, 1998). STAT3 and c-jun, on the other hand, can negatively regulate Fas expression (Ivanov *et al.*, 2002). FasL expression is also up-regulated by Forkhead transcription factor, FOXO3a, upon inhibition of the PI3 kinase/Akt signaling pathway (Suhara *et al.*, 2002). In addition to FOXO3a, other transcription factors including NF- κ B (Kasibhatla *et al.*, 1998), c-Myc

(Brunner *et al.*, 2000), NF-AT (nuclear factor of activated T-cells) [Latinis *et al.*, 1997], and SP-1 are also involved in the regulation of FasL expression (Kavurma *et al.*, 2001).

The Fas mediated cell death has a major role in the immune homeostasis by regulating two main events in the immune system. First, the Fas mediated cell death is essential for cytotoxic T-lymphocyte (CTL) killing of virally infected cells. The activated CTLs kill their targets in two ways: one is through the release of toxic perforin and granzyme B and the other is by the engagement of FasL on T-cells with the Fas on the surface of the infected cells. Both of these pathways lead to apoptosis of the virally infected cells through activation of caspases. Apoptosis was shown to be normal in mice that were defective in perforin/granzyme B release. However, CTLs from these mice could not kill cells when they lacked Fas/FasL. These two mechanisms seem to be the main ways that activated CTL kill their targets (Goldsby *et al.*, 2003). The Fas mediated cell death is also essential for deletion of activated T-lymphocytes and the self-reacting T-cells from the periphery. Deletion of the activated T-cells in the periphery by Fas reduces the immune response once the invading pathogen has been eliminated from the host cells. The self-reactive T-cells that fail to be eliminated in thymus are also removed from the periphery by Fas in order to prevent autoimmunity. The positive and negative selection of lymphocytes in the thymus, however, is not regulated by Fas since these events are normal in mice with a non-functional mutation in the Fas gene (*lpr* mice) [Nagata & Golstein, 1995].

The nonfunctional mutations in the mouse Fas (*Lpr*) and FasL (*gld*) demonstrate the vital role of Fas signaling pathway in immune cell homeostasis. The *lpr* or *gld* mutant mice have an increase in the number of activated T and B cells, and an increase in the number of double negative T-cells (Nagata & Golstein, 1995). They also display an autoimmune

syndrome which could be due to the failure of the Fas system to eliminate self-reactive T-cells from the periphery (Puck & Straus, 2004). These mice have a phenotype characterized by lymphadenopathy, splenomegaly, and glomerulonephritis. Humans with a similar phenotype and a resistance to Fas mediated apoptosis have also been described. This condition in humans is termed the autoimmune lymphoproliferative syndrome (ALPS) which can result from a mutation in Fas or FasL gene. ALPS displays many of the same symptoms as *lpr* and *gld* mice (Bleesing *et al.*, 2002).

1.3 THE BCL-2 FAMILY PROTEINS

The Bcl-2 family proteins are regulators of cell survival and death. These proteins maintain essential checkpoints in the mitochondrial mediated or intrinsic cell death pathway. Bcl-2, the first member of the family was identified as an oncogene in B-cell lymphoma, *t*(14:18). Bcl-2 is a proto-oncogene normally located on chromosome 18. In B-cell lymphoma, however, the Bcl-2 gene translocates to chromosome 14, putting Bcl-2 close to the enhancer of the immunoglobulin heavy chain locus. This enhancer is very active in B-cells (antibody producing cells) and as a result, the Bcl-2 protein is expressed at an abnormally high level in these *t*(14:18) B cell Lymphomas. How is it possible that a gene involved in cell survival contributes to oncogenesis? Until the discovery of Bcl-2 as a cell survival protein with oncogenic potential, the consensus amongst the scientific community was that oncogenesis was caused only by unregulated cell growth and the discovery of Bcl-2 gene dramatically changed this whole notion. Now it is widely recognized that mutations in genes involved in regulation of apoptosis can also contribute to tumorigenesis.

The Bcl-2 family proteins are evolutionary conserved in species as diverse as the worm *Caenorhabditis elegans* (*C. elegans*) and mammals. The family consists of members

with opposing functions. While some like Bcl-2 are pro-survival, others like Bax are pro-apoptotic and the interaction between the pro-survival and pro-apoptotic members determines whether cells will survive or die. So far more than 20 different Bcl-2 family members have been identified in mammals whereas in *C-elegans* only one pro-survival (CED-9) and one pro-apoptotic member (EGL-1) have been identified. The worm *C. elegans* is a simple, yet very well characterized model system in which detailed genetic studies have shown that these two Bcl-2 related proteins are essential for controlling developmentally programmed somatic cell deaths (Horvitz, 1999). Expression of EGL-1, the death trigger, is induced due to developmental cue or damage signals. EGL-1 in turn binds to the pro-survival Bcl-2 ortholog, CED-9, and this interaction releases the adaptor CED-4 from CED-9. CED-4 then activates the caspase-like CED-3 leading to apoptosis. Although the core apoptotic machinery is somewhat conserved between worm and mammals, the mammalian system is much more complex.

The mammalian Bcl-2 family proteins are divided into 3 main classes defined by shared features within conserved regions termed Bcl-2 homology (BH) domains (Fig 1.6). The BH domains are short motifs that mediate protein-protein interaction within the family members and are important for apoptosis regulation. The pro-survival CED-9-like members include Bcl-2, Bcl-xL, Mcl-1, A1, and Bcl-W which show conserved homology within 3 to 4 BH domains (BH1, BH2, BH3, BH4). The pro-apoptotic members are further subdivided into two groups, the multi-domain Bax-like pro-apoptotic members and the BH3-only members. The Bax like group which include, Bax, Bak, and Bok share homology within the BH1, BH2, and BH3 domains. Both the anti-apoptotic and the Bax-like pro-apoptotic members also have C-terminal transmembrane domains which target these proteins to

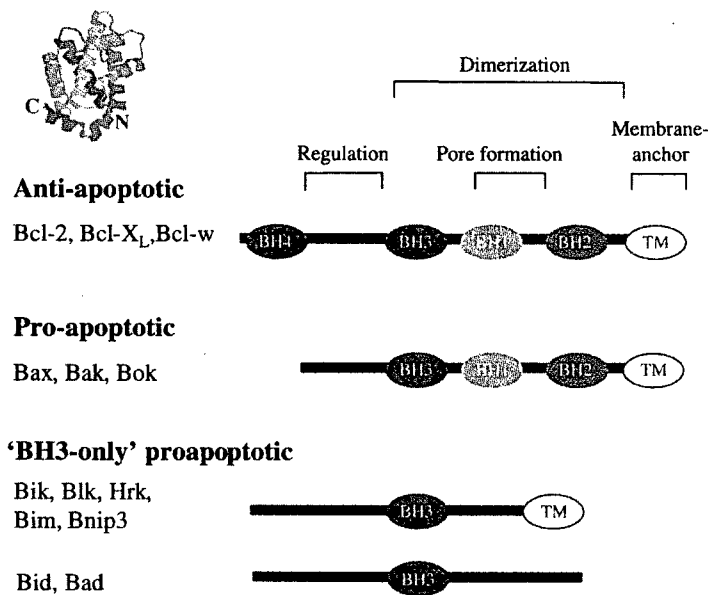


Figure 1.6- The members of the Bcl-2 family. Three subfamilies are indicated: the anti-apoptotic Bcl-2 members promote cell survival, whereas pro-apoptotic and BH3-only members facilitate apoptosis. BH1-BH4 are conserved sequence motifs. Several functional domains of Bcl-2 are shown. A membrane-anchoring domain (TM) is not carried by all members of the family.

different cellular membranes including mitochondrial outer membrane, endoplasmic reticulum and nuclear envelope. The EGL-1 like BH3 only members which have homology only in the BH3 domain include, Bad, Bid, Bim, Bik, Noxa, puma, and Hrk. These proteins are largely unrelated in sequence to either Bcl-2 or each other, except for the 16 amino acid BH3 region, which is essential for their killing function.

The regulation of cell death and survival is mediated through homo and heterodimerization reactions among the family members. The pro-apoptotic Bax-like members and the pro-survival members can homo-dimerism or homo-oligomerize and, in addition, the BH3 domain of the pro-apoptotic members can interact with the hydrophobic domain formed by the BH1, BH2, and BH3 domains of the anti-apoptotic members (Sattler *et al.*, 1997 and Minn *et al.*, 1999). The structure of Bcl-xL monomer revealed that its BH1-

BH3 domains form a hydrophobic cleft which can accommodate the BH3 amphipatic α -helix of the pro-apoptotic members (Muchmore *et al.*, 1996).

1.3.1 The pro-survival Bcl-2 family proteins

The pro-survival Bcl-2 like proteins in mammals include, Bcl-2, Bcl-xL, Mcl-1, Bcl-w and A1. They contain 3 to 4 Bcl-2 homology (BH) domains (BH1-BH4) which are essential for their pro-survival function. These domains do not have any enzymatic activity but are important in mediating the interaction between the Bcl-2-like proteins and their cellular partners. In addition, they also have C-terminal hydrophobic transmembrane domains which help localize them to three cellular membranes: mitochondrial outer membrane, endoplasmic reticulum, and nuclear membranes. Bcl-2 is permanently embedded within the intracellular membranes even in healthy cells, whereas Bcl-W and Bcl-xL are loosely associated to these membranes and become tightly bound only upon induction of apoptosis. Mcl-1 which lacks the BH4 domain is normally found associated with mitochondria.

Solution structure of Bcl-2, Bcl-xL, and the viral homologue from Kaposi sarcoma-associated Herpes virus (v-Bcl-2-KSHV) showed that the BH1-BH3 domains form a hydrophobic groove and the N-terminal BH4 domain is required to stabilize this groove (Aritomie *et al.*, 1997; Muchmore *et al.*, 1996;). A number of studies indicate that mutations in the BH regions destroys the anti-apoptotic function of the Bcl-2-like proteins (Yin *et al.*, 1994; Borner *et al.*, 1994) and loss- and gain-of-function mutations in the *C.elegans* Bcl-2 homologue, CED-9, also map to these regions (Hengartner, 1999; Hengartner and Horvitz, 1994). These studies indicate that the BH1-BH3 hydrophobic grooves are functionally essential for the pro-survival activity of the Bcl-2 like proteins.

Genetic studies indicate that without the Bcl-2 like survival guardians, most cells of

metazoa are destined to die. In *C.elegans*, for example, the Bcl-2 like protein, CED9, is required for embryogenesis because its absence allows the adaptor protein CED4 to activate the caspase-like CED-3 initiating the apoptotic program. In mammalian cells, however, deletion of the Bcl-2 like pro-survival genes leads to an increase in cell death only in specific tissues, possibly due to their redundancy and partially overlapping expression pattern. All the Bcl-2 like pro-survival proteins are more or less essential for maintaining cellular integrity and survival, however, since the focus of this thesis is on Bcl-2, Bcl-xL, and Mcl-1, these proteins will be discussed in more detail next.

1.3.1.1. Bcl-2

B-cell lymphoma/leukemia-2 (Bcl-2) is a proto-oncogene located on human chromosome 18q21. The Bcl-2 gene was originally discovered in the 1980s as a novel transcript associated with the t(18;14) chromosomal breakpoint which occurs in human lymphomas/leukemias. It was later shown that Bcl-2 is ubiquitously expressed in many tissues (Bakhshi *et al.*, 1985; Cleary *et al.*, 1986).

The Bcl-2 gene contains 3 exons and extends over at least 370 Kb. It encodes a protein of 239 amino acids with a molecular weight of 26 KDa. A few domains have been identified on the Bcl-2 protein including the BH1-BH4 domains and also a C-terminal transmembrane domain which is a stretch of 21 hydrophobic amino acids important for docking Bcl-2 protein to the outer mitochondrial membrane, ER membrane, and the nuclear envelope.

Bcl-2 gene expression is regulated by different transcription factors. Kobayashi *et al.* (2006) have recently reported that the transcription factor GATA-4 regulates Bcl-2 expression in cardiomyocytes both *in vitro* and *in vivo*. They also showed that transfection of

HEK 293 cells with GATA4 plasmids activated the Bcl-2 promoter and elevated Bcl-2 protein levels. Gomez *et al.* (1998) reported that IL-2 stimulation of the murine T-cell line, TS1 alphabeta induced expression of Bcl-2 and they suggested that this IL-2 induction of Bcl-2 might be directly or indirectly mediated by the NF-AT transcription factor. Riccio *et al.* (1999) suggested a model in which the nerve growth factor, NGF, promotes survival of neurons, in part through a mechanism involving CREB family transcription factor-dependent expression of Bcl-2 gene.

Over-expression of Bcl-2 prevents apoptosis induced by a wide range of stimuli, for example, UV and g irradiations, treatment with DNA damaging drugs, and cytokine deprivation in a number of different cell lines. In our laboratory, we have over-expressed Bcl-2 in hemopoietic cell lines including the murine mast cell line MC9, murine FDCP-1, and the human erythroleukemia cell line, TF-1, and consistently observed that Bcl-2 provides a survival signal, protecting these cells against apoptosis induced by cytokine withdrawal and a number of other cytotoxic stimuli such as staurosporine and taxol (published and unpublished findings).

Bcl-2 over-expression is common in many human malignancies including prostate, lung, renal, gastric, neuroblastoma, non-Hodgkin's lymphoma, and the acute and chronic leukemias (Selzer *et al.*, 1998). Evidence from transgenic mice that over-express Bcl-2 in B-cells strongly support the hypothesis that inhibition of apoptosis is a critical step in the development of cancer. Mice that over-express Bcl-2 specifically in the lymphoid compartment develop splenic hyperplasia and high survival rate of explanted B-cells. Later in life, these mice acquire other mutations and develop lymphoma.

The Bcl-2 knock out mice are viable, although, half of them die by 6 weeks of age.

Later in life, the animals develop polycystic kidney disease, hair hypopigmentation, and distortion of the small intestine. The kidneys from Bcl-2 knock-out animals are small and have fewer nephrons and their immune system shuts down from a loss of B and T cells due to increased apoptosis (Nakayama *et al.*, 1994; Veis *et al.*, 1993).

1.3.1.2 Bcl-x

Bcl-x, another member of the Bcl-2 family was first discovered through cross hybridization of gene libraries with the Bcl-2 probe (Boise *et al.*, 1993). The Bcl-x gene contains 3 exons and in human and mouse, two major Bcl-x mRNA splice variants with opposite biological functions have been identified, including the pro-survival variant, Bcl-xL, and the pro-apoptotic member, Bcl-xS. The pro-survival variant, Bcl-xL (L=large) is a protein of 233 amino acids which contains all the 4 BH domains (BH1-BH4), whereas, the pro-apoptotic Bcl-x molecule, Bcl-xS (S=short), is a protein of 170 amino acids that lacks the BH1 and BH2 domains (Boise *et al.*, 1993). In addition to the BH domains, the Bcl-x proteins contains a C-terminal transmembrane domain which targets them specifically to the mitochondrial outer membrane (MOM). This domain is different in Bcl-2 since the Bcl-2 C-terminal transmembrane domain targets the protein non-specifically to different intracellular membranes including the MOM, the ER membrane, and the nuclear envelope (Kaufmann *et al.*, 2003).

The transcription of the Bcl-x gene is complex and regulated by multiple factors in multiple tissues. The Bcl-x gene contains a number of tissue specific transcription start sites and a multiple consensus binding sites for both ubiquitous and tissue-specific transcription factors. Socolovsky *et al.* (1999) showed that erythropoietin-induced activation of STAT-5 mediated the immediate early induction of Bcl-xL in erythroid cells through direct binding of

STAT5 to the Bcl-x promoter. The direct induction of Bcl-xL by STAT5 provides a direct mechanism for growth factor mediated regulation of the Bcl-2 gene family. In the same line, the STAT3 transcription factor has also been linked to up-regulation of Bcl-xL (Haga *et al.*, 2003). Chen *et al* (2000) demonstrated that stimuli which activate NF-kappaB (NF-KB) also up-regulate Bcl-xL and this was mediated through direct activation of the Bcl-xL gene by NF-KB. GATA-1 has also been implicated in the activation of Bcl-xL gene expression (Yu *et al.*, 2005).

The Bcl-xL protein has a high sequence homology with its pro-survival counterpart, Bcl-2, and like Bcl-2, it inhibits apoptosis induced by a wide range of stimuli (Boise *et al.*, 1993; Grillot *et al.*, 1995; Schott *et al.*, 1995; Decaudin *et al.*, 1997; Hatano *et al.*, 2001; Rashmi *et al.*, 2004). We have shown in our laboratory that similar to Bcl-2, over-expression of Bcl-xL promotes survival against cytotoxic stimuli as diverse as ceramide treatment or cytokine withdrawal in a number of different cell lines (published and unpublished observations). In contrast, over-expression of Bcl-xS antagonizes Bcl-2 and Bcl-xL, and promotes apoptosis (Boise *et al.*, 1993; Minn *et al.*, 1996).

Increased levels of Bcl-xL are detected in a variety of human cancers including acute myelogeneous leukemia (Pallis *et al.*, 1997), Hodgkin lymphoma (Schlaifer *et al.*, 1995), non-Hodgkin lymphoma (Hermann *et al.*, 1997), Renal carcinomas (Gobe *et al.*, 2002), and pancreatic cancer (Ghaneh *et al.*, 2002). The Bcl-xL transgenic animal models provide insight into tumor promoting activity of Bcl-xL. Recently, Linden *et al.* (2004) showed that targeted over-expression of Bcl-xL in the B-lymphoid cells in mice resulted in lymphoproliferative disease and plasma cell malignancies. In a mouse model of multistage tumorigenesis of islet β -cells Naik *et al* (1996) reported that Bcl-xL over-expression

protected most oncogene expressing cells from apoptosis, enhancing progression from the angiogenic progenitor stage to solid tumor formation. In another mouse model, a Bcl-xL transgene promotes malignant conversion of chemically initiated skin papillomas (Pena *et al.*, 1998).

Analysis of the Bcl-x knock out animals revealed that the Bcl-x gene is essential for embryogenesis. In contrast to the Bcl-2 knockout, the Bcl-x knockout is embryonic lethal and Bcl-x deficient mice die by embryonic day 13 (E13) with massive apoptosis in the brain and hemopoietic tissues (Motoyama *et al.*, 1995). Ma *et al.* (1995) showed that elimination of Bcl-x specifically in the lymphoid compartment interferes with the function of immune system more extensively than when the Bcl-2 gene is deleted. The number of Bcl-x^{-/-} B and T cells drops dramatically in these animals and the mice become highly susceptible to infection. Considering that the phenotype of the Bcl-2 and Bcl-x knockouts are different, then these proteins must have overlapping but not identical functions.

1.3.1.3 Mcl-1

The pro-survival Bcl-2 family protein, Mcl-1, was originally identified by Kozapas *et al.* (1993) as an early induction gene in a differentiating myeloid leukemia cell line, ML-1. Human Mcl-1 is located on chromosome 1q21 and contains 3 coding exons. The Mcl-1 protein consists of at least 3 different isoforms. The full-length Mcl-1 consists of 350 amino acid residues and contains the BH1-BH3 but it lacks the N-terminal BH4 domain which is present in both Bcl-2 and Bcl-xL. It also contains the C-terminal transmembrane domain which targets the protein to different intracellular membranes, in particular, the mitochondrial outer membrane (Yang *et al.*, 1995). The localization of full-length Mcl-1 to the mitochondrial outer membrane is believed to contribute to the pro-survival function of

Mcl-1 through maintaining the mitochondrial membrane integrity. Alternative splicing of the full-length Mcl-1 gives rise to a shorter isoform, Mcl-1S, which contains 271 amino acid residues. Mcl-1S is generated from skipping of the second Mcl-1 exon leading to a frame shift downstream of the BH3 domain and the loss of BH1, BH2, and the C-terminal transmembrane domain in the translated protein (Bae *et al.*, 2000; Bingle *et al.*, 2000). Bae *et al.* (2000) reported that Mcl-1S functioned as a pro-apoptotic protein when over-expressed in Chinese Hamster ovary cells. This induction of apoptosis by Mcl-1S was abrogated by over-expression of full-length Mcl-1. Jamil *et al.* (2005) from our laboratory recently identified a variant of Mcl-1, snMcl-1 (short nuclear mcl-1) which is possibly generated via proteolysis of the full-length Mcl-1. The snMcl-1 protein has not yet been fully characterized but it is predicted that the protein lacks the C-terminal transmembrane domain. This Mcl-1 variant was mainly localized to the nucleus where it co-immunoprecipitated with the cell cycle regulatory protein, Cdk-1, possibly having an inhibitory effect on activity of the kinase. Since Mcl-1 has an inhibitory effect on cell growth (Fujise *et al.*, 2000; Jamil *et al.*, 2005), it is possible that inhibition of the Cdk-1 activity by snMcl-1 might account for growth inhibition by Mcl-1.

Unlike other members of the Bcl-2 family such as Bcl-2 with a half life of 10-14 hrs (Kitada *et al.*, 1993), Mcl-1 has a very short half life ranging from 30 min to 90 min. Mcl-1 has an extended N-terminal domain rich in PEST (proline, glutamic acid, serine, threonine) sequences that could account for the short half-life of the protein (Kozopas *et al.*, 1993) since the PEST sequences are known to frequently signal rapid protein turnover (Rogers *et al.*, 1986). Recent data, however, question the importance of the PEST domains in regulating Mcl-1 turn over (Akgul *et al.*, 2000). The Mcl-1 protein level rapidly decreases in response to

stimuli that induce apoptosis including cytokine withdrawal (Chao *et al.*, 1998), viral infection (Cuconati *et al.*, 2003), and UV irradiation (Nijhawan *et al.*, 2003). The disappearance of Mcl-1 is consistently associated with the onset of apoptosis and is achieved by combination of synthesis block and continuous degradation (Cuconati *et al.*, 2003; Nijhawan *et al.*, 2003). The degradation of Mcl-1 in HeLa cells can be prevented by proteasome inhibitors, suggesting a role for ubiquitin-mediated proteasome pathway in regulation of Mcl-1. Zhong *et al.* (2005) recently identified a BH3 containing E3 ubiquitin ligase Mule (Mcl-1 ubiquitin ligase E3) which interacts with Mcl-1 and mediates polyubiquitination of Mcl-1 followed by Mcl-1 degradation via the proteasome mediated pathway during DNA damage induced apoptosis in HeLa cells.

Mcl-1 expression can be stimulated by a variety of growth factors or cytokines including, IL3, IL5, IL6, IL7, stem cell factor (SCF), granulocyte-macrophage colony stimulating factors (GM-CSF), vascular endothelial growth factor, and epidermal growth factor (Altmeyer *et al.*, 1997; Chao *et al.*, 1998; Huang *et al.*, 2000; Jourdan *et al.*, 2000; Leu *et al.*, 2000). A few signal transduction pathways has been implicated in induction of Mcl-1 expression including the mitogen-activated protein kinase (MAP kinase) dependent pathway acting on SRF/ELK-1 (Townsend *et al.*, 1998; Townsend *et al.*, 1999), the PI3-K/AKT dependent pathway through phosphorylation and activation of CREB (Wang *et al.*, 1999), and the p38 MAP kinase dependent pathway via phosphorylation and activation of PU.1 (Wang *et al.*, 2003). In addition, published results from our laboratory indicate that the PI3 kinase signaling pathway is involved in the regulation of Mcl-1 protein translation (Schubert and Duronio, 2001).

Increased expression of endogeneous full-length Mcl-1 is associated with the

maintenance of cell viability (Lomo *et al.*, 1997; Moulding *et al.*, 1998). Mcl-1 can provide acute protection against apoptosis induced by a variety of stimuli including DNA damage, viral infection, cytokine withdrawal, and cytotoxic drugs (Cuconati *et al.*, 2003; Derouet *et al.*, 2004; Huang *et al.*, 2000; Le Gouill *et al.*, 2004; Nijhawan *et al.*, 2003; Zhang *et al.*, 2002; Zhou *et al.*, 1997). Over-expression of Mcl-1 results in increased cell survival as noted in murine FDCP-1 pro-myeloid leukemia cell line and Chinese Hamster Ovary cells (Reynolds *et al.*, 1994; Zhou *et al.*, 1997). In our hands, over-expression of Mcl-1 in FDCP-1 cells renders transient protection against apoptosis induced by IL-3 withdrawal, albeit to a lesser degree compared to over-expression of either Bcl-2 or Bcl-xL (our laboratory observations). In mice that express full-length Mcl-1 as a transgene in the hemopoietic compartment, lymphoid (B and T) and myeloid cells show increased survival, and the mast cells and monocytes dramatically live longer (Zhou *et al.*, 1998). When these animals were monitored for an extended period of time (Zhou *et al.*, 2001), they developed lymphoma with a long latency and a high probability (more than 85% for over two years). Over-expression of Mcl-1 has been found in a variety of human malignancies and often correlates with adverse clinical outcome (Zhang *et al.*, 2002). Increased level of Mcl-1 has been detected, for example, in prostate cancer (Krajewska *et al.*, 1996), B cell chronic lymphocytic leukemia (Kitada *et al.*, 1998), and leukemic relapse in AML and ALL (Kaufmann *et al.*, 1998).

Gene knockout studies have established an essential role for Mcl-1 in embryogenesis and lymphocyte homeostasis. Conditional knockouts of Mcl-1 in thymus and spleen results in a dramatic decrease in the number of B and T cells (Opfermann *et al.*, 2003), and Mcl-1 also plays an essential role in the survival of hemopoietic stem cells (Opfermann *et al.*, 2005). In addition, complete deletion of Mcl-1 gene in mice leads to pre-implantation

embryonic lethality (Rinkenberger *et al.*, 2000). The Mcl-1(-/-) embryos do not implant in utero but the blastocysts could be recovered at E3.5-4.0. Interestingly, these null blastocysts showed no evidence of increased apoptosis suggesting that Mcl-1 has a function beyond regulating apoptosis.

In addition to its interaction with the pro-apoptotic Bcl-2 family proteins such as Bak, Bim, Puma, and Noxa, (Chen *et al.*, 2005; Wang *et al.*, 1998), Mcl-1 has been shown to associate with the cell cycle regulatory proteins such as Proliferating Cell Nuclear Antigen (PCNA) and the Cyclin dependent kinase-1 (Cdk-1). The possible role of Mcl-1 in cell cycle regulation was first noted by Fujise *et al.* (2000) who showed that Mcl-1 over-expression significantly inhibited BrdU incorporation. This inhibition of cell cycle progression was linked to an association between Mcl-1 and PCNA since mutants of Mcl-1 which lacked the PCNA binding motif could not inhibit cell cycle progression as effectively as wild type Mcl-1. PCNA plays an important role in DNA replication through interaction with DNA polymerase Delta and directing the polymerase to RNA priming site, a process required for initiation of DNA replication. Thus, by inhibiting PCNA, Mcl-1 may partially prevent initiation of DNA synthesis required for progression through the S-phase of the cell cycle. Jamil *et al.*, (2005) identified a shortened form of Mcl-1, snMcl-1 that prevents progression through the G2/M phase of the cell cycle, possibly via its interaction with Cdk-1. The cyclin partner of Cdk-1, Cyclin B1 is absent from the immunocomplex formed between snMcl-1 and Cdk-1 and the Cdk-1 bound to Mcl-1 was found to have a lower kinase activity. The Mcl-1 over-expressing cells not only had a lower rate of proliferation, but they also had less total Cdk-1 kinase activity compared to parental cells. Given the important role of Cdk-1 in progression through the G2/M phase of the cell cycle, it was concluded that the inhibition of

Cdk-1 by snMcl-1 might account for the inhibitory effect of Mcl-1 on cell proliferation. It remains to be determined what other cell cycle regulatory proteins interact with Mcl-1 and whether the Mcl-1/PCNA and Mcl-1/Cdk-1 interactions are mechanistically linked.

In addition, an ongoing investigation in our laboratory links Mcl-1 to the regulation of the DNA damage checkpoint control. We have detected interaction between Mcl-1 and components of the DNA damage checkpoint control including the checkpoint kinase-1 (Chk-1), and the phosphorylated histone H2AX and we believe that this potential function of Mcl-1 in the regulation of DNA damage checkpoint control might help explain why Mcl-1 deficiency leads to pre-implantation embryonic lethality in mice. The mechanism of DNA damage checkpoint control will be discussed later in the introduction.

1.3.2. Multidomain Bax-like pro-apoptotic Bcl-2 family members, executioners of cell death

The Bax-like proteins, Bax, Bak and Bok are examples of the multidomain pro-apoptotic Bcl-2 family proteins containing 3 BH domains, BH1-BH3 (Adams and Cory, 2001). Interestingly, no Bax-like homologue has been identified in *C.elegans*. The lack of BH4 domain in the Bax-like group compared to the pro-survival members was initially thought to be one reason why these proteins are pro-apoptotic. Since the BH4 domain stabilizes the hydrophobic pocket formed by the BH1-BH3 domains, its absence might unfold this region leading to conformational changes in the protein that confers pro-apoptotic activity. However this notion is debatable for the following reasons. First, although the pro-survival Bcl-2 family members, A1 and Mcl-1, lack the BH4 domain, they induce cell survival very effectively (Adams and Cory, 1998). Second, structural comparison between Bcl-2 and Bax indicates Bax has a degenerate BH4 like domain at its N-terminus. Finally,

Bcl-xs, the pro-apoptotic spliced variant of Bcl-xL, retains the BH4 domain (Boise *et al.*, 1993) but induces apoptosis when over-expressed (Clarke *et al.*, 1995; Lindenboim *et al.*, 2000). Hence, the absence of BH4 domain in the Bax-like group cannot entirely explain why these proteins are pro-apoptotic. At this point it is not clear what additional mechanisms might determine that Bax-like death factors induce opposite effect than the Bcl-2 like pro-survival factors.

Bax and Bak are essential for apoptosis in many cell types although it needs to be established whether this is true for all cell types. Bax and Bak are widely distributed in many tissues whereas Bok (Bcl-2 associated ovarian killer) has limited tissue distribution being more prevalent in the reproductive tissues. Even though loss of either Bax or Bak has little consequences in mice possibly due to functional redundancy, elimination of both genes severely impairs developmental apoptosis in many tissues leading to prenatal death (Lindsten *et al.*, 2000) and cells lacking both molecules cannot be induced to undergo apoptosis mediated by BH3 only proteins (Zong *et al.*, 2001). Mice deficient in both Bak and Bax in the hemopoietic compartment show that Bak and Bax are required for thymic selection and peripheral lymphoid homeostasis (Rathmell *et al.*, 2002).

Bax-like pro-apoptotic proteins are stringently regulated at the post-transcriptional level. In healthy cells, Bax is a monomeric protein which is kept in an inactive state in the cytosol (Desagher *et al.*, 1999). In its inactive state, the hydrophobic carboxy terminal end of the molecule occludes its hydrophobic BH1-BH3 groove which is needed for Bax pro-apoptotic activity (Suzuki *et al.*, 2000).

Apoptotic signals induce conformational changes in Bax molecule causing its translocation and integration into the mitochondrial outer membrane and its subsequent oligomerization.

Since the hydrophobic carboxy terminus is essential for targeting Bax to the intracellular membranes, particularly the mitochondrial membrane, stress signals then provoke the C terminus to flip out mediating Bax integration into the mitochondria and its subsequent oligomerization (Hsu *et al.*, 1997; Wolter *et al.*, 1997). Apoptotic stimuli also induce conformational changes in the N- terminus of Bax since antibodies specific to an epitope in this region interact with Bax in apoptotic but not healthy cells (Desagher *et al.*, 1999). Whether release of the C-terminal tail, exposure of the N-terminal epitope, translocation to the intracellular membranes, and oligomerization occur simultaneously or sequentially is not yet clear. These steps are essential for initiation of apoptosis and keeping Bax dormant in the cytosol guarantees against unwanted activation of the apoptotic program. In agreement with this notion, Bax mutants constitutively localized at the mitochondrial membrane kill more potently than wild type Bax (Suzuki *et al.*, 2000) and enforced Bax dimerization induces apoptosis (Gross *et al.*, 1998). In contrast to Bax, Bak is an oligomeric integral membrane protein. Upon initiation of cell death, Bak also undergoes conformational changes to form larger aggregates. Interestingly, antibodies directed against an epitope in the N-terminus of Bak, interacted with this molecule only in the stress-induced apoptotic but not healthy cells demonstrating that, similar to Bax, the N-terminal region of Bak undergoes a conformation change triggered by stress stimuli (Griffiths *et al.*, 1999; Nechushtan *et al.*, 2001; Wei *et al.*, 2000).

It is not yet clear how these Bak and Bax homo-oligomers form. It is possible that the BH3 domain of one Bax-like molecule interact with the hydrophobic groove formed by the BH1-BH3 of another Bax-like molecule in such a way that one Bax molecule assume a "BH3 donor" conformation and another Bax molecule assume a "BH3 acceptor" conformation

within the membrane environment. Alternatively, some Bax (or Bak) molecules might anchor to the mitochondrial membrane via the C-terminal transmembrane tails and this would allow others to assemble on them through intermolecular association between the BH1-BH3 hydrophobic grooves and the extruded hydrophobic tails. In addition, some evidence suggests that reorganization of Bak requires Bax and their separate homo-oligomers may interact (Mikhailov *et al.*, 2003).

The structural similarities of Bax to the pro-survival Bcl-2 family proteins has encouraged the notion that some BH3 only pro-apoptotic Bcl-2 family members, for example, Bid and Bim might directly engage Bax, thus regulating its activity. At the moment, there is no conclusive evidence that these BH3 only proteins directly bind Bax *in vivo*. *In vitro*, however, Bid, induces a conformational change in Bax leading to exposure of Bax N-terminal epitopes (Desagher *et al.*, 1999). This conformational change in Bax might further change the three dimensional structure of the molecule, unfolding its C-terminal hydrophobic tail and exposing the BH3 domain, which are the events required for Bax translocation and oligomerization at the mitochondrial membrane. Alternatively, Bid might directly interact with Bax at the mitochondrial membrane. In agreement with this, Desagher *et al.* (1999) showed that during certain types of apoptosis, Bid translocates to mitochondria and binds to Bax, leading to a change in conformation of Bax. Kuwana *et al.* (2002) showed that peptides corresponding to the BH3 domains of Bim and Bid can directly activate Bax to permeabilize liposomes. So far, the evidence for the direct interaction of Bid and Bim with the Bax-like proteins has not been conclusive. The absence of t-Bid in the oligomeric Bax complexes led to the suggestion that Bid interacts with Bax in a “hit and run” fashion. Interestingly, in Bid deficient cells, Bax could still translocate and insert into the mitochondrial membrane

(Ruffolo *et al.* 2000), raising the possibility that some Bid-independent mechanisms might be involved in Bax regulation.

Additional mechanisms that contribute to the regulation of Bax in the cytosolic compartment have been suggested. The 14-3-3- σ has been identified as a Bax-interacting protein retaining Bax in the cytosol. The expression of 14-3-3- σ in human colorectal tumor cells caused G2 arrest and prevented apoptosis when cells were exposed to DNA damaging agents (Samuel *et al.*, 2001). Three other members of the family (θ , ϵ , ζ) were also proposed to sequester Bax in the cytosol (Nomura *et al.*, 2003). Caspase cleavage of 14-3-3 θ and phosphorylation of 14-3-3 proteins by the c-Jun NH2-terminal kinase (JNK) were suggested as mechanisms promoting the dissociation of Bax, allowing its translocation to mitochondria (Nomura *et al.*, 2003; Tsuruta *et al.*, 2004). In agreement with this, inhibition of proteins of the 14-3-3 family leads to apoptosis (Masters and Fu, 2001), but the mechanisms involved could be numerous, as they are also known to bind to several other proteins involved in survival and in death signaling, including another member of the Bcl-2 family, Bad (Zha *et al.*, 1996). Recently, two additional proteins, Humanin (HN) and KU70, have been proposed to be involved in retention of Bax in the cytosolic compartment. Humanin was shown to prevent translocation of Bax to mitochondria and conversely, reducing HN expression by small interfering RNAs (SiRNA) sensitized cells to Bax and increased Bax translocation to membranes (Guo *et al.*, 2003). In addition, the KU70 protein, also involved in DNA repair, has been shown to play an important role in inhibiting Bax translocation to the mitochondrial membrane. Sawada *et al.* (2003) who identified KU70 as a Bax inhibitor showed that in healthy cells, a large proportion of Bax was found in complex with KU70 and over-expression of KU70 prevented Bax translocation from cytosol to mitochondria under

conditions of apoptotic stress. Thus, it is possible that pro-apoptotic stimuli release 14-3-3, Humanin, KU70, or other cytosolic Bax inhibitors, from the Bax molecule, leading to conformational changes in Bax and its translocation to the mitochondrial outer membrane.

Once activated, Bax like proteins are believed to cause permeabilization of the outer mitochondrial membrane and the mitochondrial release of cytochrome C, in addition to other apoptogenic factors (for example, Smac/Diablo, Omi/HtrA2, endonuclease G, AIF), which complexes with Apaf-1 and ATP in the cytosol to activate caspases initiating the apoptotic program. Elegant in vitro experiments showed that Bax induces Cytochrome C release from isolated mitochondria (Jurgensmeier, 1998; Finucane *et al.*, 1999; Narita *et al.*, 1998). In addition, the in vitro experiments established that Bax must be in oligomerized form in order to induce outer mitochondrial membrane permeability (Antonsson *et al.*, 2000; Desagher *et al.*, 1999). Furthermore, when isolated mitochondria were incubated with recombinant Bax, it was demonstrated that Bax was able to induce the release of other apoptogenic factors, i.e., Smac/Diablo and Omi/HtrA2, from the mitochondria (Arnoult *et al.*, 2003). A few models have been proposed that describe the possible actions of Bax in induction of mitochondrial membrane permeabilization (MMP). One model which is based on the structural similarity between the Bcl2 family members and the pore forming bacterial toxins, for example diphtheria toxin, is that Bax and Bak form channels in the mitochondrial membrane large enough for the passage of cytochrome C and even much larger molecules such as AIF. *In vitro*, Bax is able to form channels in lipid membranes without any additional proteins (Antonsson *et al.*, 1997) and the “Bax alone” channels are able to permeabilize lipid membranes to cytochrome C (Saito *et al.*, 2000). The BH3 only protein, Bid, is believed to synergize with Bax to increase the size of pore formation by Bax. Kuwana *et al.* (2002)

showed that Bid, or its BH3-domain peptide, activated monomeric Bax to produce membrane openings that allowed the passage of very large (2 megadalton) dextran molecules, explaining the translocation of large mitochondrial proteins during apoptosis. This process required the mitochondrial lipid, cardiolipin, and was inhibited by anti-apoptotic Bcl-xL. Recently, a new high conductance channel, mitochondrial apoptosis-inducing channel (MAC), was identified in mitochondria from apoptotic cells (Pavlov *et al.*, 2001) and the electrophysiological properties of this channel resembles those of the high conductance Bax channel. Other models suggest that Bcl-2 family proteins might interact with the components of mitochondrial permeability transition (MPT) pore to create a large channel in the membrane. MPT is characterized by massive swelling of the mitochondria followed by rupture of the outer mitochondrial membrane, depolarization of the inner mitochondrial membrane, and the uncoupling of the oxidative phosphorylation that results in the loss of ATP synthesis (Zamzami and Kroemer, 2001). The mitochondrial permeability transition pore complex (PTP) consists of a number of molecules including the Voltage Dependent Anion Channel (VDAC) in the outer mitochondrial membrane, Adenine Nucleotide Translocase (ANT) in the inner membrane, and Cyclophilin D in the matrix. The channel is permeable to molecules up to 1500 Da and the pores in the inner and outer membranes seem to be gated. Apoptotic stimuli such as oxidative stress or calcium overload open the PTP. This in turn opens an ion channel in the inner mitochondrial membrane that deflates the proton gradient and allows water to enter the matrix swelling the mitochondria. It is hypothesized that Bax interacts with the components of the PTP complex and increases its pore size to an extent that it can release very large apoptogenic molecules such as cytochrome C (15 Kda), AIF (57 Kda), and Smac/Diablo (25 Kda) from the inter- membrane

space into the cytosol. Indeed Bax has been reported to induce MPT in cells upon induction of apoptosis (Jacotot *et al.*, 1999). Bax can physically interact with VDAC when co-expressed in yeast or mammals and it can induce opening of VDAC in synthetic liposomes (Shimizu *et al.*, 1999). In addition, inhibitors of MPT such as cyclosporine A and oligomycin inhibited Bax mediated VDAC opening (Narita *et al.*, 1998). Another member of the PTP complex, ANT, which is involved in the exchange of ATP for ADP across the inner mitochondrial membrane has also been implicated in Bax mediated apoptosis (Marzo *et al.*, 1998). Bax and Bcl-2 have been reported to bind to peptides of the ANT by yeast two-hybrid screening and the ANT inhibitors also had an effect on apoptosis (Jacotot *et al.*, 1999). Based on these models, Bax-like factors either form channels or interact with channel-forming proteins to increase the permeability of the outer mitochondrial membrane. While Bax channels may release relatively small molecules such as cytochrome *c*, mixed Bax/VDAC or Bax/ANT channels could deliver larger molecules such as Smac/DIABLO and Htr2A/Omi.

Bax and Bak are also involved in apoptosis initiated from the ER in response to stresses imposed by ER calcium overload, and they might function directly on the ER as well as the mitochondria. When Bax and Bak are over-expressed in human PC-3 cells, they induce release of calcium (Ca^{2+}) from the ER Ca^{2+} pool concomitant with an increase in the mitochondrial Ca^{2+} level (Nutt *et al.*, 2002a;). Interestingly, co-expression of Bcl-2/Bcl-xL blocks this Ca^{2+} mobilization and an inhibitor of mitochondrial Ca^{2+} uptake, RU360, blocks Bax/Bak and staurosporin-induce cytochrome C release and apoptosis (Nutt *et al.*, 2002a). Furthermore, the Bax deficient DU145 cells, are resistant to staurosporin-induced ER Ca^{2+} release, uptake of Ca^{2+} from mitochondria, and cytochrome C release, all of which is overcome by re-introduction of Bax (Nutt *et al.*, 2002b). In addition, the ER directed Bcl-2

(Bcl-cb5) as well as Bax inhibitor-1, which is an anti-apoptotic transmembrane ER protein, can both protect against Bax-induced apoptosis (Wang *et al.*, 2001; Xu and Reed, 1998). Thus Bax and Bak have dual role at the ER and mitochondria and regulate apoptosis from both sites, possibly through regulation of Ca^{2+} flux between ER and the mitochondria.

In fact, members of all 3 classes of Bcl-2 proteins are localized to the ER, including the pro-survival member Bcl-2, Bax and Bak, and the pro-apoptotic BH3 only members such as Bik. These proteins have been shown to influence ER homeostasis, possibly by influencing membrane permeability and Ca^{2+} homeostasis. For example, Bcl-2 has been reported to physically interact with the ER Ca^{2+} channel, IP3R-1, and regulate the amount of Ca^{2+} released from this channel (Oakes *et al.*, 2005). Interestingly, this physical interaction is enhanced when cell are deficient in both Bax and Bak (Oakes *et al.*, 2005). It is hypothesized that Bax and Bak may regulate ER Ca^{2+} by binding to and displacing Bcl-2 from IP3R-1. Furthermore, a number of ER proteins have been shown to influence apoptosis by interacting with Bcl-2 proteins at the ER (Tagami *et al.*, 2000; Ng *et al.*, 1998; Ng *et al.*, 1997; Torgler *et al.*, 1997). For example, both Bcl-2 and Bcl-xL interact with the pro-apoptotic ER integral membrane protein, Bap-31 (Ng *et al.*, 1997), and possibly regulate its pro-apoptotic activity. In short, the Bcl-2 proteins regulate ER homeostasis by regulating ER Ca^{2+} channels activity and also through interaction with ER resident proteins.

1.3.3 BH3 only death factors: the sensors and mediators of apoptosis

The BH3 only proteins are proximal sensors that sense developmental cues, DNA damage, or stress signals and relay the information to the downstream effectors to activate the apoptotic program. Genetic studies in *C.elegans* and mice indicate that the BH3 only proteins are essential initiators of programmed cell death. In *C.elegans*, developmental death

cues or DNA damage induce transcription of the single BH3-only protein, EGL-1, which binds to and inactivates the pro-survival Bcl-2 homolog, CED-9. This frees the adaptor protein CED-4 from sequestration by CED-9, allowing CED-4 to activate the caspase homolog CED-3 at the cytosolic face of the nuclear envelope to initiate apoptosis. In the absence of EGL-1, all developmentally programmed death of somatic cells are abrogated in *C.elegans* emphasizing on the essential role of EGL-1 in apoptosis. Mammals, however, have multiple numbers of BH3 only proteins including Bim, Bid, Bik, Bad, Bmf, Puma, Noxa, and Hrk. Hence, the multiplicity as well as tight regulation of these proteins allow for more sophisticated control over apoptosis. Specific BH3 only proteins are expressed within certain cell types. Some are believed to monitor specific cellular compartments for damage or stress, and/or to respond to particular set of cytotoxic signal. Studies in mice indicate that Bim is critical for normal homeostasis of lymphoid and myeloid cells (Bouillet *et al.*, 2002). It is also essential for apoptosis induced by cytokine withdrawal or deregulated calcium flux but not markedly for apoptosis induced by g-irradiation (Bouillet *et al.*, 1999). Bad is required for the death that follows glucose deprivation (Denial *et al.*, 2003) and to some extent for growth factor withdrawal-induced apoptosis (Ranger *et al.*, 2003). Noxa and Puma, which are both induced by the tumor suppressor p53, appear to be involved in DNA damage-induced apoptosis. Bmf is required for death induced by anoikis, the apoptosis caused by detachment of epithelial cells from the extracellular matrix (Puthalakath *et al.*, 2002). Bid acts as an amplifier of the death receptor signaling and as will be discussed later, it plays a prominent role in hepatocytes but not lymphocytes (Yin *et al.*, 1999).

To prevent unnecessary cell death, the BH3 only proteins are tightly regulated by multiple means (Cory *et al.*, 2003, Danial and and Korsmeyer 2004). Some BH3 only

proteins are subject to transcriptional control. For example Puma and Noxa are induced by P53 in response to DNA damage (Nakano and Vousden, 2001; Oda *et al.*, 2000) and Hrk/DP5 is induced in neurons upon growth factor deprivation or exposure to β -amyloid proteins (Imaizumi *et al.*, 1999). Transcription of Bim is induced in cytokine starved hematopoietic cells, in part by the FKHRL1/FOXO3a transcription factor (Dijkers *et al.*, 2000) and in cytokine starved neuronal cells through activation of JNK (Harris and Johnson, 2001; Putcha *et al.*, 2001). Other BH3 only proteins that are constitutively expressed are kept dormant until activated by post transcriptional modifications. For example, growth factors induce phosphorylation of Bad at multiple sites (Zha *et al.*, 1996) and phosphorylated Bad is subsequently sequestered away from the mitochondria by the 14-3-3 scaffold protein. Apoptotic activation of Bad requires its dephosphorylation for example by Calcineurin (Wang *et al.*, 1999). On the other hand, Bik is activated when phosphorylated possibly by a casein kinase II related enzyme and its dephosphorylation reduces its apoptotic activity (Verma *et al.*, 2001). Bid is negatively regulated by phosphorylation via Casein kinase I (CK1) and Casein kinase II (CK2) and a mutant of Bid that cannot be phosphorylated was found to be more toxic than wild-type Bid (Desagher *et al.*, 2001). Bid induces apoptosis downstream of the death receptor pathway and its full activation requires its truncation by caspase 8 or Granzyme B to expose its BH3 domain. The truncated P15 Bid (tBid) is then N-myristoylated enhancing its translocation to mitochondrial membrane to induce apoptosis (Zha *et al.*, 2000).

Bim and Bmf appear to be involved in guarding the integrity of the cell cytoskeleton. Alternative splicing in Bim gives rise to three Bim variants: BimS, BimL, BimEL (O'Connor *et al.*, 1998). In healthy cells, both BimL and BimEL, the most predominant isoforms of Bim,

are sequestered in the cytoplasm away from the mitochondria by binding to the 8-KD dynein light chain (DLC1 or LC8), a component of the microtubule-associated dynein motor complex. Certain apoptotic stimuli, cytokine deprivation for example, frees Bim (along with the LC8) from the dynein motor complex and allows Bim to translocate to mitochondria and interact with the pro-survival Bcl-2 proteins, thereby activating the apoptotic program (Puthalakath *et al.*, 1999). BimS does not bind to the dynein motor complex and hence it is a more potent inducer of apoptosis than the other two Bim isoforms; however, the level of BimS in healthy cells is not enough to cause any damage (O'Connor *et al.*, 1998). Another BH3 only protein regulated through interaction with the cell cytoskeleton is Bmf. In healthy cells, Bmf is sequestered to myosin V motor on filamentous actin by association with dynein light chain 2 (DLC2). Certain damage signals, such as loss of cell attachment (anoikis), unleashes Bmf, allowing it to translocate and bind pro-survival Bcl-2 proteins and activate apoptosis. Although similarities exist in regulation of Bim and Bmf, these proteins transduce distinct death signals caused by different forms of cell stress. For example, treatment of cells with paclitaxol which polymerizes microtubules released Bim but not Bmf (Puthalakath *et al.*, 2002), whereas, anoikis, an apoptotic stimulus that affects the actin cytoskeleton (Frisch *et al.* 1997), resulted in the release of Bmf but not Bim (Puthalakath *et al.*, 2002). Thus, different cytotoxic signals trigger activation of specific BH3 only proteins and subsequent binding of these proteins to the pro-survival Bcl-2 family members at the mitochondria neutralizing their pro-survival function, thereby initiating apoptosis

Structural studies have shown that the hydrophobic surface of the amphipathic α -helix formed by the BH3 domain inserts into the hydrophobic groove formed by the BH1, BH2, BH3 domains of the pro-survival proteins (Liu *et al.*, 2003). Previously it was thought that

once the BH3 only proteins are activated, they function similarly by targeting all the pro-survival Bcl-2 family members with equal affinity (Danial and Korsmeyer, 2004; Huang and Strasser, 2000). However, some studies have shown that although some BH3 proteins including Bim and Puma promiscuously bind to all the pro-survival Bcl-2 family members, others such as Bad and Noxa are selective in their interaction with specific pro-survival members. Bad, for example, has a high affinity for Bcl-xL, Bcl-2, and Bcl-w but only weak affinity for A1 and none for Mcl-1, whereas, Noxa binds only with A1 and Mcl-1. The most promiscuous member, Bim, is a potent cell killer since it is able to bind and neutralize all the pro-survival Bcl-2 members. The more selective members, Bad and Noxa, are weak killers by themselves since they could only neutralize certain subset of the pro-survival members. However, once co-expressed, Bad and Noxa became potent killers (Chen *et al.*, 2005). This complementary killing profile of Bad and Noxa suggests that efficient apoptosis might require neutralization of two subsets of pro-survival proteins, one comprising Bcl-2, Bcl-xL, Bcl-w and the other Mcl-1 and A1.

Studies of Bak regulation reveals why efficient killing requires neutralization of two classes of the pro-survival Bcl-2 proteins. Mcl-1 has been implicated in regulation of Bak by its formation of complexes with Bak (Cuconati *et al.*, 2003). However, targeting Mcl-1 by RNAi (Nijhavan *et al.*, 2003) or Noxa over-expression (Chen *et al.*, 2005) failed to kill cells, implicating additional pro-survival relatives. In healthy cells, Bak is kept in check by binding with Mcl-1 and Bcl-xL but not significantly by Bcl-2, Bcl-w, or A1 (Willis *et al.*, 2005). While Noxa displaced Bak from Mcl-1, Bak mediated killing also required its displacement from Bcl-xL by another BH3 only protein such as Bad (Willis *et al.*, 2005). These findings suggest that Bak is restrained solely by both Mcl-1 and Bcl-xL and it has to be freed from

both proteins in order to induce apoptosis. Hence, the BH3 only proteins may activate Bak by freeing it from the pro-survival proteins designed to guard it. The virtually equivalent function of Bak and Bax indicates that Bax might be regulated in a similar manner as well. Although Bak is normally membrane associated in complex with pro-survival Bcl-2 members, Bax is kept in a monomeric form in the cytosol (Cory *et al.*, 2003; Danial *et al.*, 2004). However, it is possible that small proportion of Bax molecule is loosely associated with membranes, particularly the mitochondrial membrane, and hence different pro-survival Bcl-2 proteins might bind with this membrane associated Bax via its BH3 domain keeping Bax in check. Although in the presence of detergents Bax has been shown to interact with all the pro-survival family members, Bax might only interact with specific subsets of the pro-survival proteins inside cells. Thus one could speculate that in order to inactivate Bax, specific pro-survival Bcl-2 family members have to be neutralized by specific BH3 only proteins.

In summary, certain BH3 only proteins are activated by specific cytotoxic stimuli in specific cell types. These proteins then regulate apoptosis through differential interaction with members of the pro-survival Bcl-2 proteins and possibly the Bax-like pro-apoptotic members at the mitochondrial outer membrane. The ratio of the BH3 only proteins to their pro-survival counterparts dictates whether the cell would survive or die.

1.4 DNA DAMAGE CHECKPOINT RESPONSE

Growing evidence suggests that in addition to the regulation of apoptosis, the Bcl-2 proteins might play a role in events such as the cell cycle control, and perhaps even DNA damage checkpoint response (review by Zinkel *et al.*, 2006; Zinkel *et al.*, 2005; Han *et al.*, 2004; Komatsu *et al.*, 2000). Certainly, some components of the DNA damage check point

machinery have been shown to interact with members of the Bcl-2 family proteins including interaction between Rad9 and Bcl-2/Bcl-xL (Komatsu *et al.*, 2000), Phosphorylation of Bid by ATM (Gross, 2006), as well as, phosphorylation of a peptide corresponding to a site in Bad by the DNA check point kinase, Chk-1 (Han *et al.*, 2004). In recent unpublished studies, our laboratory has been investigating the function of Mcl-1, and found that it may play a key role in the DNA damage checkpoint response (S. Jamil and V. Duronio, manuscript in preparation). We have shown that Mcl-1 protein level increases upon treatment of cells with etoposide, a DNA damaging agent, which is believed to initiate a DNA damage check point response. We pondered on the reason why Mcl-1 level would increase by DNA damage. Two possibilities came into mind. When the DNA is damaged, the cells' DNA damage check point response machinery try to repair the damaged DNA and an increase in the level of a pro-survival protein such as Mcl-1 would ensure that the cells would survive during this correction process. Another possibility, which we tried to explore in this thesis, is that Mcl-1 might be involved in the regulation of the DNA damage check point response by interacting with the components of the DNA damage check point machinery, which ties in with the cell growth inhibition observed when Mcl-1 is over-expressed. Here I will present a brief background regarding the components of DNA damage checkpoint control.

Genomic DNA damage is a common event in the life of a cell, which, if undetected, might lead to mutations, cancer, or cell or organism death. Since DNA damage poses a threat to the genetic integrity of organisms, once a DNA lesion is detected, the cell engages a tightly coordinated set of molecular events to either faithfully repair the damage or, if the damage is beyond repair, induce apoptosis. These set of molecular events are termed the DNA damage checkpoint response. The DNA damage checkpoints are specific points in the

cell cycle when the integrity of genomic DNA is examined before allowing progression to the cell cycle.

All eukaryotic cells have 4 phases within their cell cycle which are known as G1, S, G2, and M phases. These phases define unique cellular circumstances that are directed by distinct biochemical events taking place within each phase. The transition between G1/S, and G2/M, as well as progression through the S phase is tightly controlled and available evidence suggest that many of the same proteins involved in the progression through the cell cycle are also involved in the DNA damage checkpoint response. Thus, the DNA damage check point is not a biochemical pathway that is turned on once a damaged DNA is detected, but rather it is an active event that continues during cell cycle progression and is amplified once damage is detected. In other word, the DNA damage check point functions as a constant surveillance and response system that continuously monitors genomic integrity (Nasmyth, 1996).

In higher organisms, the presence of damaged DNA is signaled by a family of protein kinases known as the PI-3 kinase related kinases (PIKKs) which include the Ataxia telangeictasia mutated (ATM), Ataxia telangeictasia related (ATR), and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [Shiloh, 2003]. Upon detection of DNA damage, these PIKK's act as sensors as well as signal transducing molecules, which are recruited to the site of damage through physical associations with specific DNA damage binding proteins- the Mre11/Rad50/Nbs1 (MRN) complex for ATM, ATR interacting protein (ATRIP) for ATR, and a complex of Ku70 and Ku80 for DNA-PKcs (Falck *et al.*, 2005; Lee and Paul, 2005). Once at the site of damage, PIKK's are activated and subsequently transmit the damage signal to their downstream substrates. These PIKK substrates, which are themselves signal transducers, then transduce the damage signal to their downstream

substrates, which act as effectors, causing a delay in the cell cycle. This would allow the DNA damage machinery to repair the DNA damage, and if the damage is beyond repair, to eliminate the cell.

ATM and ATR are the two main checkpoint specific damage sensors in mammals. Mutations in ATM (ataxia telangiectesia mutated) cause ataxia-telangiectesia (A-T) in humans, characterized by cerebellar degeneration, immunodeficiency, genome instability and a predisposition to cancer (Shiloh, 1997). ATM is a serine/threonine (S/T) protein kinase of 350-kDa with a significant sequence homology to PI-3 kinases, but lacks the lipid kinase activity (Shiloh, 1997; Savitsky *et al.*, 1995). ATM is activated by ionizing radiation and agents that induce double stranded DNA breaks (Banin *et al.*, 1998). It is recruited to the site of damage where it preferentially binds to DNA termini (Smith *et al.*, 1999). Once activated, ATM phosphorylates a number of cellular proteins, including the check point kinase-2 (Chk2), P53 (Banin *et al.*, 1998; Canman *et al.*, 1998), and itself (Bakkenist and Kastan, 2003). Auto-phosphorylation of ATM is believed to convert the enzyme from an oligomer to a monomer, which is the active form of ATM (Bakkenist and Kastan, 2003). ATR is also a S/T protein kinase which encodes a protein of 303 KDa with moderate homology to other PIKK family members. ATR deficiency in mice results in embryonic lethality (Brown and Baltimore, 2000), and in human, partial loss of ATR activity is linked to the Seckel syndrome, which shares features in common with A-T (O'Driscoll *et al.*, 2003). ATR is activated by UV irradiation, rather than double stranded break, and is the main PIKK that initiates check point response following UV irradiation (Abraham, 2001). ATR phosphorylates Chk1, and P53 and essentially almost all of the cellular proteins that are phosphorylated by ATM.

The Chk1 and Chk2 are both S/T kinases with moderate substrate specificities. In mammalian cells, the double stranded break induces Chk2 phosphorylation by ATM (Hirao *et al.*, 2000; Matsuoka *et al.*, 2000) and the UV damage induces phosphorylation of Chk1 by ATR (Zhao, 2001). It is worthwhile mentioning that in mice, Chk1 deficiency causes embryonic lethality (Takai *et al.*, 2000), whereas, Chk2 knock-out mice are viable with seemingly normal checkpoint responses (Jack *et al.*, 2002). Since Chk1 and Chk2 have some overlapping functions, this might account for the near normal phenotype of the Chk2 knock-out mice. Once activated, Chk1 and Chk2 transduce the DNA damage signal to their downstream effectors which include p53 and the phosphatase, Cdc25.

Phosphorylation of p53 and Cdc25 by Chk1 and Chk2 results in a delay in the cell cycle with the aim that the DNA damage is repaired. The P53 transcription factor is activated by phosphorylation on two serine residues, on S20 by Chk1/Chk2 and on S15 by ATM/ATR (Banin *et al.*, 1998; Canman *et al.*, 1998; Kastan and Lim, 2000). Phosphorylation increases p53 levels, and thus p53 activity, by preventing its nuclear export and degradation (Zhang and Xiong, 2001). P53 induces the expression of the cell cycle inhibitor P21^{Cip-1} (Harper *et al.*, 1993) Bax, Puma, and Noxa (Slee *et al.*, 2004). In contrast to p53 activation by Chk1 and Chk2, phosphorylation of Cdc25 renders the phosphatase inactive. In mammals, the three Cdc25 phosphatases, Cdc25A, Cdc25B, and Cdc25C, de-phosphorylate the cyclin dependent kinases (Cdk) that are important in the regulation of cell cycle transition. Phosphorylation of Cdc25 phosphatase creates a binding site for the 14-3-3 proteins, which exports these phosphatases from the nucleus and/or subjects them to proteolytic degradation (Kastan and Lim, 2000; Falck *et al.*, 2001). Un-phosphorylated Cdc25 promotes G/S and G2/M transition by de-phosphorylating Cdk-2 and Cdk-1, respectively, on their inhibitory phosphate residues

(Bartek and Lukas, 2001).

In summary, the DNA damage checkpoint response pathways involve distinct biochemical events. The different types of DNA damage are first sensed by distinct sensors, for example the double stranded break by ATM and the UV-induce DNA damage by ATR. These sensors then transmit the damage signals to the signal transducers Chk1/Chk2, which subsequently transduce the signal to the downstream effectors such the Cdc25. The outcome of these events is a delay in the cell cycle which would allow enough time for the cell to repair the damaged DNA. The DNA damage checkpoint response not only induces cell cycle arrest in the presence of DNA damage, but it is also involved in the repair process as well. If the damage is beyond repair, the checkpoint response eliminates the cell through a P53 mediated pathway which involves up-regulation of pro-apoptotic genes.

1.5 AIM OF STUDY

In this thesis we studied the regulation of cell survival and apoptosis in hemopoietic cells. Cytokine dependent hemopoietic cell lines are a useful tool for exploring the events controlling apoptosis since they rapidly undergo cell death when a single cytokine required for growth and/ or survival is withdrawn. We have focused most of these studies on the functions of Bcl-2 related proteins.

The initial aim of the study was to investigate the mechanism by which cytokine dependent hemopoietic cells undergo apoptosis as a result of cytokine starvation. We showed that FasL expression was induced in cytokine starved cells possibly by FOXO3A transcription factor. However, a direct link between FOXO3A activation which was detected in cytokine starved cells and the increase in FasL expression level was not investigated. Although both Fas and FasL were expressed on cytokine starved cells, Fas receptor mediated

events did not appear to be activated upon cytokine starvations. However, our results supported the conclusions of previous studies that mitochondrial mediated cell death through up-regulation of the pro-apoptotic Bcl-2 family protein, Bim, might play a major role in apoptosis of these cells.

The pro-survival Bcl-2 and Bcl-xL proteins are believed to induce cell survival by localizing to mitochondria and thereby protecting mitochondrial membrane integrity. We next examined two closely related pro-survival Bcl-2 family proteins, Bcl-2 and Bcl-xL, in their ability to protect against apoptosis induced by cytokine starvation. Our results show that Bcl-2 is a more potent pro-survival protein than Bcl-xL against apoptosis induced by cytokine starvation in the cytokine dependent hemopoietic cell line, FDCP-1. In addition to the mitochondrial outer membrane, the pro-survival Bcl-2 family proteins are also localized at other intracellular membranes including the endoplasmic reticulum. We next examined the significance of subcellular localization Bcl-xL and hypothesized that localization of Bcl-xL within the cells alters its ability to inhibit apoptosis. Our results showed that a Bcl-xL mutant targeted to ER membrane was a more potent pro-survival protein compared to either the mitochondrial targeted or wild type Bcl-xL against Bad-induced apoptosis.

Finally, we studied the regulatory roles of Mcl-1, another pro-survival Bcl-2 family member, which is now known to possess functions distinct from its pro-survival effects. The investigations of Mcl-1 were intended to follow up previous work from our laboratory showing its association with Cdk-1 as well as further probing its involvement in DNA damage checkpoint response. Our results showed that Mcl-1/Cdk1 association is cytokine dependent. Moreover, we showed a possible link between Mcl-1 and the DNA damage checkpoint regulator, Chk1. We further hypothesized that Mcl-1 may be involved in the

regulation of cellular differentiation. The premise of this investigation was based on observation that HL-60 cells when induced to differentiate by treatment with PMA showed an up-regulation in Mcl-1 protein levels. However, this increase in Mcl-1 expression did not appear to play an obvious role for Mcl-1 in differentiation of HL-60 cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL LINES AND TISSUE CULTURE

MC/9, FDCP-1, and TF-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1mM sodium pyruvate, 50 nM β -mercaptoethanol. For MC/9 and FDCP-1 cells, the above medium was supplemented with 2.5% WEHI-3-conditioned medium as a source of mouse IL-3 and TF-1 cells required 1% CGMI-conditioned medium as a source of hGM-CSF. MC/9 cells over-expressing Bcl-xL and Bcl-2 (MC/9-Bcl-xL), or Bcl-2 (MC/9-Bcl-2), and FDCP-1 cells over-expressing Bcl-xL (FDCP-1/Bcl-xL) or Bcl-2 (FDCP-1/Bcl-2) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1mM sodium pyruvate, 50 nM β -mercaptoethanol, and 2.5% WEHI-3-conditioned medium. The MC/9-Bcl-2 and FDCP-1/Bcl-2 cell lines were maintained in the presence of (200 μ g/ml) hygromycin. The MC/9-Bcl-xL, and the FDCP-1/Bcl-xL were maintained in the presence of (0.5 mg/ml) G418. HL-60, Jurkat, and THP-1 cells expressing Fas Ligand were grown in RPMI 1640 supplemented with 10% calf serum, 2mM L-glutamine, 50 nM β -mercaptoethanol, and 1mM sodium pyruvate. 293T cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. Rat-1 fibroblast cell lines over-expressing Bcl-xL targeted to endoplasmic reticulum, ER, (Bcl-xL-cb5), mitochondria (Bcl-xL-ActA), or wt Bcl-xL were grown in α -MEM supplemented with 10% calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate and 3 μ g/ml puromycin. Cells were maintained at 37°C and 5% CO₂ in a humidified incubator. The MC/9-Bcl-2, MC/9-Bcl-xL, FDCP-1/Bcl-2, and FDCP-1/Bcl-xL were constructed using retroviral infection. Rat-1 fibroblast cell line

over-expressing the different Bcl-xL mutants was a kind gift from Dr. David Andrews. To direct Bcl-xL to the cytosolic face of the endoplasmic reticulum (ER), a gene fusion was created in which the cDNA encoding the insertion sequence of Bcl-xL was replaced by cDNA encoding the analogous insertion sequence of the ER-specific isoform of rat hepatic cytochrome b5 (Bcl-xL-cb5). To target Bcl-xL specifically to the outer mitochondrial membrane, a fusion protein was constructed in which the carboxy terminal hydrophobic end of Bcl-xL was replaced with the mitochondrial insertion domain of the *Listeria* protein, ActA (Bcl-xL-ActA) [Fiebig *et al.*, 2006].

2.2 REAGENTS

Recombinant murine GM-CSF and IL-3, and recombinant human GM-CSF were purchased from R&D Systems (Minneapolis, MN). Synthetic murine IL-4 was a gift from the late Dr. Ian Clark-Lewis (University of British Columbia). The inhibitors LY-294002, wortmannin and rapamycin were from Calbiochem. Etoposide was also purchased from Calbiochem. Membrane bound FasL vesicles were from Upstate Biotechnology (Lake Placid, NY). Matrix metalloproteinase inhibitor KB8301 was from BD Pharmingen (Mississauga, ON). Propidium iodide (P.I.) was from Sigma-Aldrich (Saint Louis, MO). The Caspase FLICA kits were from Immunochemistry Technologies. Caspase inhibitors were from Calbiochem. The BP and LR clonase enzymes, Zeocin and Blastocidin were from *Invitrogen*. The Effectene and Lipofectamine 200 transfection reagents were from GIBCO BRL.

2.3 ANTIBODIES (Abs)

Table 2.1: Abs used in this thesis

Antibody	Type	Concentration	Procedure	Source
α -FKHRL1 (FOXO3a)	Rb	1:1000	W	Gift from Drs. Brunet and Greenberg
α -p-Thr32 FKHRL1	Rb	1:500	W	Gift from Drs. Brunet and Greenberg

Antibody	Type	Concentration	Procedure	Source
α -p-Ser 253FKRHL1	Rb	1:1000	W	Gift from Drs. Brunet and Greenberg
α -P-Ser315 FKHRL1	Rb	1:1000	W	Gift from Drs. Brunet and Greenberg
α -P-Ser473 PKB		1:1000	W	Cell Signaling Technologies (Beverly, MA)
α -P-Thr389p70 S6kinase		1:1000	W	Cell Signaling Technologies (Beverly, MA)
α -P-Thr202/Tyr204 MAP Kinase		1:1000	W	Cell Signaling Technologies (Beverly, MA)
α -Human Bid	pAb	1:1000	W	Cell Signaling Technologies (Beverly, MA)
α -Mouse Bid	pAb (Rb)	1:1000	W	BD Pharmingen (Mississauga, ON)
α -Fas	pAb (Ms)	2 μ g/ml	Flow Cytometry	Stressgen Biotechnologies, Victoria, BC
α -Mouse FasL	pAb (Rb)	2 μ g/ml	Flow Cytometry	Santa Cruz Biotechnology (Santa Cruz, CA)
α -Human FasL	Rb	2 μ g/ml	Flow cytometry	Upstate Biotechnology (Lake Placid, NY)
α -Mcl-1	Rb	1:1000 W, 1:200 IP	W, IP	Santa Cruz Biotechnology (Santa Cruz, CA)
α -Cdk-1	Ms	1:1000 W, 1:200 IP	W, IP	Santa Cruz Biotechnology (Santa Cruz, CA)
α -Chk1	Rb	1:1000 W, 1:200 IP	W, IP	Santa Cruz Biotechnology (Santa Cruz, CA)
α -Cyclin B1	Rb	1:1000	W	Santa Cruz Biotechnology (Santa Cruz, CA)
α -ATM		1:1000	W, IP	Santa Cruz Biotechnology (Santa Cruz, CA)
α -Actin	Goat	1:1000	W	Santa Cruz Biotechnology (Santa Cruz, CA)
α -FITC-rabbit-IgG		1:250	Flow Cytometry	Molecular Probe
α -Rhodamine-mouse-IgG		1:250	Flow Cytometry	Molecular Probe
α -Bim	pAb (Rb)	1:1000	W	Cedarlane Laboratories Ltd., Hornby , ON
α -p27kip1	Rb	1:1000	W	Santa cruz
α -Caspase 8	pAb (Rb)	1:1000	W	BD Pharmingen (Mississauga, ON)
α -Caspase 9	pAb (Rb)	1:1000	W	Stressgen Biotechnologies, Victoria, BC

α =anti, P=phospho, IP=immunoprecipitation, W=Western blot, Ab=antibody, Rb=rabbit, Ms=mouse,

2.4 PLASMIDS

Plasmids CTV83 expressing human Bcl-xL and CTV87 expressing human Bcl-2 were a gift from Dr. Rob Kay. BAD-pMXpuro and Mcl-1-pMXpuro were constructed in our laboratory by Drs. Shaynoor Dramsi and Sarwat Jamil, respectively. pDONR 201, plenti4/DEST, plenti6/TR, pLP1, pLP2, and pVSVG were all from *Invitrogen*.

2.5 PROTEIN ANALYSIS

2.5.1 Cell treatments

For analysis of proteins by Western blotting, cytokine-dependent cells were starved of cytokine by overnight incubation leaving 10% of the original IL-3 containing medium, or alternatively, cells were washed three times with PBS and incubated in cytokine free medium for the indicated time. For experiments involving cytokine stimulation, cells were stimulated with 10ng/ml recombinant GM-CSF, 10µg/ml synthetic IL-3 or 10µg/ml synthetic IL-4 for 10 min, conditions previously shown to induce maximal tyrosine phosphorylation (Duronio *et al.*, 1992; Welham *et al.*, 1994; Ettinger *et al.*, 1997). In experiments involving inhibitors, cells were pretreated with 50µM LY294002, 100nM WM or 100ng/ml rapamycin prior to addition of cytokine.

2.5.2 Preparation of total cell lysate (TCLs)

Total cell lysates were obtained by lysing cells in ice-cold solubilization buffer [50 mM Tris/HCl, pH 7.7, 1% Triton X-100, 10% (v/v) glycerol, 100 mM NaCl, 2.5 mM EDTA, 10 mM NaF, 40µg/ml phenylmethylsulfonyl fluoride, 1mM pepstatin, 0.5mg/ml leupeptin, 10mg/ml soybean trypsin inhibitor, 0.2 mM Na₃VO₄, 1mM Na₃MoO₄ and 1mg/ml microcystin-L for 10 min followed by centrifugation at 13000 rpm for 10 min. The supernatants containing the total cell proteins were boiled for 5 minutes in SDS sample

buffer containing 1% β -mercaptoethanol and subsequently used for Western blotting.

2.5.3 Preparation of nuclear and cytoplasmic extracts

For extraction of cytosolic and nuclear proteins, cells were lysed in buffer A containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol plus 1 mM DTT, 0.1% Triton X-100, 0.2 mM Na_3VO_4 , 1mM Na_3MoO_4 , and the protease inhibitor cocktail for 5 minutes followed by centrifugation at 3500 rpm for 5 min to pellet primarily the nuclei. The supernatants were further centrifuged at 14000 rpm for 10 min and subsequently stored as cytosolic fractions and pellets were used for extraction of nuclear proteins. The pellets were washed twice with buffer A and the nuclei were resuspended in buffer B containing 0.2 mM EGTA (pH 8), 3 mM EDTA (pH 8), plus 1 mM DTT, RNAaseA, DNAaseA, 0.2 mM Na_3VO_4 , 1mM Na_3MoO_4 , and the protease inhibitor cocktail for 30 min followed by sonication using a Sonic Dismembrator 550 (Fisher Scientific, Nepean, ON, Canada) at setting 3 for 10 s. The extracted proteins were centrifuged at 4000 rpm for 5 minutes. The supernatants contained the nuclear proteins. The nuclear and cytosolic extracts were then boiled in SDS sample buffer containing 1% β - Mercaptoethanol for 5 min.

2.5.4 Co-immunoprecipitations (IPs) and Western blotting

Typically, 2–4 mg of protein extracts was used for immunoprecipitations. Extracted proteins were incubated overnight at 4°C with the antibodies listed in section 2.3 at the indicated concentrations. Immuno-complexes were captured with 40 μ l of protein G-Sepharose beads slurry at 4°C for 1 h. Beads were washed five times with cold lysis buffer and boiled in SDS sample buffer containing 2% β -Mercaptoethanol for 5 minutes.

TCLs and IPs were separated by SDS/PAGE followed by Western blot analysis.

Transfers were made by semi-dry blotting on to nitrocellulose membranes. The membranes were blocked for 1 h in 5% (w/v) low-fat dry milk in Tris-buffered saline with 0.05% Tween 20 followed by overnight incubation at 4 °C with the antibodies listed in section 2.3 at the indicated concentrations. Anti-rabbit, anti-mouse or anti-goat antibodies conjugated to horseradish peroxidase were used to detect the immunocomplexes by enhanced chemiluminescence (Amersham International, Oakville, ON, Canada).

2.6 ASSAYS

2.6.1 Analysis of Fas and FasL

Cells were grown in the presence or absence of cytokine and/or inhibitors for various time points (6 hrs to 24 hrs). 200 nM of the matrix metalloproteinase inhibitor was added to the cultures to block FasL cleavage from the membrane. Total Fas and FasL protein was isolated and detected by immunoblotting as described above. For Fas and FasL cell surface analysis, cells were cultured at 1×10^6 cells/ml. Cells were collected and washed three times with PBS containing 2.5% FBS. Samples were resuspended in 500 μ l PBS with 2.5% FBS and incubated on ice for 1 h with either mouse anti-Fas or rabbit anti-FasL antibodies at concentrations indicated in section 2.3. Cells were centrifuged at 2000 rpm for 5 min at 4°C, washed twice with the PBS solution and then re-suspended in 500 μ l. Samples were incubated for 30 min on ice with either Rhodamine anti-mouse for Fas detection or FITC anti-rabbit for FasL detection at 1:250 dilutions. Cells were pelleted, washed twice and resuspended in 1ml of PBS and analyzed using an Epics XL Flow Cytometer (Coulter). THP-1 cells over-expressing FasL (kind gift from Dr. C. Ong, UBC) were used as a positive control for flow cytometry.

2.6.2 Apoptosis assays

2.6.2.1. Induction of apoptosis by FasL vesicles

Cells were grown in the presence and absence of cytokine with and without 20ng/ml membrane bound Fas L vesicles. At 12 and 24 h cells were collected and stained for propidium iodide followed by flow cytometry analysis. Briefly, cells were centrifuged and fixed in 70% ethanol for 15 minutes. The cells were then pelleted and incubated in PBS containing 0.1% glucose, 100 µg/ml RNase A and 50 µg/ml propidium iodide for 30 min in the dark. The samples were analyzed by flow cytometry for the presence of subdiploid DNA as a measure of cells that had undergone apoptosis. Alternatively, following treatment with FasL, cells were collected and analyzed for caspase 8 activation using the FLICA caspase detection kit as described in section 2.6.2.2 below

2.6.2.2 Caspase activation

We used the FLICA apoptosis detection kit (Immunochemistry Technologies, Bloomington, MN) to detect active caspases. The methodology is based on the Fluorochrome Inhibitor of Caspases (FLICA).

Table 2.2: FLICA kits used in this thesis

Caspases	FLICA Peptides	Stock Concentration
Polycaspases (Z-VAD)* FLICA	FAM-VAD-FMK	30X
Caspase 2 FLICA	FAM-VDVAD-FMK	30X
Caspase 3 FLICA	FAM-DVED-FMK	30X
Caspase 8 FLICA	FAM-LETD-FMK	30X
Caspase 9 FLICA	FAM-LEHD-FMK	30X

Cells were washed 3X with PBS and then culture in the presence or absence of WEHI-3 conditioned medium (as a source of IL-3) plus/minus the inhibitors at a density of 1×10^6 cells /ml. A time course of 4-24 hours was used for this study. After the appropriate time points, the cells were pelleted and concentrated to a density of 1×10^7 cells /ml. About 300 μ l of each cell suspension was transferred to sterile tubes and 10 μ l of the 30 X FLICA stock solution was directly added to the 300 μ l cell suspensions. The cells were incubated for 1 hour in the dark at 37 C in a 5%CO₂ incubator. Cells were pelleted and washed twice with 1X wash buffer and resuspended in 1 ml of 1X wash buffer. Cells were kept on ice and were analyzed by flow cytometry using the Epics XL Flow Cytometer (Coulter).

2.6.2.3 Caspase inhibition

The effect of caspase inhibitors in preventing apoptosis in cytokine starved cells was analyzed. Cells were washed 3X with PBS and plated at a density of 1×10^6 cells/ml in the presence or absence of cytokine. To prevent caspase activation during the washing step, cells were pretreated with 100 μ M of each of the caspase inhibitors, z-VAD-FMK, z-DEVD-FMK, z-LETD-FMK, z-LEHD-FMK, z-VDVAD-FMK (Calbiochem), for 30 minutes prior to the washing step and also during washing. One or more caspase inhibitor was added to each plate at a concentration of 100 μ M for each inhibitor and the cells were incubated at 37°C for 12 and 24 hours. The cells were collected and the percentage of subdiploid DNA was analysed by flow cytometry as described in section 2.6.2.1.

2.7 RETROVIRAL INFECTION

The retroviral infection was done to over-express human Bcl-2 or Bcl-xL into cytokine dependent FDCP-1 and MC/9 cell lines. Briefly, BOSC23 packaging cells were plated onto 100 mm dish so that on the day of transfection (24 hours later) the cells would be

50% confluent. Approximately 1 µg of either CTV83-Bcl-xL or CTV87-Bcl-2 retroviral vectors were used to transfect the cells using the Effectene transfection kit (Qiagen). Twenty-four hours following transfection, the old media was replaced with fresh media and 72 hours later the viral supernatant was collected and centrifuged at high speed to remove all the cell debris. The viral supernatant was subsequently filtered through a 0.45 µm filter and the supernatant was then used to infect FDCP-1 and MC/9 cells. The cell media was replaced with the viral supernatant and 48 hours after infection the cells were selected with either G418 (0.5 mg/ml) or Hygromycin (200 µg/ml) for cells infected with CTV83-Bcl-xLretrovirus or CTV-Bcl-2 retrovirus, respectively. Cells were cloned and clones expressing medium and high levels of Bcl-2 or Bcl-xL were selected.

2.8 THE VIRALPOWER T-REX LENTIVIRAL EXPRESSION SYSTEM

The ViralPower T-Rex lentiviral expression system that we used was from Invitrogen. This system combines Invitrogen's ViralPower Lentiviral and T-Rex technologies to facilitate lentiviral based regulated expression of the target gene of interest in dividing or non-dividing mammalian cells. We used this system in order to express Mcl-1 in human promyeloid leukemia cell line, HL60, in order to investigate a possible role for Mcl-1 in cellular differentiation.

2.8.1 Components of the ViralPower T-Rex system

1. An inducible expression construct, plenti4/DEST, into which Mcl-1 was cloned. The resulting construct allows tetracycline-regulated expression of Mcl-1 under the control of a hybrid promoter consisting of the human cytomegalovirus (CMV) promoter and two tetracycline operator 2 (TetO2) sites.
2. A regulatory expression construct, plenti6/TR that facilitates high-level, constitutive

expression of the Tet repressor (TetR). In this construct, expression of the TetR gene is controlled by CMV promoter.

3. The ViralPower packaging mix, a mixture of three packaging plasmids including pLP1, pLP2, and pVSVS, required for the production of the lentivirus.

Infection with the lentivirus results in the reverse-transcribed viral RNA being integrated into the host genome. Upon addition of tetracycline, the gene of interest (i.e. Mcl-1) is induced (Hillen and Berens, 1994; Hillen *et al.*, 1983)

2.8.2 Primers

The Mcl-1 DNA was amplified by PCR, using primers containing the attB sequences. The primers were designed so that 25 bp Mcl-1 specific sequences would be in frame with 31 bp and 30 bp sequence of lentiviral attB1 (for forward primer) or attB2 (for reverse primer), respectively:

Forward primer:

GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTTTGGCCTCAAAAGAAAC

GCGG **attB1** **Mcl-1**

Reverse Primer:

GGGGACCACTTTGTACAAGAAAGCTGGGTCTCTTATTAGATATGCCAAACCAG

CT **attB2** **Mcl-1**

2.8.3 PCR

The full-length Mcl-1 was amplified using the Mcl-1 cDNA as a probe and the attB-Mcl-1 primer sets mentioned above. The PCR reaction consisted of 1X PCR buffer [200 mM Tris-Hcl (pH8.4), 500 mM KCl, 50 mM MgCl₂], 10 µM each of the forward and the reverse primers, 5 units of Taq polymerase, and 100 nM of the Mcl-1 cDNA. The PCR reaction was

carried out under a “hot start” condition.

Table 2.3: PCR condition for Mcl-1 amplification

Steps	Time	Temperature	Cycles
Initial denaturation	5 min.	94 °C	1X
Denaturation	30 sec.	94 °C	35X
Annealing	30 sec.	63 °C	
Extension	1 min.	72 °C	
Extra extension	7 min.	72 °C	1X

Min= minutes, sec= seconds

2.8.4 The BP reaction

The purpose of the BP reaction is to ligate the Mcl-1 PCR products (discussed in section 2.8.5) into an entry vector, pDONR 201. The Mcl-1 PCR product was ligated into a pDONR 201 vector to create an entry clone containing full-length Mcl-1 (pENTRY-Mcl-1) through a BP reaction. Briefly, 5 µl of the Mcl-1 PCR product was mixed with 125 ng of the pDONR 201, 1X BP buffer, and 2 µl of the BP clonase enzyme and the mixture was incubated overnight at room temperature. To stop the reaction, 2 µl of proteinase K solution was added at 37°C for 10 minutes.

2.8.5 LR reaction

The LR reaction involves the recombination between the entry clone generated through the BP reaction with a Destination vector, plenti4/DEST, to generate a lentiviral expression vector containing Mcl-1 (pLenti/DEST-Mcl-1). Briefly, 100 ng of the entry clone was mixed with 300 ng of plenti4/DEST, 1X LR reaction buffer, and 2 µl of the LR clonase enzyme mix and the reaction was incubated overnight at room temperature. To stop the

reaction, 2 μ l of proteinase K solution was added at 37°C for 10 minutes.

2.8.6 Bacterial transformation

Once the BP and LR reactions were carried out, the constructs generated from these reactions were propagated by transforming the DH5 α competent cells. Briefly, one microliter each of BP or LR reaction mix was added to 50 μ l of DH5 α competent cells. The reactions were incubated on ice for 30 minutes followed by heat shock at 42°C for 30 seconds. The cells were then put on ice for 1-2 minutes followed by addition of 450 μ l of LB medium and incubation at 37°C for 1 hr. The cells were then spread on LB plates containing 50 μ g/ml kanamycin (BP reaction) and 100 μ g/ml ampicillin (LR reaction). The kanamycin or ampicillin resistant colonies were selected and the plasmids were propagated and purified using plasmid midi-prep kit (Qiagen).

2.8.7 Transfection of the 293T packaging cell line

The 293T cell line was used as a packaging cell line to generate lentiviruses carrying either the regulatory Tet repressor construct (pLenti6/TR or the Mcl-1 expression construct (pLenti4/DEST-Mcl-1) Briefly, the day before transfection, 5×10^6 293T cells were plated in a 10 cm tissue culture plate. On the day of transfection, the culture medium was removed and was replaced with 5 ml of DMEM containing serum. The DNA-Lipofectamine 2000 complexes were prepared as follows: In a 15 ml Falcon tube, 3 μ g of either the plenti4/TO/V5-DEST-Mcl-1 or the plenti6/TR plus 3 μ g each of the ViralPower packaging mix containing pLP1, pLP2, pVSVG were mixed in 5 ml of Opti-MEM reduced serum medium. In another tube, 36 μ l of the Lipofectamine 2000 reagent was mixed with 5 ml of the Opti-MEM reduced serum medium. The mixtures were allowed to sit at room temperature for 10 minutes. The DNA was then added to the Lipofectamine 2000 mixture

and the resulting mixture was incubated at room temperature for 30 minutes. The DNA-Lipofectamine 2000 complexes were added dropwise to each plate of cells. The cells were incubated overnight at 37°C in a CO₂ incubator. The following day the medium was removed and was replaced with 10 ml of DMEM containing 10% serum. After 48 hours, the media containing the viral supernatant was collected and was filtered through a 0.45 µm filter. The virus was concentrated by centrifuging at a high speed in a Beckman centrifuge at 20,000 rpm for 2.5 hours. The supernatant was removed and the pellet was resuspended in a small volume of PBS. The viral titer was measured with the p24 ELISA assay. HL60 cells were infected with the virus at a multiplicity of infection (MOI) of 100 virus /cell.

2.8.8 Infection of the HL-60 cells

We first created a stable HL60 cell line that constitutively expressed the Tet repressor (ViralPower T-REx host cell line) and then used that cell line to create a second cell line which would inducibly express Mcl-1 from the pLenti4/DEST lentiviral construct. Briefly, 5×10^5 cells were infected with the virus carrying pLenti6/TR at a MOI of 100 viral particles per cell. The next day (day 2), the medium containing the virus was removed and replaced by fresh complete medium. The following day (day 3) the medium was removed and replaced by a fresh complete medium containing 20 µg /ml Blastocidine to select for stably transduced cells. Once the Blastocidine resistant cells were selected, 5×10^5 cells were infected with the virus carrying the pLenti4/DEST-Mcl-1 construct at a MOI of 100 viral particles per cell. The next day (day 2), the medium containing the virus was removed and replaced by fresh complete medium. The following day (day 3), the medium was removed and replaced by a fresh medium containing 200 µg/ml of Zeocin to select for stably transduced cells.

2.9 MCL-1 OVER-EXPRESSION BY ELECTROPORATION

Transfection of HL-60 cells was performed as described in manufacturer's protocol for Nucleofection from Amaxa Biosystems. Three million cells were taken and were resuspended in the buffer provided. Mcl-1 plasmid or pMXpuro empty vector and low concentration of GFP were added to the sample which was then transferred to a sterile cuvette provided. Electroporation was performed at setting T-19. Cells were transferred cells to a 6-well plate containing RPMI media and were analyzed after 48 hours of transfection.

CHAPTER 3

INVESTIGATION OF A POSSIBLE ROLE FOR FOXO3A-INDUCED FASL EXPRESSION IN APOPTOSIS OF HEMOPOIETIC CELLS FOLLOWING CYTOKINE STARVATION

3.1 INTRODUCTION

There are two major mechanisms of apoptosis in mammals: death receptor-mediated and mitochondrial-mediated, commonly referred to as extrinsic or intrinsic, respectively. One death receptor pathway is activated upon engagement of the death receptor, Fas, with its corresponding ligand, FasL. This leads to recruitment of downstream activators to the membrane which is followed by activation of caspase 8 and subsequent cell death. The mitochondrial-mediated pathway results in loss of mitochondrial integrity, release of cytochrome C into the cytoplasm, activation of caspase 9 followed by cell death. These mitochondrial events are controlled, both positively and negatively, by various members of the Bcl-2 family of proteins as discussed earlier. The mitochondrial pathway can be further divided into death receptor dependent and death receptor independent pathways. In both cases, cytochrome c is released from the inter-membrane space of mitochondria. However, the “dependent” pathway requires activation from the death receptor followed by cleavage of Bid, a pro-apoptotic Bcl-2 family protein.

Hemopoietic cell lines that are dependent upon cytokines for their growth and survival have been widely used as model systems for the study of apoptosis. Upon removal of specific cytokines from the growth medium, while still in the presence of serum, the loss of survival signals initiates the processes that lead to death of the cells by apoptosis. An important signaling pathway that blocks apoptosis is the activation of phosphatidylinositol 3-kinase (PI3 kinase) (Scheid *et al.*, 1995). The serine/threonine kinase, PKB (Akt), is a

downstream target of PI3-kinase that plays a major role in preventing apoptosis. While various targets of PKB have been identified, including GSK-3 (Cross *et al.*, 1995), the FOXO3 transcription factors (Kops *et al.*, 1999; Rena *et al.*, 1999; Madema *et al.*, 2000), and possibly the pro-apoptotic protein, Bad (Downward, 1999), the complete list of key targets that promote cell survival have still not been completely defined. Each of these substrates may play a role in mediating PKB-dependent survival in specific circumstances, but of particular interest in our studies are the primary events controlling cell death following cytokine withdrawal in hemopoietic cells.

As mentioned earlier, one of the mechanisms by which PKB promotes cell survival is by inactivating the FOXO3A transcription factor through phosphorylation. This inhibitory phosphorylation on FOXO3A keeps the protein in the cytosol away from its nuclear targets. In the absence of cytokines when the PI-3 kinase survival signaling pathway is inhibited, FOXO3A translocates to the nucleus where it induces the expression of pro-apoptotic proteins such as Bim and FasL. The role of Bim in cytokine withdrawal-induced cell death is well established. In this study, we have investigated a possible role for a FasL-mediated apoptosis in cytokine starved cells which at the onset of our studies had not been fully characterized. Our working hypothesis was that apoptosis of cytokine starved hemopoietic cells is due to Fas receptor mediated events. We found that treatment of hemopoietic cells with several different cytokines caused phosphorylation of endogenous levels of FOXO3A, at all three regulatory sites. In addition, inhibition of the PI3-kinase/PKB survival signaling pathways by either cytokine withdrawal or the PI3-kinase inhibitors blocked this inhibitory phosphorylation on FOXO3A. Furthermore, our results demonstrated that Fas and FasL (FasL) expression levels increased on the surface of cytokine deprived cells. However, by

analysis of caspase cascades and lack of sensitivity of the cells to FasL, it was found that Fas mediated events were not the key initiators of apoptosis in hemopoietic cells. It is possible that the Fas mediated signaling is impaired in the human and murine cell lines investigated, an area which needs further investigations. Based on our observations, we conclude that following cytokine withdrawal mitochondrial-mediated events are likely the primary means of causing apoptosis. The forkhead-mediated regulation could potentially play a role through its effects on Bim and/or p27kip1 expression, as has been reported by others.

3.2 RESULTS

3.2.1 Cytokines stimulate phosphorylation of FOXO3A

In order to explore a possible involvement of FOXO3A in cytokine withdrawal-induced cell death, we first tested the effect of different cytokines on FOXO3A phosphorylation. Our initial experiments utilized murine MC/9 mast cells to characterize responses to IL-3 and GM-CSF, as well as IL-4. While the first two act as complete growth factors for these cells, IL-4 is only able to maintain cell survival (Scheid *et al.*, 1995). Each of these cytokines has been shown previously to activate PI 3-kinase as well as PKB in these cells (Scheid *et al.*, 1998; Gold *et al.*, 1994) and this was the hemopoietic cell line in which activation of PI 3-kinase was shown to be a key anti-apoptosis signal (Scheid *et al.*, 1995). As shown in Fig. 3.1A, each of the cytokines was able to stimulate increased phosphorylation at the three well characterized sites of phosphorylation in FOXO3A, Thr32, Ser253 and Ser315. The ability of IL-4 to stimulate FOXO3A phosphorylation is of interest since in MC/9 cells, IL-4 can activate PI 3-kinase and PKB, without any effect on the Ras/Erk pathway (Welham *et al.*, 1992; Duronio *et al.*, 1992) or on activation of JNK (Foltz and Schrader, 1997) or p38 MAPK's (Foltz *et al.*, 1997).

Similar experiments were done to characterize FOXO3A phosphorylation in two other cell lines that are also cytokine-dependent. In FDC-P1 cells, IL-3 and GM-CSF had similar effects (Fig. 3.1B), while in human TF-1 cells, stimulation with GM-CSF was also found to cause increased phosphorylation at all three sites (Fig. 3.1C). It is worthwhile noting that these results are the first to report the ability these hemopoietic cytokines to stimulate phosphorylation of endogenous FOXO3A in different cell lines, and are significant since it is the endogenous transcription factor, rather than over-expressed protein, which is being detected. The focus in this study was on FOXO3A. FKHR, or FOXO1, was not as readily detectable in our hands and we have not determined whether that was due to lower expression levels, or due to the anti-FOXO1 antibodies being less reliable.

3.2.2 PI 3-kinase inhibitors block phosphorylation of FOXO3A

We wished to verify that the phosphorylation of the sites on FOXO3A is dependent upon PI 3-kinase activity. As shown in Fig. 3.2, prior treatment of MC/9 cells with either LY-294002 or wortmannin, at concentrations known to efficiently block PI 3-kinase activity (Scheid *et al.*, 1996), blocked GM-CSF stimulated phosphorylation at all three sites on FOXO3A. It can be seen that these inhibitors also blocked GM-CSF-stimulated phosphorylation of PKB at Ser473. When the inhibitor of m-TOR, rapamycin, was used, there was no effect on FOXO3A phosphorylation, but p70 S6K phosphorylation was inhibited as expected. Taken together, these results confirm that cytokine-dependent phosphorylation of FOXO3A in hemopoietic cells proceeds in a PI 3-kinase-dependent fashion, as has been shown in other cases.

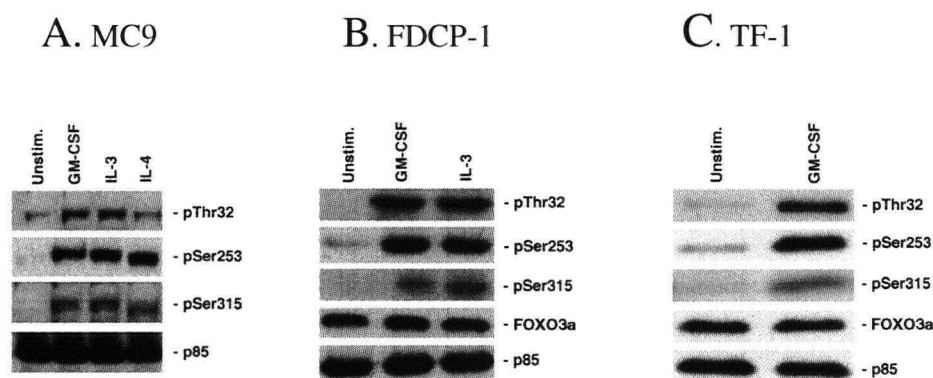


Figure 3.1- Cytokines stimulate phosphorylation of FOXO3A. A. MC/9 cells were starved of cytokine, followed by addition of either GM-CSF, IL-3 or IL-4 for 10 min. B. FDC-P1 cells were starved and treated with IL-3 or GM-CSF for 10 min. C. TF-1 cells were starved and treated with GM-CSF for 10 min. Immunoblots showing phosphorylation at Ser253, Ser315 and Thr32 of FOXO3A are shown, as well as blots for total FOXO3A protein. Immunoblots showing the p85 subunit of PI 3-kinase was used as a loading control. Results are representative of 3 separate experiments.

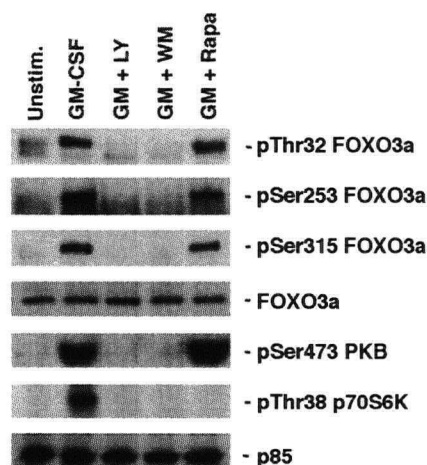


Figure 3.2- Inhibition of PI 3-kinase blocks phosphorylation of FOXO3A. MC/9 cells were starved of cytokine. Prior to stimulation with cytokine, cells were pre-incubated for 10 min with 25 μ M LY294002 or 10 nM wortmannin or 100 ng/ml rapamycin. Phosphorylation on the three sites of FOXO3A were detected by immunoblotting and total FOXO3A and p85 protein were detected as controls. Phosphorylation of Ser473 of PKB was detected to show the effectiveness of the PI3K inhibitors. Phosphorylation of Thr389 of p70 S6K was detected to show the effectiveness of rapamycin, as well as the effect of PI3K inhibitors on that pathway. Results are representative of 3 separate experiments.

3.2.3 Cytokine starvation induces cell surface expression of FasL

One of the reported targets of forkhead transcription factors is the pro-apoptotic cell surface protein, FasL (Brunet *et al.*, 1999) which at least partially accounts for the role of Forkhead transcription factor activity in inducing cell death. In hemopoietic cells undergoing cell death due to cytokine starvation, or due to inhibition of the PI 3-kinase survival signaling pathway, we asked whether these events increase FasL expression. Our results showed that in a time course of cytokine starvation of FDC-P1 cells, the expression of FasL protein was increased over an 8, 12 and 24 hour period (Fig. 3.3A-D), coincident with the time period in which these cells undergo apoptosis. In TF-1 cells, incubation with LY 294002 for 24 hours which induces apoptosis was also found to cause increased expression of FasL (Fig. 3.3E-F). Cells over-expressing FasL were used as a positive control for the antibody being used to detect cell surface FasL (Fig. 3.3G). In separate experiments, immunoblots with anti-FasL antibody were able to demonstrate that cytokine starvation or treatment of cells with the PI-3 kinase inhibitor, LY29004, resulted in increases in total FasL protein (Fig. 3.3H). Taken together these results demonstrate that inhibition of PI3-kinase signaling events either through cytokine withdrawal or the PI3-kinase inhibitor leads to activation of FOXO3A and a subsequent increase in FasL expression. However, since a direct link between FOXO3A activation and induction of FasL expression was not investigated in this thesis, we can only assume a direct connection based on other studies (Brunet *et al.*, 1999). A possible role for FOXO3A in the regulation of the FasL expression could be further studied by knocking down FOXO3A, in order to examine its functional significance in the regulation of this event during cytokine starvation. Alternatively, electrophoretic mobility shift assay (EMSA)

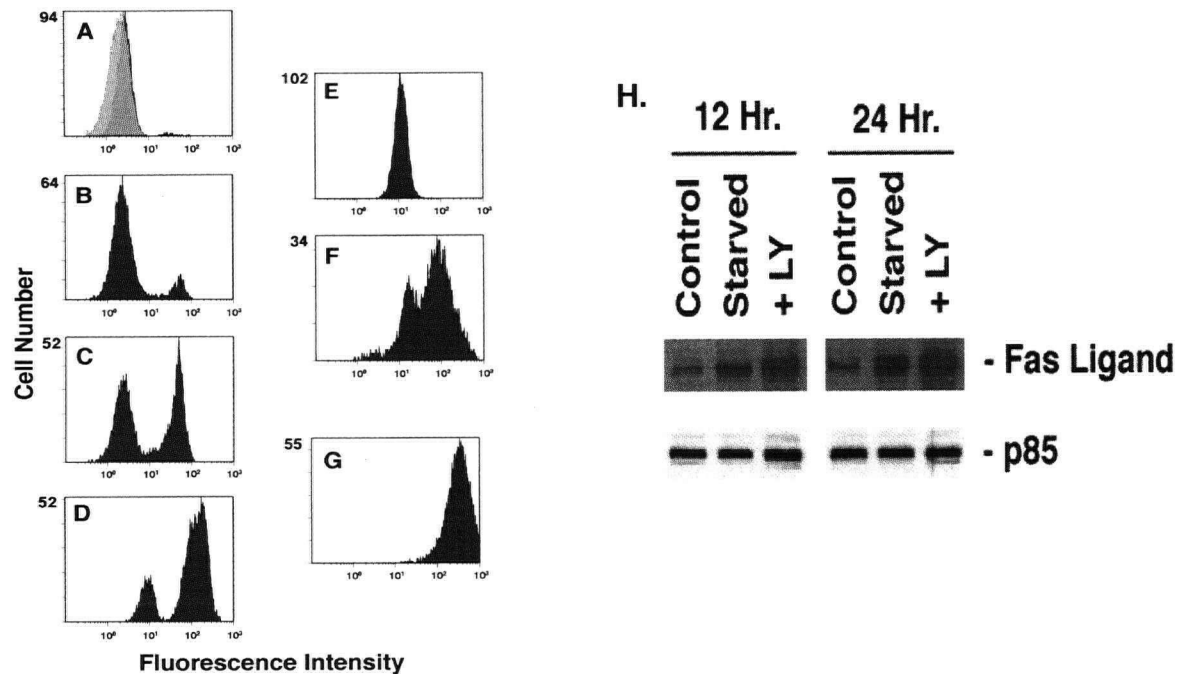


Figure 3.3- FasL is detected on the surface of cells upon inhibition of PI3 kinase survival pathway. FDC-P1 cells were in normal growth medium (A), or starved of cytokine for 8 hours (B), 12 hours (C) or 24 hours (D). FasL expression was detected at the cell surface by flow cytometry as described. The light gray peak in panel A is the superimposed tracing of background fluorescence observed with secondary antibody alone. Results shown in A-D are representative of at least 4 separate experiments. TF-1 cells were in normal growth medium (E) or incubated in the presence of 25 μ M LY294002 for 24 hours (F). As a positive control, THP-1 cells overexpressing FasL were analyzed using the same conditions as for the other cell types (G). Immunoblot in H showing an increase in the expression of FasL in FDCP-1 cells upon cytokine starvation. Results are representative of 3 separate experiments

using a radio-labeled oligo-nucleotide probe corresponding to the FOXO3A binding site on the FasL promoter could be employed to test the affinity of FOXO3A for FasL promoter in the cytokine starved cells.

3.2.4 Cytokine starvation enhances cell surface expression of Fas

Since cytokine withdrawal led to an increase in FasL expression, we wished to examine the expression level of Fas receptor (Fas) in cytokine deprived cells. Our results showed an increase in cell surface expression of Fas coincident with increased FasL expression, as seen in analysis of FDCP-1 cells at 8, 12 or 24 hours after cytokine withdrawal

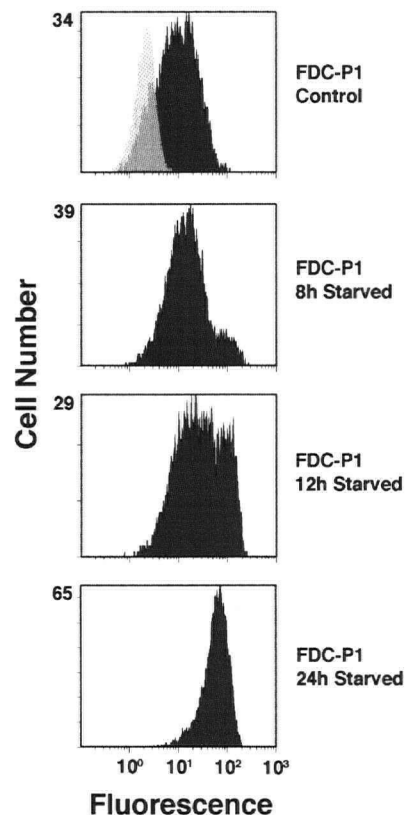


Figure 3.4- Cytokine starvation enhances surface expression of Fas. FDC-P1 cells were in normal growth medium (control), or starved of cytokine for 8, 12 or 24 hours as indicated. Fas expression was detected at the cell surface by flow cytometry as described. The background fluorescence observed with secondary antibody alone is superimposed in the first panel in a lighter shade of gray. Results are representative of 4 separate experiments.

Fig. 3.4). There was detectable Fas expression even in cells growing in the presence of cytokine, and an increased expression seen in cells under conditions of cytokine starvation. At this time, it has not been determined whether appearance of Fas is a result of cell surface localization of pre-existing protein, or whether expression of Fas is also increased.

3.2.5 Membrane bound FasL does not increase death of cytokine-starved cells

Since we established that both Fas and FasL are expressed on the surface of cytokine starved cells, we wished to determine a potential role for the death receptor Fas pathway in mediating death of cytokine-dependent hemopoietic cells. We first tested this by incubating cytokine-starved cells in the presence of exogenous membrane bound FasL at a concentration

which is known to kill cells that express Fas on their surface. As can be seen from Table 3.1, Jurkat T cells that were used as a positive control underwent FasL dependent apoptosis as expected. However, addition of FasL had no effect on FDCP-1 or TF-1 cells. Even under conditions in which these cells were beginning to undergo apoptosis, there was no significant increase in cell death caused by the presence of exogenous FasL.

This lack of sensitivity of FDCP-1 and TF-1 cells to exogenous membrane bound FasL is rather surprising and requires further investigations. It is possible that these cells secrete excessive amounts of sFasL, a metalloprotease mediated cleavage product of the membrane bound FasL. Soluble FasL is not as effective in inducing cell death as the membrane bound FasL and it might compete with the exogenous membrane bound FasL for binding to Fas receptor. This possibility could be tested by treating cells with exogenous sFas receptor to neutralize the inhibitory effects of the sFasL. Alternatively, one could block the Fas mediated signaling events by using an agonistic anti-Fas receptor antibody and examining its effects on cytokine deprivation-induced cell death.

3.2.6 Both caspase 8 and caspase 9 are activated upon cytokine starvation

Since we could not determine a role for Fas-mediated apoptosis by exogenous addition of FasL, we decided to look at activation of caspase 8 in cytokine starved cells. We assumed that if death of cytokine starved cells is induced by the Fas receptor pathway, we should be able to detect activation of caspase 8 prior to caspase 9 activation. Therefore, we looked at temporal activation of caspase 8 versus caspase 9. As shown by flow cytometry using fluorescent markers that bind specifically to each active caspase, there was no evidence of earlier activation of caspase 8 which might have been expected if death was initiated by

TABLE 3.1- Membrane bound FasL does not increase death of the cytokine starved cells:

Condition	FDC-P1 12 hrs	FDC-P1 24 hrs	TF-1 24 hrs	TF-1 30 hrs	Jurkat 24 hrs
Control	6.8 ± 0.87	7.7 ± 1.83	8.75 ± 0.88	11.3 ± 1.16	5.9 ± 0.82
+ FasL	6.5 ± 0.73	7.4 ± 1.55	8.65 ± 0.39	10.2 ± 0.53	59.2 ± 5.09
Cytokine starvation	58.8 ± 4.7	83.7 ± 2.5	36.2 ± 2.26	54.3 ± 0.81	
Cytokine starvation + FasL	54.6 ± 2.5	81.7 ± 2.4	40.2 ± 2.28	54.0 ± 1.23	

Percentage of cells carrying subdiploid DNA content- For control conditions, FDC-P1 were in the presence of WEHI-3-conditioned medium as a source of IL-3, while TF-1 cells were in the presence of GM-CSF-containing medium. Jurkat cells serve as a positive control for Fas-mediated apoptosis, but are not cytokine-responsive. Results shown are mean ± SEM of 3 separate experiments.

Fas receptor pathway (Fig. 3.5). In fact, both caspase 8 and caspase 9 appeared to be activated around 8 hours of cytokine starvation as shown in Fig. 3.5E. No major caspase activation was detected at 6 hours post cytokine starvation. Our inability to detect activation of caspase 8 prior to caspase 9 activation could be due to the fact that caspase activation is a highly rapid process and, therefore, it is possible that we were unable to capture the exact moment a particular caspase was cleaved. Experiments using shorter time intervals between the 6 and 8 hour time course of cytokine starvation are required to further confirm our findings.

3.2.7 Membrane bound FasL does not induce caspase 8 activation

It has been previously shown that exogenous addition of membrane bound FasL induces death by a Fas mediated pathway through activation of caspase 8 in cells that express Fas on their surface (Huang *et al.*, 1999). Further to our studies in section 3.2.5, we tested the

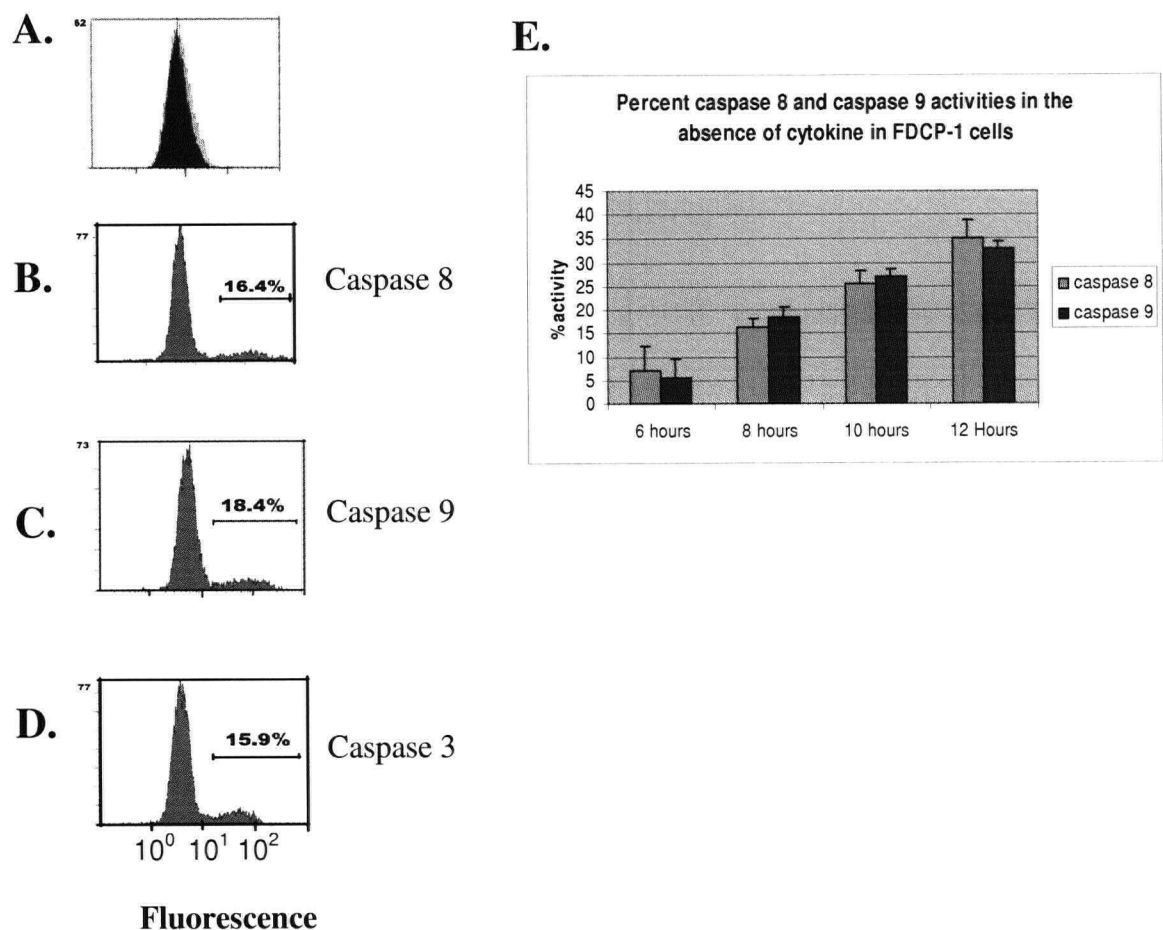


Figure 3.5- Both caspase 8 and 9 are activated within the same time frame upon cytokine starvation.
A-D) FDCP-1 cells were incubated in the presence or absence of cytokines for 8 hours. Cells were then treated with fluorescent markers that specifically bind to active caspase 8 (panel **B**), caspase 9 (panel **C**), or caspase 3 (panel **D**) and the percentage of each caspase activity was then analyzed by flow cytometry. Panel **A** is the super-imposed tracing of background fluorescent observed in normally proliferating control FDCP-1 cells stained with the caspase 8 (black area) or caspase 9 (grey area) fluorescent markers. **E)** Percentage of caspase 8 and caspase 9 activity in FDCP-1 cells in the absence of cytokine for 6, 8, 10, and 12 hours. Results in **E** are representative of 3 separate experiments.

ability of exogenous FasL to induce caspase 8 activation. When FDCP-1 cells were starved of cytokine from 8 to 24 hours, there was a noticeable activation of caspase 8 detected in cytokine starved cells, but there was no difference in the level of active caspase 8 following addition of exogenous FasL as demonstrated in Fig. 3.6 and Table 3.2. FasL, however, did induce caspase 8 activation in Jurkat cells which were used as a positive control.

Further to our discussion in section 3.2.5, it is possible that a component of the death receptor pathway is defective in FDCP-1 cells. For example, the Fas receptor or the adaptor protein FADD might be mutated and thus non-functional. Another possibility which needs to be further examined is that an inhibitor of the Fas pathway, for example, FLIP might be over-expressed in FDCP-1 cells. This last possibility could be tested by knocking down expression of FLIP by SiRNA and determining if this would allow Fas-mediated apoptosis in cytokine starved cells.

3.2.8 Bid is not activated early upon cytokine starvation

Bid is a pro-apoptotic Bcl-2 family protein which is cleaved upon activation of caspase 8. The cleaved Bid (t-Bid) then translocates to the mitochondrial membrane where it disrupts mitochondrial membrane integrity by interacting with either the pro-apoptotic or anti-apoptotic Bcl-2 family members. Thus, tBid acts as a bridge linking the Fas mediated events to the mitochondrial mediated cell death. Our specific aim was to show Bid cleavage in FDCP-1 cells by Western blotting. Since no specific antibody against t-Bid was available, we used an antibody which detected full length Bid and then, the loss of full length Bid was interpreted as Bid cleavage and activation. When FDCP-1 cells were starved of cytokine for 8, 10, 12 hours, no early cleavage of Bid could be detected in the cytokine starved cells as shown in Fig. 3.7A. However, Bid cleavage was detected at 20 hours post cytokine starvation (Fig 3.7C). In addition, treatment of cells with membrane bound FasL did not induce a further increase in Bid cleavage following cytokine starvation. Membrane bound FasL, however, did induce Bid cleavage in Jurkat cells that were used as a positive control (Fig. 3.7C). Therefore, with the exception of longer starvation of FDCP-1 cells, there was no evidence of Bid cleavage at times when the cells were undergoing apoptosis. The decrease in

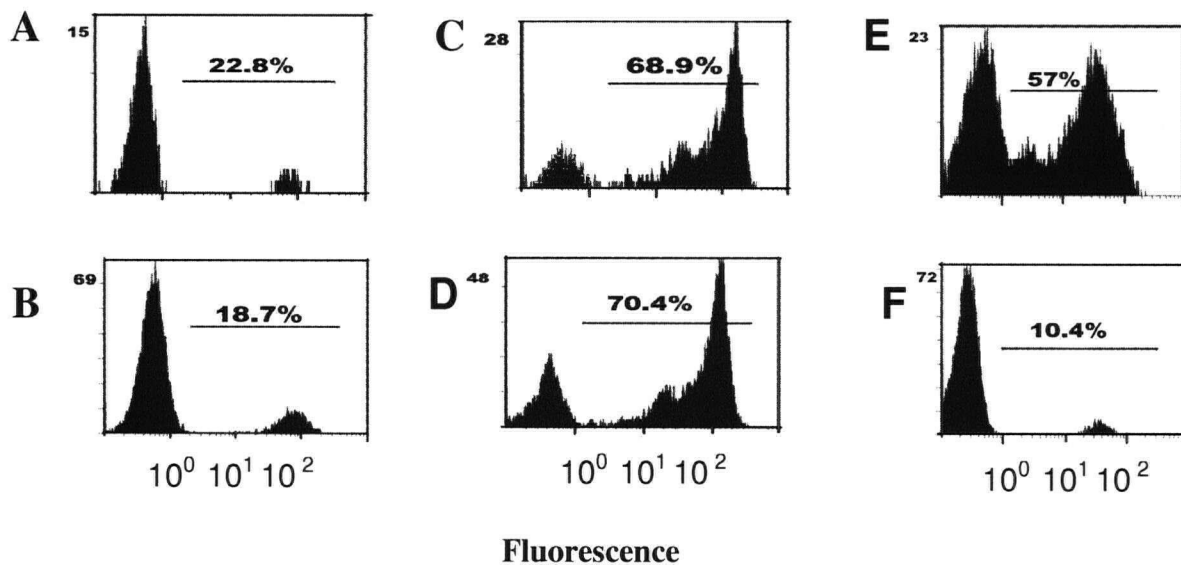


Figure 3.6- Membrane bound FasL does not induce activation of caspase 8- FDCP-1 cells were treated with membrane bound FasL in the presence or absence of cytokine for 12 hrs. Cells were then treated with a fluorescent marker that specifically binds to active caspase 8 and the percentage of caspase 8 activity was detected by flow cytometry. Panels **A** and **B** show normally proliferating FDCP-1 cells in the presence or absence, respectively, of FasL, whereas, panels **C** and **D** show cytokine starved FDCP-1 cells in the presence or absence, respectively, of FasL. Jurkat cells serve as a positive control for Fas mediated apoptosis with panels **E** and **F** showing Jurkats cells in the presence or absence of FasL.

Table 3.2- Percentage of active caspase 8 in FDCP-1 cells treated with membrane bound FasL.

Condition	FDCP-1 8 hrs	FDCP-1 12 hrs	FDCP-1 24 hrs	Jurkats 12 hrs
Control	15.5 \pm 1.56	21.3 \pm 0.75	23.1 \pm 0.61	11.6 \pm 0.76
+ FasL	13.7 \pm 1.2	18.2 \pm 1.02	20 \pm 0.5	58 \pm 0.50
Cytokine starvation	34 \pm 1.30	73.6 \pm 2.36	88 \pm 1.80	
Cytokine starvation +FasL	29.3 \pm 0.76	71.3 \pm 1.61	91.3 \pm 1.60	

For control conditions, FDC-P1 cells were cultured in the presence of WEHI-3-conditioned medium as a source of IL-3. Jurkat cells serve as a positive control for Fas-mediated apoptosis, but are not cytokine-responsive. Results shown are mean \pm SEM from 3 separate experiments.

full length Bid detected at 20 hour post cytokine starvation could be due to the fact that many of cellular proteins are non-specifically cleaved at the late stages of apoptosis when many of cellular proteases and caspases are activated. Alternatively, this decrease in Bid level could be due to an overall decrease in the rate of protein synthesis during the later stages of apoptosis.

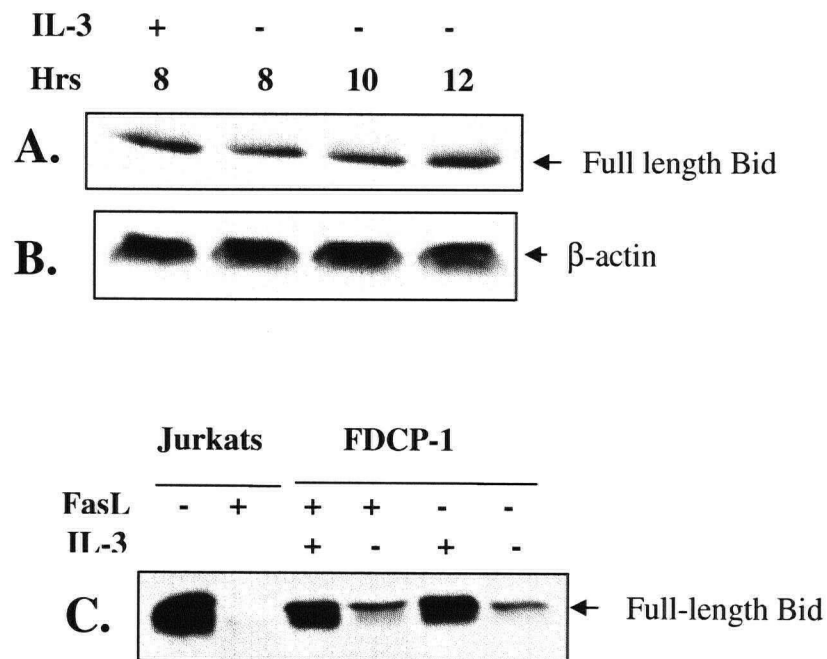


Figure 3.7- Bid activation is a late event in cytokine withdrawal-induced cell death. **A.** FDCP-1 cells were starved of cytokine for 8-12 hours (panel **A**) and the immunoblot shows the presence of full length Bid. The antibody detects the full-length Bid and, thus, a reduction in the intensity of the band corresponding to full length Bid would represent Bid cleavage and, therefore, its activation. Panel **B** represents the loading control for β-actin. FDCP-1 cells (**C**) were treated with membrane bound FasL in the presence or absence of cytokine for 20 hours. Jurkat cells treated with FasL for 20 hours were used as positive control. Results are representative of at least 3 separate experiments.

3.2.9 Caspase 8 inhibitor does not protect cells from apoptosis induced by cytokine starvation

Caspase inhibitors are cell permeable caspase substrates that irreversibly bind to active caspases and prevent activation of the downstream caspases. Numerous studies have

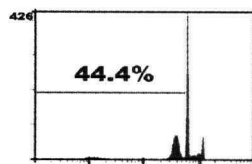
shown that caspase inhibitors are able to block apoptosis either fully or partially when added to cells (Du et al., 2005; Kazimierz et al., 2004; Gastman et al., 2000). We assumed that if caspase 8 activation initiated apoptosis in cytokine starved cells, its inhibition should protect cells from apoptosis. Thus, we tested the ability of the caspase 8 inhibitor to block apoptosis of cytokine-starved cells by adding the inhibitor to the cells in the presence or absence of cytokines. The percentage of cells with subdiploid DNA was then determined by PI staining followed by flow cytometry. As can be seen in Fig. 3.8, neither caspase 8, nor caspase 9 inhibitor alone protected cells against apoptosis induced by cytokine starvation.

Others in our laboratory have been able to show that the caspase 8 inhibitor at a concentration used in this thesis was able to inhibit camptothecin-induced caspase 8 activation in Jurkat cells (S. Jamil, personal communications). In addition, by using the same caspase 8 and 9 inhibitors, Marrota et al (2006) from our laboratory demonstrated that these inhibitors were effective in inhibiting Sulindac sulfide-induced caspase 8 and caspase 9 activation in the colonic carcinoma cell line studied. Furthermore, in other unpublished studies, these inhibitors have been used in our laboratory to inhibit caspase activation and apoptosis in other cell lines. Therefore, we feel confident that these inhibitors should have worked in FDCP-1 cells, as well. However, it is possible that many upstream caspases including caspase 2, 8, and 9 are simultaneously activated upon cytokine starvation in FDCP-1 cells and inhibition of all of these caspases might be necessary to inhibit apoptosis.

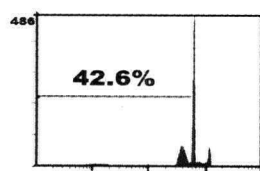
3.2.10 Caspase 2 is activated upon cytokine starvation.

Pro-caspase 2 is activated in response to a variety of cytotoxic stimuli including tumor necrosis factor- α , FasL, DNA damaging agents, and growth factor withdrawal (Harvey *et al.*, 1997; Droin *et al.*, 2001; Stefanis *et al.*, 1998). The study by

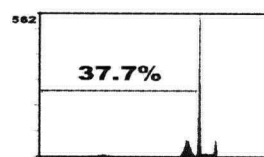
A. Control



B. Caspase 8 inhibitor



C. Caspase 9 inhibitor



D.

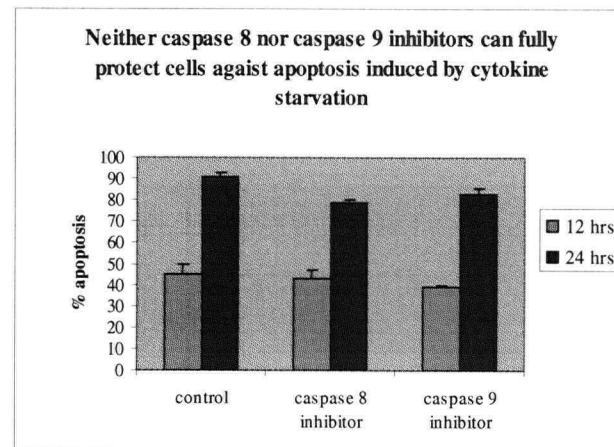


Figure 3.8- Neither caspase 8 nor caspase 9 inhibitors protect cells against apoptosis induced by cytokine starvation. FDCP-1 cells were starved of cytokine for 12 and 24 hours whilst in the presence of caspase 8 or caspase 9 inhibitors and the percentage of cell death was determined by PI staining and flow cytometry. **A**, **B**, and **C** are the representative histograms for the 12 hour time point with **A** showing the percentage of cell death in the absence of cytokine, while **B** and **C** show cytokine starved cells treated with either the caspase 8 inhibitor (**B**) or the caspase 9 inhibitor (**C**). The histogram in **D** shows percent apoptosis of cytokine starved FDCP-1 cells treated with or without the caspase 8 or 9 inhibitors for 12 and 24 hours. Results shown in **D** are from 3 separate experiments.

Kumar (1995) showed that FDCP-1 cells, in addition to many other hemopoietic cell lines, do express caspase 2 mRNA and an anti-sense against caspase 2 mRNA in FDCP-1 cells significantly inhibited cell death induced by cytokine withdrawal. Since in our hands the caspase 8 and 9 inhibitors did not appear to prevent cytokine withdrawal-induced apoptosis, we tested the possibility that caspase 2 could play a major role in initiating apoptosis due to cytokine starvation. Our results show activation of pro-caspase 2 protein upon cytokine

withdrawal by both Western blotting and flow cytometry (Figs 3.9A-D and 3.9E, respectively). However, similar to the caspase 8 and 9 inhibitors, the caspase 2 inhibitor does not appear to protect cells against apoptosis induced by cytokine withdrawal (Fig 3.9 F-G).

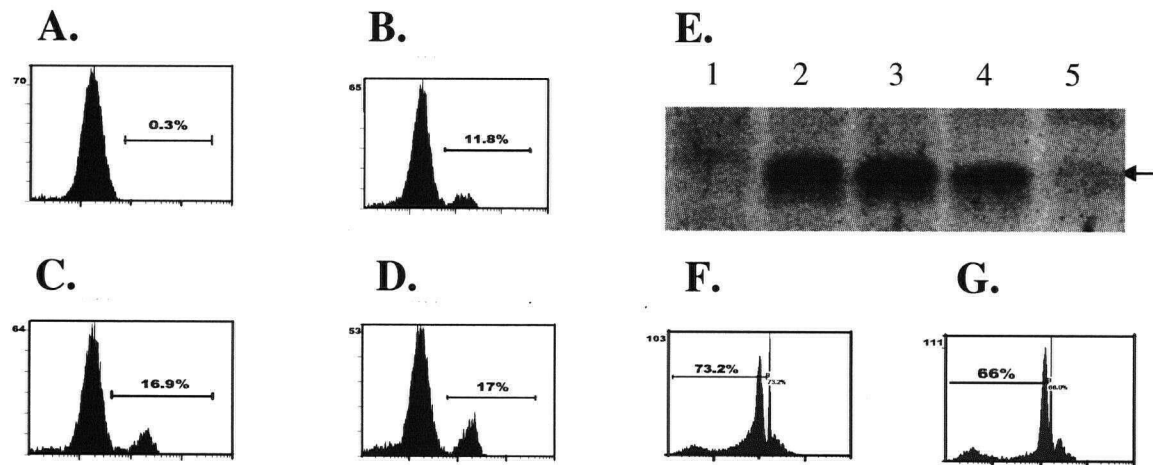


Figure 3.9- Caspase 2 is activated upon cytokine starvation. FDCP-1 cells were grown in the presence or absence of cytokine for the indicated time and the cells were then treated with a fluorescent marker which specifically binds to active caspase 2 and the percentage of caspase 2 activity was determined by flow cytometry. Panel A shows caspase 2 activity in normally proliferating cells in the presence of cytokine while panel B, C, and D show percentage of caspase 2 activity 8, 10, and 12 hours following cytokine starvation, respectively. E. Immunoblot with arrow showing the presence of active caspase 2 in FDCP-1 cells. In these cells cleaved caspase 2 was detected when the PI-3 kinase pathway was inhibited either by cytokine withdrawal (lane 2) or treatment of cells with LY29004 (lane 3) for 12 hours. Jurkat cells treated with camptothecin were used as positive control (Lane 4). No caspase 2 cleavage was observed either in normally proliferating FDCP-1 cells in the presence of cytokine (Lane 1) or normally proliferating Jurkat cells (Lane 5). F-G) FDCP-1 cells were starved of cytokine for 18 hours in the absence (F) or presence (G) of caspase 2 inhibitor and the percentage of cell death was determined by P.I. staining and flow cytometry. Results are representative of 3 separate experiments.

3.2.11 p27kip1 and Bim are induced within two hours of cytokine starvation

During the course of these studies, publications appeared which suggested that increases in both p27kip1 and Bim expression can result from activation of forkhead transcription factors, and these are likely to play a role in hemopoietic cell death (Dijkers et al, 2000a, 2000b; Dijkers et al., 2002). These studies were done on Baf-3 cells and thus we

decided to verify these findings in other hemopoietic cell lines, such as FDCP-1. We found that as little as 2 hours following withdrawal of cytokine, p27kip1 expression increased dramatically (Fig. 3.10A). Similarly, increases in Bim protein was observed within this time frame (Fig. 3.10B). The timing of both of these events supports the suggestion that they may play a crucial role in starvation-induced apoptosis.

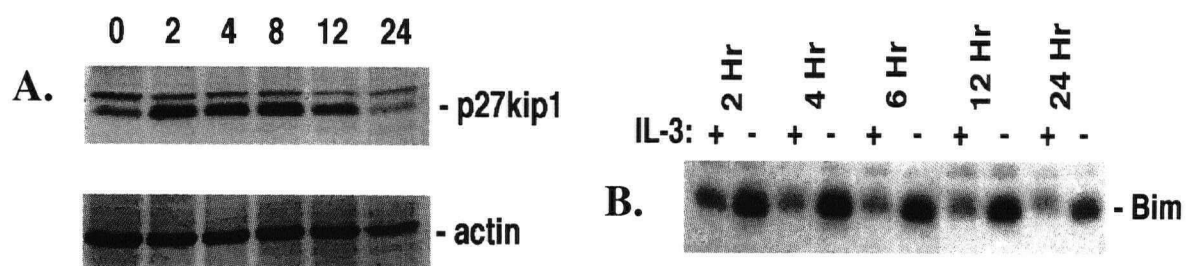


Figure 3.10- Bim and p27kip1 protein are induced following cytokine starvation. FDC-P1 cells were starved of IL-3 for the time indicated and cell extracts prepared and immunoblotted to detect the level of p27kip1 (panel A) or Bim (panel B). In panel B, samples left in the presence of IL-3 (+) at each of the time points were also run for comparison. Re-probing with anti-actin antibody was performed to indicate level of total protein loading, which was less at later times of cytokine starvation.

3.3 DISCUSSION

In the past several years, intense efforts have been made to understand the regulation of apoptosis in hemopoietic cells. Many laboratories, including ours, have used these types of cells as model systems since they rapidly (in 24 hours or less) undergo apoptosis upon cytokine withdrawal. We have been particularly interested in the signaling pathways activated by various cytokines that are important for inhibiting apoptosis. In this study, we continued probing the role of the PI3K signaling pathway in cell survival and have investigated the phosphorylation of forkhead transcription factors, in particular FOXO3A. We have shown in three different hemopoietic cell lines that IL-3, IL-4 and GM-CSF can

stimulate phosphorylation of FOXO3A at three sites including Thr32, Ser253, and Ser315, in a PI3Kdependent manner. Thus, FOXO3A appears to be phosphorylated in a number of contexts by the PI3K pathway in cytokine-dependent hemopoietic cells.

One of the reported targets of FOXO3A that we were interested in was FasL, the cell surface bound ligand for the Fas death receptor. We began these studies asking whether FasL up-regulation might be involved in induction of hemopoietic cell death via the Fas receptor when these cells were starved of cytokine. We were able to show that when cells were starved of cytokine, or when the PI3K signaling pathway was blocked, there was a clear up-regulation of FasL expression. Furthermore, the Fas receptor could be detected on these cells, and while it was at low levels in the actively growing cells, its cell surface expression was clearly increased in cells as they progressed towards apoptosis. Despite the cell surface expression of both the Fas receptor and its ligand, FasL, we could not clearly demonstrate a role for Fas signaling in the death of these cells. We attempted to show this by enhanced cell death in response to exogenously added FasL, as well as by detecting some of the hallmarks of Fas-mediated death, caspase 8 activation and cleavage of the Bcl-2 family protein, Bid. Although Fas receptor was present on the surface of normally proliferating as well as cytokine starved cells, to our surprise, addition of exogenous FasL did not induce caspase 8 activation and cell death. FasL did, however, induce cell death in Jurkats which were used as a positive control. Moreover, even though caspase 8 activation was detected in cytokine starved cells, its inhibition did not prevent cell death. The same caspase 8 inhibitor at a similar concentration used in this thesis has been previously shown to inhibit camptothecin-induced caspase 8 activation in Jurkat cells (personal communications). Furthermore, Bid cleavage could not be detected following cytokine starvation. Bid is the downstream

substrate of caspase 8 and although caspase 8 was activated around 8 hours following cytokine starvation, Bid cleavage could not be detected up to 12 hours following cytokine starvation in FDCP-1 cells. These findings led to the conclusion that perhaps FOXO3A-induced up-regulation of FasL does not play a major role in apoptosis due to cytokine starvation

Since we could not find a role for Fas, we investigated other possible mechanisms that might be involved in this process. A few studies have suggested that FOXO3A can regulate expression of p27kip1, which could contribute to cell cycle arrest (Dijkers *et al.*, 2000a; Medema *et al.*, 2000). In addition, the pro-apoptotic Bcl-2 family member, Bim, has been shown to be a target of forkhead transcription factors (Dijkers *et al.*, 2000b). Thus, we were not surprised that we could observe increases in the level of both p27kip1 and Bim proteins when cells were starved of cytokine.

Although we could not demonstrate a role for Fas in apoptosis of cytokine starved cells, we can not rule it out either. Our inability to show a role for Fas-mediated events in this process could be due to the limitations of the techniques employed. In our hands exogenous addition of FasL induced cell death in Jurkats (positive control) but not FDCP-1 cells. It is possible that FDCP-1 cells secrete excessive amounts of sFasL, a metalloproteinase-mediated cleavage product of the membrane bound FasL, which might compete with the exogenous FasL for binding to Fas receptor. sFasL is probably less toxic than the membrane bound FasL due to the incapacity of sFasL to efficiently engage with the Fas receptor. This possibility could be further explored by exogenous addition of sFas receptor (sFas) to neutralize the inhibitory effect of the sFasL. Alternatively, one could inhibit the Fas-mediated signaling events in order to show a role for Fas in apoptosis. If the death receptor pathway

plays an important role in cytokine starvation-induced cell death, then abrogation of the Fas receptor mediated signaling, for example by using SiRNA targeted to Fas receptor, or agonistic anti-Fas antibody, or over-expression of dominant negative FADD would have to impair the Fas-mediated death signaling events. These and other relevant studies are needed before we could definitively rule out a role for Fas in apoptosis induced by cytokine starvation.

It is worthwhile noting that in our hands caspase 8 activation was detected but it did not appear as an early event in cytokine deprivation-induced cell death. Since both caspase 8 and 9 were activated at approximately the same time, it is possible that caspase 8 might be activated downstream of caspase 9 through cleavage by effector caspase 3 as shown by others (Yang et al., 2006). Another possibility, which we could not rule out with certainty, is that caspase 8 might still act upstream of caspase 9 although in our hands we could not find a role for caspase 8 as an upstream initiator caspase. Activation of caspases is a highly regulated and rapid process and it is possible that we were unable to capture the exact moment a particular caspase was cleaved. Further experiments will be necessary to verify our findings.

In summary, our data demonstrates that the Fas mediated pathway might not be involved in the death of cytokine starved hemopoietic cells and the death might be mediated through activation of Bim and the subsequent loss of mitochondrial membrane integrity as shown by others.

CHAPTER 4

STUDIES OF BCL-2 AND BCL-XL FUNCTIONS

4.1 INTRODUCTION

The two pro-survival Bcl-2 family members, Bcl-2 and Bcl-xL, protect cells against apoptosis induced by a wide variety of cytotoxic stimuli. It is generally assumed that these proteins have a redundant function in providing cell survival since both proteins block BH3 only and Bax/Bak mediated mitochondrial membrane permeability. Studies using Bcl-2 and Bcl-xL knockout mice, however, show that they might have non-redundant roles in providing cell survival (Veis *et al.*, 1993; Motoyama *et al.*, 1995), although this might be a reflection of the differences in the tissue distribution of these proteins. There are also reports which indicate that Bcl-2 and Bcl-xL differentially protect cells against apoptosis induced by certain cytotoxic stimuli (Gottschalk *et al.*, 1994; Yuste *et al.* 2002; Simonian *et al.*, 1997, Fiebig *et al.*, 2006). In order to explore the differential ability of Bcl-2 and Bcl-xL in protecting cells against apoptosis induced by different cytotoxic stimuli, we over-expressed Bcl-2 and Bcl-xL in the cytokine dependent murine hemopoietic cell line FDCP-1. Since Bcl-2 is localized at both the mitochondrial and ER membranes but Bcl-xL is primarily localized at the ER membrane (Kaufmann *et al.*, 2003), we hypothesized that Bcl-2 is a more potent pro-survival protein than Bcl-xL against cytokine starvation-induced cell death. Our results supported our hypothesis that under our experimental set up, Bcl-2 was indeed a more potent pro-survival protein.

The pro-survival Bcl-2 family members inhibit apoptosis at least in part by preventing cytochrome C release from the mitochondria. Although the exact mechanism of such

inhibitions is not entirely clear at present, pro-survival Bcl-2 family proteins are believed to maintain mitochondrial membrane integrity by inhibiting activation of the Bax-like proteins on the mitochondria (Mikhailov *et al.*, 2001; Murphy *et al.*, 2000). In addition, it is not certain that the role of pro-survival Bcl-2 family members is limited to the mitochondria. Much emphasis has been placed on Bcl-2 function on the mitochondria, although it has been reported that wild type Bcl-2 localizes to the mitochondria, ER, and nuclear membranes (Akao *et al.*, 1994) and there is growing evidence that the ER is important in apoptosis. The Bcl-2 family members, for example, have been implicated in regulating ER calcium during apoptosis (Foyouzi-Youssefi *et al.*, 2000; He *et al.*, 1997; Lam *et al.*, 1994). Recent evidence suggests that pro-apoptotic Bcl-2 family members like Bik (Germain *et al.*, 2002) or Bax and Bak (Nutt *et al.*, 2002) localize on the ER to regulate apoptosis. Also, many apoptosis-regulating proteins such as caspase 12 (Nakagawa *et al.*, 2000) and Bap-31 (Ng *et al.*, 1997) reside on or in the ER. It has also been reported that Bcl-2 targeted solely to the ER is effective in blocking apoptosis (Zhu *et al.*, 1996). This ER-targeted form of Bcl-2, Bcl2-Cb5, is protective against many forms of apoptosis and has been shown to inhibit caspase activation and cytochrome *c* release (Hacki *et al.*, 2000). Others have shown that Bcl2-Cb5 can inhibit apoptosis induced by over-expression of Bax (Wang *et al.*, 2001), suggesting that Bcl-2 can still inhibit the actions of pro-apoptotic family members when localized to the ER.

Unlike Bcl-2, Bcl-xL is specifically localized to the mitochondria. However, Bcl-xL can protect cells against cytotoxic stimuli that induce ER stress response (Srivastava *et al.*, 1999; Morishima *et al.*, 2004; White *et al.*, 2005). To specifically address the physiological relevance of the membrane targeting of Bcl-xL, we used Rat-1 fibroblast cell lines over-expressing Bcl-xL mutants that were targeted to ER (Bcl-xL-cb5), mitochondrial outer

membrane (Bcl-xL-ActA), or wild type Bcl-xL (Bcl-xL-wt). We hypothesized that the ER targeted Bcl-xL is as protective as the mitochondrial targeted and wild type Bcl-xL against apoptosis induced by various cytotoxic stimuli. Our results show that the ER targeted Bcl-xL appears to be more protective against Bad-induced apoptosis compared to either the mitochondrial targeted or wild type Bcl-xL. In addition, the ER targeted Bcl-xL is as effective as the mitochondrial targeted and wild type Bcl-xL against growth factor withdrawal and ceramide induced apoptosis.

4.2 RESULTS

4.2.1 Comparing the expression of Bcl-2 family proteins in hemopoietic cell lines and the Rat-1 fibroblast cell line

The Bcl-2 family proteins are expressed at various levels in many cells throughout the body. We looked at endogenous expression of the Bcl-2 proteins in the different cell lines that were the subject of our studies in this thesis, including the hemopoietic cell lines, MC9, TF-1, FDCP-1, and the Rat-1 fibroblast cell line. As the immunoblots show in Fig. 4.1, the pro-survival family members, Bcl-2 and Bcl-xL, as well as the pro-apoptotic members, Bim, and Bax are expressed in all of these cell lines at varying levels. Bim seems to be expressed at a very low level in MC9 compared to either TF-1 or FDCP-1, whereas Bax expression is greater in MC9 compared to all of the other cell lines studied.

4.2.2 Bcl-2 appears to be a more potent pro-survival protein than Bcl-xL against apoptosis induced by cytokine withdrawal.

Bcl-2 and Bcl-xL play an important role in the regulation of hemopoietic cell survival. As shown in Figure 4.1, both Bcl-2 and Bcl-xL proteins are endogenously expressed in FDCP-1 cells.

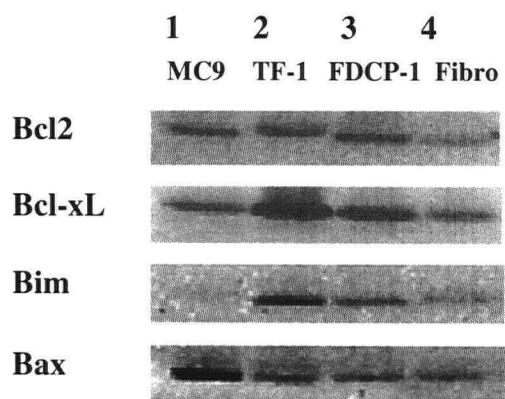


Figure 4.1- Expression of Bcl-2 family proteins in different cell lines. Normally proliferating cells were homogenized, 50 µg of lysates was loaded in each lane, and the expressions of different Bcl-2 family proteins were analyzed by western blotting: MC/9 (lane 1), TF-1 (lane 2), FDCP-1 (lane 3), and the Rat-1 fibroblast cell line (lane 4).

The aim of this study was to compare the differential ability of Bcl-2 and Bcl-xL to induce cell survival in cytokine starved cells. We stably over-expressed Bcl-2 and Bcl-xL in the IL-3 dependent FDCP-1 cell line and generated mixed populations of FDCP-1 cells expressing Bcl-2 or Bcl-xL (Fig. 4.2A-B). Apoptosis was induced by cytokine withdrawal for 24 hours. Cells were stained with propidium Iodide (P.I.), and the percentage of cells carrying subdiploid DNA was determined by FACS analysis. The 24 hour time course was chosen since FDCP-1 cells are very vulnerable to cytokine withdrawal and within 24 hours, almost all the parental cells undergo apoptosis. As can be seen in figure 4.2, almost 90% of the parental FDCP-1 cells (Fig. 4.2D) and close to 80% of cells over-expressing Bcl-xL (Fig. 4.2F) underwent apoptosis within 24 hours, whereas, the majority of cells over-expressing Bcl-2 survived the cytotoxic effect of cytokine withdrawal (only 28% cell death) [Fig. 4.2E]. These FDCP-1 cells were also used to clone out specific cell lines expressing different levels

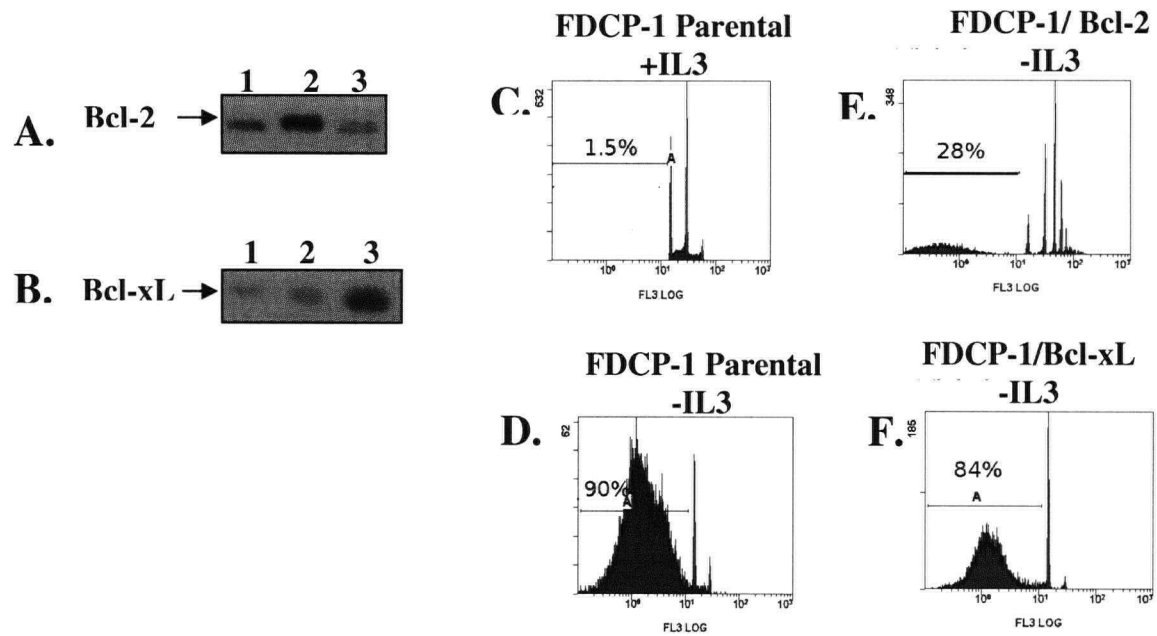


Figure 4.2- Bcl-2 and Bcl-xL over-expressing cells (Mixed population). FDCP-1 cells were retrovirally transfected with pMXpuro/Bcl-2 or pMXpuro/Bcl-xL and stable mixed population of cells were generated. Western blots in **A** and **B** show Bcl-2 and Bcl-xL levels, respectively, in mixed population of cells over-expressing either of these proteins, with lanes 1 representing parental cells, lanes 2, Bcl-2 over-expressing and lanes 3, Bcl-xL over-expressing cells. **C-F**) Parental, as well as, Bcl-2 over-expressing cells (FDCP-1/ Bcl-2) and Bcl-xL over-expressing cells (FDCP-1/ Bcl-xL) were induced to undergo apoptosis by cytokine withdrawal for 24 hours. Cells were collected, stained with P.I. and the percentage of cells with subdiploid DNA was obtained by flow cytometry. Results are representative of 3 separate experiments.

of Bcl-2 or Bcl-xL. Western blotting was performed to confirm expression of Bcl-2 and Bcl-xL in these clones (Fig. 4.3A-B). The density of the bands in Fig. 4.3A-B was measured against the corresponding β -actin and based on these values, two clones each of Bcl-2 or Bcl-xL over-expressing cells were selected for further analysis. These clones were labeled “Medium” or “High” expressing Bcl-2 or Bcl-xL clones (Fig. 4.3C). It is very well documented that the level of the pro-survival proteins expressed in cells is directly proportional to their survival function (Fiebig et al., 2006). This means that clones expressing higher levels of pro-survival proteins are better protected against apoptosis compared to clones expressing lower amounts of these proteins. Since our initial observations in mixed

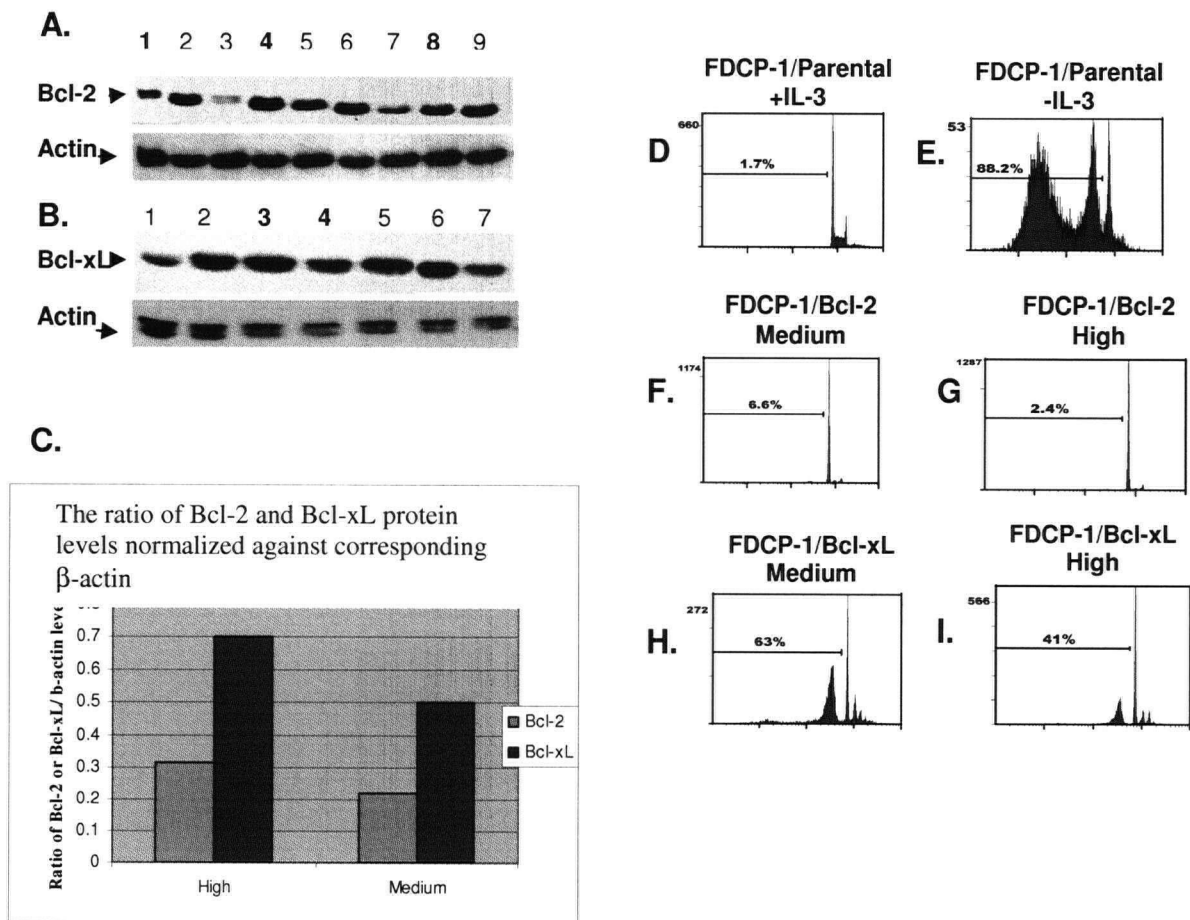


Figure 4.3- Bcl-2 is a more potent pro-survival protein than Bcl-xL. Specific clones of FDCP-1 cells that over-expressed Bcl-2 or Bcl-xL were generated. Western blots in **A** and **B** show the expression levels of Bcl-2 and Bcl-xL, respectively, in these clones. In panel **A**, lanes 1, 4, and 8 (bold) represent parental, high Bcl-2 expressing, and medium Bcl-2 expressing clones, respectively. In panel **B**, lanes 1, 3, and 4 (bold) represent parental, high Bcl-xL expressing, and medium Bcl-xL expressing clones, respectively. Anti- β -actin antibody was used as control for equal loading. **C**) The density of the bands representing clones with high or medium levels of Bcl-2 or Bcl-xL in panels **A** and **B** was normalized against the corresponding β -actin and the ratio was plotted as a histogram. **D-I**) The medium and high expressing Bcl-2 or Bcl-xL clones as well as the parental cells were induced to undergo apoptosis by cytokine withdrawal for 24 hours and percentage of cells carrying subdiploid DNA was determined by PI staining followed by FACS analysis. Panels **D** and **E** represent parental cells in the presence or absence of cytokine, respectively while panels **F** and **G** show medium and high expressing Bcl-2 clones, respectively, and panels **H** and **I** show medium and high expressing Bcl-xL clones, respectively, in the absence of cytokine. **A**, **B**, and **C** are from a single experiment while the results shown in **D-I** are representative of 3 separate experiments.

population of Bcl-2 and Bcl-xL over-expressing cells indicated that Bcl-2 was more potent than Bcl-xL, in order to rule out the possibility that Bcl-xL level might have been less than that of Bcl-2 in the mixed population, we only compared Bcl-2 and Bcl-xL over-expressing

clones, in which the Bcl-xL expression levels appeared to exceed those of the Bcl-2 by Western blotting, as can be seen in the representing histogram in Fig. 4.3C. Apoptosis was induced by cytokine withdrawal for 24 hrs and the percentage of cells with subdiploid DNA was determined by P.I. staining followed by FACS analysis. In multiple repeats of this experiment, the percentage of cell death in the absence of IL-3 in the parental FDCP-1 cells was always close to 90 percent. When Bcl-2 was over-expressed at medium or high levels, almost all cells survived the effect of cytokine withdrawal (7% cell death in medium Bcl-2 over-expressing and 2% death in high Bcl-2 over-expressing cells) [Fig. 4.3]. However, cells over-expressing medium level of Bcl-xL were barely protective (about 60% cell death) and cells expressing high level of this protein only partially protected cells against the effect of cytokine withdrawal (40% cell death).

Although we show here that Bcl-2 is more potent than Bcl-xL in providing survival in cytokine starved cells, further experiments are needed to validate our findings since our study design was not optimal. The lack of our ability to clearly demonstrate that equimolar concentrations of Bcl-2 and Bcl-xL proteins were over-expressed in the clones studied is one such flaw in the study design. The anti-Bcl-2 and anti-Bcl-xL antibodies have different affinities for their targeted proteins, a fact that cannot be quantitated by simple Western blotting. A proper way to solve this problem is to tag the genes of interest. If the over-expressed proteins are tagged, the expression levels of these proteins could be easily compared by Western blotting using a single antibody against the tag epitope.

4.2.3 Endoplasmic reticulum localized Bcl-xL is a more potent pro-survival protein than either the mitochondrial targeted or wild type Bcl-xL against apoptosis induced by Bad over-expression

Bcl-2 is found on different intracellular membranes including ER, outer mitochondrial, and nuclear membranes, whereas, Bcl-xL is specifically targeted to the outer mitochondrial membrane. In order to explore the importance of subcellular localization of Bcl-xL, Rat-1 fibroblast cell lines over-expressing Bcl-xL-cb5 (ER), Bcl-xL-ActA (mitochondria), or Bcl-xL-wt (wild type) were used. These mutant Bcl-xL over-expressing Rat-1 fibroblast cell lines were a kind gift from Dr. David Andrews laboratory (McMaster University) and the specific subcellular localization of these Bcl-xL mutants have been confirmed by their laboratory (Fiebig *et al.*, 2006). Using these Bcl-xL mutant cell lines, we hypothesized that the Bcl-xL mutant targeted to ER is as protective as the wild type or the mitochondrial targeted Bcl-xL against apoptosis induced by various cytotoxic stimuli.

We first confirmed the expression of the different Bcl-xL mutants in these cells by Western blotting as shown in Fig. 4.4A. Previously, Dr. Shaynoor Dramsi, a post doctoral fellow from our laboratory, showed that unlike Bcl-2, wild type Bcl-xL did not protect cells against death induced by the BH3 only pro-apoptotic Bcl-2 family member, Bad (unpublished observations). Others have also shown that Bad induced apoptosis specifically through its interaction with Bcl-xL. Using the cell lines expressing Bcl-xL targeted to different subcellular membranes, we asked whether specific subcellular localization of Bcl-xL would differentially affect Bad-induced apoptosis. We investigated this by transiently over-expressing Bad wild type into the different mutant Bcl-xL over-expressing Rat-1 fibroblast cell lines. Forty-eight hours following transfection, cells were collected and the percentage of cells carrying subdiploid DNA was determined by P.I. staining and FACS analysis. The results show that the ER targeted Bcl-xL is more protective than either the

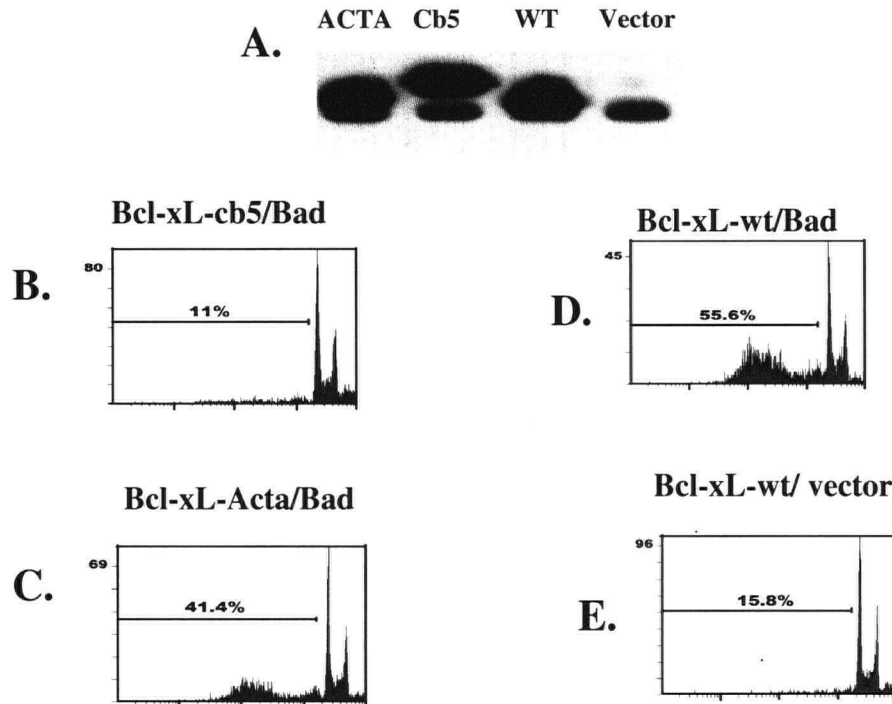


Figure 4.4- Mutants of Bcl-xL targeted to different subcellular organelles differentially protect cells against death induced by Bad over-expression. A) Rat-1 fibroblast cell lines over-expressing the different Bcl-xL mutants were homogenized, 50 μ g of the cell lysates were loaded in each lane, and the expression of the different Bcl-xL mutants was determined by Western blotting using anti-Bcl-xL antibody. B-E) Bcl-xL over-expressing Rat-1 fibroblast cell lines were induced to undergo apoptosis by transient over-expression of Bad. Forty-eight hours following transfection, cells were collected for P.I. staining and FACS analysis. Panel B (cells over-expressing ER targeted Bcl-xL transfected with Bad/pMXpuro vector), Panel C (cells over-expressing mitochondrial targeted Bcl-xL transfected with Bad/pMXpuro vector), Panel D (cells over-expressing wild type Bcl-xL transfected with Bad/pMXpuro vector), Panel E (cells over-expressing wild type Bcl-xL transfected with control pMXpuro vector alone). Results shown are representative of 3 separate experiments ($P= 0.0053$).

Bcl-xL targeted to mitochondria, or wild type Bcl-xL against Bad-induced apoptosis, with close to 10% of cells with subdiploid DNA in ER over-expressing cells compared to about 40% and 60% in cells over-expressing mitochondrial targeted or wild type Bcl-xL, respectively (Figs 4.4B-E).

4.2.4 All three Bcl-xL mutants equally protect against apoptosis induced by either ceramide treatment or growth factor withdrawal.

In addition to Bad over-expression, we used other cytotoxic stimuli, including growth factor withdrawal and treatment of cells with ceramide, to induce apoptosis in the Bcl-xL over-

expressing Rat-1 fibroblast cell lines. We then asked which Bcl-xL mutant was more potent in providing survival against apoptosis induced by these death stimuli. Apoptosis was induced by either culturing the cells in the presence of 10% serum plus 50 μ M ceramide or in the absence of serum. Cells were collected 48 hours later and the percentage of cells with subdiploid DNA was analyzed by P.I. staining followed by FACS analysis. When cells were treated with ceramide (Figs 4.5A-D), increased number of cells with subdiploid DNA was detected in the control Rat-1 fibroblast cell line which carried the vector without the Bcl-xL insert. However, Bcl-xL localized at the ER, mitochondria, or the wild type Bcl-xL were all equally protective against ceramide induced apoptosis. A similar pattern was observed when cells were cultured in the absence of serum (Figs. 4.5E-H). Thus, at least in ceramide, as well as, serum deprivation-induced cell death, Bcl-xL over-expression appears to efficiently contribute to cell survival, irregardless of its subcellular targeting.

In our hands, the Rat-1 fibroblast cell lines were very resistant to treatment with either ceramide or growth factor withdrawal. In fact, very minimal cell death was detected within 24 hours of these treatments. The 48 hour time course was chosen since cells transfected with vector alone showed moderate cell death at this time course (Figs. 4.5A and E). It would be worthwhile, however, to examine the pro-survival effects of these Bcl-xL targeted mutants in response to longer treatments with ceramide or growth factor withdrawal.

4.3 DISCUSSION

In this chapter, results were presented from several sets of experiments exploring Bcl-2 family functions and interactions in an attempt to better understand their relationships. First, it was shown that Bcl-2 has a more potent anti-apoptotic function compared to Bcl-xL in hemopoietic cells. Second, the role of ER vs. mitochondrial targeted pro-survival proteins

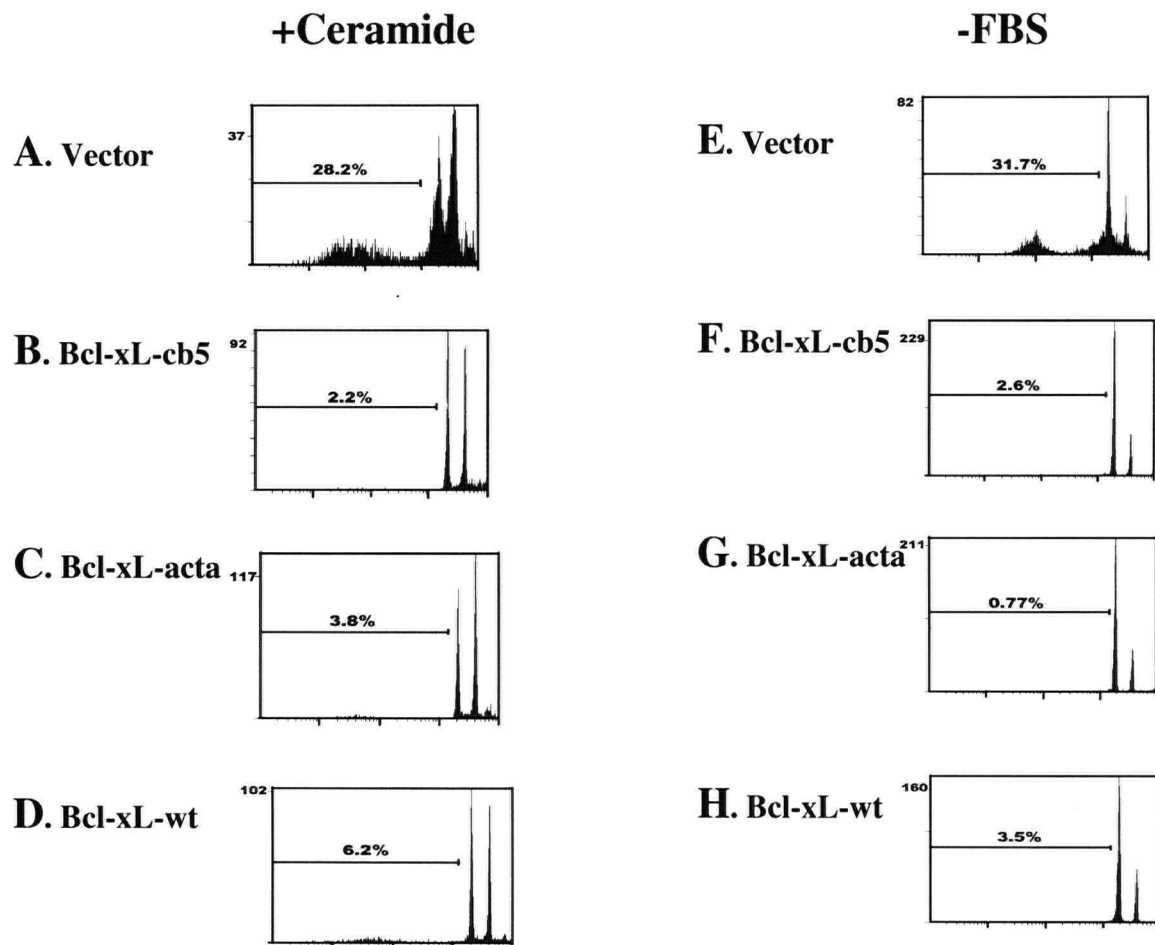


Figure 4.5- Mutants of Bcl-xL targeted to different subcellular organelles are comparable in protecting cells against apoptosis induced by ceramide or growth factor withdrawal. The Bcl-xL over-expressing Rat-1 fibroblast cell lines were induced to undergo apoptosis either with ceramide treatment or growth factor withdrawal for 48 hrs. Cells were stained with propidium Iode and analyzed by flow cytometry. First column represents ceramide treated cells and the second column represents cells grown in the absence of serum. Panels **A** and **E** (control Rat-1 fibroblast cells expressing vector), Panels **B** and **F** (cells expressing ER targeted Bcl-xL), Panels **C** and **G** (cells expressing mitochondrial targeted Bcl-xL), Panels **D** and **H** (cells expressing wild type Bcl-xL). The results are representative of 3 separate experiments.

was compared with respect to apoptosis induced by the BH3-only Bcl-2 family protein Bad, ceramide treatment, or serum withdrawal. The results showed that while the ER and mitochondrial targeted Bcl-xL equally protected cells against serum withdrawal and

ceramide-induced apoptosis, the ER targeted Bcl-xL was far more protective than the mitochondrial targeted Bcl-xL against Bad-induced apoptosis.

Our results using mixed population as well as clones of Bcl-2 or Bcl-xL over-expressing FDCP-1 cells supported our hypothesis and showed that Bcl-2 was consistently a more potent pro-survival protein than Bcl-xL. Bcl-2 and Bcl-xL are generally assumed to have redundant non-overlapping functions. They are very similar in many aspects including their structure and function and one of the very few observable differences between Bcl-2 and Bcl-xL appears to be their physiological site of localization as mentioned earlier. Perhaps cytokine withdrawal activates both ER mediated and mitochondrial mediated apoptotic events and since Bcl-2 is localized at both of these organelles, it might be more potent in blocking apoptosis compared to Bcl-xL which is presumed to be localized only at the mitochondria. Simonian *et al.* (1997) proposed that the differential ability of Bcl-2 and Bcl-xL to protect, for example, against chemotherapy-induced apoptosis in their studies seemed to be dependent on the molecular mechanism or cellular target of the drugs. For example, Bcl-2 and Bcl-xL provided similar protection against the chemotherapeutic drugs, vincristine and vinblastine, whereas Bcl-xL provided 50% greater cell viability than Bcl-2 against etoposide-induced cell death (Simonian *et al.*, 1997). Similarly, Keong *et al.* (2000) reported that the increased expression of Bcl-xL, but not Bcl-2, suppressed TRAIL-induced apoptosis in MDA-MB-231, HeLa, and Jurkat cells (Keogh *et al.*, 2000). A few others have also reported that Bcl-2 and Bcl-xL, to varying degrees, protect cells against apoptosis induced by certain cytotoxic stimuli (Gottschalk *et al.*, 1994; Yuste *et al.* 2002). Just recently, Fiebig *et al.*, (2006) reported that in human breast cancer cell line, MCF-7, as well as the Rat-1 fibroblast cell line, Bcl-xL and Bcl-2 targeted to ER (Bcl-xL-cb5, and Bcl-2-cb5,

respectively) functioned quantitatively different in protecting cells against apoptosis induced by different stress stimuli. They showed that while Bcl-xL-cb5 effectively protected cells against apoptosis induced by etoposide, TNF- α , and doxorubicin, Bcl-2-cb5 was ineffective. Thus, depending on the mode of action of the cytotoxic insult, the cell type, and the location of Bcl-2 or Bcl-xL in the cells, Bcl-2 and Bcl-xL could differentially protect against particular insults.

Although in the present study we demonstrated that Bcl-2 was more potent than Bcl-xL in protecting cells against apoptosis induced by cytokine starvation, a significant drawback of this study was our inability to ascertain that equimolar concentrations of Bcl-2 or Bcl-xL were over-expressed in the Bcl-2 and Bcl-xL over-expressing clones. It would have been ideal to have run protein standards containing known concentrations of Bcl-2 or Bcl-xL proteins on the SDS gels along with the lysates from Bcl-xL or Bcl-2 over-expressing clones. This would have allowed us to more accurately quantitate the amounts of Bcl-2 or Bcl-xL in each clone relative to the known concentrations of standards. It is very crucial to compare clones that express similar amounts of these proteins since small differences in the amount of pro-survival proteins present in the cells can lead to a large difference in cell survival. Another problem that needs to be addressed is the 24 hour time period used in this study although this time period was selected based on the observation that 90% of parental FDCP-1 cells die within 24 hours of cytokine starvation and our aim was to show a large difference in cell survival between over-expressed and the parental cells. However, further studies are needed to examine shorter and longer time periods following cytokine starvation. As mentioned above, in our hands Bcl-xL was shown to be less potent than Bcl-2 in providing short term survival; however, since long-term survival following cytokine

withdrawal was not studied, we are unable to rule out the role of Bcl-xL in such a scenario. Therefore, although we have demonstrated that Bcl-2 was a more potent pro-survival protein than Bcl-xL, further studies are needed to validate these findings due to the problems discussed above.

Our second observation was that ER targeted Bcl-xL is substantially more effective in blocking apoptosis induced by Bad over-expression compared to its mitochondrial or wild type counterparts. Furthermore, we demonstrated that targeting Bcl-xL to ER inhibits apoptosis induced by C2 ceramide or growth factor withdrawal, at a similar level as targeting Bcl-xL to either mitochondria, or wild type Bcl-xL. There are some explanations of how Bcl-xL targeted to ER might be able to protect against treatment with ceramide, growth factor withdrawal, or Bad-induced apoptosis. One possibility is that Bcl-xL might regulate ER calcium (Ca^{2+}) homeostasis and protect against apoptosis stimuli that perturb intracellular calcium levels. Both the ER and mitochondria act as Ca^{2+} stores controlling the Ca^{2+} influx and cytoplasmic Ca^{2+} homeostasis (Pozzan *et al.*, 2000). Changes in the cytoplasmic Ca^{2+} level are linked to apoptosis and, in fact, blocking Ca^{2+} signaling with chelators inhibits apoptosis (Dowd *et al.*, 1992). Release of Ca^{2+} from ER initiates a number of pro-apoptotic events including activation of Ca^{2+} dependent protease (calpain), caspase activation, and/or mitochondrial dysfunction (Smaili *et al.* 2003). Certainly there is an apoptotic cross talk between ER and mitochondria since agents that perturb ER homeostasis, for example drugs such as brefeldinA (BFA) or tunicamycin, induce apoptosis through the mitochondrial mediated pathway via the release of cytochrome C from the mitochondria (Hacki *et al.*, 2000). Interestingly, both the wild type as well as the ER targeted Bcl-2 are protective against BFA and tunicamycin-induced apoptosis. Ceramide, a lipid

second messenger, induces apoptosis through an increase in cytoplasmic Ca^{2+} level which is followed by an early loss in mitochondrial membrane potential (Pinton *et al.*, 2001) and surprisingly, Bcl-2 targeted to ER protects against ceramide-induced apoptosis (Annis *et al.*, 2001). Similar to Bcl-2, Bcl-xL also influences ER Ca^{2+} , for example, by decreasing both the expression of Ca^{2+} channel, inositol 1,4,5, triphosphate receptor (IP3R), and Ca^{2+} release following T cell stimulation (Li *et al.*, 2002). Recently White *et al.* (2005) reported that Bcl-xL regulates the inositol 1,4,5 triphosphate receptor (InsP3R) Ca^{2+} release channel in the ER, which antagonizes apoptosis. The authors used pull-down assays and co-immunoprecipitation of the endogenous protein to demonstrate that Bcl-xL interacts with all three mammalian InsP3R isoforms. Although it has been reported that Bcl-xL is specifically targeted to the mitochondrial outer membrane, there is still a possibility that some Bcl-xL might be localized to ER and this ER targeted Bcl-xL might be involved in the regulation of ER mediated Ca^{2+} homeostasis. Alternatively, Bcl-xL could interact with the InsP3R Ca^{2+} channel from the remote mitochondrial location. Thus, our observation that the Bcl-xL at the ER was protective against ceramide-induced apoptosis could be due to regulation of the Ca^{2+} signaling by Bcl-xL.

In addition to regulating the ER Ca^{2+} level, the anti-apoptotic Bcl-2 proteins localized at ER might sequester pro-apoptotic proteins such as the BH3 only Bcl-2 family members. In agreement with this notion, ER localized Bcl-2 (Bcl2-cb5) can protect against Bad-induced apoptosis (Thomenius *et al.*, 2003). Also, co-expression of Bad and Bcl2-cb5 retains Bad at ER. Bcl-2 appears to directly interact with Bad at ER since mutation of the Bad binding site on Bcl2-cb5, renders Bcl-2 ineffective in preventing Bad-induced apoptosis (Thomenius *et al.*, 2003). This direct interaction between Bcl2-cb5 and Bad argues in favor of the notion

that the pro-survival Bcl-2 members at the ER function to sequester the pro-apoptotic proteins away from their other intracellular targets such as the mitochondria. Similar to Bcl-2, Bcl-xL targeted to ER (Bcl-xL-cb5) is equally effective as the wild type Bcl-xL in blocking apoptosis by the ER stress inducers such as tunicamycin and Thapsigargin (Morishima *et al.*, 2004). Since the mechanism of action of Bcl-2 and Bcl-xL appears to be similar, Bcl-xL at ER might also function by sequestering pro-apoptotic molecules such as Bad or Bik. Thus, our observation that the ER targeted Bcl-xL was effective in protecting against Bad-induced apoptosis could be due to sequestration of Bad by ER targeted Bcl-xL, which would then prevent Bad from targeting mitochondria.

CHAPTER 5

A NOVEL ROLE FOR MCL-1 IN THE REGULATION OF DNA DAMAGE CHECKPOINT RESPONSE

5.1 INTRODUCTION

Mcl-1 (myeloid cell leukaemia-1) is a pro-survival Bcl-2 family member that was originally identified as a result of its up-regulation in a human myeloblastic leukemia ML-1 cell line that was induced to differentiate along the monocyte lineage (Kozopas *et al.*, 1993; Yang *et al.*, 1996). Interestingly, rapid up-regulation of Mcl-1 was observed when ML-1 cells differentiated along the monocyte, but not the granulocyte lineage (Yang *et al.*, 1996). Mcl-1 has been shown to have only moderate pro-survival activity compared with the significant anti-apoptotic effects of Bcl-2 or Bcl-XL (Zhou *et al.*, 1997; Zhou *et al.*, 1998). Moreover, when the Mcl-1 gene was knocked out in mice, Mcl-1^{-/-} embryos failed to implant, thus showing pre-implantation lethality. Of interest was the observation that these embryos displayed no evidence of altered apoptosis (Rinkenberger *et al.*, 2000) unlike Bcl-xL knockout mice where the embryos did not survive due to enhanced apoptosis. These observations suggest that Mcl-1 must serve other functions in addition to its activity as an anti-apoptotic Bcl-2 family member. One possibility is that Mcl-1 might have a role in regulation of cell cycle progression. Fujise *et al.* (2000) suggested a role for Mcl-1 in blocking progression through S-phase of the cell cycle through its association with the cell cycle regulator, PCNA (proliferating-cell nuclear antigen). In addition, our laboratory had previously shown that a short form of Mcl-1, which we termed snMcl-1 for short nuclear Mcl-1, may negatively regulate cell cycle progression through its interaction with the cell-cycle-dependent kinase, Cdk1, in a human promyelocytic leukemia cell line, HL-60, since

the Cdk-1 associated with Mcl-1 was shown to have a lower kinase activity (Jamil *et al.*, 2005). In addition, Cdk1 activity was greatly decreased in Mcl-1 over-expressing cells, which exhibited a delay in cell-cycle progression. We believe that this novel function of Mcl-1 might partly explain its critical role in mammalian development, defining a new mode of regulation by a member of the Bcl-2 family.

In view of the observations described above, we hypothesized that Mcl-1 plays a regulatory role in surveillance of cell cycle progression. In addition, since Mcl-1 level increases in cells undergoing DNA damage and also in cells undergoing differentiation towards monocytic lineage, we hypothesized that Mcl-1 is required for the regulation of DNA damage checkpoint response pathway as well as cellular differentiation.

Our first observation with regards to a possible role for Mcl-1 in cell cycle regulation was that in the cytokine dependent TF-1 cells, snMcl-1 interacted with Cdk-1 only in the nucleus, which confirmed the previous findings in HL-60 cells (Jamil *et al.*, 2005). However, unlike the previous findings in HL-60 cells, we showed that Cdk-1 could co-immunoprecipitate with the full length Mcl-1 in TF-1 cells. In addition, we explored a potential cytokine dependent effect on this interaction and showed that it only occurs in the presence of cytokine.

In other studies, it was observed that the level of Mcl-1 protein increases upon treatment of cells with etoposide, leading to the possibility that Mcl-1 might be involved in the DNA damage checkpoint response. It was shown that Mcl-1 co-immunoprecipitates with the checkpoint kinase-1 (Chk-1) and the phosphorylated histone H2AX. In addition, Mcl-1 over-expression in HL-60 cells was shown to induce phosphorylation of Rad 51, a protein involved in the homologous recombination repair process. These preliminary yet novel

results suggested that Mcl-1 might play a role in the regulation of DNA damage checkpoint response.

Another potential role for Mcl-1 might be its involvement in cellular differentiation based on its initial discovery in a differentiation model system, as well as its essential role in embryonic development. In order to explore this possibility in a hemopoietic differentiation model, we successfully over-expressed Mcl-1 in HL-60 cells by electroporation, however, our preliminary observations indicated that Mcl-1 might not play a role in the induction of cellular differentiation in HL-60 cells.

5.2 RESULTS

5.2.1 Mcl-1 associates with the cell-cycle regulatory protein Cdk1

As mentioned earlier, one of the interests of our laboratory is to study interactions between Bcl-2 family members and cell cycle regulatory proteins. Jamil et al. (2005) showed an interaction between snMcl-1 and Cdk-1 in HL-60 cells. The specific aim of this study was to confirm these findings in TF-1 cells and to determine whether these interactions are dependent upon the presence of cytokines. The specificity of the anti-Mcl-1 antibody was investigated by immunoprecipitating Mcl-1 in the lysates from normally proliferating TF-1 cells and the membrane was probed with the anti-Mcl-1 antibody. As can be seen in Fig. 5.1A, the anti-Mcl-1 antibody clearly detects only one band of approximately 42 KDa only in the sample immunoprecipitated with the anti-Mcl-1 antibody (lane 2). No non-specific bands were detected in the negative control lane which included the anti-Mcl-1 antibody control (lane 1). Similarly, when we tested the ability of Cdk-1 antibody to immunoprecipitate lysates from TF-1 cells, as shown in (5.1B), Cdk-1 was found to detect a 34 KDa specific Cdk-1 band. Having established the specificity of these antibodies, we

proceeded to study co-immunoprecipitation between Mcl-1 and Cdk-1. TF-1 cells were grown in the presence or absence of cytokine for 12 and 24 hours. In an attempt to show association between Mcl-1 and Cdk1, cytosolic and nuclear proteins obtained from these TF-1 cells were immunoprecipitated with anti-Cdk-1 antibody and probed for Mcl-1. The results showed that Cdk-1 immunoprecipitated 2 Mcl-1 bands, a stronger band at 42 KDa corresponding to full length Mcl-1 and a weaker band at 36 KDa corresponding to snMcl-1 only in the nuclear fractions obtained from cells grown in the presence of cytokine. No interaction was detected in the absence of cytokine (Fig 5.2A). When the same membrane was probed with an antibody to the cyclin partner of Cdk-1, Cyclin B1, as shown in Fig. 5.2B, the Cdk-1/Cyclin B1 interaction was detected only in the nucleus and only in the presence of cytokine. These data show that Cdk-1 associates both with Mcl-1 and Cyclin B1 in the nucleus. Together, these results validate previous observations made in HL-60 cells (Jamil *et al.*, 2005). In addition, use of a cytokine-dependent cell line has allowed us to show that this association is dependent upon the presence of cytokine and, therefore, might be a cell cycle stage dependent event.

5.2.2 Mcl-1 associates with the regulator of DNA damage checkpoint control, Chk-1

In a parallel project in our laboratory, Dr. S. Jamil has shown that treatment of HL-60 and HeLa cells with the DNA damaging agent etoposide caused an increase in the expression of Mcl-1. Furthermore, recent studies have supported an essential role for Mcl-1 in DNA damage checkpoint response (Jamil *et al.*, submitted). Therefore similar approaches were used to investigate these events in TF-1 cells. As shown in Fig. 5.3, in TF-1 cells, Mcl-1 level was also increased upon treatment of cells with 15 μ M etoposide for 24 hours. The

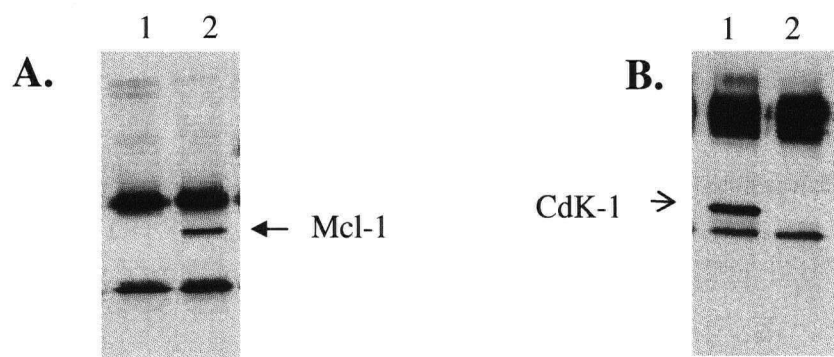


Figure 5.1- Anti-Mcl-1 and anti-Cdk-1 antibodies are specific. **A)** Normally proliferating TF-1 cell lysate was immunoprecipitated with anti-Mcl-1 antibody and the membrane was probed with anti-Mcl-1 antibody (lane 2). Lane 1 is the anti-Mcl-1 antibody control. **B)** Cdk-1 was immunoprecipitated from the TF-1 cell lysate and the membrane was probed for Cdk-1 (Lane 1). Lane 2 is the anti-Cdk-1 antibody control. The bands just above and below the Mcl-1 (**A**) and Cdk-1 (**B**) bands correspond to the immunoglobulin heavy (IgH) and light (IgL) chains. **WCL** (whole cell lysate). Blots are representative of 3 separate experiments.

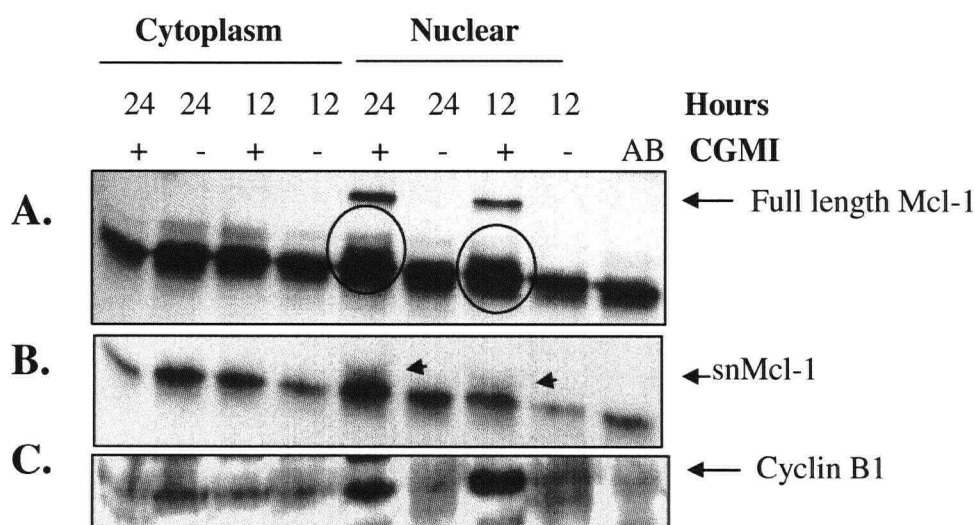


Figure 5.2- Cdk-1 co-immunoprecipitates with Mcl-1. TF-1 cells were grown in the presence (+) or absence (-) of CGMI for the indicated times and nuclear and cytoplasmic extracts were prepared for immunoprecipitation with anti-Cdk-1 antibody and the membrane was probed for either Mcl-1 (**A**) or Cyclin B1 (**C**). In Fig. **B**, a shorter exposure of the blot from **A** (circled area), the presence of snMcl-1 was also detected as shown by arrowhead. **AB** (anti-Cdk-1 antibody control). Blots are representative of 3 separate experiments.

concentration and time course of etoposide treatment in TF-1 cells were previously optimized in our laboratory and was selected based on the observations that etoposide treatment at this

concentration over a period of 18 hours induced G2/M arrest without inducing apoptosis. The role of Mcl-1 in DNA damage check point response was explored further in my studies.

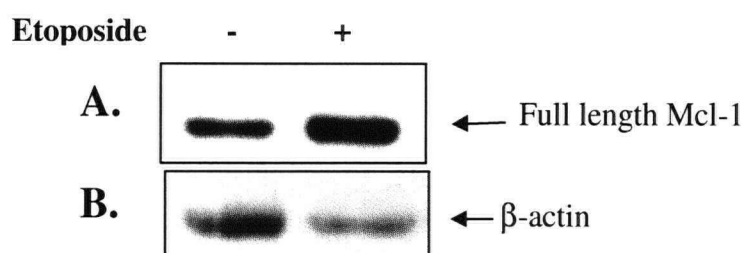


Figure 5.3- Mcl-1 level increases upon treatment of cells with etoposide. Normally proliferating TF-1 cells were treated with or without the DNA damaging agent etoposide for 24 hours and the whole cell lysates were prepared for Western blotting. The blot was probed with the anti-Mcl-1 antibody (A). Panel B represents the loading control for β -actin. Blots are representative of 3 separate experiments.

Since Chk1 plays a central role in the DNA damage check point response, its association with Mcl-1 was investigated and demonstrated in HeLa cells (S. Jamil, personal communication). We further looked for an association between Mcl-1 and Chk-1 in TF-1 cells. In order to show such association, cytosolic and nuclear extracts from normally proliferating TF-1 cells were immuno-precipitated with anti-Mcl-1 antibody and probed for Chk-1. Immunoblotting for Chk-1 as shown in Fig. 5.4A, revealed the presence of a specific band at 54 kDa, just above the heavy chain band seen with antibody alone, corresponding to Chk1 in both nuclear and cytosolic fractions. When normally proliferating TF-1 cells were treated with the DNA damaging agent etoposide, the Mcl-1/Chk1 association was predominantly detected in nuclear fraction as seen in Fig. 5.4B.

Although these results are preliminary, the DNA damage checkpoint responses are primarily activated in the nucleus. Our observations and those of others from our laboratory

(Jamil et al., submitted) that the Mcl-1/Chk-1 complex is predominantly detected in the nucleus following DNA damage appears compelling. If Mcl-1 does indeed interact with Chk-1, it would be ideal to further characterize the site of such interactions and the functional implications of these interactions. Attempts are underway in our laboratory to generate cell lines that over-express Mcl-1 mutants that lack certain BH domains that are important for Mcl-1 function as a pro-survival protein. These mutant cell lines will be used to investigate if the pro-survival effects of Mcl-1 are related to its possible role in the DNA damage checkpoint response pathway.

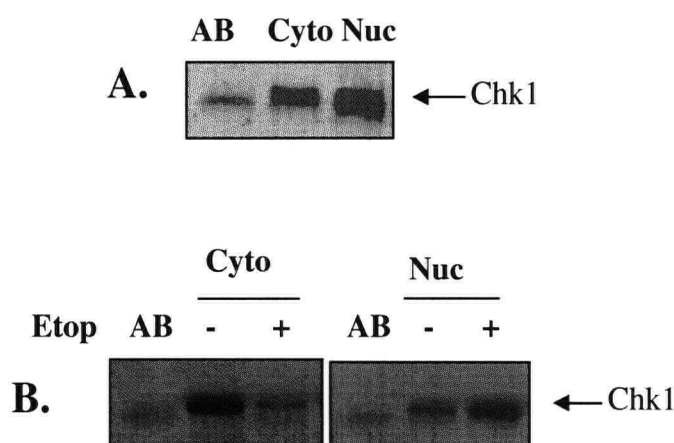


Figure 5.4- Mcl-1 associates with the DNA damage check point kinase, Chk1. **A)** Nuclear (Nuc) and cytoplasmic (Cyto) fractions from normally proliferating TF-1 cell lysates were immunoprecipitated with anti-Mcl-1 antibody and the immunoblot was probed with anti-Chk-1 antibody. **B)** TF-1 cells were treated with or without 15 μ M etoposide (Etop) for 24 hours. The nuclear (Nuc) and cytoplasmic (Cyto) fractions were immunoprecipitated with the anti-Mcl-1 antibody and the immunoblot was probed with the anti-Chk1 antibody. AB (anti-Mcl-1 antibody control). Results are representative of 3 separate experiments.

5.2.3 Mcl-1 interacts with the DNA damage response marker phospho-H2Ax

Phosphorylation of histone H2AX is considered to be a hallmark of DNA damage response. Upon induction of double stranded DNA breaks, H2Ax accumulates along with ATM/ATR at the site of damage, where it is phosphorylated on serine 139 by ATM and ATR

(Burma *et al.*, 2001; Ward *et al.*, 2001). We looked at a possible interaction between Mcl-1 and phospho-H2AX through immunoprecipitation and showed that in HL-60 cells, upon treatment with etoposide, phospho-H2AX co-immunoprecipitates with Mcl-1 only in the nuclear fraction (Fig. 5.5). No interaction was detected in the cytosolic fraction. These observations suggest possible accumulation of Mcl-1 in the foci formed at the site of DNA damage.

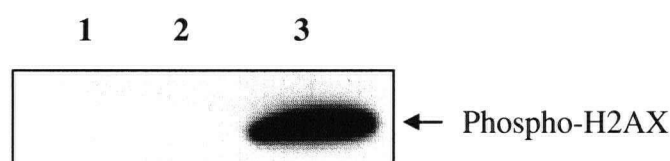


Figure 5.5- Mcl-1 associates with phosphorylated histone H2AX in the presence of DNA damage. HL60 cells were induced to undergo DNA damage by treatment of cells with etoposide and the nuclear fraction was extracted and immunoprecipitated with anti-Mcl-1 antibody. Western blotting was done using the phospho-histone H2AX antibody. Lane 1 (antibody control), lane 2 (untreated cells), lane 3 (etoposide treated cells). Results are representative of 3 separate experiments.

5.2.4 Mcl-1 level increases upon treatment with PMA

Many studies have shown that Mcl-1 expression level increases upon induction of cellular differentiation (Kozopas *et al.*, 1993). Treatment of HL-60 cells with PMA results in growth arrest and differentiation towards the macrophage lineage (Das *et al.*, 2000). Thus we wished to examine Mcl-1 expression level in HL-60 cells that were undergoing cellular differentiation with PMA. HL-60 cells were treated with 10 nM PMA and an increase in the expression of Mcl-1 and a concomitant expression of snMcl-1 was evident at 2 hr post PMA treatment, which was maintained until 24 hrs (Fig. 5.6A). Similar results were obtained in THP-1 cells (Fig. 5.6B). In addition, a decrease in the mobility of Mcl-1, 15 min following PMA treatment was also observed (Fig. 5.6C).

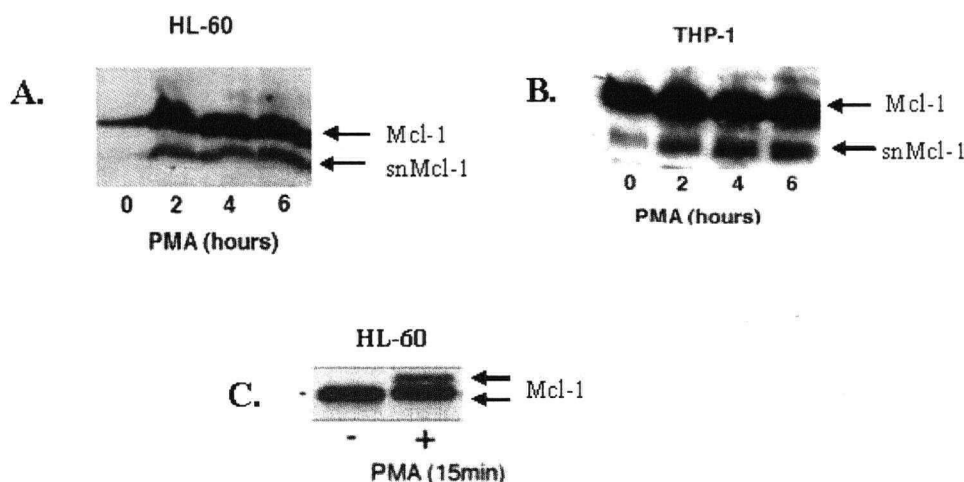


Figure 5.6- Mcl-1 level increases upon treatment of cells with PMA. HL-60 (A) and THP-1 (B) cells were induced to undergo differentiation with PMA for the indicated time and the Mcl-1 level was detected by Western blotting as shown in A and B. Also, a shift in the mobility of Mcl-1 was detected 15 minutes following PMA treatment in HL60 cells (C). Results are representative of 3 separate experiments.

5.2.5 Exploring a possible role for Mcl-1 in cellular differentiation

Since Mcl-1 level increased upon treatment of HL-60 cells with PMA, we examined the functional significance of Mcl-1 in differentiation of HL-60 cells by over-expressing Mcl-1 in these cells. HL-60 cells are notoriously difficult to transfect and after several failed efforts to transfect the Mcl-1 gene into HL-60 by using several different transfection and infection means, including effectene and lipofectamine, and also by using an inducible lentiviral expression system, HL-60 cells were successfully transfected with Mcl-1 by electroporation as shown in Fig. 5.7, and a possible role for Mcl-1 in cellular differentiation was explored. Since a great deal of work was spent on trying to establish the inducible lentiviral Mcl-1 over-expression system in our laboratory, these efforts will be described later in appendix 1.

To examine the effect of Mcl-1 over-expression on differentiating HL-60 cells, the Mcl-1 over-expressing cells were treated with PMA. The CD11b level on the cell surface was used

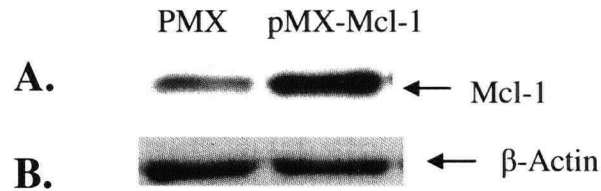


Figure 5.7- Over-expression of Mcl-1 in HL-60 cells. HL-60 cells were transiently transfected with either pMX-puro vector alone or with the pMX-puro vector carrying full length Mcl-1. Protein expression was analyzed by Western blotting on total cell lysate. **A.** Mcl-1 expression in Mcl-1 over-expressing HL60 cells. **B.** β -actin was used as a loading control.

as a marker of differentiation and the differentiation was monitored by staining the cells with PE-conjugated antibody against CD11b. Immuno-fluorescence analysis by flow cytometry showed that an 18 hour PMA treatment did not increase CD11b expression (Fig. 5.8A) and neither did a 48-hour PMA treatment (Fig. 5.8B). Since there was no change in the level of CD11b in the control versus Mcl-1 over-expressing cells, we conclude that Mcl-1 might not play a role in differentiation of HL-60 cells.

Since Mcl-1 did not appear to play a major role in differentiation of HL-60 cells and we now had access to Mcl-1 over-expressing HL-60 cells, we continued to follow up its involvement in DNA damage checkpoint response. As shown recently in our laboratory (S. Jamil, personal communication), transient transfection with Mcl-1 causes an increase in Chk1 phosphorylation in the absence of DNA damage. A downstream target of Chk1 kinase is Rad51, which is a protein involved in homologous recombination repair associated with DNA double-stranded break (Sorensen *et al.*, 2005). Thus, we looked for Rad51 phosphorylation in the Mcl-1 over-expressing cells. HL-60 cells were transfected with Mcl-1 vector by electroporation and after 48 hours of transient transfection, cells were untreated or treated with PMA for six hours. Western blotting on the total cell lysate was performed to

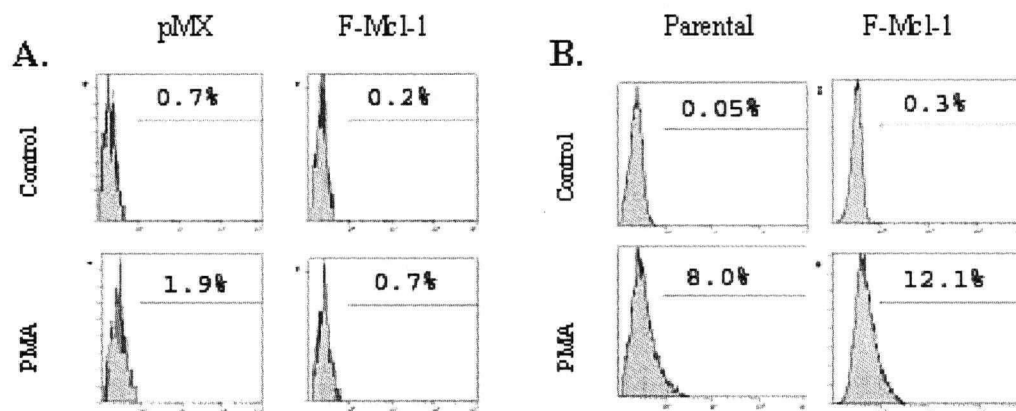


Figure 5.8- FACS analysis of CD11b expression. Mcl-1 over-expressing cells were untreated or treated with PMA for 18 (A) or 48 (B) hours. Cells were collected and were stained with PE-anti-CD11b antibody. Percentage of CD11b expression was analyzed by flow cytometry. Cells transfected with empty vector (A) or parental HL-60 cells (B) were used as the control. pMX (pMXpuro vector), F-Mcl-1 (full-length Mcl-1). Results are representative of 3 separate experiments.

look at the expression of Mcl-1 in the vector alone as well as the Mcl-1 over-expressing cells (Fig. 5.9A). When the same blot was probed for Rad51, cells treated with PMA showed a shift in the mobility of Rad51. Coincidentally, the Mcl-1 over-expressing cells in the absence of PMA also showed a shift in the mobility of Rad51, indicating possible phosphorylation of the protein as a result of activation of Chk1 (Fig. 5.9B).

The significance of Mcl-1 interaction with Chk-1, the phospho-histone H2AX, as well as a possible connection to Rad51 phosphorylation is currently not clear. Further studies are required to determine the functional relevance of these. It will be interesting to test what happens to the Chk-1, Rad51, and histone H2AX phosphorylation events when Mcl-1 is knockdown.

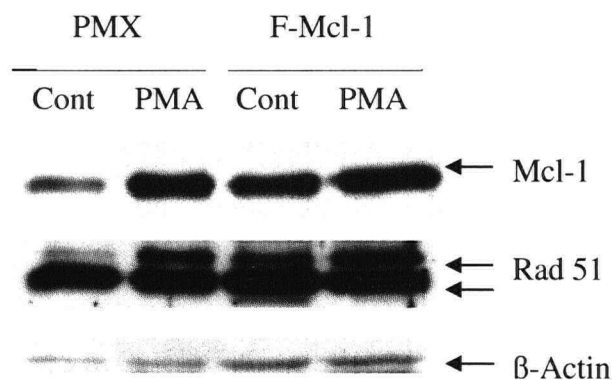


Figure 5.9- Rad 51 is phosphorylated in Mcl-1 over-expressing HL60 cells. HL-60 cells were transiently transfected with empty vector (pMX) or F-Mcl-1. Cells were untreated (Cont) or treated with PMA for 6 hours. Protein expression was analyzed by Western blotting on total cell lysate. **A)** Expression of Mcl-1 in Mcl-1-overexpressing HL-60 cells. **B)** Phosphorylation of Rad51 in Mcl-1 overexpressing cells. **C)** β -actin was used as loading control. pMX (pMXpuro vector), F-Mcl-1 (full-length Mcl-1). Results are representative of 3 separate experiments.

5.3 DISCUSSION

This chapter presents data on a project distinct from those in the previous two results chapters. The studies were undertaken to help in further characterizing the potential novel functions of Mcl-1. Thus, results presented have explored the possible involvement of Mcl-1 in cell cycle regulation, DNA damage checkpoint response, and cellular differentiation.

There is growing evidence that in addition to regulation of apoptosis, the Bcl-2 family proteins might also be involved in the regulation of cell cycle progression as well as the DNA damage check point response. For example, both Bcl-2 and Bcl-xL are believed to block cell cycle re-entry from G0 in fibroblasts, although their effect on cell survival seems to be indistinguishable from the pro-survival effect (Janumyan *et al.*, 2003). The authors also showed that the cell cycle effects of Bcl-2 and Bcl-xL were reversed by the pro-apoptotic BH3 only protein, Bad, which is believed to counter the survival function of Bcl-2 and Bcl-xL. Mcl-1 was also proposed to inhibit cell cycle progression through S phase of the cell cycle, possibly through its interaction with PCNA (Fujise *et al.*, 2000. In addition, Previous

results from our own laboratory has shown that in the human leukemia cell line, HL-60, a short form of Mcl-1, snMcl-1, delays cell cycle progression possibly through its interaction with the cyclin-dependent kinase, Cdk-1 (Jamil *et al.*, 2005).

In our current study, in order to further understand the interaction between Mcl-1 and Cdk1, we investigated the effects of cytokine on this interaction in the cytokine dependent human erythroleukemia cell line, TF-1. Our results showed that in TF-1 cells the interaction between Mcl-1 and Cdk1 was indeed cytokine dependent and occurred only in the presence of cytokine in the nucleus. In contrast to earlier findings in HL-60 cells, in TF-1 cells the association between Cdk1 and Mcl-1 was observed mainly with full length Mcl-1, however, a minor interaction was also detected with snMcl-1. The significance of the cytokine dependency of this interaction is not clear at this point.

Previous results from our laboratory indicated that the association between Mcl-1 and Cdk-1 occurred at the G2/M phase of the cell cycle and the Cdk-1 associated with Mcl-1 was shown to be inactive. These findings led to the conclusion that Mcl-1 might have an inhibitory effect on Cdk-1 during cell cycle progression and thereby provided a possible explanation for the growth inhibitory effect of Mcl-1 seen in our studies and those of others (Jamil *et al.*, 2005; Fujise *et al.*, 2000). However, in our current study, we observed Mcl-1/Cdk-1 interaction only in the cycling cells in the presence of cytokine but not in the absence of cytokine when the cell cycle progression is blocked. A possible explanation is that under normal proliferation in asynchronous conditions, cells are likely to be present at all stages of the cell cycle. It is, therefore, not surprising that this interaction was not detected upon cytokine withdrawal as starved cells normally accumulate at the junction of G1/S and the Mcl-1/Cdk-1 interaction mainly occurs at G2/M. It is also possible that Mcl-1 and Cdk-1

do not interact directly but are part of a larger complex that immunoprecipitate together. For example, Mcl-1 and Cdk-1 might be part of the larger complex which is associated with the DNA damage checkpoint response which is discussed below.

In addition to their role in cell cycle regulation, some Bcl-2 family proteins may also be linked to the DNA damage checkpoint response. Zinkel *et al.* (2005) showed that the BH3 only protein Bid plays a role in the intra-S phase checkpoint downstream of DNA damage distinct from its pro-apoptotic role. They further showed that this role is mediated through phosphorylation of Bid by the DNA damage checkpoint sensor, ATM. Another BH3 only protein, Bad, has also been linked to components of the DNA damage checkpoint response. Han *et al.* (2004) reported that the DNA damage checkpoint kinase, Chk1, associates with Bad and phosphorylates Bad at serine 155 and serine 170. The authors suggested that Chk1 may inactivate BAD by associating with and phosphorylating residues critical for BAD function in response to DNA damage.

In order to elucidate a role for Mcl-1 in DNA damage check point response, we first determined the effect of DNA damage induced by etoposide on Mcl-1 expression levels. Our results showed a modest increase in Mcl-1 expression upon treatment with etoposide and supporting data from the laboratory further showed that this increase in Mcl-1 expression was diminished by pre-treatment of cells with caffeine (S. Jamil, personal communications). Caffeine has been widely used to study ATM and ATR signaling because it inhibits the ATM/ATR associated DNA damage checkpoint responses (Cortez, 2003). Since Mcl-1 regulation appeared to be modulated by events mediated by ATM/ATR, we next determined a possible link between Mcl-1 and a key DNA damage checkpoint regulator, Chk-1 which is one of the main downstream targets of ATM/ATR. We showed that indeed Mcl-1

immunoprecipitates with Chk1 and since proteins involved in the DNA damage checkpoint response are normally active in the nucleus, we compared this association in the nuclear versus cytoplasmic compartments. Our results show that Mcl-1 interacts with Chk1 in both cytosolic and nuclear extracts. Treatment of cells with etoposide resulted in the association being observed predominantly in the nucleus. Another marker for induction of DNA damage checkpoint repair process is phosphorylation of histone H2AX (phospho-H2AX). Hence, we looked at an association between Mcl-1 and phospho-H2AX in HL-60 cells. Our results show that Mcl-1 immunoprecipitates with phospho-H2AX only in nuclear extracts and this association was only observed when cells were treated with etoposide.

Recent work from our laboratory showed that Mcl-1 over-expression resulted in phosphorylation of Chk1, while Mcl-1 knockdown resulted in loss of Chk1 phosphorylation in response to etoposide (S. Jamil, unpublished observations). Chk1, in turn, phosphorylates and activates Rad51, a protein involved in homologous recombination repair associated with DNA double-stranded break. When we looked at Rad51 expression by Western blotting, we noticed that Mcl-1 over-expressing cells showed increased levels of a slower migrating band in the absence of any exogenous DNA damage, suggesting a possible phosphorylation of Rad51. However, further experiments are needed to confirm the identity of the band shift. This finding supports our ongoing hypothesis that perhaps Mcl-1 plays a role in the regulation of DNA damage check point response. We have shown in section 5.2.2 that the endogenous Mcl-1 level increases upon induction of DNA damage. If we are to correlate this increase in the level of Mcl-1 expression with the induction of DNA damage, then the increase in Mcl-1 level due to its over-expression might induce activation of DNA damage check point response by default. Therefore, it is not surprising that we see phosphorylation of

Rad51, a DNA damage checkpoint marker in Mcl-1 over-expressing cells. Thus based on these novel data, together with other data from our laboratory, there is a potential for Mcl-1 to be functioning in DNA damage checkpoint regulation and further investigation will be required in order to elucidate the exact mechanism by which Mcl-1 is operating.

These studies are very preliminary and although we see a possible involvement of Mcl-1 in the DNA damage checkpoint repair process, we cannot make a conclusive statement regarding a role for Mcl-1 in the regulation of this process. The major short coming of this study is that we mainly used immunoprecipitation to probe for interaction between Mcl-1 and the proteins involved in the regulation of DNA damage checkpoint response. These in vitro immunoprecipitation reactions might not mimic the events that take place in vivo. An ideal scenario is to generate Mcl-1 deficient cells by knocking-down Mcl-1 and induce DNA damage, for example, by etoposide. If the DNA damage checkpoint repair pathway is perturbed in the absence of Mcl-1, one could conclude with a great certainty that Mcl-1 is involved in the regulation of this process. However, many cellular events and in particular the DNA damage checkpoint repair pathway are tightly regulated events and many of the proteins involved in these pathways might play degenerate or redundant roles. Thus, if Mcl-1 is knocked-down, other proteins in the pathway might fulfill its role and, therefore, no disturbance in the DNA damage checkpoint repair process might be detected in the Mcl-1 deficient cells. Interestingly, Jamil et al (submitted, 2007) from our laboratory have recently presented data showing that Mcl-1 knockdown results in loss of Chk-1 phosphorylation. Perhaps Mcl-1 plays a role as a chaperone by either stabilizing Chk-1 or facilitating the interaction between ATM (upstream kinase of Chk-1) and Chk-1. If Mcl-1 indeed interacts with Chk-1, it would be ideal to characterize the site of these interactions. These and other

studies are currently underway in our laboratory in order to unveil a function for Mcl-1 in the regulation of DNA damage repair process.

In addition to knocking down Mcl-1, one could also over-express Mcl-1 in order to follow its possible involvement in the regulation of DNA damage repair process. As mentioned earlier, we did see phosphorylation of histone H2AX in Mcl-1 over-expressing cells in the absence of any DNA damaging agents. Others in our laboratory have also shown that Mcl-1 over-expression induces phosphorylation of Chk-1 (Jamil et al., submitted). Thus although in this thesis we could not conclusively demonstrate a role for Mcl-1 in the regulation of DNA damage repair process, our data combined with the supporting data from our laboratory demonstrate a possible role for Mcl-1 in the regulation of DNA damage repair processes.

In addition to a possible involvement of Mcl-1 in regulation of cell cycle and DNA damage checkpoint response, we also explored a potential role for Mcl-1 in cellular differentiation. We noticed that when we treated HL-60 cells with PMA to induce differentiation into a macrophage lineage, the level of Mcl-1 increased upon treatment with PMA. In addition, Mcl-1 appeared to be phosphorylated following PMA treatment. We speculated that these changes in Mcl-1 might be directly associated with its possible involvement in differentiation. Alternatively, since Mcl-1 is a pro-survival protein, the rise in Mcl-1 level during differentiation might be involved in protecting the differentiating cells from apoptosis. Thus, we attempted to over-express Mcl-1 in HL-60 cells in order to examine its possible involvement in differentiation of these cells. The rationale for this was that if Mcl-1 has a role in differentiation, then its over-expression should facilitate differentiation of HL-60 cells either by itself or in combination with PMA treatment. HL-60 cells are

notoriously difficult to transfect. We managed, however, to over-express Mcl-1 in HL-60 cells by using a very effective electroporation system as described in section 2.9. We theorized that if Mcl-1 was involved in cellular differentiation, its over-expression in HL-60 cells would induce cellular differentiation. Likewise, if Mcl-1 played a role in cellular differentiation, then induction of differentiation by PMA might proceed in a shorter time frame in Mcl-1 over-expressing cells compared to the control. When we looked at the CD11b expression on the cell surface by FACS analysis, the level and the time frame of its expression (18 hr versus 48 hr) were very similar in the Mcl-1 over-expressing cells compared to the control (vector alone) in the PMA treated cells. Therefore, we are left with the conclusion that the change in the level of expression of Mcl-1 is not able to alter the process of differentiation of HL-60 cells.

Although we did not observe a role for Mcl-1 in cellular differentiation, we can not conclusively rule it out either since only one model system based on the ability of HL60 to differentiate towards the monocytic lineage was studied. HL60 cells are pro-lymphocytic myeloid leukemia cell line and although a convenient model for differentiation are not representative of the events that take place in primary cells undergoing differentiation. Perhaps a better model would have been to induce differentiation in primary bone marrow derived cells. An increase in Mcl-1 protein level has also been observed in primary cells undergoing differentiation (S. Wang, personal communication). Alternatively, Mcl-1 might not have a direct role in cellular differentiation and an increase in its expression level might be a reflection on its role as a pro-survival protein since cells undergoing differentiation are vulnerable and require protection from apoptosis.

These results, together with many other ongoing studies in our laboratory, support

additional function of Mcl-1 other than inhibition of apoptosis. It is now well known that over-expression of Mcl-1 has an inhibitory effect on cell growth. Mcl-1 interaction with Cdk-1, as well as its activation of Chk-1 could both play a role in cell cycle progression. While Mcl-1 clearly has pro-survival effects, its effects on cell cycle events may be just as, or even more important than its pro-survival effects. It is possible that these additional functions of Mcl-1 could control any effect it might have on cellular differentiation. Although the experiments in this thesis attempted to address a role for Mcl-1 in differentiation of HL-60 cells, our preliminary results are not sufficient to make any firm conclusions.

CHAPTER 6

SUMMARY AND PERSPECTIVE

In this thesis, a variety of experimental approaches have been used to address questions about the means by which hemopoietic cells undergo apoptosis, as well as to investigate the mechanism of action of several Bcl-2 family proteins. In Chapter 3, we investigated a potential role for the death receptor Fas-mediated pathway by hypothesizing the death receptor Fas mediated pathway is involved in the death of cytokine dependent hemopoietic cells that were induced to undergo apoptosis by cytokine starvation. Our results led to the conclusion that the Fas-mediated pathway is possibly not involved in this process. This was confirmed in murine promyelocytic leukemia cell line, FDCP-1, and human erythrocytic leukemia cell line, TF-1. Both of these cell lines require cytokines for survival and proliferation, and withdrawal of the cytokine results in apoptosis of these cells. The survival signaling is mediated through the PI-3 kinase dependent pathway and it has been shown in numerous studies that blocking of the PI-3 kinase pathway either through the PI-3 kinase inhibitor, LY294002, or cytokine withdrawal leads to apoptosis. At the time these studies were initiated, we asked whether these effects were mediated, at least in part, through activation of the Forkhead transcription factor, FOXO3A. In the presence of cytokines, FOXO3A was shown to be phosphorylated at the inhibitory sites Thr32, Ser253, and Ser315 through a PI-3 kinase/PKB dependent pathway. Inhibition of the PI-3 kinase signaling pathway leads to activation of FOXO3A transcriptional activity. One of the reported targets of FOXO3A is Fas ligand (FasL), which we showed was up-regulated by cytokine starvation or treatment of cells with LY294002. The expression of Fas receptor was also enhanced on the surface of cytokine starved cells and also upon treatment with LY294002, although we

have not investigated the mechanism by which this may be occurring.

Many studies have investigated the role of Fas and FasL in apoptosis of hemopoietic cells. In primary monocytes, addition of cytokines was not shown to affect expression of Fas or FasL, but instead acted to suppress death signaling downstream of Fas (Kiener *et al.*, 1997). Likewise, neutrophils were reported to co-express Fas and FasL and thus were committed to autocrine death (Liles *et al.*, 1996) which could be modulated by survival factors. In the case of mast cells, one study had suggested that FasL in mast cells was not localized at the cell surface, but remained intracellular (Wagelie-Steffen *et al.*, 1998). However, it is possible that lack of detection was due to cleavage of cell surface FasL. We found that the use of a metalloproteinase inhibitor was necessary to detect FasL on TF-1 or FDC-P1 cells and in its absence, little FasL could be detected. Results of an earlier study examining the potential for Fas-mediated death of mast cells (Hartmann *et al.*, 1997) demonstrated that Fas was detected at the cell surface of mast cells, and they could be induced to undergo apoptosis with addition of exogenous FasL. No analysis of FasL expression was done in that study. Our results with FDC-P1 and TF-1 cells also show that a low level of Fas is present at the cell surface, but we found an increase in Fas was also detected in cells undergoing apoptosis. Importantly, addition of exogenous FasL, which could induce caspase 8 activation and apoptosis in Jurkat cells, had no effect on apoptosis of these Fas-expressing cells.

An important question which needs to be further addressed is why the Fas receptor expressing FDCP-1 and TF-1 cells do not undergo apoptosis upon addition of FasL in the same way Jurkats do. We compared different hemopoietic cells that expressed Fas receptor on the cell surface and showed that while some cells like Jurkats underwent apoptosis upon

addition of an agonistic anti-Fas antibody, others did not. It is possible that a component of the death receptor pathway is defective in these cell lines. For example, the receptor, itself, might be mutated, and, thus non-functional. Similarly, the adaptor protein, FADD might be mutated or absent. Another possibility is that an inhibitor of the Fas receptor pathway, for example FLIP, might be over-expressed and, therefore, this might protect cells from the Fas-mediated cell. This could be further explored by knocking down expression of FLIP by SiRNA to determine if this allows Fas-mediated apoptosis.

On the other hand, we observed that the pro-apoptotic protein, Bim, and the cell cycle inhibitor, P27kip1, were up-regulated as a result of cytokine starvation. Similar to our findings, Dijkers *et al.* (2002) showed that in IL-3-dependent Baf-3 cells, FOXO3A mediated apoptosis involved up-regulation of p27kip1 and Bim. They found no activation of caspase 8 and thus did not find any role for Fas-mediated death in these cells, although no analysis of FasL was done. While Baf-3 cells are used in many studies investigating cytokine-dependent events, they show many characteristics that are different from other hemopoietic cell types. Thus, we felt that it was important to investigate these events in other cytokine-dependent cells. In fact, we used two different hemopoietic cell lines, a murine FDCP-1 cell line and a human TF-1 cell line, with comparable results observed in both. Such an approach provides assurance that the effects are not due to use of a unique cell type. An important difference in our studies from those using Baf-3 cells is that we were able to detect the activation of caspase 8, even though we could not definitively connect this to any Fas-mediated process. Therefore, we would conclude that caspase 8 is likely being activated as a result of caspase 9 and/or caspase 3 activation. Thus, our studies support the conclusion that Bim and p27kip1 expression are more likely to be key FOXO3A-dependent events in the process leading to

apoptosis as a result of cytokine starvation.

To summarize our findings in Chapter 3, our investigations into the cytokine-mediated phosphorylation of FOXO3A, which is largely mediated in a PI3K and PKB-dependent manner, confirmed that up-regulation of FasL occurs when FOXO3A phosphorylation is decreased. However, we were unable to find evidence that cytokine-deprived cells utilize the cell surface FasL and Fas to initiate an apoptosis cascade. On the other hand, we were able to demonstrate that mitochondrial mediated events through activation of the pro-apoptotic protein Bim might be involved in apoptosis of hemopoietic cells under the condition of cytokine starvation. While our studies alone were not able to definitively rule out a role for Fas-mediated apoptosis, the approaches we used could not show that FasL and Fas were functional. In light of other reports that appeared at the time these studies were done, we feel confident in suggesting that intrinsic cell death pathways play a dominant role in apoptosis of hemopoietic cells deprived of cytokines.

In chapter 4 we compared the differential ability of Bcl-2 and Bcl-xL to protect against cytokine withdrawal-induced apoptosis in the cytokine dependent hemopoietic cell lines and our results suggested that Bcl-2 might be a more potent pro-survival protein than Bcl-xL. A number of reports also indicated a differential role for Bcl-2 and Bcl-xL in protection against apoptosis induced by different cytotoxic stimuli (Simonian *et al.*, 1997; Keong *et al.*, 2000; Gottschalk *et al.*, 1994; Yuste *et al.* 2002). As suggested by others, the differential ability of Bcl-2 or Bcl-xL to protect against apoptosis induced by certain cytotoxic drugs might depend on the molecular mechanism or the cellular target of the drugs (Simonian *et al.*, 1997). Since Bcl-2 and Bcl-xL are thought to regulate similar cell survival pathways, the cytokine withdrawal-induced cell death pathways might activate certain

cellular factors that differentially regulate Bcl-2 and Bcl-xL. These death inducers, for example, might activate certain BH3 only proteins that might have differential affinity for Bcl-xL versus Bcl-2 (Chen *et al.*, 2005). In addition, Bcl-2 and Bcl-xL might differentially block certain caspases that might be activated by specific cytotoxic stimuli. We have shown in the previous section that cytokine withdrawal induces activation of Caspase 2 and, thus, Bcl-2 and Bcl-xL might differentially regulate activity of this caspase which is thought to act upstream of the mitochondrial death pathway. Additionally, it is possible that certain cytotoxic stimuli biochemically modify Bcl-2 and Bcl-xL, for example, Bcl-2 and Bcl-xL can both function as pro-apoptotic molecules upon cleavage by cellular caspases during IL-3 withdrawal-induced apoptosis. Also, the chemotherapeutic agent, taxol, and IL3, have been shown to induce phosphorylation of Bcl-2, which has been suggested to differentially modulate its anti-apoptotic activity (Halder *et al.*, 1994; Halder *et al.*, 1995; May *et al.*, 1994). Therefore, it is possible that certain cytotoxic stimuli differentially regulate cleavage, phosphorylation or dephosphorylation of Bcl-2 and Bcl-xL and selectively affect their ability to inhibit apoptosis.

In Chapter 4, we also looked into the mitochondrial and ER mediated events, in particular, the role of Bcl-2 family proteins, in regulation of apoptosis. In Rat-1 fibroblast cell lines stably over-expressing Bcl-xL targeted to ER (Bcl-xL-cb5), or the mitochondrial outer membrane (Bcl-xL-ActA), or wild type Bcl-xL (Bcl-xL-wt), we showed that all three Bcl-xL mutants are equally protective against either ceramide or growth factor deprivation-induced apoptosis. However, the ER targeted Bcl-xL was shown to be a more potent pro-survival protein than either the mitochondrial targeted or wild type Bcl-xL against Bad-induced apoptosis. Until recently, little attention had been paid to the possible role of Bcl-2

family proteins in ER homeostasis. Selective targeting of Bcl-2 at ER, provided valuable information on the anti-apoptotic activity of Bcl-2 at this location. The ER targeted Bcl-2, for example, prevented apoptosis induced by ER stress agents, ceramide, Myc or Bax over-expression, and ionizing radiation (Hacki *et al.*, 2000; Annis *et al.*, 2001; Rudner *et al.*, 2001; Wang *et al.*, 2001). Annis *et al.* (2001), reported that the ER targeted Bcl-2 differentially protected cells against, for example, Myc or C2 ceramide-induced apoptosis, but not the staurosporine-induced cell death, whereas wild type Bcl-2 was protective against all of them. Thus the ER targeted Bcl-2 appears to be restricted in its ability to provide survival against different apoptotic stimuli.

Both the pro- and anti-apoptotic Bcl-2 family proteins are localized at ER and perhaps one of the mechanisms by which they regulate apoptosis is through regulation of the ER Ca^{2+} homeostasis. Bcl-2 over-expression has been reported to reduce ER Ca^{2+} stores by increasing the passive Ca^{2+} leak from ER, whereas, over-expression of Bax and Bak promote Ca^{2+} mobilization from ER to mitochondria during apoptosis (Chami *et al.*, 2004). In fact, cells deficient in both Bak and Bax are highly resistant to Ca^{2+} dependent death stimuli (Scorrano *et al.*, 2003). The mechanism by which the Bcl-2 family proteins regulate intracellular Ca^{2+} concentrations is not clear. One possibility is that the Bcl-2 family proteins form Ca^{2+} conducting pores in the ER membrane, however, Chami *et al.* (2004) showed that the pore forming domain of Bcl-2 and Bcl-xL are not involved in the regulation of ER Ca^{2+} by these proteins. Another possibility is that the Bcl-2 proteins interact with or regulate the Ca^{2+} channels already present at the ER. To this end, both Bcl-2 and Bcl-xL have been reported to physically interact with the ER Ca^{2+} channel, IP3R (Scott *et al.*, 2005). In addition, Bcl-xL over-expression leads to reduction in the expression of the ER Ca^{2+} channel, IP3R (Li *et al.*,

2002). Therefore, through interaction with pre-existing Ca^{2+} channels, Bcl-2 family proteins might regulate apoptosis by either perturbing or maintaining ER Ca^{2+} homeostasis. Thus our findings that ER targeted Bcl-xL is protective against ceramide-induced apoptosis is not surprising considering the fact that ceramide increases the intracellular Ca^{2+} concentration through release of Ca^{2+} from intracellular stores.

Another mechanism by which the pro-survival Bcl-2 proteins could protect cells against apoptosis is by sequestering pro-apoptotic molecules such as the BH3 only Bcl-2 family proteins away from their target site. We found that ER targeted Bcl-xL was more protective against Bad-induced apoptosis possibly because it sequestered Bad away from the mitochondria, whereas with the mitochondrial targeted and wild type Bcl-xL, Bad was specifically targeted to the mitochondria through interaction with Bcl-xL. In addition to sequestering the BH3 only proteins, Bcl-2 and Bcl-xL might prevent activation of the pro-apoptotic ER membrane proteins, for example, Bap31 or the BH3 only protein spike (Mund *et al.*, 2003). Both Bcl-2 and Bcl-xL physically interact with Bap31 and prevent its cleavage and subsequent activation by caspases, and expression of Bcl-2 prevents pro-apoptotic activity of cleaved Bap31 (Ng *et al.*, 1997). Spike interacts with Bap31 and in doing so, it is able to inhibit the formation of the anti-apoptotic complex between Bap31 and Bcl-xL. Overexpression of Bcl-xL, however, prevents spike-induced apoptosis (Mund *et al.*, 2003). Thus, the ER targeted Bcl-2 and Bcl-xL might prevent apoptosis possibly through both maintaining intracellular Ca^{2+} homeostasis and/or sequestering the anti-apoptotic proteins at the ER.

In Chapter 5 we investigated potential involvement of Mcl-1 in different cellular events other than its established role in cell survival. As mentioned earlier, abrogation of the Mcl-1 gene in mice leads to pre-implantation embryonic lethality, however, these embryos

do not display any evidence of altered apoptosis (Rinkenberger *et al.*, 2000). This is interesting considering the fact that the Bcl-xL knockout is also embryonic lethal and, in this case, the failure of the embryos to survive is due to excessive apoptosis (Motoyama *et al.*, 1995). The reason why the Mcl-1 knockout embryos do not implant is not clear and it is possible that Mcl-1 has functions other than its role in cell survival and this was the rationale for our studies in this section. Previously, we and others showed involvement of Mcl-1 in cell cycle regulation (Fujise *et al.*, 2000; Jamil *et al.*, 2005). The previous work from our laboratory showed that a short form of Mcl-1, snMcl-1, co-immunoprecipitates with Cdk-1 in HL60 cells and the Cdk-1 pulled down with Mcl-1 was shown to have a reduced kinase activity (Jamil *et al.*, 2005). In the current study, we explored the cytokine dependency of this interaction in the cytokine dependent TF-1 cells and speculated that Mcl-1 and Cdk-1 might associate in the absence of cytokine, when the Cdk-1 activity is decreased. Our results were surprising, however, since Mcl-1 co-immunoprecipitated with Cdk-1 only in the presence of cytokine in the nucleus. One possibility is that cells in the presence of cytokine are continually cycling through the different phases of the cell cycle including the G2/M phase where Mcl-1/Cdk-1 interaction takes place (Jamil *et al.*, 2005). However, in the absence of cytokine, the cell cycle progression might be halted at other phases of the cell cycle and it is possible that the number of cells halted at the G2/M might not be enough to detect such interaction. Alternatively, the Mcl-1/ Cdk-1 interaction might not be involved in the regulation of cell cycle but rather the Mcl-1 and Cdk-1 might be part of a larger complex that performs other functions. Since Mcl-1 protein level increased upon treatment of TF-1 cells with the DNA damaging drug, etoposide, we examined a potential function for Mcl-1 in the regulation of DNA damage checkpoint response. We are the first to report a possible role for

Mcl-1 in the regulation of DNA damage control by showing an association between Mcl-1 and the two essential components of the DNA damage checkpoint response, Chk-1 and the phosphorylated histone H2AX. Histone H2AX is one of the several variants of the nucleosome core histone H2A. Phosphorylation of H2AX is considered to be a hallmark of DNA damage checkpoint activation. Upon induction of double stranded DNA breaks, H2AX accumulates along with ATM/ATR at the site of damage, where it is phosphorylated on serine 139 by ATM and ATR (Burma *et al.*, 2001; Ward *et al.*, 2001). The fact that Mcl-1 interacts with phosphorylated histone H2AX only upon treatment of cells with etoposide combined with our other findings that Mcl-1 associated with Chk-1 translocates to the nucleus upon etoposide treatment indicate that Mcl-1 likely plays a role in DNA damage checkpoint response. At this point it is not clear what function Mcl-1 plays in the regulation of this pathway but supporting data from our laboratory indicates that Mcl-1 might directly interact with and thus stabilize Chk-1. These supporting data shows that the half life of Chk-1 is extended up to 24 hours in Mcl-1 over-expressing cells compared to a half life of only 4 hours in control cells. Attempts in our laboratory are underway to silence Mcl-1, for example by using Mcl-1 siRNA, and check the stability of Chk-1 protein in the absence of Mcl-1. It is not surprising that Mcl-1 might be involved in the DNA damage checkpoint response pathway since other members of the Bcl-2 family proteins such as Bid and Bad have also been implicated in the checkpoint response pathway (Zinkel *et al.*, 2005; Han *et al.*, 2004).

Finally, as mentioned in Chapter 5, we successfully over-expressed Mcl-1 in HL-60 cells in order to examine a potential role for Mcl-1 in cellular differentiation. As mentioned earlier, HL-60 is an undifferentiated human leukemic cell line capable of undergoing differentiation toward monocytic or granulocytic lineages upon treatment with PMA or

DMSO, respectively. Mcl-1 was originally identified as a gene rapidly up-regulated early in the differentiation of a human myeloid leukemia cell line (ML-1) upon induction with PMA (Yang *et al.*, 1996). In our hands, treatment with PMA not only up-regulates Mcl-1 expression but it also phosphorylates Mcl-1. We speculated that Mcl-1 might be involved in the regulation of cellular differentiation, however, our preliminary findings in HL-60 cells indicate that Mcl-1 might not play a role in differentiation of these cells. Other members of our laboratory are currently exploring a potential role for Mcl-1 in the regulation of ES cell differentiation and their data would perhaps shed more light in the area of cellular differentiation by Mcl-1. As can be seen from the preliminary nature of some of the data, Chapter 5 is an exploratory chapter. Thus, we tried to explore potential roles for Mcl-1 in the regulation of various events (i.e., cell cycle, DNA damage checkpoint response, and cellular differentiation) in hemopoietic cells. The results, when combined with parallel work done by other members of our laboratory, strongly suggest a novel role for Mcl-1 in the regulation of DNA damage checkpoint response in addition to its pro-survival function.

In conclusion, our findings have revealed some new and novel functions of Bcl-2 family proteins in the regulation of hemopoietic cell apoptosis, survival, and DNA damage checkpoint response. In particular, our findings have expanded our understanding in the regulation of hemopoietic cell apoptosis due to cytokine starvation. In addition, our work on the pro-survival Bcl-2 family proteins, Bcl-2 and Bcl-xL, have shed some light into how proteins with very similar functions could respond differentially to various cytotoxic insults, and suggest that continuous exploration of this area might prove invaluable for a comprehensive understanding of the mode of action of the Bcl-2 family proteins as a whole. Moreover, our novel results with Mcl-1 provide a starting point for future studies to dissect

the exact mechanism by which Mcl-1 might regulate DNA damage checkpoint response.

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APPENDIX 1

A. The lentiviral work

The Mcl-1 gene was first amplified (Fig. 1) using the Mcl-1 cDNA as a template and the primer set described in section 2.8.2. This Mcl-1 was then cloned into an entry vector, pDONR201, via the BP reaction as described in section 2.8.4 and Fig. 2A. In order to confirm the size of the product, The BP reaction product was cut with *Apa*I as shown in

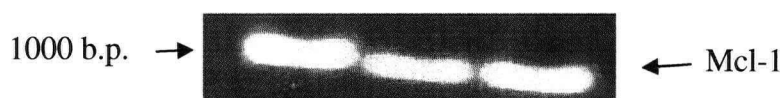


Figure 1- Mcl-1 PCR product. Mcl-1 was amplified by PCR using the Mcl-1 cDNA as a template. The expected size for the Mcl-1 gene was approximately 1000 base pare (b.p.).

Fig. 2B. The reaction product (Mcl-1/pDONR 201 construct) was sequenced using the forward and reverse primers sequences on the pDONR 201 vector (Fig. 2A). The accuracy of the Mcl-1 sequence was confirmed by DNA sequencing and this Mcl-1 was further sub-cloned into the Lentiviral expression vector, pLenti4/DEST by using the LR reaction as described in section 2.8.5 and Fig. 3A. The LR generated construct was linearized with *Cla*I in order to check the size of the product as shown in Fig. 3B. Since the product (Mcl-1/pLenti4/DEST) was only about 600 b.p. smaller than the original pLenti/DEST construct (Fig. 3A), we were not able to detect this difference on the 0.8% agarose gel as seen in Fig. 3B. However, we were able to confirm, by DNA sequencing, that Mcl-1 was indeed present in the LR reaction generated construct. The LR reaction product was sequenced using the attB primer set described in section 2.8.4.

Figure 2A- Recombination region of the expression clone resulting from pDONR 201 entry clone:

```

                                Forward priming site
293  CCTACTCTCG  CGTTAACGCT AGCATGGATC  TCGGGCCCCA AATAATGATT TTATTTTGAC
                                AGCCCGGGGT TTATTACTAA AATAAAACTG

353  TGATAGTGAC CTGTTCGTTG CAACAAATTG ATGAGCAATG CTTTTTTATA ATG CCA AGT
    ACTATCACTG GACAAGCAAC GTTGTTTAAC TACTCGTTAC GAAAAAATAT TAC GGT TCA

                                attL1
413                                     2656
412  TTG TAC AAA AAA GCA GGC TNN --- --- NAC CCA GCT TTC TTG TAC AAA
    AAC ATG TTT TTT CGT CCG ANN --- Gene --- NTG GGT CGA AAG AAC ATG TTT

2666  GTG GGC ATT ATAAGAAAGC ATTGCTTATC AATTGTGTTG AACGAACAGG TCACTATCAG
    CAC CCG TAA TATTCTTTCG TAACGAATAG TTAAACAACG TTGCTTGTC AGTGATAGTC

                                attL2
                                Reverse priming site
2725  TCAAAATAAA ATCATTATTT GCCATCCAGC TGCAGCTCTG GCCCGTGTCT CAAAATCTCT
    AGTTTTATTT TAGTAATAAA CGGTAGGTCG

2785  GATGTTACAT TGCACAAGAT AAAAATATAT CATCATGAAC AATAAAACTG TCTGCTTACA

```

Note: As a result of the recombination, about 2000 b.p. is removed from the entry clone (413-2656) and this is replaced by 1000 b.p. Mcl-I insert (Shaded area). As a result, the vector generated through the BP reaction is about 1000 b.p. shorter than the original pDONR 201 vector.

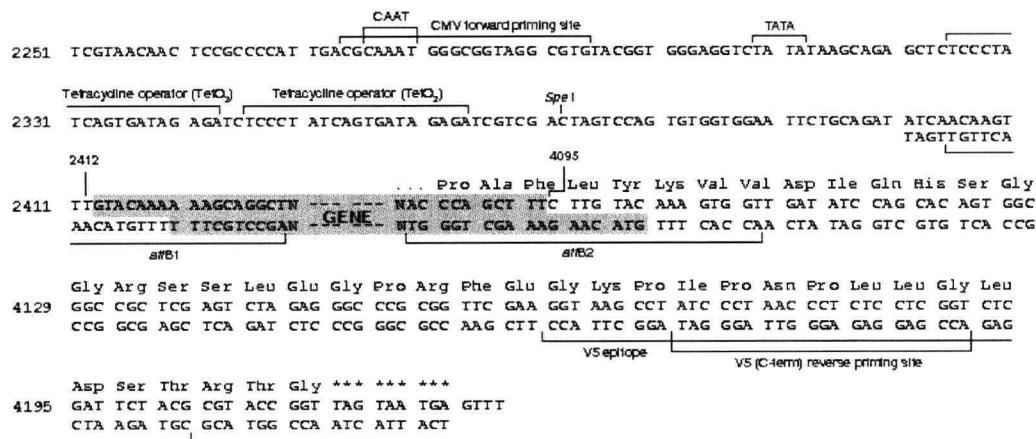


Figure 2B- The BP reaction product. The pDONR 201 vector and the BP reaction products were linearized with the restriction enzyme, ApaI, and the products were loaded on a 0.8% agarose gel followed by electrophoresis.

After the Mcl-I expression construct was generated, two sets of viruses were made (discussed in section 2.8.7): one carrying the tetracyclin (Tet) repressor (TetR) construct (pLenti6/TR) and the other set carrying the Mcl-I lentiviral expression construct (pLenti4/DEST-Mcl-1). We were able to successfully generate an HL-60 cell line over-expressing Tet repressor by selecting cells in the presence of Blastocidine. This host cell line

was then infected with the virus carrying the Mcl-1/pLenti4/DEST construct. Cells were selected in Zeocin (section 2.8.8) but two separate attempts to isolate Zeocin resistant clones failed. At this point, the work with the lentivirus system concluded and another member of the laboratory will be carrying on with this work.

Figure 3A- Recombination region on pLenti4/DEST construct generated from the LR reaction:



Note: The shaded region (~1600 b.p.) in the pLenti4/DEST construct was removed as a result of the LR reaction and this was replaced with ~1000 b.p. Mcl-1 gene. Thus the resulting Mcl-1 expression construct is about 600 b.p. shorter than the original pLenti4/DEST construct.

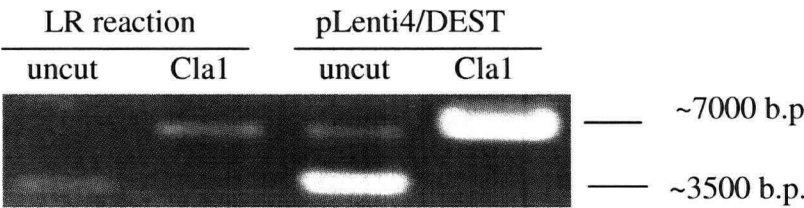


Figure 3B- The LR reaction product. The pLenti4/DEST vector and the LR reaction product were linearized with ClaI and the products from the restriction digest were loaded on 0.8% agarose DNA gel followed by electrophoresis.

